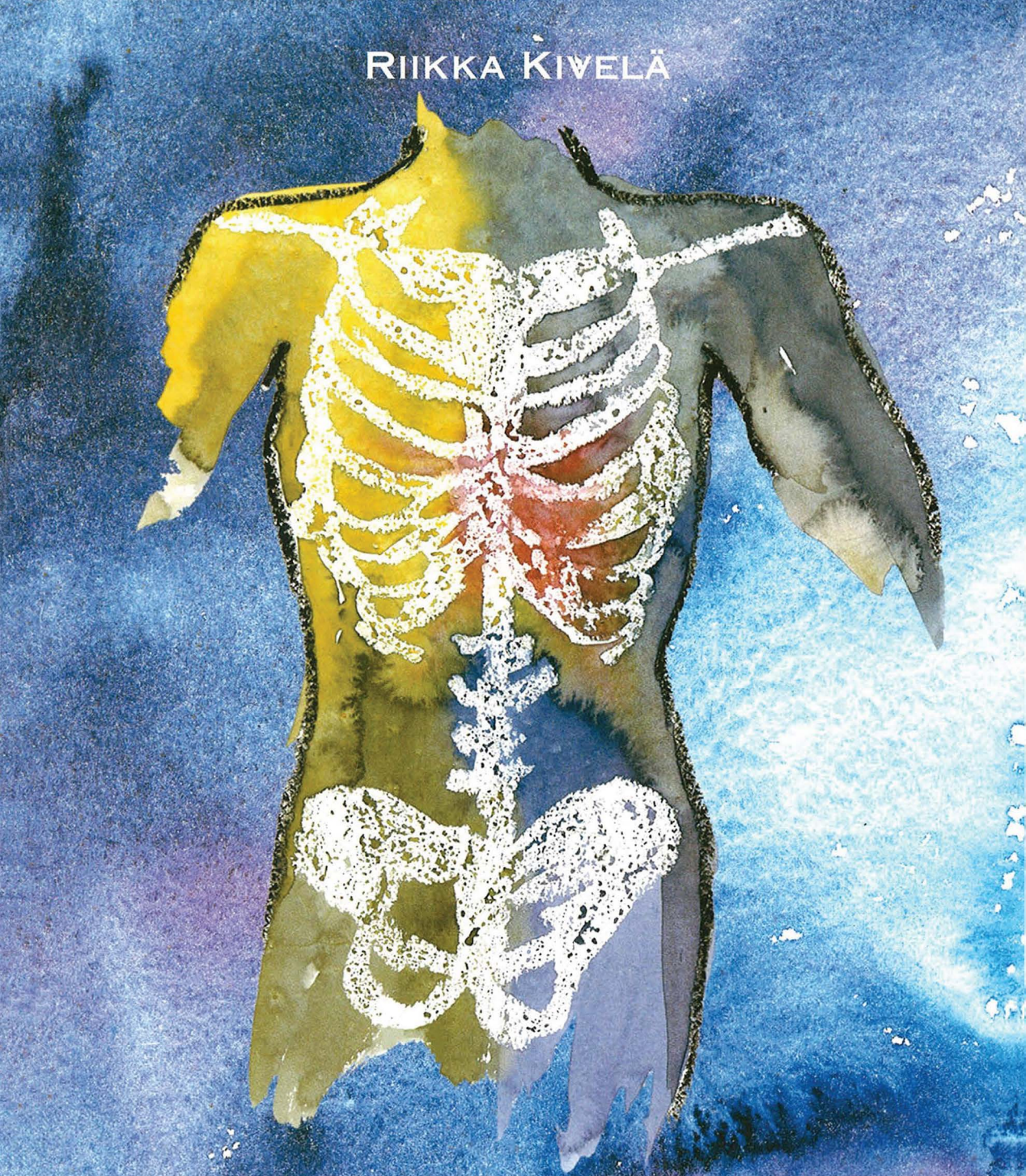
Riikka Kivelä



BLOOD AND LYMPHATIC VESSELS AND THEIR GROWTH FACTORS IN SKELETAL MUSCLE: EFFECTS OF EXERCISE AND DIABETES

LIKES - Research Reports on Sport and Health 209

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Doctoral thesis, University of Jyväskylä
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ABSTRACT

Riikka Kivelä. Blood and lymphatic vessels and their growth factors in skeletal muscle: effects of exercise and diabetes.
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The structure and function of the circulatory system are essential for the metabolism and function of skeletal muscles. In healthy muscles exercise promotes the growth of new capillaries; a process called angiogenesis, and increases the production of angiogenic growth factors. In diabetic skeletal muscles angiogenesis has been shown to be impaired. The purpose of the present study was to investigate the effects of exercise and diabetes on blood and lymphatic vessels and their growth factors in skeletal muscle. Diabetes decreased the mRNA level of many genes known to be involved in the regulation of angiogenesis, most interestingly those of VEGF-A and VEGF-B together with their receptors VEGFR-1, VEGFR-2 and neuropilin-1. Diabetes also increased mRNA levels of TSP-1, a known inhibitor of angiogenesis. The present study showed that streptozotocin-induced diabetes and the subsequent hyperglycemia reduce the mRNA levels of proangiogenic proteins and increase those of antiangiogenic ones together with decreased capillarization. This change of balance may be one of the major reasons for the markedly increased risk for peripheral vascular complications in diabetes. Endurance training alleviated some of these changes but did not fully restore the diabetes-induced defects. These training effects, seen in the mRNA levels of angiogenesis–related genes, may be one of the mechanisms responsible for the beneficial effects of regular endurance exercise in diabetic patients. This study also provided evidence that diabetes and exercise affect the production of angiogenic factors in both capillaries and muscle fibres, and that the responses are more pronounced in capillaries. With the newly found specific markers for the lymphatic endothelium, LYVE-1 and VEGFR-3, capillary-sized lymphatic vessels were visualised for the first time in human and mouse skeletal muscle capillary bed. Lymphangiogenesis growth factors VEGF-C and VEGF-D were found in skeletal muscle, but exercise did not affect their expression or the number of lymphatic capillaries. Diabetes increased the expression of VEGF-D and this seemed to be related to muscle fiber damage. Obesity, metabolic syndrome and type 2 diabetes are rapidly increasing in the western world. Human diabetes is closely related to central and peripheral cardiovascular diseases. To be able to efficiently prevent and treat diabetes-related cardiovascular problems it is important to know the mechanisms by which diabetes negatively affects the circulation. This study provided data on the mechanisms by which exercise affects capillarization also in diabetic skeletal muscle.

Key words: angiogenesis, skeletal muscle, exercise, diabetes, lymphatic vessels

TIIVISTELMÄ

Riikka Kivelä. Liikunnan ja diabeteksen vaikutukset raajalihasten veri- ja imusuonistoon ja niiden kasvutekijöihin. (Blood and lymphatic vessels and their growth factors in skeletal muscle: effects of exercise and diabetes)
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Väitöskirja

Verenkierron, verisuoniston ja erityisesti lihassyiden ympärillä olevien kapillaarien merkitys on keskeinen lihaksen aineenvaihdunnan ja toimintakyvyn kannalta. Liikunnan tiedetään lisäävän kapillaarien määrää lihaksissa terveillä, kun taas diabeteksen on havaittu vaikuttavan raajojen verenkiertoa heikentävästi. Tämän väitöskirjatutkimuksen tarkoituksena oli selvittää liikuntaharjoittelun vaikutuksia veri- ja imusuoniston rakenteeseen sekä suonten kasvutekijöiden geenien ilmenemiseen terveillä ja diabeettisilla hiirillä. Diabetes vähensi lihaksen kapillarisaatiota ja vaikutti heikentävästi monen verisuonten kasvua eli angiogeneesiä edistävän geenin ilmenemiseen (esim. VEGF-A, -B, neuropilin-1, VEGFR-1 ja -2). Lisäksi angiogeneesia estävien tekijöiden (esim. TSP-1) ilmeneminen kasvoi lihaksissa. Kestävyysharjoittelu lievensi diabeteksen vaikutuksia, muttei pystynyt niitä kokonaan eliminoimaan. Tutkimustulokset osoittivat että lihaskudoksessa sekä kapillaarit että lihassyyt ilmentävät angiogeneesiin positiivisesti ja negatiivisesti vaikuttavia tekijöitä. Tulokset osoittivat myös CCN-perheen proteiinien reagoivan kohonneeseen veren glukoosipitoisuuteen sekä kovaan fyysiseen kuormitukseen. Nämä proteiinit liittyvät mm. kudosten soluvälitilan muokkaukseen ja angiogeneesiin. Tässä tutkimuksessa osoitettiin ensimmäistä kertaa, että imusuonia esiintyy lihaksessa myös lihassyiden lomassa verisuonikapillaarien vieressä eikä ainoastaan suurten verisuonten lähistöllä lihassyykimppujen välissä kuten aiemmin on oletettu. Myös imusuonten kasvutekijöitä (VEGF-C ja -D) ilmeni lihaskudoksessa. VEGF-D:n lisääntyminen diabeteksen yhteydessä näyttää liittyvän lihassyiden degeneraatioon, makrofagien tunkeutumiseen kudokseen tai lihassyiden regeneraatioon, mikä mahdollisesti johtuu veren kohonneesta glukoosipitoisuudesta. Liikuntaharjoittelulla ei ollut vaikutusta imusuonten määrään eikä niiden kasvutekijöiden geenien ilmenemiseen. Imusuonten vaste kuormitukseen näyttäisi siis poikkeavan verisuonista, joiden on osoitettu lisääntyvän harjoittelun seurauksena. Lihavuus, metabolinen oireyhtymä ja tyypin 2 diabetes ovat sekä Suomessa että maailmanlaajuisesti yleistyneet valtavasti viime vuosina. Ihmisillä diabeteksen yhteydessä esiintyy yleisesti sydän- ja verisuonisairauksia, erityisesti raajoissa. Jotta diabetekseen liittyviin verenkierron häiriöihin voitaisiin vaikuttaa entistä tehokkaammin, on tärkeää selvittää niiden taustalla olevia mekanismeja. Tämän tutkimuksen tulokset antavat viittcitä siitä, kuinka liikunta voi vaikuttaa positiivisesti diabeettisen lihaksen verisuonistoon.

Avainsanat: angiogeneesi, lihas, liikunta, diabetes, imusuonisto

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Jyväskylä, March 2008,

Riikka Kivelä

"Those who think they have not time for bodily exercise will sooner or later have to find time for illness."

Edvard Stanley, Earl of Derby (1799-1869)

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on following original articles, which are referred to in the text by their Roman numerals.

I Kivelä R, Silvennoinen M, Touvra A, Lehti M, Kainulainen H & Vihko V (2006). Effects of experimental type 1 diabetes and exercise training on angiogenic gene expression and capillarization in skeletal muscle. *The FASEB Journal* 20: 1570-72 (E921-E930).
https://doi.org/10.1096/fj.05-4780fje

II Kivelä R, Silvennoinen M, Lehti M, Jalava S, Vihko V & Kainulainen H (2007). Expression of angiogenic growth factors in skeletal muscle and in capillaries after exercise in healthy and diabetic mice. *Submitted for publication.*
https://doi.org/10.1186/1475-2840-7-13

III Kivelä R, Kyröläinen H, Selänne H, Komi PV, Kainulainen H & Vihko V (2007). A single bout of exercise with high mechanical loading induces the expression of Cyr61/CCN1 and CTGF/CCN2 in human skeletal muscle. *Journal of Applied Physiology* 103: 1395-401.
https://doi.org/10.1152/japplphysiol.00531.2007

IV Kivelä R, Havas E & Vihko V (2007). Localisation of Lymphatic Vessels and Vascular Endothelial Growth Factors-C and -D in Human and Mouse Skeletal Muscle with Immunohistochemistry. *Histochemistry and Cell Biology* 127: 31-40.
https://doi.org/10.1007/s00418-006-0226-x

V Kivelä R, Silvennoinen M, Lehti M, Kainulainen H & Vihko V (2007). Effects of acute exercise, exercise training, and diabetes on the expression of lymphangiogenic growth factors and lymphatic vessels in skeletal muscle. *American Journal of Physiology – Heart and Circulatory Physiology* 293: H2573-9.
https://doi.org/10.1152/ajpheart.00126.2007

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ABBREVIATIONS

AGE	Advanced glycation end product
Ang-1, -2	Angiopoietin-1 and -2
CK	Creatine kinase
CS	Citrate synthase
CTGF/CCN2	Connective tissue growth factor
Cyr61/CCN1	Cysteine-rich angiogenic protein 61
ECM	Extracellular matrix
ELISA	Enzyme linked immunosorbent assay
FGF	Fibroblast growth factor
GAPDH	Glyseraldehyde-3-phosphate dehydrogenase
HGF	Hepatocyte growth factor
HIF-1	Hypoxia-inducible factor 1
LCM	Laser capture microdissection
LYVE-1	Lymphatic vessel endothelial receptor 1
MMP	Matrix metalloproteinase
MnSOD	Manganese Superoxide Dismutase
mRNA	Messenger RNA
MVC	Maximum voluntary contraction force
NGF	Nerve growth factor
NO	Nitric oxide
NOS	Nitric oxide synthase
Nrp-1, -2	Neuropilin-1 and -2
PDGF	Platelet-derived growth factor
QPCR	Quantitative polymerase chain reaction
RAGE	Receptor for advanced glycation end products
Rbl2	Retinoblastoma-like 2
ROS	Reactive oxygen species
TIMP	Tissue inhibitor of metalloproteinases
TSP-1	Thrombospondin 1
VEGF (-A, -B, -C, -D, -E)	Vascular endothelial growth factor A, B, C, D, and E
VEGFR (-1, -2, -3)	Vascular endothelial growth factor receptor 1, 2 and 3

Group abbreviations

Studies I & V

C (1, 3, 5) Healthy sedentary mice; samples taken after one, three or five weeks
D (1, 3, 5) Diabetic mice; samples taken after one, three or five weeks
T (1, 3, 5) Healthy exercise trained mice; trained for one, three or five weeks
DT (1, 3, 5) Diabetic exercise trained mice; trained for one, three or five weeks

Studies II &V

H Healthy control mice
D Diabetic control mice
HE3 Healthy exercised mice, killed 3 h post exercise
HE6 Healthy exercised mice, killed 6 h post exercise
DE3 Diabetic exercised mice, killed 3 h post exercise
DE6 Diabetic exercised mice, killed 6 h post exercise

CONTENTS

1 INTRODUCTION

The function of the circulation is to maintain optimal environment for the cells by transporting oxygen and nutrients to and waste products away from tissues. The circulatory system consists of a dense network of blood and lymphatic vessels, which penetrates most tissues in the body. Physical exercise exerts significant stress on the circulatory system, especially in skeletal muscles. Remodelling of the capillary network is essential for the physiological adaptation of skeletal muscle to exercise, and the phenomenon has been studied since 1930's. Despite the fact that lymphatic vessels were first described as long ago as 1627 by Gasparo Aselli, and that they have vital functions in the body, much less is known about the lymphatic than blood vessels. The main reason for this has been the lack of specific staining methods for lymphatic vessels. Recently, specific markers for the lymphatic endothelium have been identified in cancer research. This led to the present interest in studying the structure of the lymphatic system in skeletal muscles in more detail, and furthermore, its possible adaptations to exercise stimulus.

Cardiovascular complications are the leading cause of morbidity and mortality in patients with diabetes. In the end of 1990s it was shown that the growth of new blood vessels, a process called angiogenesis, is significantly reduced in diabetes (Abaci *et al.* 1999; Rivard *et al.* 1999). The mechanisms by which diabetes could limit the formation of new blood vessels or the survival of existing ones remain largely undefined. In the present studies diabetes-induced effects on blood and lymphatic vessels and their growth factors in skeletal muscles were investigated, and the question whether these impairments could be reversed, or at least attenuated, by physical exercise was addressed. The mechanisms behind exercise-induced angiogenesis in healthy muscle have been investigated by several groups during the last years, and considerable progress has been achieved to undercover the complex pathways regulating angiogenesis. However, the whole picture is not yet clear, and it is not known how diseases affect the exercise response.

Exercise is widely recommended in the management of both type 1 and type 2 diabetes. It is important to understand the molecular basis of exercise and muscle

adaptation to physical activity, both in health and disease. Most patients with diabetes or chronic cardiovascular diseases have markedly reduced exercise tolerance. One important question is whether their skeletal muscles are pathologically abnormal, or whether they exhibit dysfunction because of a sedentary lifestyle or because of their hypoxic or ischemic environment. Thus, in order to establish a rational basis for exercise programs for the healthy population and people with various chronic diseases, we need to know how the molecular mechanisms in muscle are affected by exercise, and are the responses similar in healthy and diseased skeletal muscle.

2 REVIEW OF THE LITERATURE

2.1 Overview of the skeletal muscle structure

Skeletal muscle is the largest organ in the human body; accounting for about 40 per cent of our body weight. The main function of the musculature is to generate force and to produce movement. All muscles are made of fibres (muscle cells), which lie parallel to each other, extending the entire length of the muscle. Fibres in turn contain numerous myofibrils, which consist of myosin and actin filaments that are responsible for muscle contraction (Guyton & Hall 2006). Each muscle fibre is surrounded by a thin layer of connective tissue, mostly collagen, and this layer is called the endomysium. Only capillaries and the finest neuronal branches are found between the individual muscle fibres in the endomysium (Ross *et al.* 2003). The perimysium is a tough and relatively thick layer of connective tissue that surrounds a group of muscle fibres to form a bundle, or fascicle. Muscle fibres are polygonal in shape in order to enable the greatest number of fibres to be fitted within the fascicle (McComas 2006). This diminishes the interstitial space between the muscle fibres. For skeletal muscle, an interstitial fluid volume of 0.1 ml/g wet weight has been calculated, which is about ¼ of that in skin (Aukland & Reed 1993). The arterioles, venules and nerves travel between the fascicles in the perimysium. The epimysium is a sheath of dense connective tissue that surrounds all the fascicles in the muscle. The largest vessels and nerves penetrate through the epimysium (Ross *et al.* 2003). Figure 1 shows a schematic representation of skeletal muscle structure.

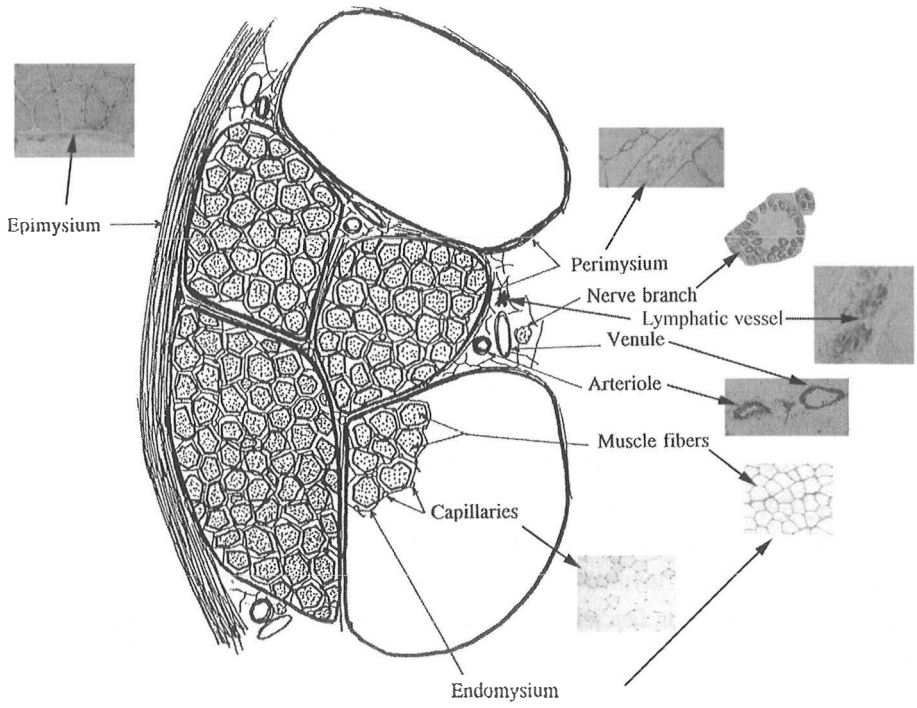


FIGURE 1. Schematic figure of skeletal muscle cross-section with microscopy images of the various structures present. The figure is modified from McComas (2006).

2.2 Blood and lymphatic vessels in skeletal muscle

2.2.1 Blood circulatory system

The circulatory system transports oxygen and nutrients to the tissues and waste products from the tissues in order to maintain an optimal environment for the function and survival of the cells. The blood vessel network comprises of arteries, arterioles, capillaries, venules and veins. The function of the arteries is to transport blood under high pressures to the tissues, and they contain strong vascular walls (Guyton & Hall 2006). The tight regulation of tissue blood flow occurs at the level of the arterioles. Contraction of the smooth muscle in the wall of arteriole can reduce or even completely shut off the blood flow to the capillaries (Ross *et al.* 2003). This tight regulation is important in directing blood flow where it is needed. Indeed, physiological blood flow in skeletal muscles is well adapted in relation to tissue needs, *e.g.*, at rest or during exercise (Saltin *et al.* 1998).

The most important function of the circulation occurs in the capillaries. The capillary walls are very thin and permeable containing only a single layer of endothelial cells and basal lamina (Ross *et al.* 2003). Thin vessel walls and close proximity to metabolically active cells enables efficient exchange of fluids, gases, nutrients, electrolytes, hormones and other substances between the blood and the interstitial fluid. Capillaries have a diameter just large enough to allow the passage of one red blood cell at a time (<10 μm) and a length of about 0.3-1 mm. The blood remains in the capillaries for about 1-3 s and all diffusion has to occur during this time (Guyton & Hall 2006). Capillaries in skeletal muscles are continuous and some of them contain pericytes in association with the endothelium (Ross *et al.* 2003). As relatively undifferentiated cells, pericytes serve to support blood vessels, but can also differentiate into fibroblasts, smooth muscle cells, or macrophages. When blood leaves the capillaries it is collected by the venules and finally the larger veins transport it back to the heart. In addition to transport, veins are also important as a major reservoir of blood (Guyton & Hall 2006). Skeletal muscles usually have multiple arterial inflow vessels and venular outflow vessels (Schmid-Schönbein *et al.* 1986). Arterioles and

venules are found in the perimysial space, while capillaries are also located between the individual muscle fibres. This space is often referred as the skeletal muscle capillary bed.

2.2.2 Lymphatic system

Lymphatic vessels were first described around the beginning of the seventeenth century by Gasparo Aselli; however, the growth factors and specific markers for lymphatic endothelium were discovered only about a decade ago. The lymphatic system drains protein-rich interstitial fluid from tissues back to the blood. Due to their high permeability, lymphatic vessels also transport large proteins and dietary lipids in the small intestine, which cannot cross the blood capillary endothelium (Guyton & Hall 2006). Before lymph is returned to the blood, it is processed in the lymph nodes, which are an integral part of the immune defence system. Thus, the lymphatic system is an important part of both the vascular and immune systems in the body (Alitalo *et al.* 2005). The interplay between blood and lymphatic capillaries and the interstitium is presented in Figure 2. The lymphatic system consists of an extensive network of initial capillaries, collecting vessels and ducts. It permeates most of the body's tissues except avascular structures such as cartilage, epidermis, hair, nails and cornea, and some vascularized organs such as the brain and retina (Ryan *et al.* 1986; Schmid-Schönbein 1990). Unlike the blood vascular system, which forms a closed system with arterial and venous vessels, the lymphatic system is an open ended, one-way transit system draining protein-rich lymph from the interstitium back into the blood. When compared with the blood vascular endothelium, the lymphatic endothelium shows specific morphological and molecular characteristics. Lymphatic capillaries have an irregular or collapsed lumen with no red blood cells, a discontinuous basement membrane and anchoring filaments, which connect the lymphatic endothelial cells to the extracellular matrix (Schmid-Schönbein 1990). Unlike blood capillaries, lymphatic capillaries do not have pericyte coverage. Thin-walled lymphatic capillaries are highly permeable and contain only a single-layer of endothelial cells (Ryan *et al.* 1986; Skalak *et al.* 1984; Schmid-Schönbein 1990).

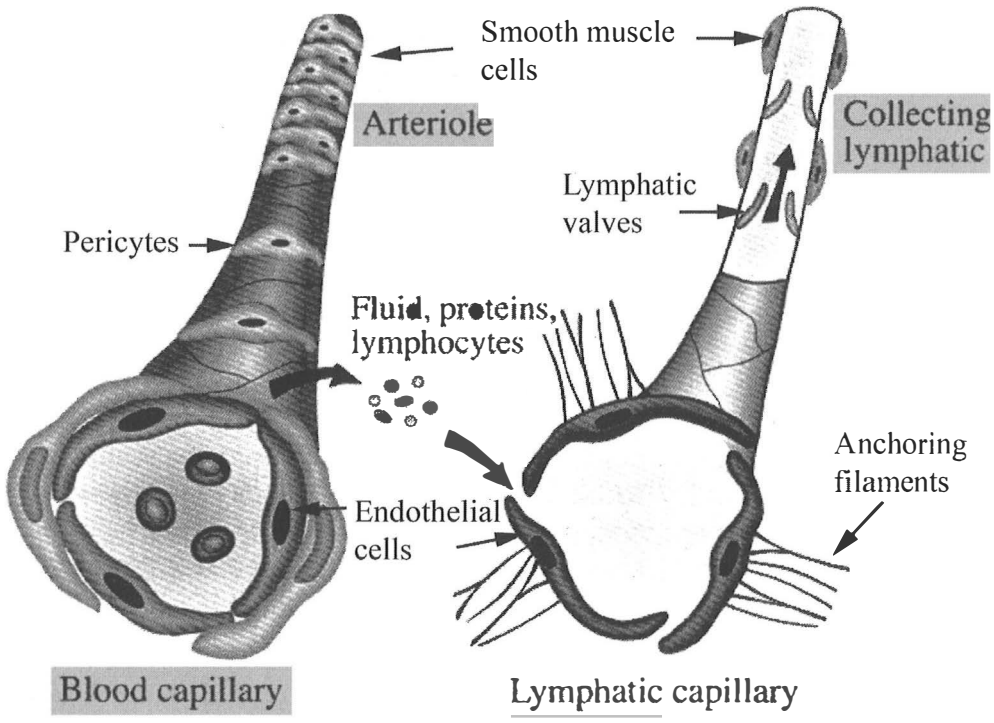


FIGURE 2. The structure and interplay of blood and lymphatic vessels. Fluid and large proteins are extravasated from blood capillaries to interstitial fluid and collected by lymphatic capillaries. Lymphatic vessels have important role in maintaining the normal interstitial pressure by removing extra fluid, proteins and cells, which cannot re-enter to the blood, from the interstitium. The figure is modified from Guyton and Hall (2006) and Mäkinen *et al.* (2007).

The present morphological picture of the lymphatic system in skeletal muscles is based on a few light and electron microscopy studies on rat spinotrapezius (Skalak *et al.* 1984; Schmid-Schönbein 1990), gastrocnemius and soleus muscles (Stingl & Stembera 1974). The lymphatic system in skeletal muscle seems to differ from that in most other tissues. It consists only of initial lymphatic capillaries, which do not contain smooth muscle cells. They follow transverse and arcading arterioles, but are much fewer in number and are not known to extend to the capillary bed (Skalak *et al.* 1984). Until

recently, histological studies of lymphatic vessels have been hampered by the lack of specific staining methods. To date, specific markers for the lymphatic endothelium have been identified, especially in connection with cancer research (Sleeman *et al.* 2001; Jussila & Alitalo 2002; Jackson 2003; Oliver & Alitalo 2005). Recent data on the diaphragmatic lymphatic system suggests that lymphatic capillaries may also be found in the capillary bed in the diaphragm (Grimaldi *et al.* 2006).

2.2.3 Mechanisms of blood and lymphatic vessel growth

2.2.3.1 Development of the vascular systems

The blood vasculature is the first organ system to develop in the embryo (Carmeliet 2003). The differentiation of hemangioblasts from the mesoderm and the formation of primitive blood vessels from hemangioblasts at or near the site of their origin are the two distinct steps during the onset of vascularization that are defined as vasculogenesis (Risau & Flamme 1995). During vasculogenesis, endothelial progenitor cells build up a primitive vascular network with arteries and veins. The growth of blood vessels, a process called angiogenesis, is essential for tissue growth and repair. Angiogenesis expands the network, and capillaries are formed between the arteries and the veins. Pericytes and smooth muscle cells appear gradually to cover the endothelial tubes, and finally a complex and organized network is formed (Carmeliet 2005).

Lymphatic vessels begin to develop in embryos around midgestation when they are needed for the regulation of interstitial pressure. The origin of the lymphatic vessels has been controversial (Jussila & Alitalo 2002). Recent experimental data from mice support the idea proposed by Florence Sabin some 100 years ago. The hypothesis states that lymphatic endothelial cells arise by sprouting from embryonic veins, and form primary lymph sacs and primary lymphatic plexus with capillary-like vessels, resulting in a network of lymphatic capillaries and collecting lymphatic vessels (Alitalo *et al.* 2005). There are morphological similarities between blood vessel and lymphatic vessel

remodelling, but these two types of vessels are connected only at few precise locations, such as at the junctions of subclavian and jugular veins (Mäkinen *et al.* 2007).

2.2.3.2 Angiogenesis, arteriogenesis and lymphangiogenesis

The term vasculogenesis is used to describe the true *de novo* development of new blood vessels, whereas arteriogenesis and angiogenesis describe the formation of blood vessels and lymphangiogenesis the formation of lymphatic vessels from the pre-existing vascular structures. To what degree true vasculogenesis takes place in the adult organism is a controversial issue (Grundmann *et al.* 2007). Postnatal vessel growth is still related to organ growth. Most blood vessels remain quiescent and angiogenesis appears to occur only in the female reproductive system. Angiogenesis can also be activated, *e.g.*, in wound healing, in tumour growth, and in response to physical activity.

Arteriogenesis is a process of formation or remodelling of arterioles and arteries. It involves the enlargement of existing arteries, and the proliferation of endothelial cells, smooth muscle cells and fibroblasts (Heil *et al.* 2006). There is strong evidence that increase in shear stress is the most important stimulus for arterial enlargement (Grundmann *et al.* 2007). Arteriogenesis is a powerful mechanism to ensure blood supply to a tissue at risk, if a main artery is chronically occluded. In skeletal muscle exercise training has been shown to increase collateral-dependent blood flow indicating enhanced arteriogenesis in rats (Lloyd *et al.* 2001). Muscle stretch also induces the growth of the arterial network. This has been shown to precede the growth of capillaries (Hansen-Smith *et al.* 2001).

Angiogenesis is the formation of new small capillaries from the pre-existing capillary network in the tissues, and is in most cases induced by local ischemia (Risau 1997; Carmeliet 2000; Carmeliet 2005). Angiogenesis can occur as sprouting or non-sprouting angiogenesis (Risau 1997). In sprouting angiogenesis, the basement membrane and extracellular matrix are first degraded, then endothelial cells proliferate,

migrate and form a new lumen branching from the original vessel (Ausprunk & Folkman 1977; Risau 1997; Carmeliet 2000; Haas *et al.* 2000; Carmeliet 2003). Non-sprouting angiogenesis is a process of splitting the pre-existing vessels by proliferation of endothelial cells inside a vessel or by extracellular material penetration (Burri & Tarek 1990; Risau 1997). Both the sprouting and non-sprouting forms of physiological angiogenesis exist in skeletal muscle (Egginton *et al.* 2001). Angiogenesis via longitudinal capillary splitting occurs after chronic elevation of shear stress by increased blood flow, and angiogenesis by capillary sprouting, in turn, can be induced by stretch as a result of muscle overload (Egginton *et al.* 2001; Williams *et al.* 2006a). After electrical stimulation, which increases blood flow and muscle loading, both types of angiogenesis take place (Egginton *et al.* 2001).

Lymphangiogenesis refers to the growth of new lymphatic vessels. Lymphatic vessel proliferation occurs via sprouting from pre-existing ones, which is suggested to occur analogous to angiogenesis (Jeltsch *et al.* 1997). During embryonic development angiogenesis and lymphangiogenesis are thought to occur in parallel (Jussila & Alitalo 2002). Postnatal lymphangiogenesis has also been demonstrated, *e.g.*, in tumours, after myocardial infarction and in wound healing, but the underlying mechanisms are not completely understood (Tammela *et al.* 2005b; Mäkinen *et al.* 2007). In addition to pathological lymphangiogenesis, the occurrence and the role of physiological lymphangiogenesis in various adult tissues is not known.

2.2.4 Effects of exercise on capillarization in healthy skeletal muscle

Capillary growth (angiogenesis) in adult skeletal muscle due to muscle activity and especially during endurance exercise training, is a well-established phenomenon (Hudlicka *et al.* 1992). It was first reported by Vanotti and Pfister already in 1933 and Vanotti and Magiday in 1934 with electrical stimulation in rabbit skeletal muscle (Vanotti & Pfister 1933; Vanotti & Magiday 1934). In humans, increased capillarization after physical training and differences between different types of muscles were demonstrated about 40 years later (Andersen 1975; Andersen &

Henriksson 1977; Brodal *et al.* 1977; Andersen & Kroese 1978; Ingjer & Brodal 1978). The increased number of capillaries facilitates oxygen delivery and utilization in the muscles by increasing the diffusion surface area, decreasing the diffusion distance, and increasing the blood transit time to allow more efficient exchange of oxygen, nutrients and waste products between muscle fibres and blood. Thus, capillary network remodelling is essential for the physiological adaptation of muscle to exercise. However, regular physical activity seems to be needed to maintain the improved capillary network, as detraining has been shown to induce capillary regression already within the 2-3 weeks after the cessation of training (Klausen *et al.* 1981; Mujika & Padilla 2001). However, it has also been shown that increased capillarization may be maintained up to three months after training has ended (Coyle *et al.* 1984).

Increased capillarization is usually coupled with an increase in the oxidative capacity of muscle, *i.e.*, higher oxidative enzyme activity and volume density of mitochondria (Hudlicka *et al.* 1992). Angiogenesis in skeletal muscle occurs in response to mechanical stress induced by increased shear stress and cell stretch (Hudlicka *et al.* 1992; Rivilis *et al.* 2002; Prior *et al.* 2004; Milkiewicz *et al.* 2007). In endothelial and muscle cells these mechanical stimuli induce chemical and molecular responses, which are further discussed below, initiating the angiogenic processes. No data exists on the effects of exercise on the remodelling of the lymphatic system.

2.2.5 Diabetes and blood vessels

2.2.5.1 Subtypes of diabetes

Diabetes can be classified into two main subtypes, insulin-dependent diabetes mellitus (type 1 diabetes) and non-insulin-dependent diabetes mellitus (type 2 diabetes). Type 1 diabetes is caused by an auto-immune reaction where the body's defence system attacks the insulin-producing cells, thus people with type 1 diabetes produce very little or no insulin. The mechanisms causing type 1 diabetes are not yet fully understood. In Finland, the incidence of type I (insulin-dependent) diabetes in children aged 14 years or under is the highest in the world, and the trend in incidence has been increasing (Tuomilehto *et al.* 1999). The age at which the Finnish population is at risk for type 1 diabetes extends also into young adulthood (Lammi *et al.* 2007). The continuing increase in the incidence of type 1 diabetes has been a global phenomenon during the last few decades (DIAMOND Project Group 2006).

Type 2 diabetes constitutes about 85% to 95% of all diabetes cases in developed countries and accounts for an even higher percentage in developing countries (International Diabetes Federation, http://www.idf.org). In 2007, it is estimated that there are 246 million people with diabetes in the adult population and by the year 2025, the figure is expected to rise to 380 million. Worldwide one of the major current public health problems is the rapid increase in the incidence of type 2 diabetes in the young adult population (Lammi *et al.* 2007). People may have a genetic predisposition to type 2 diabetes but it is closely related to lifestyle factors such as unhealthy nutrition, physical inactivity and obesity. Unlike in type 1 diabetes, people with type 2 diabetes normally secrete insulin, but its effects on the target tissues are impaired. This is called insulin resistance. In both subtypes of diabetes blood glucose levels are elevated, which is regarded as the main factor causing diabetes-related complications.

2.2.5.2 Effects of diabetes on skeletal muscle vascularization

Diabetes is an important risk factor for central and peripheral cardiovascular diseases, which significantly increase morbidity and mortality (Kannel & McGee 1979; The diabetes control and complications trial research group 1993; Lehto *et al.* 1996; Laakso & Lehto 1998). Diabetes-induced remodelling of the skeletal muscle capillary bed is observed as decreased capillary luminal diameter, decreased capillary-to-fibre ratio, and reduced proportion of capillaries supporting continuous flow, reducing the capillary diffusing capacity and disturbing regional hemodynamic regulation (Sexton *et al.* 1994; Kindig *et al.* 1998). These observations are from animals with streptozotocin-induced experimental type 1 diabetes. In addition, capillary rarefaction and thickening of the capillary basement membrane has been observed in the obese Zucker rat, which is regarded as a model of the metabolic syndrome and type 2 diabetes (Lash *et al.* 1989; Frisbee 2005). It has also been shown that diabetes impairs angiogenesis and arteriogenesis in animal models of ischemia, and in ischemic human hearts (Abaci *et al.* 1999; Rivard *et al.* 1999; Li *et al.* 2006; Li *et al.* 2007). The mechanisms by which diabetes could cause microvascular rarefaction or limit the formation of new blood vessels remain largely unknown. The impairment in skeletal muscle capillarization may be one cause for the reduced glucose uptake in muscle fibres. Effects of diabetes on lymphatic vessels have not been studied.

Studies on the effects of exercise training on diabetic muscle capillarization have produced conflicting results. In type 1 diabetic patients, exercise training for 8 weeks did not increase the capillary-to-fibre ratio or capillary density, although both variables were increased in healthy subjects (Wallberg-Henriksson *et al.* 1984). In addition, no change was observed in capillary density in another exercise training study for 20 weeks with type 1 diabetic patients (Mandroukas *et al.* 1986), whereas a significant increase in the capillary-to-fibre ratio occurred after 4 months of physical training in a group of type 1 diabetic patients (Wallberg-Henriksson *et al.* 1982). In obese Zucker rats exercise training has been demonstrated to enhance muscle capillarization (Lash *et al.* 1989; Frisbee *et al.* 2006). The differences between the findings from these studies may arise from different training protocols used, and the type and duration of diabetes.

2.3 Molecular regulation of blood and lymphatic vessel growth

Angiogenesis involves multifactorial processes with both proangiogenic and antiangiogenic factors interacting with endothelial cells, smooth muscle cells and the extracellular matrix (Carmeliet 2003). It occurs in a highly regulated manner and varies between tissues and different stimuli. Much less is known about lymphangiogenesis, but similar mechanisms are thought to be involved. The major molecular players in angiogenesis and lymphangiogenesis known today, and the reported effects of exercise and diabetes on these factors are presented below.

2.3.1 Vascular endothelial growth factors (VEGFs)

VEGF-A

Vascular endothelial growth factor-A (VEGF-A, also known as VEGF) is a major mediator and essential for physiological and pathological angiogenesis (Risau 1997; Carmeliet 2003; Ferrara *et al.* 2003; Carmeliet 2005; Lee *et al.* 2007). Its cDNA sequence was first published in 1989 (Keck *et al.* 1989; Leung *et al.* 1989). However, already in 1983, a protein named vascular permeability factor (VPF) was identified on the basis of its ability to induce vascular leakage (Senger *et al.* 1983). The sequences later revealed that VPF and VEGF-A were the same molecule. VEGF-A is expressed as several isoforms, all of which are formed from the same *vegf* gene by alternative splicing (polypeptides with 121, 145, 165, 183, 189 and 206 amino acid residues) (Ferrara *et al.* 2003). These isoforms are thought to have distinct but overlapping functions in angiogenesis, and VEGF-A_{165} is the most prominent isoform in most tissues, *e.g.*, in skeletal muscle (Jensen *et al.* 2004b; Gustafsson *et al.* 2005). It is also suggested that one isoform cannot completely replace the function of another (Ferrara *et al.* 2003). The mouse and rat isoforms have one amino acid less than those of humans. VEGF-A is essential for embryonic angiogenesis, since inactivation of only one VEGF-A allele in mice resulted in embryonic death due to defective angiogenesis (Carmeliet *et al.* 1996). In addition to proliferation of endothelial cells, VEGF-A is needed for the survival and homeostasis of blood vessels (Gerber *et al.* 1998). Very

recently it was shown that autocrine VEGF-A signalling in endothelial cells is required to maintain blood vessel homeostasis, which could not be rescued by paracrine VEGF-A (Lee *et al.* 2007).

VEGF-A mRNA expression is induced by hypoxia in physiological and pathophysiological circumstances. The effects of hypoxia are mediated through the activation of hypoxia-inducible factor HIF-1 (Shweiki *et al.* 1992; Ferrara *et al.* 2003). Also several molecules, such as other growth factors and cytokines, and hypoglycaemia and insulin are involved in the regulation of VEGF-A production (Neufeld *et al.* 1999; Ferrara *et al.* 2003; Jiang *et al.* 2003; Lerman *et al.* 2003). Nitric oxide (NO) has also been shown to upregulate VEGF-A expression and, interestingly, NO production is also upregulated by VEGF-A, indicating a positive loop between these two molecules (Neufeld *et al.* 1999). In addition to VEGF-A, the vascular endothelial growth factor protein family consists of four other proteins: VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF) (Tammela *et al.* 2005a). VEGF protein family and their receptors are presented in the Figure 3.

VEGF-B

In contrast to VEGF-A, surprisingly little is known about the precise biological role of **VEGF-B**. VEGF-B is a secreted growth factor that has strong sequence homology with VEGF-A. The VEGF-B gene yields two isoforms, VEGF-B_{167} and VEGF-B_{186} by alterative splicing. It is abundantly expressed in the myocardium, skeletal muscle, and in brown fat tissue. It is also present in the brain, pancreas and smooth muscle cells of blood vessels (Olofsson *et al.* 1996). Unlike VEGF-A knock-out mice, which die during embryogenesis, VEGF-B knock-out mice are healthy and fertile, but may have smaller hearts and a dysfunctional coronary vasculature (Bellomo *et al.* 2000). In another model of VEGF-B gene-targeted mice, prolonged PQ-interval in ECG was observed in VEGF-B null mice (Aase *et al.* 2001). Gene transfer of VEGF-B has also been shown to induce angiogenesis *in vivo* in skeletal muscle (Silvestre *et al.* 2003). Sun and colleagues found evidence that VEGF-B may be involved in the regulation of adult neurogenesis (Sun *et al.* 2006). In contrast to VEGF-A, VEGF-B is not upregulated by hypoxia or several growth factors (Enholm *et al.* 1997). Despite the recent findings regarding the functions of VEGF-B, its precise role *in vivo* is still not

known. Its abundant expression in skeletal muscle and in the myocardium suggests that its main functions are executed in these tissues.

VEGF-C and VEGF-D

VEGF-C and **VEGF-D** are primary lymphangiogenic factors, inducing the growth of lymphatic vessels during development and in adults (Jussila & Alitalo 2002; Tammela *et al.* 2005b). Human VEGF-C was first cloned in 1996 and its mature form is about 30 % identical to VEGF-A_{165} (Joukov *et al.* 1996). VEGF-D was cloned in 1997, and it has 48 % amino acid sequence identity with VEGF-C (Yamada *et al.* 1997; Achen *et al.* 1998). They are both synthesised as large precursor proteins, which after secretion are proteolytically processed into mature forms comprising the VEGF homology domain (Joukov *et al.* 1997; Achen *et al.* 1998). The long unprocessed forms are considered to be mainly lymphangiogenic and the mature short forms to be more angiogenic (Jeltsch *et al.* 1997; Joukov *et al.* 1997).

The development of lymphatic vessels during embryogenesis is dependent on vascular endothelial growth factor-C, as VEGF-C knock-out mice die before birth due to the lack of lymphatic vessels (Kärkkäinen *et al.* 2004). In contrast to VEGF-C-deficient mice, VEGF-D-deficient mice are viable, have normal body weight and no defects in lymphatic function (Baldwin *et al.* 2005). Baldwin and co-workers have suggested that the biological functions of VEGF-D are more likely to be revealed in response to diseases or tissue damage in adult tissues (Baldwin *et al.* 2005).

VEGF-C stimulates the proliferation and migration of endothelial cells (Joukov *et al.* 1997). Several studies using transgenic mice overexpressing VEGF-C and adenoviral VEGF-C transduction have demonstrated the potency of VEGF-C to induce lymphangiogenesis *in vivo* (Tammela *et al.* 2005a). Recently Kärpänen and co-workers showed that overexpression of VEGF-C in skin induced lymphatic hyperplasia during embryogenesis in mice, and similar expression of VEGF-D resulted in lymphangiogenesis predominantly after birth (Kärpänen *et al.* 2006). This finding further supports the idea that VEGF-C is important during development and VEGF-D during postnatal life. In addition to lymphangiogenesis, VEGF-C is also able to

regulate physiological and pathological angiogenesis *in vivo* (Witzenbichler *et al.* 1998; Cao *et al.* 2004). Unlike VEGF-A, the expression of VEGF-C does not appear to be regulated by hypoxia (Enholm *et al.* 1997), but is induced by proinflammatory cytokines (Ristimäki *et al.* 1998). VEGF-D also stimulates the proliferation of endothelial cells, and it has angiogenic properties both *in vitro* and *in vivo* (Marconcini *et al.* 1999). The mature form of VEGF-D delivered by gene transfer is able to induce strong angiogenesis in addition to lymphangiogenesis in skeletal muscle (Byzova *et al.* 2002; Rissanen *et al.* 2003). In adult human tissues, VEGF-D mRNA is most abundant in the heart, skeletal muscle, lungs, colon, and small intestine (Achen *et al.* 1998), suggesting that VEGF-D also has a role in skeletal muscle physiology, although as yet largely unknown.

VEGF receptor -1, VEGFR-2, and VEGFR -3

VEGF receptor-1 (VEGFR-1/Flt-1) and **VEGFR-2** (Flk-1, KDR) are receptor tyrosine kinases, which are important in blood endothelial cell proliferation, migration and survival (Neufeld *et al.* 1999; Ferrara *et al.* 2003). VEGFR-1 binds VEGF-A, VEGF-B and PlGF with high affinity, while VEGFR-2 binds VEGF-A and the mature forms of VEGF-C and VEGF-D (Ferrara *et al.* 2003; Tammela *et al.* 2005a). VEGFR-1 and VEGFR-2 knock-out mice die during early development due to the defects in vasculogenesis and angiogenesis, and excess growth of endothelial cells (Ferrara *et al.* 2003). VEGFR-1 alone induces only weak mitogenic signals, but it can heterodimerize with VEGFR-2 to transmit stronger signals in endothelial cells (Tammela *et al.* 2005a). VEGFR-2 is the primary receptor mediating the effects of VEGFs to induce angiogenesis, endothelial cell survival and vascular permeability (Ferrara *et al.* 2003; Tammela *et al.* 2005a). Hypoxia seems to upregulate both VEGFR-1 and VEGFR-2; however, this may occur indirectly, since VEGF-A induces the expression of its receptors (Neufeld *et al.* 1999).

VEGFR-3 (flt-4) is essential for lymphatic endothelial cell proliferation and survival (Mäkinen *et al.* 2007). VEGFR-3 is initially expressed in all embryonic vasculature, but its expression becomes gradually restricted to lymphatic endothelial cells (Kaipainen *et al.* 1995; Mäkinen *et al.* 2007). There are two different human VEGFR-3 splice variants, of which the long form is predominant in most tissues (Jussila &

Alitalo 2002). VEGFR-3 binds full-length VEGF-C and VEGF-D (Joukov *et al.* 1997; Stacker *et al.* 1999). Further proteolytic processing of these proteins enables them to induce angiogenesis in humans by activating VEGFR-2. In mice, VEGF-C activates both VEGFR-2 and –3 receptors, as in humans, but VEGF-D activates only VEGFR-3 (Baldwin *et al.* 2001). In certain pathological conditions, such as cancer, VEGFR-3 can be re-activated in the blood vessel endothelium (Valtola *et al.* 1999; Jussila & Alitalo 2002). Since VEGFR-3 is mainly restricted to the lymphatic endothelium in adult tissues, it has been used as a specific marker for lymphatic vessels (Jussila *et al.* 1998; Sleeman *et al.* 2001; Jussila & Alitalo 2002).

Neuropilins

Neuropilins are transmembrane receptors, which act as co-receptors for VEGFRs. Neuropilin-1 (Nrp-1) and neuropilin-2 (Nrp-2) were originally described in axon guidance and neuronal development, but subsequently they have also been shown to be involved in angiogenesis (Neufeld *et al.* 2002; Tammela *et al.* 2005a). In addition to several class 3 semaphorins, Nrp-1 binds VEGF-A_{165}, VEGF-B and PlGF, while Nrp-2 binds VEGF-A_{145}, VEGF-A_{165}, VEGF-C, VEGF-D and PlGF (Staton *et al.* 2007). Nrp-1 amplifies the VEGF-A – VEGFR-2 interactions and signalling, and it is suggested that Nrp-1 is required in cardiovascular development since it regulates VEGF-A levels (Kawasaki *et al.* 1999). Nrp-2 is also expressed in lymphatic endothelial cells, and the mutated form of Nrp-2 induces abnormal development of small lymphatic vessels (Yuan *et al.* 2002). On the basis of these findings and its ability to bind VEGF-C and –D, Nrp-2 is thought to play a role in lymphatic vessel growth.

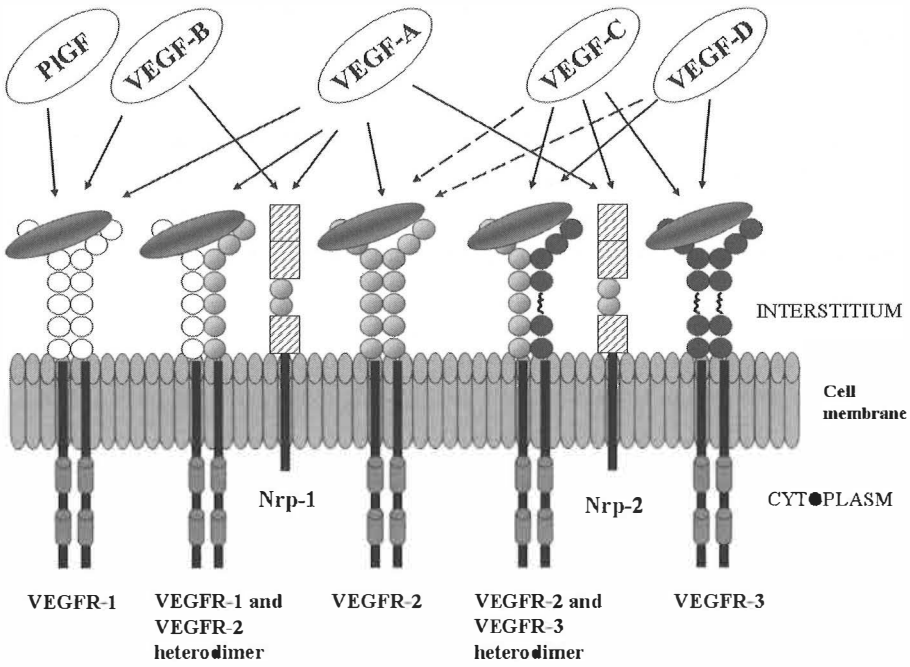


FIGURE 3. The vascular endothelial growth factor (VEGF) family ligands and their receptors. VEGF-A, VEGF-B and placental growth factor (PlGF) bind to VEGF receptor-1 (VEGFR-1). VEGF-A binds also VEGFR-2 and the VEGFR-1/VEGFR-2 heterodimer. VEGF-C and VEGF-D primarily bind VEGFR-3 on the lymphatic endothelium, but their processed forms can also bind VEGFR-2. VEGF-A and VEGF-B can also bind neuropilin-1 (Nrp-1) receptor and neuropilin-2 (Nrp-2) interacts with VEGF-A and VEGF-C. Depending on the receptors with which VEGFs interact, they can either induce angiogenesis or lymphangiogenesis. (Neufeld *et al.* 1999; Jussila & Alitalo 2002; Ferrara *et al.* 2003; Tammela *et al.* 2005a; Mäkinen *et al.* 2007)

2.3.2 Other angiogenic growth factors and extracellular matrix remodelling

To date, angiogenic properties have been found in tens of molecules. In addition to VEGFs, the proteins described below, have been found to induce angiogenesis in different tissues or settings. However, it should be kept in mind that this list is not complete, and more factors are likely to be discovered in the near future.

VEGF-A is produced when O_2 availability becomes restricted. This effect is mediated by hypoxia-inducible factors (HIFs), of which the isoform **HIF-1α** seems to be the most important. HIFs act as transcriptional factors for variety of genes, including VEGF-A, VEGFR-1, erythropoietin, inducible nitric oxid synthase (NOS2), insulin-like growth factor-2, and plasminogen activator inhibitor-1 (Semenza 2000; Wenger 2002). It has been shown that the most important step in the regulation of HIF-1α levels occurs in the stabilisation of the protein, although increased mRNA expression, nuclear localization and post-translational protein modifications are also involved (Wenger 2002).

Angiopoietin-1 and –2 (Ang-1 and Ang-2) compete for binding to Tie-2, a tyrosine kinase receptor, on endothelial cells. Only Ang-1 can activate the receptor directly, and its functions seem to be related to stabilizing and maintaining the vessel wall. Ang-2, in turn, is considered as an antagonist for Tie-2. In the presence of endogenous VEGF-A, Ang-2 promotes a rapid increase in capillary diameter, remodelling of the basal lamina, proliferation and migration of endothelial cells, and stimulates the sprouting of new blood vessels. When VEGF-A is absent, Ang-2 promotes endothelial cell death and vessel regression (Lobov *et al.* 2002). The Ang-2/Ang-1 expression ratio is thought to affect the stability of the blood vessel wall.

Fibroblast growth factors (FGFs) are multifunctional proteins stimulating the proliferation of several cell types, including endothelial cells, smooth muscle cells and myoblasts. They have been found to be involved in embryonic development, tissue

regeneration and angiogenesis. Among the at least 22 different members of the FGF family, FGF-1, FGF-2, FGF-4, and FGF-5 have been more widely investigated with respect to angiogenesis (Presta *et al.* 2005). **The platelet-derived growth factor** (PDGF) family of proteins consists of four members: PDGF-A, -B, -C and –D. PDGF-B has recently been described as a potent angiogenesis-inducer in hindlimb ischemia in diabetic muscles (Tanii *et al.* 2006).

The **extracellular matrix** (ECM) plays a critical role in angiogenesis by providing biochemical and mechanical cues, thus influencing endothelial cell behaviour (Krishnan *et al.* 2007). Of the ECM-associated CCN family proteins, **cystein-rich protein 61** (Cyr61/CCN1) and **connective tissue growth factor** (CTGF/CCN2) are important regulators of angiogenesis, endothelial cell function and ECM modulation (Brigstock 2002; Perbal 2004). They also regulate the activity and production of other angiogenic proteins such as VEGF-A (Perbal 2004; Zhou *et al.* 2005). In the myocardium, Cyr61 expression is increased in response to pressure overload and ischemia (Hilfiker-Kleiner *et al.* 2004). ECM degradation is important in angiogenesis. New sprouting vessels require degradation of the vascular basement membrane and remodelling of the ECM in order to allow endothelial cells to migrate and invade the surrounding tissue. **Matrix metalloproteinases** (MMPs) are a family of enzymes that proteolytically degrade various components of the extracellular matrix. Certain MMPs have also been shown to enhance angiogenesis by helping to detach pericytes from vessels undergoing angiogenesis and by releasing ECM-bound angiogenic growth factors (Rundhaug 2005). MMPs can also contribute negatively to angiogenesis through the generation of endogenous angiogenesis inhibitors by proteolytic cleavage of certain collagen chains and plasminogen (Rundhaug 2005). **Endogenous tissue inhibitors of metalloproteinases** (TIMPs) reduce excessive proteolytic ECM degradation by MMPs. The balance between MMPs and TIMPs plays a major role in ECM remodelling.

2.3.3 Inhibitors of angiogenesis

As in the case of proangiogenic factors, several antiangiogenic molecules have been found, most of them very recently (Karagiannis & Popel 2007; Tabruyn & Griffioen 2007). Below, the most widely studied proteins with known ability to inhibit angiogenesis *in vivo* are described.

The **thrombospondin** family of proteins consists of a group of five prototypical members (TSP-1, -2, -3, -4, -5). Thrombospondin-1 was the first endogenous inhibitor of angiogenesis to be identified, and it has been shown to play a critical role in inhibiting neovascularization (Good *et al.* 1990; Iruela-Arispe *et al.* 1996). Thrombospondin-1 inhibits the proliferation and migration of endothelial cells both *in vitro* and *in vivo*. Conversely, matrix-bound TSP-1 supported vessel formation in organ culture (Nicosia & Tuszynski 1994). The angiostatic activity of TSP-1 has been localized to the procollagen domain and type-1 repeat sequence of the molecule (Tolsma *et al.* 1993). This region has been found to interact with several candidate receptors. It is suggested that CD36, a cell-surface receptor, mediates the inhibitory effects of TSP-1 on endothelial cells (Primo *et al.* 2005; Tabruyn & Griffioen 2007). Diminished TSP-1 levels have been reported during physiological angiogenesis (Iruela-Arispe *et al.* 1996).

Endostatin is an angiostatic 20 kDa internal fragment of type XVIII collagen (O'Reilly *et al.* 1997). It has been demonstrated that endostatin interferes with VEGFR-2, inhibits MMP-2 activity, and arrests the endothelial cell cycle, leading to reduced cell motility, proliferation and survival (Tabruyn & Griffioen 2007). **Angiostatin** is a cleavage product of plasminogen. Angiostatin induces apoptosis, which is strictly restricted to proliferating endothelial cells (Tabruyn & Griffioen 2007). The apoptotic pathways activated by angiostatin and endostatin show similar features. Karagiannis and Popel recently identified 18 novel antiangiogenic peptides that were derived from proteins containing type 1 thrombospondin motifs, and showed that these peptides could inhibit the proliferation and migration of endothelial cells *in vitro* (Karagiannis & Popel 2007).

2.3.4 Effects of exercise

The importance of VEGF-A in skeletal muscle physiology and exercise capacity has been demonstrated with tissue-specific knock-out mice, which lack VEGF-A protein in skeletal muscles. These mice have significantly reduced capillarization and increased apoptosis in their muscles (Tang *et al.* 2004). In addition, the endurance capacity of VEGF-A deficient mice was only 20% that of wild-type mice (Wagner *et al.* 2006). These data implicate VEGF-A as an essential survival factor for muscle capillarity, and also demonstrate that insufficient VEGF-dependent signalling leads to apoptosis in mouse skeletal muscle.

Increased mRNA and protein expression of VEGF-A in skeletal muscle after an acute bout of exercise has been demonstrated in several animal and human experiments (Breen *et al.* 1996; Gustafsson *et al.* 1999; Richardson *et al.* 1999; Gavin *et al.* 2000; Brutsaert *et al.* 2002; Hudlicka *et al.* 2002; Gavin *et al.* 2004; Jensen *et al.* 2004a; Jensen *et al.* 2004b; Gustafsson *et al.* 2005; Gavin *et al.* 2006; Gavin *et al.* 2007; Gustafsson *et al.* 2007). In healthy muscle this acute response is, however, attenuated as the training is continued (Richardson *et al.* 2000; Gavin & Wagner 2001; Lloyd *et al.* 2003), although elevated basal levels may be found after the training period (Gustafsson *et al.* 2002). This exercise-induced VEGF-A expression has recently been shown to be required for both the splitting and sprouting models of angiogenesis. Williams and colleagues inhibited the action of VEGF-A by using VEGF Trap, and this inhibition abolished the exercise-induced increase in skeletal muscle capillarization (Williams *et al.* 2006b). The expression of all three receptors for VEGF-A; VEGFR-1, VEGFR-2 and Nrp-1, may also be upregulated in skeletal muscle by aerobic exercise (Gavin & Wagner 2002; Gavin *et al.* 2004; Gavin *et al.* 2005; Gustafsson *et al.* 2005). The effects of exercise on other VEGFs have not been studied.

Messenger RNA expression of angiopoietins-1 and -2 and their receptor Tie-2 can also be affected by exercise training. In rats, acute treadmill exercise with ischaemia increases Ang-2 mRNA and the Ang-2/Ang-1 mRNA ratio together with Tie2 mRNA (Lloyd *et al.* 2003), favouring destabilisation of the capillary wall and promoting angiogenic processes. The increased Ang-2/Ang-1 ratio after exercise was also

observed in a recent human study (Gustafsson *et al.* 2007). Endothelial nitric oxide synthase (eNOS) expression is also increased by exercise in the beginning phase of training, becoming gradually attenuated thereafter, in much the same way as observed for VEGF-A and Ang-2 (Lloyd *et al.* 2003).

HIF-1α and HIF-2α mRNA and protein levels have been demonstrated to increase post exercise or after muscle stretch, although, discrepancies between studies exist (Ameln *et al.* 2005; Lundby *et al.* 2006; Milkiewicz *et al.* 2007). In general, the current consensus seems to be that in addition to the well-recognized stimulus of hypoxia, mechanical forces alone could also induce the expression of HIF-1α and HIF-2α. It was recently suggested that in skeletal muscle these transcription factors may play a central role in stretch-induced, but not shear-stress induced, angiogenesis (Milkiewicz *et al.* 2007).

Matrix metalloproteinase activity is required for activity-induced angiogenesis. MMP-2 and (MT-1)-MMP have been shown to be activated in response to electrical stimulation and muscle stretch, which induce the sprouting type of angiogenesis, and this activation precedes capillary growth (Haas *et al.* 2000; Rivilis *et al.* 2002). Splitting angiogenesis induced by increased shear stress, however, does not seem to require MMP activation.

2.3.5 Effects of diabetes

Until the last couple of years, only a few studies have evaluated the mechanisms by which diabetes affects angiogenesis in skeletal muscle. Contradictory findings exist in the literature on the effects of type 1 and type 2 diabetes or hyperglycemia on the expression of VEGF-A. Several studies have shown decreased expression of VEGF-A in diabetic skeletal muscle or in the myocardium (Chou *et al.* 2002; Emanueli *et al.* 2004; Chung *et al.* 2006; Shoji *et al.* 2006). Some studies have reported no change in the expression of VEGF-A due to hyperglycemia (Larger *et al.* 2004; Tanii *et al.*

2006), while others have even shown increased expression compared to a healthy myocardium or healthy skeletal muscle (Sasso *et al.* 2005; Li *et al.* 2007). Together with decreased cardiac expression of VEGF-A, reduced expression of its receptors VEGFR-1 and 2 has also been found in the diabetic rat heart and in diabetic human myocardium (Chou *et al.* 2002). In cell cultures, high glucose levels inhibited VEGF-A production (Lerman *et al.* 2003), while insulin, in turn, enhanced VEGF-A levels (Jiang *et al.* 2003). High glucose treatment of endothelial cells also decreased the expression of MMP-1 and hepatocyte growth factor (HGF), suggesting that dysregulation of these proteins might also play a role in impaired angiogenesis in diabetes (Taniyama *et al.* 2001). In addition, reduced nerve growth factor (NGF) levels have been reported, and NGF supplementation was shown to be effective in reparative angiogenesis after ischemia in diabetic muscles (Salis *et al.* 2004).

Increased expression of angiogenesis inhibitor TSP-1 in the vessel wall of diabetic Zucker rats has been reported (Stenina *et al.* 2003). Moreover, TSP-1 was upregulated by high glucose in cultured endothelial cells, vascular smooth muscle cells, and fibroblasts. It has been proposed that hyperglycaemia-induced TSP-1 in vascular cells could be a link between diabetes and atherosclerotic complications. A recent study demonstrated that high glucose activates the hexosamine metabolic pathway, which directly mediates TSP-1 upregulation in smooth muscle cells (Raman *et al.* 2007). Increased expression of another angiogenesis inhibitor, angiostatin, has also been observed in diabetic arterial vasculature (Chung *et al.* 2006). Glycation of ECM components is a consequence of prolonged exposure of matrix proteins to elevated glucose levels. Advanced glycation end-products (AGEs) affect ECM degradation and impair angiogenesis, probably by disturbing matrix degradation processes (Tamarat *et al.* 2003). Furthermore, a receptor for AGEs (RAGE) was shown to be involved in impaired angiogenic response in diabetes, but the mechanisms were not uncovered (Shoji *et al.* 2006).

3 AIMS OF THE STUDY

The aim of this thesis was to study the effects of exercise, diabetes and their combination on the molecular mechanisms of angiogenesis and lymphangiogenesis in skeletal muscle. Furthermore, the recently discovered markers for lymphatic vessel endothelium were applied to identify lymphatic vessels and to study their localization and quantity in human and mouse skeletal muscles.

The specific aims were to study:

1. The changes in angiogenic and lymphangiogenic gene expression after experimentally induced type 1 diabetes, exercise training and their combination in mouse skeletal muscle. (I, V)

2. The effects of a single bout of running and jumping exercises on the expression of angiogenic and lymphangiogenic growth factors in healthy and diabetic skeletal muscle. (II, III, V)

3. The effects of diabetes and exercise on the expression of VEGF-A and TSP-1 in blood capillaries and in pure muscle fibres collected from skeletal muscle. (II)

4. The localization of lymphatic vessels and their growth factors VEGF-C and VEGF-D in skeletal muscles using specific antibodies. (IV)

5. The effects of diabetes and exercise training on capillarization and lymphatic vessel density. (I, V)

4 MATERIALS AND METHODS

4.1 Animals and human subjects

All the experimental procedures for the present animal studies were approved by the Animal Care and Use Committee of the University of Jyväskylä, Finland. For the exercise training study (studies I and V), ten to fifteen-week-old male NMRI mice (n = 60, Harlan, The Netherlands) were used. Mice were randomly assigned into healthy and diabetic groups. The diabetic group received a single peritoneal injection of streptozotocin (STZ, Sigma-Aldrich, France, 180 mg/kg) dissolved in sodium citrate buffer solution (0.1 mol/l, pH 4.5) to induce experimental type 1 diabetes (21). The other group received a sham injection of an equal volume of the buffer. Diabetes was confirmed 72 hours after the injection by a urine glucose test (Glukotest®, Roche, Germany). Mice were characterized as diabetic when their urine glucose values were greater than 200 mg/dl. Another group of NMRI mice (n = 48, Harlan, The Netherlands) was used for the acute running experiment in studies II and V. Diabetes was induced as described above and confirmed three days after the injection by a blood glucose test (HemoCue B-Glucose analyzer, Sweden). Mice were not treated with insulin during either of the experiments.

Eight healthy males volunteered for study III. The mean age of the subjects was 26 ± 4 yrs, height 179 ± 7 cm and body mass 78 ± 9 kg (mean ± SD). They had different training backgrounds but none of them was involved in any systematic training during the study period or the year preceding it. All subjects were informed of the purpose, nature and potential risks of the study, and they gave their written consent prior to entering the study. The study was conducted according to the Declaration of Helsinki and the ethical committee of the University of Jyväskylä approved the study protocol. Control muscle samples were also used in study IV.

4.2 Exercise protocols

For the exercise training study, diabetic and healthy mice were randomly assigned into 12 groups (n = 5 per group), which were either sedentary or trained for one, three or five weeks. Groups were named as follows: sedentary healthy mice (C1, C3, C5), trained healthy mice (T1, T3, T5), sedentary diabetic mice (D1, D3, D5) and trained diabetic mice (DT1, DT3, DT5). Training groups performed 1 hour per day of treadmill running at 21 m/min (= 1260 m/h) and at an uphill inclination of 2.5° for five days a week. Training was started one week after the streptozotocin injection. Trained mice were killed 24 hours after the last training bout together with their sedentary controls. Sample collection was performed 24 h after the last training session to focus on the effects of training and not effects resulting from the last exercise bout. Muscle samples from sedentary mice were also used in study IV.

For the acute exercise bout study (II and V), mice were divided into three healthy and three diabetic groups (n = 8) ten days after the injections. One healthy and one diabetic group served as control groups without exercise (H and D), and four groups performed one-hour of running exercise on a treadmill (speed 21 m/min, inclination of 2.5°). One healthy and one diabetic exercise group were sacrificed three hours after the exercise bout (HE3 and DE3), and the other two groups six hours post exercise (HE6 and DE6).

After a 5-min warm-up period on a bicycle ergometer maximum voluntary contraction force (MVC) of both legs was measured unilaterally. Thereafter, the study subjects were seated on a special sledge ergometer (Kyröläinen & Komi 1995) inclined at 20.3 degrees from the horizontal. After determination of optimal dropping height, subjects performed 100 maximal, unilateral drop jumps every five seconds. Maximal jumps were immediately followed by continuous jumping to a submaximal height representing 50% of their maximum until exhaustion. All the jumps were performed by the right leg while the left leg served as a control. The average number of jumps (maximal + submaximal) performed during the exercise protocol was 876 ± 609 (mean ± SD, range 403-2032). The mean duration of jumping was 19.3 ± 13.2 min. Unilateral MVC of both legs was measured again immediately and 48 h post exercise.

4.3 Muscle tissue preparation

For the histochemical analyses the proximal part of the left *quadriceps femoris* muscle (studies I and IV) and proximal part of the right *gastrocnemius* muscle (studies II and V) were used. Muscle samples were mounted in Tissue-TEK O.C.T. embedding medium (Miles Inc., USA) under a microscope to orientate muscle fibres transversally, and snap frozen in isopentane (-160°C) cooled with liquid nitrogen. For the biochemical analyses the calf muscles (*soleus*, *gastrocnemius* and *plantaris*) were snap frozen and all samples were stored at -80°C for further analysis.

Needle biopsies were obtained 30 min and 48 h post exercise from the middle region of the *vastus lateralis* muscle of the exercised leg under local anaesthesia in study III. A control biopsy was taken from the non-exercised leg. One part of the muscle specimen was transversally oriented under a microscope, mounted with embedding medium, and frozen in isopentane similarly to the mouse samples. The other part was immediately frozen in liquid nitrogen, and both parts were stored at –80°C.

4.4 Immunohistochemistry (I, III, IV)

4.4.1 Muscle samples

In study I, *quadriceps femoris* muscles from mice trained for five weeks and their respective controls were used for the immunohistochemical analyses. In studies II and V, mouse *gastrocnemius* muscles were studied, and in study III the analysed muscle was the human *vastus lateralis* muscle. In study IV both the mouse *quadriceps femoris* muscle group and the human *vastus lateralis* muscles were used. Transverse sections of the muscle samples (6-10 μm) were cut in a cryomicrotome and used for immunohistochemical stainings.

4.4.2 Staining protocols and antibodies

Sections were air-dried, fixed with ice-cold acetone or 4 % paraformaldehyde, and incubated with 2 % bovine serum albumin or 5 % normal serum to block non-specific binding. Light microscopic samples were also pre-incubated with 0.3 % H_2O_2 to quench endogenous peroxidase activity. Samples were then incubated with optimised dilutions of primary and secondary antibodies. The primary antibodies used are presented in Table 1. For the light microscopy, peroxidase staining was performed with a Vectastain Elite ABC kit (Vector Laboratories, USA), and DAB or AEC (Sigma, France) were used as a chromogen. Samples were counterstained with Meyer's hematoxylin. For the fluorescence and confocal microscopy, secondary antibodies labelled with Alexa Fluor-dyes 488 and 555 (Molecular Probes, the Netherlands) were used, and nuclei were stained with Hoechst. Negative controls were done by omitting the primary antibody and using an irrelevant antibody of the same isotype.

TABLE 1. Antibodies for immunohistochemistry.

Protein	Antibody type	Manufacturer
VEGF-A	goat-anti-mouse IgG	Santa Cruz Biotechnology, Santa Cruz, USA
VEGF-B	rabbit-anti-mouse IgG	Santa Cruz Biotechnology, Santa Cruz, USA
VEGF-C	goat-anti-mouse IgG	Santa Cruz Biotechnology, Santa Cruz, USA
VEGF-D	goat-anti-mouse IgG	R&D Systems, Minneapolis, USA
VEGFR-2	goat-anti-mouse IgG	R&D Systems, Minneapolis, USA
VEGFR-3	mouse-anti-human IgG rat-anti-mouse IgG	From prof. Kari Alitalo, University of Helsinki, Finland
Neuropilin-2	goat-anti-mouse IgG	R&D Systems, Minneapolis, USA
LYVE-1	goat-anti-human IgG	R&D Systems, Minneapolis, USA
CD31/PECAM-1	mouse-anti-human IgG rat-anti-mouse IgG	BD Biosciences Pharmingen, San Diego, USA
PAL-E	mouse-anti-human IgG	Monosan, Uden, the Netherlands
Dystrophin	mouse-anti-human IgG	Novocastra, Newcastle upon Tyne, UK
Cyr61	goat-anti-human IgG	Santa Cruz Biotechnology, Santa Cruz, USA
CTGF	goat-anti-human IgG	Santa Cruz Biotechnology, Santa Cruz, USA

4.4.3 Microscopy

Stained sections were studied with light or fluorescence microscopy (Olympus BX-50F, Olympus Optical, Japan) and images were captured and processed with AnalySIS software (Olympus, Germany). TEMA image analysing software (Scan Beam, Denmark) was used to analyse the cross-sectional area of muscle fibres (CSA), capillary density (number of capillaries per mm^2) and capillary-to-fibre (C:F) ratio. Olympus FV1000 confocal imaging system (Olympus Optical) was used for the analyses of the fluorescent double-stained samples.

4.5 Protein measurements

4.5.1 VEGF-A ELISA (I)

A part of the right calf muscle complex (study I) was homogenised in RIPA buffer. VEGF-A was determined in duplicate by a commercial high-sensitivity ELISA kit according to the manufacturer's instructions (R&D Systems, USA). VEGF-A levels were obtained by use of a microplate reader at 450 nm and corrected by readings at 540 nm. VEGF-A concentration was related to the total protein content of the homogenate. Dissolved muscle protein concentration was measured using the Bio-Rad Protein Assay (Bio-Rad, USA).

4.5.2 Western blotting for Cyr61 and CTGF (III)

Muscle samples were weighed and homogenised in RIPA buffer. Total protein concentration was measured with a D_C Protein Assay (Bio-Rad, USA) according to the protocol. Samples were analysed by 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and blotted onto PVDF membranes (GE Healthcare Biosciences, Sweden). Filters were blocked with rabbit normal serum in 1% BSA in PBS, and

incubated with primary antibodies (Cyr61 and CTGF). After washes, membranes were incubated with horseradish-peroxidase-conjugated antigoat IgG (GE Healthcare), washed, and detected using Amersham ECL Plus chemiluminescence reagents (GE Healthcare). The amount of Cyr61 and CTGF proteins was determined by densitometry (KemEnTec Aps, Denmark) and normalised to total protein.

4.7 Messenger RNA expression (I-V)

4.7.1 RNA extraction

Total RNA was isolated from the muscle samples with Trizol Reagent (Invitrogen, USA) and samples for microarray were further purified with a RNeasy kit (Qiagen, Germany). The concentration and purity of RNA was determined spectrophotometrically at wavelengths 260 and 280 nm. Integrity was checked with agarose gel electrophoresis. RNA was reverse transcribed to cDNA with a High Capacity cDNA Archive Kit (Applied Biosystems, USA).

4.7.2 Reverse transcription-PCR (IV)

Primer pairs for VEGF-C (forward CTTGCTGTGCTTCTTGTCTC, reverse GTCCCC TCTCCTGGTATTG) and VEGF-D (forward GCGGCAACTTTTCTATGACAC, reverse CACTAACTCGGGCACTGATG) were designed using Oligo Explorer software and synthesized by TAG Copenhagen (Denmark). Reverse transcription-PCR was done with an Enhanced Avian HS RT-PCR kit (Sigma, France). RNA from heart muscle was used as a reference sample as it is known to contain both VEGF-C and -D mRNAs (Achen *et al.* 1998; Kukk *et al.* 1996). The specificity of each primer pair was evaluated using 2% agarose gel electrophoresis; only a single product of appropriate size was produced for each PCR reaction.

4.7.3 Real-Time Quantitative PCR (I, II, III, and V)

The ABI Prism 7700 and 7300 Sequence Detection Systems (Applied Biosystems, USA) were used to perform TaqMan probe or SYBR Green-based real-time PCR reactions. TaqMan primer and probe sets were designed and synthesized by Applied Biosystems. Primer pairs were designed so that they overlapped an exon-exon boundary to avoid interference from possible genomic DNA contamination. Primers for SYBR Green reactions were adopted from Thijssen *et al.* (2004) and produced by TAG Copenhagen (Denmark). The primers and probes used in the present experiments are shown in Table 2. Target genes in the sample were quantified according to the corresponding gene-specific standard curve. As an endogenous control to correct for potential variation in RNA loading or amplification efficiency, GAPDH mRNA was used. In the microarray data (study I), GAPDH showed the steadiest expression in all conditions when the normally used housekeeping genes were compared. GAPDH is also considered the most stable internal control in exercise studies (Jemiolo & Trappe 2004). All samples were analyzed in triplicate.

TABLE 2. Primers and probes used for real-time QPCR.

	TaqMan primers and probes	SYBR Green primers (5'-3')
VEGF-A		
Mouse	Mm00437304_m1	
Human	Hs00173626_m1	
VEGF-B		
Mouse	Mm00442102_m1	
Human	Hs00173634_m1	
VEGF-C		
Mouse	Mm00437313_m1	Fwd: GTAAAAACAAACTTTTCCCTAATTC
	(NM_010228)	Rew: TTTAAGGAAGCACTTCTGTGTGT
Human	Hs00153458_m1	
VEGF-D		
Mouse	Mm00438965_m1	Fwd: GCAAGACGAGACTCCACTGC
	(NM_010612)	Rew: GGTGCTGAATGAGATCTCCC
Human	Hs00189521_m1	
VEGFR-1		
Mouse	Mm00438980_m1	
VEGFR-2		
Mouse	Mm00440099_m1	
VEGFR-3		
Mouse	Mm00433337_m1	Fwd: GCAGGAGGAGGAAGAGGAGC
	(XM_129720)	Rew: TGCATGCTGGGTGGACTATCA
TSP-1		
Mouse	Mm00449022_m1	
CTGF		
Mouse	Mm00515790_g1	
Human	Hs00170014_m1	
Cyr61		
Mouse	Mm00487498_m1	
Human	Hs00155479_m1	
HIF-1α		
Human	Hs00153153_m1	
GAPDH		
Mouse	Mm99999915_g1	
Human	Hs99999905_m1	

TaqMan probe-based assays are gene expression assays from Applied Biosystems.
Primer sequences for SYBR Green are adopted from Thijssen *et al.* 2004.

4.7.4 Microarray analysis (I)

Oligonucleotide array analyses for pooled RNA samples were performed using the Affymetrix Gene Chip MG U74Av2 (Affymetrix, Inc., USA), which represents 6000 known genes and 6000 ESTs. The Finnish DNA Microarray Centre at the Turku Centre for Biotechnology conducted the microarray analyses according to the instructions supplied by Affymetrix. Arrays were scanned using a GeneArray Scanner G2500A (Agilent, USA) and images were analyzed with Microarray Suite 5.0 software (Affymetrix Inc., USA). All samples were quality-checked according to the recommendations of Affymetrix before comparison analysis to determine differentially expressed genes. The following comparisons were performed using Microarray Suite 5.0: trained (T) vs. control (C), diabetic (D) vs. control, trained diabetic (DT) vs. control and trained diabetic vs. diabetic. The comparisons were made at each time point. Transcripts had to meet multiple criteria before they were regarded as differentially expressed. Changes in expression had to be significant (increased or decreased) according to Microarray Suite 5.0 algorithms and the two based log ratio of expressions >0.3 or < -0.3. Transcripts had to be at least marginally present in one of the two compared samples and they also had to be present in at least three samples. GeneSpring 6.1 (Silicon Genetics, USA) software was used in applying the last-mentioned filter when drawing up gene lists and preliminary result tables. The complete data set is publicly available in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/; accession number GSE1659).

4.7.5 Cyr61 and CTGF mRNA in pure muscle fibres (III)

To verify the localization of Cyr61 and CTGF in muscle fibres, which was seen with immunohistochemistry, single muscle fibres were separated from mouse *flexor digitorum brevis* muscle. Fibres were incubated with collagenase, purified and cultured on a Matrigel-coated dish (Rahkila *et al.* 1996). Total RNA was extracted with a Pico Pure RNA isolation kit (Arcturus, USA) and transcribed to cDNA with a Sensiscript RT kit (Qiagen, Germany). Real-time PCR reactions were performed as described

above with TaqMan primers and probes for mouse Cyr61 and CTGF (Applied Biosystems, USA).

4.7.6 Laser capture microdissection (LCM) and real-time PCR (II)

LCM was used to collect capillaries and pure muscle fibres from the *gastrocnemius* muscle. Three mice from groups C (healthy control), D (diabetic control), HE6 (healthy exercised, killed 6 h post exercise) and DE6 (diabetic exercised, killed 6 h post exercise) were studied, amounting to a total of 24 LCM samples (capillaries and muscle fibres from each 12 animals). The procedures used to collect capillaries and muscle fibers from skeletal muscle were adopted from Milkiewicz & Haas (2005). Cryosections (8 μm) were cut on uncoated glass slides, immediately fixed with cold acetone and stored in a freezer at -80°C. Samples were further processed within two days. All equipment was treated with RNase Away solution (QBiogene, USA) to prevent RNA degradation. Immediately the samples were taken out of the freezer, capillaries were stained with Isolectin GS-IB_4 from Griffonia simplicifolia Alexa Fluor 488 conjugate (Molecular Probes, the Netherlands), which was diluted in sterile PBS with RNAase inhibitor (SUPERaseIn, Ambion, USA). Laser capture microdissection for capillaries and pure muscle was performed with a Veritas microdissection system (Arcturus Engineering, USA). Capillaries were distinguished by size (<10 μm) from larger blood vessels. RNA was extracted with a PicoPure RNA isolation kit (Arcturus Engineering) according to the protocol. RNA was transcribed to cDNA with a Sensiscript reverse transcription kit (Qiagen) using both random and oligo (dT) primers (Ambion) at 42°C for 2 h.

From the LCM samples mRNA expression of VEGF-A and TSP-1 was analysed. The comparative C_T method was utilized as outlined in Applied Biosystems User Bulletin 2 (Applied Biosystems) to detect differences between groups. All samples were analyzed in triplicate.

4.8 Citrate synthase analysis (I)

Muscle samples (~30 mg) were homogenised in cold 0.2 M NaCl Tris-buffered solution (pH 7.5). The supernatants were used for the assay of citrate synthase activity as previously described (Kainulainen *et al.* 1984). Enzyme activities were expressed as units per mg of dissolved protein.

4.9 Statistical methods

Data were analysed with SPSS for Windows statistical software release 13.0.1 (SPSS Inc., USA), and differences were considered significant at $P < 0.05$. Depending on the normal distribution of the data, one-way ANOVA or non-parametric Kruskall-Wallis with Mann-Whitney U tests were used to analyze differences in mRNA and protein levels, capillarization and fibre CSA. Pearson's correlation coefficient was used to study the associations between the responses in mRNA expression and physiological characteristics.

For the oligonucleotide array, the one-sided Wilcoxon's signed rank test (WSR) was applied to the perfect match (PM) and mismatch (MM) intensities of each probe set to determine which genes were expressed above the background. Genes were called as significantly expressed at $P \leq 0.04$. Statistical algorithms based on the WSR test were also used to determine significant differential expression in the comparative analyses between treatment groups. Gene expression was considered significantly increased at $P \leq 0.0025$ and decreased at $P \geq 0.9975$. Calculation of the magnitude of the change in expression was based on differences between the intensities of the corresponding probe pairs (PM-MM) across the two arrays and one-step Tukey´s biweight estimate statistics.

5 RESULTS

5.1 Effects of streptozotocin-induced diabetes and exercises on general physiological parameters

In the exercise training studies (I, V) diabetes decreased body weight and increased serum glucose significantly. Skeletal muscle citrate synthase (CS) activity was decreased in the sedentary diabetic compared to sedentary healthy mice. Exercise training increased CS activity in both healthy and diabetic mice compared to their respective sedentary groups. The data are shown in Table 3.

TABLE 3. Effects of diabetes and endurance training on body weight, blood glucose and citrate synthase (CS) activity (studies I and V). Each group consists of mice from all three time points (n=15 in each group). Values are presented as mean ± SD.

	Healthy Control	Healthy Trained	Diabetic Control	Diabetic Trained
Change in BW (%)	0.1 ± 5.1	2.0 ± 5.5	-23.4 ± 10.2***	-19.7 ± 10.6***
Serum glucose (mmol / l)	10.3 ± 1.5	9.9 ± 1.3	54.9 ± 5.7***	50.5 ± 6.8***†
CS Activity (nmol x min^{-1} x mg^{-1})	469 ± 99	553 ±140*	400 ± 74*	501 ± 60†††

* $P < 0.05$ vs. healthy control; *** $P < 0.001$ vs. healthy control; ††† $P < 0.001$ vs. diabetic control; † $P = 0.07$ vs. diabetic control

In the single-bout running experiment (studies II and V), blood glucose was significantly higher in the diabetic than healthy mice (35.6 ± 1.4 vs. 8.1 ± 0.2 mmol/L, $P < 0.001$). Diabetic and healthy mice did not differ in body weight in the beginning of the experiment (36.8 ± 0.5 vs. 36.9 ± 0.7 g), but after ten days of diabetes, the diabetic mice weighed significantly less than the healthy mice (33.1 ± 0.8 vs. 37.9 ± 0.7 g, $P <$ 0.001).

A single bout of strenuous jumping exercise caused a significant ($P < 0.05$) decrease in isometric maximal voluntary contraction force (MVC) of the exercised leg 30 min post exercise (847 ± 92 vs. 584 ± 86 N). Two days after the exercise bout MVC had returned close to pre-exercise levels (727 ± 125 N, $P = 0.051$). No change occurred in the maximal force of the control leg. Lactate increased from 1.4 ± 0.2 mmol L^{-1} before exercise to 9.9 ± 3.5 mmol L^{-1} post exercise ($P < 0.001$).

5.2 Angiogenesis and angiogenic growth factors

5.2.1 Capillarization and muscle fibre cross-sectional area (I)

Both the sedentary and trained diabetic mice showed a significant decrease in muscle fibre cross-sectional area (CSA) after five weeks compared to the healthy controls ($P < 0.05$). Capillary density (cap x mm^{-2}) was not significantly changed due to diabetes, although it was slightly elevated due to the reduced fibre cross-sectional area. Instead, the capillary-to-fibre ratio, which is commonly used to describe capillary supply to muscle fibres, showed a decrease in both diabetic groups compared to the healthy control group ($P < 0.05$), but did not differ between the trained and sedentary diabetic mice. The healthy trained mice tended to have greater capillary-to-fibre ratio than the healthy control mice, but the difference was not statistically significant. Representative images from healthy and diabetic muscles are shown in Figure 4.

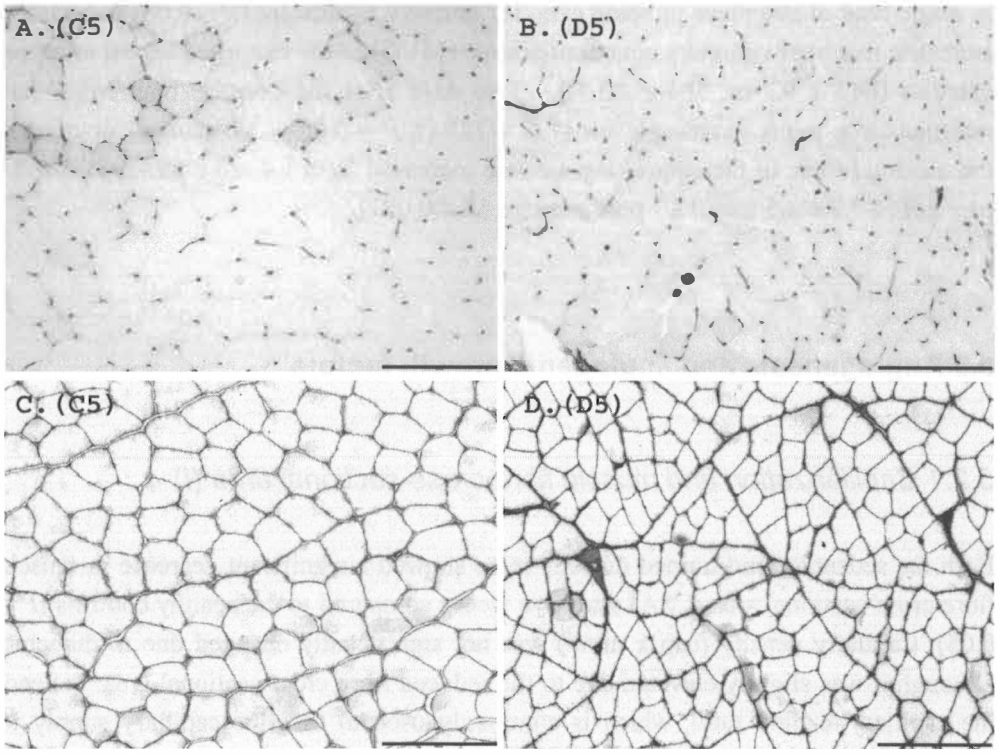


FIGURE 4. Representative images from healthy and diabetic skeletal muscles. Diabetes decreased muscle fiber area and the capillary-to fiber ratio significantly. Capillaries are stained with CD31 antibody in images A (healthy) and B (diabetic). Muscle fibers are visualized with dystrophin staining in images C (healthy) and D (diabetic). Scale bars are 200 μm.

5.2.2 VEGF-A, VEGF -B and VEGF receptors 1 and 2 (I, II, III)

In the exercise training study (I) the microarray results showed that mRNA levels of the angiogenic growth factors VEGF-A and VEGF-B and their receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1), and neuropilin-1 were downregulated in diabetic muscles (Figure 5). Endurance training alleviated the diabetes-induced changes in the

mRNA levels of VEGF-A, VEGF-B, neuropilin-1, but did not completely restore them. VEGF-A protein content was reduced in the sedentary diabetic mice after three and five weeks of diabetes compared to healthy controls (P <0.05). In the endurance-trained diabetic groups VEGF-A protein concentration showed a significant decrease only after five weeks. After three weeks, the trained diabetic animals tended to have higher VEGF-A content than the sedentary diabetic mice (P = 0.086).

In study II, basal mRNA levels of VEGF-A, VEGF-B and VEGFR-2 were not significantly reduced in the diabetic mice compared to healthy mice after ten days of diabetes. The acute bout of running exercise significantly increased (P < 0.05) VEGF-A and VEGFR-2 mRNA expression in healthy mice 6 h post exercise. A similar trend was also observed in VEGF-B at 6 h post exercise, but the increase was not statistically significant (P = 0.08). No significant change was observed in the diabetic mice in the expression of VEGF-A, VEGFR-2 or VEGF-B post exercise; however, the levels of VEGF-A and VEGFR-2 were 17 % and 20 % higher in DE6 than in the diabetic control mice. VEGFR-1 expression was not affected by exercise in either the healthy or diabetic mice.

The expression of VEGF-A and VEGF-B was also studied 30 min and 48 h after the strenuous jumping exercise in humans. The mRNA levels of VEGF-A and VEGF-B did not change significantly at either 30 min or 48 h post exercise. However, there were large individual differences in the responses of VEGF-A and VEGF-B mRNA post exercise, and the intragroup variation increased markedly at both 30 min and 48 h post exercise compared to the control leg values. No change was observed in the expression of HIF-1α mRNA.

Accession	Gene name	Gene / Protein	D1	D3	D5	DT1	DT3	DT5	T1	T3	T5
U43836	Vegfb	vascular endothelial growth factor B	0,87	0,71	0,54	1,15	0,87	0,81	1,32	1,23	1,23
M95200	Vegfa	vascular endothelial growth factor A	0,62	0,62	0,44	0,76	0,76	0,66	0,93	1,15	1,07
D50086	Nrp	neuropilin	0,66	0,54	0,57	0,81	0,57	0,87	1,07	1,00	1,07
X70842	VEGFR-2/Flk1	kinase insert domain protein receptor	0,47	0,71	0,71	0,62	0,71	0,93	0,66	1,15	1,15
D88689	VEGFR-1/Flt1	FMS-like tyrosine kinase 1	0,93	0,71	0,81	1,00	0,87	1,15	1,15	1,07	1,32

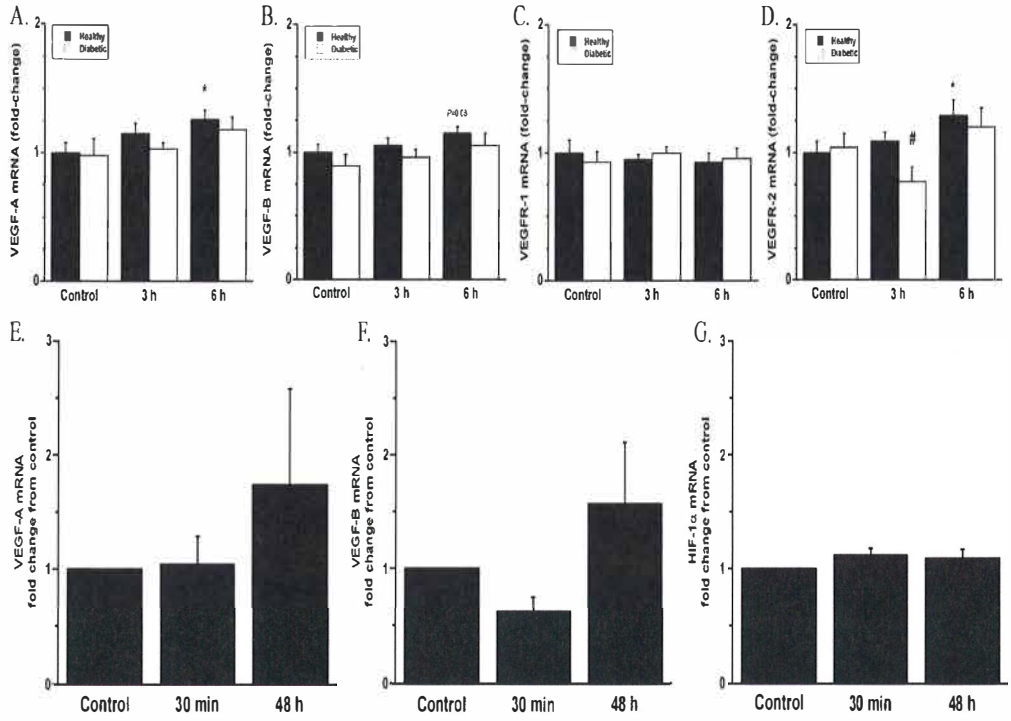


FIGURE 5. *Upper panel*: Changes in the VEGF- and VEGF receptor-genes due to diabetes and exercise training in study I. Gene expressions are expressed in relation to the control group (=1) at the same time point. Statistically significant changes are color-coded as follows: red=up-regulated, green=down-regulated, yellow=diabetes-induced change in expression was attenuated by exercise. Group abbreviations: D=diabetic, DT=diabetic trained and T=healthy trained, numbers=duration of treatment in weeks. *Middle panel (A-D):* Messenger RNA expression of VEGF-A, VEGF-B, VEGFR-1 and VEGFR-2 3 h and 6 h after acute running exercise in healthy and diabetic mice (study II). Filled bars=healthy mice, open bars=diabetic mice. *Lower panel (E-G):* Effects of jumping exercise with high mechanical loading on expression VEGF-A, VEGF-B and HIF-1α (study III).

The localization of the VEGF-A, VEGF-B and VEGFR-2 proteins was studied with immunohistochemistry, and representative images are presented in Figure 6.

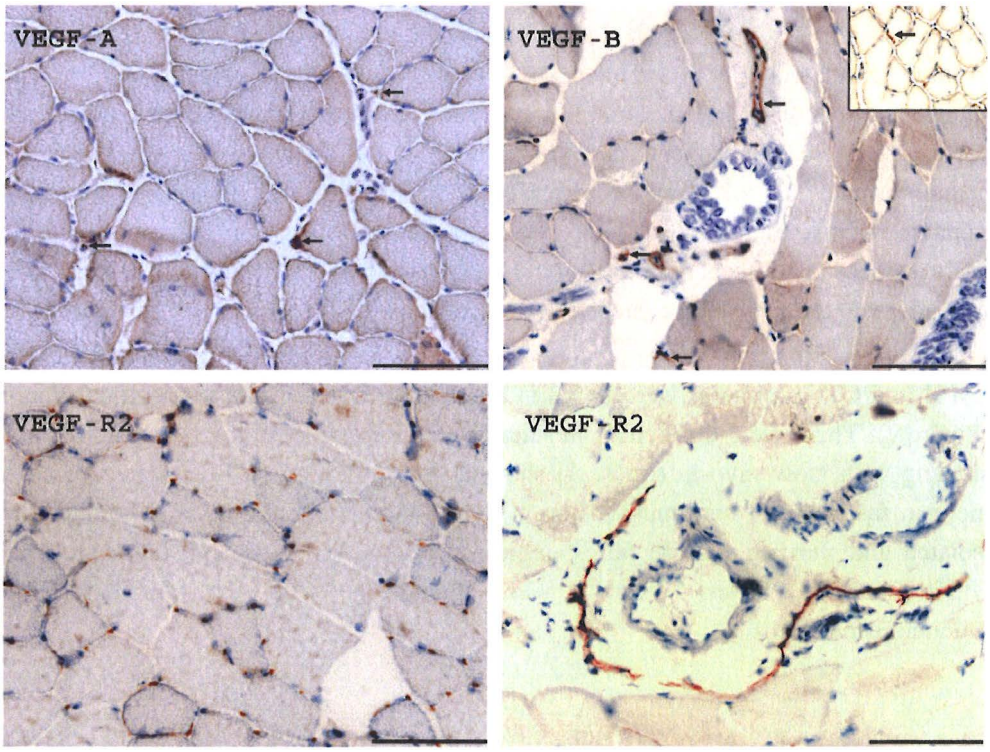


FIGURE 6. Localization of VEGF-A, VEGF-B and VEGFR-2 proteins in mouse skeletal muscle. VEGF-A protein was localized under the sarcolemma in some of the muscle fibres, in the endothelium of larger blood vessels and in some capillaries (upper left), as has also been reported in earlier studies. As a novel finding, VEGF-B staining is shown in the larger blood vessels, in certain interstitial cells and in only few muscle fibres (upper right). Weak staining was also observed in some capillaries. The insert shows the myofibrillar localization of VEGF-B. VEGFR-2 protein was expressed in most of the capillaries (lower left) and in vessels without a thick smooth muscle cell layer, which were situated close to arteries and large veins (lower right). There was no difference in protein localization between the healthy and diabetic mice. Scale bars are 200 μm all except in the lower right image, where it is 100 μm.

5.2.3 Angiogenic CCN proteins Cyr61 and CTGF (I, II, III)

Diabetes increased the expression of Cyr61 and CTGF mRNA significantly in the exercise training experiment (I). In the acute running study (II) Cyr61 was also found to be up-regulated in both sedentary and exercised diabetic muscles. CTGF was not studied in this experiment. Jumping exercise with high mechanical loading and eccentric muscle actions increased Cyr61 and CTGF mRNA expression significantly in healthy humans. Cyr61 was also increased at the protein level. In contrast, the acute running exercise bout with moderate intensity did not affect Cyr61 expression in the healthy mice. The effects of exercise on the expression of Cyr61 and CTGF seem to be dependent on the type and/or intensity of exercise.

Cyr61 and CTGF proteins were found in muscle fibres and in the extracellular matrix (Figure 7). There were differences in staining intensity between the fibres, and double staining with slow myosin (MHC 1) showed that Cyr61 and CTGF were expressed more in fast than in slow muscle fibres. Cyr61 and CTGF mRNA was produced by isolated and purified muscle fibres, indicating that CTGF and Cyr61 are produced within muscle fibres. This is in agreement with the findings from immunohistochemistry.

Accession	Gene name	Gene / Protein	D1	D3	D5	DT1	DT3	DT5	T1	T3	T5
M70642	Ctgf	connective tissue growth factor	1,41	1,41	1,52	0,93	0,93	1,87	0,93	0,66	0,87
M32490	Cyr61	cysteine rich protein 61	1,87	2,46	1,52	1,87	1,52	1,23	1,41	0,93	0,66

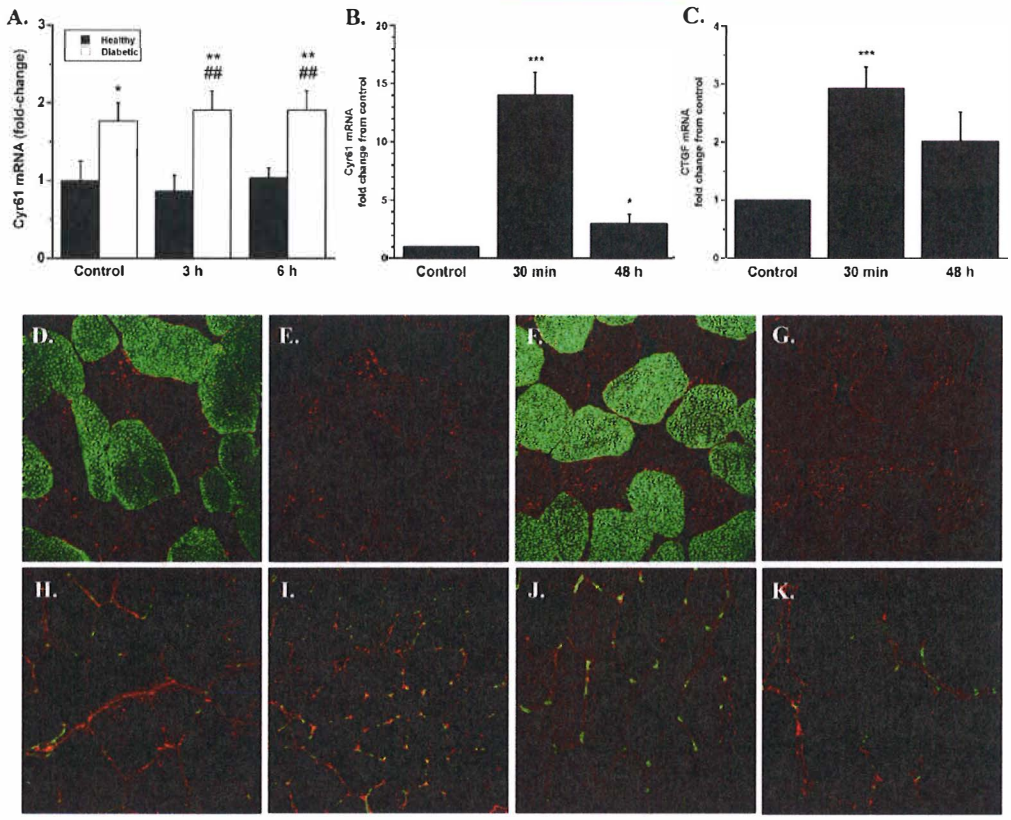


FIGURE 7. The effects of diabetes and exercise training on the expression of Cyr61 and CTGF (study I) are shown in colour-coded table (colours as in Fig.5). A) Diabetes significantly upregulated the expression of Cyr61 in mice (study II). Running exercise had no effect in either healthy or diabetic animals. B-C) High mechanical loading increased markedly the immediate expression of Cyr61 and CTGF in the human skeletal muscle (study III). Cyr61 expression remained elevated also at 48 h post exercise. D-K) Localization of CTGF and Cyr61 in human skeletal muscle. Confocal microscopic images showing Cyr61 (D, E) and CTGF (F, G) with red colour and slow muscle fibres with green. Cyr61 and CTGF localized to skeletal muscle fibres and surrounding extracellular matrix. Fast muscle fibres expressed more CCN proteins than slow fibres. H-I) Cyr61 double staining with CD31 (capillaries, green). In addition to muscle fibres, Cyr61 staining was also found around capillaries in ECM. J-K) CTGF double staining with CD31. CTGF was mainly found in ECM and in the muscle fibres.

5.2.4 Other angiogenic factors (I)

The microarray results showed that also many other angiogenesis-related genes were affected by diabetes (Table 4). The mRNA levels of myoglobin, MnSOD (SOD2), integrin alpha V, secreted acidic cysteine-rich glycoprotein (Sparc) and several collagens, *e.g.*, type I and type IV collagens, were downregulated in diabetic muscles. The levels of HIF-1α and metallothionein 1 and 2 were, in turn, increased in the muscles of diabetic mice. Exercise training alleviated diabetes-induced changes, at least to some extent, in myoglobin, HIF-1α, and metallothioneins. In the healthy mice one, three or five weeks of endurance-type exercise training did not induce significant changes in the basal mRNA levels of most pro- or antiangiogenic factors.

TABLE 4. Angiogenesis-related genes, other than VEGFs, which were significantly affected by diabetes and/or exercise training in the study I are shown. Gene expressions are expressed in relation to the control group at the same time point (=1). Statistically significant changes are colour-coded as follows: red=up-regulated, green=down-regulated, yellow=diabetes-induced change in expression was attenuated by exercise. Group abbreviations: D=diabetic, DT=diabetic trained and T=healthy trained, numbers=duration of treatment in weeks.

Accession	Gene name	Gene / Protein	D1	D3	D5	DT1	DT3	DT5	T1	T3	T5
L35528	Sod2	superoxide dismutase 2	0,57	0,62	0,66	0,81	0,81	0,76	1,07	1,15	1,15
X04405	Mb	myoglobin	0,35	0,50	0,62	0,71	0,66	0,76	1,07	1,15	1,15
M15832	Col4a1	procollagen, type IV, alpha 1	0,47	0,54	0,66	0,57	0,54	0,76	0,66	0,93	1,00
X04647	Col4a2	procollagen, type IV, alpha 2	0,57	0,76	0,76	0,66	0,76	0,87	0,81	1,15	1,07
AI840158	Angptl2	angiopoietin-like 2	0,44	0,76	0,62	0,62	0,81	0,76	0,81	1,00	1,00
AI843901	Itgav	integrin alpha V	0,62	0,76	0,93	0,66	0,71	0,76	0,76	0,81	0,93
Z50013	Hras1	Harvey rat sarcoma virus oncogene 1	0,57	0,66	0,76	0,81	0,71	0,76	0,87	0,93	0,81
X04017	Sparc	secreted acidic cysteine rich glycoprotein	0,47	0,57	0,81	0,57	0,71	0,81	0,76	1,07	1,15
U88327	Socs2	suppressor of cytokine signaling 2	0,93	1,07	1,15	0,87	0,62	0,81	0,87	0,81	0,87
AF003695	Hif1a	hypoxia inducible factor 1, alpha	0,93	1,23	1,23	0,81	0,93	1,00	0,81	0,81	0,87
D88791	Csrp3	cysteine and glycine-rich protein 3	3,73	2,83	2,14	1,87	1,00	2,46	0,93	1,00	1,15
V00835	Mt1	metallothionein 1	11,31	8,00	2,30	6,06	3,25	7,46	3,48	1,41	0,87
K02236	Mt2	metallothionein 2	10,56	8,00	4,00	7,46	4,00	8,57	4,93	1,23	1,00
AF064088	Tieg1	TGFB inducible early growth response 1	2,83	1,52	1,74	1,74	1,32	1,15	1,23	0,87	0,54
AW049795	Tbrg1	TGF beta regulated gene 1	2,14	1,52	1,32	1,23	1,32	1,32	1,07	0,93	0,93
AW120719	Eif2b1	eukaryotic translation initiation factor 2B 1a	1,87	1,32	1,23	1,15	1,32	1,32	1,07	0,93	1,00
AI849838	Cul1	cullin 1	1,74	1,23	1,32	1,23	1,23	1,32	1,23	1,00	1,00
U42384	Fin15	fibroblast growth factor inducible 15	1,52	1,63	1,23	1,23	1,41	1,07	1,07	1,41	1,15
AI843709	Il6st	interleukin 6 signal transducer	1,63	1,32	1,41	1,41	1,23	1,23	1,15	0,93	0,87
AF061503	Bop1	block of proliferation 1	1,63	1,87	0,93	1,63	1,74	1,52	1,23	1,52	1,32

5.2.5 Anti-angiogenic factors (I, II)

Diabetes markedly increased the expression of thrombospondin-1 (TSP-1) mRNA, an inhibitor of angiogenesis, in diabetic muscles (Figure 8). The levels of retinoblastoma-like-2 (Rbl-2), another angiogenesis inhibitor, were also increased by diabetes in the training experiment. Exercise did not affect the expression of TSP-1 or Rbl-2 either in healthy or diabetic muscles.

Accession	Gene name	Gene / Protein	D1	D3	D5	DT1	DT3	DT5	T1	T3	T5
M62470	Thbs1/Tsp1	thrombospondin 1	1,74	2,14	2,83	1,63	1,87	2,64	0,71	0,81	0,66
U36799	Rbl2	retinoblastoma-like 2	1,23	1,32	1,41	1,32	1,32	1,52	1,07	0,87	1,00

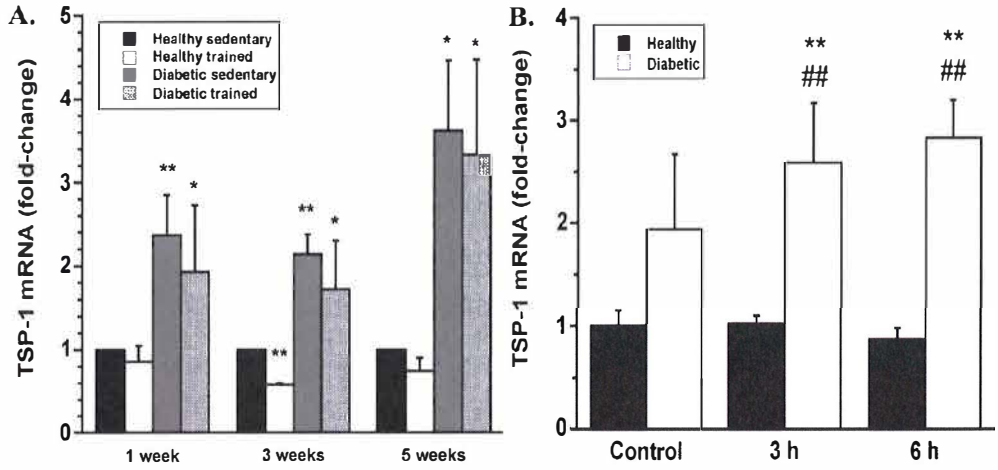


FIGURE 8. The expression of angiogenesis inhibitors thrombospondin-1 and retinoblastoma-like 2 was increased in diabetic muscles. The colour-coded table shows the expression patterns in study I. A) TSP-1 expression in study I analysed by real-time PCR. The results concur well with the microarray data. In addition, TSP-1 expression was decreased in the trained healthy mice compared to controls after three weeks (* different from the control group). B) Thrombospondin-1 expression was increased in the exercised diabetic mice compared to healthy controls (*) and the respective exercised groups (#) in study II.

5.2.6 Laser capture microdissection (II)

VEGF-A mRNA expression in the capillaries collected from the gastrocnemius muscle with LCM increased significantly in the healthy exercised mice (HE6) compared to the healthy controls (C) (Figure 9). VEGF-A expression in the capillaries of the exercised diabetic mice (DE6) did not differ significantly from diabetic controls (D), although it tended to be higher in the exercised mice. Capillary expression of TSP-1 decreased in the healthy exercised mice and increased in both diabetic groups compared to healthy controls.

In the pure skeletal muscle fibres obtained with LCM, VEGF-A tended to increase in both the healthy and diabetic exercise groups compared to sedentary groups, but these differences were not significant. TSP-1 expression in muscle fibres was significantly increased in the exercised diabetic mice compared to diabetic controls, and it was also more than 4-fold higher in DE6 compared to the healthy groups, although the difference was not statistically significant.

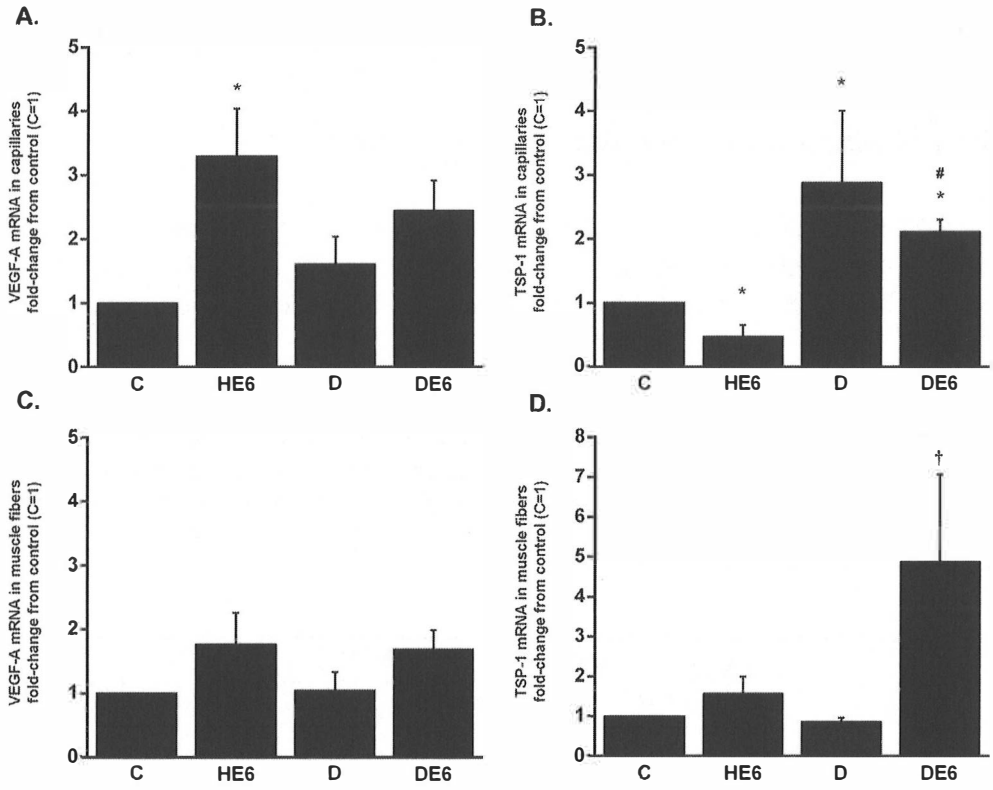


FIGURE 9. Capillaries and muscle fibres collected from the gastrocnemius muscle with laser capture microdissection were analyzed for the mRNA expression of VEGF-A and TSP-1. Effects of exercise and diabetes in capillaries are shown in images A and B and in muscle fibres in C and D. Groups: C=healthy control, D=diabetic control, HE6=healthy exercised, killed 6 h post exercise, DE6=diabetic exercised, killed 6 h post exercise. * $P < 0.05$ vs. healthy control, # $P < 0.05$ vs. respective healthy exercised group, † $P < 0.05$ vs. diabetic sedentary.

5.3 Lymphatic vessels and lymphangiogenic growth factors (III-V)

5.3.1 Identification of blood and lymphatic vessels

For the reliable differentiation of blood and lymphatic vessels the combined use of different endothelial markers was employed. The specificity of these endothelial markers, as reported in the literature and as found in the present study, is presented in Table 5.

TABLE 5. The expression patterns of the endothelial markers used to distinguish blood and lymphatic vessels in the present study. By combining these markers, it was possible to identify blood and lymphatic vessels in human and mouse skeletal muscle with light and fluorescence microscopy (+ = protein is expressed in endothelia, – = protein is not found in endothelia, number of + or – indicates the staining intensity).

	CD31 (Sleeman *et al.* 2001)	**PAL-E** (Schlingemann *et al.* 1985)	**VEGFR-3** (Jussila *et al.* 1998; Paavonen *et al.* 2000; Valtola *et al.* 1999)	**LYVE-1** (Jackson 2003)
Blood vessel endothelia	+++	+++ (Except arteries)	– (+)	–
Lymphatic vessel endothelia	+ / –	–	++	+++

5.3.2 Lymphatic vessels in skeletal muscle (IV, V)

Light microscopy (IV)

In human and mouse muscles, VEGFR-3 staining was found in larger vessels, which were negative for PAL-E and located in the perimysial interstitium between muscle bundles close to arteries and veins. In the whole mouse muscles VEGFR-3 positive

vessels were also found in the epimysium, which covers the whole muscle group. This cannot be seen in specimens from human muscle biopsies, since they represent only a small portion of muscle. Only those VEGFR-3-positive vessels which had thin vessel walls and were negative for PAL-E staining were classified as lymphatic. VEGFR-3 staining was also observed in the capillary bed, but it was not possible to definitely determine without double staining, whether it was a blood or lymphatic vessel. Vessels positive for LYVE-1 antibody were found both within the connective tissue next to arteries and veins and in small vessels between the muscle fibres.

Confocal microscopy (IV)

With LYVE-1 and CD31 double staining and confocal microscopic examination we were able to confirm the finding that larger blood and lymphatic vessels situated next to each other in the extracellular matrix in human skeletal muscle. In addition to the presence of larger LYVE-1 positive vessels in the perimysial space, LYVE-1 and CD31 double staining also revealed small capillary-sized vessels positive only for LYVE-1 in the capillary bed between individual muscle fibres. These vessels were located next to CD31 positive blood capillaries, but were much fewer in number. The LYVE-1 antibody used in the present study only stained vessels which were negative for CD31 antibody. Thus, CD31 specifically stained the blood vessel endothelium and LYVE-1 only the lymphatic endothelium. To verify the detection of the lymphatic endothelium with the LYVE-1 antibody, we used human skin samples, because skin is known to contain a large number of lymphatic vessels. In skin, CD31 and LYVE-1 similarly stained two different vessel populations, confirming the findings from the muscle samples and the specificity of the antibodies.

Lymphatic vessel density (V)

Lymphatic capillaries were not evenly distributed throughout the mouse muscles. Some areas had a markedly larger number of small lymphatic capillaries than others. The mean lymphatic capillary density in the mice varied from 17 to 54 (cap x mm^{-2}), which is more than 10-fold less than the number of blood capillaries. These numbers do not include larger lymphatic vessels in the perimysium. No differences were observed in lymphatic capillary density between the healthy and diabetic or sedentary and trained groups.

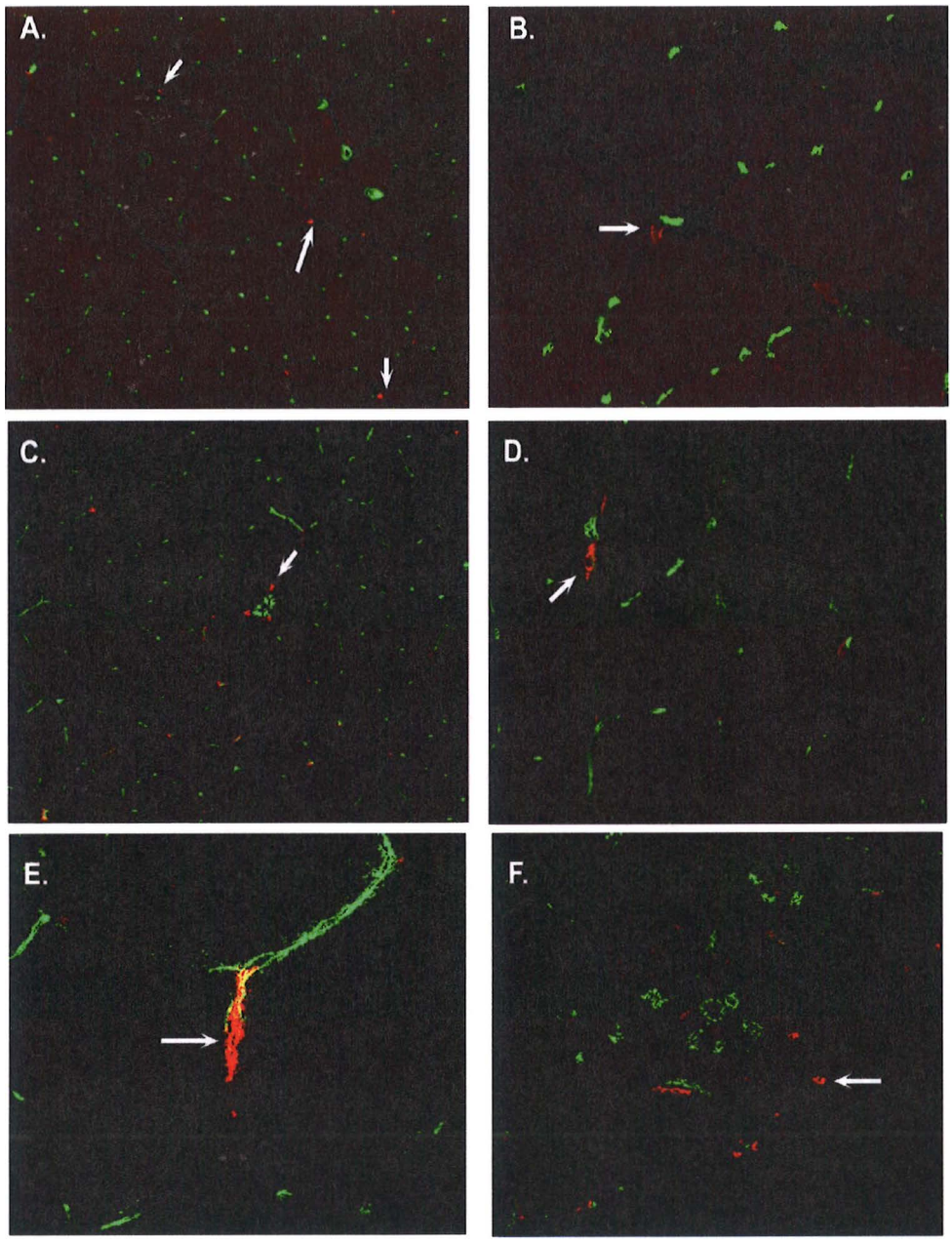

A.
B.
C.
D.
E.
F.

FIGURE 10. (previous page) A-B) Blood (green) and lymphatic (red) capillaries in mouse skeletal muscle. Blood capillaries are stained with CD31 antibody and lymphatic vessels with LYVE-1 antibody. A) Lymphatic vessels were not equally distributed around the muscle fibres, as blood capillaries were, and they were ~10-fold fewer in number. B) Confocal microscopic image showing thin endothelial layer of lymphatic vessels. C-D) A similar distribution of lymphatic capillaries was found in human skeletal muscle as was found in mouse muscles. E) With confocal microscopy it was possible to visualize blood and lymphatic vessels traversing in parallel between muscle fibres. Shown here is the image stack from several individual layers. A video clip is included in the supplementary material appended to the original article. F) The LYVE-1 staining of lymphatic vessels in human skin was used as a positive control, as skin is known to contain a large number of lymphatic vessels.

5.3.3 Effects of exercise and diabetes on lymphangiogenic growth factors (III, V)

Acute running experiment (V)

Skeletal muscle VEGF-C mRNA expression was lower in the healthy control group than in the healthy exercised or any of diabetic groups. VEGF-C expression in the exercised diabetic mice did not differ from that in their diabetic control group. Exercise had no effect on VEGF-D mRNA expression in healthy or diabetic mice. However, VEGF-D expression was significantly greater in the exercised diabetic mice than in their respective healthy exercised groups or in the healthy control group. VEGFR-3 mRNA expression, in turn, was not affected by either exercise or diabetes.

Exercise training experiment (V)

One, three or five weeks of exercise training induced no effects on the mRNA expression of VEGF-C, VEGF-D or VEGFR-3 in the healthy or diabetic mice. VEGF-D expression was significantly increased in the diabetic mice compared to the healthy mice at all the time points studied ($P < 0.001$). The variation in the diabetic groups was large, individual increases varying from 1.2 to 18.9-fold compared to those in their respective controls. After one and three weeks of training, VEGF-D expression was lower in the trained diabetic groups than in the respective sedentary diabetic groups ($P < 0.05$). Diabetes did not affect the expression of VEGF-C or VEGFR-3 mRNA. In the

both acute and training exercise experiments the mRNA levels of VEGF-D correlated positively with blood glucose levels (Acute: $r = 0.567$, Training: $r = 0.549$, $P < 0.001$).

VEGF-D protein concentration was measured from serum and muscle homogenates. In serum, VEGF-D protein concentration was above the level of detection of the ELISA kit in only 1/3 of the samples, and there was no difference between the healthy and diabetic mice in serum VEGF-D values. It was not possible to perform a statistical comparison between each time point and group because of the low sample number with detected levels of VEGF-D. VEGF-D concentration in the skeletal muscle homogenates was greater than in serum, and above the detection level in all samples. However, no significant difference was observed between the healthy and diabetic or exercise trained and sedentary groups in muscle homogenate VEGF-D protein concentration.

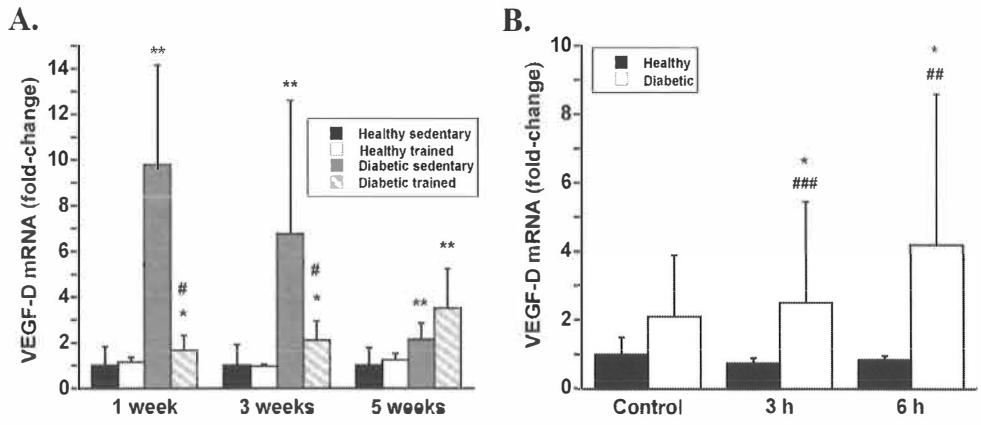


FIGURE 11. VEGF-D mRNA expression was increased in diabetic muscles in both A) exercise training and B) acute exercise experiments. Exercise training reduced the expression in diabetic mice after one and three weeks of training. In the acute exercise experiment the running bout did not affect VEGF-D expression.

Jumping exercise with high mechanical loading (III)

In the jumping exercise study (III), mRNA levels of VEGF-C and VEGF-D did not change either 30 min or 48 h post exercise in healthy human skeletal muscle.

5.3.4 Localization of VEGF-C and VEGF-D in skeletal muscles (IV, V)

Immunohistochemical staining of VEGF-C and VEGF-D showed different localization of these two growth factor proteins in skeletal muscle. Intensive VEGF-C staining was found in nerves, muscle spindles and interstitial cells (possibly fibroblasts), but was not found in myofibres or larger vessels. VEGF-D was located under the sarcolemma in some of the muscle fibres. VEGF-D was also found in fibroblasts and in the endothelium of larger blood vessels (Fig. 12). Both VEGF-C and –D were not evenly distributed across the whole muscle group, but were expressed in or around clusters of 2-10 muscle fibres. Neither VEGF-C nor –D were found in capillaries. All the studied skeletal muscle and heart samples contained at least small amount of both VEGF-C and –D mRNAs, showing that both proteins are produced in skeletal muscles.

Immunohistochemical staining of VEGF-D in diabetic muscles showed diffuse staining inside muscle fibres that were swollen or infiltrated by macrophages, *i.e.*, in damaged fibres (in study V). Of the ten diabetic muscles studied two contained more than 30 VEGF-D-positive fibres while the remainder had between 1 and 10. Damaged fibres were not found in healthy muscles. One diabetic mouse had an unusually large number of damaged fibres that were positive for VEGF-D. This mouse also had quantitatively one of the highest mRNA expressions of VEGF-D. In addition to diffuse staining of VEGF-D in swollen fibres, necrotic fibres with a large number of infiltrated macrophages stained intensively for VEGF-D. Larger vessels in the perimysium were also stained intensively for VEGF-D, but this staining was similar in healthy and diabetic mice. The staining pattern or intensity of VEGF-C and VEGFR-3 did not differ between healthy and diabetic or sedentary and exercise trained mice.

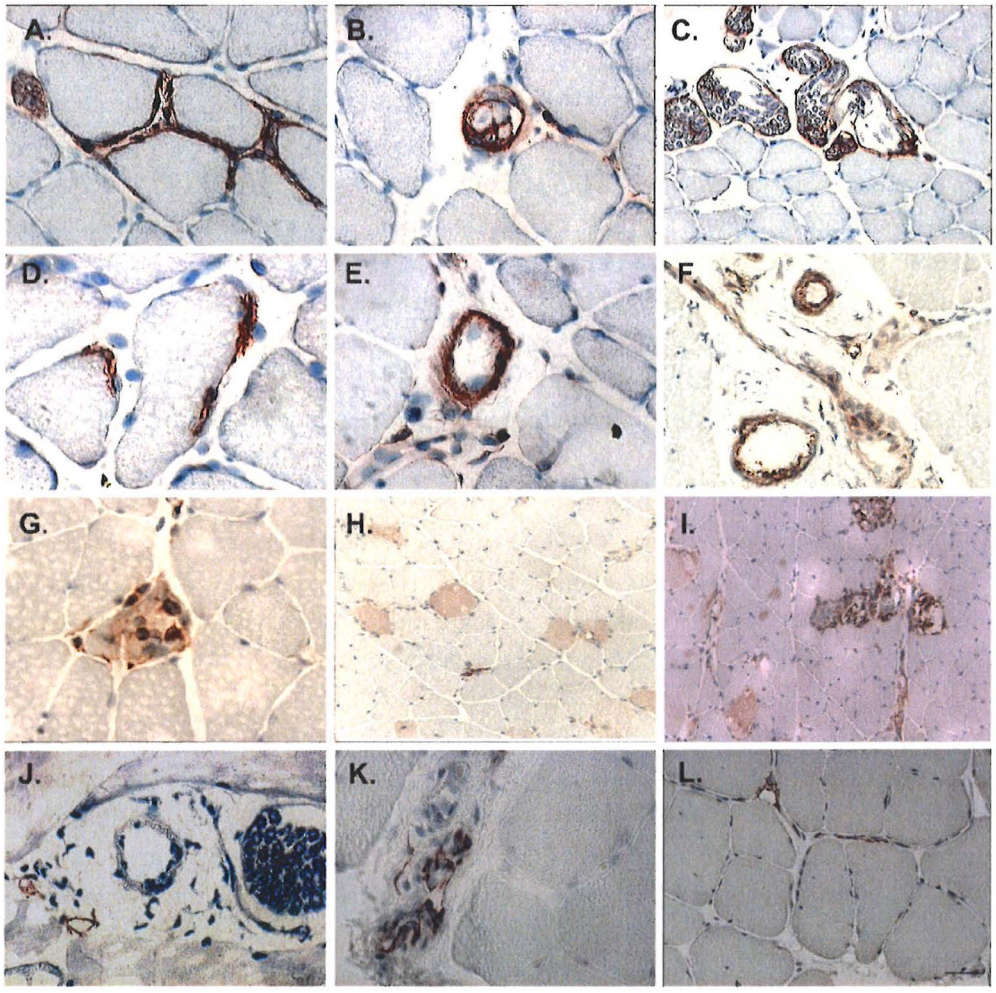


FIGURE 12. VEGF-C protein was found A) in the interstitium between the muscle fibres, B) in muscle spindles and C) in nerves in mouse skeletal muscle. The localization was not affected by either diabetes or exercise training. VEGF-D was found D) in some of the muscle fibres under the sarcolemma, E) in arterioles and F) in larger vessels between the muscle fibre bundles. This type of expression was found in both healthy and diabetic muscles. In addition, in diabetic muscles VEGF-D was also localized G) in muscle fibres with centralised nuclei (infiltrated macrophages), H) in swollen fibres and I) in damaged muscle fibres. This staining pattern was not found in any of the healthy muscles. J-L) VEGFR-3 was found in the endothelia of smaller and larger lymphatic vessels with thin endothelia and close to arteries and veins. It was also found in the capillary bed between muscle fibres.

5.4 Summary of the results

In this thesis the mRNA expression and protein localization of vascular endothelial growth factors, their receptors and other angiogenesis-related molecules were studied in relation to exercise and diabetes. Below is a summary table showing the main results of the mRNA expression (Table 6). This study also demonstrated the presence and quantity of small lymphatic vessels between muscle fibres in the skeletal muscle capillary bed. Immunohistochemical stainings showed the localization of the VEGF family proteins in different cell types in skeletal muscle.

TABLE 6. Summary of the main mRNA expression results. ↓ = downregulated, ↑ = upregulated, ↔ = no change. Two arrows in the same box mean that there were differences in the responses between the acute or training studies.

Protein name (mRNA expression)	Effect of diabetes in muscle	Effect of exercise in healthy muscle	Effect of exercise in diabetic muscle
VEGF-A	↓ ↔	↑	↑ ↔
VEGF-B	↓ ↔	↑ ↔	↑ ↔
VEGF-C	↔	↔	↔
VEGF-D	↑	↔	↔ ↓
VEGFR-1	↓ ↔	↔	↔
VEGFR-2	↓ ↔	↑	↑ ↔
VEGFR-3	↔	↔	↔
Neuropilin-1	↓	↔	↑
TSP-1	↑	↓(cap) ↔ (skm)	↔
Rbl2	↑	↔	↔
Myoglobin	↓	↔	↑
Cyr61	↑	↑ ↔	↔
CTGF	↑	↑	↓
Metallothioneins	↑	↑	↓
MnSOD (SOD2)	↓	↔	↑

6 DISCUSSION

6.1 Capillarization and angiogenic gene expression

Angiogenesis is modulated by several pro- and antiangiogenic factors, which form a complex angiogenesis-regulatory network. In this thesis the purpose was to determine the separate and combined effects of diabetes and exercise training or an acute exercise bout on the expression of pro- and antiangiogenesis genes. The objective was to gain more insight into the mechanisms by which diabetes affects peripheral circulation, and whether the positive effects of exercise on angiogenesis are mediated by the same or partly overlapping pathways.

In the training experiment, diabetic mice exhibited significant atrophy of the muscle fibres, which is a common phenomenon in type 1 diabetes without insulin treatment. Because of the smaller fibres, capillary density (cap x mm^{-2}) tended to increase in diabetic muscles; however, the capillary-to-fibre ratio decreased significantly in both sedentary and trained diabetic mice compared to healthy mice. This indicates that diabetes caused capillary rarefaction in the muscles, which concurs with earlier findings from animal models of type 1 diabetes and metabolic syndrome (Kindig *et al.* 1998; Frisbee 2005). Data on humans come from rather few studies with low numbers of subjects. Type 2 diabetes is associated with either decreased capillarization (Mathieu-Costello *et al.* 2003) or no difference compared to healthy controls (Zierath *et al.* 1996). Type 1 diabetes has been shown to induce thickening of the capillary basement membrane (Leinonen *et al.* 1982; Yokoyama *et al.* 1997). However, no differences in the capillary-to-fibre ratio has been observed (Leinonen *et al.* 1982; Wallberg-Henriksson *et al.* 1984). Exercise training in the present study did not increase capillarization statistically significantly in either healthy or diabetic muscles, but tended to do so in healthy mice. This is similar to the findings of Wallberg-Henriksson and co-workers who showed increased capillarization in healthy subjects after exercise training, but no change in type 1 diabetic patients (Wallberg-Henriksson *et al.* 1984). The effects of endurance training are dependent on mode of exercise, training intensity, frequency, and duration, which complicate the comparison of results

from different studies. In addition, the glycaemic state of the patients may play an important role, if hyperglycaemia and subsequent AGE formation are among the major factors in capillary rarefaction or angiogenic responses to exercise. In diabetic patients with coronary or peripheral artery disease, the formation of new blood vessels is significantly reduced (Abaci *et al.* 1999; Rivard *et al.* 1999). This may contribute to severe limb ischemia, which in diabetic patients often results in foot ulceration and lower extremity amputation. On the other hand, diabetes induces proliferative retinopathy, which includes excess angiogenesis. Many questions remain unanswered about the mechanisms by which diabetes could both limit the formation of new blood vessels in most organs and simultaneously induce angiogenesis in the retina. Below the diabetes-induced changes in the regulation of angiogenesis in skeletal muscle are discussed.

Angiogenic VEGFs

In the exercise training study (I) diabetes decreased the mRNA and protein levels of VEGF-A by 30-50 % depending on duration of diabetes (2-6 weeks). The levels of VEGF-B, VEGFR-1, VEGFR-2, and neuropilin-1 were significantly reduced as well. In the experiment with the single bout of exercise (II) the basal mRNA levels of VEGF-A, VEGF-B, VEGFR-1 or VEGFR-2 did not differ between healthy and diabetic mice after ten days of diabetes, which can be considered as acute diabetes. In accordance with the present findings from the training experiment, several studies have shown decreased expression of VEGF-A in diabetic skeletal muscle or myocardium (Chou *et al.* 2002; Emanueli *et al.* 2004; Chung *et al.* 2006; Shoji *et al.* 2006). There are also studies with findings similar to those of our acute exercise study that have reported no change in the expression of VEGF-A due to hyperglycemia (Larger *et al.* 2004; Tanii *et al.* 2006), while others have even shown increased expression compared to healthy muscle or myocardium (Sasso *et al.* 2005; Li *et al.* 2007). In diabetic myocardium increased VEGF-A expression was accompanied by decreased expression of VEGFR-1 and VEGFR-2 and downregulation of its signal transduction (Sasso *et al.* 2005). It may be that long-term hyperglycaemia induces dysregulation in various parts of the VEGF-A signalling in both myocardium and skeletal muscle, resulting in impaired angiogenesis and maintenance of the vasculature. In the present study (II) the expression of angiogenic growth factors VEGF-A and VEGF-B strongly correlated with each other and with their receptors, especially VEGFR-2. This suggests that the

expression of angiogenic growth factors and their receptors are similarly regulated in response to exercise or elevated blood glucose. Interestingly, the present results show high similarity with the findings by Schiekofer and co-workers, who showed dysregulation of the angiogenic network in skeletal muscle of leptin-deficient mice, which is a model of type 2 diabetes (Schiekofer *et al.* 2005). These similar findings suggest that hyperglycaemia may be the common factor inducing the dysregulation of angiogenesis.

Exercise training did not induce many changes in the basal mRNA levels of angiogenesis-related genes in healthy skeletal muscle. This indicates the existence of a good balance between pro- and antiangiogenic factors. In diabetic mice increased VEGF-A, VEGF-B and myoglobin mRNA levels after exercise training may enhance capillary survival and oxygen delivery to muscle fibres compared to sedentary diabetic mice. The effects of exercise training on angiogenic growth factors in diabetic muscle have not been reported earlier. Results from the acute exercise study showed that in healthy mice the mRNA expression of VEGF-A and its receptor VEGFR-2 were significantly increased 6 h post exercise in the skeletal muscle homogenates. VEGF-B expression also showed a similar trend. Increases in VEGF-A and VEGFR-2 after exercise have been demonstrated earlier in healthy muscles, *e.g.*, (Breen *et al.* 1996; Gavin *et al.* 2004; Jensen *et al.* 2004b; Gustafsson *et al.* 2005), but the effects of exercise on the expression of VEGF-B have not been reported earlier. In diabetic mice there was also a trend to increased expression of VEGF-A (17 %) and VEGFR-2 (20 %) 6 h post exercise, but these changes did not reach statistical significance. This suggests that angiogenic responses to a single bout of exercise in skeletal muscle are reduced in diabetic compared to healthy mice. The expression of angiogenic growth factors in diabetic muscles may, nevertheless, be upregulated by exercise to some extent. The observed increase in VEGF-A expression was modest compared to those found in previous studies. VEGF-A mRNA expression has been shown to increase 0-6 hours after an exercise bout in both animal and human studies, and in many studies the increases have been about 2 to 5-fold (Breen *et al.* 1996; Gustafsson *et al.* 1999; Gavin *et al.* 2006; Gavin *et al.* 2007; Gustafsson *et al.* 2007; Hellsten *et al.* 2007). One reason could be the choice of exercise intensity, which was moderate in our study, especially for the healthy mice, since all the mice ran at the same absolute intensity for 1 h. Also

methodological differences (Northern blot *vs.* qPCR) may account for some of the differences in the magnitude of fold-change.

Exercise training has earlier been demonstrated to improve endothelial function in patients with type 1 diabetes (Fuchsjager-Mayrl *et al.* 2002). The direct mechanisms behind the improvement were not studied, but they could be related to the present observations that the expression of many genes responsible for the maintenance and survival of the endothelium were increased in the trained compared to the sedentary diabetic mice. Both of these training effects could be mediated at least partly by nitric oxide (NO), since both vasodilation and angiogenesis are regulated by NO (Benoit *et al.* 1999; Gavin *et al.* 2000; Hudlicka *et al.* 2000; Lloyd *et al.* 2001; Gavin & Wagner 2002; Milkiewicz *et al.* 2005). NO is generated by the activation of neuronal nitric oxide synthase (nNOS) by muscle activity or endothelial NOS (eNOS) by increased blood flow and capillary shear stress. NO may increase capillary proliferation through upregulation of VEGFR-2 and VEGF-A (Milkiewicz *et al.* 2005). If hyperglycaemia reduces NOS expression and activity or NO bioavailability, this could lead to reduced VEGF-A signalling in diabetic muscles. There are recent findings suggesting that nNOS may be reduced in diabetic muscles (Bradley *et al.* 2007), and that impaired ischemia-induced angiogenesis is related to reduced phosphorylated eNOS/total eNOS ratio indicating reduced downstream VEGF-A signalling (Hazarika *et al.* 2007).

The reduced capacity in diabetic mice to enhance angiogenic mRNAs and capillarization in skeletal muscle could be also related to recent findings from gene transfer studies, where diabetic mice showed impaired response to angiogenic gene therapy (Emanueli *et al.* 2007). Although the effects of gene therapy may be reduced in diabetic compared to healthy animals (Roguin *et al.* 2003; Emanueli *et al.* 2007), there are experiments showing the potential of angiogenic gene transfer also in diabetic muscles (Rivard *et al.* 1999; Li *et al.* 2007). However, in these studies the magnitude of the effects has not been compared with the effects in healthy mice. The results of placebo-controlled human clinical trials with VEGF or other growth factors have not been as encouraging as those found in animal studies (Rissanen & Ylä-Herttuala 2007), indicating that more than a single growth factor may be needed for adequate angiogenesis in pathological skeletal muscle. This makes "exercise therapy" an

interesting option in the prevention and treatment of peripheral vascular problems in diseases such as diabetes.

Other angiogenic factors

Several other angiogenesis-related factors were affected by diabetes in skeletal muscles. The expression of myoglobin, MnSOD, integrin alpha V and secreted acidic cysteine-rich glycoprotein (Sparc) were downregulated, and the levels of metallothioneins 1 and 2 were increased in diabetic muscles. Reactive oxygen species (ROS) are increased in hyperglycemic tissues and this could be one of the main mechanisms leading to diabetic complications (reviewed in Brownlee 2001 & 2005). MnSOD is effective in degrading superoxide, and thus preventing increased ROS in diabetic muscle. The cytoprotective effects of VEGF-A have been linked to its ability to induce MnSOD (Abid *et al.* 2004). Thus, the reduced MnSOD expression found in the present study may be related to the decreased VEGF-A levels, and may further predispose myofibres and endothelial cells to oxidative damage. Myoglobin and MnSOD mRNA levels were increased in trained diabetic mice compared to sedentary diabetic mice at least in some phase of the training period, showing the positive effects of exercise. Metallothioneins, in turn, were markedly increased in diabetic muscles. This has been shown earlier in diabetic myocardium and endothelial cells (Apostolova *et al.* 2001; Cai *et al.* 2005; Song *et al.* 2005). Metallothioneins are also involved in protecting from cellular damage by ROS. Recently, metallothioneins were shown to rescue HIF-1 transcriptional activity in diabetic cardiomyocytes, which may lead to upregulation of angiogenic growth factors (Feng *et al.* 2007). In exercise trained diabetic mice metallothioneins were also increased but to lesser extent than in sedentary diabetic mice. This may be related to either reduced ROS production or better degrading of ROS by increased MnSOD.

The expression of Cyr61 and CTGF was also increased in diabetic muscles (studies I and II). In addition, the present results showed that high mechanical loading on muscle fibres and connective tissue induced a marked increase in the mRNA and protein levels of Cyr61 and CTGF in healthy skeletal muscle. These CCN-family proteins are important regulators of angiogenesis, endothelial cell function and ECM modulation (Brigstock 2002; Perbal 2004). Cyr61 is classified as an immediate early gene, which is rapidly induced in response to externally applied cyclic mechanical strain in cardiac

and smooth muscle cells and in fibroblasts (Tamura *et al.* 2001; Schild & Trueb 2004; Zhou *et al.* 2005). It also regulates the activity and production of other angiogenic proteins such as VEGF-A (Perbal 2004; Zhou *et al.* 2005). CTGF has earlier been shown to be up-regulated by mechanical stress in fibroblasts (Schild & Trueb 2004). The observed increase in the mRNA and protein expression of Cyr61 after exercise are in line with the observations by Hilfiker-Kleiner and colleagues (Hilfiker-Kleiner *et al.* 2004), who showed that Cyr61 is induced in the myocardium by pressure overload and in cardiomyocytes after mechanical stretch. It has been suggested that Cyr61 might play an important role in the adaptation of the heart to cardiovascular stress. The present results show that this occurs also *in vivo* in skeletal muscle. It is interesting that both high eccentric muscle loading and hyperglycaemia induce significant expression of CCN proteins in skeletal muscle. Also denervation of muscles could induce increased expression of Cyr61 and CTGF (Magnusson *et al.* 2005). These findings together suggest a role for CCN proteins in skeletal muscle adaptation to either physiological or pathological conditions.

It has been proposed that mechanical stretch -induced Cyr61 expression stimulates genetic reprogramming of angiogenic, adhesive and structural proteins, and that CTGF promotes extracellular matrix accumulation, especially type 1 collagen (Brigstock 2002; Zhou *et al.* 2005; Chaqour & Goppelt-Struebe 2006). The potential functions of Cyr61 and CTGF in healthy skeletal muscle after exercise include the induction of angiogenic responses. Fataccioli and co-workers showed with gene transfer that Cyr61 was effective in improving perfusion in ischemic hindlimb model (Fataccioli *et al.* 2002). Its role in vascular development has also been shown with knockout mice, which suffer embryonic death due to vascular defects in placenta (Mo *et al.* 2002). Other possible mechanisms of CCN proteins in skeletal muscle include modulation of ECM, which is known to occur due to exercise (Kjaer 2004). Cyr61 has also been shown to have both pro- and antiapoptotic effects in different cell types (Chen & Du 2007). Recently, in diabetic retina advanced glycation end products were shown to induce the expression of Cyr61 and CTGF, and this seems to be related to the thickening of the capillary basement membrane (Hughes *et al.* 2007). If this is also the case in skeletal muscle, it may lead to excess accumulation of extracellular matrix components and endothelial cell death, leading to dysfunctional capillaries and capillary rarefaction. CTGF is also linked to diabetic nephropathy by inducing fibrosis

in the kidney (Guha *et al.* 2007). However, it is important to notice that the effects of hyperglycaemia may be completely different in different organs, such as skeletal muscle and retina. The signalling pathways mediated by CCN proteins are being intensively studied at the moment, but their role in skeletal muscle is yet to be defined. Cyr61 binds to at least five integrins and heparan sulphate proteoglycans (Chen & Du 2007). Through these receptors it can activate Wnt pathway, NFkB pathway, MAPK pathway or Akt pathway depending on the cell type and receptor. To date the downstream signalling of Cyr61 has not been studied in skeletal muscle or myoblasts. It may be that CCN proteins have either positive or negative effects, depending on the target tissue or the (patho)physiological environment.

Angiogenesis inhibitors

The expression of thrombospondin-1 (TSP-1), a known inhibitor of angiogenesis (Jimenez *et al.* 2000), was significantly increased in the muscles of diabetic mice in both of the present experiments (studies I and II). Our results concur with those of Stenina and co-workers, who showed increased TSP-1 expression in the arterial vessel wall of diabetic Zucker rats (Stenina *et al.* 2003). Recently, the hexosamine pathway was found to mediate the hyperglycaemia-induced upregulation of TSP-1 in human aortic smooth muscle cells (Raman *et al.* 2007). This pathway has earlier been shown to mediate hyperglycaemia-induced pathologic changes in gene expression (Brownlee 2001 & 2005). It has been proposed that increased TSP-1 in blood vessels could be a direct response of vascular cells to increased glucose levels and, thus, a link between diabetes and atherosclerotic complications (Stenina *et al.* 2003; Raman *et al.* 2007). On the basis of findings of the present studies, TSP-1 could also lead to capillary rarefaction in skeletal muscle microcirculation, as was found in the present study, and also in obese Zucker rats (Frisbee 2005). The possible mechanisms behind this could be increased apoptosis of the existing endothelial cells and inhibition of the new capillary formation.

In the present experiments exercise training had only minor or no effects on the diabetes-induced expression of TSP-1. It is possible that exercise could affect TSP-1 expression in diabetic muscles by decreasing blood glucose levels, and via this inhibit the activation of the hexosamine pathway. In streptozotocin-induced diabetes without insulin treatment, as in the present experiments, the blood glucose values remained

high also in the exercise-trained groups, which may have blunted the possible effects of exercise. Interestingly, it was recently shown that a single exercise bout increased TSP-1 mRNA in healthy rat skeletal muscle, but after 3 consecutive days of exercise this response was ablated (Olfert *et al.* 2006). In our exercise training study (I) TSP-1 expression was decreased after 3 weeks of training in the healthy trained mice compared to sedentary mice. In the acute exercise study (II) no effect of exercise was observed in muscle homogenates, but in capillaries TSP-1 expression was downregulated 6 h post exercise. In the study by Olfert and colleagues, the peak expression was detected 1 h after the cessation of exercise, and the increase was no longer statistically significant 2 h post exercise. It may be that immediately after exercise TSP-1 is upregulated and proangiogenic factors downregulated, as has been found for the VEGF-A protein immediately after exercise (Gavin *et al.* 2004). Thus, when capillary shear stress induced by increased blood flow has returned to normal levels after cessation of exercise, proangiogenic factors are gradually produced and TSP-1 expression is reduced, shifting the balance towards the proangiogenic state. A similar pattern is observed in protein metabolism: immediately after exercise skeletal muscles are in a catabolic state, whereas a few hours later protein synthesis exceeds protein breakdown.

There was a strong positive correlation between blood glucose concentration and the expression of TSP-1 in the muscle homogenates, indicating that the expression of TSP-1 is related to the level of hyperglycaemia.

Messenger RNA expression in capillaries and muscle fibres

Earlier studies, including ours, exploring the effects of exercise or diabetes on the expression of angiogenic factors in skeletal muscle have used skeletal muscle homogenates, which, in addition to muscle fibres, contain endothelial cells, fibroblasts and macrophages. Thus, it has been impossible to say which cell types have been responsible for the production of the growth factor. *In situ* hybridisation has given some idea, but especially in the interstitium it is difficult to distinguish between the different cell types. Cell culture studies have also provided some evidence, but they do not reflect the *in vivo* situation in the muscle. Milkiewicz and colleagues showed the feasibility of the laser capture microdissection method (LCM) to study within the muscle separate responses of endothelial cells to skeletal muscle overload (Milkiewicz

& Haas 2005). This technique was applied in this thesis to study the production of VEGF-A and TSP-1 separately in endothelial cells and muscle fibres after an acute exercise bout in healthy and diabetic muscles. On the basis of the present LCM results it seems that the mRNA expression of VEGF-A and TSP-1 is affected by diabetes and exercise more in capillaries than in skeletal muscle fibres. The expression of VEGF-A in capillaries was increased in the healthy exercised mice compared to controls. In the muscle fibres the increase was more modest and non-significant. The expression pattern found in capillaries and muscle fibres was similar to that in the muscle homogenates. Capillary TSP-1 expression was reduced in the healthy exercised group and increased in both the sedentary and exercised diabetic groups compared to healthy controls. These changes in capillary TSP-1 also resemble the changes detected in the muscle homogenates, although the reduced expression in the healthy exercised mice was not detected in the homogenates. If this reduction occurs only in capillaries and not in muscle fibres, it is evident that it is very difficult to detect the change in muscle homogenates. This demonstrates the importance of studying the cell types separately. The muscle fibres from the exercised diabetic mice showed increased expression of TSP-1 indicating that also muscle fibres respond to hyperglycaemia by producing TSP-1. Taken together, these LCM findings indicate that diabetes- and exercise-induced effects on angiogenic factors are stronger in capillary endothelial cells than in muscle fibres. This observation is supported by a recent report by Lee and co-workers, who showed that autocrine VEGF-A signalling in endothelial cells is required for vascular homeostasis and endothelial cell survival, and that this could not be compensated for by paracrine VEGF-A (*e.g.* from skeletal muscle fibres) (Lee *et al.* 2007). The reduced ability of the diabetic endothelial cells to increase VEGF-A production in response to exercise stimulus compared to the endothelial cells from healthy muscles may be related to the impaired vascular function and capillary rarefaction seen in diabetic skeletal muscle.

Downstream signalling pathways of VEGF-A and TSP-1

The possible downstream signalling pathways of VEGF-A and TSP-1 are presented in Figure 13 (Primo *et al.* 2005; Isenberg *et al.* 2007; Tabruyn & Griffioen 2007; Roy *et al.* 2008). The effects of exercise and diabetes on these pathways in skeletal muscle are not yet completely understood. Reactive oxygen species and redox regulation has been implicated to play a role in both exercise- and diabetic-induced effects in various

tissues (Brownlee 2005; Roy *et al.* 2008) The existing data suggests that, in addition to the upstream regulation of these angiogenic factors, which was discussed above, the signalling may also be affected at several points downstream, which makes therapeutic targeting even more complicated (Hazarika *et al.* 2007). Endothelial NOS may be involved in both up- and downstream (Hazarika *et al.* 2007; Roy *et al.* 2008). It is interesting that TSP-1 binding to CD36 not only inhibits angiogenesis but also inhibits the fatty acid translocase activity of CD36, which may be related to dysregulated processing of fatty acids in diabetic muscles (Isenberg *et al.* 2007).

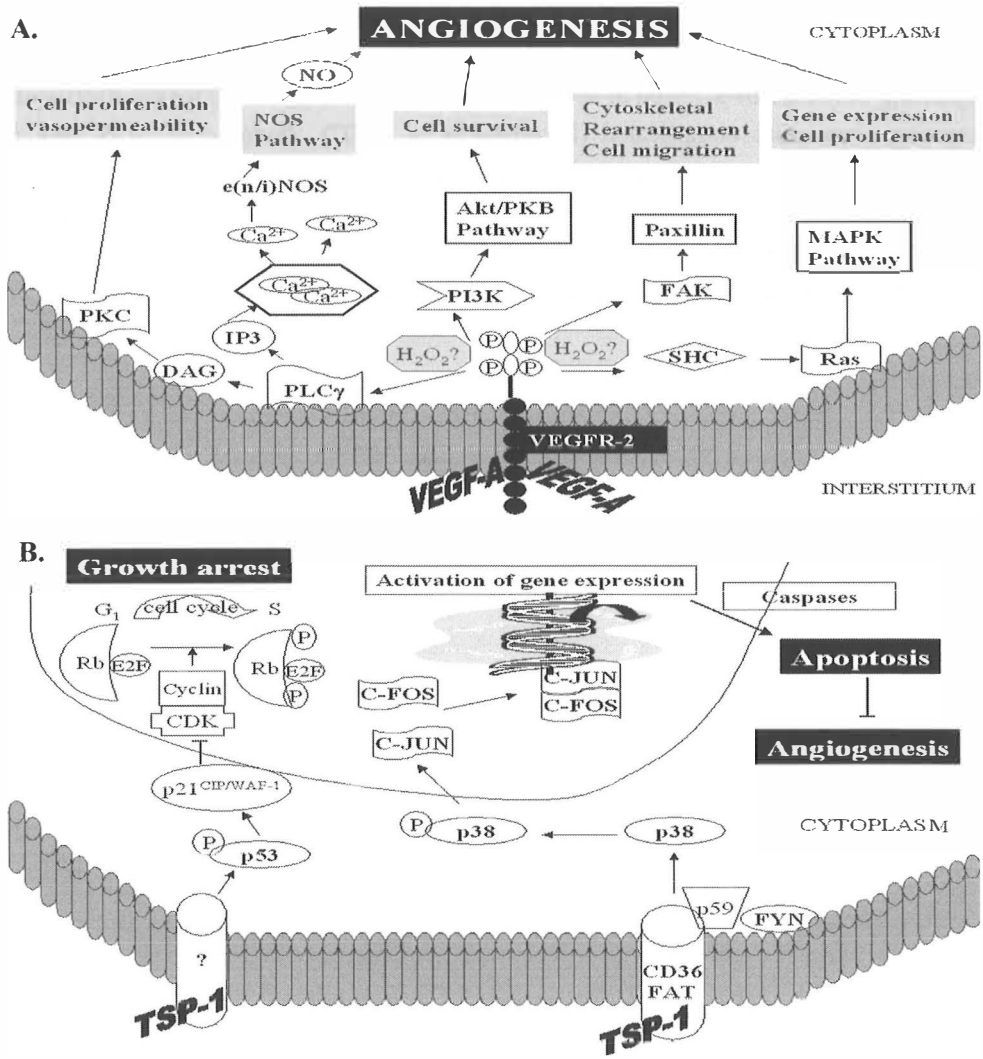


FIGURE 13. Possible downstream signalling pathways of A) VEGF-A and B) TSP-1 (modified from, Isenberg *et al.* 2007, Tabruyn & Griffioen 2007, and Roy *et al.* 2008).

6.2 Lymphatic vessels and lymphangiogenic gene expression

Until the last decade, lymphatic vessels have received relatively little attention compared to blood vessels. The discovery of lymphatic endothelium-specific markers, such as VEGFR-3, LYVE-1, podoplanin and prox1 has led to major advances in the study of the lymphatic system and lymphangiogenesis (Jussila & Alitalo 2002; Oliver & Alitalo 2005; Tammela *et al.* 2005b). Microarray analyses have shown that gene expression of these proteins is markedly increased in isolated lymphatic vessel endothelial cells as compared with blood vessel endothelial cells (Podgrabinska *et al.* 2002; Hirakawa *et al.* 2003). Studies on VEGF-C and VEGF-D and their interaction with VEGFR-3 have shown their importance in the normal development of lymphatic vessels (Jeltsch *et al.* 1997; Jussila & Alitalo 2002; Tammela *et al.* 2005b; Mäkinen *et al.* 2007).

In exercise physiology, increased skeletal muscle blood flow and capillarization have been acknowledged playing a very important role in the adaptation to training, while the lymphatic part of the circulatory system has been largely ignored. Various physiological and pathological factors, such as exercise and inflammation, induce hyperaemia and increase net capillary filtration in muscles. These changes result in increased hydrostatic and colloid osmotic pressure in the interstitial fluid, and address the need for increased lymph flow to maintain optimal conditions in the tissues (Aukland & Reed 1993). Exercise has been shown to increase skeletal muscle lymph flow markedly in both animal (Jacobsson & Kjellmer 1964; Bach & Lewis 1973; Reed *et al.* 1985; Coates *et al.* 1993) and human (Olszewski *et al.* 1977; Havas *et al.* 1997; Havas *et al.* 2000) studies, especially at the onset of exercise. It has been shown that in cancer both angiogenesis and lymphangiogenesis are vital for the growth and survival of tumours. These findings prompted the idea that also the lymphatic system in skeletal muscle may adapt in response to exercise. It has been proposed that lymphatic vessels in skeletal muscles are only located in the perimysium next to arterioles and veins (Schmid-Schönbein 1990). This concept is based on a few electron and light microscopy studies in animal skeletal muscles (Stingl & Stembera 1974; Skalak *et al.* 1984). However, most of the tissue fluid exchange in skeletal muscle occurs in the capillary bed between the muscle fibres, which suggests a need for initial lymphatic capillaries close to blood capillaries. With the newly found specific markers for the

lymphatic endothelium, LYVE-1 and VEGFR-3, we could stain both larger and capillary-sized lymphatic vessels in human and mouse skeletal muscles. To our knowledge, this is the first study to show that small lymphatic capillaries exist in the human skeletal muscle capillary bed. At the same time as our results were published, Grimaldi and co-workers reported small initial lymphatic capillaries between the muscle fibres in the rat diaphragm, also using LYVE-1 antibody for identification of the lymphatic endothelium (Grimaldi *et al.* 2006). After the discovery of lymphatic capillaries between the muscle fibres, their localization and density was studied in more detail in healthy and diabetic muscles. In addition, the possible effects of exercise training on lymphangiogenic growth factors and lymphatic vessel density were examined.

Mean lymphatic capillary density was more than 10-fold less than the density of blood capillaries in skeletal muscle, some areas of the muscles having more lymphatic capillaries than others. Lymphatic capillary density did not change after exercise training in the present study, and was similar in both healthy and diabetic muscles. Furthermore, we showed that in mouse skeletal muscle the expression of the two main lymphangiogenic growth factors, VEGF-C and VEGF-D, and their receptor VEGFR-3 is not affected by an acute running exercise bout or exercise training for one, three or five weeks. The only exception was found after the acute running exercise, where VEGF-C mRNA expression was slightly increased 3 h and 6 h post exercise in healthy exercised muscle compared to control. All the diabetic groups also had higher VEGF-C mRNA levels than the healthy control group. These findings are not supported, however, by the results of the exercise training experiment, where VEGF-C mRNA expression was similar in both the healthy and diabetic groups. VEGF-C expression is low in skeletal muscle, which increased the variation in the results. In human skeletal muscle, neither VEGF-C nor VEGF-D mRNA expression was affected post exercise.

On the basis of the present findings, the responses of lymphatic vessels and growth factors to exercise seem to be different from the responses of blood vessels and angiogenic growth factors. Skeletal muscle blood perfusion may be increased up to 100-fold during exercise (Saltin *et al.* 1998), whereas lymph flow has been shown to be elevated about 5-fold at the onset of exercise and thereafter declining to 2-3-fold compared to rest when exercise is continued (Havas *et al.* 2000; Lane *et al.* 2005). It

seems that the lymphatic part of the circulation, unlike blood vessels, is not challenged to its limit during exercise, and this could, at least partly, explain the observed differences in the growth factor responses. Given the structure of the thin lymphatic vessel wall and the present results on the number of vessels and their growth factor responses in skeletal muscle, it is suggested that lymphatic vessels may rather enlarge than proliferate in response to exercise stimulus.

Interestingly, a significant increase in VEGF-D mRNA expression was observed in the diabetic skeletal muscle in studies I and II. In adult tissues, VEGF-D mRNA is most abundant in the heart, skeletal muscle, lungs, colon, and small intestine (Achen *et al.* 1998), suggesting that VEGF-D also has a role in skeletal muscle physiology, although it is yet largely unknown. In contrast to VEGF-C-deficient mice, VEGF-D-deficient mice are viable, have normal body weight and no defects in lymphatic function (Baldwin *et al.* 2005), indicating that VEGF-C is more important than VEGF-D for the development of the lymphatic system during embryonic development. Baldwin *et al.* (2005) have suggested that in adult tissues the biological functions of VEGF-D are be more likely to be revealed in response to diseases or tissue damage, and the present findings support this idea well. Serum glucose concentration and VEGF-D mRNA expression correlated positively, suggesting that VEGF-D expression increases along with the severity of diabetes.

6.3 Localization of the proteins

In both healthy and diabetic muscles the VEGF-A protein was localized under the sarcolemma in muscle fibres, in the endothelium of larger blood vessels and in the interstitium, most likely in capillaries. The VEGFR-2 protein was expressed in the capillaries between muscle fibres and in larger vessels without a thick smooth muscle cell layer, which located close to arteries and veins. A similar expression pattern has been demonstrated earlier in healthy rodent and human muscle (Milkiewicz *et al.* 2001; Hudlicka *et al.* 2002; Jensen *et al.* 2004a). The immunohistochemical finding of VEGF-A localizing to capillaries was supported by the mRNA results from LCM-extracted muscle capillaries, which expressed VEGF-A mRNA abundantly.

Furthermore, the importance of the endothelial colocalization of VEGF-A and VEGFR-2 in endothelial cell survival has been shown recently (Maharaj *et al.* 2006; Lee *et al.* 2007). VEGF-B was stained strongly in the larger blood vessels, in certain interstitial cells and in a few muscle fibres. Weak staining was also observed in some capillaries. This is the first report to show VEGF-B protein localization in skeletal muscles, indicating that in addition to VEGF-A, VEGF-B plays a role in the maintenance of blood vessels in skeletal muscle. No differences were observed between the healthy and diabetic mice in the localization of these proteins.

VEGF-C and VEGF-D mRNA were shown to be produced in skeletal muscle, and endogenous VEGF-C and D proteins were stained in various structures within the muscle. Gene transfer studies have shown earlier that these proteins could induce both angiogenesis and lymphangiogenesis in muscles (Witzenbichler *et al.* 1998; Byzova *et al.* 2002; Rissanen *et al.* 2003). VEGF-D was located in some of the muscle fibres under the sarcolemma, similarly to VEGF-A, and in the endothelium of arteries. In addition, VEGF-D staining in interstitial cells suggests that fibroblasts and/or macrophages could produce VEGF-D in skeletal muscle. Byzova and colleagues have shown earlier that after adenovirus gene transfer of VEGF-D, the protein can be detected in muscle fibres, fibroblasts and blood vessel walls (Byzova *et al.* 2002). However, their control muscles did not express VEGF-D in detectable quantities. Rutanen and co-workers proposed that VEGF-D may have a role in maintaining the normal function and homeostasis of blood vessels (Rutanen *et al.* 2003). This is supported by the findings of this thesis, since the VEGF-D protein was strongly expressed in larger blood vessels in skeletal muscles.

In concert with the high VEGF-D mRNA levels in diabetic muscles, increased VEGF-D protein expression was observed in some of the muscle fibres in diabetic muscles. Closer examination revealed that fibres which were positive for VEGF-D were damaged (swollen, necrotic or contained centralized nuclei). The fibre damage observed in the diabetic mice was similar to that found in exercise-induced muscle damage already a few decades ago (Vihko *et al.* 1978). Damaged fibres were found in both the sedentary and exercised diabetic mice, but not in the healthy mice. Thus, the fibre damage is more likely due to hyperglycaemia or lack of insulin than to the exercise protocol. Whether VEGF-D expression is related to muscle fibre degeneration

and inflammation processes in general, or specifically when hyperglycaemia and/or hypoinsulinemia are present, needs to be studied further. Interestingly, also VEGF-A protein expression has been reported to increase in regenerative muscle fibres after ischemia (Rissanen *et al.* 2002). Together these findings provide additional evidence that VEGF family proteins are also involved in myofibre growth/regeneration, as has been suggested (Arsic *et al.* 2004).

VEGF-C protein was expressed in the interstitial connective tissue, nerves and muscle spindles, but not in muscle fibres. VEGF-C, unlike VEGF-D, was not found in arterioles or in capillaries, although we cannot completely rule out its presence in endothelial cells, since it may have been expressed in amounts too small to be detected. Fibroblasts could be one source of VEGF-C production in skeletal muscle, since pro-inflammatory cytokines induced VEGF-C production in lung fibroblasts (Ristimäki *et al.* 1998). The present results showed colocalization of VEGF-C and neuropilin-2 in nerves and muscle spindles in skeletal muscle. Neuropilin-2 is a receptor which is localized in nervous tissue and can bind VEGF-C (Jussila & Alitalo 2002). The role of VEGF-C as a trophic factor for neural progenitor cells has been reported earlier (Le Bras *et al.* 2006), supporting the present findings of VEGF-C in nervous tissues. However, because both VEGF-C and D are diffusible factors, we could not determine whether they are produced in the cell types where they were found to be localized. After production they can relocate and bind to their receptors in a paracrine manner. Thus, the present results describe the cell types which either produce these growth factors or are the target cells for their functions.

The findings that Cyr61 and CTGF mRNA are upregulated by both hyperglycaemia and high mechanical loading in skeletal muscle initiated the question about their localization in skeletal muscle. In the myocardium Cyr61 is localized to myocytes and blood vessels (Hilfiker-Kleiner *et al.* 2004), and vascular smooth muscle cells have been shown to produce Cyr61 (Grote *et al.* 2004). CTGF, in turn, is produced at least in fibroblasts in response to mechanical stress (Schild & Trueb 2002). In this thesis the localization of Cyr61 and CTGF proteins in muscle fibres and in the ECM is presented. Fast muscle fibres were found to express more Cyr61 and CTGF compared to slow fibres. Since CCN proteins contain a signal peptide for secretion (Perbal 2004), it is possible that these proteins are produced in muscle fibres and secreted into the

interstitial space to perform their functions in the ECM, *e.g.* in blood vessels. Cyr61 is able to execute its functions through autocrine and paracrine mechanisms by interacting with integrins and heparan sulfate proteoglycan (Chen & Du 2007).

6.4 Effectiveness of the treatments and limitations of the study

The high blood glucose concentration and considerable loss of body weight confirmed that streptozotocin had induced diabetes in the studied mice. The significant atrophy of the muscle fibres seen in study I is also normal in diabetic animals, if they are not treated with insulin. The effectiveness of the exercise training in the study I was evaluated by the increased activity of citrate synthase in both healthy and diabetic trained mice. Also the blood glucose concentration tended to decrease in trained diabetic animals compared to sedentary, suggesting a positive effect of physical activity to blood glucose. In the human exercise study (III), the significantly increased blood lactate concentration post exercise, the large increases in creatine kinase activity, and decreased maximal force production demonstrate that the jumping exercise was strenuous. The present exercise model was chosen to exert high mechanical loading on muscle fibres and connective tissue in the thigh muscles, and it allowed exercise of only one leg while the other served as a control. In jumping exercise muscle fibres undergo a repeated stretch-shortening cycle with both eccentric and concentric phases, as in natural human locomotion, but the loading was higher compared to, *e.g.*, walking.

The exercise intensity in both the exercise training and single exercise bout was chosen so that the diabetic mice were also able to complete the one-hour running bout. Thus, the relative intensity was probably higher for the diabetic mice than for the healthy mice, as their exercise capacity was decreased due to diabetes. This, together with constant exercise load during the training period, could explain why not many changes were seen in basal angiogenic gene expression in the healthy mice after training periods. However, after the acute exercise bout the changes in the angiogenic gene expression were greater in the healthy mice than diabetic mice. This further supports the suggestion that angiogenic responses in skeletal muscle are reduced in diabetic mice compared to healthy mice.

Numerous studies have shown that type 1 diabetes is the predominant feature of mice and rats treated with streptozotocin (Rees & Alcolado 2005), and thus diabetes and subsequent hyperglycemia were presumably the main causes of the observed changes in the present study. Other non-diabetic effects cannot, however, be completely ruled out. It has been suggested that animal models can be used successfully to study specific aspects of diabetic processes, but they should not be considered to represent the clinical disease (Leiter & von Herrath 2004; Roep & Atkinson 2004; Roep 2007). Skeletal muscle is a conservative tissue, and it is structurally and functionally analogous between humans and mouse. A high degree of similarity has also been demonstrated between the pathological changes in the venous wall during human diabetes and streptozotocin-induced diabetes in rats (Mompeo *et al.* 1999). These findings justify the use of mouse models of diabetes, but it has to be kept in mind that the results, without further verification in humans, cannot be directly generalized to human diabetes.

7 MAIN FINDINGS AND CONCLUSIONS

The main results and conclusions of the present thesis can be summarised as follows:

1. Diabetes impairs capillarization and affects the expression of several genes involved in angiogenesis in skeletal muscle. The expression of many proangiogenic genes (VEGF-A, VEGF-B, VEGFR-2, Nrp-1) is downregulated and the expression of angiogenesis inhibitors (TSP-1, Rbl2) is upregulated in diabetic muscles, leading to the dysregulation of complex angiogenesis processes. Endurance training alleviates some of the diabetes-induced changes already after few weeks of training. These training effects may be one of the mechanisms behind the beneficial effects of regular exercise in diabetic patients.

2. The present findings also show that the response of the angiogenic growth factors to acute bout of exercise may be attenuated in diabetic compared to healthy muscle. Analysis of the capillaries and muscle fibres collected from skeletal muscle by laser capture microdissection show that diabetes and exercise affect the production of VEGF-A and TSP-1 both in capillaries and in muscle fibres, the responses being more pronounced in capillaries.

3. In addition to traditional angiogenic proteins, the CCN family proteins Cyr61 and CTGF are produced in skeletal muscle in response to both hyperglycaemia and high mechanical loading of muscle fibres. These proteins may play an important role in the remodelling of the skeletal muscle vasculature, extracellular matrix and myofibres, as they have both direct and indirect effects on various cell types within skeletal muscle.

4. This thesis provides the first evidence of the presence and the amount of lymphatic capillaries in the skeletal muscle capillary bed in both human and mouse muscles. The localization of the lymphangiogenic vascular endothelial growth factors –C and -D in skeletal muscle is also reported.

5. Acute exercise and exercise training have only a minor or no effect on lymphatic vessel density and the mRNA expressions of lymphangiogenic VEGF-C, VEGF-D and VEGFR-3, which is in contrast to the angiogenic responses to exercise.

6. Another novel finding is the increased expression of VEGF-D in diabetic muscle fibres, which may be related to muscle fibre damage, infiltration of macrophages and fibre regeneration.

The mechanisms by which diabetes and exercise could affect skeletal muscle capillarization, and how these mechanisms are connected are presented in Figure 14. In contrast to gene therapy studies with a single angiogenic growth factor, which have not thus far been very successful, exercise could positively affect angiogenic signalling by stimulating the whole signalling pathway and thus be effective in the prevention and treatment of peripheral vascular problems in diseases such as diabetes.

Based on the present findings and recent literature there are growing evidence that factors affecting angiogenesis and lymphangiogenesis may play an important role also in cells other than endothelial cells. In the future connections between vascular and myofibre growth and cellular aerobic metabolism should be studied in more detail. It is tempting to speculate a close relationship in the regulation of vascular supply and oxygen metabolism in skeletal muscles. Both of these are adversely affected by diabetes and enhanced by exercise training.

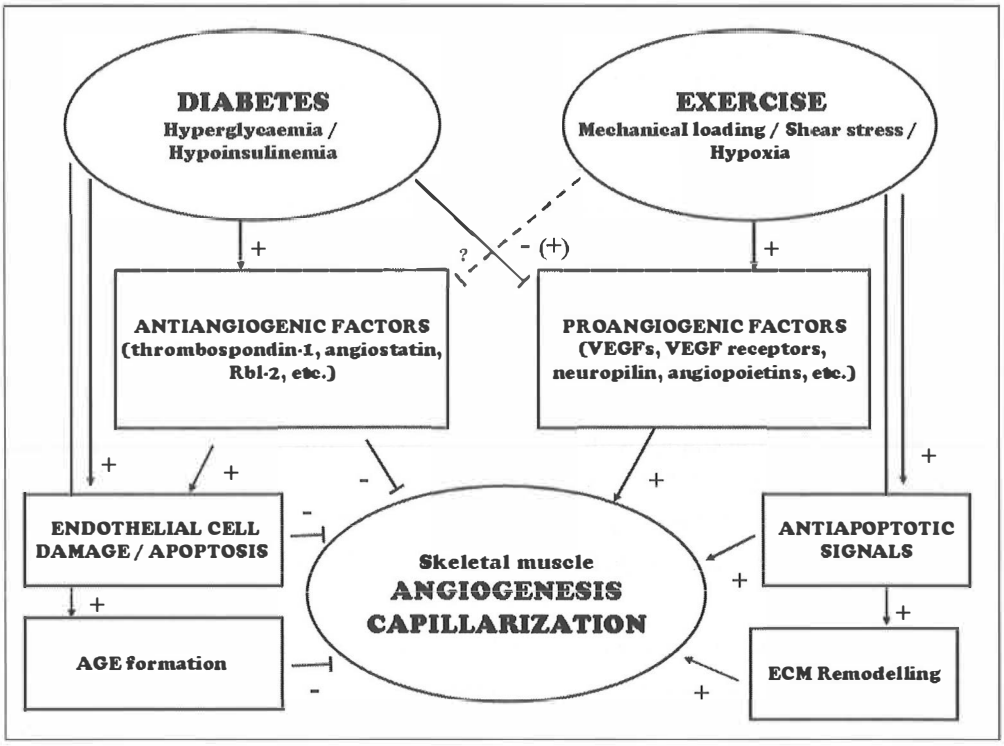


FIGURE 14. Mechanisms by which diabetes and exercise could affect skeletal muscle capillarization and angiogenesis (modified from Kivelä *et al.* 2006).

8 YHTEENVETO

Veri- ja imusuonet ovat elintärkeitä lähes kaikille elimistömme kudoksille, ja verenkiertoelimistö onkin sikiön ensimmäisenä kehittyvä elin. Luurankolihakset muodostavat elimistön suurimman kudoksen, ja niiden päätehtävänä on raajojen liikkeet ja niiden säätely. Lihakset osallistuvat myös elimistön aineenvaihdunnan säätelyyn merkittävästi. Kestävyysharjoittelun tiedetään lisäävän lihasten verisuonten määrää terveillä, kun taas diabeteksen on puolestaan havaittu vaikuttavan haitallisesti lihasten verisuonten kasvuun eli angiogeneesiin. Näiden muutosten taustalla olevia mekanismeja ei vielä tarkkaan tunneta. Liikunnan vaikutuksista diabeettisten lihasten verisuonistoon on olemassa vielä melko vähän tietoa. Tämän väitöstutkimuksen tarkoituksena oli tarkastella liikunnan ja diabeteksen vaikutuksia raajalihasten veri- ja imusuonistoon. Tavoitteena oli selvittää, mitkä mekanismit vaikuttavat diabeteksessa verisuonten kasvua heikentävästi ja toisaalta kestävyysharjoittelun seurauksena verisuonten kasvua edistävästi. Lisäksi tavoitteena oli tarkastella voidaanko diabeteksen aiheuttamia muutoksia angiogeneesin signalointireiteissä ehkäistä liikuntaharjoittelun avulla. Vaikka Gasparo Aselli löysi imusuonet jo 1600-luvulla, on niistä oleva tieto ollut huomattavasti verisuonia vähäisempää. Imusuoniston tutkimus on kuitenkin viime vuosina lisääntynyt merkittävästi, mm. syöpätutkimuksessa, koska uudet menetelmät ovat mahdollistaneet imusuonten paremman tunnistuksen kudosnäytteistä. Lihasten imusuonistoa on kuitenkin tutkittu erittäin vähän. Tämän tutkimuksen yhtenä tavoitteena oli tuottaa lisätietoa raajalihasten imusuoniston rakenteesta sekä imusuonten kasvutekijöistä lihaksissa.

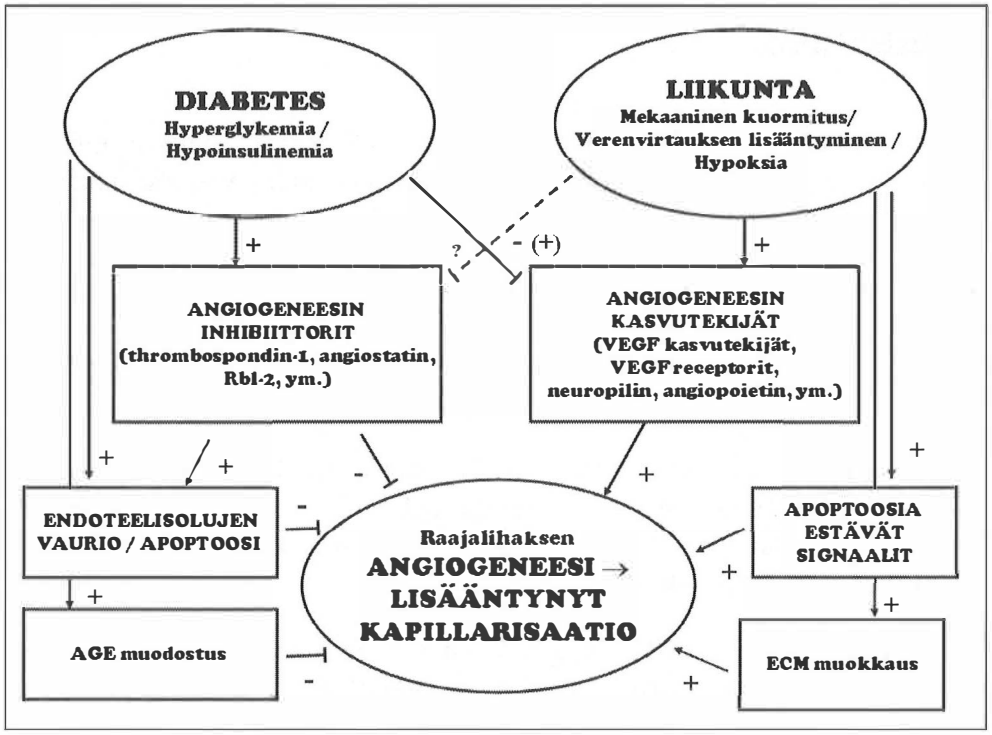
Tämän tutkimuksen tulokset osoittavat, että kokeellinen tyypin 1 diabetes heikentää lihasten kapillarisaatiota ja vaikuttaa monien verisuonten uudismuodostukseen (angiogeneesi) liittyvien geenien ilmenemiseen. Useiden angiogeneesiä lisäävien geenien ilmeneminen on vähentynyt diabeettisessa lihaksessa (esim. VEGF-A, VEGF-B, VEGFR-2, Nrp-1) ja toisaalta angiogeneesiä estävien geenien (TSP-1, Rbl2) ilmeneminen on lisääntynyt. Tämä osoittaa diabeteksen vaikuttavan haitallisesti angiogeneesiä säätelevien monimutkaisten signaalireittien toimintaan. Kestävyysharjoittelu vähensi diabeteksen aiheuttamia muutoksia geenien ilmenemisessä, mikä voi osaltaan selittää liikunnan aikaansaamia tunnettuja hyötyjä

diabeteksessa. Tulokset osoittivat myös, että liikunnan ja diabeteksen vaikutukset angiogeneesiä sääteleviin geeneihin (VEGF-A ja TSP-1) olivat voimakkaampia verisuonikapillaarien endoteelisoluissa kuin lihassoluissa.

Tässä väitöskirjassa havaittiin, että raajalihasten imusuonisto ulottuu myös yksittäisten lihassolujen väliin eikä ainoastaan lihassolukimppujen välissä sijaitsevien suurten verisuonten lähettyville, kuten oli aiemmin esitetty. Havainnot osoittivat, että imusuonikapillaarit sijaitsevat verisuonikapillaarien läheisyydessä, mutta niiden lukumäärä on vain noin kymmenesosa verrattuna verisuonikapillareihin. Myös imusuonten kasvutekijöiden (VEGF-C ja VEGF-D) läsnäolo ja sijainti lihaksissa raportoitiin ensimmäistä kertaa.

Akuutti kuormitus juoksumatolla lisäsi verisuonten kasvutekijöiden määrää lihaksissa, mutta sillä ei ollut vaikutusta imusuonten kasvutekijöihin, kuten ei myöskään viiden viikon kestävyysharjoittelulla. Harjoittelu ei myöskään lisännyt imusuonten määrää lihaksissa. Diabetes lisäsi VEGF-D:n ilmenemistä lihaksissa, mikä näyttäisi liittyvän lihassolujen vaurioon tai vaurionkorjausmekanismeihin, koska VEGF-D proteiinia oli runsaasti soluissa, jotka olivat joko turvonneita, makrofagien valtaamia tai uudistuvia.

Yhteenveto mekanismeista, joiden kautta diabetes ja liikunta vaikuttavat raajalihasten kapillarisaatioon ja angiogeneesiin on esitetty kuvassa 15. Geeniterapian uskotaan tulevaisuudessa olevan tehokas hoitokeino sairauksissa, joissa raajalihasten verisuonitus on heikentynyt. Kliiniset tutkimukset, joissa lihaksiin on virusvektorien avulla viety verisuonten kasvutekijöitä, eivät kuitenkaan vielä ole olleet kovinkaan lupaavia. Tämä voi johtua siitä, että yksittäinen geeni ei ole riittävän tehokas lisätäkseen verisuonten kasvua, koska taustalla olevat sairaudet (esim. diabetes) ovat voineet vaikuttaa heikentävästi useisiin angiogeneesin geeneihin. Liikuntaharjoittelu lisää usean angiogeneesiä edistävän geenin ilmenemistä lihaksissa yhtäaikaisesti, mikä voi selittää sen tehoa verisuonten kasvussa. Liikunta näyttäisi siis olevan tehokas keino ennaltaehkäistä ja jopa hoitaa moniin sairauksiin liittyviä perifeerisiä verisuoniongelmia.



KUVA 15. Liikunta ja diabetes voivat vaikuttaa raajalihasten kapillarisaation ja verisuonten kasvuun useiden mekanismien kautta. (muotoiltu lähteestä Kivelä ym. 2006).

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Expression of angiogenic growth factors in skeletal muscle and in capillaries after exercise in healthy and diabetic mice.

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