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EXERCISE AND ANGIOGENIC GROWTH FACTORS IN HUMAN SKELETAL MUSCLE

by

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Errata Thomas Gustafsson

1. ***Page 8 figure 3:*** Fal should be Fat
2. ***Page 18 first paragraph:*** Forty-six should be Fifty-six
3. ***Page 20 second paragraph first line:*** Second sentence should be changed to: " Four subjects were randomly selected to perform the exercise bout with restricted leg blood flow and five subjects to perform non-restricted leg blood flow first.
4. ***Page 21 Firs paragraph line 4.*** Second sentence should be changed to "Blood samples were drawn at rest, at 17, 37 and 57 min of exercise, and 120 min after exercise…."
5. ***Page 27 between first and second paragraph:*** Fibre-type-specific expression of VEGF-A and splice variants of VEGF-A was analysed using Wilcoxon matched pair test. Because of correlation for within subjects changes, the relative distribution of specific splice variants was evaluated using one-way Anova (time)
6. ***Page 27 second paragraph:*** In study II should be In study III
7. ***Page 31 figure 16:*** following sentence should be included in the figure text; ‡ Denotes significantly increase from 2h *vs*. 6h ($p<0.05$)
8. ***Page 34 figure 20 (and in ms of paper IV):*** † should be ‡ in figure text
9. ***Page 34 first paragraph:*** Following sentence should be excluded "A concurrent increase in VEGF-A mRNA and protein expression was observed (figure 21)"
10. ***Page 34 second paragraph line 3:*** (figure 21) should be (figure 22)
11. ***Page 34 second paragraph*:** Following sentences should be included at the end. The basal level of VEGF-A protein increased between pre-training and 10 days in the R-leg and showed a trend to further increase after five weeks. Basal VEGF-A protein level did not change after training in the NR-leg.
12. ***Page 37 Figure 22:*** T $P<0.05$ should be included in figure showing Ang-2 protein levels
13. ***Page 34 fourth paragraph last line:*** last sentence should be changed to "The basal levels of Ang-2 mRNA and protein…. "
14. ***Page 41 first paragraph line 8:*** Following references should be included: Esbjornsson et al. 1993
15. ***Subheading 6:1:4*** should be modified to; VEGF-A and endostatin muscle protein levels.
16. ***Page 42 fourth paragraph:*** should be modified to; "…..which not support this factor to be involved in the initial regulation of exercise induced angiogenesis"
17. ***Subheading 6:1:5*** should be modified to; Changes in plasma levels and exchange of VEGF-A
18. ***Page 47 first paragraph last line:*** Following references should be included (thus also in the reference list): Milkiewicz, M., Pugh, C.W., & Egginton, S. (2004). Inhibition of endogenous HIF inactivation induces angiogenesis in ischaemic skeletal muscles of mice. *J Physiol* **560**, 21-26
19. ***Paper IV page 7 line 12***. Following sentences should be excluded " The mix of 18 S rRNA was prepared in accordance to ……

ABSTRACT

Long-term electrical stimulation and endurance exercise increase the amount of capillaries in skeletal muscle. VEGF-A is a well-characterized stimulatory angiogenic growth factor and has shown to play an important role in angiogenesis in pathological conditions in humans and in physiological conditions in animal models. A close relationship has recently been observed between VEGF-A and another group of endothelial specific growth factors, angiopoietins, during development and during angiogenesis in adult animal tissue. In situations with increased expression of Ang-2 and high availability of VEGF-A a favourable impact exists on the angiogenic process through Ang-2 induced endothelial destabilization, which facilitates the effects of VEGF-A on endothelial activation. The general aim of this thesis was to study the influence of exercise on angiogenic growth factors. The levels and regulation of VEGF-A, its splice variants and receptors as well as Ang-1 and Ang-2 and their receptors were studied. The specific aims were to study 1) effects of single and repetitive bouts of endurance exercise on the expression of angiogenic growth factors in skeletal muscle, 2) acute effects of a single bout of endurance exercise on the exchange of angiogenic growth factors over the working leg, and 3) effects of restricted leg blood flow, and thereby reduced oxygen delivery to the working leg on the expression of angiogenic growth factors in skeletal muscle. The main hypotheses were that endurance exercise increases the expression of angiogenesis growth factors and their receptors and that this response is enhanced by reduced oxygen delivery to the exercising muscles in a fashion that facilitates the angiogenic process. A single bout of endurance exercise increased the expression of VEGF-A mRNA, but not of FGF-2 mRNA in human skeletal muscle, as measured in biopsy samples from vastus lateralis. Metabolic perturbation, induced by exercise with restricted leg blood flow, enhanced the VEGF-A response. Also the expression of the VEGF receptors, VEGFR-1 and VEGFR-2 increased at the mRNA level in response to a single bout of exercise. The recognized splice variants in skeletal muscle were VEGF-A_{121}, VEGF-A_{165} and VEGF-A_{189}, which all increased in response to a single bout of exercise. However, the relative distribution of VEGF-A splice-variant expression changed after exercise; initially with a relative increased expression of VEGF-A_{165}, followed by a later relative increase of VEGF-A_{189}. There was no uptake of VEGF-A protein into exercising skeletal muscle from the circulation, which indicates that the blood stream is not a source for the observed increase in skeletal muscle VEGF-A protein after exercise. Repetitive bouts of endurance exercise with restricted blood flow increased a) basal VEGF-A protein levels, but not basal VEGF-A mRNA, which increases only temporarily in association to each single bout of exercise, b) the basal level of VEGFR-2 mRNA, but not of VEGFR-1, which suggests a selective contribution of the VEGF-A receptors in exercise-induced angiogenesis c) basal level of Ang-2 protein and the mRNA ratio Ang-2 to Ang-1. Several components of the HIF-1 pathway were activated in response to acute changes in oxygen demand in human skeletal muscle. However, the present data also shows a relationship between the exercise-induced increase in venous lactate concentration and VEGF-A mRNA expression, which indicates that energy metabolites may be involved in the regulation of exercise-induced VEGF-A expression. The results presented in this thesis support that oxygen sensitive pathways may be relevant for adaptation to physical activity in human skeletal muscle. The present data also shows that the exercise-induced increase in VEGF-A is more complex than activation of HIF-1 alone. Finally, it is suggested that the concurrent changes observed in the VEGF-A and angiopoietin systems may have exerted a permissive effect on angiogenesis.

Keywords: adapatation, angiogenesis, angiogenic growth factors, blood flow, gene expression, gene regulation, human, ischaemia, skeletal muscle, training

List of papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. **Gustafsson, T., Puntschart, A., Kaijser, L., Jansson E., and Sundberg, C.J.** Exercise-induced expression of angiogenesis related transcription and growth factors in human skeletal muscle. *Am J Physiol* **276**, H679-H685, 1999

II. **Gustafsson, T., Ameln, H., Fischer, H., Sundberg C.J., Timmons, J.A., and Jansson, E.** Increased VEGF-A gene expression preceedes modulation of VEGF receptors gene expression in exercising human skeletal muscle. *J Appl Physiol* **98**, 2137-2146, 2005

III. **Ameln* H., Gustafsson* T., Sundberg C.J., Okamoto K., Jansson E., Poellinger L., and Makino Y.** Physiological Activation of Hypoxia Inducible Factor - 1 in Human Skeletal Muscle. *Accepted in FASEB J. Published online April 5, 2005*

* Equally contributing authors

IV. **Gustafsson, T., Ameln, H., Rullman, E., Sundblad, P. and Sundberg C.J., Jansson E.** Exchange of angiogenic growth factors in exercising human skeletal muscle. *In manuscript*

V. **Gustafsson, T., Ameln, H., Norrbom, J., Jansson, E., Sundberg, C.J.** The influence of physical training on VEGF and angiopoietin systems in human skeletal muscle. *In manuscript*

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1. ABBREVIATIONS

Ang-1	Angiopoietin 1
Ang-2	Angiopoietin 2
ANOVA	Analysis of variance
ARNT (HIF-1β)	Aryl hydrocarbon nuclear translocater
ECM	Extracellular matrix
ELISA	Enzyme linked immunosorbent assay
FGF-1 (aFGF)	Fibroblast growth factor-1 (acid fibroblast growth factor)
FGF-2 (bFGF)	Fibroblast growth factor 2 (basic fibroblast growth factor)
HIF-1	Hypoxia inducible factor 1
HR	Heart rate
HRE	Hypoxic response element
HuR	Hu protein R
L-RPE	Local rate of perceived exhaustion
MMP	Matrix metalloproteinase
N	Number of subjects
NO	Nitric oxide
NR-condition	Non-restricted leg blood flow
NRP-1	Neuropilin 1
NRP-2	Neuropilin 2
PKB/Akt	Protein kinase B
PO_2	Oxygen partial pressure
R-condition	Restricted leg blood flow
SAP	Systolic arterial blood pressure
sVEGFR-1	Soluble vascular endothelial growth factor-1
Tie-1	Tyrosine kinase with Ig and EGF homology domains –1
Tie-2	Tyrosine kinase with Ig and EGF homology domains –2
VEGF-A	Vascular endothelial growth factor A
VEGFR-1 (Flt-1)	Vascular endothelial growth factor receptor 1
VEGFR-2 (KDR)	Vascular endothelial growth factor receptor 2
VEGFR-3 (Flt-4)	Vascular endothelial growth factor receptor 3
VO_2max	Maximal oxygen uptake

2. INTRODUCTION

2.1 Exercise, health and muscle capillarization

Physical activity favourably influences multiple cardiovascular risk factors. Increased physical activity improves total serum lipoprotein concentrations, serum low-density lipoprotein concentrations, lipid handling, total body fat content (Dufaux et al., 1982), and insulin sensitivity, glucose disposal, and glucose tolerance (Helmrich et al., 1991). Physical activity improves the control of blood pressure in individuals with borderline or overt hypertension (Paffenbarger et al., 1983, Blair et al., 1984, Hagberg, 1988), and has favourable effects on systemic haemostatic parameters associated with the presence and progression of atherosclerotic disease (Estelles et al., 1989). Increasing evidence shows that several of the positive effects on cardiovascular health of regular exercise are mediated through chronic adaptations in skeletal muscle. Many of the metabolic pathways linked to favourable or unfavourable risk factor levels in humans may be affected on the surface membrane of vascular endothelial cells in skeletal muscle (Bassett, 1994). For example, several independent studies support the theory that an increased endothelial surface area increases glucose uptake, lipoprotein metabolism and insulin sensitivity (Rasio, 1982, Lillioja et al., 1987, Marin et al., 1994). Thus, an increase in vascularity could be an important mechanism underlying the improvements in cardiovascular health.

In addition to improving cardiovascular health, exercise training increases work performance, although it has not yet been established whether these events are related to each other. Evidence supports the concept that the central circulation (i.e., cardiac output) is the major determinant of exercise tolerance during exercise using a large muscle mass (Saltin, 1985, Rowell, 1986). Experimental models using one-legged endurance training illustrate however, the role of peripheral factors in limiting oxygen uptake. Peak oxygen uptake is higher during exercise with the trained leg than in the contra-lateral untrained leg (Saltin et al., 1976). It is likely that the increased exercise capacity and peak oxygen uptake of the trained leg is associated with peripheral adaptations associated with increases in oxygen extraction and utilization, such as increased muscle capillarization or oxidative capacity. This suggests that peripheral factors cannot be discounted as determinants of skeletal muscle oxygen uptake and performance during exercise. Increasing the number of capillaries facilitates oxygen uptake and utilization by providing a larger surface area available for diffusion, decreased diffusion distance, and increased transient time for exchange of oxygen, substrates, and waste products (Saltin et al., 1976, McAllister and Terjung, 1991, Bebout et al., 1993). Although, it has been suggested that improved peripheral oxygen uptake and utilization are more likely to be caused by increased oxidative capacity than by increased capillarization (Robinson et al., 1994, Blomstrand et al.,

1997). However, indirect support for the importance of increased capillarization comes from models of exercise under hypoxic and hyperoxic conditions, which indicate that the tissue O_2 diffusion capacity is an important factor determining peripheral oxygen uptake (Hogan et al., 1989, Wagner, 1992, Richardson et al., 1999, Wagner, 2000).

2.2 Postnatal vessel growth

Postnatal vessel growth occurs naturally in the female reproductive system, in wound healing, and in skeletal muscle in response to exercise. It may occur as arteriogenesis or angiogenesis (described below), in involving new blood vessels growth from existing vessels. Endothelial precursor cells were recently found in adult humans bone marrow and peripheral circulating blood (Isner and Asahara, 1999, Takahashi et al., 1999, Murasawa and Asahara, 2005) and these cells are recruited to angiogenic-activated sites (Asahara et al., 1999). These findings suggest that vessel growth from undifferentiated stem cells (vasculogenesis) may also occur in the adult organism in contrast to previous ideas that this was restricted to embryonic cardiovascular development. At present, some controversy exists, however, about the function and the importance of these cells in postnatal vessel growth (Castro et al., 2002, Wagers et al., 2002).

2.2.1 Arteriogenesis

Arteriogenesis is a functional modification from existing arteries (i.e., the growth of pre-existing vessels into functional collateral arteries). Arteriogenesis involves remodelling of endothelial cells, smooth muscle cells, and fibroblasts and lead to enlargement of the arterial vessels (Carmeliet, 2000, van Royen et al., 2001, Helisch and Schaper, 2003, Heil and Schaper, 2004). Convincing data suggests that biomechanical effects of increased blood flow, shear stress and wall tension, on the vascular wall are of primary regulatory importance. Simplified, the mechanisms for shear stress to induce arteriogenesis is for example through activation of several transcription factors with shear stress elements in their promoter region and to increase in nitric oxide (NO) production (Resnick and Gimbrone, 1995, Nagel et al., 1999, Yang et al., 2002). Moreover, increased mechanical stretch may also directly affects proteases of importance of extracellular remodeling, metalloproteinase (O'Callaghan and Williams, 2000)

Arteriogenesis in response to increased muscle activity has been reported in animal models (Mackie and Terjung, 1983, Laughlin et al., 1996, Lloyd et al., 2001); only a few reports indicate that arteriogenesis may occur in response to exercise in healthy humans. However, two investigations have reported that the dimension of the common femoral artery is greater in high-performance athletes than in untrained control subjects and that this dimension increases with training (Huonker et al., 1996, Miyachi et al., 1998). These reports suggest that the size and blood flow volume of the proximal limb arteries are adjusted to the metabolic needs of the

musculature. However, it has been questioned why this does not seem to occur in patients with peripheral vascular disease (Stewart et al., 2002, Wahlberg, 2003). One possible explanation is that the blood flow is limited in patients, to such a degree that the exercise-induced increase in blood flow is insufficient to induce remodelling of the vessel into greater dimension

2.2.2 Angiogenesis

Angiogenesis is the establishment of new capillaries from an already established capillary network (Risau, 1997, Carmeliet, 2000). The British surgeon John Hunter first used the term 'angiogenesis' in 1787, to describe blood vessels growing in the reindeer antler. and Arthur Tremain Hertig first described angiogenesis in the placenta of pregnant monkeys in 1935. Folkman hypothesized in 1971 that tumor growth is dependent on angiogenesis (Folkman, 1971). Understanding the importance of angiogenesis in tumour progression (Folkman, 1971, Folkman, 1995), led to intensive research on the basic mechanisms underlying angiogenesis and its regulation (Carmeliet, 2003), which was aimed at discovering the underlying mechanisms and using this knowledge to prevent tumour growth through inhibition of angiogenesis (Folkman and Ingber, 1992, O'Reilly et al., 1994, O'Reilly et al., 1997, Kuo et al., 2001, Marx, 2003). The first inhibitor of angiogenesis was discovered in 1975 (Brem and Folkman, 1975).

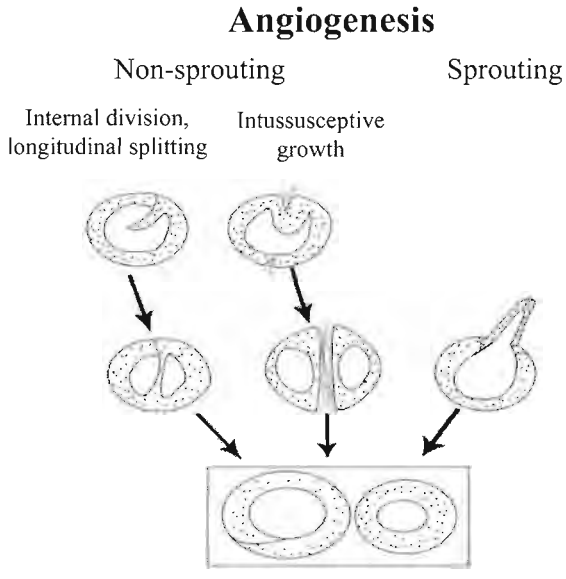


Figure 1. Schematic diagram of non-sprouting and sprouting angiogenesis. Non-sprouting angiogenesis can be dived into subgroups; internal division or longitudinal splitting; cytoplasmic invagination and bridging over the lumen that occurs without infiltration; and intussusceptive that occurs with infiltration of interstitial cells. Sprouting angiogenesis is illustrated with the classic hallmarks of endothelial proliferation and migration.

Later efforts have focused on ways to stimulate angiogenesis (also known as therapeutic angiogenesis) to treat pathological conditions characterized by reduced blood flow to peripheral tissues, such as ischemic heart disease and peripheral arterial vascular insufficiency (Freedman and Isner, 2001, Yla-Herttuala and Alitalo, 2003, Khan et al., 2003). The first stimulator of angiogenesis, basic fibroblast growth factor (bFGF or FGF-2), was purified in 1984 (Shing et al., 1984), and the current best-characterized stimulator of angiogenesis, vascular endothelial growth factor-A (VEGF-A) was described 1989 (Ferrara and Henzel, 1989, Plouet et al., 1989). However, VEGF-A turned out to be identical to a molecule called vascular permeability factor that was already discovered 1983 (Senger et al., 1983).

Based on the work in the past few decades, it is now accpeted that there are at least two different types of angiogenesis; sprouting and non-sprouting angiogenesis or intussusception growth (Risau, 1997). Sprouting angiogenesis is regarded as the major type of growth, and has been studied more than non-sprouting angiogenesis, although non-sprouting angiogenesis may, occur more frequently than previously believed (Djonov et al., 2003, Kurz et al., 2003).

Sprouting angiogenesis is a complex process involving many cell types, signalling pathways, growth factors, and receptors (Risau, 1997, Han and Liu, 1999, Conway et al., 2001, Carmeliet, 2003). Briefly, it is initiated by activation of endothelial cells and vasodilatation of the parent vessel, followed by increased vascular permeability. The basement membrane and extracellular matrix (ECM), which sequesters growth factors, are degraded through activation of various proteases. The degradation of the ECM releases numerous growth factors but maybe more importanly allows the endothelial cells to migrate to the site where new capillaries are needed and, it may be assumed, from where the angiogenic process is initiated.

The best-characterized type of non-sprouting angiogenesis is intussusception (growth within itself), which was first described in the pulmonary capillary network (Caduff et al., 1986, Burri and Tarek, 1990) but is now known to occur in many other tissues. In brief, the process occurs as follows. Initially, the opposing microvascular walls protrude into the capillary lumen creating a contact zone between endothelial cells. The endothelial bilayer is perforated, intercellular contacts are recognized, and a transluminal pillar with an interstitial core is formed. The core is invaded by interstitial cells leading to its rapid enlargement through deposition of collagen fibrils (Djonov et al., 2003). An important advantage of intussusception capillary growth is that it permits rapid expansion of the capillary network and thus enlargement of endothelial surface for metabolic exchange. Moreover, it does not initially need proliferation of endothelial cells but rather rearrangement and plastic remodeling of existing cells. The non-sprouting angiogenesis observed in the skeletal muscle resembles intussusception capillary growth, at least the initial process. Although it has clearly been shown that in skeletal muscle the non-sprouting angiogenesis is not to be followed by infiltration of interstitial cells. Thus,

this type of non-sprouting angiogenesis has alternatively been named luminal division, longitudinal splitting or bridging to distinguish it from intussusception capillary growth (Zhou et al., 1998a, Egginton et al., 2001).

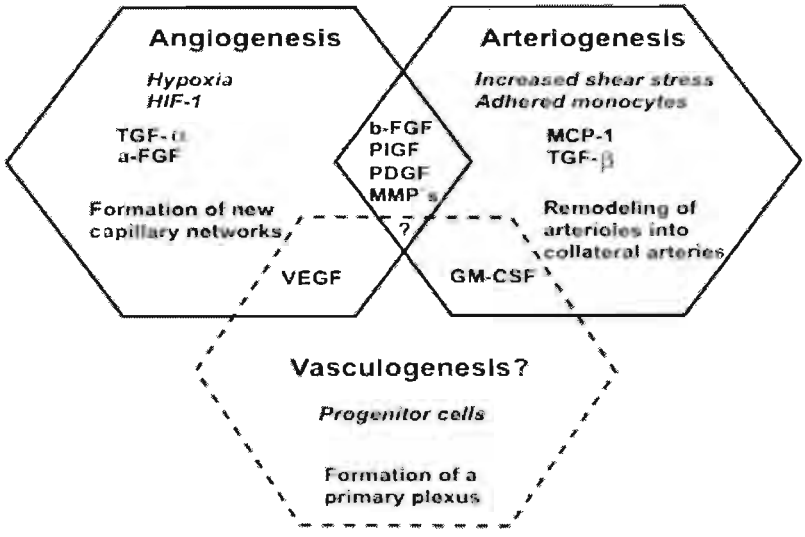


Figure 2. Three forms of postnatal growth; angiogenesis, arteriogenesis and vasculogenesis. It indicates the suggested differences in underlying mechanism of induction as well as the involved mediators and role of growth factors. There is a clear overlap between the forms of postnatal growth.

2.3 Changes in capillarization in response to physical activity

Vanotti and Magiday (1934) first documented the increased capillarization after muscle activation alone (Vanotti and Magiday, 1934). Using direct transcutaneous electrical muscle stimulation five minutes a day for four to six weeks, they observed a doubling in the capillary-to-fibre ratio in stimulated muscle compared with a non-stimulated control muscle. Moreover, as early as 1937, Petrén et al. (Petrén et al., 1937) demonstrated that in muscle from rat and guinea pigs the number of capillaries increases with voluntary endurance exercise training. (1937) More recent studies have confirmed the potential of electrical muscle stimulation and exercise-training to induce increased capillarization in skeletal muscle in different animal species (Carrow et al., 1967, Mai et al., 1970, Cotter et al., 1973, Brown et al., 1976).

In human skeletal muscle, the re-introduced percutaneous biopsy technique (Bergström, 1962) has made it possible to estimate the number of capillaries and their relation to fibre area and fibre type in cross-sections of the muscle. Unfortunately, the methods used to measure the number of capillaries developed for animal studies, such as alkaline phosphates staining, were not suitable for humans because of species differences in enzyme abundance. Not until the 1970s, human studies were published showing an increase in capillarization in response

to increased physical activity (Andersen, 1975, Andersen and Henriksson, 1977, Brodal et al., 1977, Ingjer, 1979). Later studies reported that cessation of exercise training induces a rapid regression in capillarization (Klausen et al., 1981, Salmons and Henriksson, 1981). However, the exact relation between the increases in muscle activity and capillarization is not fully known. For example, it is not known to what extent the increased capillarization in human muscle depends on the exercise training intensity, frequency and duration. Nevertheless, the increased capillary number in skeletal muscle after long-term electrical stimulation or endurance exercise is an established and well-characterized phenomena on of skeletal muscle adaptation in humans and animals (Saltin and Gollnick, 1983, Hudlicka et al., 1992).

In addition, animal preparations using other techniques, such as electron microscopy and measurements of activated endothelial cells labeled with bromodeoxyuridine, have provided more mechanistic information about capillary growth in response to various exercise stimuli (Hansen-Smith et al., 1996, Zhou et al., 1998a, Zhou et al., 1998b, Egginton et al., 2001). These studies have shown that both sprouting and non-sprouting angiogenesis occur in skeletal muscle.

2.4 Exercise induced regulatory stimuli -angiogenesis

The cellular homeostasis in skeletal muscle cells is perturbed during and after physical activity and exercise (Booth and Thomason, 1991, Neufer and Williams, 1996). Many of the changes in the internal milieu have been shown in different models to be possible regulators involved in skeletal muscle adaptation, including changes in intracellular levels of ATP, Ca^{2+}, O_2, glycogen, and cAMP as well as changes in mechanical stretch and fibre recruitment (Neufer and Williams, 1996, Chin et al., 1998, Musi et al., 2001, Pilegaard et al., 2002, Booth, 1998, Cameron-Smith, 2002, Fluck and Hoppeler, 2003). However, it is difficult to distinguish between these possible stimuli because many of these changes occur concurrently or in response to changes in each other. Moreover, changes in nerve activation, autocrine and paracrine substances, hormones, temperature and circulatory factors are likely to be involved in the adaptation to exercise (Neufer and Williams, 1996, Fluck and Hoppeler, 2003). Thus, the exercise adaptation is probably not due to a single mechanism but rather depends on the integration of multiple stimuli at both the systemic and local levels.

Protein expression is a function of the balance between the rates of synthesis and degradation. Increased muscle activity is predominantly associated with increased protein synthesis, whereas decreased muscle activity causes decreased protein synthesis. The major rate-limiting step for the synthesis of many proteins is at the transcriptional level (Neufer and Williams, 1996, Cameron-Smith, 2002, Ookawara et al., 2002, Fluck and Hoppeler, 2003). However, changes in other processes, such as mRNA stability or translational efficiency may

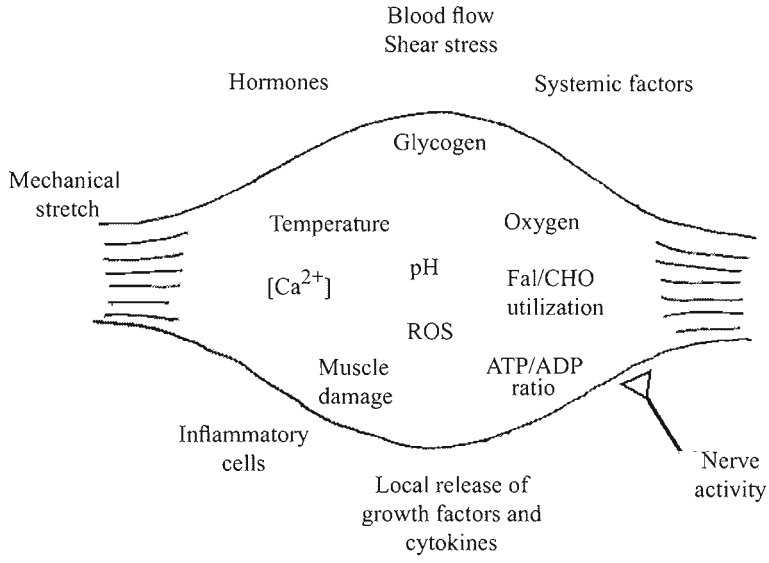


Figure 3. Proposed factors involved in exercise-induced adapation in skeletal muscle. These factors are of extra- as well as intracellular origin. Exercise responses are probably due to a combination of several of these factors. CHO-carbohydrates, ROS-reactive oxygen species.

also influence the expression of a specific protein. Several kinases involved in initiating protein synthesis, including protein kinase B/Akt and p38 increase in response to exercise (Widegren et al., 1998, Nader and Esser, 2001, Sakamoto et al., 2004). In addition, recent work has shown the role of protein degradation through ubiquitination, which is now believed to be more actively regulated and selective for protein degradation than previously thought (Tanaka et al., 2004). For example, chronic motor nerve stimulation of the rabbit tibialis anterior muscle for 28 days markedly increases total proteoasome activity (Ordway et al., 2000). In addition, binding to the ECM or plasma proteins can inactivate many potent growth factors and this binding may be influenced by muscle contraction or by other exercise induced changes in the homeostasis.

Hudlicka, and colleagues have provided important data about exercise-induced increases in blood flow and muscle stretching as possible stimuli for angiogenesis in skeletal muscle (Hudlicka et al., 1992, Harada et al., 1994, Zhou et al., 1998a, Zhou et al., 1998b, Koffi et al., 1999, Saleh and Jurjus, 2001). 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 (Risau, 1997). Moreover, mechanical factors, such as shear stress and tissue stretching, induce endothelial cell proliferation, changes in cell-cell and cell-matrix interactions, and the up-regulation and release of angiogenic factors (Resnick and Gimbrone, 1995, Resnick et al., 2000, Milkiewicz et al., 2001, Rivilis et al., 2002, Brown and Hudlicka, 2003, Milkiewicz et al., 2005).

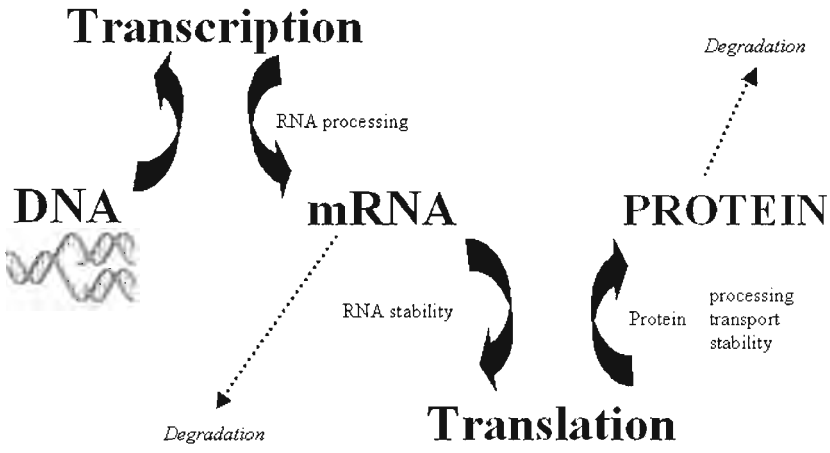


Figure 4. Different steps in the regulation of gene expression. After transcription, RNA is further processed to mRNA, followed by translation, processing, transportation and stabilization.

It has been argued that exercise induced lowered oxygen tension or related metabolic alterations stimulate angiogenesis in skeletal muscle (Adair et al., 1990, Sundberg, 1994). This theory is supported by many studies reporting that reduced oxygen tension in tumour cells is crucial for tumour angiogenesis (Folkman, 1995). In skeletal muscle tissue, augmentation exercise-induced angiogenesis have been observed with experimentally reduced blood flow to the exercising leg in both animals (Yang et al., 1991, Yang et al., 1994, Roberts et al., 1997) and healthy humans (Esbjornsson et al., 1993), observations that supports the role of oxygen tension as regulatory stimulus for angiogenesis.

Thus, exercise-induced angiogenesis probably results from the integrated responses of multiple systems and to different types of angiogenesis. Hypoxia and mechanical stretch appear to lead to sprouting angiogenesis and shear stress seems to induce the non-sprouting type of angiogenesis. Regardless of the differences in the initiating stimuli and the type of angiogenesis, angiogenic growth factors are crucial in both sprouting and non-sprouting angiogenesis and similar factors are also shown to be involved in these two principle different types of angiogenesis (Milkiewicz et al., 2001, Rivilis et al., 2002, Brown and Hudlicka, 2003, Prior et al., 2004)

2.5 Angiogenic switch

It is now believed that the angiogenesis process is regulated and mediated by diffusible angiogenic factors, through the increased synthesis or release from intracellular and/or extracellular storage (Folkman and Klagsbrun, 1987, Folkman, 1995, Risau, 1996, Risau 1997). The development of *in vivo* bioassays, *in vitro* analytic techniques, and murine transgenic

models has made it possible to identify and characterize several angiogenic factors (Jain et al., 1997). From these studies, it has become apparent that such factors mediate angiogenesis, either directly or indirectly through secondary factors. To date, more than 30 factors, that directly or indirectly stimulate angiogenesis have been described (Conway et al., 2001).

In 1996, Hanahan and Folkman introduced the term "angiogenic switch", which reflects the balance between angiogenic stimulators and inhibitors (Hanahan and Folkman, 1996) They proposed that angiogenesis could be induced by an increase in angiogenic stimulators or a decrease in angiogenesis inhibitors (angiostatic factors) or both, which later have been confirmed to be correct (Risau, 1997, Carmeliet, 2000, Conway et al., 2001). Numerous angiostatic factors have been described, most of which are produced through the proteolytic processing of extracellular matrix molecules (Conway et al., 2001, Sottile, 2004).

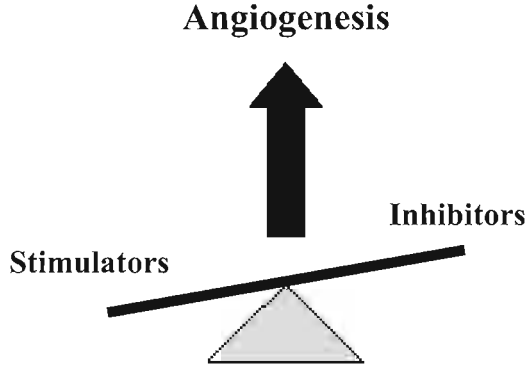


Figure 5. Angiogenic switch. The balance between stimulators and inhibitors will determine whether angiogenesis will occur or not.

2.6 Factors regulating angiogenesis

2.6.1 Vascular endothelial growth factor-A

Numerous studies have shown that vascular endothelial growth factor-A (VEGF-A) is expressed in spatial and temporal association with physiological events during angiogenesis, and that it plays an important role in tumor angiogenesis, development of the cardiovascular system, wound healing as well as in diseases with pathologic angiogenic responses (Folkman, 1995, Folkman and D'Amore, 1996, Risau, 1997, Neufeld et al., 1999, Carmeliet, 2003). The VEGF-A splice variants and their receptors are major regulators of angiogenesis, and have specific but overlapping roles in controlling angiogenesis. Switches in the expression of specific VEGF-A splice variants and VEGF-A receptors have been described during the accelerated angiogenesis accompanying the progression of malignant tumours (Zhang et al., 2000, Yuan

et al., 2001). At least five different VEGF-A splice variants, $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$, have been identified and shown to be generated through alternative splicing; these also have different biological characteristics (Houck et al., 1991, Neufeld et al., 1999, Ferrara et al., 2003). Splice variants containing the domain encoded by exon 6, such as $VEGF_{189}$, are bound tightly to cell-surface heparin-sulfate proteoglycans, whereas splice variants lacking this domain, such as $VEGF_{121}$ and $VEGF_{165}$, are diffusible (Houck et al., 1991, Neufeld et al., 1999, Ferrara et al., 2003). $VEGF\text{-}A_{165}$ is regarded as having the greatest intrinsic capacity for promoting vascular growth (Carmeliet et al., 1999, Stalmans et al., 2002).

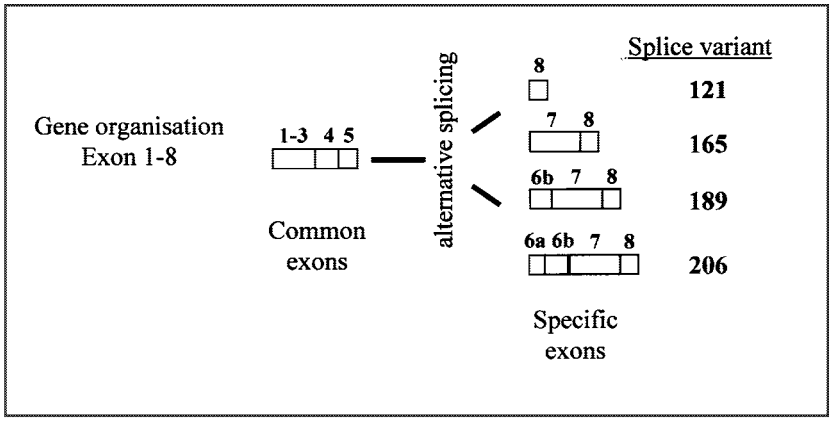


Figure 6. The major splice variants of vascular endothelial growth factors VEGF-A, including exons of each splice variant.

Several factors, including cytokines, growth factors, and tumour suppressor factors, seem to be involved in the regulation of VEGF production (Neufeld et al., 1999, Ferrara et al., 2003). Hypoxia and hypoglycaemia are other well-described stimulators of VEGF gene expression (Shweiki et al., 1992, Minchenko et al., 1994, Neufeld et al., 1999, Ferrara et al., 2003). Hypoxia regulates VEGF-A expression mainly through the activation of the hypoxia inducible factor HIF-1 (Forsythe et al., 1996, Neufeld et al., 1999, Ferrara et al., 2003), which acts through a hypoxic response element (HRE) sequence (Forsythe et al., 1996, Semenza, 2001) in the VEGF-A promoter (Forsythe et al., 1996, Semenza, 2001). HIF-1 is a heterodimeric transcription factor complex consisting of HIF-1α and HIF-1β (also named ARNT) (Wang and Semenza, 1995, Semenza, 1999). The oxygen-regulated component of this complex is the HIF-1α subunit, which is rapidly degraded in normoxia (Huang et al., 1998) initiated by proline hydroxylation, which at the end lead to ubiquitination and finally protein degradation. In contrast, hypoxia suppresses its degradation causing HIF-1α to accumulate rapidly and to translocates into the nucleus (Jiang et al., 1996a, Jiang et al., 1996b, Kallio et al., 1997, Huang et al., 1998, Jewell et al., 2001).

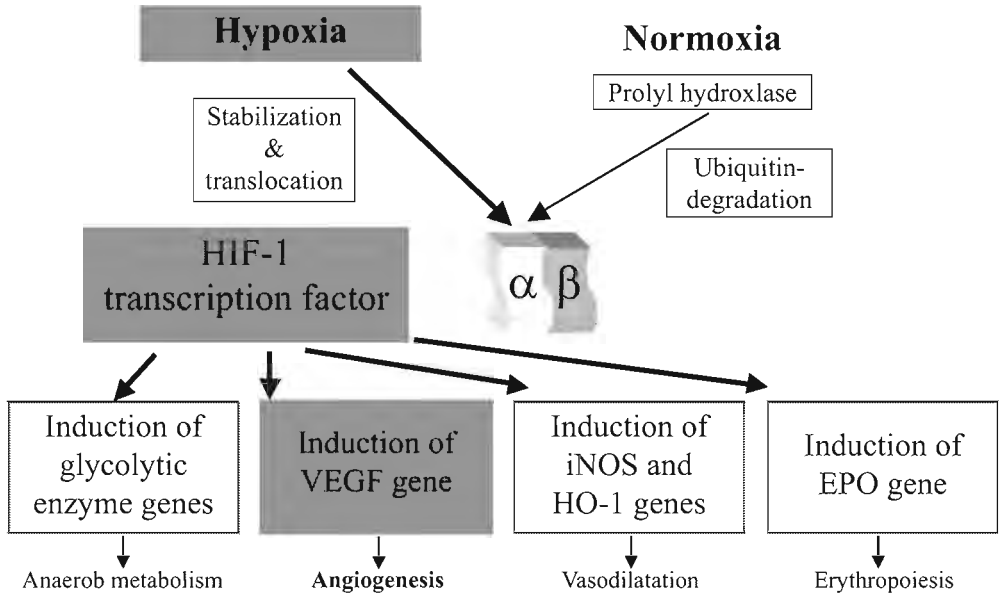


Figure 7. Regulation of the transcription factor, hypoxia inducible factor 1 (HIF-1) and its target genes. Indicated in red is the pathway investigated in the *studies* in this thesis.

Other mechanisms, such as mRNA stabilization, may also be involved in the increased level of VEGF mRNA in response to hypoxia (Stein et al., 1995, Levy et al., 1996, Neufeld et al., 1999, Tang et al., 2002, Ferrara et al., 2003). For example, Hu protein R stabilizes VEGF-A mRNA in response to hypoxia by interacting with the VEGF-A-3′ untranslated region (Tang et al., 2002). Other factors involved in blood flow regulation, such as nitric oxide (NO) and adenosine can also induce VEGF expression (Hashimoto et al., 1994, Burns and Wilson, 2003, Jensen et al., 2004c). However, the effect of NO on VEGF expression is controversial (Tsurumi et al., 1997, Jensen et al., 2004c). Recent studies using animal experimental models show that inhibition of NO production attenuate the increase in VEGF-A expression (Gavin et al., 2000, Baum et al., 2004, Milkiewicz et al., 2005). In contrast other studies has observed that shear stress not induce VEGF-A in endothelial cells during controlled experimental settings (Gan et al., 2000) and that NO does not seem to have a major impact on VEGF-A expression in skeletal muscle cells (Jensen et al., 2004c). VEGF is a potent stimulator of vasodilatation and vessel permeability, which seems to be mediated through NO and prostacyclins (Hood et al., 1998, Murohara et al., 1998). NO has also been suggested to act as a downstream mediator in VEGF-stimulated angiogenesis (Ziche et al., 1997, Shizukuda et al., 1999).

Other VEGF family growth factors have been identified, such as VEGF-B, VEGF-C, and VEGF-D. Although not as well characterized as VEGF-A, these factors seem to have some

angiogenic potential; in contrast to VEGF-A, none of these factors is regulated by hypoxia (Li and Eriksson, 2001).

2.6.2 Vascular endothelial growth factor-A receptors

Three different vascular endothelial growth factor-A receptors, all with tyrosine kinase activity, have been described: VEGFR-1 (also known as Flt-1), VEGFR-2 (KDR), and VEGFR-3 (Flt-4). VEGFR-1 and VEGFR-2 are high-affinity receptors for VEGF-A (Mustonen and Alitalo, 1995, Hanahan, 1997, Shibuya et al., 1999, Ferrara et al., 2003, Neufeld et al., 1999). VEGF-A activity is mainly localized to the endothelial cells, because that is the predominately location of these receptors (Neufeld et al., 1999, Shibuya et al., 1999, Ferrara et al., 2003). Although, activation of VEGFR-1 and VEGFR-2 induces different biological effects (Shibuya et al., 1999, Waltenberger et al., 1994), it is now agreed that VEGFR-2 to be the major mediator of the angiogenic effects of VEGF-A. VEGFR-2 undergoes dimerization and ligand-dependent tyrosine phosphorylation, resulting in a mitogenic, chemotactic and survival signal for the endothelial cells (Neufeld et al., 1999, Shibuya et al., 1999, Ferrara et al., 2003). Despite the differences in activation and biological effects, both the receptors are important in the regulation of angiogenesis. For example, homozygous knockout of the VEGFR-1 or VEGFR-2 genes leads to underdeveloped cardiovascular system and embryonic death in mice (Fong et al., 1995, Shalaby et al., 1995). However, the VEGFR-1 knockout model exhibits an excessive proliferation of angioblasts, which contrasts with the lack of vasculogenesis observed in the VEGFR-2 knock-out model. It has been suggested that the function of VEGFR-1 in the regulation of angiogenesis is that it act mainly as a decoy receptor for VEGF-A (Park et al., 1994, Ferrara et al., 2003). A soluble VEGFR-1 has also been identified and characterized (Kendall et al., 1996). Because it has no stimulatory effects on vessel growth, it is also believed to act as a decoy receptor for VEGF-A (Clark et al., 1998). Consistent with the hypothesized role as a decoy receptors the observation that the tyrosine kinase activity can be deleted from the receptor without any adverse effects on the vascular development (Hiratsuka et al., 1998). Although, for monocyte the VEGFR-1 tyrosine kinase activity seem to be necessary for VEGF-A induced migration (Hiratsuka et al., 1998). In addition, both VEGFR-1 and VEGFR-2 are present at the haematopoietic stem cells and are involved in the recruitment of these cells from the bone marrow (Rabbany et al., 2003). This is an additional mechanism by which the VEGF-A system may stimulate vessel growth besides the better-characterized localized angiogenic effects.

New receptors, neuropilin (NRP-1 and NRP-2) have been identified recently (Soker et al., 1998). NRP-1 facilitates the binding of $VEGF_{165}$ to VEGFR-2, but does not act on other diffusible VEGF isoforms, such as $VEGF_{121}$ (Soker et al., 1998, Neufeld et al., 1999, Ferrara et al., 2003). It has been suggested that binding to NRP-1 may partly explain the greater mitogenic

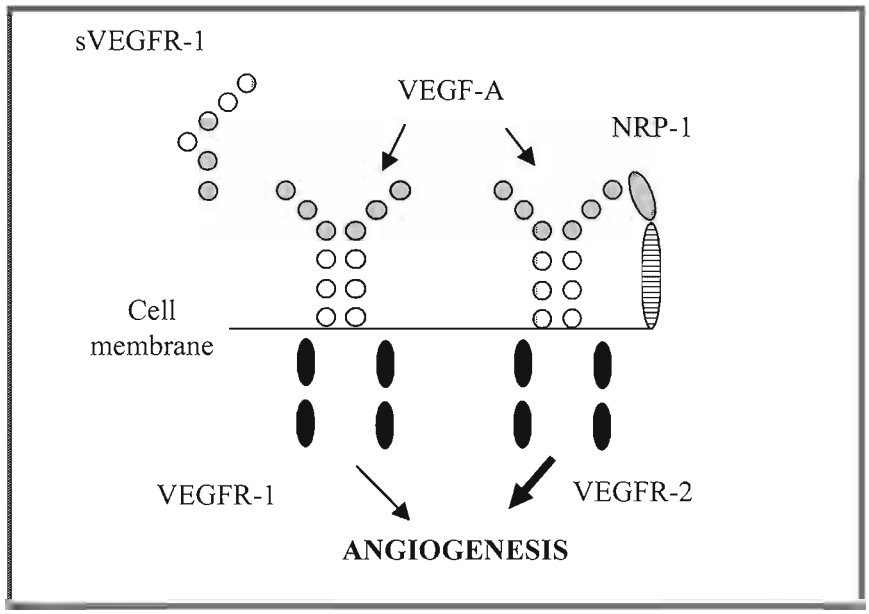


Figure 8. Vascular endothelial growth factor receptors (VEGFR). VEGF-A interacts with VEGR-1 and VEGFR-2. In addition, the splice variant $VEGF_{165}$ interacts also with NRP-1 receptor and faciliates the binding of $VEGF_{165}$ to VEGFR-2. A soluble VEGFR-1 receptor exists, possibly acting as a decoy receptor for VEGF-A.

potency of $VEGF_{165}$ relative to the $VEGF_{121}$ splice variant (Ferrara et al., 2003)

The VEGFR-1 promoter includes a binding site for HIF-1, and is regulated by hypoxia in a similar manner to VEGF-A (Detmar et al., 1997, Gerber et al., 1997, Kremer et al., 1997). Hypoxia also induces the expression of VEGFR-2 in endothelial cells from the postnatal brain, (Detmar et al., 1997) although this occurs as an indirect effect through stimulation of increased levels of VEGF-A, rather than through effect of hypoxia *per se* (Kremer et al., 1997)

2.6.3 Angiopoietins

VEGF-A also associates with a relative newly identified group of factors, the angiopoietins (Ang-1 and Ang-2), during development and angiogenesis in adult tissue (Maisonpierre et al., 1997, Holash et al., 1999). Ang-1 and Ang-2 are vascular endothelial cell-specific factors, that share and compete for a common receptor, the tyrosine kinase receptor Tie-2. Similar to VEGF-A, the angiopoietins are specific for endothelial cells because of the restricted location of the Tie-2 receptor (Dumont et al., 1994, Sato et al., 1995). Ang-1 acts as an agonist on Tie-2, and seems to maintain and stabilize mature vessels by promoting the interaction between endothelial and their surrounding cells. Ang-2 is thought to block the Tie-2 receptor; in the absence of VEGF-A this leads to vessel degradation. Conditions that causes increased expression of Ang-2 and high

availability of VEGF-A, induces a favourable effects on the angiogenic process through Ang-2 induced endothelial destabilization, which facilitates the effects of VEGF-A on endothelial activation (Lobov et al., 2002).

The functions of the other Tie family member, Tie-1, are not completely known. Neither Ang-1 nor Ang-2 binds to Tie-1, but Tie-1 physically interacts with Tie-2 in endothelial cells suggesting that this receptor participates in some aspect of Tie-2 signalling (Marron et al., 2000). Together with Ang-2, Tie-1 has been observed to be intensely expressed during conditions with enhanced angiogenesis (Brown et al., 2000). However, it is unknown whether an increase in Tie-1 induces angiogenic activity or if it represents a negative feed-back, and thus may represent a maturation signal (Chen-Konak et al., 2003).

2.6.4 Fibroblast growth factors

The angiogenic properties of growth factors of the fibroblast growth factor (FGF) family have been studied extensively. The best-characterized members are FGF- 1 (also known as acidic FGF or aFGF) and FGF-2 (basic FGF or bFGF) (Bikfalvi et al., 1997, Gospodarowicz et al., 1987). FGF-1 and FGF-2 stimulate angiogenesis and their biological effects are mediated through at least four different receptors, all with tyrosine kinase activity (Fernig and Gallagher, 1994, Friesel and Maciag, 1995). Their biological effects are less spatially restricted than those of VEGF-A (Gospodarowicz, 1987, Fernig and Gallagher, 1994, Bikfalvi et al., 1997).

Both FGF-1 and FGF-2 lack conventional leader sequences for cellular secretion, and it has been questioned whether secretion via the endoplasmic reticulum occurs *in vivo*. Other mechanisms have been proposed to explain FGF release and its activation of the angiogenic response, such as cell damage, tissue stretching, and the subsequent release from extra-cellular binding to heparin (McNeil et al., 1989). It is unclear whether hypoxia induces FGF activation. No hypoxic response element has been found in the promoter region of FGF-1 or FGF-2, and hypoxic exposure in vitro does not induce FGF-2 expression (Brogi et al., 1994).

2.6.5 Extracellular matrix

The extracellular matrix (ECM) is involved in the regulation of various aspect of the vascular growth in different ways. Remodelling of the ECM can have either have pro- or anti-angiogenic effects. Degradation of the ECM by proteases promotes cell migration, a critical event in the formation of sprouting angiogenesis. This process also promotes angiogenesis by the release of stimulatory angiogenic matrix-bounds factors, such as FGF-2 (Pepper, 2001). Several families of enzymes can activate proteolysis of the basement membrane, such as matrix metalloproteinases (MMPs), plasminogen, and urokinase and tissue plasminogen activators (Pepper, 2001). MMPs, a major enzyme family, that mediates degradation of various

components of the ECM (Birkedal-Hansen, 1995, Massova, 1998) and have been suggested as key factors in the regulation of ECM remodelling.

The action of proteases can also be anti-angiogenic, because of their ability to generate fragments of ECM molecules that have anti-angiogenic properties not possessed by the intact molecules. A number of ECM angiostatic fragments have been recognized, although it is not known whether all of these fragments are produced in vivo (Sottile, 2004). Endostatin is a 20 kD C-terminal fragment of collagen XVIII (O'Reilly et al., 1997, Zatterstrom et al., 2000) which is the best characterised ECM-derived anti-angiogenic factor. Many proteasess, seem to be able to cleave and release the endostatin, including cathepsin L, elastase and MMP (Felbor et al., 2000, Zatterstrom et al., 2000). First isolated from a murine haemangioendothelioma, endostatin was observed to almost completely suppresses tumour-induced angiogenesis through inhibition of endothelial cell migration, although it has no effect on proliferation (O'Reilly et al., 1997).

2.7 Summary

It can be argued the surface membrane of vascular endothelial cells in skeletal muscle affects many of metabolic pathways and that changes in the surface membrane may be linked to changes in the risk factor levels in humans. Peripheral factors are involved as determinants of skeletal muscle oxygen uptake and performance during exercise. An increased number of capillaries should facilitate oxygen uptake and exercise performance by providing a larger surface area available for diffusion, decreased diffusion distance, and increased transient time for substrate exchange. Both long-term electrical stimulation and endurance exercise lead to increase in the number of capillaries in skeletal muscle and at least two different types of angiogenesis occur in skeletal muscle: true sprouting of capillaries from pre-existing vessels and non-sprouting angiogenesis. Hypoxia and mechanical stretching appear to lead to sprouting angiogenesis and shear stress seems to induce non-sprouting type angiogenesis. Regardless of the differences in the initiating stimuli, similar angiogenic growth factors are crucial to both types of angiogenesis. VEGF-A is the best-characterized stimulatory angiogenic growth factor and plays an important role in angiogenesis in both normal and pathological conditions. VEGF-A effects was recently shown to related to expression of the angiopoietins, during development and angiogenesis in adult tissue. Conditions that increase the expression of Ang-2 and the availability of VEGF-A have a favourable effects on the angiogenic process through Ang-2 induced endothelial destabilization which facilitates the effects of VEGF-A on endothelial activation.

3. AIMS

The general aim of this thesis was to study the influence of exercise on angiogenic growth factors and their regulating pathways in human skeletal muscle. The levels and regulation of VEGF-A, its splice variants and receptors as well as Ang-1 and Ang-2 and their receptors were studied in response to exercise under normal and reduced oxygen delivery to the leg.

The specific aims were to study

1) the acute effects (up to 6 h post exercise) of a single bout of endurance exercise on the expression of angiogenic growth factors in skeletal muscle,

2) the acute effects of a single bout of endurance exercise on the exchange of angiogenic growth factors over the working leg,

3) the accumulated effects of repetitive bouts of endurance exercise (5 weeks of training) on the basal expression (24 h post exercise) of angiogenic growth factors in skeletal muscle and

4) the effects of restricted leg blood flow, and the thereby reduced oxygen delivery to the working leg on the expression of angiogenic growth factors in skeletal muscle.

The main hypotheses were that endurance exercise increases the expression of angiogenesis growth factors and their receptors and that this response is enhanced by reduced oxygen delivery to the exercising muscles in a fashion that facilitates the angiogenic process.

4. METHODS

4.1 Subjects

Forty-six healthy men, aged 18 to 37 years, 170 to 194 cm tall and weighing 58 to 94 kg were examined in *studies I-V*. The subjects were given oral and written information about the purposes of the work and about the possible risks before giving their consent to participate. The Ethics Committee at Karolinska Institutet approved the experimental procedures and protocols before the start of each study.

Table 1. Background characteristics of the subjects.

Study	Subjects (N)	Age (year)	Heigh (cm)	Weight (kg)
I	16	24 (20-32)	184 (175-194)	79 (70-94)
II-III	9 10	22 (18-26) 24 (20-29)	180 (170-188) 180 (174-190)	74 (65-84) 74 (65-92)
IV	10	25 (18-37)	180 (170-190)	77 (58-82)
V	11	24 (20-27)	181 (173-190)	75 (63-90)

4.2 Exercise models

4.2.1 One legged cycling

In *studies I-III* and *V*, subjects exercised in the supine position using a dynamic constant-load knee-extension exercise (45 min, 60 rpm) on an electrodynamically loaded cycle ergometer in a manner similar to that described by Andersen and Saltin (1985). Each voluntary contraction extended the leg from 70 to 150 degrees knee angle. Flexion was performed passively using the ergometer flywheel momentum to reposition the leg for the next extension. At least three weeks before the first experiment in *studies I-III* and *V*, each subject was familiarized twice with the experimental model and procedures. During familiarization, the subjects maximal one-legged performance capacity was determined with non-restricted leg blood flow. The starting workload was 5 W, and workload increased by 5 W each minute until the subjects indicating volitional fatigue.

4.2.2 Two-legged cycling

In *study IV*, subjects exercised in an upright position on an electrodynamically loaded cycle ergometer (65 min, 60 rpm). Two-legged cycling was also used to determine VO_2max in *studies IV* and *V*.

4.2.3 Restricted and non-restricted leg blood flow

In *studies I-III* and *V*, a method first described by Eiken and Bjurstedt (1987) was used to induce of restricted blood flow during exercise. Controlled local application of external pressure over the working leg was used to reduce blood flow. The subject was positioned supine in the opening of a large pressure chamber with both legs inside the chamber and with a pad strapped to the calf of one leg. The pad was connected, via a metallic bar to the crank-arm of an electrically braked cycle ergometer with locked flywheel, with the centre of rotation at the level of the heart. The chamber opening was sealed at the level of the groin by a rubber diaphragm with holes and self-sealing sleeves for the legs. Shoulder supports were used to prevent craniad displacement of the body as the chamber pressure was increased. For exercise with restricted blood flow, the chamber pressure acting on the exercising leg was elevated to 50 mm Hg above atmospheric pressure a pressure known to reduce leg blood flow during one-legged cycle exercise by 15%-20% (Sundberg and Kaijser, 1992). Exercise under the non-restricted blood flow condition was performed using the same experimental arrangements but at normal atmospheric pressure.

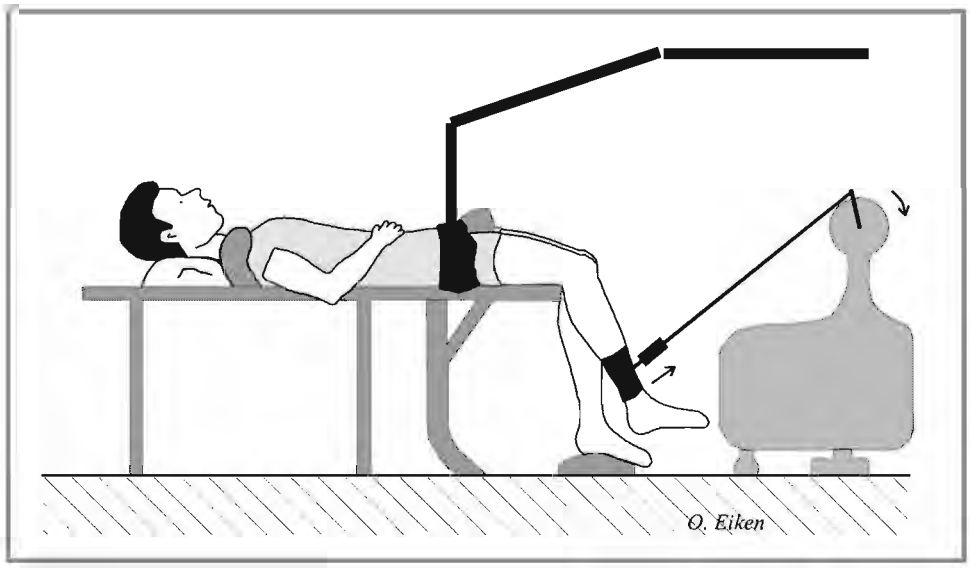


Figure 9. Restricted leg blood flow. Exercise and training set-up enabling the subject to perform one-legged knee exetension with the legs exposed to supraatmospheric pressure giving rise to restricted leg blood flow.

4.3 Experimental procedures

4.3.1 Exercise protocol study I

A total of 16 subjects participated in *study I.* Eight of the subjects exercised with the leg blood flow restriction and eight subjects without leg blood flow restriction. During the experiment, Teflon catheters were inserted one hour before exercise into the brachial artery and the femoral vein. The relative workload in the experiments was 24 ± 3 % of the one-legged peak load. The absolute workloads were 9 ± 1 and 10 ± 1 W during exercise in the restricted and non-restricted blood flow conditions, respectively. To evaluate the exercise-induced differences between the two conditions heart rate (HR) was continuously recorded, and systolic arterial blood pressure (SAP) determined in the supine position. The local rate of perceived exertions (L-RPE) was estimated by Borg´s 6-20 RPE scale (Borg, 1982). To measure arterio-venous difference of venous lactate concentration blood sample were drawn simultaneously from the brachial artery and the femoral vein at rest, at 15, 30 and 45 min of exercise and 20 min after exercise. Muscle biopsies were performed from the vastus lateralis muscle at rest and 30 min after exercise. In an additional study six of the subjects performed two 5-min exercise bouts at the same workload as in the main study and oxygen saturation, oxygen tension and pH of the effluent femoral venous blood were measured during exercise with restricted and non-restricted leg blood flow.

4.3.2 Exercise protocol studies II-III

Nine subjects performed 45 min of one-legged exercise. Five subjects were randomly selected to perform the exercise bout with restricted or non-restricted leg blood flow first. The two exercise bouts test were separated by 10 days. The relative workload used in the experiments was 10 ± 1 W or 26 ± 4 % of the one-legged peak load in the non-restricted blood flow condition. To evaluate exercise-induced differences between the two conditions HR was continuously recorded, SAP was determined in the supine position and L-RPE was estimated by Borg´s 6-20 RPE scale, as described above for *study I.* Anticubital venous blood samples were obtained at rest and after 15, 30, and 45 min of exercise to determine lactate concentration. Muscle biopsies were performed from the vastus lateralis muscle at rest and at 0, 30, 120 and 360 min after exercise in the restricted and non-restricted blood flow condition. To control for the biopsy procedure on gene expression, muscle biopsies were obtained at each of the time points described above from additional 10 healthy male control subjects who did not exercise.

4.3.3 Exercise protocol study IV

Ten subjects performed two-legged upright cycling. The average VO_2max in the participating subjects was: 51 (43-64) ml kg/min. The subjects cycled for the first 20 min at workload equivalent to 50% of VO_2max (125 ±20 W), after which they cycled a further 40

min at a higher workload equivalent to 65% of VO_2max (165 ± 30 W). Finally, the subjects were instructed to cycle at the highest tolerable workload (175 ± 50 W) that could be sustained for 5 min. Teflon catheter were inserted in the femoral artery and into the ipsilateral femoral vein, at the level of the inguinal ligament. Blood samples were drawn at rest, at 20, 40, 60 min of exercise, and 120 min after exercise and used to calculate the arterio-venous differences. Muscle biopsies were performed from the vastus lateralis muscle of one leg at rest, at 65 min of exercise and 120 min after exercise.

4.3.4 Exercise protocol study V

Eleven subjects performed one-legged exercise four times per week, over a five-week period. The average VO_2max in the participating subjects was 51 (40–66) ml kg/min. The subject first performed one-legged exercised with restricted leg blood flow for 45 min, after which the subject performed similar amount of exercise with non-restricted blood flow using the other leg. The subjects were instructed to exercise at the highest tolerable workload for 45 min, taking into account that they must complete the full 45-min session. Compared with the workload in the first week, the average workload increased by 30 % ± 22 in week 2, 52 % ± 39 in week 3 and 84 % ± 7 % in week 4. Muscle biopsies were performed from the vastus lateralis muscle of both legs before and 120 min after the first exercise bout. Post-training samples were performed after five weeks at rest and 120 min after the last exercise bout; the resting was performed 24 h after the previous exercise session. A further resting biopsy (24 h after the latest exercise bout) was performed from each leg 10 days after the start of training period.

4.3.5 Muscle biopsy

In *all studies*, biopsies were performed from the vastus lateralis muscle, using the percutaneous needle biopsy technique (Bergström, 1962). All muscle biopsy samples were frozen in liquid nitrogen within 10-15 s and stored at –80°C until further analysis. The variables of interest and their sample times are shown in Table 2.

Table 2. Measured variables in skeletal muscle and plasma in *studies I-V*.

		Skeletal muscle		Plasma
Study	Biopsy time points	mRNA	Protein	Protein
I	Pre-ex Post-ex (0, 30 min)	VEGF-A, FGF-2		
II	Pre-ex Post-ex (120, 360 min)	VEGF-A,VEGFR-1, VEGFR-2, VEGF- isoforms		
III	Pre-ex Post-ex (0, 30, 120, 360 min)	VEGF-A, HIF-1α	HIF-1α	
IV	Pre-ex Post-ex (0, 120 min)	VEGF-A	VEGF-A Endostatin	VEGF-A Endostatin
V	Pre-ex (before training, after 10 days and 5weeks) Post-ex (0, 120 min)	VEGF-A, VEGFR-1, VEGFR-2 Ang-1, Ang-2, Tie-1, Tie-2	VEGF-A Ang-2	

4.4 Laboratory methods

4.4.1 Oxygen measurements

In *study I*, oxygen tension and oxygen saturation was measured with an automatic spectrophotometric technique (OSM-3 Hemoximeter, Radiometer, Copenhagen).

4.4.2 Plasma lactate

In *studies I, II,* and *III*, plasma lactate concentration was determined using the principles of Lowry and Passoneau (Lowry and Passonneu, 1972).

4.4.3 RNA extraction

In *studies I-V* total RNA was prepared by the acid phenol method (Chomczynski and Sacchi, 1987) and quantified spectrophotometrically by absorbance at 260 nm. The integrity of the total RNA was determined by 1% agarose gel electrophoresis. Two micrograms of RNA was reverse transcribed by Superscript reverse transcriptase (Life Technologies, Stockholm, Sweden) using random hexamer primers (Roche Diagnostics GmbH, Mannheim, Germany) in a total volume of 20 µl.

4.4.4 mRNA quantification

4.4.4.1 Study I

Quantification of specific RNAs was performed using a statistical PCR approach (Puntschart et al., 1998). The designed primers are shown in Table 3. The specificities of the primers were tested by diagnostic restriction cuts of the PCR products. The amounts of PCR products were quantified by hybridization of specific biotinylated probes and detection by ELISA.

Table 3. The primers and probe sequences used in A) study I and B) study II-V. In B, all oligonucleotide sequences are written in the 5' to 3' direction.

Gene	Forward primer	Reverse primer	Biotin probe
VEGF-A	bases 140–166	bases 296–270	bases 215–234
FGF-2	bases 1358– 1385	bases 1593–1566	bases 1390–1409
28S	bases 4535–4564	bases 4667–4638	bases 4283–5006

Gene	Forward primer	Reverse primer	Probe
VEGF-A	ACTGCCATCCAATCGAGACC	GATGGCTTGAAGATGTACTCGATCT	TGGTGGACATCTTCCAGGAGTACCCTGA
VEGF-A_{121}	AGCTTCCTACAGCACAACAAATG	CTCCTGCCCGGCTCAC	CATTTTTCTTGTCTTGCTCTATCTTTCTTTGGTCTG
VEGF-A_{165}	TGTGAATGCAGACCAAAGAAAGA	TGCTTTCTCCGCTCTGAGC	AGGCCCACAGGGAACGCTCCAG
VEGF-A_{189}	CGCAAGAAATCCCGGTATAAGT	TGCTTTCTCCGCTCTGAGC	AGGCCCACAGGGAACGCTCCAG
VEGFR-1	GCATATGGTATCCCTCAACCTACAA	CATCCAGGATAAAGGACTCTTCATTAT	TAACCATAATCATTCCGAAGCAAGGTGTGACT
VEGFR-2	TCTTCTGGCTACTTCTTGTCATCATC	TGGACAAGTAGCCTGTCTTCAGTT	ACGGACCGTTAAGCGGGCCAAT
Ang-2	TGAACCAAACAGCTGAGCAAAC	TGTCGAGAGGGAGTGTTCCAA	TGGAAGCCCAAGTATTAAATCAGACCACGAGA
Tie-2	GCCGCTACCTACTAATGAAGAAATGA	AATGATCCGTATGGTTAAAGTCTTTTG	AGCCCCATGGGACAGTGCTCCAT

4.4.4.2 Study II-V

In *studies II-V,* mRNA was detected on an ABI-PRISM 7700 Sequence Detector (Perkin-Elmer Applied Biosystems Inc, Foster City, CA, USA). Oligonucleotide primers and TaqMan probes were designed by using Primer Express version 1.5 (Perkin-Elmer Applied Biosystems Inc.) or ordered as gene assay on demand (Perkin-Elmer Applied Biosystems Inc.). The primers and probes are shown in Table 3. Tie-1 and Ang-1 were ordered as gene assay by demand (Hs00178500_m1, Hs00919197_m1 Perkin-Elmer Applied Biosystems Inc).

The probes were designed to cover exon–exon boundaries to avoid amplification of genomic DNA. As an endogenous control to correct for potential variations in RNA loading B-actin (B-actin Part No. 310881E (TAMRA), 4326315E (DQ) was selected in *study II-IV* and 18S rRNA was selected in *study V* (18S 4310893E, Perkin-Elmer Applied Biosystems Inc.). All reactions were performed in 96-well MicroAmp Optical plates, using the ABI-PRISM 7700 Sequence Detector (Perkin-Elmer Applied Biosystems Inc.). For every gene, all samples were amplified simultaneously in duplicate in one assay run. A dilution series was performed and used as a standard curves for all mRNA samples. The threshold cycle was determined for both the experimental gene and the endogenous control gene, and was used to calculate the

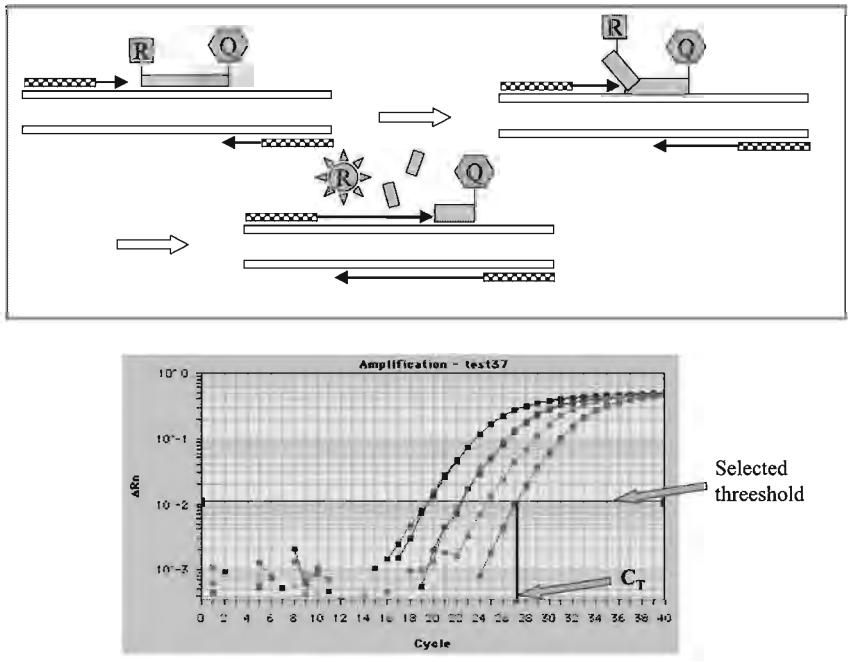


Figure 10. Real time PCR Taqman®. The amount of PCR product is quantified in real time by using special probes, "reporter dye" that fluorescense when the "quencher" is removed from the mRNA fragment during the PCR extension cycle. The number of PCR cycles (termed C_T value) needed to reach a given fluorescence signal (treshold) is related to the mRNA in the sample.

relative expression based on the respective standard curve equation. The expression level was normalized to the endogenous control gene.

4.4.5 Muscle protein quantification

4.4.5.1 HIF-1α

In *study III*, muscle samples were homogenized in accordance to Bergeron et al. 1999. The protein content was determined by Bradford assay using BSA as a standard. Muscle homogenate was mixed with 2 X loading buffer and separated on a 6% SDS-polyacrylamid gel (50 µg of protein per lane). Prestained molecular weight markers from BioRad (Biorad, USA) were used. Immunoblotting was performed using a murine anti-human HIF-1α antibody (Novus Biological, USA) and HRP-linked horse anti-mouse antibody (New England Biolabs, USA). Immunocomplex on the membrane were detected by an enhanced chemiluminescense system using SuperSignal® West Femto Maximum Sensitivity Substrate (Pierce, USA) according to the manufacturer's protocol.

4.4.5.2 VEGF and Endostatin

In *study IV* and *V* part of the skeletal muscle biopsy (40 µl buffer/mg wet muscle) was homogenized in ice-cold buffer containing 0.1 M potassium phosphate (pH 7.7), 0.05% BSA, 20 µg/ml leupeptin, 50 µg/ml aprotinin and 40 µg/ml PMSF. The homogenate was rotated for 60 min at 4 °C and centrifuged at 15.000 g for 15 min at 6 °C. Two hundred microlitres of the 1:5 diluted supernatant was used to quantify VEGF protein using sandwich ELISA (Quantikine R&D Systems, USA). All techniques and materials in this analysis were used according to manufacturers' protocols. Optical density was quantified on a micro plate-reader *Quant (Bio-Tek® Instruments Inc., USA). All samples were assayed in duplicate.

4.4.6 Electrophoretic mobility shift assay

In *study III* muscle homogenate were dialysed against 1000 × excess volume of a buffer containing 20 mM Hepes, 0.05 mM EDTA, 0.3 mM MgCl2, 2 mM DTT, and 1 mM PMSF for 1 hr at 4 °C. Oligonucleotide probe containing the HRE was end-labeled with 32P dCTP using the Klenow fragment of DNA polymerase I. Eighty micrograms of dialysed muscle homogenate was incubated with the 32P labeled Epo HRE probe for 20 min and separated on 4% polyacrylamid gel in 0.5 × Tris-borate-EDTA buffer at 200 V, 4 °C. The image was visualized by autoradiography.

4.4.7 Immunohistochemistry

4.4.7.1 HIF-1α

In *study III* cross sections (8 µm) of biopsy samples were cut at – 20 °C, placed on glass slides and immediately fixed in ice-cold acetone for 10 min, followed by incubation with 1% hydrogen peroxide (H_2O_2) in PBS to inactivate the endogenous peroxidase activity. The slides

were incubated with PBS containing 3% BSA for 1 h at room temperature, and a murine anti-human HIF-1α antibody (Novus Biologicals, USA) diluted 1:100 in PBS with 1% BSA was applied to the sections and incubated overnight at 4 °C. The sections were then incubated with biotinylated anti-mouse secondary antibody (DAKO A/S, Denmark) diluted 1:100 in PBS with 1% BSA for 30 min at room temperature, followed by incubation with HRP-conjugated avidin-biotin (DAKO A/S) at room temperature for 30 min. The antigen-antibody-enzyme complex was visualized using diaminobenzidine (DAB) as the chromogenic substrate for peroxidase. Nuclei were counterstained with haematoxylin.

4.4.7.2 VEGF-A

Cryocross-sections (8 μm) of the muscle biopsies were fixed in 2% paraformaldehyde and endogenous peroxidase activity was quenched with 1% H_2O_2. To block non-specific binding the sections were treated with normal donkey serum, (Jackson ImmunoResearch Laboratories Inc, MD, USA), diluted 1:20 in PBS containing 1% BSA for 20 min. The expression of VEGF–A was detected with a polyclonal goat antibody (A-20, Santa Cruz Biotechnology, Inc, USA) diluted 1:100 in PBS containing 1% BSA, followed by a biotinylated donkey anti-goat antibody (Jackson ImmunoResearch Laboratories Inc) diluted 1:200 in PBS containing 1% BSA for 40 min. The sections were incubated with extrAvidin® peroxidase (Sigma-Aldrich Inc, USA) for 45 min. The antigen-antibody-enzyme complex was visualized using DAB as the chromogenic substrate for peroxidase.

4.4.7.3 VEGFR-2

Paraffin sections of 3-4 μm thick, from the muscle biopsies were deparaffinised, rehydrated and processed in citrate buffer for antigen retrieval by microwave treatment. The endogenous peroxidase activity was quenched with H_2O_2. The VEGFR-2 (R&D Systems) was detected with a polyclonal goat antibody diluted 1:20 in PBS-Triton. The sections were incubated over night at 4 °C rinsed in PBS, and incubated in biotinylated donkey anti-goat antibody, diluted 1:2000 in PBS –Triton for 40 min. The sections were then incubated in EAP diluted 1:200 for 45 min (Sigma-Aldrich, Inc.). The antigen-antibody-enzyme complex was visualized using DAB as the chromogenic substrate for peroxidase.

4.4.8 Plasma proteins

In *study IV* VEGF and endostatin plasma concentrations were quantified by sandwich ELISA (VEGF-A Quantikine R&D Systems, USA, Endosatin Cytimmune Science Inc, USA) using similar procedures as those described for tissue VEGF-A. All techniques and materials used in this analysis were used in according to the manufacter's protocol. Plasma albumin was analysed at the Karolinska University Laboratory using the Modular P2 technique.

4.5 Statistics

In *studies I-III* a two-way ANOVA for repeated measures was used to evaluate the effect on hemodynamic parameters, venous lactate, L-RPE, and mRNA levels (time and exercise condition)

In *study II* exercise-induced changes in protein levels of HIF-1α were analysed with the Wilcoxon matched-pairs test.

In *study IV* a one-way ANOVA for repeated measures was used to evaluate the effect of time on mRNA and protein expression and a two-way ANOVA for repeated measures was used to evaluate the effects of time and localization (artery or vein) on the plasma concentrations of VEGF-A and endostatin, and on the concentration of plasma albumin.

In *study V* the analysed were conducted on log-transformed ratios (target: endogenous control) in order to to fulfill the assumption for parametric testing. Two-way ANOVA for repeated measures was applied to evaluate the effect of training (before, and after 10 days and 5 weeks of training) in the two exercise conditions (restricted and non-restricted condition) on basal mRNA and protein contents. Three-way ANOVA for repeated measures was applied to evaluate the mRNA response to a single bout of exercise (pre exercise and 120 min post exercise), training (before and after 5 weeks) in the two exercise conditions (restricted and non-restricted condition).

In *all studies* planned comparison (i.e., *post hoc* tests) were used to locate the differences corresponding to the significant interactions in the ANOVA models. Differences were considered to be significant at $P < 0.05$.

5. RESULTS

5.1 Haemodynamic, metabolic and oxygen parameters

During one-legged exercise, the increase in heart rate, systolic arterial blood pressure and local rates of perceived exertions were greater ($p<0.05$, $p<0.01$ and $p<0.001$, respectively) with restricted leg blood flow (R-condition) than with non-restricted leg blood flow (NR-condition). In *study I*, the venous oxygen saturation, oxygen tension and pH were lower after 5 min of exercise. These variables were also lower during exercise in R-condition than in the NR-condition (Figure 11). In *study I*, at the onset of exercise, the plasma lactate concentrations in the arterial and femoral venous samples and the arterial-femoral venous lactate concentration increased with exercise in the R-condition but not in the NR-condition. In *studies II-III* venous lactate concentration in the anticubital increased with R-condition, but not in the NR-condition. These differences between R- and NR-condition remained throughout the 45 min exercise period (Figure 11).

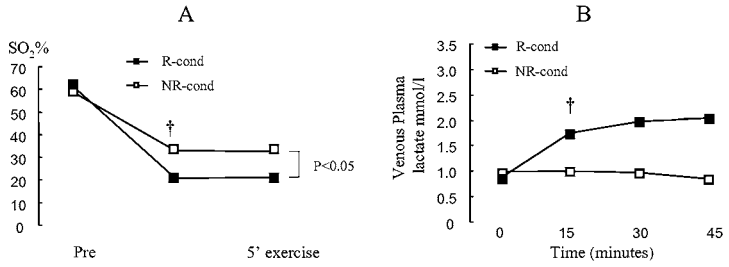


Figure 11. ***A)*** Oxygen saturation, SO_2, in the femoral vein draining the exercising muscle during exercise with restricted and non-restricted leg blood flow. ***B)*** Anticubital venous plasma lactate levels during exercise with restricted and non-restricted leg blood flow. † denotes significant difference ($P<0.05$) between preexercise and 15 min) between restricted and non-restricted conditions. Values are presented as means (A; N=6, B; N=10).

5.2 Response to a single bout of exercise

5.2.1 Study I

A single bout of exercise increased the VEGF-A mRNA expression in the vastus lateralis 30 min after exercise. VEGF-A mRNA expression did not differ significantly between the R- and NR-conditions, although there was a trend toward a greater increase in the R- than in the NR condition ($P = 0.1$). The exercise-induced increase in VEGF-A mRNA levels, correlated with the exercise-induced increase in the femoral venous lactate concentration (Figure 12). FGF-2 mRNA expression did not change in response to a single bout of exercise. The exercise-induced changes in FGF-2 mRNA did not correlate with the exercise-induced increase in femoral venous lactate concentration.

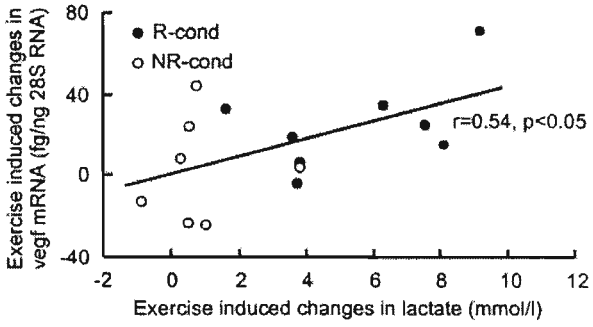
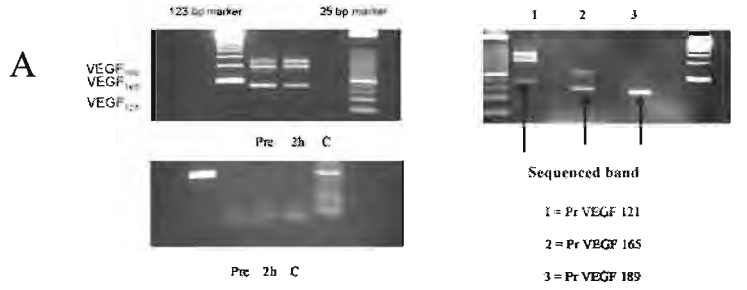


Figure 12. Relationship between individual exercise-induced changes in VEGF mRNA and exercise-induced change in lactate concentration.

5.2.2 Study II

The splice variants VEGF-A_{121}, VEGF-A_{165} and VEGF-A_{189} were evident, whereas the splice variants VEGF-A_{145}, VEGF-A_{206} and VEGF-A_{165B} were not detected in any of the muscle sample (Figure 13).



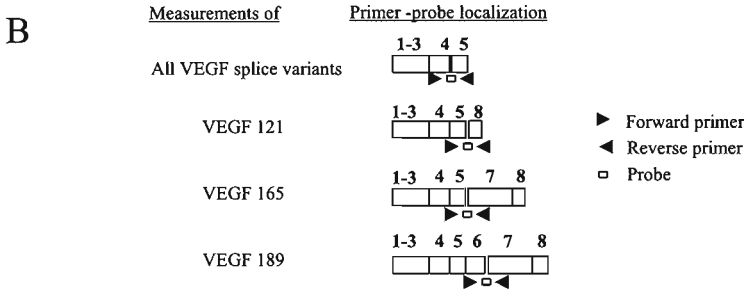


Figure 13. A) The PCR product from skeletal muscle biopsies obtained with the designed specific primers for each splice variant. Each band was sequenced to ensure the specificity of the designed primers.
B) The exon-exon locations for the primers and probe used in the mesurements of specific variants.

Following exercise in the R-condition, expression of "total" VEGF-A mRNA and the three splice variants increased 120 min after exercise and to a greater extent in the R-condition than in the NR-condition. An increase in the relative proportion of VEGF-A_{165} was observed 120 min after exercise, followed by an increase in the relative proportion of VEGF-A_{189} 360 min after exercise (Figure 14). The pattern of change was similar following exercise with or without restricted blood flow.

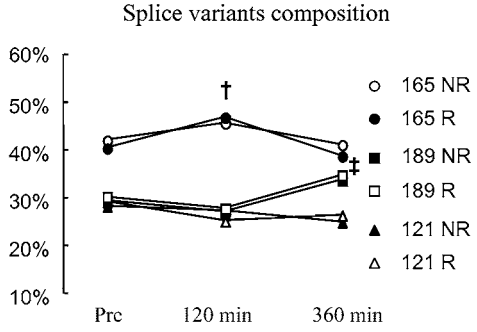


Figure 14. The percentage distribution of $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ splice variants, where $VEGF_{121}$ was the reference from which the percentage distribution was calculated for each splice variant. Measurements made at rest and post exercise (120 min and 360 min) with restricted and non-restricted leg blood flow. † denotes significantly change pre exercise vs. 120 min after exercise for $VEGF_{165}$ ($P<0.05$). ‡ denotes significantly increase from 120 vs. 360 min ($P<0.05$) for $VEGF_{189}$. Values are presented as medians (N=9).

The expression of total VEGF-A and VEGF-A_{121}, VEGF-A_{165} and VEGF-A_{189} increased to a similar extent in type I and type II muscle fibres 120 min after exercise in the R-condition (Figure 15).

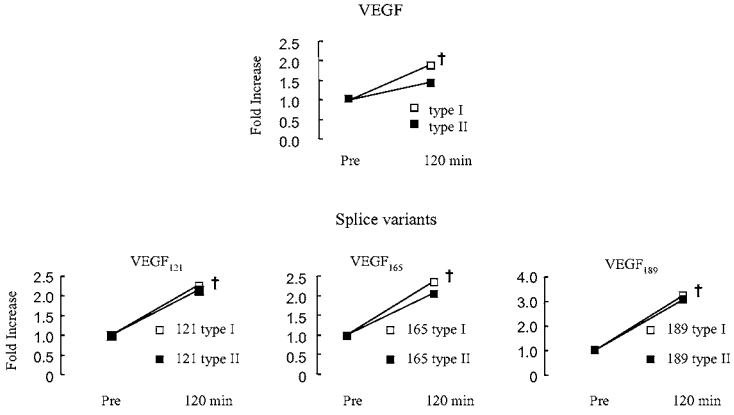


Figure 15. Exercise induced changes in type I and II muscle fibers of the detectable VEGF splice variants. $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ pre exercise and at 120 min after exercise in the restricted blood flow condition only. † denotes significantly change pre exercise and 120 min after exercise. Values are presented as medians (N=5).

For all the receptors, VEGFR-1, VEGFR-2 and NRP-1 mRNA increased more after exercise in the R- than in the NR-condition (Figure 16).

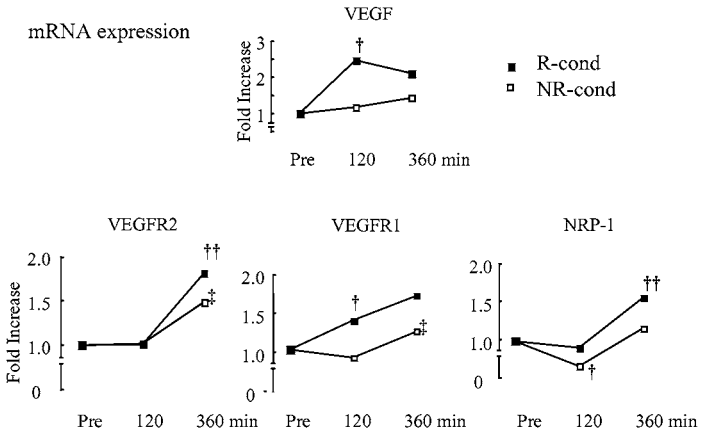


Figure 16. mRNA levels of VEGFR-1, VEGFR-2 and NRP-1 in m. vastus lateralis after exercise with restricted and non-restricted leg blood flow. † denotes significantly differences between the two exercise conditions (P<0.05) between pre exercise and 120 min after exercise. †† denotes significantly differences between the two exercise conditions (P<0.05) between 120 min and 360 min after exercise. Values are presented as medians (N=9).

5.2.3 Study III

VEGF-A mRNA expression increased 30 min after exercise, and reached its peak expression 120 min after exercise (Figure 17). At this time point, VEGF mRNA was three times higher in the R- than in the NR-condition. There were no changes in VEGF mRNA steady-state levels in the biopsies from the control group, who did not exercise (Figure 17).

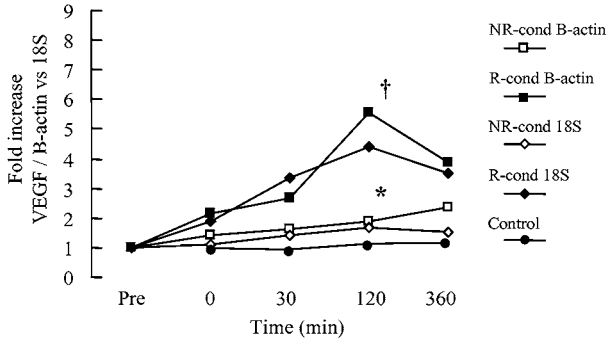


Figure 17. Exercise induced increase in VEGF-A mRNA levels with restricted and non-restricted leg blood flow condition and VEGF-A mRNA in control biopsies. † denotes significant difference (P<0.05) between pre exercise and 120 min between R- and NR-condition. * denotes significant difference (P<0.05) between pre exercise and after 120 min after exercise within same conditions. Values are presented as medians (N = 9)

HIF-1α protein levels increased by 83% above pre-exercise levels immediately after the 45-min exercise bout (Figure 18). The increase in HIF-1α protein levels did not differ significantly between conditions (73% increase in the R-condition and 97% increase in the NR-condition). HIF-1α mRNA expression did not change after exercise and did not differ between the two conditions. Before exercise, the myonuclear HIF-1α specific staining signal was low, but a stronger staining was observed both immediately and 30 min after exercise (Figure 18). Incubation of the labelled HRE probe with cellular extracts from biopsies revealed an enhanced DNA binding activity of expressed HIF-1 complex after exercise compared with before exercise (Figure 18).

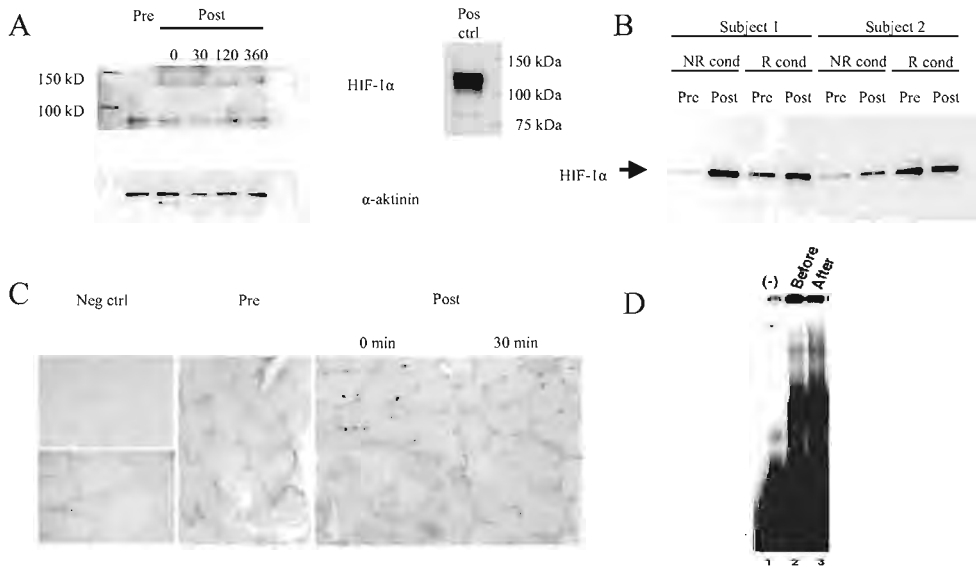


Figure 18. HIF-1α and protein in human skeletal muscle. ***A)*** Immunoblot analyses of HIF-1 α protein levels before (Pre) and 0, 30,120, and 360 min after (Post) a 45-min bout of 1-legged knee-extension exercise (lanes 1–5). Whole cell extract from hypoxic (1% O_2) HeLa cells was used as a positive control (lane 6). Lower molecular weight band represents non-specific immunoreactivity. ***B)*** Immunoblot analyses of HIF-1α protein levels from 2 subjects, before (Pre) and immediately after (Post) 45 min of 1-legged knee-extension exercise in restricted and non-restricted condition (R- or NR-cond). **C)** Immunohistochemical analysis of HIF-1α protein in human skeletal muscle. In the negative control, primary antibody replaced by mouse serum. ***D)*** DNA binding activity (EMSA) of HIF-1α in human skeletal muscle. Muscle homogenate was mixed with HRE sequence of 3´ enhancer of Epo gene. Lane 1 shows the mobility of free probe. Lane 2 includes homogenate from resting biopsy and shows some DNA binding. Homogenate from 0 min after exercise (lane 3) shows a stronger band that migrates similarly to HIF-1 DNA binding complex previously seen in hypoxic cells.

5.2.4 Study IV

VEGF-A mRNA expression decreased immediately after exercise and then increased 120 min after exercise (Figure 19). Compared with pre-exercise levels the VEGF-A protein expression did not change immediately after exercise, but increased 120 min after exercise (Figure 19). Endostatin protein expression did not change significantly at either time after exercise.

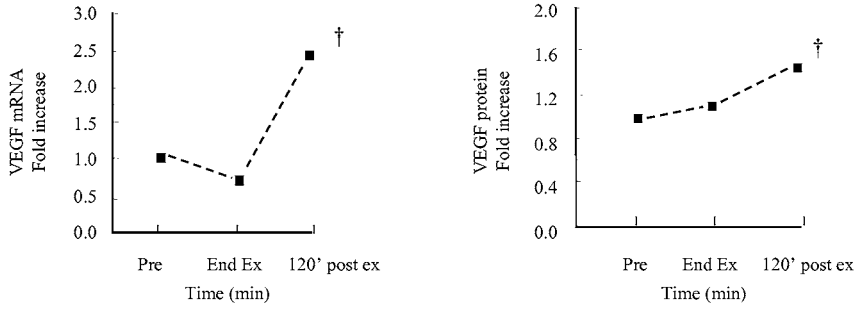


Figure 19. VEGF-A mRNA and VEGF-A protein levels in vastus laterialis pre-exercise, immediately after and 120 min after exercise. † denotes significantly difference between pre exercise and 120 min after exercise ($P<0.05$). Values are presented as means (N=10).

The venous concentration of VEGF-A increased significantly above the pre-exercise value at 17 min of exercise, whereas the arterial concentration remained unchanged. At 17 min of exercise, the VEGF-A concentration was significantly higher in the venous blood than in arterial blood, indicating a release of VEGF-A from the muscle into the circulation (Figure 20). At 57 min of exercise, the venous and arterial concentrations of VEGF-A did not differ significantly. The arterial and venous VEGF-A concentrations were significantly lower than pre-exercise values 120 min after exercise.

Both arterial and venous concentrations of endostatin increased above pre-exercise values at 17 and 57 min of exercise. At 17 min of exercise, venous endostatin concentration was significantly lower than the arterial concentration, indicating an uptake from the circulation into the muscle. At 57 min of exercise, venous endostatin concentration was significantly higher than the arterial concentration, indicating a release from the muscle into the circulation. Arterial and venous endostatin concentrations did not differ significantly from pre-exercise values 120 min after exercise.

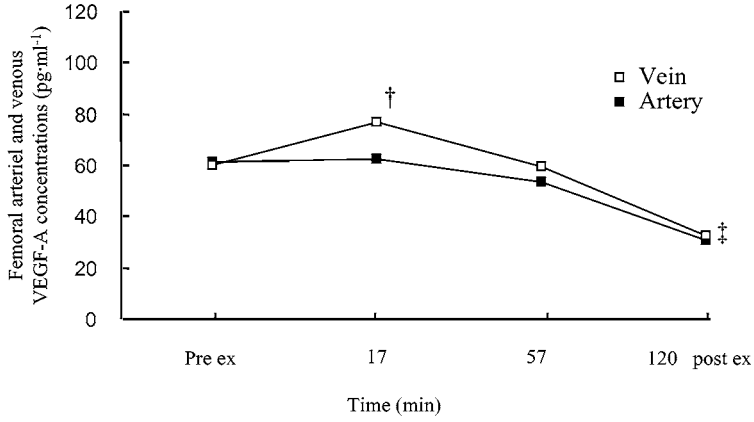


Figure 20. Arterial and venous plasma VEGF-A concentrations pre-exercise, during exercise and post-exercise. † denotes significantly differences between arterial and venous concentration at the same time point ($P<0.05$). †† denotes a significant differences compared to arterial and venous pre-exercise concentrations, respectively. Values are presented as means (N=10).

5.2.5 Study V

The expression of VEGF-A, VEGFR-1 and VEGFR-2 mRNA increased more in the R-condition than in the NR-condition 120 min after exercise (Figure 21). A concomitant increase in VEGF-A mRNA and protein was observed (Figure 21). The expression of Ang-1, Ang-2, Tie-1 and Tie-2 mRNA expression, in Ang-2 protein concentration did not change in response to a single bout of exercise, in either of exercise condition (Figure 21).

5.3 Response to training - basal levels

In *study V* basal levels of VEGF-A mRNA and FGF-2 mRNA (data not shown) did not change significantly throughout the training program in either of the two exercise conditions (Figure 21).

The basal level of VEGFR-1 mRNA did not change with training in the R-condition. However, the basal level of VEGFR-1 mRNA decreased at 10 days in the NR-condition and remained at this level throughout the 5 weeks of training (Figure 22). The basal level of VEGFR-2 mRNA increased, after 10 days in the R-condition, and increased further after 5 weeks of training. Basal VEGFR-2 mRNA levels did not change with training in the NR-condition.

Basal Ang-1 mRNA levels decreased after 10 days of training independent of exercise condition (Figure 22). The basal level of Ang-2 mRNA increased, independent of exercise

condition, after 10 days of training and remained elevated after 5 weeks of training (Figure 22). In the R-condition, but not in the NR-condition, the ratio of basal Ang-2 to Ang-1 mRNA increased after 10 days of training and remained elevated after 5 weeks of training.

The basal level of Tie-1 mRNA increased after 5 weeks of training, independent of exercise condition (Figure 22). The basal level of Tie-2 mRNA did not change significantly throughout the training program in either of the two exercise conditions.

5.4 Effects of training on the response to a single bout of exercise

In *study V,* VEGF-A mRNA expression increased 120 min after a single bout of exercise in the R-condition independent of training (Figure 21). In the NR-condition, VEGF-A mRNA expression did not increase after exercise before training, but there was a tendency toward an increase after training (interaction term between single bout of exercise and training, P =0.08)

VEGFR-1 mRNA expression increased 120 min after a single bout of exercise in the R-condition independent of training. Before training, VEGFR-1 mRNA expression did not increase after exercise in the NR-condition, but there was a tendency toward an increase after training (interaction term between single bout of exercise and training, P =0.12).

VEGFR-2 mRNA expression increased 120 min after a single bout of exercise in the R-condition before training and there was a tendency toward attenuated response after the 5 weeks of training (interaction term between single bout of exercise and training, P=0.10) (Figure 21). Before training, VEGFR-2 mRNA expression did not increase after exercise in the NR-condition, but there was a tendency toward an increase after training (interaction term between single bout of exercise and training, P =0.12). No changes in FGF-2 (data not shown), Ang-1, Ang-2, Tie-1 and Tie-2 mRNA expression were observed in response to a single bout of exercise in either of the two exercise conditions (Figure 21).

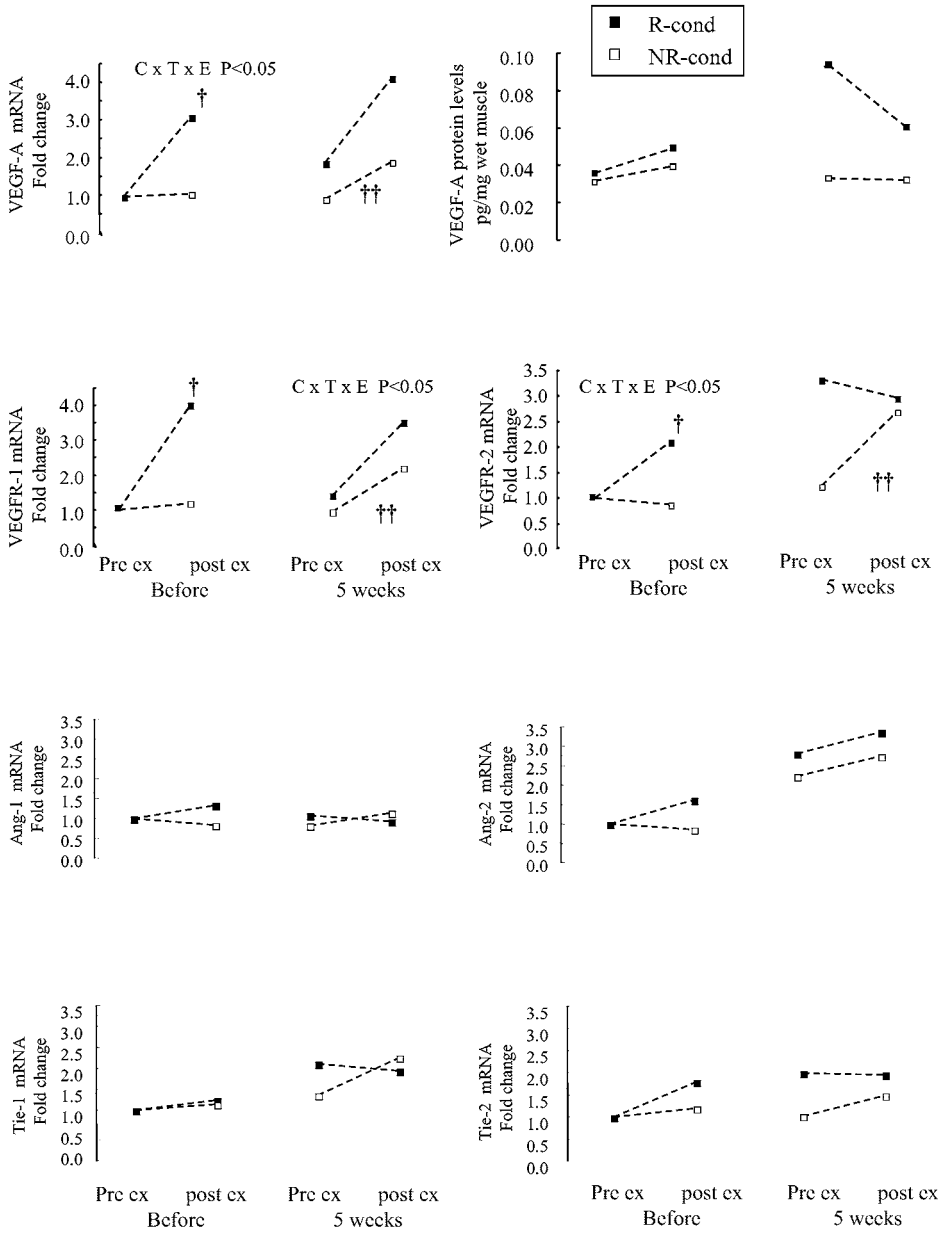


Figure 21. VEGF-A mRNA and protein levels and VEGFR-1, VEGFR-2, Ang-1, Ang-2, Tie-1, and Tie-2 mRNA in vastus lateralis muscle after acute exercise before and after 5 weeks of training. † denotes difference (P<0.05) between the two exercise conditions between pre exercise and 120 min post exercise. †† denotes the interaction term in the NR-condition between (VEGF-A, P=0.08, VEGF-R1, P=0.12 and VEGFR-2, P=0.12), C x T x E depicts the three-ways interactions between exercise condition (C), training (T) and a single bout exercise (E). Values are presented as means (N=11, except for the protein measurements where N=8).

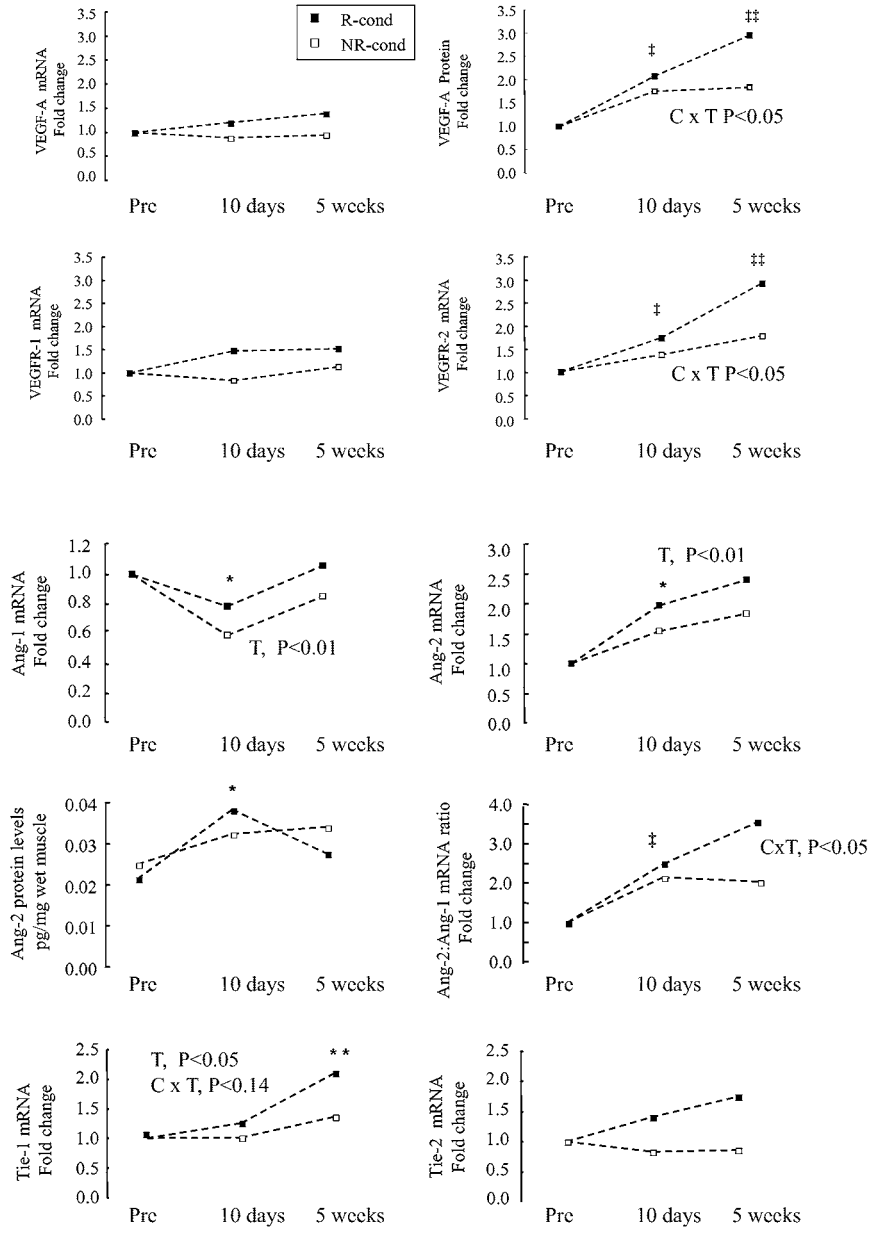


Figure 22. VEGF-A and Ang-2 mRNA and protein levels, VEGFR-1, VEGFR-2, Ang-1, Tie-1, and Tie-2 mRNA levels in vastus lateralis muscle over 5 weeks of training. ‡ denotes difference (P<0.05) between pre exercise and 10 days. *denotes difference (P<0.05) between pre exercise and 10 days without any difference between the two exercise conditions. ‡‡ denotes differences between 10 days and 5 weeks and between the two exercise conditions. ** denotes difference (P<0.05) between10 days and 5 weeks without any difference between the two exercise conditions. A two way interaction between condition (C) and training (T) is indicated by C x T. A main effect of training is indicated by T. Values are presented as means (N=11, except for the protein measurements where N=8).

5.5 Protein localization

Immunohistochemical evaluation showed that the expression of VEGF-A protein was detected within the muscle fibres and localized close to the plasma membrane and nuclei, but not within the nuclei (Figure 23). It was not possible to determine whether VEGF-A was expressed in the endothelial cells. VEGFR-2 protein had a myocellular localization most probably representing endothelial cells (unpublished data: T. Gustafsson, M. Esbjörnsson, M. Ekman and O. Johansson).

The localization of VEGF-A and VEGFR-2 was mainly the same comparing biopsies obtained at rest and after exercise. However, the limited amount of biopsy material obtained for immunohistochemistry did not allow for any detailed analysis of exercise-induced differences in amount or localization of VEGF-A and of VEGFR-2.

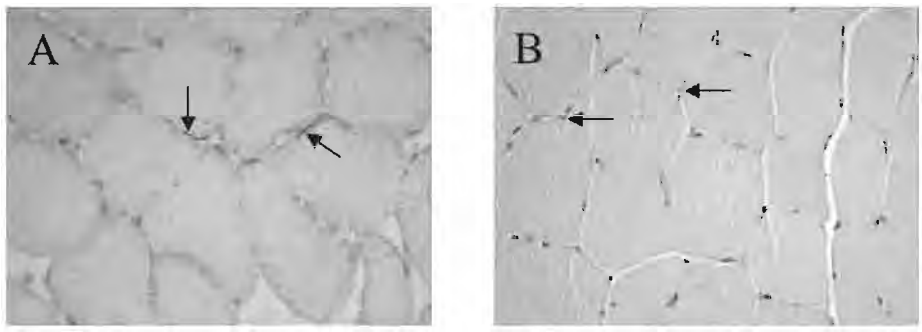


Figure 23. Immunohistochemical staining of two different human transverse muscle tissue sections. In ***A)*** the arrows points at brown areas which indicate expression of VEGF-A. In ***B)*** the arrows points at brown areas, which indicates expression of VEGFR-2. Nuclei were stained blue with haematoxylin.

6. GENERAL DISCUSSION

Endurance exercise increases the expression of angiogenesis growth factors and their receptors and that this response is enhanced by reduced oxygen delivery to the exercising muscles in a fashion that facilitate in angiogenic process.

6.1 The VEGF-A system

6.1.1 VEGF-A mRNA expression

Recent attention has focused on vascular endothelial growth factor VEGF-A, as a major factor regulator of the process of angiogenesis. Many studies have shown that, during angiogenesis, VEGF-A expression is associated, both spatially and temporally, with tissue capillarization (Folkman, 1995, Neufeld et al., 1999, Carmeliet, 2003, Ferrara et al., 2003). In *study I*, VEGF-A mRNA expression increased in skeletal muscle tissue in response to a single bout of exercise and in contrast to the other major suggested angiogenic factor, FGF-2. Others have now reported similar findings in humans and in animals (Brown et al., 1998, Richardson, 1999, Richardson et al., 2000, Gavin and Wagner, 2001).

In *study III*, the time course of changes in VEGF-A mRNA expression, which peaked mRNA 120 min after exercise, indicate that the temporal pattern of gene expression differs between animals and humans (Breen et al., 1996). In this thesis VEGF-A mRNA expression either did not change (*study III*) or declined (*study IV*) immediately following exercise, suggesting that pre-translational changes in VEGF-A occurred only in the recovery phase after exercise. This has been reported for other gene transcripts in response to various types of exercise (Cameron-Smith, 2002). Such findings may reflect down regulation of the transcription machinery to allow for other energy-consuming processes during exercise. It is also possible that the exercise durations in *studies III* and *IV* were too short to induce detectable changes in gene expression immediately after exercise. A previous study reported that the VEGF-A mRNA expression increased during 3 h of exercise (Hiscock et al., 2003) and continued to increase further during recovery after exercise which is consistent with both possibilities.

Summary: *Study I* was the first report in humans showing that exercise increases the expression of VEGF-A but not FGF-2 mRNA, which now can be regarded as an established finding. The increase in mRNA expression was observed only in the recovery phase after exercise in humans but later than the time course found in other species.

6.1.2 mRNA expressions of VEGF-A splice variants

VEGF-A exists as multiple splice variants and each splice variant can produce vessels with different structural characteristics (Carmeliet et al., 1999, Stalmans et al., 2002). This

may reflect differences in the localization, accessibility, and receptor-binding affinity between individual VEGF-A splice variants (Houck et al., 1991, Neufeld et al., 1999, Ferrara et al., 2003). Three splice variants, VEGF-A_{121}, VEGF-A_{165} and VEGF-A_{189}, were detected in human skeletal muscle in *study II*, which all increased in response to a single bout of exercise. Splice variants containing the domain encoded by exon 6, such as VEGF-A_{189}, are bound tightly to cell-surface heparin sulfate proteoglycans, whereas splice variants lacking this domain, such as VEGF-A_{121} and VEGF-A_{165}, are diffusible (Houck et al., 1991, Neufeld et al., 1999, Ferrara et al., 2003). The temporal pattern of the relative changes between the different splice-variants expression after a single bout of exercise observed in *study II* may, represent early endothelial activation followed by a later chemo-attractant and differentiation role for the VEGF-A system.

The present results are somewhat in contrast to those from a recent report (Jensen et al., 2004b), which concluded that the $VEGF_{165}$ splice variant is the main variant that increases in response to exercise. However, only $VEGF_{165}$ was measured separately; the other VEGF splice variants ($VEGF_{145}$, $VEGF_{189}$, and $VEGF_{205}$) were measured together in a single analysis and, $VEGF_{121}$ was not measured at all. In *study II*, VEGF-A_{165} was the most abundantly splice variant expressed, followed by VEGF-A_{121} and VEGF-A_{189}, which agrees with the observed splice-variant rank order in adult murine skeletal muscle (Ng et al., 2001). Limited modulation of the less abundant $VEGF_{121}$ and $VEGF_{189}$ variants would be difficult to detect with the strategy used by Jensen at al. (2004) and could be one reason behind the observed lack of changes in these splice variants in that study.

The splice variant $VEGF_{165b}$ inhibits VEGF-A_{165}-mediated endothelial cell proliferation and migration (Bates et al., 2002, Cui et al., 2004). In *study II*, the $VEGF_{165b}$ splice variant could not be detected, in resting or exercising human skeletal muscle, indicating that $VEGF_{165b}$ is not relevant in human skeletal muscle, in contrast to its suggested role in tumour angiogenesis.

Summary: The VEGF-A splice variants VEGF-A_{165},VEGF-A_{121} and VEGF-A_{189} all increase but to different degrees in response to exercise, which is a novel finding. This may indicate a selective and specific contribution of each splice variant to events such as early endothelial activation (VEGF-A_{165}) followed by a later chemo-attractant and differentiation role (VEGF-A_{189}). These observations also demonstrate that exercise is a useful experimental model to study the mechanisms regulating VEGF-A splicing in humans.

6.1.3 VEGF-A and splice variants and their relationship to muscle fibre types

In *study II*, the distribution pattern for the VEGF-A splice variants was not related to "resting" human muscle fibre types I or II. The increase in VEGF-A mRNA expression after exercise in the R-condition in *study II* was also similar in type I and II fibres. This is consistent

the observation that capillarization increase to a similar extent around type I and II muscle fibres after high intensity endurance training (Jensen et al., 2004a). It was recently also reported, that exhaustive exercise in the mouse recruits glycolytic fibres, that glycolytic fibres is the first fibre type to demonstrate a rapid increase in capillary number, which is preceded by an increase in VEGF-A mRNA and protein levels (Birot et al., 2003, Waters et al., 2004). This suggests that the VEGF-A response is related to fibre recruitment rather than fibre characteristics. Glycogen is depleted from a high proportion of type II fibres with exercise using the present experimental model during exercise with restricted blood flow, indicating that type II fibres are recruited. Thus the observed changes in VEGF-A mRNA levels in type II fibres are consistent with recent reports in the rat and mouse (Birot et al., 2003, Waters et al., 2004).

If the pre-translational regulation of VEGF-A mRNA is influenced by the metabolic disturbance in the myocytes, type II fibres would have increased the VEGF-A mRNA expression more than the type I fibres. However, the response in type I fibre during exercise in the R-condition might have had a greater effect on the metabolism in type I fibres than in type II fibres (Greenhaff et al., 1993). This would ensure a similar amount of metabolic disturbance in both 'fibre-type' populations with reduced blood flow. Thus, if metabolic factors influence VEGF-A gene expression and reduced blood flow 'equalized' the metabolic responses in type I and type II fibres, then one might expect similar increases in VEGF-A mRNA expression, if both fibres are recruited.

Summary: This is the first time that human skeletal fibre specific VEGF-A and its splice variants have been measured. It was shown that the basal mRNA levels of VEGF-A and VEGF-A splice variants were equally abundant in the different human muscle fibres types and that they increased to a similar extent in response to a single bout of exercise. This shows that transcriptional regulation of VEGF-A is not related to the characteristics of muscle fibre type.

6.1.4 VEGF-A muscle protein levels

Basal VEGF-A mRNA remains unchanged after eight weeks of training (Richardson et al., 2000). Because VEGF-A expression is assumed to be regulated at pre-translational level, it has been questioned whether basal VEGF-A expression increases only in the early phase of adaptation to repeated bout of exercise (Gustafsson et al., 2002). In *study V*, repeated bouts of exercise increased the basal VEGF-A protein levels without concomitant change in basal VEGF-A mRNA expression. However, transient increases in VEGF-A mRNA levels most likely occurred with each exercise bout in the R-condition since a single bout of exercise increased VEGF-A mRNA expression both before and after 5 weeks of training. One possible explanation for the increased basal protein levels, observed in the R-condition, is that the exercise-induced increase in VEGF mRNA expression is followed by an increase in VEGF-A translation. This is

a novel and potentially very important observation because it provides one possible explanation of the regulation of VEGF-A expression in exercising human skeletal muscle. This observation is supported by the findings that increased VEGF mRNA expression induced by electrical stimulation of skeletal muscle is followed by a subsequent increase in protein secretion into the surrounding medium (Kanno et al., 1999), and that a concurrent increase in mRNA and protein expression occurs in electrically stimulated rat muscle (Tang et al., 2004).

It was reported recently that an increases in VEGF-A protein levels could be initiated in cell culture through enhanced translation without any changes in mRNA levels (Feliers et al., 2005). The involvement of similar processes cannot be discounted in the present study, given the importance of the activation of Akt in response to exercise in humans (Sakamoto et al., 2004). Activation of Akt phosphorylates 4E-BP1, which permits dissociation of the eIF4E/4E-BP1 complex and thus allows eIF4 to promote the cap-dependent translational of VEGF-A (Mamane et al., 2004; Segrelles et al., 2004; Feliers et al., 2005). If Akt had influenced the VEGF-A protein translation in *study V*, it would mean that this kinase was activated to a greater extent during exercise in the R-condition than during exercise at the same absolute workload in the NR-condition; these variables were not measured and it is unknown whether this occurred or not.

How rapidly any change in VEGF mRNA expression is translated into protein in human skeletal muscle has been largely unknown but in the rat a concurrent increased VEGF-A mRNA expression and protein levels has been observed following electrical stimulation (Tang et al., 2004). VEGF-A mRNA expression and protein levels increased concomitantly 120 min after a 60 min two-legged exercise bout in *study IV* and in response to a single bout of one-legged exercise with restricted blood flow in *study V*. However, in *study V* these responses did not differ statistically between the two exercise conditions and it is therefore not allowed to statistically handle them separately.

Summary: That repetitive exercise bouts increase VEGF-A protein but not VEGF-A mRNA levels and that the latter increases only in association to acute exercise are novel findings. These findings are potentially important since it further supports the involvement of VEGF-A in human exercise-induced angiogenesis and clarifies how the changes in VEGF-A mRNA and protein levels are related to each other in response to repetitive exercise bouts. The unchanged endostatin level in skeletal muscle in humans, also a novel finding, suggests that this factor is not involved in the regulation of exercise-induced angiogenesis.

6.1.5 Changes in plasma levels and exchange of VEGF-A and endostatin

A recent study reported a decrease in muscle VEGF-A protein levels immediately after exercise (Gavin et al., 2004). The authors suggested that this decrease could be explained by a

release of VEGF-A from exercising muscle. Interstitial VEGF-A concentration increase during exercise, especially in the first 30 min of a 60 min exercise bout (Hoffner et al., 2003). A small but significant release of VEGF-A was observed in the initial phase of exercise in *study IV*. It may be assumed that VEGF-A protein synthesis cannot increase during such a short time i.e., 17 min after the onset of exercise in the present *study IV* and during the first 30 min of exercise in the study by Hoffner et al. (2003). These findings may therefore reflect a release of pre-existing VEGF-A, consistent with the hypothesis of Gavin et al. (2004).

To our knowledge, only one previous study has measured the arterio-venous differences of VEGF-A in the exercising leg (Hiscock et al., 2003). It was reported that VEGF-A was released 120 min after a 180 min single exercise bout. The longer exercise duration in this previous study, may have provided a greater stimulus and more time for the induction of VEGF-A transcription and, possibly, for VEGF-A translation. In fact in the study by Hiscock et al. (2003), the skeletal muscle VEGF-A mRNA expression was increased before any VEGF-A protein was released into the circulation. Thus, it is suggested that two different mechanisms exists for exercise-induced release of VEGF-A from skeletal muscle tissue into the circulation; an initial release during exercise from pre-existing VEGF-A and a later release after prolonged exercise resulting from increased VEGF-A protein expressions and secretion from VEGF-A-producing cells.

Consistent with earlier reports (Asano et al., 1998, Gunga et al., 1999, Gustafsson et al., 2002) a decrease in plasma VEGF-A concentration was observed 120 min after exercise in *study II*. It has been suggested previously that, during muscle contraction, a peripheral uptake of VEGF-A from the circulation by the skeletal muscle tissue is one possible mechanism for the decrease in plasma VEGF-A concentrations and could thus be a mechanism for the exercise-induced stimulation of the angiogenic process. However, no uptake of VEGF-A from the circulation into skeletal muscle tissue was observed neither during nor after exercise in *study II*. It is therefore more likely that the observed exercise-induced decrease in plasma VEGF-A concentration involves other mechanisms, such as changes in the binding to soluble VEGFR-1 or uptake into other tissues or organs.

Summary: Angiogenesis-related factors were exchanged between the muscle and circulating blood during exercise before any changes in mRNA expression were observed and, most likely, before any changes in protein expression. The lack of uptake of VEGF-A into skeletal muscle from the circulation indicates that the blood stream is not a source for the observed increase in skeletal muscle VEGF-A in the recovery phase after exercise.

6.1.6 Expression of VEGF-A receptor mRNA

VEGF receptors are important for capillarization and exercise-induced angiogenesis.

For example, exercise-induced angiogenesis in ischemic rat skeletal muscle is blunted by administration of the VEGF receptor inhibitor (ZD4190) during treadmill training (Lloyd et al., 2005). In *study II*, exercise induced an increase at the mRNA level of all three receptors involved in VEGF-A signalling. Co-expression of the VEGF-A, NRP-1, and VEGFR-2 receptors is important for effective tissue angiogenesis (Ishida et al., 2000). In *study II* and *V*, it was shown that VEGFR-1 and VEGFR-2 increase in response to a single bout of exercise in humans, which previously also has been reported by Gavin et al. (2004). Moreover in *study V*, with training in the R-condition the receptor VEGFR-2 expression also increases, whereas VEGFR-1 mRNA expression does not. This is an important finding because recently it was reported that angiogenesis in response to electrical stimulation of ischaemic rat muscle occured only when an increase in VEGFR-2 was observed (Milkiewicz et al., 2003).

Summary: The increased VEGFR-2 and the unchanged VEGFR-1 mRNA levels after five weeks of training is novel in humans and suggest a selective contribution of the VEGF-A receptors in exercise-induced angiogenesis and thus a further support that the VEGF-A system plays an important role in exercise-induced skeletal muscle angiogenesis.

6.1.7 VEGF-A and VEGFR-2 protein localization

The source of VEGF-A in skeletal muscle has not been clearly identified. In addition to skeletal muscle cells other cell types present in skeletal muscle tissue, such as endothelial cells, macrophages, and fibroblast, are known to express VEGF-A (Berse et al., 1992, Steinbrech et al., 1999). Exercise-induced changes in VEGF-A expression might occur in any of these cells. VEGF-A protein expression was initially found mainly in the interstitial tissue between the rat muscle fibres (Annex et al., 1998). However, later observations using experimental models of pharmacologically increased blood flow or electrical stimulation in the rat skeletal muscle, showed that VEGF-A protein is expressed both within the skeletal muscle fibres and within the interstitial tissue (Milkiewicz et al., 2001). The data from this thesis support the concept that VEGF-A protein is expressed in both the skeletal muscle fibres and in the interstitial tissue between the muscle fibres in humans. A similar finding was recently reported in human skeletal muscle where it also was observed that the distribution pattern of VEGF-A protein expression did not change with training (Jensen et al., 2004a).

These observations strongly suggest the skeletal muscle fibres are one source for the exercise induced increase in VEGF-A. This is further illustrated by the findings that VEGF-A mRNA expression is localised into the skeletal muscle fibres (Breen et al., 1996, Milkiewicz et al., 2001, Gustafsson et al., 2002) and that electrical muscle stimulation induces expression of VEGF-A mRNA in skeletal muscle fibres, which causes a subsequent increase of protein secretion into the surrounding medium (Kanno et al., 1999). Additional support for skeletal

muscle fibres as a source for VEGF-A expression in response to exercise comes from single fibres, controlled for absence of endothelial, and primary skeletal muscle cells. A greater VEGF-A expression is observed from fibres and cells obtained after voluntary exercise and electrical stimulation, respectively (Birot et al., 2003, Jensen et al., 2004c).

The VEGFR-2 was localized to the endothelial cells in the skeletal muscle biopsies confirming its specificity to these cells.

Summary: The observation that VEGF-A is expressed with skeletal muscle fibres is an important confirmation of previous findings and supports that muscle fibres may be one source for the increased VEGF-A expression following exercise.

6.2 The angiopoietin system

6.2.1 Expression of Ang-1 mRNA, Ang-2 mRNA and protein expression

The importance of the integrated functions between the VEGF-A and angiopoietin systems in angiogenesis has recently been highlighted (Asahara et al., 1998, Holash et al., 1999, Lobov et al., 2002, Visconti et al., 2002).

In *study V*, basal Ang-1 mRNA expression decreased and basal Ang-2 mRNA expression increased and thus the Ang-2:Ang-1 mRNA ratio increased in response to repeated exercise bouts. The Ang-2 mRNA expression changes were reflected in similar changes at the protein level. This should have a permissive effect by allowing the increase in VEGF-A to initiate angiogenesis. (Lobov et al., 2002). A recent report explored the changes in the expression of the VEGF-A and angiopoietin systems with exercise training in the rat (Lloyd et al., 2003) showing similar changes in Ang-1 and Ang-2 mRNA expression. The similarity between the data of Lloyd et al. (2003) and those in *study V* further supports the notion that exercise induces changes in the angiopoietin system could allow the exercise-induced increase in VEGF-A to initiate angiogenesis.

Summary: That exercise changed expression of the angiopoietin system factors, e.g. increased Ang-2 protein and the Ang-2:Ang-1 mRNA ratio, is novel in exercising human skeletal muscle. This might have a "permissive" effect on angiogenesis by allowing the increase in VEGF-A to stimulate endothelial migration and proliferation.

6.2.2 Tie-1 and Tie-2 mRNA expression

The Tie family consists of Tie-1 and Tie-2. The functions of Tie-2 is reasonably well characterized with Ang-1 and Ang-2 acting as an agonist respectively as an antagonist and Ang-2 induceing endothelial destabilization through inhibition of the Tie-2 receptor (Lobov et al., 2002). The cellular functions of Tie-1 are largely unknown. In *study V*, a single bout of exercise

or repetitive exercise bouts did not cause any change in pre-translational Tie-2 expression. In contrast Tie-1 mRNA levels increased after repetitive exercise bouts. Even though Tie-1 at present not has a clearly defined function it has been shown to physically interact with Tie-2 in endothelial cells (Marron et al., 2000, Chen-Konak et al., 2003). Moreover, studies using target disruption of Tie-1 in transgenic mice clearly show that Tie-1 is required for normal vascular development as well as for vessel maintenance, and endothelial cell survival (Tallquist et al., 1999).

Summary: This is the first report that specifically investigates the exercise response of the Tie-receptors in humans. The absence of exercise induced changes in the angiopoietins receptor, Tie-2, suggests that this receptor is not a major factor in the regulation of exercise induced angiogenesis. However, the observed increase in Tie-1 mRNA suggests that exercise can modulate the Tie receptor system and since Tie-1 has been reported to interact with Tie-2 signalling such a change may be involved in the regulation of exercise induced angiogenesis.

6.3 Possible regulating stimuli

In the experimental models used in *studies I-III,* and *V*, the major differences between the two legs arise mainly because of differences in the exercise-induced reductions in oxygen tension, the related metabolic perturbation and associated physiological consequences (Sundberg, 1994). One-legged exercise training with this model has shown that during restricted leg blood flow induces a greater increase in capillarization and oxidative enzyme activity than in training with non-restricted leg blood flow (Esbjornsson et al., 1994).

6.3.1 VEGF-A

6.3.1.1 Exercise-induced reduction in tissue oxygen tension

The observations in *studies II, III,* and *V*, showing significantly greater VEGF mRNA expression in skeletal muscle exercise in the R-condition supports the role of exercise-induced reductions in oxygen tension and the related metabolic perturbation as stimuli in skeletal muscle angiogenesis. Moreover, the enhanced expression of VEGF-A mRNA expression followed by an increased protein expression in the leg trained in R-condition further strengthens this line of reasoning. However, *study I* showed no difference between exercise in the R-condition and NR-condition. The peak in VEGF-A mRNA occurred 120 min after exercise in *study III* and, measuring mRNA expression only 30 min after exercise as in *study I* might have blunted the possibility to detect any differences between exercise in the R- and NR-condition. The decision to obtain the biopsy 30 min after exercise in *study I* was due to that in voluntary running (60 min) rats the expression peak in VEGF-A mRNA was observed at 30 min after end of exercise (Breen et al., 1996). This finding highlights the importance of the sampling times for biopsies to measure mRNA in relation to exercise and also as earlier discussed the species differences in

the temporal pattern of gene expression.

In exercising skeletal muscle, a substantial reduction occurs in intracellular PO2, reflected by decrease in venous oxygen saturation (Richardson et al., 1995), to levels known to induce the major transcription factor for hypoxic gene expression HIF-1 (Jiang et al., 1996b). HIF-1 acts through the HRE sequence (Forsythe et al., 1996, Semenza, 2001). In *study III*, it was shown that exercise stabilizes the HIF-1α protein levels, induces translocation of the protein into the nuclei, and enhances the HIF-1α dependent DNA binding activity. Taken together, these data strongly suggest that exercise activates HIF-1α activity. In *studies II* and *III* the two genes with HRE's in their promoter region, VEGF-A and VEGFR-1 (Forsythe et al., 1996, Gerber et al., 1997), behaved similarly in response to a single bout of exercise, with a greater response after exercise in the R-condition, indirectly supporting the involvement of HIF-1 in the enhanced expression during exercise in the R-condition. A recent study in skeletal muscle HIF-1α knocked out mice has shown attenuation of the increase in VEGF-A mRNA in response to exercise, which further supports the involvement of HIF-1 signalling in the exercise-induced increase in VEGF-A mRNA (Mason et al., 2004). Moreover, inhibition of endogenous HIF-1 activation inactivation in mice induces angiogenesis in ischaemic skeletal muscle, further establishing HIF-1 as an important pathway for skeletal muscle angiogenesis in these conditions.

Surprisingly, the HIF-1α protein levels after exercise did not differ between exercise in the R-condition and NR-condition in *study III*, despite that a significantly reduced oxygen tension and saturation was observed in *study I* the femoral vein draining the restricted leg exercising indicating a lower oxygen tension in the muscle (Richardson et al., 1995) in the R-condition with the present experimental model. A possible explanation for this finding is that that oxygen tension was already sufficiently low to fully stabilize HIF-1α during exercise in the NR-condition. In fact, the decrease in oxygen tension and saturation from rest to exercise in the NR-condition was greater than the difference between R- and NR-conditions. In contrast, several co-regulatory mechanisms can modulate HIF-1α stabilization. One example is mechanical stretch, presumably rather similar in the two exercise conditions (discussed further below), which induces HIF-1 activity (Kim et al., 2002). Many other exercise-associated events can also influence HIF-1 activity (Semenza, 2002, Kasuno et al., 2004, Yuan et al., 2005). Moreover, the HIF-1α protein levels after exercise did not differ between the two exercise conditions, suggesting therefore that other mechanisms are involved in enhancing the VEGF expression observed after exercise in the R-condition. For instance, in addition to the HIF-1-mediated pathway, other mechanisms for the regulation of VEGF expression have been documented (Neufeld et al., 1999, Burns and Wilson, 2003, Ferrara et al., 2003). This notion is supported by Tang et al., (2004), who reported that VEGF-A mRNA and HIF-1α protein levels increases in proportion to each other, but only during specific exercise conditions. The data of Tang et al.

(2004) suggest the existence of HIF-1 independent as well as HIF-1 dependent mechanisms in the regulation of VEGF-A in response to exercise. An alternative HIF-1 independent regulation mechanism is mRNA stabilization of VEGF-A (Stein et al., 1995, Levy et al., 1996). One possible factor is Hu protein R, which stabilizes VEGF-A mRNA in vitro, and increases in ischaemic rat skeletal muscle in parallel with increased expression of VEGF-A mRNA (Tang et al., 2002).

Summary: A single bout of exercise with restricted leg blood flow enhanced the VEGF-A mRNA expression. This is the first data from humans showing that reduced oxygen tension in the muscle or the associated metabolic perturbation or some combination thereof might be important regulatory stimuli for exercise induced VEGF-A expression. This report provides also the original evidence that human HIF-1α can be fully activated under physiological conditions, supporting the general importance of this pathway. The present data also suggests that the mechanisms responsible for the exercise-induced increase in VEGF-A are more complex than activation of HIF-1 alone.

6.3.1.2 Exercise induced metabolites

Metabolites, such as lactate, adenosine, ATP, and ADP produce angiogenic responses *in vitro* (Murray and Wilson, 2001 Jensen, 2004), which can be inhibited by anti-VEGF-A neutralizing antibodies (Burns and Wilson, 2003). The relation between exercise-induced increase in venous lactate and VEGF-A mRNA in *study I* and the fact that the exercise induced lactate concentration was higher during exercise in the R-condition in *studies I-III*, suggest that lactate concentration or other metabolites may partly account for the obtained differences in VEGF-A mRNA expression between the two exercise conditions.

A previous study showed that eight weeks of exercise training attenuates the increase in VEGF-A mRNA expression after a single bout of exercise. This indirectly supports the influence of metabolic perturbation on the expression of VEGF-A since the subjects exercised at the same absolute workload before and after training (i.e., the relative workload was lower after the training program) (Richardson et al., 2000). Although, from these data one can not discount the possibility that training downregulates the pathways stimulating VEGF-A expression, which could be an alternative explanation for the attenuated response. The absence of any attenuated VEGF-A mRNA response to maximal exercise after training in *study V*, suggests that the observed attenuated decrease in VEGF-A mRNA expression by Richardson et al. (2000) was due to a reduced exercise stimulus because of skeletal muscle adaptation. The involvement of metabolites is supported by other studies. In rabbit and rat skeletal muscle, the highest VEGF mRNA expression are observed during the earliest stages of electrical stimulation when muscle lactate concentration is highest as the muscle respond to the sudden increase in contractile activity (Hang et al., 1995, Skorjanc et al., 1998).

Summary: The relationship between the exercise-induced increase in venous lactate concentration and VEGF-A mRNA expression and the higher lactate concentration and VEGF-A mRNA expression after exercise in the R-condition indicate that energy metabolites may be involve in the regulation of exercise induce VEGF-A expression.

6.3.1.3 Execise induced changes in shear stress, wall tension and mechanical stress

Hudlicka and co–workers (Hudlicka et al., 1992, Hudlicka, 1998, Brown and Hudlicka, 2003) have clearly shown that both increased blood flow and muscle stretch induces angiogenesis in skeletal muscle (Ziada et al., 1984, Dawson and Hudlicka, 1989, Egginton et al., 1998, Zhou et al., 1998a, Zhou et al., 1998b). Shear stress and increased wall tension to stimulate non-sprouting angiogenesis and mechanical factors stimulate sprouting angiogenesis (Zhou et al., 1998a, Zhou et al., 1998b, Egginton et al., 2001). Both sprouting and non-sprouting angiogenesis occur in response to electrical stimulation suggesting roles of shear stress and mechanical stretch or other stimuli acting from the abluminal side (Egginton et al., 2001, Brown and Hudlicka, 2003). Regardless of the type of angiogenesis, VEGF-A expression in skeletal muscle is induced by angiogenesis induced by both shear stress and mechanical factors (Rivilis et al., 2002) although each probably act through different mechanisms.

Shear stress induces NO and inhibition of NO attenuates, at least part the increase in VEGF-A expression in response to exercise (Gavin et al., 2000, Baum et al., 2004, Milkiewicz et al., 2005), suggesting that NO is an inducing factor for VEGF-A expression in exercising skeletal muscle. One may assume that shear stress was lower during exercise in the R-condition because of lower blood flow and probably greater vasodilatation, both of which reduce shear stress, than during exercise in the NR-condition. Thus, an increase in shear stress in the capillaries could not likely explain the enhanced expression during exercise on the R-condition in *studies I-III* and *V*. Furthermore, in contrast to the reports mentioned previously, other studies have observed that shear stress does not induce VEGF-A in endothelial cells (Gan et al., 2000). Mechanism other than shear stress may induce NO production in skeletal muscle. Skeletal muscle produces NO at low rates and at higher rates during muscle contraction (Reid, 1998, Reid, 2001). Previous studies using an experimental model similar to that used in this work have shown that plasma nitrate, the main metabolite of NO in humans (Wennmalm et al., 1992), increases during exercise in the R-condition but not during exercise in the NR-condition (Sundberg and Kaijser, 1992). It must, however, be noted that NO does not seem to have a major impact on VEGF-A expression in skeletal muscle cells (Jensen et al., 2004c).

It is difficult to know whether wall capillary tension differs between exercise in the R-condition compared to in the NR-condition. The observed increase in systemic arterial blood pressure and a likely increase in capillary diameter during exercise in the R-condition should increase the wall tension. In contrast, the pressure applied pressure over leg should act to

reduce wall tension because the increased pressure is transmitted to the soft tissue in a nearly 1: 1 relationship (Lundvall and Lanne, 1989).

Mechanical stretch, induced by overloading acts as an abluminal activator for sprouting angiogenesis (Egginton et al., 1998, Zhou et al., 1998b, Egginton et al., 2001), and VEGF-A expression increases in response to muscle stretch (Rivilis et al., 2002). The repetitive muscle stretch-shortening cycle that occurs during exercise, induces VEGF-A expression in the cardiomyocytes (Seko et al., 1999, Zheng et al., 2001). In the exercise model used in *studies I-III* and *V*, the two legs performed the same workload, and it is unlikely that mechanical stretch differed between the legs to such extent to explain the observed differences in VEGF-A expression between the two exercise conditions. Activation of skeletal muscle under the levels needed for muscle contraction has been reported to increase VEGF-A mRNA, suggesting that muscle activation per se may induce VEGF-A expression (Kanno et al., 1999). Exercise in the R-condition causes a greater total activation of muscle fibres (Sundberg 1994), thus the obtained differences in VEGF-A expression between exercise in the R-condition and the NR-condition may therefore be influenced by differences in the total amount of fibre recruited in the two conditions.

Summary: It is not likely that shear stress, wall tension or mechanical stress could explain the differences in the enhanced VEGF-A expression following exercise with restricted blood flow. However, as earlier shown, the greater muscle recruitment in the R-condition may have contributed to enhance VEGF-A expression after exercise in the R-condition compared to NR-condition.

6.3.2 VEGF receptors

VEGF-A and VEGFR-1, two genes with HRE element in their promoter sequences (Forsythe et al., 1996, Gerber et al., 1997) behaved similarly in response to a single bout of exercise in *studies II* and *V*, indirectly supporting the concept that HIF-1 is involved in the expression of VEGFR-1. Both endothelial cells and skeletal muscle cells express VEGF-A, but VEGFR-1 is predominantly expressed on endothelial cells (Shibuya et al., 1999).

Endothelial specific von Willebrand factor is present in the RNA extracted from skeletal muscle biopsies (data not shown), suggesting that part of the RNA analysed is derived from endothelial cells, and thus is the likely cell which the measured VEGF receptors mRNA originate from. Thus, one may question if the endothelial cells is exposed to enough of a reduction of oxygen tension during exercise bout to induce VEGFR-1 expression through a HIF-1 dependent mechanism but *study I* showed that the oxygen tension on the venous side declines substantially during exercise, why such a mechanism cannot be excluded. The similarity in VEGF-A and VEGFR-1 expression pattern might also result from stimuli

associated to reduced oxygen tension, i.e., oxygen tension in other cells may influence the VEGF-R1-expression in the endothelial cells through paracrine stimulation. In fact, one such example is that VEGFR-2 is shown to be induced through autocrine or paracrine stimulation by increased levels of VEGF-A (Gerber et al., 1997). In *study V*, VEGFR-2 mRNA expression and VEGF-A protein levels were observed in the R-condition, suggesting that VEGF-A is involved in the regulation of VEGFR-2 in response to exercise.

Study V provide additional support for the importance of metabolic stimuli in the regulation of the VEGF-receptor system in the exercising skeletal muscle. The mRNA levels of the VEGF receptors tended to increase in response to a single bout of exercise in the NR-condition after 5 weeks of training in the leg trained in the R-condition. Given the experimental design, it may be possible that the training workload after 5 weeks reached a level that reduced oxygen tension and caused metabolic perturbation. If so, it adds further support for a role of oxygen tension and metabolic perturbation in the regulation of the VEGF-receptor system in exercising skeletal muscle.

The inhibition of NO suppresses the VEGFR-2 expression induced by electrical stimulation (Milkiewicz et al., 2005). Since shear stress and NO may influence the expression of VEGF-A, and VEGF-A is a potent activator of VEGFR-2 (discussed above) it is not known whether the changes in VEGFR-2 reflect changes in VEGF-A expression induced by shear stress. Nevertheless, as argued above one might assume that shear stress was lower during exercise in the R- than in the NR-condition and hence differences in shear stress cannot explain the observed differences in VEGFR-2 expression between the two exercise conditions with training as observed in *study V*.

Summary: The similarity between VEGF-A and VEGFR-1 mRNA expression both to a single bout of exercise and repetitive exercise bouts suggest that they might have associated regulating stimuli, e.g. through HIF-1 pathways since both factors have a hypoxic response element in their promoters. The observation that basal levels of VEGFR-2 mRNA in human skeletal muscle increased only with repetitive exercise bouts with restricted leg blood flow is novel, and suggests that VEGF-A is a possible regulator of VEGFR-2 expression during this condition.

6.3.3 Angiopoietin and Tie system

Many cell types in the skeletal muscle express Ang-1 and Ang-2, and thus could influence the expression in skeletal muscle tissue of these factors in skeletal muscle tissue (Kelly et al., 2003). The effects of exercise on angiopoetin and Tie has only been studied in this thesis work and in the study by Lloyd et al., who explored the effects of these factors in the rat in response to voluntary exercise (Lloyd et al., 2003). Therefore, much less is known about the

regulation of Ang-Tie systems compared with VEGF-A system. Although, the expression of Ang-1 mRNA decreases in response to hypoxia in skeletal muscle and in response to ischaemia in myocardial muscle cells (Kelly et al., 2003, Sandhu et al., 2004). The present finding of decreased Ang-1 mRNA expression after 10 days is in part consistent with the data of Kelly et al. because a significant reduced oxygen tension was observed with exercise in both R- and NR-condition. Although, no differences were observed between R-condition and NR-condition in Ang-1 mRNA expression, which might challenge the role of an exercise-induced reduction of oxygen tension in the regulation of Ang-1.

The regulating mechanisms behind the increase in Tie-1 are unclear but it has been shown that VEGF-A increases the expression of Tie-1. However, the increase in Tie-1 expression occurred in exercise both in the R-condition and in the NR-condition, whereas the VEGF-A protein increased only in the R-condition. These findings indicate that other regulatory mechanisms must contribute to the increase in Tie-1.

Summary: This is the first study on the effects of exercise on the Angiopoietin and Tie systems during different environmental condition. The weak relationship (only increase in mRNA ratio Ang-2 to Ang-1) to exercise conditions suggests that oxygen levels and metabolic perturbation maybe less important for the regulation of these factors than it is for VEGF-A.

7. SUMMARY AND CONCLUSION

A single bout of endurance exercise increased the expression of VEGF-A mRNA, but not of FGF-2 mRNA in human skeletal muscle. Metabolic perturbation induced by exercise with restricted leg blood flow, enhanced the VEGF-A mRNA response. Also the expression of the VEGF receptors, VEGFR1 and VEGFR-2 increased at the mRNA level in response to a single bout of exercise.

The recognized splice variants in skeletal muscle were VEGF-A_{121}, VEGF-A_{165} and VEGF-A_{189}, which all increased in response to a single bout of exercise. However, the relative distribution of VEGF-A splice-variant expression changed after exercise, initially with a relative increased expression of VEGF-A_{165}, followed by a later relative increase of VEGF-A_{189}. Metabolic perturbation did not influenced this shift. It is tempting to speculate that this reflects different events such as early endothelial activation (VEGF-A_{165}) followed by a later chemo-attractant and differentiation role (VEGF-A_{189}).

Repetitive bouts of endurance exercise with restricted blood flow increased basal VEGF-A protein levels, but not basal VEGF-A mRNA, which increased only temporarily in association to each single bout of exercise. This finding is potentially important since it further supports involvement of VEGF-A system in human exercise-induced angiogenesis and shows how the changes in skeletal VEGF-A mRNA and protein levels are related to each other in response to exercise bouts in humans.

Repetitive bouts of endurance exercise with restricted blood flow also increased the basal level of VEGFR-2 mRNA, but not of VEGFR-1, which suggests a selective contribution of the VEGF-A receptors in exercise-induced angiogenesis. The concurrent increase in levels of VEGF-A protein and VEGFR-2 mRNA levels after exercise with restricted blood flow, suggest that VEGF-A is a possible regulator of VEGFR-2 expression during this condition as reported from other experimental settings.

The lack of uptake of VEGF-A into skeletal muscle from the circulation indicates that the blood stream is not a source for the observed increase in skeletal muscle VEGF-A after exercise.

Repetitive bouts of endurance exercise increased the basal level of Ang-2 protein and the mRNA ratio Ang-2 to Ang-1 in skeletal muscle. This might have a "permissive" effect on angiogenesis by allowing the increase in VEGF-A to stimulate endothelial migration and proliferation. The weak relationship between the exercise-induced enhancement of the angiopoietin system on the one hand and leg blood flow condition on the other, suggests that

oxygen levels and metabolic perturbation may be less important for the regulation of factors in the angiopoietin than in the VEGF-A system.

Several components of the HIF-1 pathway were activated in response to acute changes in oxygen demand in human skeletal muscle, which is the first evidence that human HIF-1 can be fully activated under physiological conditions, supporting the concept that oxygen-sensitive pathways are relevant for adaptation to physical activity, including the expression of VEGF-A and thus in exercise-induced angiogenesis.

The present data show that the exercise-induced increase in VEGF-A is more complex than activation of HIF-1 alone. The relationship between the exercise-induced increase in venous lactate concentration and VEGF-A mRNA expression and the higher lactate concentration and VEGF-A mRNA expression after exercise with restricted blood flow indicates that energy metabolites may be involve in the regulation of exercise induce VEGF-A expression.

8. FINAL REMARKS

This thesis has shown that the VEGF-A and angiopoietin systems are regulated in human skeletal exposed to exercise. This together with the observation that the VEGF-A system was influenced more, when oxygen delivery was reduced to and/or the related metabolic perturbation was larger in the exercising leg, provide some new insight into the complex regulation of angiogenic factors under physiological condition in humans. Since this thesis work focused only on the most well-characterized angiogenic factors, in particular the VEGF-A system, it can only provide a simplified version. Future studies in humans are needed to integrate the growing basic knowledge from animal studies regarding various angiogenic factors and their interaction.

Human skeletal muscle is an optimal tissue to study the molecular mechanisms in angiogenesis as well as other mechanisms underlying adaptive processes and signalling pathways in humans because it is accessible in amounts needed for analysis at the mRNA and protein levels. Newer methods, such as laser dissection of tissue sections provide ways to analyse the role of different types of cells. Physical exercise is also an ideal model for studying angiogenesis because it is one of the few conditions in which angiogenesis occurs in healthy humans and because it can be applied in a controlled manner (e.g., by varying intensity, duration and frequency), which makes it possible to study the effects of different metabolic and environmental conditions (e.g., oxygen, substrate and metabolic availability) on angiogenesis as well as on other adaptation mechanisms.

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10. RELATED PUBLICATIONS by Thomas Gustafssson

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