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THE IGF-IGFBP SYSTEM IN AEROBIC EXERCISE-WITH FOCUS ON SKELETAL MUSCLE

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Stockholm 2007

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Published by Karolinska Institutet. Printed by [name of printer]

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ISBN 978-91-7357-379-5

To Emil and Emmy, the true researchers

ABSTRACT

Activity induced skeletal muscle adaptation has been suggested to be mediated largely by intrinsic factors, such as insulin-like growth factor (IGF)-I. IGF-I stimulates muscle glucose and amino acid uptake and promotes anabolism. IGF-I is bound to a family of high affinity IGF binding proteins (IGFBP 1-6) which regulate access of systemic IGF-I to tissues and determines local IGF-I bioavailability. These functions are modulated by posttranslational modifications of IGFBPs such as proteolysis and phosphorylation. Levels of unbound bioavailable IGF-I have not previously been explored in muscle or other tissues.

This thesis focuses on IGF-I protein levels in the human skeletal muscle interstitial fluid. In models of endurance exercise in both sexes and *in vitro*, we explored factors expected to regulate local muscle IGF-I activity including circulating IGF-I and IGFBPs under the influence of interleukin-6 (IL-6) and sex hormones. This was possible by the development of a microdialysis approach. Unbound IGF-I in human skeletal muscle interstitial fluid was detected in microdialysate (md-IGF-I) collected from an intramuscular probe. Basal md-IGF-I at rest was in the same concentration range as free (unbound) IGF-I in serum and correlated with total (bound plus unbound) IGF-I. Endurance exercise with one leg (45 or 60 min), decreased md-IGF-I in the resting leg concomitantly with increased circulating IGFBP-1. This is the first evidence to support that increasing circulating IGFBP-1 decreases local muscle IGF-I bioavailability. Exercising leg md-IGF-I did not decrease and free IGF-I was released to the regional circulation (v-a difference) but with lack of correlation to systemic changes. We conclude that the regulation of unbound IGF-I in the exercising muscle is less affected by systemic factors than the resting muscle. Proteases partially cleave IGFBPs into distinct fragments and increase IGF bioavailability. This process may contribute to increased local IGF-I in exercising muscle. IGFBP-3 was cleaved during extended ultra endurance exercise. Since this may reflect local IGFBP-3 proteolysis we examined a Ca^{2+} - activated muscle protease m-calpain. *In vitro*, m-Calpain cleaved IGFBP-2 and -3 into fragments that we identified by N-terminal amino acid sequencing. Gonadal function was suppressed by ultra endurance exercise but with no major sex differences and no correlation to changes in IGF-IGFBP. For the first time, we demonstrated a net release of IL-6 from exercising muscle in women. The role of IL-6 was specifically addressed by a 3 h IL-6 infusion that increased IGFBP-1 concentrations with no effect on circulating free IGF-I or IGFBP-3.

The results from these studies shed new light on the regulation of skeletal muscle tissue IGF-I bioavailability which may be of importance for exercise adaptation and resting metabolism and anabolism.

ISBN 978-91-7357-379-5

LIST OF PUBLICATIONS

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

I. **U. Berg**, J.K. Enqvist, C.M. Mattson, C. Carlsson-Skwirut, C.J. Sundberg, B. Ekblom, P. Bang. Lack of sex differences in the IGF-IGFBP response to ultra endurance exercise. *Submitted manuscript.*

II. **U. Berg**, T. Gustafsson, C.J. Sundberg, C. Carlsson-Skwirut, K. Hall, P. Jakeman, P. Bang. Local changes in the Insulin-like Growth Factor system in human skeletal muscle assessed by microdialysis and arterio-venous differences technique. *GH and IGF Research 2006; 16; 217-223.*

III. **U.Berg**, T.Gustafsson, C.J. Sundberg, L.Kaijser, C. Carlsson-Skwirut, P. Bang.
Interstitial IGF-I in exercising skeletal muscle in women. *European Journal of Endocrinology 2007; 157; 427-435.*.

IV. S. Pihl, C. Carlsson-Skwirut, **U. Berg**, K. Ekström, P. Bang. Acute IL-6 infusion increases IGFBP-1 but has no short-term effect on IGFBP-3 proteolysis in healthy men. *Hormone Research 2006; 65; 177-184.*

V. **U. Berg**, C. Carlsson-Skwirut, P. Bang. Calpain proteolysis of IGFBP-2 and IGFBP-3, but not of IGFBP-1. *Biological Chemistry 2007; 388; 859-863.*

CONTENTS

LIST OF ABBREVIATIONS

(v-a) difference	venous concentrations-arterial concentrations
ADAM 12	A Disintegrin And Metalloprotease
ALS	Acid Labile Subunit
AMP-K	AMP activated protein Kinase
DELFIA	Delayed Enhanced Lanthanide Fluorescent ImmunoAssay
ECLIA	Electrochemiluminiscence Immunoassay
ECM	ExtraCellular Matrix
ELISA	Enzyme-Linked Immunosorbent Assay
Ex-leg	Exercising leg
f-IGF-I	Free IGF-I determined by DSL ELISA in this thesis
FSH	Follicle Stimulating Hormone
GH	Growth Hormone
GLUT	Glucose transporter
IGF	Insulin-Like Growth Factor
IGF1R	IGF type 1 receptor
IGFBP	Insulin-Like Growth Factor Binding Protein
IGFBP-3 PA	IGFBP-3 Protease Activity
IL-6	Interleukin-6
IR	Insulin Receptor
K_D	Dissociation constant
kDa	kiloDalton
LH	Luteinizing Hormone
LID mouse	Liverspecific IGF-I Knock Out mouse
md-IGF-I	IGF-I concentrations in microdialysate (corrected for calculated IGF-I recovery)
md-IGF-$I_{absolute}$	IGF-I concentrations in the microdialysate ("raw data")
MGF	Mechano Growth Factor
MMP	Matrix MetalloProtease
N + LP IGFBP-1	Non and Lesser Phosphorylated IGFBP-1
NLS	Nuclear Localization Sequence
PAPP-A	Pregnancy-Associated Plasma Protein A
PPAR-γ	Peroxisome Proliferator-Activated Receptor γ
Rest-leg	Resting leg
RIA	Radioimmunoassay
rpm	revolutions per minute
RR	Reverse Recovery
RXR	Retinoid X Receptor
SD	Standard Deviation
SDS	Standard Deviation Score
t-IGF-I	Total IGF-I determined by RIA or DELFIA in this thesis
VEGF	Vascular Endothelial Growth Factor
VO_2	Oxygen uptake
WIB	Western Immunoblotting
WLB	Western Ligand blotting

1 INTRODUCTION

The dynamic and metabolically active skeletal muscle tissue rapidly adapts to functional demands, contributing to several of the beneficial effects of exercise. Repeated exercise bouts (physical training) protects against type 2 diabetes (121), cardiovascular disease (199) and all cause mortality (27). Each single exercise bout (acute exercise) involves activation of processes that are dependent on factors such as exercise type, the intensity and duration of exercise, the amount of muscle mass involved, the nutritional status and (less investigated) the sex of the subject. Aerobic endurance exercise is often defined as low-moderate intensity exercise performed during a prolonged time period (in this thesis > 45 minutes). Activity-induced skeletal muscle adaptation is considered to be mediated largely by intrinsic mechanisms. Local growth factors have been claimed to play an important role with Insulin-like growth factor-I (IGF-I) being one of the major players. However, studies of the actual changes in local concentrations of IGF-I in the muscle during acute exercise are few and before this thesis work, unbound bioavailable IGF-I levels had not been explored. In this thesis, we explore such changes in the skeletal muscle interstitial fluid during and after single bouts of aerobic endurance exercise. An optimized microdialysis methodology is applied. In the circulation and in an *in vitro* model, factors including IGF-binding proteins (IGFBPs) and IGFBP proteases expected to regulate the biovailability of IGF-I are explored.

1.1 SKELETAL MUSCLE AND THE ADAPTATION TO EXERCISE

The studies in the present thesis focus on acute aerobic endurance exercise involving a small or a large muscle mass. The effects of repeated bouts of aerobic endurance exercise (endurance training) have previously been more extensively investigated than the effects of single exercise bouts. Aerobic endurance training results in an increased sensitivity to insulin, increased mitochondrial biogenesis and vascularisation, increased lipid utilization at rest and during exercise, skeletal muscle tissue remodelling (e.g. fibre type transformation), satellite cell activation and adaptations in the intramuscular connective tissue (reviewed in (126, 128)). Possibly, many of these adaptational processes are initiated already in connection with the first bout of exercise, but the time course remains to be explored.

Acute aerobic exercise has been demonstrated to result in an insulin independent increase in skeletal muscle glucose uptake and is followed by an increase in insulin

sensitivity which persists 16 h after exercise or more (reviewed in (28, 113)). This is observed in healthy subjects, as well as subjects with type 2 diabetes (28). The exercise induced increase in skeletal muscle energy demand, results in elevated energy consumption and the mobilization of total body lipid stores. Furthermore, factors known to stimulate vascularisation are activated by an acute bout of aerobic exercise (101). As already mentioned, activity-induced skeletal muscle adaptation is considered to be mediated largely by intrinsic mechanisms, such as local growth factors.

1.2 INSULIN-LIKE GROWTH FACTORS

Insulin-like growth factors (IGF-I and-II) are polypeptides (7.5 kDa), structurally similar to insulin. They exert anabolic, differentiating, anti-apoptotic and metabolic effects and are expressed in a large range of tissues (55). The major source of circulating IGF-I is the liver, as confirmed by studies of liver-specific IGF-I knockout mice which have 20 % of wild type circulating IGF-I concentrations (229). Growth Hormone (GH) stimulates IGF-I production in the liver. In the skeletal muscle, GH also stimulates IGF-I production but other regulators are important as well (63, 202). In a negative feedback loop, IGF-I inhibits pituitary GH production and release. Gonadal hormones increase IGF-I production: estradiol via increased GH secretion, and testosterone via aromatization to estradiol and/or relaxation of IGF-I negative feedback on pituitary GH production (223). Nutrition is essential for maintenance of circulating IGF-I concentrations which decrease in prolonged fasting in spite of elevated GH concentrations (212). Unlike insulin, IGF-I and –II are bound to a family of high-affinity binding proteins (IGFBP-1 to -6) present in various proportions in all body fluids, including serum. IGFBPs may be soluble, cell surface associated and/or ECM localized. IGFBP-2 and -3 have also been demonstrated to be present in the nucleus of various cell types (110, 138, 147). Post-translational modification of the IGFBPs including proteolysis or phosphorylation, affect IGF affinity, tissue distribution and bioactivity. In addition, both intact and proteolytic fragments of IGFBPs exert IGF-independent actions on cell proliferation, apoptosis and glucose uptake (75, 110, 188). The effects of IGF-I and-II are mediated through the IGF type 1 receptor (IGF1R), a tyrosine kinase receptor structurally similar to the insulin receptor (IR). The human IR exists in two isoforms (IR-A and IR-B), generated by alternative splicing of the IR gene. IGF-I and- II also bind to the IR and insulin binds to the IGF1R but with 100-fold lower affinity than the specific ligand. The IR and IGF1R are composed of two α and β subunits. The ligand binds to the α-subunit, whereas the β-subunit contains an intrinsic

tyrosine kinase activity. Upon ligand binding, the receptor autophosphorylates its opposing β-subunit and transphosphorylates intracellular substrates such as insulin receptor substrate (IRS) -1 to -4. In tissues with high expression of both IR and IGF1R, such as skeletal muscle, hybrid receptors are formed. Hybrid receptors (one half from the IGF1R and one half from the IR) can bind both IGF-I,-II and insulin. The binding affinity of IGF-I to the IGF-I-IR-B hybrid receptor is substantially higher than that of insulin and therefore these receptors are preferentially stimulated by IGF-I (144). Hybrid receptors has been reported to represent 44 (70) or 75 (8) % of the IGF-I receptors in skeletal muscle. In liver and adipose tissue IGF1R are sparse, whereas IR are abundant. IGF-II also binds to the IGF type 2 receptor (IGF2R), identical to the mannose-6-Phosphate Receptor mainly associated with internalization and clearance of IGF-II.

Alternative splicing of the IGF-I gene results in different transcripts encoding different IGF-I precursor proteins. They all give rise to mature IGF-I but the E-domain peptide derived from the cleavage of the precursors may also give rise to biologically active peptides. A specific splice variant of IGF-I has been given the name mechano growth factor (MGF) and will be further described below (reviewed in (96)).

1.3 EFFECTS OF IGF-I IN SKELETAL MUSCLE

IGF-I stimulates glucose uptake in human skeletal muscle preparations *ex vivo* (Dohm et al 1990) and human muscle cell cultures *in vitro* (44) and has hypoglycemic effects in humans *in vivo* (99). Transgenic mice with functional inactivation of both IGF1R and IR in skeletal muscle show a marked reduction in IGF-I mediated glucose uptake into skeletal muscle and develop type 2 diabetes at an early age (72). This supports the importance of skeletal muscle and IGF-I in whole body glucose homeostasis. Interestingly mice lacking "only" IR in skeletal muscle develop compensatory mechanisms to clear glucose (32). Such mechanisms could be signalling through remaining functional IGF1R. The effect of IGF1R inactivation on the sustained increase in glucose uptake after exercise has not been studied.

Unlike other growth factors, IGF-I stimulates satellite cell proliferation, differentiation to myoblasts and fusion with existing myofibers (106). Local intra-arterial IGF-I infusion increases protein synthesis and inhibits protein breakdown in human skeletal muscle (reviewed in (189)). Mice overexpressing IGF-I in muscle develop muscle hypertrophy (198) and so do rats in which exogenous IGF-I has been infused specifically into the skeletal muscle (4). Mice with functional inactivation of the IGF1R

in skeletal muscle exhibit impaired postnatal growth and muscle hypoplasia and reduced protein content during the early postnatal stages (71). In contrast to wild-type mice, endurance exercise training failed to increase muscle fibre diameter and satellite cell proliferation in these mice.

1.4 ENDOCRINE VERSUS LOCAL (AUTOCRINE/PARACRINE) IGF-I

IGF-I is not only a circulating "endocrine" hormone primarily derived from the liver under the stimulation of GH (54). It is also a local factor regulated in an autocrine/paracrine manner. The relative role of circulating vs local IGF-I for anabolic, developmental and metabolic processes has been explored in human and animal models. Global IGF-I KO mice show pre- and postnatal growth retardation and the postnatal survival rate is generally low. In the case they survive, they are infertile and fail to undergo a peripubertal growth spurt (reviewed in (231)). One investigated human subject with IGF-I gene deletion has been reported to show severe pre- and postnatal growth retardation as well as insulin resistance and mental retardation. Mice with a conditional liver specific IGF-I KO (LID) (and 20 % of circulating total IGF-I concentrations as compared to normal mice) develop skeletal muscle insulin resistance and diabetes. However, tissue anabolism (bodyweight and length) is less affected and sexual maturation and fertility are normal (reviewed in (228)). This suggests that endocrine IGF-I plays a role in skeletal muscle glucose metabolism while locally produced IGF-I is important for anabolism and development. The insulin resistance in these mice is suggested to be mediated by the elevation in GH levels associated with decreased circulating IGF-I concentrations (and a reduction of negative feedback on GH production). Elevated GH levels have been demonstrated to result in impaired insulin signalling and insulin sensitivity in both liver and skeletal muscle (105, 152). Patients with poorly controlled type 1 and 2 diabetes display low circulating IGF-I levels. Restoration of normal IGF-I levels by IGF-I treatment has been demonstrated to increase insulin sensitivity and decrease insulin needs (1, 46, 194). The beneficial effect of IGF-I administration on insulin resistance in LID mice and patients with low circulating IGF-I may partly be mediated by the lowering of circulating GH concentrations. However, studies in acromegalic patients demonstrate that IGF-I has effects on insulin sensitivity not simply mediated by suppressing the effect of GH (169).

In the present thesis, the relative changes in circulating vs local skeletal muscle concentrations of IGF-I during exercise are explored. An increase in local interstitial

IGF-I concentrations may be caused by an increased IGF-I mRNA transcription and protein synthesis. However, more acute changes are unlikely to be a transcriptional event but is rather caused by a mobilization of tissue bound IGF-I stores or IGF-I from the circulation. Studies of interstitial IGF-I concentrations may add to our understanding of the endocrine and paracrine/autocrine regulation and role of IGF-I activity.

1.5 FACTORS REGULATING IGF-I BIOAVAILABILITY AND ACTION

Determinations of IGF-I in serum aims to determine total concentrations, which are the sum of IGF-I in IGFBP bound and unbound form. In contrast, free IGF-I or more correctly expressed free dissociable IGF-I is the concentration of unbound IGF-I. The average total IGF-I concentration in the circulation in adults is ~ 200 µg/L (25 nM) Total IGF-I alone does not provide information on the bioactivity of IGF-I. The availability of the IGF ligands to the receptors is determined by the concentration and distribution of the IGFBPs. Furthermore, posttranslational modifications of IGF-I and IGFBPs modulate IGF-IGFBP binding. Attempts to assess the bioactivity of IGF-I in target tissues, have involved various methodological approaches to determine the "free dissociable", or "free" IGF-I concentrations in serum or plasma. In this thesis, we use the term "free" IGF-I concentrations. As discussed in the methods section and (12), the results vary largely among different methods. Less than 1 % of total IGF-I in the circulation has been reported to be free IGF-I. This figure largely exceeds the expected free IGF-I concentrations estimated from the reported equilibrium constants of IGF-I binding to IGFBPs *in vitro*. Importantly, methods for the determination of free dissociable IGF-I in peripheral tissues have been lacking. In the present thesis, the microdialysis methodology was optimized and validated for the determination of unbound IGF-I concentrations in skeletal muscle interstitial fluid. In order to study possible factors in the circulation regulating local IGF-I bioavailability, we also determined circulating concentrations of total and free IGF-I as well as IGFBPs.

1.5.1 IGFBPs

The availability of IGFs to the receptors is regulated by the IGFBPs, with high-affinity binding that equals or exceeds IGF binding to the IGF1R. Structurally the IGFBPs consist of three distinct regions of approximately equal size; the N- and C-terminal highly conserved domains and the central variable linker domain. The molecular mass of the intact IGFBPs ranges from 22.8 (IGFBP-6) to 43 kDa (glycosylated IGFBP-3).

A generalized diagram of IGFBP structure, illustrating various functional domains and post-translational modification is shown in Figure 1.

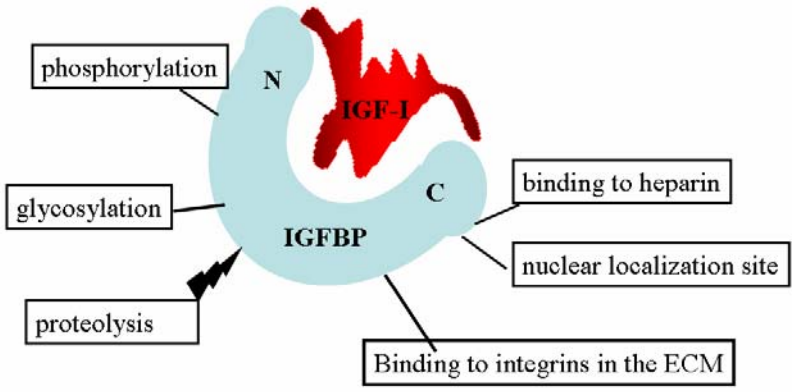


Figure 1. Generalized diagram of IGFBP structure illustrating various functional domains and post-translational modification.

In the circulation, most of the IGF-I circulates bound to IGFBP-3 and an acid labile subunit (ALS) in a large 150 kDa ternary complex. IGF-I in the ternary complex is restricted to the circulation which reduces IGF-I bioavailability and prolongs the half-life of IGF-I. GH stimulates hepatic IGFBP-3 production and results in increased circulating IGFBP-3 levels. IGFBP-3 inhibits IGF induced glucose uptake in rat and mouse skeletal muscle *in vitro* (Ahlsen et al, unpublished data) and inhibits the hypoglycemic effects of IGF-I *in vivo* (78). IGFBP-3 is highly susceptible to proteolysis. Intact as well as fragmented IGFBP-3 has been demonstrated to have IGF independent effects (188). IGFBP-3 has a number of interacting partners (75). It can bind to the extracellular matrix (ECM) and the cell surface. It is thought to be taken up by the cell after association with transferrin or caveolin (138) and it has a nuclear localization sequence (NLS), enabling transport to the nucleus of various cell types (185). IGFBP-3 has also been shown to bind to the nuclear transcription retinoid factor retinoid X-receptor-α (RXR- α) and to modulate RXR-α signalling (188) and PPAR-γ signalling with potential effects on insulin sensitivity.

IGFBP-1 sequesters IGF-I and inhibits IGF induced glucose uptake in human skeletal muscle preparations *ex vivo* (237) and protein synthesis in cultured human muscle cells *in vitro* (85). *In vivo* in rats, supra-pysiological levels of IGFBP-1 inhibit the

hypoglycemic effects of IGF-I (143). IGFBP-1 levels comparable to those observed in critically ill humans decrease protein synthesis in skeletal muscle in rats (135). In resting fasting humans, circulating IGFBP-1 and free IGF-I have been reported to correlate negatively (87-89). The phosphorylation state of IGFBP-1 affects the affinity for IGFs (see below). IGFBP-1 is the only IGFBP in the circulation undergoing a circadian variation due to its regulation by portal insulin, thus affected by nutrition. Insulin inhibits hepatic IGFBP-1 production and is the most important regulator during non-stress conditions. Several other factors have also been demonstrated to affect IGFBP-1 production and/or serum concentrations in different situations. Changes in IGFBP-1 in serum are to a large part determined by hepatic production but changes in IGFBP-1 transport to the tissues may contribute. In a rat heart *ex vivo* model, transcapillary transport of IGFBP-1 has been demonstrated (19) and reported to be enhanced by insulin (18). However, it has not yet been proven that IGF-I and -II are bound to IGFBP-1 when crossing the endothelium (45). IGFBP-1 contains an Arg-Gly-Asp (RGD) sequence that interacts with α5β1 integrin on the cell membranes. Integrins are important for cell interactions with the ECM and activates intracellular signalling cascades (188). The IGF-independent actions of IGFBP-1 in the placenta are mediated by the interaction of the RGD sequence with α5β1 integrin (114).

In general, IGFBP-2 appears to inhibit IGF actions *in vivo* and *in vitro* (75). Interestingly, it binds IGF-II with slightly higher affinity (~ 2 fold) than IGF-I and is the dominant IGFBP in fetal serum and at birth. IGFBP-2 is not subject to postprandial change and is more stable than IGFBP-1 although it increases with fasting (45)). Transcapillary transport of IGFBP-2 has been reported in rat hearts although this transport is not stimulated by insulin (18).

In general, the IGFBPs may be considered inhibitory to IGF actions in myoblasts *in vitro* (75). The roles of the IGFBPs in the postnatal development of human skeletal muscle remain unclear. Expression and production of IGFBP-2, -3, -4 and -5 have been reported in prepubertal and adult human skeletal cell lines (50) and have been shown to support myoblast differention (79). We recently observed a suppression of insulin stimulated glucose uptake in rodent muscle *ex vivo* preparations by IGFBP-3 (Ahlsen et al, unpublished data). This has previously been reported in adipocytes (40).

1.5.2 Post translational modifications of IGFBPs modifying their effects

1.5.2.1 IGFBP proteolysis

Proteolytic cleavage has been demonstrated for all six IGFBPs and is considered the predominant mechanism to increase the release of IGF-I from the IGFBPs. IGFBP proteolysis yields fragments with reduced or no affinity for IGFs. In addition, some IGFBP-fragments may exert IGF- independent effects on cell proliferation, apoptosis and glucose uptake (75, 109, 188). IGFBP proteases may be activated by other proteases and the activity is balanced by endogenous protease inhibitors such as α-1-macroglobulin (153). They may target only one or a few of the IGFBPs, while others are less specific. As shown in Figure 2, they may be present in blood and extracellular fluids (e.g. ADAM-12S and PAPP-A), associated with cell membranes or the ECM (e.g. plasmin) or they may be localized to certain cell organelles (e.g. cathepsin D) or in the cytoplasm (e.g. calpains). Depending on their chemical structure and inhibitor profile, they may be classified as serine proteases (e.g. plasmin), cystein proteases (e.g. calpain), aspartatic acid proteases (e.g. cathepsin D) or metalloproteinases (e.g. MMPs, PAPP-A, ADAM 12-S). Certain proteases (e.g. plasminogen) are activated after association with cell membranes. Following activation, cleavage of IGFBPs bound to the ECM enhance IGF-I delivery to its receptors. A comprehensive review of IGFBP proteases is provided in (153).

Clinical states in which increased proteolysis of IGFBP-2 and/or IGFBP-3 have been observed in the circulation include pregnancy (95, 116), severe illness (155), after surgery (51), and type 2 as well as adolescent type 1 diabetes mellitus (14, 22, 64, 236). These proteases have not yet been identified, but IGFBP-3 proteolytic activity (IGFBP-3 PA) in the circulation at rest have been reported to correlate positively with circulating free IGF-I concentrations (17, 168). Local regulation of IGF activity by proteolysis of IGFBPs has been shown in follicular development and implantation (94, 120). The increase in proteolysis observed in different conditions has been suggested to be due to a decrease in IGFBP protease inhibitors, rather than an increase in levels of proteases (154). IGFBP proteolysis in skeletal muscle tissue *in vivo* has not yet been investigated.

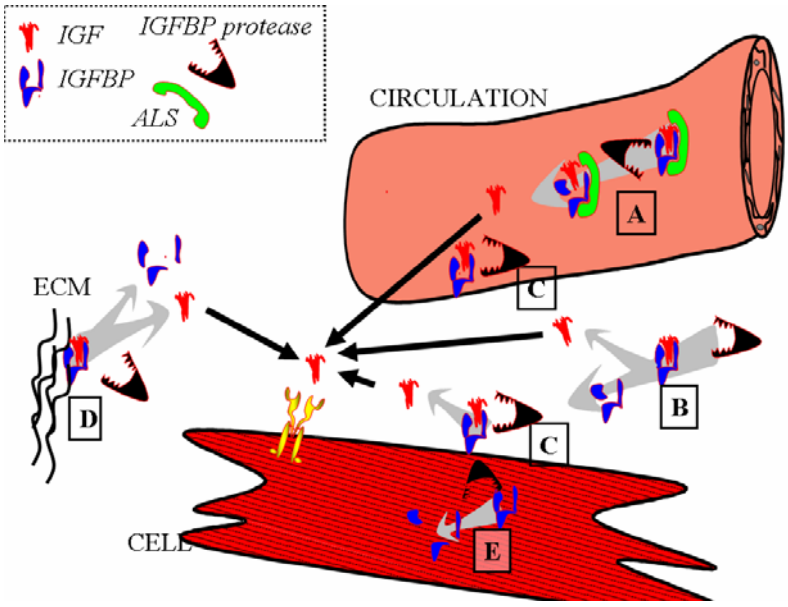


Figure 2. IGFBP proteases may be present in blood and extracellular fluids (A-D) or intracellularly (E). Interstitial IGFBP-3 proteases may be present in the interstitial fluid and/or associated with cell membranes (C) or the ECM (D).

1.5.2.1.1 m-Calpain – a possible skeletal muscle tissue IGFBP protease

The calpains are nonlysosomal, Ca^{2+}- activated cysteine proteases. They are regulatory proteases, i.e. they exert limited proteolysis on their substrates, thereby changing their function (84). They are involved in the cleavage of specific substrates essential for multiple cellular functions, including signal transduction, cell migration, proliferation, and apoptosis. They play a role in the cleavage of cytoskeletal/membrane attachments and they may be involved in the complex and dynamic pattern of integrin signaling in various cell systems (97). In the skeletal muscle, calpains have been suggested to play a role in the remodelling process after excessive exercise (21). At present, 14 human genes of the calpain family have been identified and demonstrated to have tissue-specific or ubiquitous expression related to proliferative and metabolic diseases, such as muscular dystrophies, type 2 diabetes and insulin resistance (97, 218). Two calpains, μ- calpain and m-calpain (also called -1 and -2) are well-characterized ubiquitously expressed proteins activated by micro- and millimolar concentrations of Ca^{2+}, respectively. Recently, μ- calpain was shown to proteolyze IGFBP-4 and -5 in the midregion of the peptides (93).

Skeletal muscle contains not only μ- calpain and m-calpain, but also a muscle specific calpain known as p94 or calpain-3 (203). Activation of calpain activity may occur in contracting muscle associated with increased intracellular Ca^{2+} concentrations. Furthermore, resting intracellular Ca^{2+} concentrations may persist increased for hours

(9, 119) and even days (151) following contraction. Calpains are autolytic enzymes with a short half-life after Ca^{2+}- activation. An increase in m-calpain mRNA has been observed after exercise in humans (67) and in rats an increased m-calpain activity has been demonstrated immediately after endurance running exercise (20) as well as after 2 days of reloading of previously unloaded muscle (204).

The localization of m-calpain in the cytoplasm of cells suggests that the IGF-I independent effects of IGFBPs would be more affected than the IGF-dependent effects. Such IGF-I independent effects may have an impact on metabolic as well as anabolic processes.

1.5.2.2 IGFBP phosphorylation

The affinity of IGFBP-1 for IGF-I is dependent on the degree of phosphorylation of this binding protein. Phosphorylated IGFBP-1 has a 4-6 fold higher affinity for IGF-I *in vitro* than the dephosphorylated form (125). The phosphorylation pattern of the IGFBP-1 produced differs in between different tissues. Human hepatoma (HepG2) cells have been demonstrated to secrete predominantly the phosphorylated form and in serum from normal subjects, the major part of IGFBP-1 (90%) is highly phosphorylated (225). During fetal life, the placenta is an important site for IGFBP-1 production. Amniotic fluid and fetal serum contain large proportions of lesser and non (L + N) phosphorylated IGFBP-1 as well as phosphorylated IGFBP-1. IGFBP-1 phosphorylation may be an important post-translational modification that regulates the capacity of IGFBP-1 to modulate IGF-I bioactivity. Prior to the present thesis, no studies on changes in the proportions of IGFBP-1 phosphoisoforms during exercise had been published.

1.5.2.3 IGFBP glycosylation

Glycosylation of IGFBPs is not reported to affect the affinity to IGF-I or ALS (76, 77). It has been suggested that the glycosylation of IGFBP-3 may determine the susceptibility to proteolysis by serum proteases (47) and recent findings in our group have confirmed and further developed this idea (Ahlsen et al, manuscript).

1.5.3 IGF-I proteolysis

Des (1-3) IGF-I is a naturally occurring truncated form of IGF-I which lacks the aminoterminal peptide. Des (1-3) IGF-I binds to IGFBPs with markedly lower affinity than intact IGF-I. In the presence of IGFBPs, des (1-3) IGF-I has a higher potency than intact IGF-I in rats *in vitro* ((39). Des (1-3) IGF-I has been identified in human brain (38, 193), bovine colostrum (81) and porcine uterus (170). An acid protease activity

generating des (1-3) IGF-I from intact IGF-I has been found in serum and tissue extracts from rats (232) and in human urine (233). It has not been explored in human skeletal muscle. The pH optimum is 5.5.

1.6 IL-6 AND THE IGF-IGFBP SYSTEM

IL-6 is a cytokine, a biologically active protein that is a known product of the immune system and classified as both a pro- and anti-inflammatory cytokine (215). It is expressed in skeletal muscle as well as several other tissues (monocytes, fibroblasts, vascular endothelial cells, adipose tissue, peritendinous tissue and brain) as reviewed in (69). There is an increase in interstitial IL-6 concentrations in exercising muscle (190) and a release of IL-6 from exercising leg into the circulation has been reported in men (reviewed in (69)). IL-6 has been suggested to be a "myokine" released from the exercising skeletal muscle. Thereby, IL-6 may constitute a signalling link from working muscle to other organs such as the adipose tissue, the liver and the vascular compartments. IL-6 may be a factor contributing to the increase in endogenous glucose production as well as the metabolic clearance rate of glucose during exercise in healthy humans (68).

Chronically elevated IL-6 concentrations have been observed in clinical states such as critical illness (61), arthritis (59), type 2 diabetes (177) and post-operatively (214). Interestingly, these states are characterized by hepatic and skeletal muscle insulin resistance and an increased IGFBP-3 proteolysis (56, 58, 61). Children with juvenile arthritis suffer from impaired growth. The observations that chronically elevated IL-6 concentrations coincided with metabolic and anabolic disturbances led to assumptions that some of the IL-6 effects may be mediated by the GH-IGF-IGFBP system. It was e.g. demonstrated, that juvenile arthritis in children is associated with high IL-6 concentrations, low circulating total IGF-I and serum IGFBP-3 proteolysis (60). IL-6 exerts several effects on the IGF-IGFBP system, as summarized in Table 1. The listed *in vivo* effects have been demonstrated in states with chronically elevated IL-6 concentrations. Prior to study IV described in the present thesis, studies of connections between an acute increase in circulating IL-6 and the IGF-IGFBP system, such as observed during exercise, were lacking.

In vitro		**REF**
IGF-I	↑/↓ expression (liver)	(141, 213)
IGFBP-1	↑ expression (liver)	(141, 192)
IGFBP-3	↑ expression, unchanged proteolysis (liver)	(141)
IGFBP-4	↑ expression (liver)	(141)
GH	↓ GH inducible gene expression (liver)	(5)
insulin	↓ signalling (liver) ↓ of insulin-induced glycogen synthesis (liver) ↑/→/↓ insulin-induced glucose uptake (adipocytes) → insulin signalling and insulin sensitivity at physiological doses/↑ insulin sensitivity at supraphysiological doses (skeletal muscle)	(197) (197) (35) (36, 92)
In vivo (chronic IL-6 exposure)		
IGF-I	→/↓ expression, ↓ in serum	(60, 61, 146)
IGFBP-1	↑ in plasma	(61)
IGFBP-3	↑ serum proteolysis	(60, 61)
GH	Impaired GH signalling (↑ SOCS3) Normal serum levels	(145, 146)
Insulin	↓ in insulin sensitivity (liver) →/↑ insulin sensitivity and signalling (skeletal muscle)	(129) (129, 221)

Table 1. The effects of IL-6 on the IGF-system (GH included).

1.7 THE IGF-IGFBP SYSTEM IN EXERCISE

1.7.1 The IGF-IGFBP system in the circulation

Circulating components of the IGF-system have been extensively studied in humans, although mostly in men. Blood samples have typically been obtained from an antecubital vein before and after exercise for determination of systemic (circulating) concentrations. In some, but not all studies, samples have also been drawn during exercise. In Table 2 the studies exploring total and free IGF-I concentrations in association with one single exercise bout are summarized. The studies differ in intensity, duration, type of exercise, nutritional state (fasted or not?). Furthermore, training status, sex and age of the subjects also differ. In some studies several different modes of exercise have been explored. The complexity of these studies are likely to explain differences among study findings. However, some general conclusions can be drawn: Short term (< / = 30 min) exercise with moderate/high intensity is associated with a transcient increase or unchanged total circulating IGF-I concentrations. Fasting prior to exercise does not appear to affect the response. The increase in total IGF-I

concentration in some, but not all of these studies may be explained by an increase in hemoconcentration associated with acute exercise. Whether the increased total IGF-I concentrations in the circulation (regardless of the cause of this increase) results in a higher tissue levels of IGF-I has not been explored. It has been suggested that the increase in circulating total IGF-I concentrations observed during exercise of short duration may be the result of a release of IGF-I from the exercising skeletal muscle (30). In an attempt to explore such a regional release, IGF-I concentrations in the artery to (a) as well as in the vein from (v) exercising skeletal muscle have been determined in two studies (23, 30). In our study we observed no significant (v-a) differences in total IGF-I over exercising muscle (30). Brahm et al reported a mean (v-a) difference of 16 µg/L over the exercising leg at the end of 30 minutes aerobic exercise with gradually increasing workload. The (v-a) difference did not reach significance. When it was multiplied by individual by leg blood flow, the resulting net release of IGF-I over the exercising leg appeared to be significant. We remain sceptic to this interpretation of data as there was no significant (v-a) difference. These data illustrate the need of a complementary method for the determination of local IGF-I concentrations, such as the microdialysis methodology applied in this thesis. At the end of or after acute bouts of prolonged exercise (in Table 2 defined as > 45 minutes) total IGF-I concentrations are unchanged or decreased. Repeated bouts of prolonged exercise during several days such as military training or ultra endurance exercise competition result in reduced circulating total and free IGF-I concentrations (83, 98, 118, 158, 165). Although the availability of food is not always restricted, the participants are likely to develop an energy deficient state that may contribute to the observed reduction.

As shown in Table 2, circulating free IGF-I has been reported to be increased (24), unchanged (24, 127, 164, 220) or decreased (53) shortly after exercise (duration < 3h). Bermon et al reported an increase in free IGF-I concentrations after resistance exercise (duration 75 minutes including rests) in resistance trained elderly subjects, whereas it was unchanged in untrained subjects. Dall et al (53) investigated free IGF-I concentrations after a high intensity rowing exercise (duration 20 minutes) in young subjects. The differences may reflect differences in subject age, exercise type or methodology for free IGF-I determinations (discussed in method section 3.4. in this thesis). The (v-a) differences of free IGF-I over exercising muscle had not been determined prior to the investigations in the present thesis. Circulating IGFBP-1 increases in exercise with a duration exceeding 20 minutes, further increases after the cessation of exercise and remains elevated 1-2 h into recovery (23, 131, 161, 171, 208,

220). IGFBP-1 has been suggested to restrict IGF-I bioavailability to the tissues and inhibit hypoglycemia during and after prolonged exercise (208). The effect of circulating IGFBP-1 on muscle tissue levels of unbound IGF-I has not been explored. The increase in IGFBP-1 has been suggested to be caused by decreased insulin levels. However, when insulin and glucose levels are maintained by glucose ingestion during prolonged cycling exercise, IGFBP-1 still increases although the response is attenuated (115). Some other regulatory mechanism may be activated during exercise. In the present thesis, the effect of interleukin-6 (IL-6) on circulating IGFBP-1 concentrations is explored. Increased IGFBP-3 fragmentation in the circulation has been reported in some human exercise studies (160, 196) but not in others (53, 127). No correlation with circulating free IGF-I concentrations have been detected in exercise (160). The source of the IGFBP-3 protease in exercise is unknown.

	Exercise duration	**Change in circulation**	**Reference**
Total IGF-I	Duration < / = 30 min	↑	(13, 34), (196)*, (161, 220)[c)], (53)[a)] (23)[a), b)]
		→	(227)[c)], (30)[c)], (29) * , (134)[d)]
	Duration >30 min – 45 min	→	(127)*, (161)[c)], (164)[d)]
	Duration > 45 min – 3h	↑	(24)[d), e)]
		→	(11, 122)[c)], (208)*, (24)[d)], (181)[e)]
		↓	(23, 131, 160, 195), (161, 208)[c)]
Free IGF-I	Duration < / = 30 min	↓	(53)
	Duration >30 min – 45 min	→	(127) *, (164)[d)]
	Duration > 45 min – 3h	↑	(24)[d),e)]
		→	(24) [d)] , (160)

*Table 2. Changes in total and free IGF-I in the systemic circulation during and/or at the end of exercise in humans. * Subjects fasted prior to exercise*

a) increase non significant after correction for hemoconcentration.

b) increase at 10 minutes of exercise during an exercise bout with total duration of 2h.

c) data not given: fasted/non-fasted prior to exercise.

d) resistance exercise.

e) previously resistance exercise trained group.

1.7.2 Local changes in the IGF-IGFBP system in skeletal muscle

In several *in vivo* studies, skeletal muscle IGF-I mRNA expression has been explored hours to days after exercise.

1.7.2.1.1 IGF-I mRNA expression

In animals, increased loading, stretch and contractions are known to result in increased IGF-I mRNA and IGF-I expression in skeletal muscle cells (reviewed in (3)). This increase is GH-independent as it is also present in hypophysectomised rats (63). Exercise training (repeated exercise bouts for weeks/months) has been demonstrated to result in an increase in IGF-I mRNA in human skeletal muscle (2, 216). However, reports on acute (within hours after one single exercise bout) changes in human skeletal muscle IGF-I mRNA and/or protein expression are sparse and, to some extent contradictory. Most of the human studies have been performed in skeletal muscle tissue samples from men. Skeletal muscle IGF-I mRNA has been reported to be increased (10) or transiently decreased (25) 24 to 48 hs after one bout of resistance exercise. The mRNA expression for IGF1R, IGFBP-4 and IGFBP-5 have been explored 12-24 hs after resistance exercise and the results are contradictory (10, 25). Furthermore, expression of the specific splice variant of IGF-I (MGF) has been reported in response to changes in the loading state in both animal and human skeletal muscle ((96). The MGF peptide has not been isolated but it has been dectected by immunohistochemistry. *In vitro*, a predicted peptide with 24 of the 40 amino acids of the MGF carboxy peptide sequence has been synthesized and has been reported to have biological functions in the muscle, not identical to those of mature IGF-I (235).

1.7.2.2 IGF-I protein expression

IGF-I protein expression in skeletal muscle tissue has been determined by immunohistochemistry immediately after one week of military training in men and was reported to be increased (107). Interestingly, an increase in IGF-I protein expression in spite of the lack of increased IGF-I mRNA expression has been reported in rat skeletal muscle shortly after exercise (234). This indicates that local IGF-I concentrations may be regulated by other factors than changes in gene expression. Such factors may be the release of IGF-I from IGFBP bound stores in the tissue and/or the circulation. In two previous studies attempts have been made to study exercise induced changes in the components of the IGF-IGFBP system in skeletal muscle interstitial fluid at the protein level. In one microdialysis study (3000 kDa probe; microdialysis principle described in method section 3.3. of this thesis), the skeletal muscle interstitial fluid concentrations of total IGF-I, IGFBP-3 and -4 protein were reported to be unchanged 24 h after

endurance exercise (156). In a recent study IGFBP-1 increased in the circulation as well as in the microdialysate (3000 kDa probe) from peritendinous tissue after 3h of aerobic exercise. Skeletal muscle interstitial IGF-IGFBP concentrations were not explored (171). Acute changes in the concentrations of unbound IGF-I in skeletal muscle interstitial fluid have not been determined. In this thesis, we have applied the microdialysis approach to explore them.

2 HYPOTHESIS AND AIMS

General hypothesis

In women and men, endurance exercise is associated with increased local muscle unbound IGF-I protein concentrations which are determined by local as well as circulating changes in the IGF-IGFBP system.

Aims

To optimize and validate the microdialysis methodology to enable determination of unbound IGF-I in human skeletal muscle (II, III).

To assess acute changes in interstitial IGF-I concentrations in resting and exercising muscle by microdialysis in relation to the circulating components of the IGF-IGFBP system and IL-6 (II, III).

To explore the short-term direct effects of moderately elevated circulating IL-6 levels on circulating IGF-I bioavailability by actions on the IGFBPs, such as proteolysis of IGFBP-3 and IGFBP-1 concentrations (III, IV).

To explore interactions between the IGF-IGFBP system in the circulation and the pituitary-gonadal axis in women and men during ultra endurance exercise (I).

To explore the involvement of the intracellular skeletal muscle protease m-calpain in IGFBP-1, -2 and -3 proteolysis *in vitro* (V).

3 MATERIALS AND METHODS

In the present thesis, study I-IV are *in vivo* studies in humans. As shown in table 3, the components of the IGF-IGFBP system and/or pituitary gonadal axis and/or IL-6 were determined at three levels: the systemic level, the regional level and the local level. Study V is an *in vitro* study.

Site of determination	**Method**	**Study**	**Determinations (endocrine)**
Systemic circulation	Antecubital vein	I	IGF-IGFBP system Pituitary gonadal axis
		IV	IGF-IGFBP system IL-6 Cortisol
	Femoral artery	II III	IGF-IGFBP system IGF-IGFBP system IL-6
Regional circulation (over exercising muscle)	(v-a) differences	II III	IGF-IGFBP system IGF-IGFBP system IL-6
Local (skeletal muscle interstitial fluid)			
Resting muscle	microdialysis	III	IGF-I
Exercising muscle	microdialysis	II III	IGF-I IGF-I

Table 3. The determination of hormonal components in the in vivo studies (I-IV)

3.1 SUBJECTS

Sixteen endurance trained elite athletes (7 women, 9 men) were investigated in study I. They participated in, and completed, the Adventure Racing World Championship (ARWC) in Hemavan, Sweden. Their mean (range) age was 34 (25-42) years. Fifteen men (mean age 24 (21-32) years) were included in study II. They were physically active at a moderate intensity level approximately 3 h/week. The 7 women in study III were 28 (23-39) years and had regular menstrual cycles. The experiment was performed during the follicular phase of the menstrual cycle, confirmed by measurements of LH, FSH, estradiol and progesterone. They were physically active at a moderate intensity level approximately 4 h/week. Twelve men (mean age 27 (21-34) years) were included in study IV. All subjects included in the studies were healthy and did not take any medication (except from two women in study I who were on

contraceptive medication). All subjects gave informed consent to participate and the studies were approved by the local ethics committee.

3.2 EXPERIMENTAL MODELS

3.2.1 Exercise models

3.2.1.1 Ultra endurance exercise – extreme long duration endurance exercise with large muscle groups (I)

Ultra endurance exercise can be defined as exercise with moderate to high intensity and a duration that exceeds 6 h (e.g. mountain marathons, triathlons and adventure racing). Although the availability of food may not be restricted, the participants are likely to develop an energy deficient state. The subjects performed mixed ultra endurance exercise (running, trekking, kayaking, cycling and climbing). The race was held on a predetermined course of more than 800 km, and the subjects competed in teams consisting of three men and one woman. Median duration of the race was 6.3 (range 5.2-7.3) days (n = 16). Median intensity was 38 (33-54) % of $VO_{2\ peak}$ including sleep and rest periods (n = 6 men and 3 women), established by heart rate recordings and related to the relationship between heart rate and oxygen uptake determined before the race (as described in study I). Sleeping (average 2h/24h), resting, eating and drinking were *ad libitum*. Blood samples were drawn in the morning the day before the race (PRE), immediately after the end of the race (END) and 24 hs into recovery (POST24h). The nature of the race did not allow frequent sampling and the two latter samples were drawn on random time of the day depending on the individual time point for completion of the race.

3.2.1.2 One-legged knee extension – a model for study of isolated exercising muscle (II, III)

In study II and III, subjects performed dynamic constant load one-legged knee extension exercise (60 rpm) in the supine (II) or sitting (III) position using a modified cycle ergometer (7). The subjects familiarized twice with the experimental apparatus and the maximal one-legged performance capacity was determined at least one week before the experiment. The advantage of the model is that exercise can be localized to a single muscle group. The blood flow in the femoral vein is representative of the active muscles (7). Teflon catheters were inserted in the femoral artery to and the femoral vein from the exercising leg prior to exercise.The concentration differences of substrates and metabolites (e.g. glucose, lactate) between the femoral artery and vein (v-a) differences

reflect tissue metabolism. Thus the measurements in the regional circulation may be used to assess the release or uptake of hormones over the muscle, reflecting local changes. In studies II and III, components of the IGF-IGFBP system and IL-6 (III) were explored in (v-a) differences over the exercising muscle before, during and after exercise with one leg. One limitation of the methodology is that a substantial quantity of IGF-I has to be released or taken up to impact the large circulating pool of total IGF-I and to be detected as a significant (v-a) difference. This is supported by a study in which catheterization of the hepatic vein failed to show any significant release of total IGF-I from the liver, the major source of circulating IGF-I (73). This illustrates the need of a complementary method for the determination of local IGF-I concentrations, such as the microdialysis methodology applied in the present thesis.

In studies II and III, the experiments started 1-2 hs after breakfast. The total duration of the experiments (including insertion of catheters and microdialysis probes) was 4 h (study II) and 6 h (study III). During the experiments, water intake was *ad libitum* but food intake was not allowed.

3.2.1.2.1 Description of experimental procedures and characteristics in Study II

In this study, we were invited to analyze samples from experiments originally designed to study the response of VEGF to exercise performed under different blood flow conditions. The methodology is described in detail in paper II and in (101). The microdialysis methodology is described in section 3. All subjects performed endurance exercise with one leg during 45 minutes. They performed one of three experiments: low intensity exercise under restricted blood flow (LR) obtained by application of external pressure over the working leg (absolute workload 10 +/- 0 W), low intensity exercise under non-restricted blood flow conditions (LN; 10 +/- 0 W) or high intensity exercise under non-restricted blood flow conditions (HN; 28 +/- 2 W). The model has been shown to reduce leg blood flow during one-legged cycle exercise by 15-20 % (209). In the same study, it was shown that in spite of markedly higher leg release of lactate in the ischemic than in the non-ischemic condition, there was no difference in submaximal oxygen uptake between the two. Thus, the exercise is to the largest extent aerobic in all three experiments although in LI and HN the small anaerobic component contributed to increased venous lactate concentrations (101). Components in the IGF-IGFBP system were explored in the regional circulation (through (v-a) differences) over exercising leg and in the systemic circulation (the femoral artery). Microdialysis probes were inserted in the exercising leg as described in section 3.

3.2.1.2.2 Description of experimental procedures and characteristics in Study III

This study was specifically designed to assess IGF-I concentrations in microdialysate from exercising and resting skeletal muscle. The microdialysis methodology is described in section 3. Furthermore, circulating components of the IGF-IGFBP system, as well as IL-6 and glucose metabolism were explored. These determinations were performed in the regional circulation (by (v-a) differences)) over exercising leg and in the systemic circulation (the femoral artery). The subjects performed exercise with one leg (Ex-leg) in the sitting position during one h permitting the collection of the minimal required microdialysis sampling volume (see section 3). The workload was moderate, 22 (3) W or 60 (6) % of the previously assessed maximal one-legged work-load, allowing for a high carbohydrate utilization by the exercising muscle. The resting leg (Rest-leg) was allowed to move freely in order to avoid a workload associated with counteracting or balancing contractions while the Ex-leg was kicking. This resulted in simultaneous passive movements in the knee joint of the Rest-leg with an amplitude of approximately half that of the Ex-leg.

Blood flow: Blood flow was determined in order to enable calculations of the flux of substances over the muscle (such as glucose uptake and the release of hormones). The Ex-leg blood flow was assessed according to the indicator-dilution technique (219). The calculations are described in detail in paper III.

3.2.2 IL-6 infusion – mimicking circulating IL-6 concentrations in exercise (IV)

This study was a collaborative study with Bente Klarlund Pedersen and collaborators at Rigshospitalet in Copenhagen (Denmark). Our interest in the study was to study the effects of moderately elevated IL-6 levels on circulating IGF-IGFBP components. The protocol was designed to expose subjects to IL-6 levels comparable to those observed during and after aerobic endurance exercise with large muscle groups and several h duration (176). Subjects were randomized to receive an intravenous IL-6 infusion (n = 6) or saline infusion (n = 6) for 3 h. The experiments were conducted at 08.00 h after an overnight fast. This was different from our exercise studies, in which the subjects were not fasted prior to the experiments. The femoral artery was cannulated and used for infusion. Human recombinant IL-6 (Sandoz Pharmaceuticals Corp.; Basel, Switzerland) in NaCl with 20 % albumin was infused at a rate of 5 µg/h (25 ml/h) for 3h. Control subjects received a saline (NaCl with 20 % albumin) infusion for 3h. Blood samples were collected before, during and until 8 h after infusion. Plasma IL-6

concentrations (approximately 100 ng/L) and the IL-6 expression in abdominal fat have been previously reported (Keller et al 2003).

3.2.3 IGFBP proteolysis by m-calpain *in vitro* – exploring potential effects of a local protease (V).

In this *in vitro* study, we explored the involvement of m – calpain in IGFBP-1, -2 and -3 proteolysis according to the methodology summarized below. The methods are described in detail in paper V.

3.2.3.1 Degradation of [^{125}]-labeled IGFBP-1, -2 and -3 by Ca^{2+} activated m-calpain

Trace amounts of [^{125}]-labeled native human IGFBP-1, recombinant human IGFBP-2 and recombinant human glycosylated IGFBP-3 (final concentration of each IGFBP ~0.1 nM) were each incubated at 37 ^{o}C for 1 h in a reaction mixture containing HEPES, $CaCl_2$, BSA, and, when indicated, 0.25 μM m-calpain with or without EDTA. The reaction was stopped by addition of non-reducing SDS sample buffer. Reaction mixtures were separated by SDS-PAGE, whereafter gels were dried and exposed to X-ray film.

3.2.3.2 Determination of degradation pattern, dose and time dependency of IGFBP-2 and -3 proteolysis by Ca^{2+} activated m-calpain

Recombinant human IGFBP-2 and recombinant human non-glycosylated rhIGFBP-3 at final concentrations of 2 μM and 10 μM, respectively, were incubated at 37^{o} C with various concentrations of m-calpain (0-0.6 μM) for 0, 20, 40, or 60 minutes in HEPES, NaCl, Surfactant P20, and 5 mM $CaCl_2$. The reactions were stopped by addition of EDTA. Aliquots of the reaction mixtures were separated by SDS-PAGE. Gels were either stained with Coomassie R-250 using standard procedures or further processed (see below).

3.2.3.3 Determination of primary cleavage sites in IGFBP-2 and -3 for Ca^{2+} activated m-calpain

IGFBP fragments, generated by incubation of IGFBP-2 or -3 with 0.2 μM m-calpain, were separated by SDS-PAGE (see above) and electroblotted onto PVDF membranes, stained with Commassie R-250, cut out and subjected to N-terminal amino acid sequence analysis (Edman degradation).

3.2.3.4 Monitoring of binding pattern of m-calpain and IGFBP-3

Real time BIA (Biomolecular Interaction Analysis) uses continuous flow technology to monitor biomolecular interactions in real time. Biosensor analyses were performed on a BIACORE X (GE Healthcare Biosciences). In principle, the basis for measurements with BIA is an optical phenomenon (the resonance angle). Molecules from a solution (here: m-calpain or IGF-I) flows over a sensor surface where the reactant is immobilized (here: glycosylated IGFBP-3). As molecules from the solution binds to the reactant, the resonance angle changes and a response is registered. The result is shown in sensograms, i.e., the signal measured representing the mass of protein bound to the chip as a function of time. As described in detail in paper I, The impact of Ca^{2+} activation of m-calpain on the binding to immobilized glycosylated rhIGFBP-3 was explored. Furthermore, the integrity of immobilized IGFBP-3 was assessed by binding of IGF-I to rhIGFBP-3 before and after exposure to Ca^{2+} activated m-calpain.

3.3 MICRODIALYSIS (II, III)

3.3.1 Introduction to the microdialysis methodology

Microdialysis monitors the chemistry of the extracellular space and has been used for *in vivo* determination of glucose and metabolites in brain and adipose tissue. In a few recent studies, it has been applied to determine interstitial levels of e.g. insulin (200), vascular endothelial growth factor (VEGF) (111), IL-6 (136) and Prostaglandin $F_{2\alpha}$ (217). In principle, a probe with a membrane is inserted into the muscle (figure 2). The probe is connected to a pump by the inlet tubing and is continuously perfused by a physiological solution. The perfusion solution (perfusate) passes the microdialysis probe at a low flow rate (0.1-5 μL/min) and equilibrates (partly or totally) with the surrounding extracellular tissue fluid. The fluid, now named "microdialysate" (md), is pumped via the outlet tubing to the collecting vial.

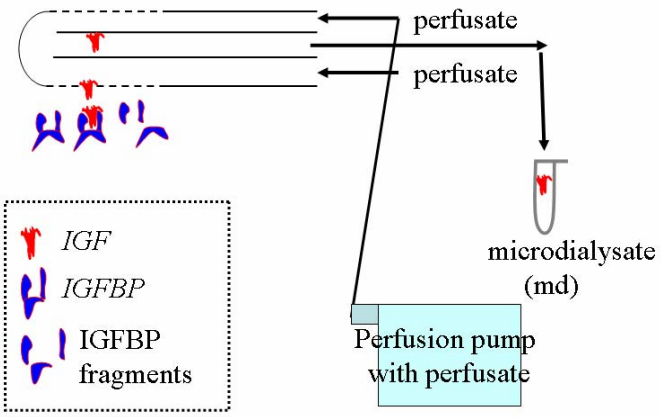


Figure 3. The microdialysis methodology in the present thesis. Only unbound IGF-I (7.5 kDa) was demonstrated to pass the microdialysis probe membrane (20 kDa) to be collected in the microdialysate (md). IGFBPs, IGF-IGFBP complexes or larger IGFBP-fragments do not pass do not pass (see below).

3.3.1.1 Recovery

The exchange of molecules over the probe membrane occurs in both directions. The recovery of a substance may be explained as the efficacy of the exchange of that molecule from the extracellular space over the membrane and into the microdialysate. The reverse recovery (RR) is the efficacy of the exchange of that molecule from the perfusate into the extracellular space. The exchange of molecules over the membrane is mainly determined by the cut-off of the membrane, the concentration gradient of the substance, the perfusion flow rate, the length of the membrane and the diffusion coefficient of the compound through the extracellular fluid. Proteins, such as IGF-I tend to adhere unspecifically to plastic materials which reduces recovery. The low recovery, combined the with small sample volumes require sensitive methods for peptide analyses. Muscle contraction and blood flow are known to affect recovery and represent an additional challenge (184).

3.3.1.2 Assessment of recovery: the internal reference method (III)

The internal reference method is based on the concept that the ratio of the *in vivo* recovery of any two compounds is equal to the ratio of the recovery of the same two compounds *in vitro* (148, 207). A reference substance is added to the perfusion fluid. The reverse recovery (RR) of the reference substance equals the loss of the substance from the perfusate and reflects the exchange of the substance over the membrane probe.

The internal reference method is expressed in the following formula:
Substance A-R A *in vitro* / Substance B-RR *in vitro* = Substance A-R *in vivo* / substance B-RR *in vivo*. The calculations are described in further detail in paper III. The internal reference method introduces an additional source of variation has been questioned especially for larger "sticky" molecules such as proteins. The molecular weight is not the only determining factor for the recovery and it may be difficult to find a reference substance with similar properties.

3.3.2 Microdialysis methodology applied in the current thesis

3.3.2.1 Microdialysis materials

In both studies II and III, microdialysis probes, perfusion pumps and perfusion fluid were all purchased from CMA Microdialysis (CMA, Solna, Sweden). The polyamide probe (CMA 60, 0.5 · 30 mm, cut-off 20 kDa) was perfused with a solution (CMA perfusion fluid). This solution does not have any buffer capacity and adjusts to the pH of the surrounding tissue. The perfusion flow rate was 2 μL/min. Prior to study III, the methodology was optimized and validated *in vitro*. The methodological differences between study II and III are summarized in Table 4. In study III, 0.05 % human serum albumin, HSA (Pharmacia, Stockholm, Sweden) was added to the perfusion fluid in order to avoid loss of IGF-I to the plastic materials. Furthermore, instead of using the collecting microvials from CMA (as in study II) the distal end of the outlet microdialysis tubing was cut off and inserted into a TreffLab polypropylene tube (Treff AG, Degersheim, Switzerland). In study II, IGF-I recovery *in vivo* was not determined. In study III, ^{14}C inulin (0.05 μCurie/mL; 5kDa; Amersham Biosciences, UK) was chosen as an internal reference substance for assessment of changes in probe recovery of IGF-I (7.5 kDa) related to muscle contractions and blood flow. Inulin has previously been used as a reference substance for insulin (5.8 kDa) due to similarity in molecular weight, lack of tissue binding and metabolism of inulin and the availability of a safe and stably labelled molecule (148, 200, 207).

	Study II	Study III	Note / Motivation for difference
Microdialysis probe	CMA 60	CMA 60	Validated
Perfusion fluid	CMA	CMA	
		HSA (0.05 %)	Increases the IGF-I recovery
		^{14}C inulin	Assessment of IGF-I probe recovery
Perfusion	2 µL/min	2 µL/min	Validated, optimized.
Perfusion pump	CMA 107	CMA 107	
Collection vial	CMA microvial	TreffLab tube	Avoid loss of IGF-I to plastic materials
Insertion	1 h prior to exercise	2.5 h before onset of exercise 1.5 h before onset of basal determination	Minimize impact of insertion trauma Allow equilibration time
Control leg	Md catheters in Ex-leg only	Md-catheters in Ex-leg and Rest-leg.	Control leg for determinations in resting skeletal muscle during one-legged aerobic exercise
Collection of microdialysate	45 minutes intervals	1 h intervals Vials weighed.	Allowing a larger sample volume Controlling for loss of perfusion fluid.
Determination of md-IGF-I	RIA (detection limit 0.1 µg/L)	DELFIA (detection limit 0.007 µg/L)	Higher sensitivity, md-IGF-I detectable in all subjects at all times.

Table 4. Microdialysis methodology applied in the current thesis.

The differences between the methodology in study III compared to study II are shown.

3.3.2.2 Insertion and handling of microdialysis catheters in vivo

In both studies II and III, microdialysis probes were inserted into the vastus lateralis muscle after inducing local anesthesia down to the muscle fascia (Mepivacainhydrochloride; Carbocain ® 10 mg/mL, Astra Zeneca, Södertälje, Sweden). The probes were inserted in a cranial direction, 45 degrees relative to the sagittal plane of the muscle. This direction was chosen in order to follow the direction of the muscle fibers, minimizing trauma to the muscle and the microdialysis probe. In study II, microdialysis probes were inserted 1 h before onset of exercise. In study III, we minimized the possible impact of skeletal muscle tissue response to the insertion trauma by inserting the microdialysis probes 2.5 hs before onset of exercise and allowing 1.5 h of equilibration before the collection of microdialysate for basal IGF-I determinations. This approach has been taken in previous microdialysis studies (82, 111). In study III, two microdialysis catheters

were inserted in each leg to secure at least one complete sample series in each leg. The collection of microdialysate from the resting control leg (Rest-leg) was unique to study III. In study II, catheters were inserted only in the exercising leg.

3.3.2.3 In vitro validation

Exp 1) IGF-I recovery *in vitro*:

In a series of *in vitro* experiments, microdialysis probes were submerged in a polyethylene tube containing an experimental "interstitial fluid" consisting of a modified Krebs Henseleit solution with 0.05 % human serum albumin (HSA) and 0.19 (0.05) μg/L (0.0248 (0.006) nM) of human recombinant IGF-I (kindly provided by Genentech Inc, South San Francisco, CA, USA) with or without IGFBP-3 (Upstate, NY, USA). The composition of the perfusion fluid was the same as that *in vivo* described above (perfusion speed 2 μL/min). Microdialysis of the "interstitial fluid" was performed for up to 24 h at 37°C under gentle shaking. Microdialysate was collected at 1-h intervals during 4 h for IGF-I determination by DELFIA (n = 4 CMA 60 catheters). The mean relative recovery of IGF-I *in vitro* IGF-$R_{in\ vitro}$ was 16 (6) %. The mean reverse recovery of ^{14}C inulin *in vitro* $(I\text{-}RR)_{in\ vitro}$ was 55 (4) %.

Exp 2) Demonstration that only unbound IGF-I passes the microdialysis probe membrane: described in paper I.

Exp 3) Demonstration that IGFBPs do not pass the microdialysis probe membrane: described in paper III.

Exp 4) Demonstration that larger IGFBP-3 fragments or IGF-IGFBP complexes (30-50 kDa) do not pass the microdialysis probe membrane:
described in paper II.

3.4 HORMONES AND SUBSTRATES

The methods for hormonal and substrate determinations are summarized in Table 5 and 6. Hormonal determinations were performed in serum (I and III), heparin plasma (II), EDTA plasma and serum (IV) and microdialysate (md) from skeletal muscle (II and III). In studies II-IV, patency of the catheters was maintained by intermittent flushing with a saline solution. Serum samples were kept on ice for one h to allow for clotting before obtaining serum by centrifugation. Serum samples were stored at –70 °C. All samples were stored at – 70 °C prior to analysis.

	Method (study)	**note**	**Ref/provider**
t- IGF-I in circulation	RIA (I, II)		(16)
	DELFIA (III, IV)		Slightly modified from (90). Described in III and IV.
Free IGF-I in circulation	ELISA (I, III, IV)		DSL Inc.
md-IGF-I ***absolute***	RIA (II)	Detection limit 0.1 µg/L	(16)
	DELFIA (III)	Detection limit 0.007 µg/L	Slightly modified from (90). Described in III.
t- IGFBP-1	RIA (I, IV)		Modified from (225).
	ELISA 6301 (II)		Described in II.
	ELISA (III)		DSL Inc.
N + LP IGFBP-1	ELISA 6305 (II)		Described in II.
IGFBP-2	WLB (I)	^{125}[I]-labeled IGF-I and IGF-II as ligands	(117)
t-IGFBP-3	ELISA (III)	Detects intact and fragmented IGFBP-3	DSL Inc.
IGFBP-3 ***in vivo*** **fragmentation**	WIB (I- IV)		(17)
IGFBP-3 PA ***in vitro***	IGFBP-3 protease assay (I, IV)		Described in IV.
IL-6	ELISA (III)		R&D Systems
FSH	DELFIA (I)		PerkinElmer
LH	DELFIA (I)		PerkinElmer
Testosterone	Competitive binding immunoenzymatic technique Access 33560 (I)		Beckman Coulter Inc.
SHBG	DELFIA (I)		PerkinElmer
Estradiol	DELFIA (I)		PerkinElmer
Progesterone	Competitive binding immunoenzymatic technique Access 33550 (I)		Beckman Coulter Inc.

Table 5. Methods for the determination of components in the IGF-IGFBP system and the pituitary-gonadal axis (I-IV.

Insulin	ECLIA (I)		Karolinska Hospital Lab. for Blood Chemistry
	RIA (IV)		Amersham Biosciences
Glukagon	RIA (I)		
Cortisol	RIA (IV)		Diagnostics Products
Glucose	Hexokinase method (Gluco-quant®) (III)		Roche Diagnostics
^{14}C inulin in microdialysate	Radioactivity determined in Beta counter (III)		Beckman Coulter Inc.
Hemoglobin	Photometer (I)		HemoCue
	Spectophotometric technique (III)		ABL 50

Table 6. Methods for the determination of glucoregulatory hormones and other components (I-IV).

3.4.1 IGF-I

In studies I and II, circulating total IGF-I was determined after ethanol extraction using des- (1-3) as a radioligand in a radioimmunoassay (RIA). The advantage with this method is the availability of a large reference material in healthy humans (16). Therefore, we were able to express basal total IGF-I concentrations in the elite athletes in paper I in SDS scores. A slight improvement of the detection limit to 0.1 μg/L was obtained using a dilution of the first antibody of 1/90 000 and a tracer dilution of 4500 cpm/100 μL. In study II, IGF-I in microdialysate (md-IGF-$I_{absolute}$) was determined as described for total IGF-I in the circulation. Determinations below the detection limit were set to this value (0.1 μg/L).

In both study II and III, (md-IGF-$I_{absolute}$) was determined directly in microdialysate. The IGF-I assay ethanol extraction procedure was excluded since exclusively unbound IGF-I and not IGF-IGFBP complexes (30-50 kDa) were demonstrated to pass the microdialysis probe membrane (described above). The low IGF-I concentrations in microdialysate required the establishment of a more sensitive IGF-I assay. In study III, we applied an IGF-I DELFIA (dissociation-enhanced lanthanide fluorescence immunoassay) to obtain more sensitive determinations of IGF-I in the microdialysate. A DELFIA originally described by Frystyk (90) was reported to have a 10-100-fold higher sensitivity than our IGF-I RIA. This method was modified as described in detail

in paper III and IV. The DELFIA is not only more sensitive than the RIA, it does not require handling of radioactive substances which is an advantage.
For the determination of "free" IGF-I in the circulation (the term "free dissociable" IGF-I may also be used) a commercially available and widely used or ELISA (DSL Inc., USA) was used (I, III, IV). The methods for the assessments of circulating free IGF-I have been developed in an attempt to assess the IGF-I bioavailability of IGF-I to the receptors in the body. However, this is problematic for several reasons. To mention some, tissues have different concentrations of e.g. binding proteins. Furthermore, the passage of IGF-I into the tissues may be differentially regulated. Extraction of IGF-I from tissues does not provide information as to the the local levels of unbound IGF-I in the extracellular fluids. Determinations of IGF-I mRNA are for obvious reasons of even less use. Attempts to determine IGF-I concentrations in lymph have been made, with the risk of blood contamination (31). For the determination of free IGF-I, the ELISA method provided by DSL uses antibodies that selectively bind to unbound IGF-I most likely because the binding determinant of the monoclonal antibody in this assay is overlapping with IGFBP binding sites. The method has been observed to be very sensitive to incubation time employed which suggests that a steady state equilibrium is not obtained and that the assay interferes with the IGF-IGFBP equilibrium. The recently developed IGF-I KIRA, (IGF-I kinase receptor activation assay) is a method based on cells transfected with the human IGF1R gene (41). It has been developed in the attempt to determine IGF-I bioactivity and has to be further evaluated. Finally, the ultrafiltration methodology is theoretically advantageous by not disturbing the equilibrium between free IGF-I and IGF bound to IGFBPs in the sample. However, it has been demonstrated to be practically complicated, and to date a functional method has only been established at one laboratory (90). The sample is ultrafiltrated through a membrane (~ 25 kDa) and the ultrafiltrate is analysed in an ultrasensitive IGF-I DELFIA. We have found that the DSL assay and the ultrafiltration assay markedly differ in their ability to detect the changes in free IGF-I which are expected with increased IGFBP-3 proteolysis in serum (Bang, unpublished data). We modified the ultrasensitive DELFIA established by Frystyk et al to assess free IGF-I in our microdialysis samples, where the interstitial fluid had been "filtrated" through the microdialysis probe membrane. In the microdialysate, no IGFBPs were present. The ultrafiltration method has been used to assess changes in circulating free IGF-I in mouse KO models of liver IGF-I, ALS or the combination. The results

do not correspond to the demonstrated changes in mitogenic or metabolic parameters considered to be IGF-I dependent (230). This further underlines the need of methods that determine free IGF-I concentrations in the actice tissues rather than in the circulation.

3.4.2 IGFBP-1

Circulating total IGFBP-1 was determined with a RIA in study III and IV. The method was established in our laboratory modified from (225). The first antibody (MAb 6303, Medix Biochimica, Finland) captures all phopshovariants of IGFBP-1. The method is described in detail in paper IV. In study II separate ELISA:s used two phosphospecific monoclonal antibodies. In study I a commercially available ELISA kit was used (DSL Inc, USA), unaffected by the state of phosphorylation.

3.4.3 IGFBP-3

Immunoreactive total IGFBP-3 from the microdialysis *in vitro* and *in vivo* experiments in study III was determined by ELISA, which is known to detect intact as well as fragmented IGFBP-3 (DSL, Webster, Texas, USA).

3.4.3.1 IGFBP-3 fragmentation in the circulation (I-IV)

Western immunoblot (WIB) was used to explore the *in vivo* fragmentation in the circulation. It detects the different molecular forms of IGFBP-3 resulting from post-translational processing including IGFBP-3 proteolysis. Serum is separated on SDS-PAGE and transferred to nitrocellulose membranes. The primary polyclonal antibody used (Upstate biotechnology, NY, USA) recognizes intact IGFBP-3 as well as IGFBP-3 fragments (although predominantly the 30 kDa fragment). The affinity for smaller IGFBP-3 fragments is low. The antibody cross-reacts with human serum albumin (HSA) resulting in an additional band of 60 kDa on the gel. WIB is a semi-quantitative method suitable for studying relative differences over time. For this purpose, bands are quantified on the scanned gels using computer programs. We used Image J (National Institute of Health, USA). The method is described in detail in papers II and IV.

3.4.3.2 IGFBP-3 protease activity in the circulation

The presence of IGFBP-3 protease activity in serum can be estimated by studying the *in vitro* degradation of recombinant ^{125}I-labeled IGFBP-3 incubated with serum for 5h at 37 °C. The sample mixture is then separated on SDS-PAGE and the radioactivity is detected on film. Different proteolytic fragments can be detected, scanned and

quantified. This is also a semi-quantitative method, described in detail in paper IV. We have demonstrated that IGFBP-3 proteolysis is not affected by venous cannulation and sampling (100).

3.4.4 IGFBP-2

IGFBP-2 was determined in serum by western ligand blotting (WLB) developed by (117). Serum was separated on SDS-PAGE and proteins are transferred to nitrocellulose membranes. Human recombinant IGFBP-2 (Novartis, Basel, Switzerland) was also applied on the gel. A mixture of ^{125}I-labeled IGF-I and II were used as ligands. The IGFBPs with conserved structure allow binding to the ligands. The radioactivity was detected on films and quantified. The method allowed us to detect intact IGFBP-2 as a band with a molecular weight of 31 kDa as confirmed by the recombinant IGFBP-2 applied on the gel.

3.5 STATISTICS

Results are given as mean (SD) if normally distributed, otherwise as median (range), unless otherwise stated. The statistical methods employed are described in detail in study I-IV. $P < 0.05$ was considered significant.

4 RESULTS

In tables 7 and 8, the main changes in the IGF-IGFBP system during and after exercise or IL-6 infusion *in vivo* are summarized. The components were explored at the systemic level (systemic circulation), at the regional level (reflected as venous (v) and arterial (a) concentrations and (v-a) differences over skeletal muscle) and at the local level (reflected as microdialysate concentrations from skeletal muscle). The *in vitro* study exploring the involvement of the skeletal muscle protease m-calpain in the proteolysis of IGFBP-1, 2 and -3 is summarized in table 9.

	Basal state (rest) (I – IV)	**Ultra endurance exercise (I)**	**Exercise with one leg (II, III)**	**IL-6 infusion at rest (IV)**
duration		5-7 days	45 or 60 min.	3h
Systemic circulation				
Total IGF-I	+1.2 SDS in endurance trained females	↓during ex. ↓ 24h after ex.	↑ early in ex.	→ during inf
	+ 0.1 SDS in endurance trained male athletes.	No corr with testosterone	↓ 1h after ex.	→ after infusion
Free IGF-I	No correlation with IGFBP-1	↓during ex. ↓ 24h after ex.	↓ early in ex. Back to basal at the end of ex.	→ during inf → after infusion
		No correlation with IGFBP-1	↓ one hour after exercise.	No correlation with IGFBP-1
			No correlation with IGFBP-1.	
IGFBP-1	↑ at the end of ex.	↑ during ex. Back to basal after exercise.	↑ at the end of ex. Furthet increase after exercise.	→ during infusion
				↑ after infusion
			→ phosphorylation state (II).	
IGFBP-3 PA/ fragmentation	Higher in female than male athletes	↑ during ex. Back to basal after ex.	→	→
IGFBP-2	Lower in female than male athletes	↑ in females after ex.		

Table 7. The IGF-IGFBP system in the systemic circulation during and after exercise or IL-6 infusion. Main findings.

	Basal state (rest) (I – IV)	**Ultra endurance exercise (I)**	**Exercise with one leg (II, III)**	**IL-6 infusion at rest (IV)**
Regional circulation ((a-v) differences over exercising muscle)				
Total IGF-I	Uptake over skeletal muscle (III).			
Free IGF-I			Release from muscle at the end of and shortly after ex.	
			No correlation with release of IL-6.	
IGFBP-1			→ phosphorylation state (II).	
IGFBP-3 PA/ fragmentation			→	
Local concentrations (skeletal muscle interstitial fluid)				
Free IGF-I	~ 0.4 % of total circulating IGF-I		Ex-leg: ↑ md-IGF-I $_{absolute}$ during ex. → md-IGF-I during ex unchanged after ex	
	Higher in younger subjects		Rest-leg (III): →md-IGF-I $_{absolute}$, → md-IGF-I during ex with Ex-leg ↓ after ex with Ex-leg	

Table 8. Regional and local changes in the IGF-IGFBP system during and after exercise. Main findings.

	Proteolysis	**Primary cleavage site**	**Binding pattern of m-calpain and IGFBP**
IGFBP-1	No		
IGFBP-2	Yes - Calpain dose dependent - No time dependency	In non-conserved linker region. Not previously reported	
IGFBP-3	Yes - Calpain dose dependent - No time dependency	In non-conserved linker region. Not previously reported	Rapid on-/rapid off-rate Ca^{2+} - dependent kinetics

Table 9. In vitro study of the involvement of Ca^{2+}-activated m-calpain in IGFBP proteolysis. Main findings.

4.1 SUBJECT CHARACTERISTICS IN ULTRA ENDURANCE EXERCISE (I)

Body weight or BMI did not change significantly. Body fat including subcutaneous and visceral adipose tissue decreased in the 9 men ($P = 0.0027$) and the 3 women (non-significant; $P = 0.083$) where it was determined. The approximated average total energy expenditure during the race was 77 000 (64 000-114 000) kcal or 12 330 (10 144-17 249) kcal/d (n = 6 men). As suggested by determinations of energy intake in three of the men, the race resulted in an energy deficit of ~ 40 000 kcal (Enqvist & Mattson, unpublished data). In all women except for one, who was amenorrheic and low in estradiol from start, a modest vaginal bleeding was reported on the second day of the race.

4.2 TOTAL AND FREE IGF-I IN THE CIRCULATION BEFORE, DURING AND AFTER EXERCISE (I-III)

In the basal state, circulatory t-IGF-I concentrations in the endurance trained athletes in study I were not lower than those of an age-matched reference material (16). In contrast, median t-IGF-I was +1.2 SDS in women and +0.1 SDS men. In the 7 women in study III, where (v-a) differences of t- and f-IGF-I were determined, basal t-IGF-I was significantly lower in the vein from (202 (SD 38) µg/L) than in the artery to (230 (SD 42) µg/L; $P < 0.05$) the Ex-leg. Such an uptake of t-IGF-I over the muscle was not observed in the 15 men (II) where no significant (v-a) differences in t-IGF-I were observed at any point in time. No significant flux of f-IGF-I was detected in the basal state (III).

During the first 10-45 minutes of endurance exercise with one leg, t-IGF-I in the circulation increased significantly by ~ 11 % (II and III). The increase reached significance in the vein (III) or the artery (II). In the 7 women in study III, venous f-IGF-I was decreased early in exercise concomitant with the increase in t-IGF-I concentrations. At the end of, and shortly after exercise, f-IGF-I was significantly higher in the vein than in the artery in all the women (III). This release was not detectable as an increase in circulating t-IGF-I. Interestingly, among subjects with a calculated net f-IGF-I release over the Ex-leg at the end of exercise, the highest release was found in those with the highest md-IGF-I (Ex-leg) during exercise. At the end of ultra-endurance exercise a decrease in both t-IGF-I and f-IGF-I, by 33 (SD 38) and 54 (19) %, respectively (I) was observed, without sex related differences. The decrease in

t-IGF-I appeared to be associated with the total energy deficit during the race (n = 3 men).

The t- and f-IGF-I concentrations were still significantly lower than basal levels 24h into recovery after ultra endurance exercise. One h after exercise with one leg (duration 1h), arterial t-IGF-I was decreased compared to basal levels (III).

4.3 MICRODIALYSIS: CALCULATED RECOVERY OF IGF-I *IN VIVO* (III)

The *in vivo* recovery of IGF-I was calculated in study III, according to the internal reference technique. Both absolute values (md-IGF-$I_{absolute}$) and values recalculated for IGF-I recovery (md-IGF-I) are given in Table 10. Basal *in vivo* ^{14}C inulin reverse recovery I-RR was 30 (7) % in Ex-leg and 39 (3) % in Rest-leg, markedly lower than 55 (4) % *in vitro*. Basal mean IGF-I recovery was calculated to be 8 (1) % in Ex-leg, significantly lower than 11 (1) % in Rest-leg. Ex-leg I-RR increased from 30 (6) % at rest to 48 (7) % during exercise; $P < 0.001$). The relative increase was 68 (49) %. Calculated mean IGF-I recovery increased to 14 (2) % in the Ex-leg ($P < 0.001$ compared to basal) and returned back to basal levels during the first h after exercise. I-RR or calculated IGF-I recovery did not change significantly over time in the Rest-leg. There was no correlation between changes calculated IGF-I recovery or I-RR and blood flow.

4.4 SKELETAL MUSCLE INTERSTITIAL IGF-I CONCENTRATIONS

4.4.1 Skeletal muscle interstitial IGF-I concentrations at rest (II, III)

In paper II, basal md-IGF-$I_{absolute}$ was below the detection limit of the RIA (0.10 µg/L) in most of the 14 male subjects (Table 10). In paper III the microdialysis methodology had been validated and optimized. The md-IGF-$I_{absolute}$ was detectable in all subjects (7 women) at all times and values in the two legs correlated (R = 0.82; $P = 0.04$). The md-IGF-$I_{absolute}$ as well as IGF-I concentrations corrected for calculated IGF-I recovery (md-IGF-I) are given in Table 10. Basal md-IGF-I was 0.87 (0.4 – 1.5) µg/L equal to 0.4 (0.2) % of t-IGF-I determined in arterial serum and in the same concentration range as f-IGF-I. Furthermore, basal md-IGF-$I_{absolute}$ correlated with arterial t-IGF-I concentrations (R = 0.79; $P = 0.04$). The correlation was no longer significant when md-IGF-$I_{absolute}$ was corrected for calculated IGF-I recovery (R = 0.57; $P = 0.18$). Basal md-IGF-I was higher in younger individuals (R = -0.77, $P = 0.04$).

4.4.2 Skeletal muscle interstitial IGF-I concentrations during exercise (II, III)

In study II, the md-IGF-$I_{absolute}$ increased during exercise in 14 of the 15 men. The median (range) md-IGF-$I_{absolute}$ was significantly higher than at rest ($P < 0.01$). There was no significant difference between the different exercise groups. The md-IGF-$I_{absolute}$ was not corrected for probe recovery and Rest-leg md-IGF-I concentrations were not determined. In study III, we attempted to control for probe recovery by applying the internal reference method. The seven women performed endurance exercise for 1 h with one leg (Ex-leg). The resting leg (Rest-leg) served as a control. Ex-leg md-IGF-$I_{absolute}$ increased in every subject (range + 6-80 %) and was significantly higher in the Ex-leg than in the Rest-leg ($P = 0.02$). Although there was an increase in mean calculated IGF-I recovery in Ex-leg during exercise, the relative changes in IGF-I recovery did not correlate with the relative changes in md-IGF-$I_{absolute}$ in each individual probe. After correction for calculated IGF-I recovery, changes in Ex-leg md-IGF-I did not reach significance.

4.4.3 Skeletal muscle interstitial IGF-I concentrations after exercise (II, III)

In study II, Ex-leg md-IGF-$I_{absolute}$ remained significantly elevated during recovery as compared to basal levels ($P < 0.01$). In study III, Ex-leg md-IGF-$I_{absolute}$ did not differ from basal levels after exercise. Ex-leg md-IGF-I (values corrected for calculated IGF-I recovery) were not significantly changed during the first h after exercise and declined during the second h after exercise although only at the limit of significance ($P = 0.05$). The decrease in Rest leg md-IGF-$I_{absolute}$ reached significance during the second h after exercise ($P < 0.01$). When corrected for calculated IGF-I recovery, Rest-leg md-IGF-I was decreased by 58 % already during the first h after exercise (0.54 (0.4-0.8) µg/L, $P = 0.02$).No correlations were detected between skeletal muscle glucose uptake and md-IGF-I concentrations at any point in time.

Collection period	**md-IGF-I (µg/L) absolute values**		**md-IGF-I (µg/L) values corrected for IGF-I recovery**	
STUDY II	Ex-leg		Rest-leg	
(-60-0)	0.10 (0.10-0.90)		Not explored	
(Ex0-Ex45)	0.27 (0.10-1.90) **		Not explored	
(Ex45-P60)	0.20 (0.10-3.10) **		Not explored	
STUDY III	**md-IGF-I (µg/L) absolute values**		**md-IGF-I (µg/L) values corrected for IGF-I recovery**	
Collection period	Ex-leg	Rest-leg	Ex-leg	Rest-leg
(-60-0)	0.07 (0.02)	0.09 (0.03) †	0.87 (0.4-1.5)	0.77 (0.4-1.2)
(Ex0- Ex60)	0.12 (0.04) ** †	0.07(0.03)	0.92 (0.4-1.2)	0.55 (0.2-0.8)
(Ex60-P60)	0.07 (0.04)	0.06(0.02)	0.58 (0.4-1.0) (n = 6)	0.54 (0.4-0.8) * (n = 5)
(P60-P120)	0.06 (0.01)	0.06 (0.02) **	0.50 (0.3-0.6) (n = 4)	0.50 (0.2-0.7) * (n = 6)

Table 10. IGF-I concentrations in microdialysate (md) from the skeletal muscle of 15 men (II) and 7 women (III) before, during and after one-legged endurance exercise during 45 min (II) or 60 min (III).

The md-IGF-$I_{absolute}$ (absolute values) are given as median (range) for men and mean (SD) for women. The md-IGF-I (corrected for calculated IGF-I recovery) are given as median (range).

** P < 0.05, ** P < 0.01, *** P < 0.001 vs basal (-60-0) values in the same microdialysis probe and leg.*

† P < 0.05 Ex-leg vs Rest-leg.

n = 7 women and 15 men, unless otherwise stated.

4.5 RELATION BETWEEN CIRCULATING AND LOCAL IGF-I

4.5.1 IGFBPs in the circulation during exercise

4.5.1.1 IGFBP-1 (I-III)

At the end of one-legged exercise (duration 1 h), circulating total IGFBP-1 was significantly increased and had increased further 1 h after exercise (III). It remained significantly elevated 2 h after exercise. Similar results were found in study II. At the end of an ultra endurance exercise race IGFBP-1 was increased but had returned to basal levels 24 h into recovery (I). No significant (v-a) differences of IGFBP-1 were

detected (II). The proportion of phosphoisoforms of IGFBP-1 did not change during one-legged exercise (II).

No significant correlations between changes in f-IGF-I and total IGFBP-1 in the circulation were found during or after exercise (I, II, III) or IL-6 infusion (IV). There was no significant correlation between changes in IGFBP-1 and md-IGF-I in the Ex-leg and / or Rest-leg during or after one-legged exercise (III). However, the relative change in the Rest-leg md-IGF-$I_{absolute}$ during exercise was inversely correlated with the relative change in arterial IGFBP-1 (R = -0.8; P = 0.02).

4.5.1.2 IGFBP-2 (I)

In the endurance trained subjects basal circulating IGFBP-2 was significantly higher in men than in women (P = 0.01). IGFBP-2 did not change significantly during the ultra endurance exercise race but was elevated in women 24 h into recovery and there was no longer any gender difference. IGFBP-2 did not correlate with circulating f-IGF-I at any single time point. However, the changes in IGFBP-2 after exercise correlated negatively with the changes in circulating t-IGF-I (R = -0.73; P = 0.008; n =12).

4.5.1.3 IGFBP-3 (I -III)

In the endurance trained subjects, IGFBP-3 PA in the circulation was significantly higher in women than in men at all time points (I). There was no sex difference in IGFBP-3 fragmentation. At the end of ultra-endurance exercise, IGFBP-3 fragmentation was increased by 26 (16) % in both sexes and returned back to basal levels 24 h into recovery (I). There was no correlation with the increase in CKMB during the race (n = 6). No changes in IGFBP-3 fragmentation were observed during or after one-legged exercise (II, III) or during/after IL-6 infusion.

4.5.2 Interactions between the pituitary-gonadal axis and the IGF-IGFBP system during ultra endurance exercise (I)

In ultra endurance exercise, pituitary-gonadal hormones in the circulation and components of the IGF-IGFBP systems were evaluated concomitantly. In women estradiol was undetectable at the end of the race without compensatory increase in FSH and/or LH. In men, testosterone decreased to prepubertal levels and FSH decreased significantly. The changes (%) in testosterone or testosterone/SHBG did not correlate with the changes in t-IGF-I.

4.5.3 IL-6 concentrations in the circulation during endurance exercise with one large muscle group (III)

In women exercising with one leg for one h, IL-6 increased and peak venous IL-6 concentrations were attained 10 minutes after exercise (14.2 (7.9 - 44.5) ng/L). There was a release of IL-6 from the leg already at rest before exercise. The (v-a) difference was significantly increased from basal values after exercise, remaining elevated one h into recovery. There was no correlation between the changes in circulating IL-6 and md-IGF-I in either Ex-leg or Rest-leg.

4.5.4 Effects of IL-6 the IGF-IGFBP system in the circulation at rest (IV)

A 3 h systemic infusion of IL-6 in humans resulted in IL-6 concentrations reaching 100 ng/L, in the same range as those observed during aerobic endurance exercise with large muscle groups (176). There was no significant difference between groups in total IGF-I or f-IGF-I at any of the single time points. IGFBP-1 was unchanged during the infusion but increased significantly after the end of infusion as compared to the control subjects (saline infusion). The peak was reached two h after the infusion (102 (33) compared to 52 (34) ng/mL in the control subjects; $P < 0.01$). IL-6 infusion did not have any acute effects on IGFBP-3 proteolysis or total and free dissociable IGF-I in the circulation. The mean concentration of insulin was unchanged. Cortisol was significantly increased 1 h, 2 h and 3 h after the start of IL-6 infusion. No correlation between the individual changes in IGFBP-1 and cortisol was found.

4.6 LOCAL MUSCLE IGFBP PROTEOLYSIS

4.6.1 Investigation of skeletal muscle components with potential impact on IGF-I biovailability

4.6.1.1 Involvement of m-calpain in IGFBP proteolysis in vitro (V).

After activation of m-calpain with Ca^{2+}, intact [^{125}]I-labeled IGFBP-2 was cleaved into two major bands of 21 and 10 kDa, and [^{125}]I-labeled IGFBP-3 was totally degraded into several smaller fragments. Labeled IGFBP-1 was not degraded.

The primary cleavage sites in both IGFBP-2 and -3 were characterized by N-terminal sequence analysis and observed to be located in the non-conserved central linker region of the IGFBP molecule. When IGFBP-2 (2 μM) or non-glycosylated IGFBP-3 (10 μM) were incubated with varying concentrations of Ca^{2+}-activated m-calpain (0.02 – 0.6 μM), a dose-response effect could be observed. At the lowest m-calpain concentration

IGFBP-2 was degraded into three fragments with apparent molecular weights of 21, 19 and 12 kDa. At higher m-calpain concentrations the quantity of the 21kDa IGFBP-2 fragment decreased, whereas the quantity of the 19 kDa IGFBP-2 fragment increased. The N-terminal amino acid sequences obtained for the 21 kDa and19 kDa IGFBP-2 fragment were EVLFR in both cases, indicating that the firstly formed 21 kDa fragment is further degraded at the C-terminus. The N-terminal sequence for the 12 kDa fragment of IGFBP-2 was RQMGK, corresponding to a fragment starting at amino acid 164 in the central linker-region of the molecule. This specific cleavage site between H^{163} and R^{164} in IGFBP-2 has not previously been reported.

At the lowest m-calpain concentration, non-glycosylated IGFBP-3 was degraded into two major fragments with the apparent molecular weights of 16 kDa and 14 kDa, respectively. These IGFBP-3 fragments were further degraded into smaller peptides at higher concentrations of m-calpain. No change was observed with increasing time; the m-calpain cleavage patterns of IGFBP-2 and -3 observed at 20, 40, and 60 minutes were the same.

The N-terminal amino acid sequence of the 16- kDa IGFBP-3 fragment was GASSA corresponding to a fragment starting at the N-terminus and SKIII for the 14-kDa fragment, corresponding to amino acid 143 in the central linker region of IGFBP-3. The 13-kDa fragment appearing at higher m-calpain concentrations, simultaneously with the disappearance of the N-terminal 16-kDa fragment, was also an N-terminal fragment with the amino acid sequence GASSA, presumably arising from further cleavage of the C-terminus of the 16-kDa fragment. The specific cleavage site between H^{142} and S^{143} in IGFBP-3 has not been reported before.

When the binding pattern of m-calpain and glycosylated IGFBP-3 was monitored in real time by biosensor analysis, only low binding of m-calpain to IGFBP-3 could be detected in the absence of Ca^{2+}. Ca^{2+} activation resulted in a fast association of m-calpain to IGFBP-3, but also a fast dissociation, and the binding pattern was affected by the duration of Ca^{2+} activation. The decreasing mass amplitude of m-calpain binding with time of Ca^{2+}-activation was most likely due to autolysis of m-calpain rather than to proteolysis of IGFBP-3 as suggested by unaltered IGF-I binding to IGFBP-3 over time.

5 DISCUSSION

In the present thesis, local interstitial IGF-I concentrations in human skeletal muscle were assessed by microdialysis and related to the circulating components of the IGF-IGFBP system. This approach has not been taken before. Our data sheds new light on the acute regulation of IGF-I concentrations in skeletal muscle tissue.

5.1 LOCAL IGF-I IN MUSCLE AT REST AND DURING EXERCISE: ASSOCIATIONS WITH REGIONAL AND SYSTEMIC LEVELS

The local IGF-I response differed in exercising compared to resting skeletal muscle. There was a significant increase in Ex-leg md-IGF-$I_{absolute}$ during endurance exercise in both women and men. In study III a significant difference in md-IGF-$I_{absolute}$ between Ex- and Rest-leg was observed during exercise. When Ex-leg md-IGF-$I_{absolute}$ was corrected for calculated IGF-I recovery (md-IGF-I), the rise in Ex-leg during exercise was attenuated and did not reach significance. In Rest-leg md-IGF-$I_{absolute}$ as well as md-IGF-I decreased significantly after exercise. Recently, it has been recommended that microdialysate concentrations of peptides should be given as $md_{absolute}$ (raw data) instead of concentrations "corrected" for calculated recovery according to the internal reference technique (Fourth International Conference on Microdialysis, Sep 19-21 2007, unpunblished data and (52)). Our first submission of paper III was in accordance with this consensus, however, the reviewers were not supportive of this approach. Methodological considerations regarding the ^{14}C inulin recovery calculations are presented in the Methods section (3.2.). Although the increase in ^{14}C inulin reverse recovery in Ex-leg during exercise in study (III) is likely to reflect an increase in IGF-I recovery, the lack correlation between the changes in I-RR and md-IGF-$I_{absolute}$ during exercise suggests that probe recovery does not fully account for the increase in md-IGF-$I_{absolute}$. We suggest that the apparent delayed decrease in Ex-leg md-IGF-I reflects the increase in md-IGF-$I_{absolute}$ that was also observed in men (study II).

Basal skeletal muscle md-IGF-I levels were in the same range as f-IGF-I in serum. However, no correlation was found at rest or during exercise. In contrast, basal md-IGF-$I_{absolute}$ correlated significantly with t-IGF-I concentrations at rest. Our conclusion from these findings is that circulating and tissue bioactivity is of similar magnitude and

is related. However, the relationship is not simple and may reflect the complex and not fully understood mechanisms regulating transport of endocrine IGF-I to the tissues (12). Two other approaches have been reported to assess interstitial IGF-I in resting muscle. When skeletal muscle extracellular fluid was collected with a 3000 kDa probe (156), total IGF-I was reported to be 10-fold higher than md-IGF-I in the present study. The impact of IGFBPs collected along with IGF-I was not assessed by these authors. In the other approach, microdialysate from a 60 kDa probe, also likely to contain IGFBPs was analyzed using a free IGF-I kit (62). Microdialysate free IGF-I was 10-fold higher than free IGF-I in serum, suggesting the presence of a steep gradient of unbound IGF-I from the muscle to the circulation. The methodological difficulties associated with the determination of free IGF-I are discussed in the method section (3.4.1) in this thesis. This would imply that endocrine IGF-I does not contribute to local tissue levels. This conclusion is in contrast to the marked effects of endocrine IGF-I on glucose metabolism and insulin sensitivity in mice (229) and humans (33, 194). Such endocrine IGF-I effects are in accordance with our findings. Furthermore, the finding of a net uptake of t-IGF-I over the leg suggests that IGF-I is cleared in the resting muscle possibly following stimulation of the IGF1R.

In this thesis, exercise induced changes in interstitial unbound IGF-I concentrations were not reflected by circulating t-IGF-I concentrations. However, a release of f-IGF-I into the circulation over the Ex-leg was observed at the end of and shortly after endurance exercise. The highest net f-IGF-I release over the Ex-leg at these time points was found in those with the highest md-IGF-I (Ex-leg) during exercise, supporting that the source of IGF-I was the muscle itself. The release of f-IGF-I was not detectable in the large pool of t-IGF-I in the circulation which questions the validity of (v-a) determinations of total IGF-I for assessments of local changes (see method section 3.2.1). The increase in circulating t-IGF-I early in endurance exercise (II, III), although it may be associated with hemoconcentration, appears to have physiological relevance given the increase in Ex-leg md-IGF-$I_{absolute}$.

5.2 LOCAL IGF-I AT REST AND DURING EXERCE: REGULATION BY IGFBP-1.

The different responses in local IGF-I in exercising and skeletal muscle (study III) may be related to IGFBP-1. Rest-leg md-IGF-I decreased concomitantly with the increase in

circulating IGFBP-1. In the Ex-leg, md-IGF-I was unchanged. Different paradigms have been proposed for the actions of IGFBP-1 on IGF-I activity in the circulation and peripheral tissues (Figure 4). In our study, the changes in circulating IGFBP-1 appeared to affect Rest-leg md-IGF-$I_{absolute}$. It is conceivable that IGFBP-1 also impacts Ex-leg md-IGF-$I_{absolute}$ but with local release of IGF-I over-riding this effect during and shortly after exercise. Our data relating circulating IGFBP-1 and local tissue IGF-I are new although attempts to address this relationship have been made in studies of lymph vs. circulating levels (31). Circulating free IGF-I concentrations and IGFBP-1 have been reported to correlate negatively (87-89). After exercise, a decrease in circulating free IGF-I has been reported concomitantly with increased IGFBP-1 concentrations in one (53) but not all (127, 220) studies. Importantly, the data in this thesis suggest that changes in skeletal muscle tissue concentrations of unbound IGF-I are poorly reflected by free circulating IGF-I concentrations unless they are determined as venous and arterial concentrations ((v-a) differences).

If there is a temporal relationship between circulating IGFBP-1 and local IGF-I bioavailability it may not be as simple as first assumed. Changes in IGFBP-1 in serum are to a large part determined by hepatic production but insulin regulated changes in IGFBP-1 transport to the tissues (Paradigm II, Figure 4) may contribute (19). Consequently tissue changes in IGFBP-1 may not be completely reflected by the changes in circulating IGFBP-1. In human peritendinous connective tissue 3h after exercise, microdialysate IGFBP-1 concentrations have been reported to be elevated concomitantly with circulating IGFBP-1 (171). In that study, skeletal muscle was not explored. Interestingly we have previously reported a significant uptake of circulating IGFBP-1 over exercising muscle immediately after endurance exercise (2 h) with large muscle groups concomitant with a transient increase in insulin (23). This was not observed in the present thesis. The exercise induced increase in IGFBP-1 has been suggested to protect from hypoglycaemia by restricting the access of circulating IGF-I to skeletal muscle during and after endurance exercise (208) (Paradigm I, figure 4). Theoretically, a co-transport of IGF-I/IGFBP-1 into the tissues may also be able to cause the decrease in circulating free IGF-I observed in several studies (Paradigm II, figure 4). The exact cascade of events from changes in circulating IGFBP-1 to changes in local IGFBP-1 is unknown. Possibly, transcapillary transport of IGFBP-1 alone may inhibit local IGF-I activity. IGFBP-1 may also exert IGF-I independent effects by

interactions with integrins on the cell membranes as mentioned in the introduction of this thesis.

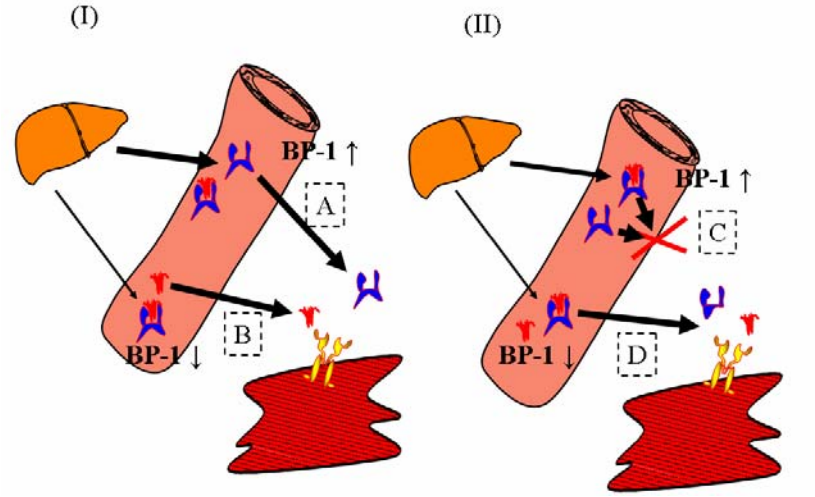


Figure 4. IGFBP-1 paradigms. ***I)*** *Circulating IGFBP-1 restricts the access of IGF-I to the tissues by binding IGF-I. High IGFBP-1 also allows more IGFBP-1 to reach the tissues further inhibiting IGF-I action (A). At low IGFBP-1, free IGF-I reaches the tissues and is not inhibited by IGFBP-1 (B).* ***II)*** *The transport of IGFBP-1 and IGFBP-1/IGF-I binary complex into the tissues is regulated (possibly by insulin). High circulating IGFBP-1 reflects accumulation of IGFBP-1 or IGFBP-1/IGF-I complex in the circulation and does not reflect tissue IGFBP-1 concentrations (C). Low IGFBP-1 on the other hand reflects higher clearance of IGFBP-I or IGFBP-1/IGF-I complex. The equilibrium between IGF-I and IGFBP-1 or other IGFBPs determines receptor bioavailability (D).*

Posttranslational modification of IGFBP-1 (i.e. IGFBP-1 phosphorylation state) may be one factor regulating the effect of IGFBP-1 on IGF-I bioavailability. In this thesis, IGFBP-1 phosphorylation state was unchanged after endurance exercise with one leg. The lack of such changes may be related to the small amount of muscle mass involved. An increase in the proportion of the lower IGF-I affinity N+LP form of IGFBP-1 have been observed in exercise with large muscle mass and 3 h duration and higher total IGFBP-1 concentrations (Keogh and Jakeman , unpublished data). In this thesis, IGFBP-1 phosphorylation state was not investigated in ultra endurance exercise.

Several factors are known to regulate IGFBP-1, insulin being the strongest regulator at rest but stimuli that may be relevant include IL-6, cortisol, adrenalin and glucagon which all increase in exercise. Other factors such as exercise intensity, muscle mass involved, duration and nutrition may affect IGFBP-1 via changes in these hormones (91, 175) or directly. In table 11, putative factors regulating circulating IGFBP-1 concentrations during exercise are listed (from available data at rest).

	Effect on circulating IGFBP-1	**reference**
In vivo studies (acute exposition)		
Insulin	↓ at rest	Reviewed in (140)
Cortisol	↑ at rest	(49)
Glukagon	↑ at rest	(108)
Epinephrine Norepinephrine	↑ at rest ↑ at rest	(74)
IL-6	↑ at rest	Study IV (thesis), (159)
Decreased liver glycogen content	↑ response in exercise (rats)	(137)
In vitro studies	**Effect on IGFBP-1 (liver cells)**	
AMPK	↑ in liver cell secretion	(142)
Insulin + IL-6	↑ in liver cell secretion	(192)
Hypoxia	↑ in IGFBP-1 gene expression	(211)

Table 11. Putatvive candidate factors regulating IGFBP-1 production in exercise-suggested by the present author based on available data at rest.

The increase in IGFBP-1 in endurance exercise was initially suggested to be caused by the decrease in insulin concentrations during exercise. However, when insulin and glucose levels are maintained by glucose ingestion during prolonged cycling exercise, IGFBP-1 still increases although the response is slightly reduced (115). This has led to the conclusion that other factors than insulin may be involved in the regulation of IGFBP-1 during exercise. In this thesis we demonstrate that moderately elevated circulating levels of IL-6 such as those observed in endurance exercise significantly elevates IGFBP-1 concentrations after the end of infusion. No changes in circulating f-IGF-I were observed. IGFBP-1 increased in spite of the lack of change in insulin concentrations. Insulin in combination with IL-6 increases IGFBP-1 in hepatocytes *in vitro* (192) suggesting that the induction of IGFBP-1 by IL-6 overrides the suppression by insulin. IL-6 has been shown to impair hepatic insulin signalling *in vitro* (197). During IL-6 infusion in resting subjects (IV), we observed an increase in cortisol

approximately 3 h before the rise in IGFBP-1, suggesting that cortisol may increase IGFBP-1 by affecting transcription. The promoter region of the IGFBP-1 gene has a regulatory element for cortisol (210). However, we could not demonstrate any correlation between the individual changes in IGFBP-1 and cortisol in the limited number of subjects that were studied. It can therefore not be excluded that a moderate increase in IL-6 directly stimulates hepatic IGFBP-1 expression. Apart from possible effects of circulating hormones on IGFBP-1 levels during and after exercise, hepatic factors such as a decrease in hepatic glycogen content have been suggested to modulate IGFBP-1 concentrations during and after exercise (137). These events may be linked either through an extrahepatic event (e.g. endocrine) or/and an event in the hepatocyte. A suggested intrahepatic event linking a decrease in glycogen content to an increase in IGFBP-1 concentrations may be the stimulation by AMPK (142), a metabolic sensor of the AMP-to-ATP ratio reported to increase in hepatocytes in exercise (37).

5.3 LOCAL IGF-I IN MUSCLE AT REST AND DURING EXERCISE-REGULATION BY PROTEOLYSIS OF IGFBP-3

IGFBP proteolysis is a potentially important mechanism to increase IGF-I bioavailability. Rapid local exercise-induced mobilization of tissue-bound IGF-I stores in the exercising muscle may, at least partly explain the increase in local skeletal muscle IGF-$I_{absolute}$ response in the exercising leg not observed in the resting leg (study III). A release of IGF-I from local stores may be induced by proteolytic cleavage of IGFBPs rather than de novo synthesis which likely to take hours to peak (139). The absence of IGFBP-3 fragmentation in the circulation in endurance exercise with one leg does not exclude that proteases are activated locally in the muscle, mobilizing IGF-I from IGFBP - bound stores. In fact, IGFBP-3 proteolysis has been suggested to be more extensive in the tissues than in the circulation where protease inhibition dominates in the normal healthy subjects (154). This may also explain why prolonged endurance exercise was required before a locally active protease had impact on IGFBP fragmentation in the circulation. In this thesis the *in vivo* IGFBP-3 degradation at the end of ultra endurance exercise was not associated with an increase in serum IGFBP-3 proteolytic activity. This suggests that IGFBP-3 fragmentation by local proteases in skeletal muscle tissue, possibly extending its activity to the vasculature may be reflected in the systemic circulation. Local muscle IGFBP proteases such as the MMP:s (Matrix Metalloproteinases) degrade IGFBPs *in vitro* in different cell systems

(reviewed in (80)) and have been demonstrated to increase in biopsies from exercised rat skeletal muscle (132). MMP-9 has been demonstrated to be activated in human skeletal muscle after endurance exercise (191). Another interesting IGFBP-3 protease candidate in exercise is plasmin. Endurance exercise activates both fibrinolytic and coagulatory pathways (reviewed in (65)). Tissue plasminogen activator (t-PA) activated by exercise is released from the vascular endothelial cells. Increased circulating t-PA levels as well as markers for plasmin generation and fibrin degradation have been reported in endurance exercise (157, 179, 222).

Theoretically, extracellular skeletal muscle IGFBP proteases may release IGF-I from IGFBP bound stores in the interstitial fluid or attached to the ECM (or possibly the vasculature). Intracellular IGFBP proteases, such as m-calpain, are likely to play a role in the regulation of the IGF-I independent effects of the IGFBPs in the muscle, unless exercise leads to disruption of muscle integrity and release of proteases. The cleavage of IGFBP-2 and -3 into distinct fragments by Ca^{2+} – activated m-calpain *in vitro* suggests that this may be a mechanism for regulation of IGFBP functions *in vivo*. No change in the Ca^{2+}-activated m-calpain cleavage patterns of IGFBP-2 and -3 was observed with increasing time. Furthermore, the mass amplitude of m-calpain binding to IGFBP-3 decreased with time of Ca^{2+}-activation. This was most likely due to autolysis of m-calpain rather than to proteolysis of IGFBP-3 as suggested by unaltered IGF-I binding to IGFBP-3 over time. This is in agreement with the view that calpains are autolytic enzymes with a short half-life after Ca^{2+}-activation. The intracellular location of m-calpain suggests that IGF-I independent effects of IGFBP-2 and -3 that require intracellular transport (138) and, in some cases, subsequent translocation to the nucleus would be the more affected. In muscle strips and adipocytes, exposure to calpain inhibitors reduces insulin-mediated glucose transport as well as glycogen synthesis (174). Future studies should clarify the possibility that calpains may affect the actions of IGFBP-3 such as the IGFBP-3 induced suppression of insulin stimulated glucose uptake in adipocytes (40) and in rodent muscle *ex vivo* preparations (Ahlsen et al, unpublished data).

Repeated exercise bouts with large muscle groups are likely to result in a more extensive local IGFBP-3 proteolysis. The difference in duration and involvement of muscle mass between our studies may explain why IGFBP-3 fragmentation was only observed in ultra endurance exercise. During ultra endurance exercise, CKMB reflects

the loss of muscle proteins into the circulation as a result of muscle damage (226). By such a mechanism, muscle proteases may get access to the circulation. The lack of a correlation between CKMB and IGFBP-3 fragmentation in the subjects performing ultra endurance exercise in our study (I) may reflect individual variation in the expression of skeletal muscle proteases and circulating protease inhibitors. In contrast to what we expected, there was a negative correlation between the changes in IGFBP-3 fragmentation detected in the circulation and the changes in circulating f-IGF-I.

5.4 IL-6 RELEASE FROM EXERCISING SKELETAL MUSCLE AND POSSIBLE INTERACTIONS WITH THE IGF-IGFBP SYSTEM

We hypothesized that IGFBP-3 proteolysis in the circulation during exercise may be induced by the increase in IL-6. In study III, we demonstrated an increase in the release of IL-6 over Ex-leg in women in association with endurance exercise, confirming previous studies in men (69). Peak venous IL-6 concentrations were markedly lower (14 ng/L) than those previously reported in endurance exercise with large muscle groups (100 ng/L), confirming that involved muscle mass and duration has an impact on skeletal muscle IL-6 production (176). No (v-a) differences in IGFBP-3 fragmentation were detected over the Ex-leg and no correlation with changes in md-IGF-I were observed in the 7 women included in study III. However a local IL-6 induced IGFBP-3 proteolysis in exercising skeletal muscle cannot be excluded. As previously mentioned, several of the MMPs are induced in rat and/or human skeletal muscle after exercise. IL-6 has been demonstrated to induce MMPs *in vitro* ((123, 133). MMP:s play a role in ECM remodelling and exercise induced angiogenesis in skeletal muscle (103). IL-6 may be an exercise induced factor regulating local skeletal muscle IGFBP proteolysis of importance for skeletal muscle adaptation.

The model in study IV was chosen to isolate one of the factors in exercise (acutely elevated circulating IL-6 concentrations) and explore the effects on the IGF-IGFBP system in resting men. An acute IL-6 infusion had no detectable effect on the fragmentation of circulating IGFBP-3. We did not determine local IL-6 concentrations in skeletal muscle in study IV and cannot exclude that they were lower than those observed in exercise. In fact, local interstitial IL-6 levels in skeletal muscle and/or peritendinous tissue have been shown to be higher than circulatory levels during and after endurance exercise (136, 190). Interstitial IL-6 concentrations in skeletal muscle (m.trapezius) reached concentrations as high as 2 000 pg/mL shortly after exercise

although the circulating IL-6 concentrations did not change significantly (190). The reliability of these md-concentrations are however difficult to judge since only "corrected" md-IL-6 values (and not the absolute values) were reported (methodology discussed in the method (section 3.3) and the discussion (section 5.1) in this thesis).

5.5 THE IGF-IGFBP SYSTEM IN ULTRA ENDURANCE EXERCISE – THE IMPACT OF NUTRITION AND THE PITUITARY-GONADAL AXIS

Energy deficit has been suggested to explain the decrease in circulating total and free IGF-I concentrations after endurance exercise. Local tissue concentrations of unbound IGF-I after endurance exercise have not been explored previously. In our study (III), circulating t- and f- IGF-I concentrations were decreased by 16 and 29 %, respectively one h after endurance exercise with one leg in women, concomitantly with significantly decreased concentrations in Rest-leg md-IGF-I. At the end ultra endurance exercise t- and f- IGF-I were decreased by 33 % and 54 %, respectively, without sex difference. Interstitial unbound IGF-I concentrations were not determined. Depressed circulating IGF-I concentrations have been suggested as a marker for endurance exercise induced energy deficit and overstrain likely to result in catabolism (158, 162). This is suggested to explain the finding of a decrease in circulating t-IGF-I after endurance exercise with one leg in women, but not in men. The exercise duration was shorter in men (45 min) than in women (60 min). Furthermore, the duration of the whole experiment when they were not eating was shorter in men (4 h in men vs 6 h in women). The observed decrease in circulating t- and f-IGF-I concentrations in ultra endurance exercise in both sexes are in accordance with previous reports from men/boys performing "acute" endurance exercise and/or exercise training associated with restricted energy intake (83, 158, 162, 163). Despite *ad libitum* nutritional intake in our study (I), the large median energy expenditure (77 000 kcal) is likely to be related to negative energy balance in all subjects. However, since energy deficit was only determined in three men, our observation of an association between the decrease in t-IGF-I at the end of the race and total energy deficit has to be interpreted with caution. In our study of ultra endurance exercise (I), fat mass expressed as relative to total body weight decreased significantly only in men. A decrease in women cannot be ruled out, since the number of women studied was low. The lack of significant changes in body weight may not correlate with energy deficit since oedema may significantly attribute to total body weight after a race of this duration and intensity (130).

Although energy deficit is suggested to be important for the reduction in total IGF-I concentrations with endurance exercise, the exact mechanism is unknown. Growth Hormone (GH) which stimulates hepatic IGF-I production is increased in both energy restriction (167) and endurance exercise (165, 205). However, there is an uncoupling of the GH/IGF-I axis in the liver observed as high GH and low total IGF-I concentrations in the circulation (66, 165, 167). It may be argued that ultra endurance exercise results in similar changes in the IGF-IGFBP system as those observed in fasting and starvation. However, fasting and starvation are not associated with IGFBP-3 proteolysis (15, 206) and/or increased insulin sensitivity (26). Such differences in between ultra endurance exercise and fasting/starvation suggest that the decrease in circulating IGF-I concentrations during ultra endurance exercise are not merely markers of energy deficit. An increase in renal excretion of proteins including IGF-I may be involved (178, 183). Furthermore, sleep deprivation has been reported to eliminate nocturnal GH pulses (57). Given the increase in GH during exercise (205) this effect appears to be minor and is not likely to explain decreased IGF-I production.
In the basal state before the race, women but not men had elevated basal IGF-I SDS. Interestingly, basal total IGF-I concentrations have been reported to be elevated in fit individuals in some studies. The large exercise training volume preceding an ultra endurance race did not result in decreased basal t-IGF-I concentrations in women but possibly in men.

The possible impact of ultra endurance exercise on local IGF-I mRNA and protein expression in skeletal muscle and gonads during exercise remains to be further investigated. In the men testosterone decreased markedly, confirming previous observations during endurance exercise in men (104, 165, 172, 173). A decrease in local tissue IGF-I in the testis may theoretically contribute to decreased testosterone production in ultra endurance exercise (48). In the women, estradiol and progesterone decreased regardless of menstrual cycle phase. Amennorhea in female athletes is secondary to a disruption in GnRH pulse activity (149, 224) but requires an energy deficit (150) as in the present study. The lack of a compensatory increase in gonadotropins may further add to the decrease in gonadal hormones and is in accordance with some (reviewed in (104)) but not all (165) endurance exercise studies. In study I, the interpretation of data at one single time point at the end of the ultra endurance race is complicated by the well known pulsatility and diurnal variation of the pituitary-gonadal hormones. Furthermore, these hormones may display different

patterns in the response to exercise/recovery. Serial sampling would have provided more information about the integrated levels of the hormones in the pituitary-gonadal axis than one single sample. However, this was not feasible in this study (I) and the data need to be further confirmed.

The majority of the observed changes in the IGF-IGFBP system in the circulation associated with ultra endurance exercise were independent of sex. IGFBP-2 was the only component to demonstrate sex related differences. Basal IGFBP-2 was significantly higher in men than in women in accordance with one previous report (166) and increased after exercise but only in women. In a previous study of repeated endurance exercise bouts during 6 days in men, IGFBP-2 was demonstrated to increase from the fifth day of exercise regardless of energy balance (186). IGFBP-2 is increased in resting subjects regardless of sex from the ninth day of fasting (Reviewed in ((212))). We only assessed energy balance in three men but negative energy balance was likely also in women. Possibly, the increase in IGFBP-2 observed after ultra endurance was related to a decrease in estradiol. Recent data has suggested that estrogen is a negative regulator of circulating IGFBP-2 concentrations (187). Basal IGFBP-3 PA was slightly higher in women than in men. This did however not result in a higher basal IGFBP-3 fragmentation and/or higher f-IGF-I concentrations in the circulation and needs to be confirmed. Local interstitial IGF-I concentrations were not determined.

5.6 POTENTIAL PHYSIOLOGICAL ROLE OF ACUTE CHANGES IN IGF-I IN EXERCISING SKELETAL MUSCLE

Changes in unbound IGF-I concentrations in the interstitial fluid can be assumed to have an impact on IGF-I effects in the skeletal muscle. Acute bouts of endurance exercise result in the activation of factors known to stimulate vascularisation. Interestingly, recent data demonstrates than the VEGF protein response to exercise training is a transcient process occurring with every single bout of exercise rather than the result of a constant upregulation of VEGF mRNA (101, 102). IGF-I stimulation of IGF1R is permissive for VEGF signalling through its own receptor (201). IGF-1R and hybrid insulin/IGF-I receptors are present in endothelial cells in several tissues (42, 43). We hypothesize that IGF-I may act indirectly by stimulating VEGF in skeletal muscle endothelial cells which is in line with the findings in other cells such as retinal pigment

epithelial cells (180) and osteoblasts (6). IGF-I plasmid therapy promotes angiogenesis in regenerating rat skeletal muscle (182).

Another potential role for IGF-I is the increase in glucose uptake and insulin sensitivity observed in association with exercise (reviewed in (28, 113)). A distinction should be made between an acute phase (during exercise and 1-2 h thereafter) and a late phase (persisting 16 h after exercise or longer). For the recruitment of GLUT4 vescicles to the muscle cell membrane in the acute phase, the energy-sensing AMP-activated protein kinase (AMPK) and the increase in intracellular Ca^{2+} have been demonstrated to be important. Furthermore, signalling pathways involving nitric oxide (NO), bradykinin and Akt may be involved (reviewed in (124)). In the late phase after exercise an increase in GLUT4 protein has been suggested to contribute to an increased sensitivity to insulin and other stimuli such as hypoxia. Both insulin and IGF-I stimulated glucose transport are enhanced in isolated rat muscle 9 h after endurance exercise (112). The possible role of IGF-I activity for the early and/or late phases of exercise induced increase in skeletal muscle uptake needs to be further explored. Microdialysis will need to be combined with other techniques such as muscle biopsy techniques and studies on intracellular signalling.

IGF-I has been demonstrated to stimulate protein synthesis as well as inhibit protein degradation in human forearm at rest (86). The possible role of local IGF-I in the prevention of lean body mass loss during ultra endurance exercise needs to be further investigated.

Although unbound IGF-I concentrations in the interstitial fluid can be assumed to have an impact on IGF-I effects in the skeletal muscle it may not linearly reflect IGF-I activity as discussed by Holly and Perks (114). IGF-I can indeed be sequestered away from cell receptors by IGFBPs restricting IGF-I activity. However the IGFBPs can also enhance activity at the cellular level by mechanisms such as cell membrane association increasing IGF-I concentrations close to the receptors or prevention of receptor down-regulation (75). The impact of such IGF-I enhancing effects by IGFBPs in skeletal muscle need to be further explored. Furthermore the biological effects of an increase in unbound IGF-I concentrations (as determined by microdialysis) need to be investigated *in vivo*.

6 SUMMARY AND CONCLUSION

The microdialysis methodology described in this thesis can be used to explore unbound IGF-I concentrations in human skeletal muscle.

Basal md-IGF-I at rest was in the same concentrations range as free (unbound) IGF-I in serum and correlated with total (bound plus unbound) IGF-I.

Endurance exercise with one leg (45 or 60 min) decreased md-IGF-I in the resting leg concomitantly with increased circulating IGFBP-1. Exercising leg md-IGF-I did not decrease and free IGF-I was released to the circulation (v-a difference) but with lack of correlation to changes in t-IGF-I, IGFBP-3 fragmentation or IGFBP-1 phosphorylation state.

The skeletal muscle protease m-calpain proteolyses IGFBP-2 and -3 *in vitro* and is suggested to be involved in the regulation of IGF-I independent IGFBP effects in this tissue.

Endurance exercise with one leg in women increases circulating IL-6 and IGFBP-1 but has no impact on IGFBP-3 proteolysis in the circulation. A 3h IL-6 infusion to resting subjects increased IGFBP-1 concentrations but had no effect on circulating free IGF-I or IGFBP-3.

Ultra endurance exercise results in similar IGF-IGFBP responses in men and women reflecting a catabolic state, including fragmentation of IGFBP-3 in the circulation. IGFBP-2 was the only exception. The gonadal hormones decreased but with no correlation to changes in the IGF-IGFBP system in the circulation.

In conclusion, this is the first evidence to support that increasing circulating IGFBP-1 concentrations decrease local skeletal muscle tissue IGF-I bioavailability at rest. In the exercising muscle, systemic factors have less impact on the regulation of unbound IGF-I. Local factors, such as IGFBP proteases may play a role.

7 FUTURE DIRECTIONS

The microdialysis methodology may be applied to explore the regulation of the local IGF-I and IGFBP activity. Specifically, with the availability of microdialysis probes with a larger cut-off, local IGFBP proteolytic fragmentation may now be explored *in vivo*. Such research may enhance our understanding of IGF-I dependent as well as independent effects in skeletal muscle. These effects are likely to be important for whole body glucose metabolism and anabolism. The effect of a local change in unbound IGF-I concentrations (as determined by microdialysis) on processes such as e.g. glucose uptake and/or protein synthesis should be explored *in vivo*. This would simplify the interpretation of md-IGF-I concentrations into IGF-I actions. The biological effects of the microdialysate *in vitro* should be explored by the use of additional techniques such as cell and tissue cultures, animal models and tools for the investigation of intracellular signalling. Furthermore, the local IGF-IGFBP system in conditions as e.g. diabetes mellitus, muscle disease, cancer and catabolic states may be investigated *in vivo* in humans of all ages in the search for treatment of such diseases.

8 POPULÄRVETENSKAPLIG SAMMANFATTNING

8.1 HORMONET IGF-I OCH DESS BINDARPROTEINER VID MUSKELARBETE

Resultaten i den här avhandlingen tyder på att nivåerna av det insulinliknande hormonet IGF-I är högre i arbetande än i vilande muskulatur. Nivåerna tycks inte regleras likadant. Metoden som utvecklats öppnar nya möjligheter att undersöka IGF-I i olika vävnader i kroppen och ta reda på mer om regleringen. Kunskaperna kan få betydelse för motionärer i alla åldrar och för människor som lider av t.ex sockersjuka och ledinflammationssjukdomar.

Det mesta av blodets IGF-I bildas av levern, men IGF-I bildas även ute i vävnaderna. Leverproduktionen av IGF-I stimuleras av tillväxthormon (GH) från hypofysen i hjärnan. Ute i vävnaderna stimuleras bildningen även på andra sätt. IGF-I ökar sockerupptaget i muskeln och ökar proteinbildning och celltillväxt. Sådant ser man också i olika grad i samband med muskelarbete. IGF-I är viktigt för längdtillväxten hos barn och det vävnads-producerade IGF-I kan ha extra stor betydelse. IGF-I binds till sex IGF-bindarproteiner (IGFBP 1-6) i blodet och i vävnaderna. Bara det IGF-I som är fritt/obundet kan binda till mottagarstrukturerna (receptorna) och utöva effekter. Det är okänt hur mycket som är obundet ute i vävnaderna där receptorerna finns. Mindre än 1 % är obundet i blodet. Vi ville undersöka snabba förändringar i obundet IGF-I i muskeln. Sådana förändringar skulle kunna bero på frisättning från bindarproteinerna (i blodet eller vävnaderna).

Mikrodialys är en metod som vi vidareutvecklade och utvärderade i studie II) och III). Efter bedövning förs ett litet membranförsett plaströr (3 cm långt) in i muskulaturen. Membranet har små hål genom vilka obundet IGF-I samlas upp medan IGFBP inte kan passera. På så vis kunde vi mäta nivåerna av obundet IGF-I i muskelvätska. Vi mätte nivåerna av IGF-I och IGFBP i blodet också, i artären till det arbetande benet och venen från benet. Då kunde vi undersöka om muskeln frisatte eller tog upp olika hormoner och andra ämnen, som socker t.ex. Försökspersonerna sparkade med ena benet mot ett motstånd i en särskild apparat i cirka en timme (”uthållighetsarbete”). **Vi fann** att nivåerna av obundet IGF-I sjönk i det vilande benet medan nivåerna var

oförändrade eller ökade i det arbetande benet. Nivåerna i den vilande muskeln visade ett samband med IGFBP-1 i blodet. Vi tror att det i arbetande muskulatur kan ske en frisättning av IGF-I från IGFBP på grund av förändringar i klyvning av IGFBPs i muskelvävnaden.. I framtida mikrodialys-studier ska vi undersöka detta.

I en provrörsstudie i studie V) **fann** vi att ett ämne i muskeln (m-calpain) som aktiveras vid muskelarbete klyver sönder två av IGFBP. Fyndet kan betyda att m-calpain reglerar effekterna av IGFBP-2 och -3 i muskelceller (de effekter som är oberoende av IGF-I).

I arbete IV) undersökte vi blodets IGF-IGFBP nivåer i samband med en infusion av IL-6 (Interleukin-6). IL-6 tillfördes i blodet (motsvarar ett ”dropp”). Det är känt att IL-6 ökar vid inflammation men också vid muskelarbete. IL-6 tycks ha betydelse för ämnesomsättningen vid muskelarbete (ökat sockerupptag i muskeln och ökad bildning av socker i levern, fettanvändning). **Vi fann** att ökade IL-6 nivåer hos vilande försökspersoner ökade IGFBP-1 i blodet. IL-6 kan alltså ha betydelse för regleringen av detta IGFBP vid muskelarbete.

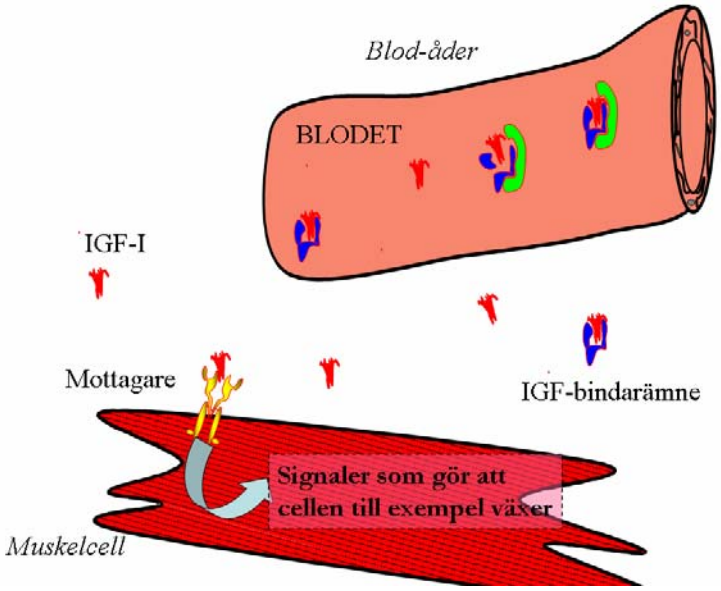
I arbete I) undersökte vi blodets nivåer av IGF och IGFBP samt könshormoner i samband med extremt uthållighetsarbete som pågick i cirka 5 dagar. Deltagarna sprang, cyklade, klättrade, vandrade och paddlade. De sov bara några timmar/dygn och utvecklade en extrem energibrist. **Vi fann** att IGF-I och IGFBP förändrades som vid svält men med ett viktigt undantag vad gällde IGFBP-3 som hade klyvts i bitar. Detta fynd antyder att förändringarna inte bara kunde förklaras av energibristen. Specifika processer aktiveras vid extremt uthållighetsarbete och syftet är möjligen att skydda från kraftig nedbrytning av muskelprotein. Vi såg inga könsskillnader mellan kvinnor och män. Könshormonerna östrogen och testosteron sjönk kraftigt men vi fann inget säkert samband med förändringarna av IGF-I och IGFBP. Vi kunde inte undersöka vävnaders obundna IGF-I nivåer i denna extrema studie.

Smmanfattningsvis tyder våra undersökningar på att nivåerna av det uppbyggande hormonet IGF-I är högre i arbetande än i vilande muskel och att regleringen är annorlunda. Fynden och den använda metodiken kan bana väg för en ökad kunskap om hur IGF-I regleras i vävnaderna. Det i sin tur kan få betydelse för hur vi väljer att behandla exempelvis sockersjuka.

8.2 SAMMANFATTNING FÖR BARN

När du hoppar, skuttar, cyklar och springer blir kroppen glad och mår bra. Du har säkert märkt att du orkar mera och blir starkare om du rör sig ganska ofta och mycket. Det beror på att musklerna förändras och hjärtat orkar mera. Jag blev nyfiken på det här, så jag började forska. Vill du hänga med en stund?

Bilden visar en muskelcell och en blodåder. Cellerna är de små små bitar som varje del av kroppen är uppbyggd av. Kan du se blodådern? Det är bara en liten bit som visas, blodådrorna är som rör som slingrar sig genom kroppen. IGF-I på bilden är ett hormon. Det säger till vad muskelcellerna ska göra. Muskelcellerna kanske ska växa eller suga upp socker från blodet så de kan arbeta hårdare. IGF-I talar om detta för cellen genom att sätta fast sig på ”mottagaren”. Då får cellen meddelande av IGF-I. Kan du hitta mottagaren på bilden?



Hormonet IGF-I finns både i blodet och ute i muskeln. Vi ville veta mer om när IGF-I väljer att vara kvar i blodet istället för att ta sig ut i muskeln. Om IGF-I är kvar i blodet kan det ju inte ”prata” med cellen genom mottagaren!
Vi forskade massor och kom på att man kan undersöka IGF-I i muskeln med en liten plastslang som fångar upp det. Det finns bindarämnen som håller kvar IGF-I i blodet. Det verkade vara mer IGF-I i en muskel som arbetar än en som vilar. Bindarämnena i blodet ändrades också då muskeln arbetade.

Människorna som vi undersökte idrottade på olika sätt. Några sparkade i en särskild maskin med bara ena benet i en hel timme! Andra var ute i bergen i 5 dagar och sprang, klättrade, paddlade och cyklade! Vi undersökte IGF-I och bindarämnena hos dem. Det var spännande.
Kanske kan det vi kommit fram till hjälpa sjuka människor. Kanske inte lika många blir sjuka. Kanske kan man ge goda råd till idrottare så att de inte skadar sig av all träning? Det får vi se när vi lärt oss ännu mer om det här!
Kul att du hängde med hela vägen hit! Hälsningar Ulrika

9 ACKNOWLEDGEMENTS

This work was supported by the Swedish National Center for Research in Sports, the Samariten Foundation, the Wera Ekstrom Foundation, the Frimurare Barnhuset Foundation in Stockholm, the Swedish Society of Medicine, the Society for Child Care, the H.R.H Crown Princess Lovisa Society for Medicine.

There have been so many people involved on the journey to complete this thesis: supervisors, colleagues, subjects in the studies, students, friends and family. It is impossible to mention all of you! Thank you! I would like to mention and thank some of you specifically below:

Peter Bang, my main supervisor. Thank you for sharing your great knowledge with me, and for all the stimulating and challenging scientific discussions we have had. You really have listened carefully and you have not hesitated in commenting and asking for more, making me develop intellectually. I also appreciate your generous behaviour at international meetings, sharing your contacts and introducing me to knowledgeable and nice researchers in the field. Finally, thank you for your "joie de vivre" and the delicious sushi dinners at your home!

Carl Johan Sundberg, co-supervisor for your enthusiasm and warmth. You ask the smart questions, with fresh intellect. Thank you also for being a role model when it comes to teaching activities, and for inviting me to teach different groups of students all through the years.

Christine Carlsson-Skwirut, co-supervisor for being such a warm, supporting, intelligent and skilled person. You have taught me lab-work, letting me think and try but you have always been there to answer questions (also the strange ones). I would never have made this without you. In addition, you are warm and understanding and a nice travel companion!

Kerstin Hall, my former supervisor at the "adult" Endocrinology Department at Karolinska Institutet. Thank you! You did not only opened the doors to your lab enabling me to analyze the samples from my first exercise study. You also shared your broad knowledge in endocrinology with me and Bengt Saltin and you have always believed in me and my project. Thanks to Kerstin Brismar, Inga-Lena Wivall and

Elisabeth Bucht for your friendly and supportive style. **Bengt Saltin**, thank you for introducing me to exercise physiology.

Thomas Gustafsson at the Clinical Phyisology Unit at Karolinska University Hospital (Huddinge) for being supportive and for thrilling scientific discussions and nice project collaborations. You are a "quality guy", thanks for being there! Thank you also to **Lennart Kaijser** , **Eva Jansson**, **Mona Esbjörnsson-Liljedahl** and **Barbara Norman** for nice collaborations.

Jan Henriksson, **Urban Ungerstedt** and **Michel Goiny** at the Department of Physiology and Pharmacology for being people knowledgeable in microdialysis , willing to discuss methodological issues in depth.

Lars Hagenäs at the Pediatric Endocrinology Unit for thrilling scientific (and other!) discussions with new, sometimes provocative "angles" on things. Thank you for giving me the opportunities to give lectures about things that are only "touching" my main scientific area, making me stretch out and develop intellectually.

I want to thank **Olle Söder** and **Martin Ritze´n** for being open and dynamic, drawing a lot of nice people from all over the world to the lab. And all these nice people themselves-my colleagues at the lab all through the years! You have all been around for talks, laughs and support: **Mona-Lisa Strand, Aida Wahlgren, Cecilia Zetterström, Cecilia Pedersen, Emma Eriksson, Paula Fernandez, Vichit Suporsilchai, Konstantin Svechnikov, Irina Svechnikov, Andrei Chagin, Eva Horemuzova, Ola Nilsson, Mi Hou, Kirsi Jahnukainen, Klas Ekström, Jenny Salemyr, Shahzad Ahkram, Elham Karimian, Britt Maseroni, Lena Sahlin, Theri Heino, Katja Sundström, Lin Ma, Carolina Bianchi, Therese, Nina Renlund, Dionisisos Chrysios, Giedre GGrigelioniene, Yvonne Löfgren, Susanne Hallberg.** Thanks **Lars S** for attending the PhD seminars with enthusiasm and interesting questions. Thanks to **Farasat** and **Damreen Zaman** for delicious Pakistani food at your home and **Farasat** for very interesting discussions at the lab. on culture, identity and life choices. Thank you **Eugenia Colon** for sharing your journey into the Swedish "läkarliv" with me. Thanks to **Sara Pihl** for being warm, human and interesting to discuss science with. Thanks to **Maria Ahlsen** for support (computer and life realted matters!), discussions and good laughs!

Astrid Häggbladh – thank you for always having answers to administrative questions and for being structured and helpful when papers were not always in order. And **Mikael Dahn**, the computer emergency guy, always calm and doing your very best to help as fast as possible! That kept my adrenalin-levels down!

Thanks to my new enthousiastic and energetic and smart collaborators during the last few years at the Swedish School of Health Sciences: **Mikael Mattson**, **Jonas Enqvist**, **Björn Ekblom** and the most recent of them **Örjan Ekblom**.
Thanks to **Maria Berner** and **Eva Örtqvist** at the Diabetes Unit at Astrid Lindgren Children´s Hospital.

Thanks to all the inquisitive and enthusiastic students I have met all through the years. Some of you have also contributed scientifically and/or practically to the present project: **Elisabeth Kuldkepp** and **Henning Wallerstedt**.

Thanks to all the **subjects** who participated in the studies!

My dear friends: Thanks to **Karin Billing** for having been there since 1971, "I vått (!) och torrt", always in contact, for sharing laughs and sorrows. **Jenny Hygins** for believing in me and asking uncomfortable questions. **Åsa Holgren** for being you and so positive towards my research project. **Gunilla v Perner** for being a thoughtful and humoristic person in combination. **Karin Aurelius** for being direct, courageous and fun. **Gi Broman** for introducing me to the crazy world of exercise physiology research and for our deep friendship. **Gunnar Edvardsson** for all the new sources of joy you introduced in my life. **Erica Fosse** for being you! **Hans Lindgren** for nice "walks and talks". Thanks to the dancers who made me leave "science" behind and relax-some of you also became friends outside the dance floor: **Claes Schumann**, **Torbjörn Hygins, Göran Winther**, **Malin Hallberg**, **Thomas Allander**, **Micke Spetz, Göran Flank** and **John Änggård**. Thanks to all other friends who make my life so joyful and interesting.

Thanks to **Anna-Karin Wikström,** physiotherapist and friend and **Peter Försth**, ortopedic surgeon, for helping me back from my disc herniation to a wonderful life without neurological pain.

Peder, Anniqa, Ella and **Hugo:** Thank you for being so generous and welcoming and for very good communication.

Thanks to my relatives **Marianne** and **Stefan** with families, **"Moster Lotta"** and **Bengt-Olov** and **Katinka, Emmanuel** and **Nils** ("open-door and always delicious food-policy"). Thanks to my neighbour Alice for your help and all nice chats.

Thanks to my beloved parents **Ingrid** and **Ulf**. You always believe in me and support my ideas and my values in life. You gave me a valuable attitude to learning: that it is joyful and fun and that anything can be learned if you really want it. **Magnus**, the best big brother I could ever wish for: supportive, creative, enthusiastic and sincere and thanks to **Pernilla**. Thanks to my wonderful sister **Fredrika** for being always there as my "kloka gumma" with warmth and tenderness and thanks to **Göran** and **Melker**.

Last but not really not least: Thank you **Emmy** for being the most lovely and creative angel girl in town. And **Emil**, thank you for every second I share with you. You taught me to be "here and now". I love you, your tricky questions and your warm hugs.
TACK!

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