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SKELETAL MUSCLE MATRIX METALLOPROTEINASE AND EXERCISE IN HUMANS

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ABSTRACT

Skeletal muscle is a highly plastic tissue; it has a great capacity to adapt to environmental demands throughout life. The structural and functional changes that occur in response to exercise training are well characterized whereas much less is known about these adaptive processes at the cellular and molecular levels. A possibly underestimated aspect of skeletal muscle adaptation to exercise is the remodeling of the extracellular matrix (ECM). Degradation and processing of the extracellular matrix are carried out by a specific category of proteases, especially the matrix metalloproteinase family (MMPs). Such remodeling is of crucial importance for successful extravasation of circulating cells and for the migration of cells between the compartments of the tissue. Furthermore, degradation products of ECM components are not always mere debris; several fragments of structural proteins have biological activity after proteolytic processing, and MMP activity may also release growth factors stored in the ECM. Little is known about these enzymes in skeletal muscle of humans and how physiological stimuli such as exercise and exercise training affect their expression and activity.

Therefore, the aim of this thesis was to characterize: 1. skeletal muscle MMP activation in response to a single bout of exercise and exercise training with regard to gene expression and enzyme activity, 2. exchange of factors associated with MMP activity between exercising leg and the circulation during exercise, 3. possible cellular sources of MMP in skeletal muscle tissue and blood, 4. the effects of restricted leg blood flow, and thereby reduced oxygen delivery, to the exercising leg on skeletal muscle and circulating levels of MMP and 5. the effects of the myokine interleukin-6 on MMP levels in skeletal muscle and in the circulation.

MMP-9 is activated and transcriptionally upregulated in human skeletal muscle after a single bout of exercise. In contrast, MMP-2 is activated and transcriptionally upregulated in human skeletal muscle by exercise training but not after a single bout of exercise. Factors possibly linked to proteolytic processing of MMP-9, such as collagen IV and VEGF-A, are released from the leg to the circulation during a single bout of exercise in humans. Circulating levels of MMP-9 increase during and after a single bout of exercise in humans but do not seem to originate from the skeletal muscle. The myokine interleukin-6 induces an increase in circulating MMP-9 in parity with what is seen after a single bout of exercise in humans, interleukin-6 also induces gene-expression and release of MMP-9 from the human moncyte cell-line THP-1, but not from human myoblasts, myotubes or endothelial cells indicating that monocytes could be the source of the interleukin-6 induced increase in circulating MMP-9.

The results from this thesis show that both MMP-2 and MMP-9 are expressed in skeletal muscle and upregulated by a physiological stimulus such as exercise but probably through different mechanisms. Furthermore, it indicates that remodeling of extracellular matrix and release of growth factors in the skeletal muscle occur after only a few minutes of exercise. Overall, the results support MMPs to play a role in the adaptation of the skeletal muscle to physical activity in humans.

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A single bout of exercise activates matrix metalloproteinase in human skeletal muscle.
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III. Rullman E., Ohlsson K., Gustafsson T
Circulating MMP-9 during exercise in humans
Manuscript

IV. Rullman E., Wågsäter D., and Gustafsson T.
Il-6 induced increase in serum MMP-9 in man
Manuscript

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LIST OF ABBREVIATIONS

A/V-diff	Arterial concentration minus venous concentration
ADAM	A disintegrin and metalloproteinase
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
AP-1	Activator protein 1
APMA	p-aminophenylmercuric acetate
ATP	Adenosine tri-phosphate
BSA	Bovine serum albumin
CD31	Cluster of differentiation 31
CRE	cAMP response element
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
ERK	Extracellular signal-regulated kinases
FNII	Fibronectin II
h	hour
HIF-1	Hypoxia inducable factor 1
HIT	High intensity training
HPX	Hemopexin
HUVEC	Human umbilical chord endothelial cells
IGF-1	Insulin-like Growth Factor
Il-6	Interleukin 6
kDa	KiloDalton
MAPK	Mitogen-activated protein kinase
MDX	Muscular dystrophy
MHC	Myosine heavy chain
min	Minutes
ml	Milliliter
MMP	Matrix Metalloproteinase
mRNA	Mesenger RNA
MT-MMP	Membrane type MMP
NF-kappaB	Nuclear factor kappa Beta
ng	Nanogram
NGAL	Neutrophil gelatinase-associated lipocalin
PBS	Phosphate buffered saline
PCr	Phosphocreatine
PEA3	polyomavirus enhancer activator 3
PGC1	peroxisome proliferator γ coactivator 1
PMA	Phorbol 12-myristate 13-acetate
SaC	Satellite cells
SD	Standard Deviation
SDS	Sodium dodecyl sulfate
SEM	Standrard Error of Mean

SP1	Specificity protein 1
SR	Sarcoplasmatic reticulum
TGF-beta	Transforming growth factor beta
TIMP	Tissue Inhibitor of Metalloproteinase
TNF-alpha	Tumor necrosis factor alpha
ug	Mikrogram
VEGF-A	Vascular Endothelial Growth Factor A
VO2	Oxygen uptake
Vo2max	Maximal oxygen uptake
W	Watt
Wk	Week

1 BACKGROUND

1.1 SKELETAL MUSCLE STRUCTURE AND FUNCTION

Skeletal muscle, the largest organ of the human body, has numerous functions, ranging from force generation and movement to mechanical protection of underlying soft tissue and maintenance of body temperature. The structure of skeletal muscle is well characterized. Muscle fibers constitute the main cellular component of muscle. Other cell types present in skeletal muscle are endothelial cells, satellite cells, pericytes, fibroblasts, macrophages and mast cells.

Each fiber contains several nuclei because of its embryological development pattern. The skeletal muscles of the limbs develop from somites, which migrate to the limb buds where they differentiate into spindle-shaped, elongated myoblasts. The first evidence of limb musculature is observed during the seventh week of development. During elongation of the limbs, muscle fibers develop through proliferation and fusion of myoblasts. Contractile myofibrils and their typical cross-striations appear by the end of the third month of development (1). In the adult, each muscle fiber is surrounded by connective tissue, the endomysium, which loosely interconnects adjacent muscle fibers. The endomysium contains mononuclear cells called satellite cells. Both the embryonic origin and the functional relevance of these cells have been debated since they were first described in 1961, but during the past decade, it was established that satellite cells are activated by exercise and muscle damage (2-4). Activated satellite cells proliferate and differentiate into new myotubes or new segments of fibers. The endomysium also contains capillaries and nerves, which provide the muscle fibers with oxygen and nutrients and regulate muscle contraction, respectively. Each muscle fiber is innervated by a motor neuron attached to the myofiber at the motor endplate(5).

Skeletal muscle is divided into a series of compartments called fascicles, each of which contains a bundle of muscle fibers. Each fascicle is surrounded by a perimysium, which contains the larger branches of blood vessels and nerves. The entire muscle is surrounded by the epimysium, which separates it from other tissues and organs. The extracellular matrix (ECM) helps to form the architecture of skeletal muscle and is a support system for nerves and blood vessels (5, 6).

Different muscle fiber classification systems have been established based on either functional or biochemical oxidative properties of muscle fibers. The most common system used for humans involves classification of fibers as type I or type II. Initially, classification was achieved using histochemical staining of ATPase, an enzyme responsible for ATP cleavage and energy release in the contractile process and related to the speed of contration (7). Various muscle fibers express different amount and isoforms of ATPase, which relate to the maximal contraction velocity of the fiber. Later the expression of myosin heavy chain (MHC) isoforms has been used as classifiers of fiber type. MHC type I fibers are characterized by slow-contractile velocity and MHC type II by fast-contractile velocity. Type II fibers can be further divided into type IIA and IIB/IIX (8, 9). There is a good agreement between the ATPase and the MHC classifications systems. The fiber types also differ in respect of oxidative and glycolytic capacity and the density of mitochondria and capillaries. Type I fibers are more oxidative and fatigue resistant than type II fibers, whereas the latter are more glycolytic and are more sensitive to fatigue than the former (10, 11). Despite

the association between the contractile and metabolic characteristics of muscle fibers, changes in their contractile and metabolic properties may occur independently of one another under circumstances such as adaptation to exercise training. These studies have reported unaltered contractile properties with exercise training but an increase in the oxidative capacity of muscle fibers after exercise training (12-14). This clearly suggests that different regulatory pathways underlie the contractile and biochemical properties of muscle fibers.

1.2 SKELETAL MUSCLE PLASTICITY

The skeletal muscle is a highly plastic organ; it has a great capacity to adapt to environmental demands, even in the adult. The structural and functional changes that occur in response to exercise training are well characterized. Most of these changes are related to processes in the actual muscle fiber and include altered contractile properties and increased mitochondrial density, reflected in an increase in levels of mitochondrial enzymes and oxidative enzymes of the citric acid cycle, elevated cytochrome and respiratory chain activity and increased fatty acid beta-oxidation (15, 16). Even though the plasticity of skeletal muscle fibers is best characterized, other cell types are involved in the adaptive processes of muscle. For example, changes in capillarization after exercise training involve the activation, migration and proliferation of endothelial cells (17-19). During the past decade, the importance of both circulating and resident progenitor cells as well as other cell types located in skeletal muscle tissue has been recognized in respect of their participation in skeletal muscle remodeling (20). The main resident stem-cell in the skeletal muscle is the satellite cell. It is generally assumed that 3–8% of the cell nuclei in the skeletal muscle tissue of healthy adult humans is of SaC origin (2). It is assumed that SaCs are activated by muscle injury, after which they proliferate and differentiate into muscle fibers (21, 22). Animal studies have suggested that, in addition to SaCs, pluripotent circulating hematopoietic cells and resident pericytes are involved in the adaptive processes of skeletal muscle (23). It has been suggested that these cells participate in angiogenesis and affect skeletal muscle fibers via paracrine signaling (24, 25).

In the context of exercise and exercise training, the degree of adaptive response is related to the duration, intensity and frequency of the exercise bouts, albeit with very large interindividual variation (26-28). Exercise performance, and ultimately adaptation to exercise, depends on the contraction of the muscle fiber (29). A muscle contraction is induced through the release of acetylcholine from the motor neuron, which activates nicotinic acetylcholine receptorsin the myofiber. Calcium is released from the SR into cytoplasm of the muscle fiber and interacts with troponin, enabling myosin to interact with the actin skeleton of the myofiber. Force develops when a moving part of the myosin molecule interacts with actin filaments in the crossbridge cycle, which requires ATP. Relaxation occurs when calcium is pumped out of the cytoplasm by an ATP-dependent calcium pump in the SR membrane (30). Factors altered with muscle contraction and shown to influence adaptation to exercise are mechanical stretch, fiber recruitment and changes in intracellular levels of ATP, Ca++, O2, glycogen and cAMP (16, 31-36). Importantly, skeletal muscle adaptation in response to exercise is probably not associated with any one factor but with the integration of multiple stimuli at systemic and local levels. Autocrine and paracrine substances, hormones, temperature and circulatory factors are likely involved in adaptation to exercise (29, 33, 35). The

disturbance in muscle homeostasis differs depending on the type of exercise, which results in differences in adaptive responses and, ultimately, a skeletal muscle phenotype related to the nature of the exercise being performed (16, 29).

1.2.1 Regulating processes

Structural and functional changes in skeletal muscle fibers depend on changes in protein level or activation, which may be pretranslational, translational, posttranslational, or a combination of all three (16, 35). Protein level is a function of the balance between the rate of synthesis and the rate of degradation. Increased muscle activity is predominantly associated with increased protein synthesis, whereas decreased muscle activity causes decreased protein synthesis (31, 33, 35, 37).

The adaptation of the muscle, as described above, is specific to the nature of the exercise stimulus, and different types of exercise activate different signaling pathways in the tissue. During the past few decades, transgenic animals in combination with traditional animal and human exercise models have provided crucial information on possible regulatory pathways and processes underlying skeletal muscle adaptation to exercise. With resistance exercise training, fiber hypertrophy is achieved via increased synthesis of contractile proteins (38). Compared with endurance exercise, little mitochondrial biogenesis and neovascularization has been reported with resistance exercise (33, 39). Recent evidence indicates that resistance exercise activates specific signal transduction pathways such as Akt and mTOR, which regulate protein synthesis and presumably regulate the degree of protein degradation (38, 40). Furthermore, activation of satellite cells, with proliferation and differentiation into new myonuclei, has been reported to be more associated with resistance exercise than with other exercise regimes (39, 40). This provides a larger protein synthesis machinery to accommodate the increasing needs of the growing myofibers (38, 40). In contrast, endurance exercise activates signal transduction pathways such as AMPK, PGC1 and HIF-1, which in turn result in increased expression of mitochondrial and other oxidative genes and angiogenic growth factors, ultimately resulting in increased capillary density and oxidative capacity (19, 41, 42). In the middle of this spectrum is sprint and high-intensity interval exercise. It has been known since the 1970s that such exercise training regimens increase both oxidative capacity, strength and muscle mass (43). More recent studies have further established that high-intensity interval training (HIT) activates signaling of AMPK and p38 MAPK to PGC-1a, which could explain, in part, the metabolic adaptations induced by HIT, including mitochondrial biogenesis and an increased capacity for glucose and fatty acid oxidation (44). Thus, exercise-induced skeletal muscle remodeling represents a continuum both in respect of functional and structural changes, ranging from those observed after repetitive high-intensity, short-duration bouts to those observed after low-intensity, long-duration bouts.

A possibly underestimated process in the adaptation to exercise training is exercise-induced changes in protein bioavailability. An effect of exercise on growth factor bioavailability is evident in observations of an increase in interstitial and circulating levels of vascular growth factor A (VEGF-A) after a few minutes' exercise (45, 46). This, in my opinion, must reflect release of preexisting protein rather than increased transcription or translation. Protein release from granules or binding protein are two possibilities underlying an immediate increase in bioavailable proteins.

Another aspect of skeletal muscle plasticity that may be of greater importance than is generally appreciated is the proteolytic degradation of the basal lamina and the extracellular matrix. The satellite cells (SaC) resides below the basal lamina of the skeletal muscle fiber (3, 4). Proliferation, differentiation and fusion with muscle fibers (21, 22) seems to be dependent on ECM remodeling as demonstrated by the report in which migration of myoblasts after a crush injury required proteolytic processing of the ECM (47). Further, in exercise-induced angiogenesis, proteolytic degradation of the capillary basal lamina is crucial for angiogenesis (48).

1.3 EXTRACELLULAR MATRIX AND SKELETAL MUSCLE PROTEASE FAMILIES

1.3.1 Extracellular matrix

The ECM immediately outside the myocyte cell membrane (sarcolemma) is the basal lamina. The basal lamina mainly contains type IV collagen, laminins and fibronectin, but also contains minor components such as entactin/nidogen-1, perlecan and agrin. The collagens are a class of at least 25 different proteins that are localized in the ECM (49).

Collagen molecules have three α chains that form a triple helix (50). Collagens types I, II, III, V and XI are termed 'fibrillar collagens' because of their capacity to form elongated fibers. They are the predominant components of the interstitial stroma (51). Collagen types IV, VI, VIII and X do not form fibrils but are cross-linked into a three-dimensional network. They are major components of the basal lamina (52, 53). Collagens provide mechanical stability and strength by interacting with one another or with other ECM components. Gelatin is denatured collagen and is not present under physiological circumstances, but is produced when collagen is denatured to gelatin under highly acidic conditions or when mammalian collagen is heated to more than 70 °C (54). Laminin, together with collagen type IV, is one of the main components of the basement membrane (55). Laminin is a family of glycoproteins that are composed of α, β and γ chains that generate several different isoforms that vary in size, composition and structure Laminins are synthesized by a wide variety of cells and appear to have a variety of biological functions such as cell adhesion, cell migration and cell differentiation (56, 57). Several laminin isoforms are upregulated threefold by endurance exercise training for three weeks (58). In animal models of muscle, levels of unloading laminins have been shown to be decreased and become degraded in the ECM along with collagen IV (59).

Fibronectin is a multifunctional glycoprotein that is found in two different forms: soluble and solid. The soluble form, which is produced by hepatocytes, is found in body fluids. The solid form, which is produced by many different cell types, including fibroblasts, endothelial cells and others, is found in loose connective tissue and most basement membranes (60, 61). Fibronectin acts through several distinct domains to promote cell adhesion, cell migration and matrix assembly(60). In skeletal muscle, fibronectin is upregulated by endurance exercise and has been suggested to play a role in the stretch-induced increase in muscle blood flow (62).

1.3.2 Skeletal muscle proteases

Based on the biochemical mechanism of proteolysis, there are five families of proteases: serine-, threonine-, cysteine-, aspartate- and metalloproteases (63). The serine protease family is vast, with 245 described members, both intra- and extracellular. The most well-characterized members of the extracellular serine proteases are the digestive enzymes trypsin and chymotrypsin, the members of the coagulation cascade and neutrophil elastase (64). The threonine family consists of 35 intracellular proteases including members of the proteasome, which is thought to play a role in muscle atrophy with unloading (38). The cysteine family of proteases has 163 members including the cathepsin family, the calpain family and the caspase signaling cascade (64). There are also some extracellular proteases in the cysteine family but none has been reported in the context of skeletal muscle. Intracellular cysteine proteases in the skeletal muscle are members of the calpains, which have been associated with degradation of intracellular structural proteins after muscle damage (65). Calpains escaping to the extracellular milieu has also been suggested to play a part in degradation of IGF-binding proteins after muscle damage (66). Levels of the intracellular proteases calpain 1, 2 and 3 and cathepsin B and L have been reported to increase in human skeletal muscle with eccentric exercise (67). Pepsin and renin are extracellular proteases of the aspartate family but there are no reports on these proteases in skeletal muscle.

Even though there are extracellular members of the serine and cysteine protease families, the most renowned and probably most important protease family for ECM remodeling is the MMP family. MMPs are involved in and are in many cases rate limiting for processes such as cell migration and the release of sequestered growth factors, in general and probably also in the skeletal muscle (68).

1.4 MATRIX METALLOPROTEINASES

1.4.1 Structure

Matrix metalloproteinases (MMPs) are a family of zinc- and calcium-dependent enzymes consisting of 23 family members in humans. MMPs are grouped into five different classes according to their structure and substrate specificity; these are collagenases, gelatinases, stromelysins and membrane-type MMPs (MT-MMPs). Seventeen of the MMPs are secreted whereas there are six cell membrane-associated MMPs (MMPs 14–17 and 24–25) (69).

All MMPs share a common domain structure: an N-terminal signal peptide that directs the enzymes to the secretory pathway, a prodomain with a conserved PRCGXPD sequence that confers the latency of the enzymes and a catalytic domain in which the catalytic zinc moiety is localized in the active site cleft. In addition, all but MMP-23 contain a hemopexin-like (HPX) domain linked to the catalytic domain through a hinge region. MT-MMPs have an additional domain, the transmembrane domain (70, 71). The various domains, modules and motifs of the MMPs are involved in interactions with other molecules, and hence affect or determine substrate specificity, cell and tissue localization and activation of MMPs. For instance, MMP-2 and MMP-9 contain a module of three fibronectin II (FnII)-like inserts (72). These inserts can interact with elastin, types I, III, IV, V, X and XI collagens, as well as with gelatins.

This may facilitate the localization of these enzymes to connective tissue matrices. This interaction appears to be of importance for the degradation of macromolecules e.g. elastin, gelatin and collagens IV, V and XI, but does not influence the degradation of chromogenic substrates or other macromolecules (73).

The best-characterized overall biological function of the MMP family is degradation of extracellular matrix proteins, and collectively, the MMPs can degrade all extracellular proteins (74). Such extracellular matrix remodeling occurs not only under physiological conditions such as bone remodeling, embryological development and the menstrual cycle, but also under numerous pathological conditions such as stroma remodeling and metastasis in cancer development, responses to ischemia-reperfusion insults and inflammation (71, 75-80). MMPs not only create a migration path for migrating cells, but also release ECM-sequestered growth factors and cytokines (81). There are also reports providing evidence that MMP substrates also include non-ECM molecules ranging from growth factor binding proteins and receptors to cell surface adhesion molecules (71, 75-80). There are three MMP family members that have been recognized in a skeletal muscle context: MMP-2, MMP-9 and MMP-14.

1.4.2 Regulatory factors

MMPs, synthesized as prozymogens, are either secreted or membrane-bound and are subsequently processed and activated, enabling localized degradation of their substrates (73). Regulation of MMP activities is achieved at multiple levels: transcriptionally, translationally, by activation of the zymogen, and by inhibition of the active enzyme (74).

The MMP-9 gene contains several regulatory cis elements. It contains a TATA box, an AP-1, an NF-KB and a PEA3-binding site (82). Increased transactivation through AP-1 or PEA3 is achieved largely through mitogen-activated protein kinase (MAPK)-mediated phosphorylation, but activated p38 kinases also increase the cellular amount of AP-1 protein indirectly by increasing transcription from the c-jun and c-fos promoters (72). Nuclear NF-KB levels are regulated by an inflammatory cytokine-activated pathway and, in particular, TNF-alpha is a well-established inducer of MMP-9 expression in this fashion (83-85). The promoters of MMP-2 and MMP-14 do not contain a TATA box, and expectedly, transcription from these promoters starts at multiple sites. The ubiquitous Sp-1 family of transcription factors, which bind to a proximal GC box, mainly determines the gene expression of these MMPs (73). Consequently, expression of these MMPs is largely constitutive with no distinct inducibility by growth factors or cytokines (74). In addition, protein levels of MMPs are affected by posttranslatory mechanisms under certain circumstances. TGF-beta increases both MMP-2 and MMP-9 levels through an increase in mRNA half-life. In contrast, the pharmacological inhibitor of MMP, doxycycline, acts both as a chelating agent (see below) and by decreasing MMP mRNA stability (86, 87).

Activation of the zymogen of MMPs is achieved by two mechanisms. One is through binding of regulatory molecules to allosteric sites and the other is proteolytic removal of the prodomain of the zymogen. Factors able to activate MMPs allosterically are organomercurials such as APMA, reactive oxygen species and detergents such as SDS. Interactions of this kind obviously occur mainly in vitro and are utilized in laboratory methods such as zymographic estimation of enzyme activity (see below) (73). There are also allosteric interactions of MMPs that have relevance in

in vivo conditions. Both pro-MMP-2 and pro-MMP-9 have been shown to become activated by mere interaction with gelatin, and more importantly, collagen IV (73, 88). A special case of allosteric interaction is binding of MMP-9 to neutrophil gelatinase-associated lipocalin (NGAL) (89). NGAL is known to form a covalent heterodimer with proMMP-9. The full function of such binding is not known but the binding of MMP-9 to NGAL hinders binding of MMP-9 to its main endogenous inhibitor, TIMP-1 (see below) and thus increases or prolongs its activity. NGAL was originally isolated from human neutrophils in a monomeric and a homodimeric form, and in addition, as an NGAL–MMP-9 complex (90). There are secretory granules in human neutrophils containing NGAL with no MMP-9 and vice versa, but there are also granules containing preformed heterodimers of NGAL-MMP-9 (91). NGAL appears to be expressed exclusively by neutrophils and the gene is expressed in myelocytes and metamyelocytes in the bone marrow and then stored in secretory granules until activation (92, 93). The level of NGAL is often used as a marker of inflammation and leukocyte activation and has recently been accepted as a biomarker for acute kidney injury(94).

A large number of proteases are involved in proteolytic activation of proMMPs. Both MMP-2 and MMP-9 have been shown to be activated in vivo, not only by serine proteinases such as chymase and trypsin, but also by circulating plasmin. The activation of MMP-2 mainly occurs at the cell surface via a cascade mediated by MMP-14. First, cell surface MMP-14 binds a TIMP-2 molecule, forming a receptor complex for MMP-2. In this complex, TIMP-2 functions as an adaptor molecule, mediating the binding of MMP-2 to MMP-14 by hydrophobic interactions between the carboxy terminal domain of TIMP-2 and the hemopexin-like domain of MMP-2. In this manner, MMP-2 is concentrated at the cell surface and brought into proximity of MMP-14 for proteolytic activation (73, 88).

1.4.3 Endogenous inhibitors

TIMPs are the major natural inhibitors of MMPs. In addition, other proteins with MMP inhibitory activity have been discovered, such as the procollagen C-terminal proteinase enhancer and tissue factor pathway inhibitor 2. However, these proteins have modest inhibitory capacity against MMPs compared with TIMPs (69, 95).

Until now, four different human TIMPs have been discovered: TIMP-1, -2, -3 and -4. TIMPs are glycoproteins with molecular weights of 20–30 kDa, which function as important physiological regulators of the activity of MMPs. TIMP molecules form high-affinity, noncovalent inhibitory complexes with MMPs in a 1:1 enzyme:inhibitor ratio. TIMPs have different specificities for different MMPs. For instance, TIMP-1 is the only TIMP that binds with high affinity to MMP-9. TIMP-1 binds modestly with MMP-2, whereas TIMP-2 interacts with high affinity with MMP-2. The relationship between MMP-2 and TIMP-2 is not a mere inhibitory one, as TIMP-2 is highly involved in the activation of MMP-2 as mentioned above. TIMP-1 is an inducible protein, as opposed to TIMP-2, which has a constitutive expression pattern (69, 88, 96).

1.4.4 Targets

Several hundred target proteins have been identified for both MMP-2 and MMP-9. However, most of these substrates are either theoretical and based on protein sequences or were established under optimal in vitro conditions. Methodological advances such as mass-spectrometric identification of proteins after 2D gel electrophoresis and ultra-high pressure liquid chromatography have resulted in the verification and identification of several new substrates under in vivo conditions (97, 98).

MMP-2 and MMP-9 both degrade the major component of the basal lamina, collagen IV. Such degradation is of crucial importance for successful extravasation of circulating cells and for the migration of cells between the compartments of the tissue (99, 100). Consequently, blockade of MMP activity suppresses various forms of cellular movement and has been shown to abolish skeletal muscle angiogenesis during exercise in the rat (48). In vitro studies have also revealed diminished migration of myoblasts after blockade of MMP-9 and upregulation of gene expression and activity during migration (101-107). In addition, MMP-2 and MMP-9 both digest other major components of the ECM such as collagen III, laminin and fibronectin (64, 72, 91). The degradation products of these ECM components are not always mere debris; several fragments of structural proteins have biological activity after proteolytic release. The best-characterized active fragment is endostatin, which is a cleavage product of collagen XVIII. It was discovered as an antiangiogenic agent about a decade ago by Folkman et al. and has been tested as an antiangiogenic agent because of its inhibitory effects on endothelial cell proliferation and migration (108, 109). Endostatin can be generated by several proteases, including MMP-9, and during the past decade, several other protein cleavage products have been shown to play potential roles in angiogenic processes. Two such cleavage products are tumstatin and angiostatin, which are generated by MMP-9 from collagen IV and fibrin, respectively (100, 109-113).

Regulation of signaling molecules by MMPs is not limited to cleavage products. In one of the first reports on MMP-9 in cancer progression, it was illustrated that MMP-9 can release preformed VEGF-A from the ECM and thereby initiate an angiogenic response (114). Furthermore, the bioavailability of another growth factor relevant to skeletal muscle plasticity, IGF-1, has been shown to be influenced by MMP-9 degradation of IGF-binding proteins (115, 116).

1.4.5 Possible physiological roles for MMP in human skeletal muscle

It is clear that the extracellular proteolytic activity of MMPs is of importance for many biological processes. Based on cell and cancer model studies and to some extent on physiological models, MMP-2 and MMP-9 have been established as key players in processes ranging from degradation of structural components during cell migration to regulation and generation of growth factors. Many of these processes are probably of major importance for skeletal muscle remodeling and adaptation to exercise, and it is known that MMP-activity is important for skeletal muscle angiogenesis in the rat, that MMP-9 is upregulated in the human tendon after exercise, and that circulating levels of MMP-9 increase after downhill running. However, little is known about these enzymes in the skeletal muscle of humans and how physiological stimuli such as exercise and exercise training affect their expression and activity.

2 AIMS

The overall aim of this thesis was to characterize expression and activity of MMPs in humans undergoing exercise and to investigate the underlying stimuli.

The specific aims were to further elucidate:
1. Skeletal muscle MMP activation in response to a single bout of exercise and exercise training with regard to gene expression and enzyme activity.
2. Exchange of factors associated with MMP activity between exercising leg and the circulation during exercise.
3. Possible cellular sources of MMP in skeletal muscle tissue and blood.
4. The effects of restricted leg blood flow, and the thereby reduced oxygen delivery to the exercising leg on skeletal muscle and circulating levels of MMP
5. The effects of the myokine interleukin-6 on MMP levels in skeletal muscle and in the circulation.

3 METHODS AND METHODOLOGICAL CONSIDERATIONS

3.1 EXPERIMENTAL DESIGN

3.1.1 Subjects

In total 32 healthy men participated in study I-IV.
In study I and III, 10 subjects participated. The mean (range) age, height and weight of the subjects were 25 (18-37), 180 (170-190) cm and 77 (58-82) kg.
In study II, 10 subjects were included. Their mean (range) age, height, and weight were 24 (20–27) yr, 181 (173–190) cm, and 75 (63–90) kg.
In study IV, 12 healthy human male subjects were inlcuded. The mean (range) age of the subjects was 27 (21-34).
All subjects included in the studies were healthy and did not take any medication. All subjects gave informed consent to participate and the local ethics committee approved the studies.

3.1.2 Experimental settings

3.1.2.1 Exercise protocol study I and III
Ten subjects performed two-legged upright cycling. The average VO2max in the participating subjects was: 51 (43-64) ml kg/min. The subjects cycled for the first 20 min at workload equivalent to 50% of VO2max (125 ±20 W), after which they cycled a further 40 min at a higher workload equivalent to 65% of VO2max (165 ± 30 W). Finally, the subjects were instructed to cycle at the highest tolerable workload (175 ± 50 W) that could be sustained for 5 min. Teflon catheter were inserted in the femoral artery and into the ibsilateral femoral vein, at the level of the inguinal ligament. Blood samples were drawn at rest, at 20, 40, 60 min of exercise, and 120 min after exercise and used to calculate the arterio-venous differences. Muscle biopsies were performed from the vastus lateralis muscle of one leg at rest, at 65 min of exercise and 120 min after exercise.
3.1.2.2 Exercise protocol study II
Eleven subjects performed one-legged exercise four times per week, over a five-week period. The average VO2max in the participating subjects was 51 (40–66) ml kg/min. The subject first performed one-legged exercised with restricted leg blood flow for 45 min, after which the subject performed similar amount of exercise with non-restricted blood flow using the other leg. Blood-flow restriction was achieved by exposing the exercising leg to 50 mm Hg supraatmospheric pressure in an exercise model first described by Eiken (117). This has been shown to reduce blood-flow during exercise with 15-20% (118). The subjects were instructed to exercise at the highest tolerable workload for 45 min, taking into account that they must complete the full 45-min session. Compared with the workload in the first week, the average workload increased by 30 % ± 22 in week 2, 52 % ± 39 in week 3 and 84 % ± 7 % in week 4. Muscle biopsies were performed from the vastus lateralis muscle of both legs before and 120 min after the first exercise bout. Post-training samples were performed after five weeks at rest and 120 min after the last exercise bout; the resting was performed 24 h after the

previous exercise session. A further resting biopsy (24 h after the latest exercise bout) was performed from each leg 10 days after the start of training period.

3.1.2.3 Experimental protocol study IV

This study was a collaborative study with Bente Klarlund Pedersen and collaborators at Rigshospitalet in Copenhagen (Denmark). At the experimental day, subjects arrived at 0800 h following an overnight fast. The femoral artery of one limb was cannulated and used for the infusion. Subjects (n=6) were infused with recombinant human Il-6 (rhIl-6) for 3-h at a rate of 5 μg/h, in a volume of 25 ml/h. The rhIl-6 was administered in 20% albumin. Control subjects (n=6) were infused with 20% albumin for 3-h. Plasma Il-6 concentrations (approximately 100 ng/L) and the Il-6 expression in abdominal fat have been previously reported (Keller et al 2003).

3.2 MUSCLE SAMPLES

3.2.1 Biopsies

In all studies, biopsies were performed from the vastus lateralis muscle, using the percutaneous needle biopsy technique (Bergström, 1962). All muscle biopsy samples were frozen in liquid nitrogen within 10-15 s and stored at –80°C until further analysis.

3.2.2 Arteriovenous differences

Teflon catheters were inserted into the femoral vein and artery at the level of the inguinal ligament. Blood samples were drawn simultaneously from the femoral artery and the ipsilateral femoral vein at rest before exercise, during, and 120 min after the end of exercise. The arteriovenous concentration differences were calculated from the measured arterial and venous concentrations.

3.2.3 In vitro experiments

3.2.3.1 Monocytes

The human THP-1 acute monocytic leukaemia cell line (THP-1) was cultured in RPMI + Glutamax (Invitrogen) supplemented with; 10% foetal calf sera (FCS) (Invitrogen), pencillin streptomycin (1%, Invitrogen), 1mM sodium pyruvate and 0.05mM b-mercaptoethanol. The cells were maintained at 37°C with 5% CO2 and seeded in 24-well plates with a cell density of 1x10^6/mL. The cells were stimulated for either one or three hours with Il-6 concentration 0 (ctrl), 1, 10 or 50 ng/mL. Each experiment was conducted with three replicates.

3.2.3.2 Macrophages

THP-1 –cells seeded in 24-well plates as described above were treated with 5% phorbol 12-myristate 13-acetate (PMA) for 24 hours to differentiate to a macrophage phenotype. Cells were washed three times prior to any further experiments in order to evade non-adhering cells and debris. Subsequently cells cells were stimulated for either one or three hours with Il-6 concentration 0 (ctrl), 1, 10 or 50ng/mL. Each experiment was conducted with three replicates.

3.2.3.3 Myoblasts

The satellite cells were extracted from fresh muscle: Immediately following the biopsy procedure approximately 100 mg of muscle tissue was placed in sterile PBS containing

1% of streptomycin in 4° C over night. The sample was incubated in 5 ml 0.25 % Trypsin EDTA in 37° C, 5% CO2 with gentle agitation for 20 minutes. Undigested tissue was allowed to settle for 5 minutes and the supernatant was collected in 5 ml Dulbecco's Modified Eagle's Medium (DMEM-F12), with 20 % fetal calf serum and 1 % Penicillin Streptomycin. The cells were cultured until reaching 60% confluence. Cells were seeded in 6-well plates and were kept proliferating prior to experiment. The cells were stimulated for one or three hours with 0, 1, 10 or 50ng/mL Il-6 in serum free DMEM media. Each experiment was conducted with three replicates. After stimulation the cells were taken for RNA extraction.

3.2.3.4 Myotubes

Myoblasts were differentiated to myotubes by serum starvation for >72 hours prior to the experiment. The cells were stimulated for one or three hours with 0, 1, 10 or 50ng/mL Il-6 in serum free DMEM media. Each experiment was conducted with three replicates. After stimulation the cells were taken for RNA extraction.

3.2.3.5 Human umbilical chord endothelial cells

Primary cultures of HUVEC were obtained from fresh umbilical cords. The umbilical vein was incubated with 0.1% dispase for 30 min. After isolation cells were kept in DMEM. Cells were seeded in 24-well plates. The cells were stimulated for one, two or four hours with Il-6 concentration 0 (ctrl), 10 or 150ng/mL in 20% FCS DMEM. Each experiment was conducted with three replicates. After stimulation the cells were taken for RNA extraction.

3.3 LABORATORY METHODS

3.3.1 Laser capture and microdissection

Cryosections (8 μm thick) were freshly cut, placed on uncoated glass slides, and immediately immersed in cold acetone for 3 min followed by transfer to a –80°C freezer, where they were stored for a maximum of 1 wk. To prevent RNA degradation during histological staining, RNAase inhibitor was added to all sterile PBS solutions (50 U/100 μl; SUPERaseIn, Ambion). The laser capture and microdissection was performed using the P.A.L.M. system (Carl Zeiss microimaging). From muscle tissue sections, larger areas of muscle tissue covering all cell types and individual muscle fibers were dissected from the same section. After capturing the sum of 100 muscle fibers and subsequently an area of tissue of the same size as the fibers from each section, the caps were inserted into 0.5-ml microcentrifuge tubes filled with 50 l of extraction buffer (PicoPure RNA isolation kit, Arcturus Engineering).

3.3.2 Enzyme-linked immunosorbent assay

In study I skeletal muscle protein levels of VEGF-A and endostatin was analyzed with ELISA. Part of the skeletal muscle biopsy (40 μl buffer/mg wet muscle) was homogenized in ice-cold buffer containing 0.1 M potassium phosphate (pH 7.7), 0.05% BSA, 20 μg/ml leupeptin, 50 μg/ml aprotinin and 40 μg/ml PMSF. The homogenate was rotated for 60 min at 4 °C and centrifuged at 15.000 g for 15 min at 6 °C. Two hundred microlitres of the 1:5 diluted supernatant was used to quantify protein utilising sandwich ELISA (Quantikine R&D Systems, USA). All techniques and materials in this analysis were used according to manufacturers' protocols. Optical density was

quantified on a micro plate-reader *Quant (Bio- Tek® Instruments Inc., USA). All samples were assayed in duplicate.
Circulating VEGF, endostatin, MMP-9 and NGAL levels were quantified by sandwich ELISA (VEGF-A Quantikine R&D Systems, Endostatin Cytimmune Science Inc) using similar procedures as those described for tissue VEGF-A. All techniques and materials used in this analysis were used in according to the manufacturer's protocol. Plasma elastase was quantified by competetive ELISA (KA0163, Abnova) according to manufacturer's protocol.

3.3.3 Zymography

Zymography supplies were purchased from Invitrogen (Carlsbad, CA). MMP-2, pro-MMP-9, and active MMP-9 distribution was determined from muscle biopsy homogenate using the samne procuder as described for ELISA. Subsequently gelatin substrate zymograms were prepared using precast 10% SDS-polyacrylamide gels contain- ing 1 mg/ml of gelatin. Equal volumes of experimental media samples (25 ug) were diluted into 2 x Tris-glycine SDS sample buffer and electrophoretically separated under nonreducing conditions. Proteins were incubated in renaturing buffer (Invitrogen) for 30 min at room temperature. The gels were incubated overnight at 37°C in developing buffer (Invitrogen). After 1 h staining with Coomassie blue and destaining for 2 days with 10% acetic acid and 40% methanol in water, gelatinase activity was evident by clear bands against a dark blue background. Quantification of the bands was performed using digital camera Fujifilm LAS-1000 or Canon 40d and densiometry software Fujifilm Image gauge version 3.46.

3.3.4 Immunohistochemistry

Frozen biopsy samples were embedded in OCT (Tissue-Tek, Sakura Finetek) and kept frozen at 80°C until further analysis. Cross sections (5 m) of biopsy samples were cut at 21°C, placed on glass slides. Sections for MMP-9 staining were put in 4% formaldehyde, and sections for MMP-2 staining were put in 99% ethanol for 10 min for fixation in room temperature. After washing in PBS for 3 5 min, the sections were incubated with PBS containing 3% BSA and 0.1% Triton X-100 (Dako) for 30 min at room temperature. A rabbit anti-human MMP-9 antibody (Sigma-Aldrich) diluted 1:500 in PBS with 1% BSA and 0.3% Triton X-100 was applied to the sections and incubated at 37°C for 60 min, and subsequently the sections were incubated overnight at 4°C with a rabbit anti-human MMP-2 antibody (Sigma-Aldrich) diluted 1:250 in PBS with 1% BSA. Following washing, the sections were incubated for 60 min with a cyanine-3- conjugated secondary antibody donkey anti-rabbit (Jackson Immu-noResearch) diluted 1:500 in 1% BSA at 37°C for 60 min. The sections were mounted with Vectashield (Vector Laboratories). Double staining was achieved by coincubation of the primary antibody together with sarcolemmal marker, a mouse anti-human caveolin-3 antibody (Santa Cruz Biotechnology) diluted 1:500 or an endothelial cell marker mouse anti-human CD31 (Dako) diluted 1:100. A FITC- conjugated donkey anti-mouse secondary antibody (Jackson Immuno Research) diluted 1:500 was added in the double-staining protocols. As a negative control, the primary antibodies were excluded from the protocol.

3.3.5 Reverse transcribed real-time PCR

In studies I-III total RNA was prepared by the acid phenol method (Chomczynski and Sacchi, 1987) and quantified spectrophotometrically by absorbance at 260 nm. The integrity of the total RNA was determined by 1% agarose gel electrophoresis. Two micrograms of RNA was reverse transcribed by Superscript reverse transcriptase (Life Technologies, Stockholm, Sweden) using random hexamer primers (Roche Diagnostics GmbH, Mannheim, Germany) in a total volume of 20 µl.
RNA isolated by LCM was kept in extraction buffer (PicoPure RNA isolation kit, Arcturus Engineering). After incubation in 42°C for 30 min, the extraction buffer was collected by a brief spin (800 g for 2 min), the caps were removed, and the microcentrifuge tubes were stored in 80°C. Total RNA was extracted from the cell lysate using the PicoPure RNA isolation kit according to the manufacturer's protocol. The isolated RNA was stored frozen in 80°C until use. Isolated RNA was reverse transcribed by Superscript reverse transcriptase (Life Technologies, Solna, Sweden) using random hexamer primers (Roche Diagnostics) in a total volume of 30 ul.
In studies I-III, mRNA was detected on an ABI-PRISM 7700 Sequence Detector (Perkin- Elmer Applied Biosystems Inc, Foster City, CA, USA). Oligonucleotide primers and TaqMan probes was ordered as gene assay on demand (Perkin-Elmer Applied Biosystems Inc.). As an endogenous control to correct for potential variations in RNA loading 18S rRNA was selected in study I-II (18S 4310893E, Perkin-Elmer Applied Biosystems Inc.) and GAPDH (Hs99999905-m1) was selected in study IV. All reactions were performed in 96-well MicroAmp Optical plates, using the ABI-PRISM 7700 Sequence Detector (Perkin-Elmer Applied Biosystems Inc.). For every gene, all samples were amplified simultaneously in duplicate in one assay run. The threshold cycle was determined for both the experimental gene and the endogenous control gene, and was used to calculate the relative expression.

3.4 STATISTICS

The data were analyzed using logarithmic-transformed ratios for mRNA, and logarithmic-transformed densiometric values for zymographies. In the analysis of plasma and serum concetrations over time concentrations was corrected for hemoconcentration by normalisation to plasma albumin. For analysis of arteriovenous differecenes raw data was used.

A one-way analysis of variance (ANOVA) for repeated measures was used to evaluate the effect of time on mRNA and protein expression. Post hoc analysis used planned comparisons to locate the points of interaction. A two-way ANOVA for repeated measures evaluated the effects of time and localization (artery or vein) in arteriovenous differences and to evaluate the effects of training (before, 10 days, and five weeks) in the two exercise conditions on basal mRNA and protein content. Planned comparison was used (i.e. post hoc-test) to locate differences corresponding to significant interactions or when no interaction was found to locate differences corresponding to significant main effects in the ANOVA models. MMP-activity was analyzed by a nonparametric method for comparison of multiple dependent samples (Friedman ANOVA, Kendall coefficient of concordance and Wilcoxon matched pair test). Cell culture gene-expression was analyzed with unpaired students t-test. Data are

presented as mean and SEM unless otherwise stated. For all tests, $P < 0.05$ was considered significant.

4 RESULTS AND DISCUSSION

4.1 SKELETAL MUSCLE MMP

4.1.1 Skeletal muscle MMP activity and expression

In study I, skeletal muscle MMP-2 mRNA and gelatinolytic activity were estimated before, immediately after, and 2 hours after a single bout of cycling exercise. MMP-2 levels remained stable at all three times. Study II supports the findings from study I in that no change in MMP-2 mRNA or activity was detected after a single bout of exercise, but both mRNA and enzyme activity increased after 10 days of exercise training (i.e., four exercise bouts). Koskinen et al reported that MMP-2 mRNA and enzyme activity increased in rats 2 days after a bout of downhill running (119, 120). The notion of an exercise-induced increase in MMP-2 in the skeletal muscle was confirmed by Carmeli et al, who showed increased expression of MMP-2 in rats performing exercise bouts at high intensity (70% of VO_{2max}) but not at moderate intensity (50% of VO_{2max}) for 2 weeks (121-123).

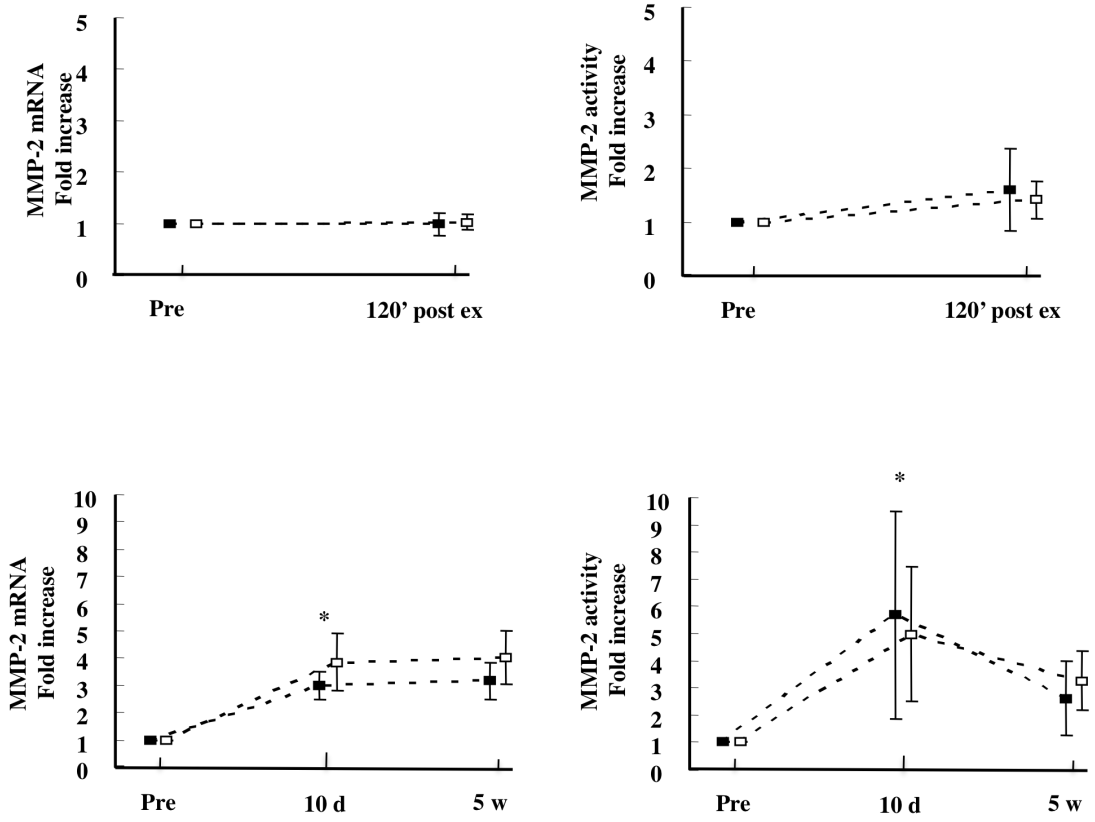

Figure 1: MMP-2 mRNA and activity in human vastus lateralis muscle before and after a single bout of exercise (a) and over five weeks of training (b). Pre denote biopsy taken before the first exercise bout. 10 d denote biopsy taken after 10 days of exercise training and 5 w denote biopsy obtained after 5 weeks of training. * denotes significant differences ($P < 0.05$) between before exercise and 10 days in both conditions. Values are as presented as fold change means and standard error of means SEM (mRNA n = 10; protein n = 7).

Heinemeier et al also investigated MMP-2 in rat skeletal muscle and compared the response to concentric, eccentric, and isometric physical activity. In that study, skeletal muscle MMP-2 increased 24 hours after 4 days of repeated bouts of exercise. (124).

Hellsten et al estimated MMP-2 after 90 minutes of passive stretch and, consistent with the findings in this thesis, did not detect any changes in the MMP-2 transcription or enzyme activity (125). Based on the consistent findings of concomitant increases in MMP-2 mRNA and activity in this thesis and in other studies, it appears that MMP-2 is upregulated after repeated bouts of exercise, presumably regulated mainly at the transcriptional level.

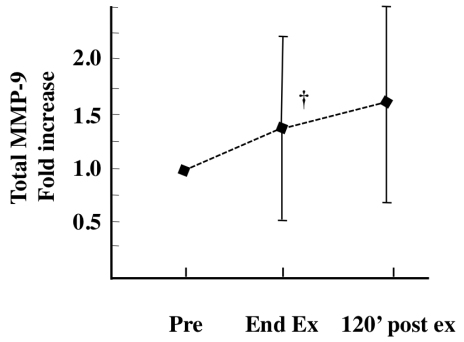

Figure 2a: MMP-9 activity levels in m. vastus laterialis pre exercise, immediately after and 120 min post exercise. † denote significant differences between pre exercise and end of exercise ($P < 0.05$). Values are presented as means and SEM (n=10).

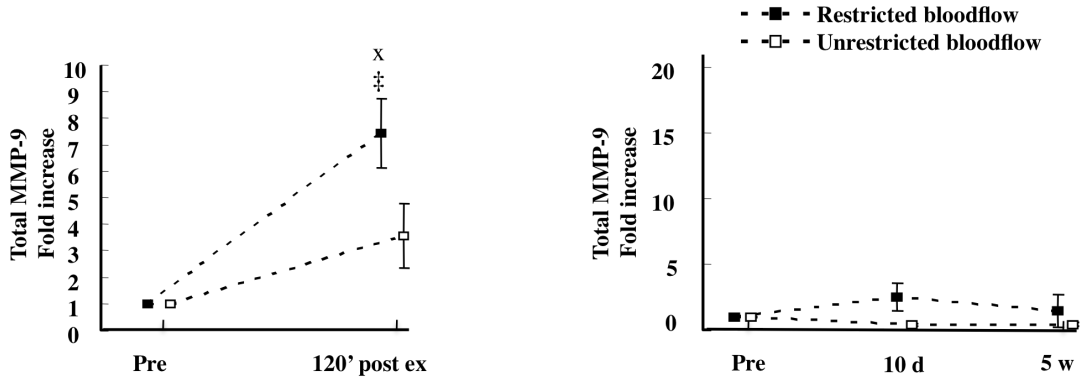

Figure 2b: MMP-9 activity (active + pro-MMP) in human vastus lateralis muscle before and after a single bout of exercise (a) and over five weeks of training (b). Pre denote biopsy taken before the first exercise bout. 10 d denote biopsy taken after 10 days of exercise training and 5 w denote biopsy obtained after 5 weeks of training. ‡ denotes significant differences ($P < 0.05$) between before and post-exercise in both conditions. Two outliers are marked with x in the figure. Values are presented as means and SEM (n=7).

In contrast to MMP-2, MMP-9 mRNA and enzyme activity increased after 60 minutes of cycling exercise in study I. The protein levels of MMP-9 increased significantly by about twofold immediately after exercise, whereas the increase in MMP-9 mRNA reached significance 2 hours after exercise. In study II, skeletal muscle MMP-9 levels increased after a single bout of exercise, both at the protein and mRNA levels. In study II, samples were obtained before and 2 hours after the exercise bout, and steady-state levels of MMP-9 were assessed by analyzing mRNA and zymography at least 48 hours after the last training session after 10 days and 5 weeks of exercise training. After 10 days of exercise, MMP-9 mRNA levels increased and remained elevated after 5 weeks of exercise training. By contrast, the MMP-9 protein levels did not increase significantly after 10 days or 5 weeks of exercise training. In resting muscle (i.e., in the preexercise biopsies) in both studies I and II, the MMP-9 mRNA levels were low, and these values were reflected in threshold cycle values > 35 in all but two subjects; MMP-9 mRNA was undetectable in two subjects despite high

concentrations of cDNA. In all other samples, MMP-9 was detectable albeit at relatively high cycle threshold values in many cases. This led to a high variance in the increase with time and prohibited the possibility of investigating interactions between the two exercise conditions using two-way ANOVA in study II; instead, a Wilcoxon matched-pair test was used.

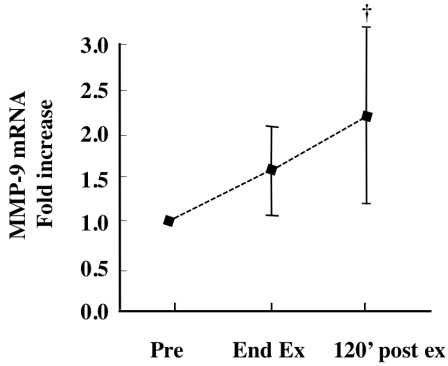

Figure 3a: MMP-9 mRNA levels in m. vastus laterialis pre exercise, immediately after and 120 min post exercise. † denote significant differences between pre exercise and 120 min post exercise ($P < 0.05$). Values are presented as means and SEM (n=10).

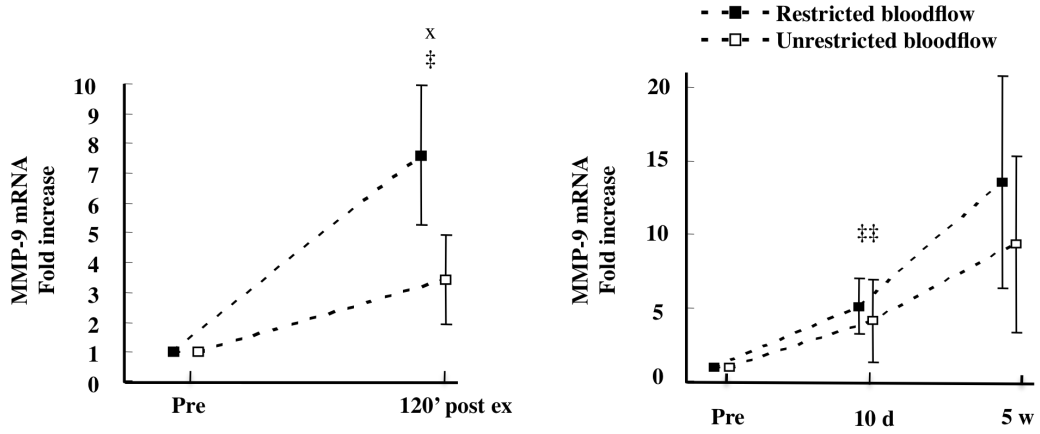

Figure 3b: MMP-9 mRNA in human vastus lateralis muscle before and after a single bout of exercise (a) and over five weeks of training (b). Pre denote biopsy taken before the first exercise bout. 10 d denote biopsy taken after 10 days of exercise training and 5 w denote biopsy obtained after 5 weeks of training. ‡ denotes significant differences ($P < 0.05$) between before and post-exercise in both conditions. ‡‡ denotes significant differences ($P < 0.05$) between before exercise and 10 days in both conditions using non-parametric statistics. Subjects with CT-values over 35 in the pre sample are excluded from the figure (n=2) and two outliers are marked with x in the figure. Values are as presented as means and SEM (n = 10).

The difference between mRNA and activity in MMP-9 immediately after exercise in study I probably reflects activation of preformed MMP-9. By contrast, in study II, the basal expression of MMP-9 mRNA was increased throughout the training program but without any evidence of increased activity in resting muscle. Taken together, the current observations indicate that MMP-9 activity is not regulated only at the translational level.

In studies I and II, TIMP-1 and MMP-14 mRNA increased with the increase in MMP-2 (i.e., with training), which is consistent with previous reports in the rat (48, 124). MMP-14 is a membrane-associated MMP and in terms of skeletal muscle expression, it appears to be upregulated in parallel with MMP-2 in injury models, during mechanical overload, and after exercise (126, 127). Consitent with the results in

this thesis, TIMP-1 has been shown to increase with exercise training in rat skeletal muscle, and has also been reported to increase in plasma immediately and up to two weeks after after eccentric work in human subjects (124, 128, 129).

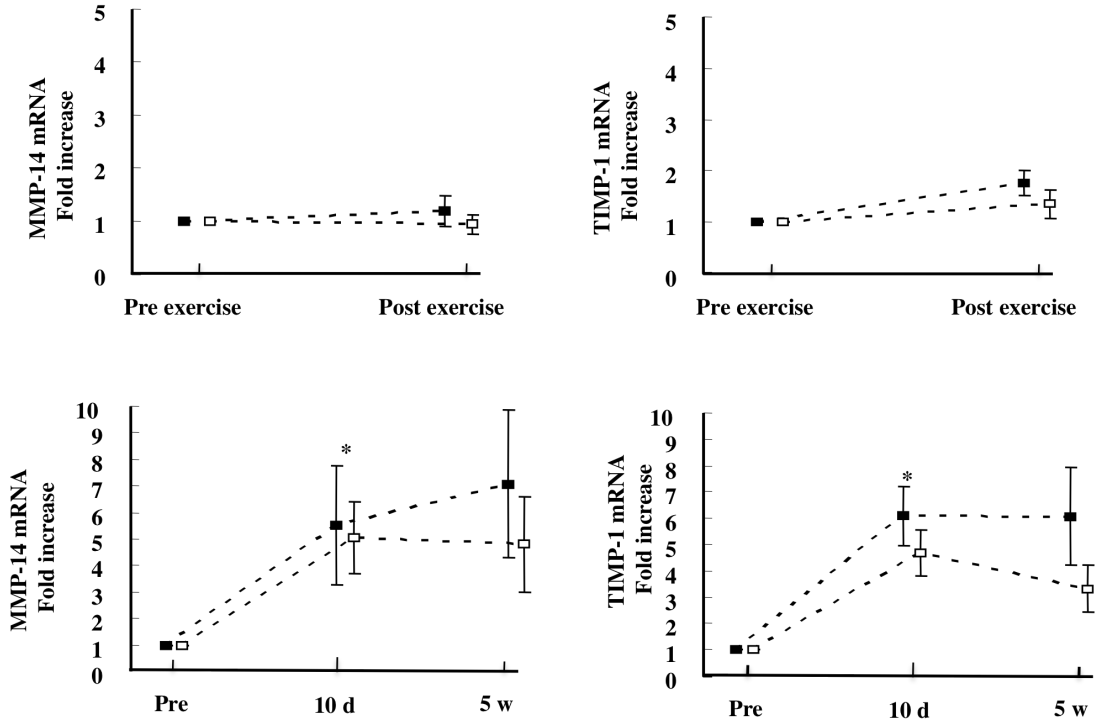

Figure 4: MMP-14 and TIMP-1 mRNA in human vastus lateralis muscle before and after a single bout of exercise (a) and over five weeks of training (b). Pre denote biopsy taken before the first exercise bout. 10 d denote biopsy taken after 10 days of exercise training and 5 w denote biopsy obtained after 5 weeks of training. * denotes significant differences ($P < 0.05$) between before exercise and 10 days in both conditions. Values are as presented as means and standard error of means SEM (n = 10).

4.1.2 MMP localization

In study II, immunohistochemistry and laser capture microdissection were used to identify the localization and cellular source of MMP-2 in skeletal muscle. Such information can provide indirect insight into the regulating stimuli and possible targets of a factor of interest. In the immunohistochemical investigation, sections were double stained with antibodies directed against MMP-2, CD31 (an endothelial cell marker), and caveolin 3, which was used as a sarcolemmal marker. Sections were also counterstained with DAPI. A generalized distribution pattern was seen in the immunohistochemistry: clear positive areas between all muscle fibers and staining around the $CD31^+$ endothelial cells. The localization of MMP-2 in the muscle tissue is consistent with previous findings, although no study had reported that MMP-2 is expressed in adult muscle fibers (130, 131). Satellite cells also express MMP-2, but studies on SaC MMP-2 expression are usually in vitro studies (47, 101, 102, 106).

As seen in immunohistochemistry scattered MMP-2-positive areas appear within the muscle fibers. Because no double staining of the protein synthesis machinery such as ribosomes, Golgi or exocyme proteins was performed, it is not possible to conclude whether this reflects actual intracellular MMP-2 or a staining artifact. Interestingly, recent studies show that there are intracellular forms of MMP-2.

Intracellular MMP-2 is thought to become activated by skeletal muscle atrophy and digestion of both intracellular and extracellular proteins, which reduce muscle mass and the thickness of the ECM(132-136). These findings suggest that an intracellular MMP-2 is present in muscle fibers.

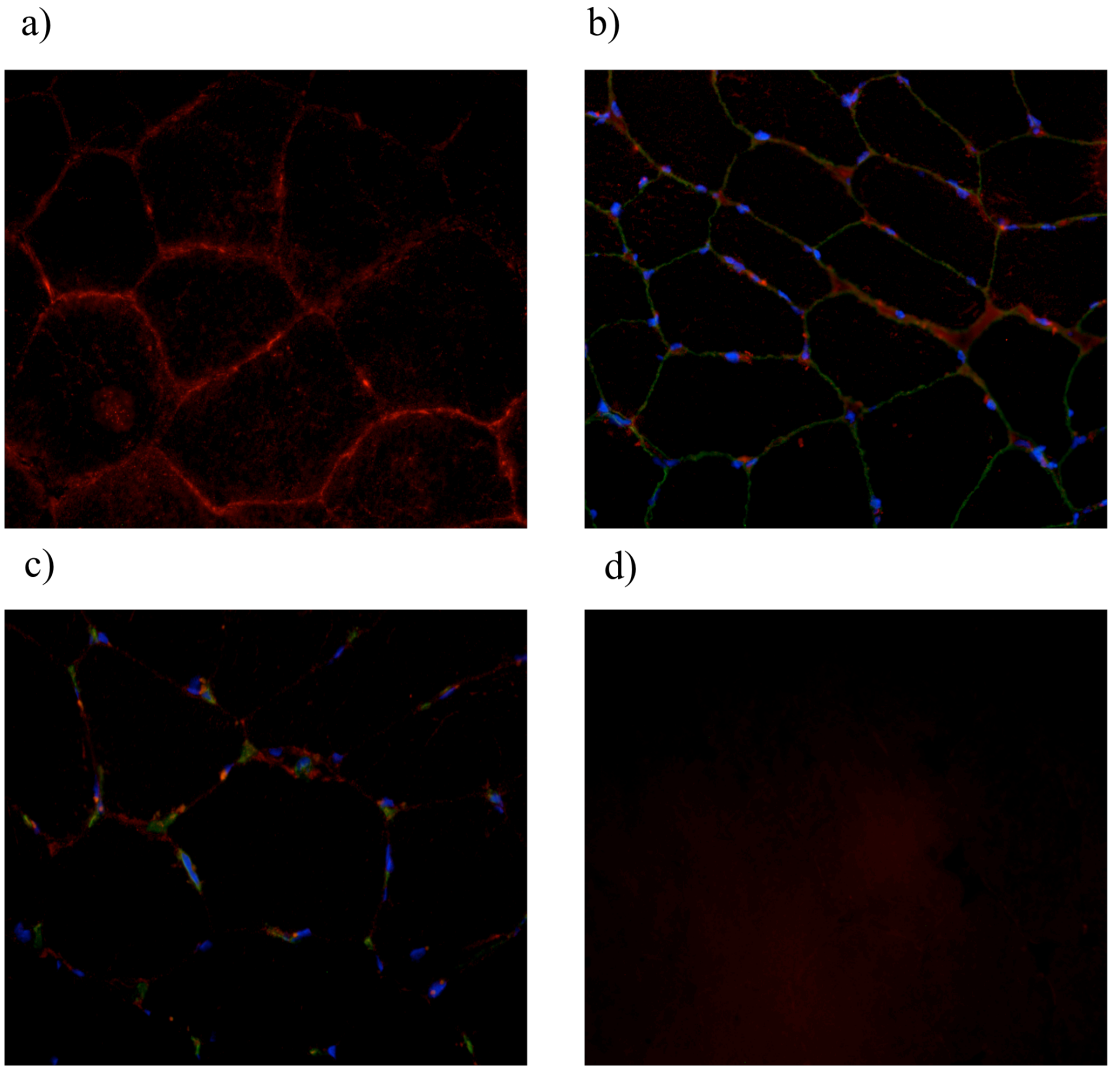

Figure 5: Immunohistochemical staining of a) MMP-2. b) MMP-2 (red) + sarcolemmal marker caveolin-3 (green). c) MMP-2 (red) + endothelial cell marker CD31 (green). d) Negative control. All in human skeletal muscle sections before exercise. b and c are counterstained with DAPI (blue). The distributions of the staining indicate MMP-2 to be distributed in the extracellular matrix around muscle fibers as well as capillaries and also within skeletal muscle fibers.

Laser capture microdissection of muscle fibers with subsequent analysis of MMP-2 mRNA was used to further address the question of whether MMP-2 is expressed in skeletal muscle. This technique was used to first inspect tissue sections under a microscope and then, using a focused laser beam, to cut out areas of interest for further analysis. Skeletal muscle fibers were cut and captured, and the MMP-2 mRNA was analyzed. The purpose was to determine whether the skeletal muscle fibers express MMP-2 and hence contribute to the MMP-2 deposited throughout the ECM of the muscle. Milkiewicz et al used the LCM technique to isolate skeletal muscle endothelial cells in the rat and reported that endothelial MMP-2 gene expression increased in vivo and in vitro with muscle overload (137, 138). The data from the LCM in study II add to

this observation by showing that the muscle fiber is another likely source of MMP-2. Samples from before and after 10 days of exercise training were analyzed, albeit only from three subjects because of the limited biopsy specimens available and because optimization of the experimental protocol consumed vast amounts of material. Despite this limitation, the mRNA level of MMP-2 normalized to 18S was higher after 10 days of exercise in all three subjects. This is the same pattern of expression as in the muscle as a whole, and this observation supports the idea that the muscle fiber contributes to the overall increase in MMP-2 after exercise training.

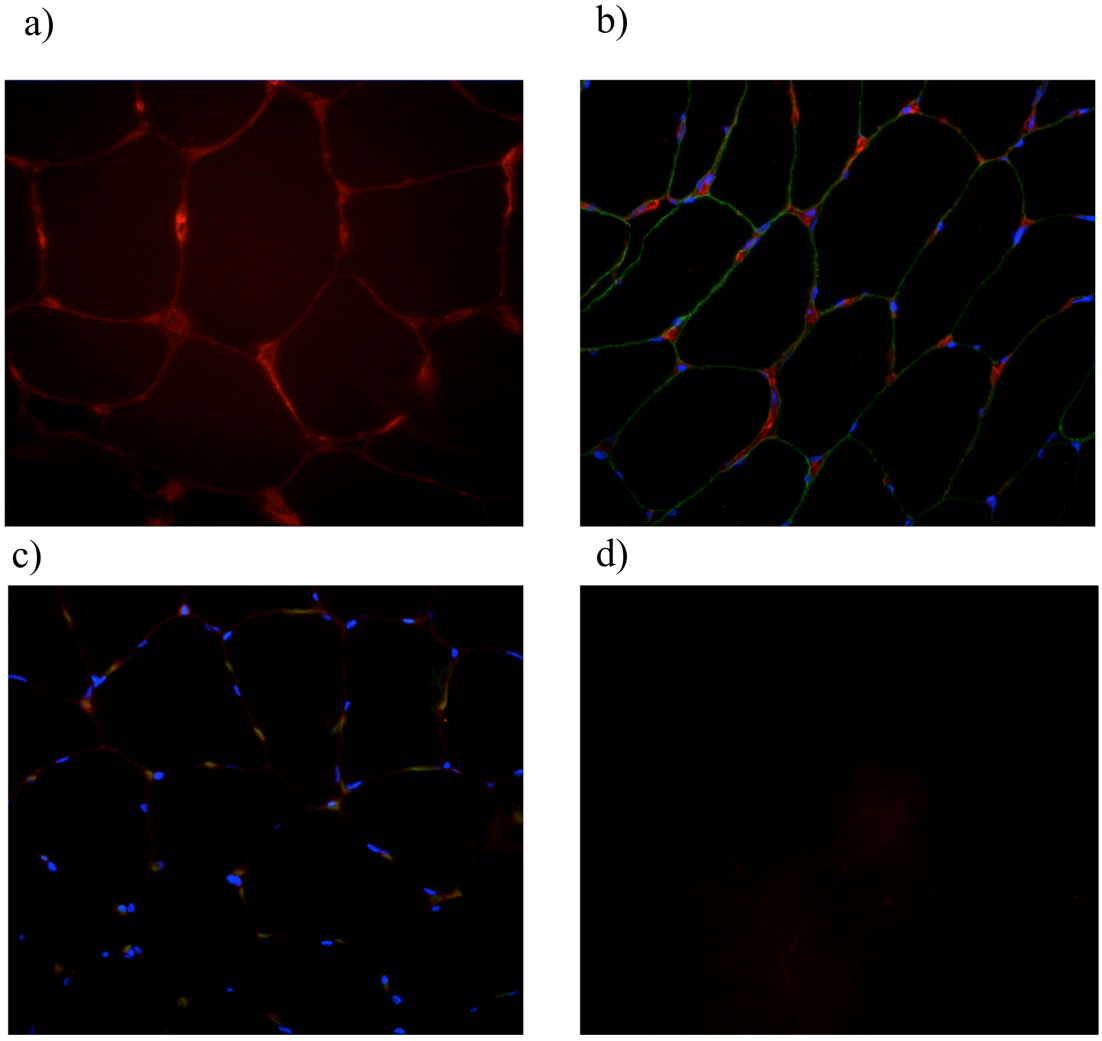

Figure 6: Immunohistochemical staining of a) MMP-9. b) MMP-9 (red) + sarcolemmal marker caveolin-3 (green). c) MMP-9 (red) + endothelial cell marker CD31 (green). d) Negative control. All in human skeletal muscle sections before exercise. b and c are counterstained with DAPI (blue). The distributions of the staining indicate MMP-9 to be distributed in the extracellular matrix around capillaries and muscle fibers but with no expression within skeletal muscle fibers.

Immunohistochemical staining in study II suggested an even distribution of MMP-9 throughout the ECM of resting skeletal muscle. This was also reported by Zimowska et al in a rat study and by Singh et al in human masseter muscle (47, 131). These findings and the current observations contrast with a report that MMP-9 expression is restricted to atrophic fibers in patients with myositis (139). No MMP-9 mRNA was detected in laser-dissected muscle fibers or in endothelial cells (unpublished data) in resting or in exercised muscle. This likely reflects that the MMP-9 mRNA level acquired with laser dissection is below the detection limit for the real-time PCR measurement. There are methodological difficulties associated with protein

quantification by immunohistochemistry. However, MMP-9 was detectable throughout the ECM of the muscle despite the low abundance of mRNA at rest, suggesting that in skeletal muscle there is a low turnover of MMP-9, with only minor translation and degradation of the MMP-9 deposited in the ECM.

In study IV, basal levels of MMP-9 mRNA were observed in both myoblasts and myotubes. The measured levels were low, and thus were similar to those noted in skeletal muscle as a whole at rest in studies I and II. Myoblasts express MMP-9 during migration and differentiation in vitro (101, 103-107). Less is known about expression of MMP-9 in vivo. Zimowska et al suggested that MMP-9-positive mononuclear cells localize around destroyed muscle fibers in a rat muscle injury model where they are activated to become satellite cells, although this was not verified by control staining or dissection (47). In muscle from patients with myositis of various etiologies, mononuclear cells expressing MMP-9 comprise macrophages and neutrophils (130, 131). This observation agrees with the general view that MMP-9 is associated mainly with invading immune cells under inflammatory conditions (92).

4.1.3 Possible regulation of skeletal muscle MMP

The expression patterns of MMP-2, MMP-9, MMP-14, and TIMP-1 in studies I and II indicate that these factors are regulated by different stimuli or through dissimilar mechanisms during exercise. This is consistent with differences in the promoter regions of these molecules (140). For example, the MMP-2 gene lacks a TATA box and is expressed constituently in most tissues; few studies have reported upregulation of the MMP-2 gene by growth factors and cytokines (140). There is little or no basal expression of MMP-9, but its promoter region contains a variety of response elements that are sensitive to a wide range of growth factors and cytokines (140).

As argued above, physical exercise is a complex process involving several factors, such as reduced tissue oxygen level, increased muscle tension, and increased shear stress and wall tension in blood vessels, that could each affect the expression levels of MMP-2 and MMP-9 in skeletal muscle (141-143). The purpose of the experimental design in study II was to identify the factors responsible for the activation of MMPs. Several aspects of the exercise stimulus can differ between exercise with and without restricted blood flow. This is reflected in differences in the activation of canonical pathways and gene expression and, ultimately, in differences in skeletal muscle adaptation. Exercise with restricted blood flow reduces oxygen tension in skeletal muscle, increases glycogen depletion, and causes metabolic perturbation (e.g., increased lactate level and decreased ATP–PCr concentration) (118, 144). Another difference is the greater total activation of muscle fibers during exercise with restricted blood flow compared with unrestricted blood flow. It is very likely that reduction in the total blood flow over the leg also decreases capillary flow velocity and hence shear stress in the microvascular endothelial cells (118, 144). The importance of these differences is demonstrated by a more robust angiogenic response, greater mitochondrial biogenesis, and a larger increase in oxidative enzyme activities and number of capillaries after 4 weeks of exercise training with restricted blood flow compared with exercise with unrestricted blood flow (118, 144-146).

In study II, MMP-2 levels increased after 10 days of exercise training without any difference between the two exercise conditions. These observations do not support the idea that oxygen tension or metabolic perturbation is critical to the

regulation of the expression or activation of MMP-2. By contrast, the stimuli that are similar in the two exercise conditions are muscle mechanical load and stretch. The expression of MMPs in skeletal muscle endothelial cells is affected by mechanical stretch (137, 138, 147), and MMP-2 expression in muscle tendon is increased by mechanical loading (124, 148). Consistent with the observations in study II, repeated bouts, but not a single bout, of passive stretching of the leg increases skeletal muscle MMP-2 (125, 149). Taken together, these findings suggest that mechanical load or stretch is the stimulus responsible for upregulation of skeletal muscle MMP-2 expression. Eccentric exercise induces a greater mechanical load compared with concentric work (150), and the report by Heinemeier et al showed a trend to a larger increase in MMP-2 following eccentric exercise. Even though the MMP-2 gene is expressed constitutively in many tissues, a few transcription factors, such as SP1, have been reported to increase its gene expression (151). Interestingly, mRNA stability, DNA binding, and nuclear translocation of SP1 increase after repeated bouts but not after a single bout of electrical stimulation of C2C12 myotubes (152, 153). This is consistent with the increase in MMP-2 expression after exercise training but not after a single bout of exercise in studies I and II, and after repeated passive stretch reported by Hoier et al (149).

Based on the MMP-9 gene promoter sequence and as observed in *in vitro* studies, there is a range of plausible mechanisms to explain the prompt increase in MMP-9 gene expression observed in studies I and II. In general, MMP-9 expression and activity are associated with inflammation and cytokine signaling, often through MAPK pathways such as p38 and ERK (92, 154-156). Several of the MAPK-pathways are activated by exercise in human skeletal muscle (157-159), and several stimuli such as metabolic perturbation and mechanical stretch can activate these pathways in animal and in vitro models (85, 160, 161).

TNF-α is one inflammatory cytokine shown to induce both gene expression and activity of MMP-9 in cultured myoblasts and in skeletal muscle of mice infused with TNF-α (83, 85, 106). Even though TNF-α is upregulated markedly in traumatically injured skeletal muscle, there is little evidence for an increase in TNF-α in the skeletal muscle by exercise (162-166). It has also been suggested that an inflammatory response to the biopsy procedure might induce MMP-9 in the skeletal muscle. To determine whether this could explain the findings in studies I and II, MMP-9 mRNA was measured in two muscle biopsies taken from the same leg at 2-hour intervals in the absence of exercise in eight subjects. The MMP-9 mRNA level did not change with time ($P = 0.3$, data not shown).

Importantly, oxygen tension and other factors related to exercise with restricted blood flow cannot be excluded as the underlying reasons for MMP-9 because the mean increase in MMP-9 was much higher in the leg exercised with restricted blood flow than in the leg exercised without blood flow restriction. The nonsignificant difference between the two exercise conditions could be related to lack of statistical power. Earlier reports have shown that MMP-9 is induced in ischemic muscles and that the promoter region of MMP-9 is responsive to many cellular signals and growth factors associated with metabolic stress (140, 162, 167). This is the case both for acute experimental limb ischemia and for steady-state levels in muscles of patients with peripheral artery disease (141). However, there is a large difference in the magnitude of perturbation in ischemic models versus physical exercise. Although oxygen tension in the muscle decreases during exercise and even more so in exercise with restricted blood

flow, the oxygen tension during femoral artery ligation or severe peripheral artery disease reaches tissue-damaging levels, which is evident by invasion of inflammatory cells and the appearance of necrotic muscle fibers. Therefore, the increased levels of MMP-9 under ischemic conditions do not necessarily reflect the reduced oxygen tension per se but might be the consequence of tissue damage. In study I, MMP-9 activity did not increase significantly before the appearance of a significant increase in mRNA levels, indicating activation of preformed MMP-9.

Nitric oxide has received attention as a potential MMP-activating factor in the skeletal muscle (83, 168, 169). Both MMP-2 and MMP-9 can become activated through allosteric interaction with nitric oxide. Nitric oxide is a free radical and, as such, it has biologically relevant effects beyond the activation of guanylyl cyclase. It is well established that skeletal muscle produces nitric oxide at low rates at rest and at higher rates during muscle contraction (170, 171). Both endothelial cells and muscle fibers can release nitric oxide; the main stimuli for nitric oxide release are shear stress in endothelial cells and mechanical stretch in muscle fibers. As argued above, shear stress tends to be lower during restricted blood flow compared with unrestricted blood flow. Previous studies using an experimental model similar to that used in this work have shown that the plasma concentration of nitrate, the main metabolite of nitric oxide in humans (172), increases during exercise with restricted blood flow but not during exercise without restricted blood flow (118). If nitric oxide was a critical factor for the observed exercise-induced activation of MMP, the activity patterns of MMP-2 and MMP-9 should also be similar and, presumably, higher following exercise with restricted blood flow.

TIMP-1 upregulation occurs primarily at the transcriptional level and involves AP-1, SP1, NF-κB, and CRE transcription factors, providing regulation that is similar to that of MMP-9. Several studies have reported skeletal muscle upregulation of TIMP-1 expression without expression of MMP-9 (58, 124, 173). This indicates that TIMP-1 and MMP-9 are not regulated by identical stimuli even though they are often induced in parallel. The overall effect of an increase in the activating factor MMP-14 and the inhibiting factor TIMP-1 as observed in study II remains to be elucidated.

Finally, the results of a rat skeletal muscle injury model suggested that both MMP-9 and MMP-2 can become activated through interaction with proteases released from circulating leukocytes (142). Even though the invasion of leukocytes into the skeletal muscle after exercise is thought to be a minor event, one might speculate that proteases released from circulating cells contribute to the activation of skeletal muscle MMP-9 after exercise.

4.2 CIRCULATING MMP

In study III, the femoral arteriovenous differences for MMP-9, collagen IV, NGAL, and elastase were calculated in the leg before, during, and after 60 minutes of cycling exercise.

In study III, the circulating levels of MMP-9 increased above the preexercise levels after 30 minutes of cycling exercise, remained elevated during the exercise bout, and decreased to preexercise levels 2 hours after the end of the exercise bout. This increase was not accompanied by any release or uptake of MMP-9 over the exercising leg.

Earlier studies demonstrated an increase in MMP-9 concentration in the circulation after muscle-damaging strenuous exercise. Studies using eccentric exercise and an exhaustive incremental step test reported increased serum MMP-9 concentration immediately following the end of exercise (128, 174). The data from study III do not support the assumption that circulating MMP-9 is derived from skeletal muscle or tendons because no MMP-9 appeared to have been released from the exercising leg. These findings support the idea that the circulating MMP-9 noted during and after an exercise bout comes from a source other than the skeletal muscle or tendon. One possible link between the exercising muscle and circulating MMP-9 is skeletal muscle-derived cytokines.

Based on the rapid kinetics of the elevation in MMP-9 level after 30 minutes of exercise, one might presume that the increase in circulating MMP-9 level resulted from the release of preformed MMP-9 from secretory granules rather than from a transcriptional mechanism. Neutrophils carry preformed MMP-9 in secretory vesicles, which can be released quickly under inflammatory conditions such as activation by lipopolysaccharide and cytokines or by adhesion to integrins (175). Neutrophil degranulation causes the release of several factors other than MMP-9, such as neutrophil gelatinase-associated lipocalin and elastase. In contrast to MMP-9, elastase is released exclusively from neutrophils and can therefore be used as a marker of neutrophil degranulation (89, 90). Study III showed an uptake of NGAL over the exercising leg but neither circulating NGAL nor elastase levels changed with exercise. The concordance with regard to stable NGAL and elastase levels in study III contradict the idea that neutrophils are a likely source of the observed increase in MMP-9 level during and after exercise.

4.2.1 Possible mechanism underlying the release and the cellular source of circulating MMP

Contracting skeletal muscle expresses a number of cytokines and releases them into the circulation; these cytokines may have both paracrine and endocrine effects. Il-6 has attracted much attention because many studies have reported exercise-induced increases in circulating Il-6 levels and within the exercising muscle. Circulating Il-6 may increase up to 100-fold following exercise and as much as 8000-fold after ultraendurance-type exercise (165, 176). Study IV tested whether Il-6 causes an increase in circulating MMP-9 level by infusing recombinant Il-6 in healthy subjects until the Il-6 level in the circulation was similar to that reported with exercise. After 3 hours of Il-6 infusion, serum MMP-9 level was almost 5-fold higher than the

preinfusion level; in control subjects, MMP-9 levels were unchanged. After cessation of the Il-6 infusion, MMP-9 level decreased and reached the preinfusion level 5 hours after the end of the infusion. The peak serum MMP-9 level during the Il-6 infusion was similar to the levels observed in study III and in previous reports on the response of circulating MMP-9 during exercise (128, 129, 174, 177, 178).

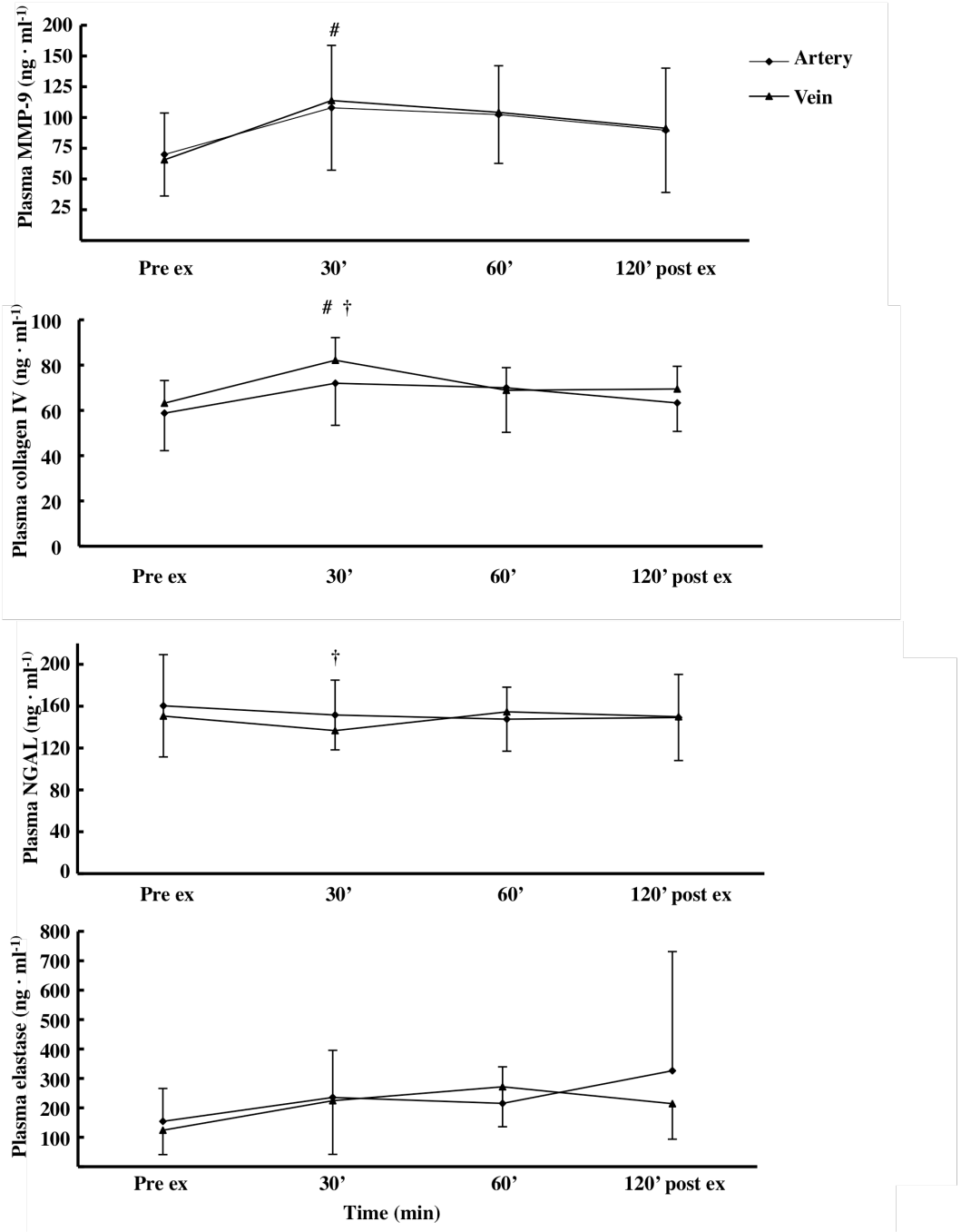

Figure 7: Femoral arterial and venous plasma MMP-9, Collagen IV, NGAL and Elastase concentrations pre exercise, during exercise and 120 min post exercise. # denote a significant difference compared to arterial and venous pre exercise concentrations, respectively ($P < 0.05$). † denote significant difference between arterial and venous concentration at the same time point ($P < 0.05$). Values are presented as means and SEM (n=10)

Previous reports on Il-6 infusion in healthy subjects have reported increased circulating neutrophil numbers (84, 89, 91, 92, 175, 179). However, in study IV, NGAL and elastase levels remained unchanged during and after Il-6 infusion,

indicating that there was no degranulation of neutrophils. Maybe more importantly, these data open for Il-6 as a possible agent behind the increase in circulating MMP-9 seen during exercise in study III. An additional observation was an uptake of NGAL over the exercising leg in study III. This uptake was however, based on the stable levels of NGAL overall, quantitatively small. Interestingly, in study I, NGAL–MMP-9 complexes were detectable in the zymograms from the skeletal muscle in six subjects, and there was a slight, although nonsignificant, increase in skeletal muscle NGAL level in four of these six subjects. Based in these findings, one might speculate that, during exercise, there is a slight uptake of unbound NGAL from the circulation to the tissue where it binds to active MMP-9.

Another possible source of circulating MMP-9 are monocytes: Monocytes express the two Il-6 receptors: Il-6R and GP-130. Stimulation by Il-6 has several effects on monocytes in vitro (180-183). In the human monocyte cell-line THP-1, we detected an increase in MMP-9 mRNA after Il-6 treatment, suggesting that circulating monocytes are a possible source of MMP-9.

Many other cell types located in the peripheral tissue can express and release MMP-9 if stimulated appropriately (83, 106, 184). To test whether cells present in skeletal muscle that are known to express MMP-9 were involved in the observed increase in MMP-9 level following Il-6 stimulation, we stimulated cells with Il-6 for one and three hours. This experimental design also allowed us to study whether Il-6 is involved in the increased
MMP-9 level observed within the skeletal muscle tissue during and after exercise. The panel of cells selected included the major cellular components of the skeletal muscle: myoblasts, myotubes, and endothelial cells (HUVECs). In addition, THP-1 cells differentiated to a macrophage phenotype were included because of their known expression of MMP-9 and suggested role in skeletal muscle remodelling (185-187).

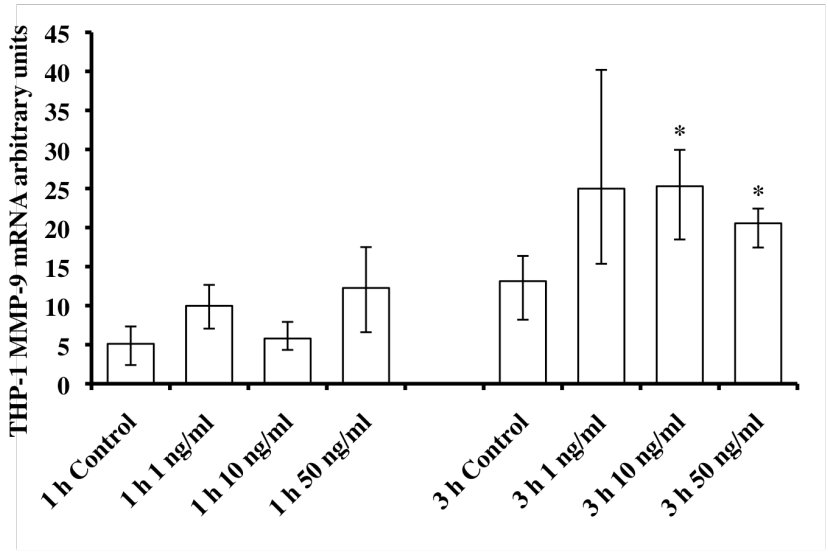

Figure 9: MMP-9 mRNA levels in the human monocyte cell line THP-1 after stimulation with Il-6 in 3 different doses for 1 and 3 hours respectively. * denote significant difference between stimulated cells and control ($P < 0.05$). Values are presented as means and range (n=3).

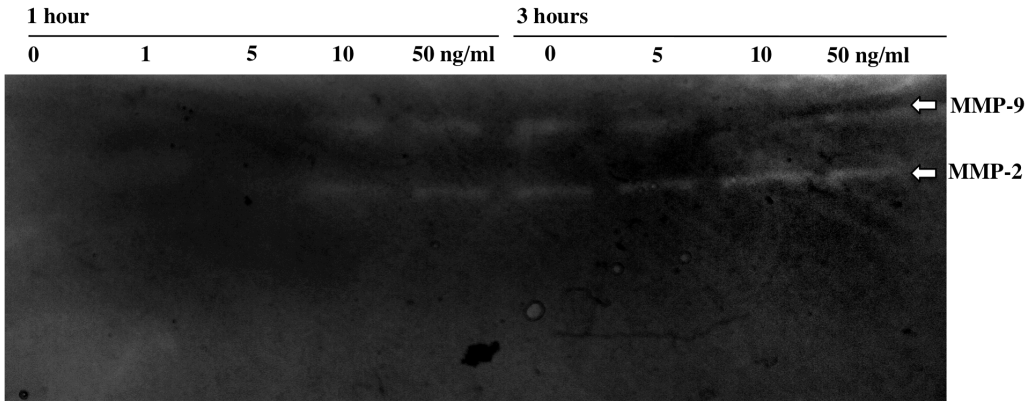

Figure 10: Zymogram of THP-1 cells treated with Il-6 for 1 and 3 hour respectively. Enzyme activity is evident by clear band against dark background. Zymogram shows an increase in the band corresponding to pro-MMP-9 after treatment with Il-6 for 1 and possibly 3 hours as compared with control.

Cells were stimulated with Il-6 in vitro for 1 or 3 hours. Myoblasts showed a low but detectable level of MMP-9 mRNA, but we detected no change after stimulation with Il-6. In differentiated myotubes, MMP-9 level was barely detectable in RT-PCR, and this was unaltered by Il-6. The difference in the basal expression of MMP-9 in myoblasts versus myotubes agrees with previous data showing that the detectable level of MMP-9 in myoblasts decreases following differentiation (101, 106). In the studies of this thesis, a low or undetectable level of MMP-9 was noted in resting skeletal muscle, but MMP-9 level increased quickly following an exercise bout (studies I and II). Hence, the present data do not support the idea that Il-6 stimulation is responsible for the exercise-induced gene expression of MMP-9 in skeletal muscle.

In HUVECs, we detected MMP-9 in unstimulated cells, but as in myoblasts, 1 or 3 hours of Il-6 treatment had no effect on MMP-9 level. This finding contrasts with the findings in human cerebral endothelial cells, in which Il-6 increases the expression and release of MMP-9 (184). In that study, MMP-9 level was measured after 24 hours but not after short-term stimulation, which might explain the differences between our results. Taken together, the data from that study and the present data do not support the idea that endothelial cells are the source of MMP-9 after short-term stimulation with Il-6.

Macrophages have a high basal expression of MMP-9, but the expression was unaffected by stimulation with Il-6. Thus, the responses of monocytes and macrophages differ. Interestingly, studies at the cell level have suggested that MMP-9 plays a pivotal role in the activation of monocytes and their differentiation into macrophages in peripheral tissues (180, 183, 188). MMP-9 degrades components of the ECM and basal lamina; such degradation is necessary for leukocyte invasion and migration (181, 189). Immunohistochemical studies in MDX mouse models and in human studies of myopathies have shown that invading macrophages secrete MMP-9 (139, 190-192).

Considering the lack of response to short-term Il-6 stimulation in the three cell types comprising most of the skeletal muscle cells, we propose that the cellular source of the serum MMP-9 observed during and after exercise is unlikely to be within the skeletal muscle tissue. This is further supported by a previous study that used Affymetrix to analyze gene expression in skeletal muscle biopsies from vastus lateralis obtained after an Il-6-infusion protocol similar to that used in the present study. This study detected no skeletal muscle MMP-9 gene expression after Il-6 infusion

(193). It does further contradict Il-6 as the mediator behind the activation of MMP-9 seen in study I and II.

4.3 POSSIBLE ROLES OF MMP

The best characterized overall biological function of the MMP family is the degradation of ECM proteins; collectively, the MMPs can degrade all extracellular proteins. Type IV collagen is a key component of the basal lamina (194) and comprises a major substrate of MMP-9 (143). In study III, arteriovenous differences in collagen IV were measured over the exercising leg. An arteriovenous difference in collagen IV corresponding to its release from the exercising leg was noted after 30 minutes of exercise. The circulating level of collagen IV increased during exercise, suggesting that the release from the leg was robust, or alternatively that collagen IV is also released from other tissues during exercise.

Collagen XVIII is another component of the basal lamina (195). Endostatin is a C-terminal cleavage product of collagen XVIII and was one of the first naturally occurring antiangiogenic factors described. Endostatin inhibits endothelial cell proliferation and migration in vitro and interferes with VEGF-A signaling, and has been tested as an antiangiogenic agent in several clinical trials (100, 109-111). The enzymatic cleavage of endostatin from collagen XVIII is a process mediated by several proteases, including MMP-9 (110, 113, 196). In study I, the femoral arteriovenous difference and skeletal muscle level of endostatin were analyzed to provide a marker of possible antiangiogenic processes occurring during exercise and as a possible MMP-9-generated factor. Endostatin protein levels in the skeletal muscle did not change significantly at either time after exercise. Both arterial and venous levels of endostatin increased above the preexercise values at 17 and 57 minutes of exercise. There was also a significant turnover of endostatin over the exercising leg, with an uptake of endostatin at 17 minutes of exercise but a release of endostatin at 57 minutes of exercise. Arterial and venous endostatin levels returned to preexercise levels by 120 minutes after the end of exercise.

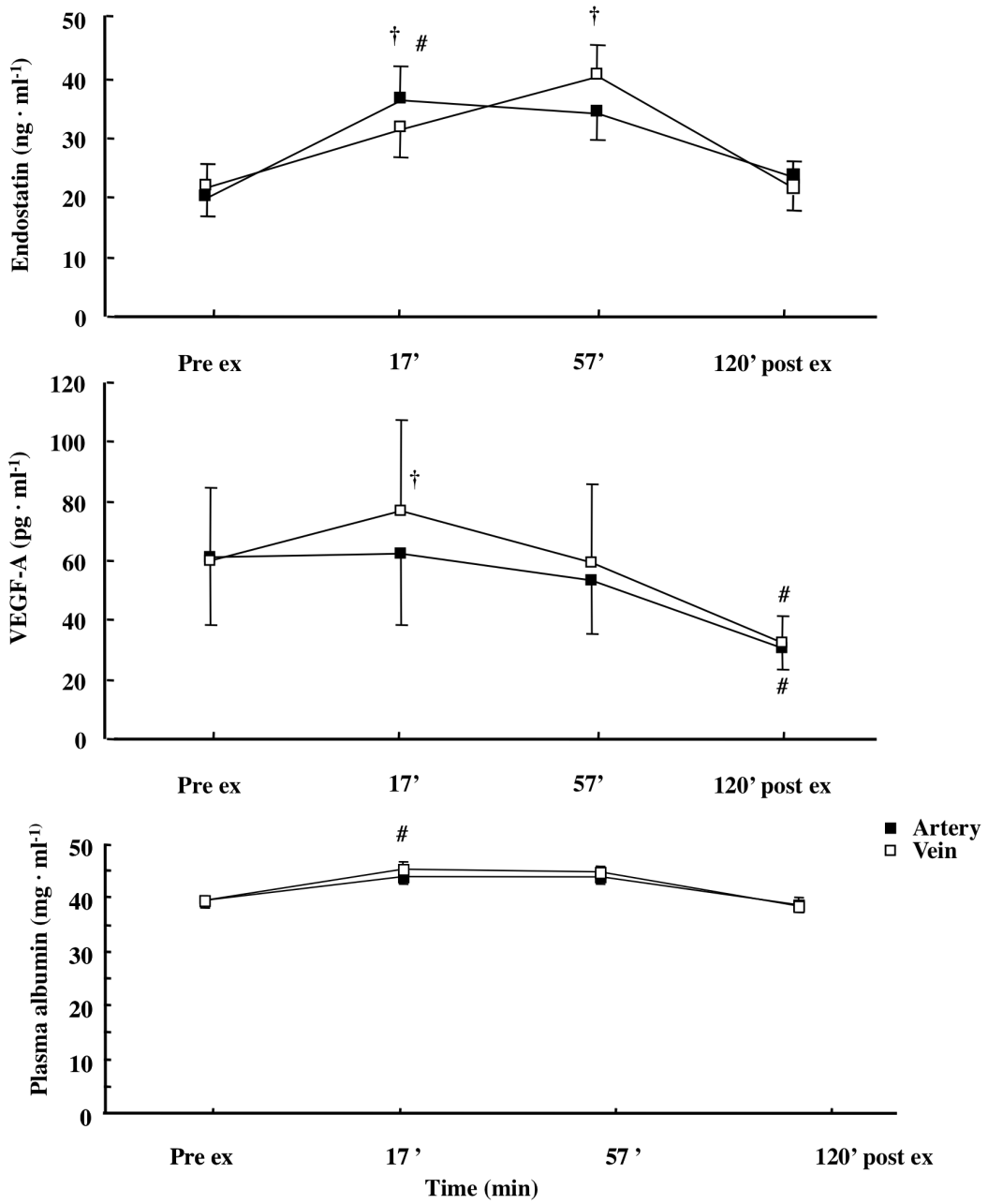

Figure 11: Femoral arterial and venous plasma endostatin, VEGF-A and albumin concentrations pre exercise, during exercise and 120 min post exercise. # denote significant differences compared to arterial and venous pre exercise concentrations, respectively ($P < 0.05$). † denotes significant differences between arterial and venous concentration at the same time point ($P < 0.05$). Values are presented as means and SEM (n=10)

The increase in circulating endostatin level before any release could be detected over the exercising leg suggests that the endostatin is not derived from the exercising leg. The decline in endostatin 2 hours after exercise is consistent with recent reports from Suhr et al, who also reported a slight increase in endostatin level immediately after exercise and a fast decline after exercise (174). In clinical trials, the plasma half-life of administered endostatin was a few hours (197). Taken together, these data suggest that endostatin is released during but not after exercise; presumably, it is released from a tissue other than the exercising leg and then removed from the circulation quickly after the end of exercise. The alternating uptake and release of endostatin over the exercising leg in study I had no effect on skeletal muscle levels, indicating that this turnover was of minor significance. It is not known if the increase in

circulating endostatin during exercise is due to MMP-activity. Still, one may speculate that release of an anti-angiogenic factor such as endostatin during ECM-remodelling plays a role in hindering activation of endothelial cells in adjacent areas and remote organs in an endocrine fashion.

VEGF-A is a growth factor that has been shown to be regulated posttranslationally by MMP-9 under certain circumstances; by sequestering ECM components, VEGF-A deposited in the ECM is made diffusible and is then released. This phenomenon was first described by Bergers et al in a model of pancreatic cancer and has since been reported in several other pathologies (111, 114, 198). In physiological contexts, such as exercise, the transcriptional regulation of VEGF-A is the most well-established mechanism, but studies have reported an increase in VEGF-A protein before any increase in VEGF-A mRNA (45, 46, 62, 198-200). Study I analyzed skeletal muscle content and exercising leg arteriovenous differences of VEGF-A. Skeletal muscle VEGF-A protein level was not significantly increased immediately after exercise but was significantly higher 120 minutes after exercise compared with preexercise levels. By contrast, VEGF-A mRNA level was significantly elevated immediately after exercise and remained elevated for 120 minutes after exercise. After 17 minutes of exercise, there was a significant arteriovenous difference that corresponded to the release of VEGF-A from the exercising leg. At the end of the exercise bout, there were no detectable arteriovenous differences and no significant change in VEGF-A level compared with the preexercise level. By contrast, 120 minutes after the exercise bout, circulating VEGF-A level was lower than the preexercise levels in both the artery and the vein.

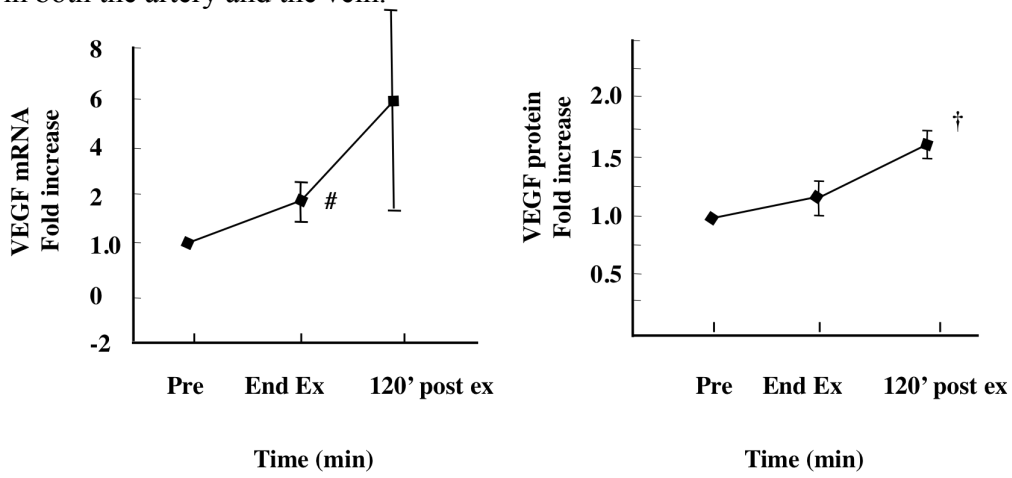

Figure 12: VEGF-A mRNA and VEGF-A protein levels in m. vastus laterialis pre exercise, immediately after and 120 min post-exercise. # and † denote significantly differences between pre exercise and end of exercise and between pre exercise and 120 min post exercise, respectively ($P < 0.05$). Values are presented as means and SEM (n=10)

The fact that collagen IV and VEGF-A were released from the exercising leg in studies III and I, together with the findings of an increase in active MMP-9 in the skeletal muscle immediately after exercise in studies I and II point to a possible connection: MMP-9 in the skeletal muscle during exercise cleaves collagen IV in the basal lamina of the muscle, which leads to a release of collagen IV and VEGF-A from the muscle. However, it is not possible to exclude a role of other collagen IV-degrading proteases, such as cathepsin B or MMP-3, in the collagen IV release during exercise. However, based on the known activation of skeletal muscle MMP-9 by exercise, MMP-9 is a plausible candidate.

5 FINAL REMARKS

The skeletal muscle is an almost ideal model for studying the physiology and molecular biology of adaptive processes in humans: the skeletal muscle tissue is accessible percutaneously in large enough quantities for thorough analysis with minimal risk and discomfort for the donor. The skeletal muscle is a highly plastic organ with relatively well-characterized phenotypical and structural changes in response to stress such as physical exercise, changes that can be related to the molecular findings and phenomenology. Human physiology research plays an important part in understanding the processes and mechanisms that may reflect the pathophysiological changes occurring in cell and animal models. The study of human physiology may help elucidate the role of these mechanisms in the homeostasis of life. Even though research on human models is descriptive and has a lesser ability to isolate a stimulus or to knock down a mechanism of interest, human experimental models are essential for our understanding of both physiology and pathophysiology.

Little is known of the role MMPs play in the adaptive processes within skeletal muscle. Increasing evidence points toward a role for the MMPs in skeletal muscle remodeling. For instance, a recent study in MMP-9-null mice reported decreased overall muscle mass and fiber-type shifts compared with wild-type mice (201). This thesis demonstrate that endurance type of exercise activate members of the MMP family in skeletal muscle and increase MMP-9 levels in the circulation. The thesis also provides insight of an endocrine effect of the myokine Il-6. The data also provide insight into one possible activating mechanism of the MMP family with physical exercise. It further suggest proteolytical processing of skeletal muscle ECM to occur during an exercise bout and that this processing possibly release preformed VEGF-A to the circulation. All together the current findings support involvement of MMPs in the adaptation to endurance exercise in humans.

Further knowledge about the effects of exercise on the substrate panel of MMPs and characterization of the proteolytically processed fragments of both structural proteins and possibly growth factors in the skeletal muscle and in the circulation are important for elucidating the role MMPs play in muscle remodeling. Thorough studies using a morphological approach or in vitro experimentation in isolated mononuclear cells are needed to understand the importance of cell migration to skeletal muscle remodeling and to determine whether the MMPs are essential.

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