MECHANO-DEPENDENT SIGNALING PATHWAYS CONTROL PROTEIN SYNTHESIS IN SKELETAL MUSCLE

Graduate School for Cellular and Biomedical Sciences

University of Bern

PhD Thesis

Submitted by

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Accepted by the Faculty of Medicine, the Faculty of Science and the Vetsuisse Faculty of the University of Bern at the request of the Graduate School for Cellular and Biomedical Sciences

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Prof. Dr. Andreas Zurbriggen

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B Projects

My PhD period was characterized by being involved in many research projects of Martin Flück as well as of Hans Hoppeler and his group. The account below specifies my input to the different studies. At the end of the thesis in the appendix, copies of the published manuscripts and of those in preparation are attached.

Main studies

The following 3 main studies have been the principal focus of my PhD work. They are therefore also the basis of the introduction, summarized in the results and discussed at the end of the thesis.

1) Eccentric exercise

Topic:

Adaptations of the muscle transcriptome to a mild eccentric ergometer exercise

Project leader:

Prof Dr Martin Flück

Grant:

Eidgenössische Sportkommission (ESK) 2004-2007 to Prof Dr Martin Flück and Prof Dr Hans Hoppeler

My involvement in the project:

A) Realization and development of physical tests and training protocol (Pmax entry test, squat and counter movement jump, CK measurements, e-bike protocol)

B) Morphometry (assistance in biopsy collection, muscle sample processing, light microscopy, stereological analysis, morphometric calculations)

C) RNA processing (sectioning biopsies, RNA isolation, gel electrophoresis, reverse transcription, RT-PCR)

D) Microarray (performing muscle-specific microarray, hybridization experiments)

E) Data analysis (statistical analysis of microarray data)

F) Publishing (data interpretation, manuscript writing, review process as corresponding author)

Publication:

Klossner S, Däpp C, Schmutz S, Vogt M, Hoppeler H, Flück M

Muscle transcriptome adaptation with mild eccentric ergometer exercise

Pflügers Arch - Eur J Physiol, DOI 10.1007/s00424-007-0303-6

2) FAK overexpression and hindlimb suspension

Topic:

Mechano-transduction towards an increased muscle protein synthesis is modulated by overexpressed FAK

Project leader:

Prof Dr Martin Flück

Grant:

Swiss National Science Foundation (SNF) 310000-112139 to Prof Dr Martin Flück

My involvement in the project:

A) Learning and applying technical surgical hindlimb suspension experiments

B) Protein analysis (Isolation, SDS-PAGE, Western Blot and immuno-precipitations)

C) Immunohistochemistry with different antibodies

D) Data analysis and interpretation

E) Publishing (data interpretation, paper writing, review process)

Publication:

Klossner S, Durieux AC, Freyssenet D, Flück M

Mechano-transduction to muscle protein synthesis is modulated by FAK

Eur J Appl Physiol 2009, DOI 10.1007/s00421-009-1032-7

3) Mechano-transduction with tenotomy

Topic:

Activation of signaling molecules towards increased protein synthesis with tenotomy

Project leader:

Prof Dr Martin Flück

Grant:

SNF 310000-112139 to Prof Dr Martin Flück

My involvement in the project:

A) Setting up and writing an application for the animal experiments to the “Kantonale Tierschutzkommission”

B) Learning and applying surgical tenotomy experiments

C) Protein analysis (Isolation, SDS-PAGE, Western Blot and immuno-precipitations)

D) Data analysis and interpretation

Publication: Klossner S, Hoppeler H, Flück M.; Tenotomy activates FAK and S6K signaling in rat *soleus* muscle; Manuscript in preparation

Other Studies

The following studies are projects I have been involved during my PhD. I performed additional experiments necessary for publication of previous work, assisted in writing reviews, or performed experiments for a new study on PMP22 mice, which is not yet ready for publication.

4) PMP22

Topic:

Muscle loading overrules PMP22-mediated and nerve-dependent gene regulation

Project leader:

Prof Dr Martin Flück

Grant:

SNF 310000-112139 to Prof Dr Martin Flück

My involvement in the project:

A) Performing hindlimb suspension experiments

B) RNA processing (RNA isolation, gel electrophoresis, reverse transcription, RT-PCR)

C) Microarray (performing muscle-specific microarray, hybridization experiments)

D) Fiber-typing (ATPase reaction, light microscopy, stereological analysis, morphometric calculations)

Publication:

Klossner S, Durieux AC, Giraud MN, Sancho S, Flück M

Muscle loading overrules nerve-dependent gene regulation

Manuscript in preparation

5) Eccentric exercise in senior subjects

Topic:

Functional and structural response of eccentric, concentric and cognitive exercise in the elderly and its consequence on the risk of falling

Project leader:

Prof Dr Hans Hoppeler

Grant:

SNFP53 405340-104718.1 2004-2007 to Prof. Dr. Hans Hoppeler and Prof. Dr. Walter Perrig

My involvement in the project:

A) Development and realization of functional tests (ramp test with ergospirometry, force measures for leg strength, dosage on the eccentric bike, risk assessment for falling)
B) Testing (timed-up-and-go test) and assistance in trainings
C) Data analysis (functional data)
Publication:
Lötscher F, Löffel T, Steiner R, Vogt M, Klossner S, Popp A, Lippuner K, Hoppeler H, Däpp C.
Biologically relevant sex differences for fitness-related parameters in active octogenarians
Eur J Appl Physiol 2007, DOI 10.1007/s00421-006-0368-5

6) Review on muscle plasticity
Project leader:
Prof Dr Hans Hoppeler
My involvement in the project:
A) Assistance in writing the review
B) Interpretation and incorporation of recently published work
Publication:
Hoppeler H, Klossner S, Flück M.
Gene expression in working skeletal muscle
Hypoxia and the Circulation + Adv Exp Med Biol 2007, Chapter 21, Springer, New York

7) Review on hypoxic training
Project leader:
Prof Dr Hans Hoppeler
My involvement in the project:
A) Assistance for Prof Dr Hans Hoppeler in writing the review
B) Interpretation and incorporation of recently published work
Publication:
Hoppeler H, Klossner S, Vogt M.
Training in hypoxia and its effects on skeletal muscle tissue
Scand J Med Sci Sports 2008, DOI: 10.1111/j.1600-0838.2008.00831.x

8) Tenascin-C and muscle repair
Topic:

Involvement of Tn-C, a mechano-regulated, morphogenic, extracellular matrix protein that is associated with tissue remodeling, in muscle repair

Project leader:

Prof Dr Martin Flück

Grant:

SNF 31-65276.01 and SNF 310000-112139 to Prof Dr Martin Flück

My involvement in the project:

A) Hosting of animals

B) RNA processing (RNA isolation, gel electrophoresis, reverse transcription, RT-PCR)

C) Protein analysis (Isolation, SDS-PAGE, Western Blot)

D) Data analysis and interpretation

Publication:

Flück M, Mund S, Schittny J, Klossner S, Durieux AC, Giraud MN

Mechano-regulated Tenascin-C orchestrates Muscle Repair

PNAS 2008, 105(36):13662–13667

9) FAK and FRNK overexpression with reloading

Topic:

FAK signaling in mechano-regulated differentiation of slow-oxidative muscle and FAK in the dominant mechano-regulator motor performance via control of gene expression

Project leader:

Prof Dr Martin Flück

Grant:

SNF 310000-112139 to Prof Dr Martin Flück

My involvement in the project:

A) RNA processing (RNA isolation, gel electrophoresis, reverse transcription, RT-PCR)

B) Protein analysis (Isolation, SDS-PAGE, Western Blot)

C) Data analysis, interpretation and assistance in paper writing

Publication:

Durieux AC, D'Antona G, Desplanches D, Freyssenet D, Klossner S, Bottinelli R, Flück M.

Focal adhesion kinase is a load-dependent governor of the slow contractile and oxidative muscle phenotype

J Physiology 2009, DOI: 10.1113/jphysiol.2009.171355

10) Eccentric exercise in senior subjects

Topic:

Structural response of eccentric, concentric and cognitive exercise in skeletal muscle of elderly subjects

Project leader:

Prof Dr Hans Hoppeler

Grant:

SNFP53 405340-104718.1 2004-2007 to Prof. Dr. Hans Hoppeler and Prof. Dr. Walter Perrig

My involvement in the project:

A) Development and realization of functional tests (ramp test with ergospirometry, force measures for leg strength, dosage on the eccentric bike, risk assessment for falling)

B) Data analysis (functional data)

Publication:

Mueller M, Breil FA, Vogt M, Steiner R, Klossner S, Hoppeler H, Dapp C

Different response to eccentric and concentric training in older men and women

Eur J Appl Physiol 2009, DOI 10.1007/s00421-009-1108-4

11) Meta-analysis and review of hypoxic training

Project leader:

Prof Dr Hans Hoppeler

My involvement in the project:

A) Collecting data, setting up master file for the meta-analysis

B) Interpretation and assistance in writing

Publication: von Elm E, Klossner S, Jüni P, Vogt M, Hoppeler H.; Meta-analysis on hypoxic training; Manuscript in preparation

12) Review on the role of IGF1 in regulating muscle hypertrophy

Project leader:

Prof Dr Martin Flück

My involvement in the project:

A) Collecting previously published data and interpretation

B) Assistance in writing

Publication:

Flück M, Klossner S, Goldspink G

IGF-1 is not the major physiological regulator of muscle mass

Manuscript in preparation

13) Review on muscle plasticity with acute and chronic exercise

Project leader:

Prof Dr Hans Hoppeler

My involvement in the project:

A) Collecting previously published data and interpretation

B) Assistance in writing

Publication:

Hoppeler H, Lurman G, Mueller M, Klossner S, Baum O

Molecular mechanisms of muscle plasticity with acute and chronic exercise

Manuscript in preparation for “Handbook of Physiology”

C Abstract

The aim of training is to provide an overload stimulus provoking specific molecular responses to enhance the adaptive phenotype of skeletal muscle. In this regard, the identification of key regulatory factors of skeletal muscle adaptation, which are likely to contribute in promoting the specificity of training responses and lead to the desired muscle adaptations, is important. Resistance training therefore should up-regulate the translational machinery and satellite cell activity, increasing protein synthesis and muscle cross-sectional area. Conversely, endurance training should activate pathways to promote adaptation towards enhanced oxidative capacity and resistance to fatigue during prolonged contractile activity.

Investigating the temporal response of muscle gene expression to a single bout of eccentric exercise, I could not identify a major up-regulation of transcripts relevant for processes supporting muscle growth. I demonstrated that eccentric exercise has a molecular time-course which is different from concentric exercise, where within the first 24 hours a major up-regulation of genes might be observed. This basic difference between the molecular responses of the two training regimes leads to the hypothesis that upon mechanical stimulation the adaptations are mediated through alterations in translation instead of transcription.

With the use of overexpressing the mechano-transducer FAK in combination with reloading of atrophied muscle, I could identify an induction of protein synthesis rate through an activation of S6K. This activation was dependent on FAK and was not mediated through Akt. These results were confirmed with a much greater mechanical stress where S6K was shown to be activated via Akt, shortly after the high mechanical load, and FAK, within 24 hours and almost “normal” muscle activity. These findings expand our current understanding of muscle biology in response to hypertrophic stimuli and establish FAK as an important upstream element in mechano-transduction towards increased protein synthesis rate.

The adaptation continuum of skeletal muscle provides a framework to assess the molecular bases of adaptation to training. However, this simplified approach of characterizing the adaptation to endurance and resistance training does not address the multifaceted nature of training specificity. This is undoubtedly complicated by the addition

of other training modes, differences in the genetic background, nutritional interventions and recovery modalities. Nonetheless, continued discovery of mechanisms involved in regulating the adaptive response will enhance our understanding of the specificity of training adaptations. Greater knowledge regarding exercise-induced adaptation in skeletal muscle requires the application of innovative training interventions to promote and extend our current understanding of adaptive events that may ultimately translate to novel training practices for athletic endeavor. Understanding the specificity of training adaptation is not only important for sport and exercise scientists, but may also provide therapeutic targets for the treatment of acute and chronic skeletal muscle diseases.

1 Introduction

1.1 Structure and function of skeletal muscle

Running, walking, swimming and flying all depend on the ability of skeletal muscle to contract and transmit tension through tendons to the skeleton.

Skeletal muscle fibers are multinuclear syncytia that are derived from the fusion of individual muscle precursor cells. Each skeletal muscle fiber is connected to a single motor-neuron and is surrounded by extracellular matrix (ECM). This meshwork of protein filaments and proteoglycans, called the endomysium, contains also capillaries and nerve fibers. Muscle fibers form bundles and these bundles together with the whole muscle are further surrounded by sheets of ECM named perimysium and epimysium, respectively. The muscle fibers merge into tendons that transmit the mechanical force, produced by muscle contraction, to the skeleton (Billeter *et al* 1994).

1.1.1 Muscle structure

Eighty percent of the fiber volume is packed with contractile elements, the myofibrils. The myofibrils of striated muscles are connected to a network of cytoskeletal proteins, which are involved with the sarcolemma and the nuclear membrane. Each myofibril consists of a chain of contractile units, or sarcomeres, which give the skeletal muscle its striated appearance. Each sarcomere extends from one Z disc to the next, where the thin actin filaments are attached. Troponin, tropomyosin and nebulin are proteins associated with actin. While the first two play a part in the control of contraction and relaxation, the latter is thought to act as a molecular ruler and therefore to regulate the length of the thin actin filament. The thick myosin filaments are attached to the M line, which is held in the middle of the sarcomere by titin. Part of each titin molecule is closely associated with myosin molecules, the rest of the molecule is elastic and changes length as the muscle contracts and relaxes. The myosin filaments have side arms, or cross-bridges, that make contact with the adjacent actin filaments.

The sarcoplasmatic reticulum (SR) is a network of internal membranes in the cytoplasm of muscle cells surrounding each myofibril. It consists of terminal (t-) and longitudinal (l-) tubuli. The t-tubuli are inversions of the SR and their inside constitutes extracellular space while the l-tubuli are internal to the muscle fibers and equivalent to the endoplasmatic reticulum of eukaryontic cells.

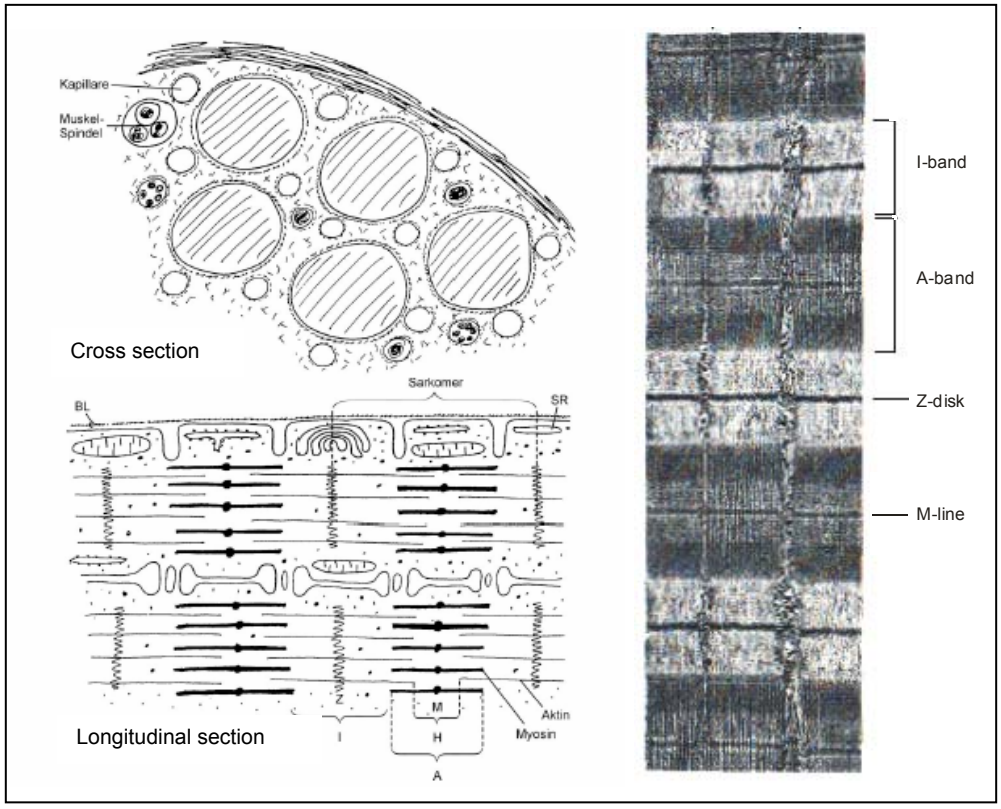

Figure 1: Sarcomeres, the contractile unit of muscle
A sarcomere is defined as the region between two subsequent Z-disks. At these Z-disks the actin filaments are attached. In the middle of each sarcomere lies the M-line where the myosin molecules are attached. In the microscope two regions within a sarcomere are clearly distinguishable: a light region called the I-band and a dark region, the A-band. (Copyright, University of Berne, 2005)

1.1.2 Fiber types

Muscle fibers can be classified into fast and slow fibers. The main components of the contractile apparatus are the actin and the myosin filaments. Each myosin molecule is a hexamere comprised of two heavy chains (MHC) and four light chains (MLC) which are assembled to form two globular heads with a long tail. In normal adult human skeletal muscle fibers, three MHC isoforms may be expressed: one slow, the MHC-I, and two fast, MHC-IIa and MHC-IIx. The latter is the fastest myosin isoform. Myosin contains the motor- and the ATPase-activity domains and differences in the kinetics of these ATPases are responsible for the different contraction velocities of the fiber types. Fibers may contain more than one MHC isoform and in addition to the heavy chain components it contains also two pairs of MLC. These MLCs also exist as fast and slow isoforms and together with the fast and slow isoforms of troponin, tropomyosin and other proteins they provide a multiplicity of contractile and regulatory protein isoform expression in muscle (Schiaffino and Reggiani 1996).

Individual muscles differ in their fiber type composition depending on their function and there is virtually no muscle that would contain only one fiber type. Muscles involved in supporting the posture have high proportions of slow, type I fibers, (i.e. *M. soleus*). They generate their energy predominantly from oxidative phosphorylation and produce tension at low energetic cost (i.e. with low ATP turnover rate). By contrast, fast muscles that contain mostly type II fibers are used in short and intense work (i.e. *M. iliopsoas*). They have a greater ATP turnover rate and derive energy more from conversion of carbohydrates by glycolysis (Harridge *et al* 1996). As a general rule, limb muscles close to the bone have higher percentage of type I fibers than muscles situated more superficially. This is also a consequence of the recruitment pattern of motor-units.

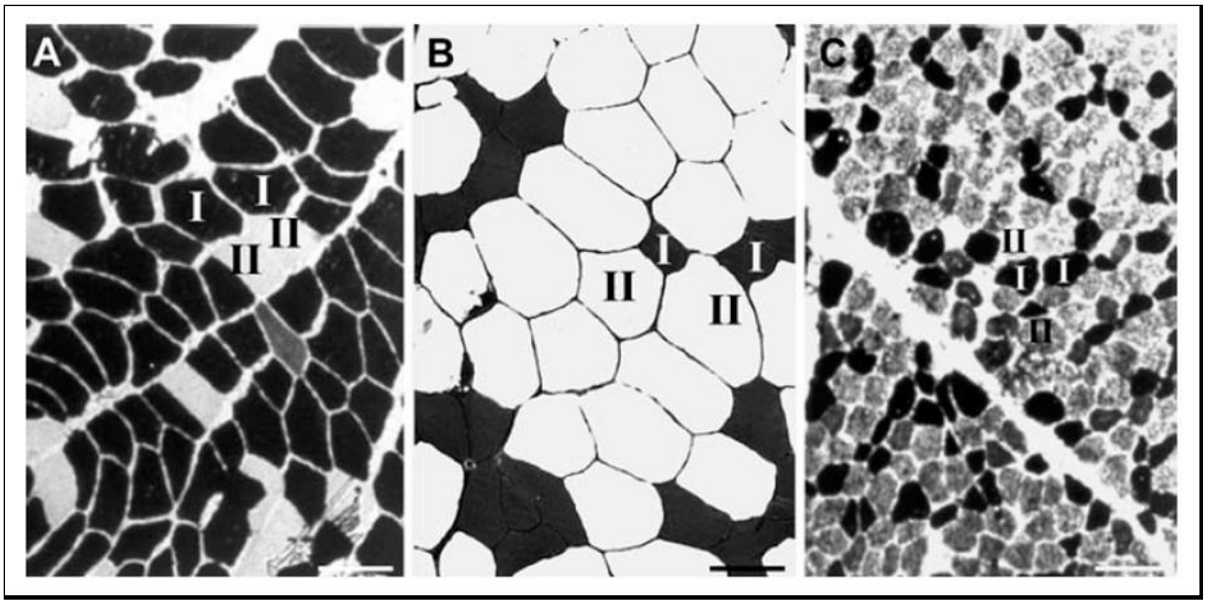

Figure 2: Plasticity of human skeletal muscle fiber types
Histochemical pictures showing adaptations of fiber types in human *m. vastus lateralis* with endurance exercise (A), resistance training (B) and denervation subsequent to spinal cord injury (C). Slow- (I) and fast-type (II) fibers are indicated. Bar, 100 µM (Billeter *et al* 2003).

1.1.3 Muscle contraction

A single motor-neuron connects hundreds of muscle fibers and controls their contraction via depolarization of the membrane. When an action potential is triggered, all the fibers innervated by a given motor-neuron are activated and all these fibers have the same contraction characteristics (Pette 2001).

A nerve impulse reaches the associated muscle fibers and leads to the release of acetylcholine (ACh) at the neuromuscular junction between the nerve and the muscle fiber (motor end-plate). ACh diffuses to the nicotinic acetylcholine receptors on the surface of the muscle fiber and these open sarcolemmal Na^+ channels. If the change in Na^+ levels is sufficient to trigger a depolarization, an action potential spreads along the surface of the

muscle. The electrical excitation stumulates the transverse tubules, or T-tubules, that extend inward from the plasma membrane around each myofibril. The signal is relayed across a gap to the sarcoplasmic reticulum. In the junctional region the ryanodine receptors (RyR), Ca^{2+} release channels, extend from the sarcoplasmic reticulum (SR) to make contact with the voltage-sensitive dihydropyridine receptors (DHPR) in the T-tubules. With the incoming action potential, DHPR triggers some of the RyR (probably by mechanical coupling) to open. This causes the release of Ca^{2+} ions and leading to further opening of Ca^{2+} channels.

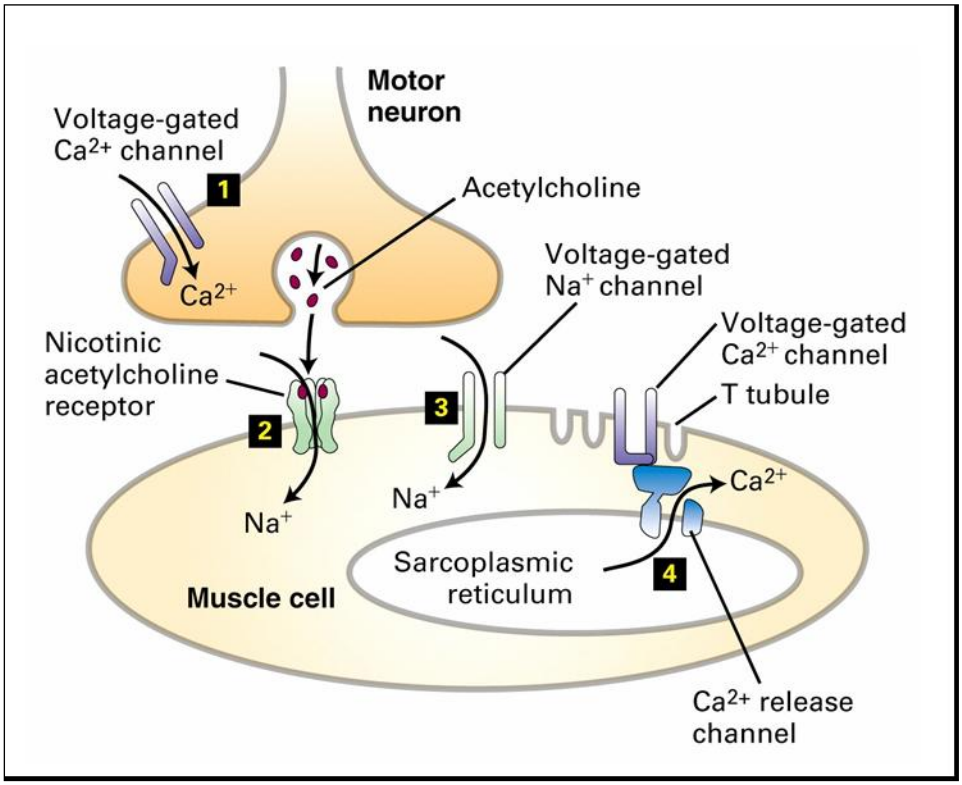

Figure 3: Sequential activation of gated ion channels at a neuromuscular junction
Arrival of an action potential at the terminus of a pre-synaptic motor neuron induces opening of voltage-gated Ca2+ channels (step 1) and subsequent release of acetylcholine (ACh), which triggers opening of the ligand-gated Ach receptors in the plasma membrane (step 2). The resulting influx of Na+ produces a local depolarization of the membrane, leading to opening of the voltage-gated Na+ channels and generation of an action potential that spreads along the sarcolemma (step 3). When the spreading depolarization reaches T tubules, it is sensed by voltage-gated Ca2+ release channels in the sarcoplasmatic reticulum membrane, releasing stored Ca2+ into the cytosol (step 4).
Molecular Cell Biology, Lodish et al 2003; WH Freeman and Company, New York

Cytoplasmic Ca^{2+} binds to the regulatory unit troponin, which is associated in each sarcomere with the actin filaments. This makes the tropomyosin slide into a position that exposes the myosin-binding sites on the actin filaments and therefore allows for interaction between actin and myosin. Calcium also activates the myosin ATPase. The ATPase splits

ATP and releases energy for contraction. ATP is broken down to ADP and a phosphate group. Both remain associated with the myosin head. The myosin heads are energized by this change. Since myosin binding sites are uncovered, the energized myosin heads of the thick myofilaments are able to grasp the thin myofilaments, forming cross-bridges. The phosphate group is released in this step. Myosin heads release ADP, rotate and pull the actin myofilaments and the attached Z-disks towards the center of the sarcomere (M-line) thereby shortening the sarcomere. The entire process is called a power stroke, which is rapidly repeated over and over, incrementally pulling the thin myofilaments further toward the sarcomere center. The myosin head action continues as long as ATP is available and Ca^{2+} levels remain high. If Ca^{2+} is pumped back into the sarcoplasmic reticulum, tropomyosin slides back and the interaction between actin and myosin stops.

1.1.4 Substrate supply

For performing repetitive contractions, muscle cells need a constant supply of adenosine triphosphate (ATP) as an energy source. ATP is regenerated from adenosine diphosphate (ADP) via the creatine phosphate kinase or the glycolytic pathway, or through oxidative phosphorylation. Creatine is a naturally occurring compound that helps to supply energy to the muscle cells and has a much longer half-life time than ATP. Half of the creatine comes from the food we eat (mainly from meat and fish), while the other half is synthesized from certain amino acids (glycine, arginine) in the liver, pancreas and kidneys. In the muscles, creatine binds to phosphate and then reacts directly with ADP forming one molecule of ATP. In glycolysis one molecule of glucose is turned into pyruvate, allowing the rapid regeneration of 2 ADP molecules to ATP. The resulting pyruvate molecules can either be converted to lactate without the use of oxygen, or oxidized in the Krebs cycle. Pyruvate (as well as fatty acids) may be transported into the mitochondrial matrix and broken down to acetyl CoA. Acetyl CoA enters the Krebs cycle where the energy of the carbohydrates and fats is harvested via high energy electrons to nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD) forming NADH and $FADH_2$. This central process called oxidative phosphorylation consists of a series of reactions that make use of the energy of the electrons of NADH and $FADH_2$ and lead to the production of ATP. Electrons are transferred to oxygen (O_2) forming H_2O. This energy is used to generate a proton (H^+) gradient over the inner mitochondrial membrane. The ATP synthase uses this electrochemical proton gradient to produce ATP. Per glucose molecule a total number of 36 ATPs can be generated in this oxygen consuming (aerobic) process.

Constant supplies of oxygen and substrates, mainly glucose and fatty acids, have to be sustained to maintain contraction. While O_2 must be supplied continuously to the muscle cells, the availability of substrates for the muscle cells depends on intracellular stores, transporters and on the absorbative surface, i.e. the capillary network density.

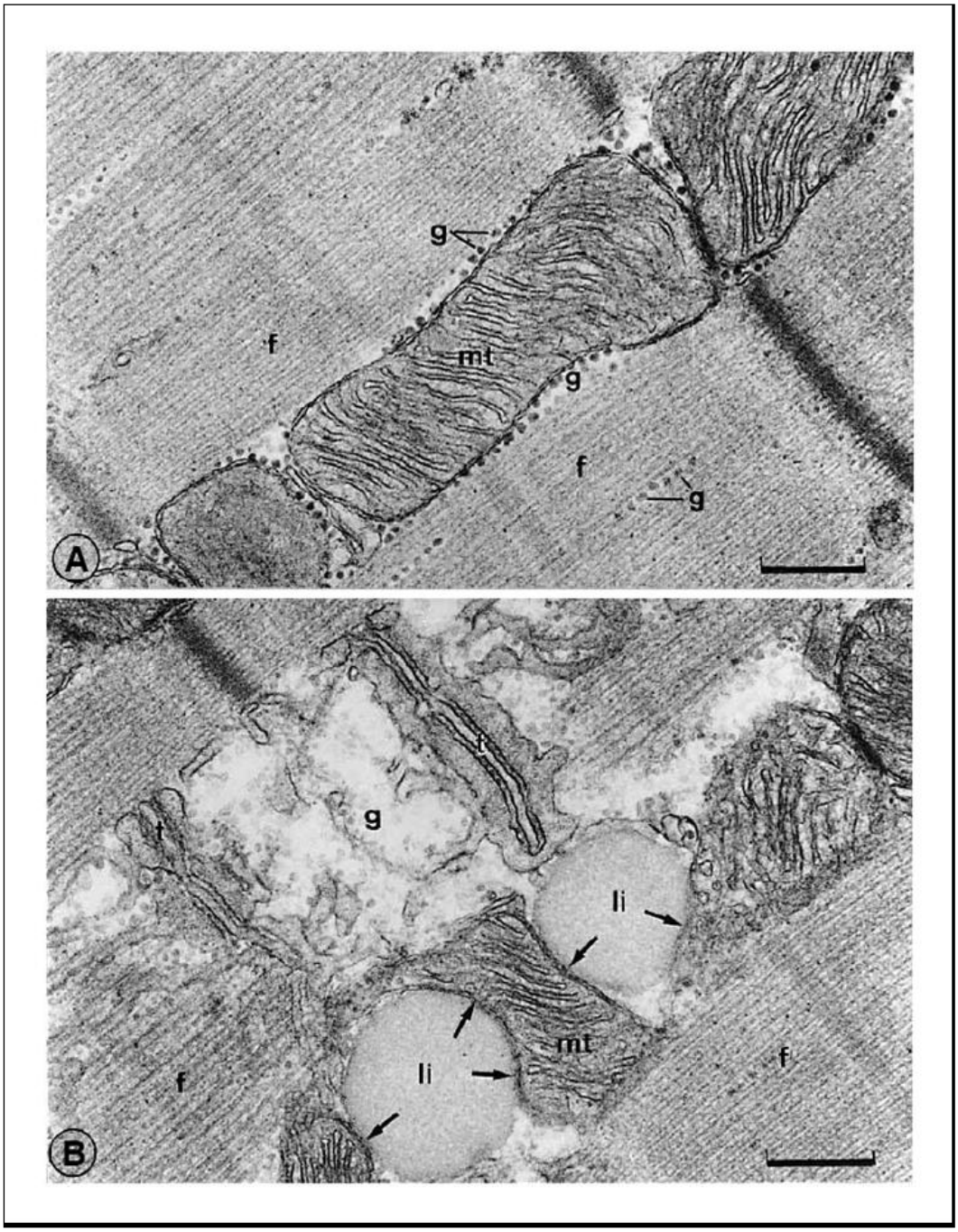

Figure 4: EM picture of muscle myofibrils, mitochondria, lipids and glycogen
Electron micrographs from a muscle fiber showing myofibrils (f), mitochondria (mt), intracellular lipid droplets (li) and glycogen granules (g). In A, the black glycogen granules are located near the mitochondria and in or around myofibrils. In B, some of the glycogen granules in the region of the tubular system (t) look 'washed out', whereas the lipid droplets are gray and show a dark surface contour. Note the dense contact surface between lipid droplets and the indented mitochondria (arrows). Scale bars, 0.3µm. (Copyright, University of Berne, 2005)

1.2 Structural and functional adaptations to training

Skeletal muscle shows an enormous plasticity of altering its type and amount of protein in response to different stimuli. The complex processes of exercise-induced adaptation in skeletal muscle involve signaling mechanisms initiating gene expression and subsequent translation. The stimuli leading to muscle adaptations can be broadly divided into four different important stressors: mechanical load, metabolic disturbances, neuronal activation and hormonal alterations (Fluck and Hoppeler 2003). All these stressors provoke intracellular responses and lead to specific adaptation within the muscle tissue and after each training session muscle performance may improve through these adaptations. The main stressor I investigated during my PhD was mechanical load. Therefore this introduction focuses primarly on the transduction of mechanical stimuli within the skeletal muscle tissue.

Skeletal muscle's main mechanical functions are to produce force, to generate power and to act as a brake. It needs to maintain the integrity of the skeleton, which allows us to walk, run, jump, and so on. As diverse as the requirements of muscle are, the mechanism of its contraction remains essentially the same. However, within the basic functional unit of contraction, the sarcomere, there are many different structural, regulatory and contractile proteins that exist as different isoforms (Schiaffino and Reggiani 1996). The ability to increase the number of sarcomeres (i.e. muscle size), together with an ability to alter protein isoform expression, gives muscle the ability to adapt to the different challenges. Therefore, the term 'muscle plasticity' refers basically to changes in muscle size and to changes in protein isoform composition. The evaluation of mechanisms, which regulate the ability of muscle to produce force and speed of movement and how changes, primarily in physical activity, may alter these properties of muscle is essential to the understanding of muscle plasticity. In recent years, developments in molecular and cell biology have been applied to study human muscle by analyzing muscle biopsy samples.

1.2.1 Force-velocity relationship

Muscle exhibits three different contraction types: isometric contraction (by maintaining constant length), concentric contraction (shortening), or eccentric contraction (lengthening). These basic mechanical properties of muscle can be described by two relationships, namely the length–tension and force–velocity relationships. The latter provides a suitable basis to study muscle plasticity in the context of its function. At a velocity of zero (i.e. maximal isometric force) the strength of a muscle can be denoted: the

force is determined by the physiological cross-sectional area (CSA) of the muscle, or the muscle fiber and is ultimately a reflection of the number of sarcomeres working in parallel with one another. At the point where the force is at its minimum, the speed potential of a muscle can be denoted: that is where the velocity of shortening is at its maximum (Vmax). With equal parameters (e.g. temperature and muscle length) Vmax is a reflection of the isoform the myosin cross-bridge contained in the fiber. The faster a muscle shortens during a concentric contraction, the lower the maximum force will be that can be generated. Conversely, the maximum force that a muscle achieves occurs during eccentric contraction. Eccentric contraction is largely unaffected by changes in the speed of the lengthening.

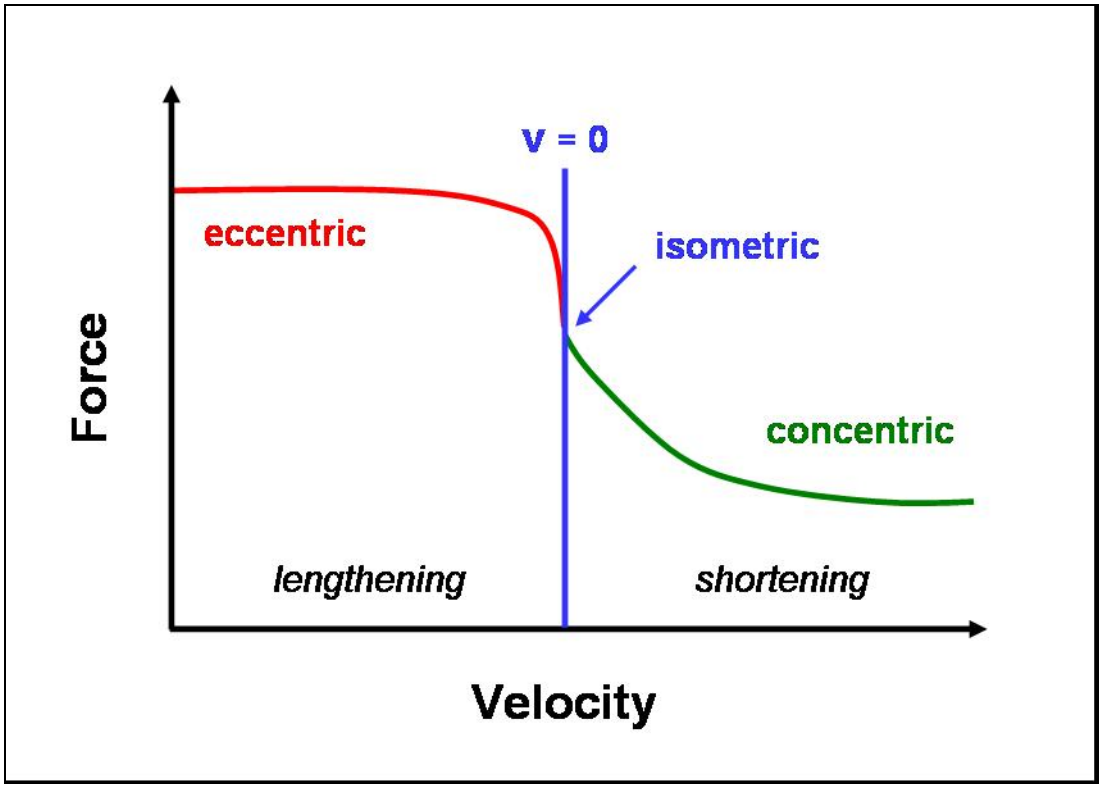

Figure 5: Force-velocity relationship
The variation in maximum muscle force as a function of muscle velocity.

1.2.2 Muscle hypertrophy

Muscle is the largest reserve of protein and may be considered a dynamic metabolic store. This may be required in extreme situations, such as during starvation and illness. Normally muscle mass is constant having an equal rate of muscle protein synthesis and protein degradation. For a muscle to enlarge (hypertrophy), there must be a net gain in protein, either from an increase in the rate of protein synthesis or a decrease in the rate of degradation or both (Rennie *et al* 2004). Muscle is very sensitive to the mechanical loads. Removing mechanical signals of everyday living e.g. through prolonged bed rest, cast

immobilization, spinal cord injury or prolonged exposure to microgravity will weaken the muscle and induce wasting (atrophy). In contrast, overloading a muscle e.g. with high-resistance strength-training exercise increases muscle size and strength (Favier *et al* 2008). These effects are quite specific to the muscle that has either been disused or loaded. Muscle strength is defined as the maximal amount of isometric force that can be generated during a single maximal voluntary contraction. In order to gain strength, the muscle needs to have more sarcomeres in parallel and consequently CSA has to be greater, e.g. resulting from repetitive strength training. However, much of the early functional adaptation to strength training does not result from an increase in muscle size but from improved activation of the muscle, as exercise-induced increases in muscle protein synthesis are greater in untrained compared with trained individuals (Phillips *et al* 1999).

Measuring hypertrophy is not trivial. While magnetic resonance imaging (MRI) provides good resolution of the CSA of an entire muscle, allowing a distinction between muscle, fat and connective tissue, it does not tell us anything about the alignment of the muscle fibers. Recent developments in ultrasound imaging allow fascicles to be identified and measured in terms of their lengths and pennation angles (Morse *et al* 2005). Following strength training an increase in the quadriceps CSA of about 10% may be expected (Aagaard *et al* 2001). In vitro studies of single human muscle fibers have shown a higher force per unit area for MHC-IIa and -IIx fibers compared with MHC-I fibers (Bottinelli *et al* 1996). Cross-sectional (Shoepe *et al* 2003) and longitudinal (Widrick *et al* 2002) single fiber studies following strength training suggest that fiber force increases in proportion to the CSA. Together with the greater sensitivity to hypertrophy of type II fibers, this would provide a mechanism for increasing force per unit area in a whole mixed muscle after strength training. The direct masuring of the rates of muscle protein synthesis is achieved through pulse-chase techniques, where labeled amino acid tracers incorporation into muscle are measured (Rennie 1999). It was shown that strength training initially causes an increase in protein breakdown, which is maintained in the absence of feeding (Phillips *et al* 1999). However, during recovery and with feeding, muscle protein synthesis increases again (Tipton *et al* 1999) and results in a net gain in protein and thus increased muscle mass when repetitively executed (Figure 6). While feeding alone increases protein synthesis for up to 3 hours (Tipton *et al* 1999), resistance exercise may elevate protein synthesis for much longer periods.

From basic cellular biology we learn that the creation of new protein depends on the transcription of DNA into mRNA followed by translation of mRNA into an amino acid

sequence, and ultimately by forming a protein. Nutrition, exercise and hormones affect both transcription and translation in muscle. Studying gene expression, it has been suggested that both strength and endurance exercise change the concentration of many hundreds of specific mRNAs (Zambon *et al* 2003,Timmons and Sundberg 2006). Therefore an increase of mRNA is likely to provoke an increase in protein synthesis and lead to a specific adaptation of muscle (Figure 6). Protein synthesis involves the processes of translation initiation, elongation and termination. These processes are regulated directly by signaling proteins, primarily via phosphorylation and de-phosphorylation. Additionally, the number of nuclei is another essential requirement and regulator of muscle hypertrophy (Kadi *et al* 1999).

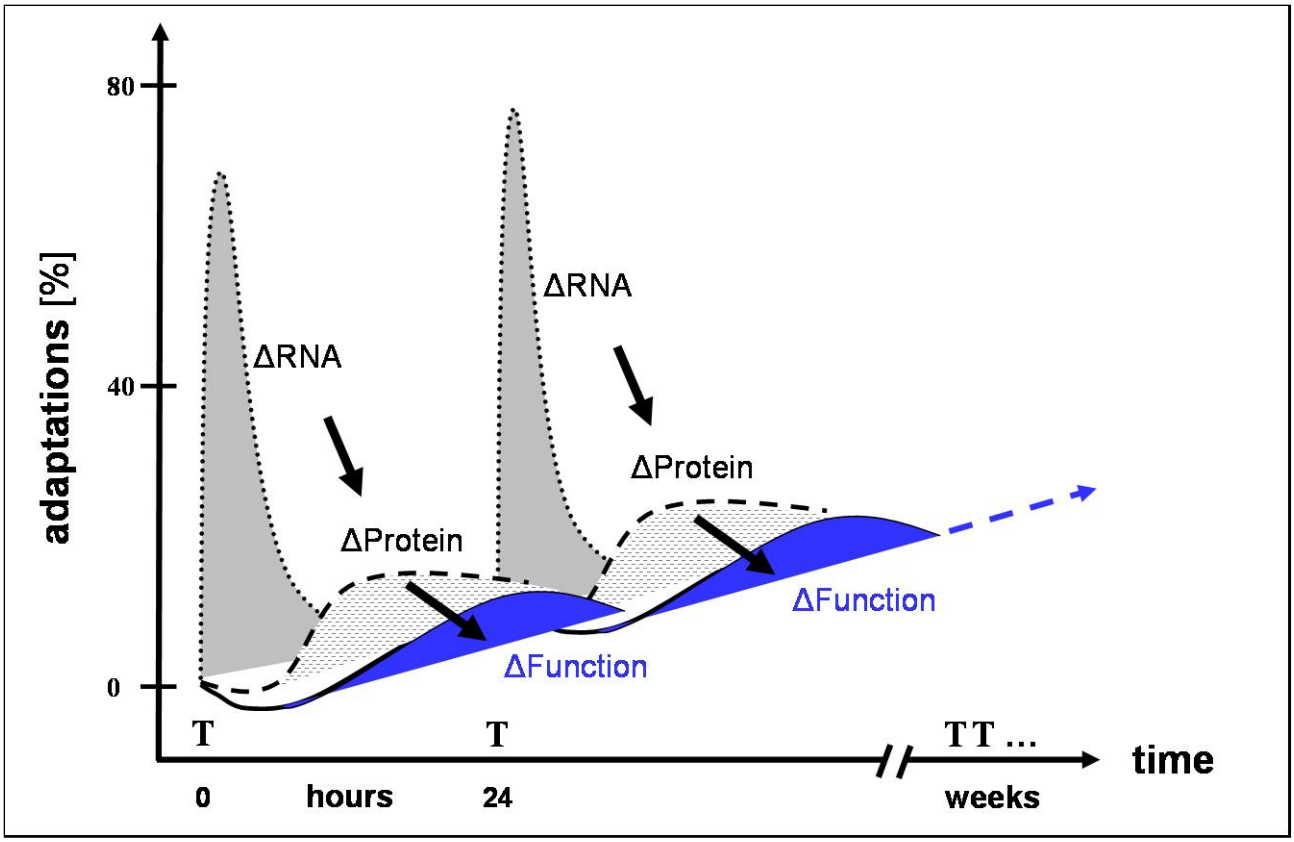

Figure 6: Molecular adaptations to training
Adaptation to training through consecutive adaptations of mRNA and protein levels towards improved function. Each training session (T) provokes an increase in specific mRNA molecules that are further translated into proteins. This adaptation in proteins leads to an increase in the specific function of muscle. Additional training sessions produce similar responses and the accumulation of mRNA and protein adaptations after each exercise bout increase muscle performance. Adapted from (Fluck 2003).

1.2.3 Satellite cells

Although muscle shows great plasticity to exercise, it is a rather stable tissue when no specific training interventions are applied. Muscle consists of differentiated muscle fibers each containing many post-mitotic nuclei. Growth and repair of muscle is linked to the

activation of myogenic precursor cells, called satellite cells. These cells are distinct from the myonuclei, being located between the basal lamina and the sarcolemma of a muscle fiber. The muscle satellite cell fulfils the basic definition of a stem cell as it can differentiate and maintain itself by self-renewing (Zammit *et al* 2006). Upon activation, most satellite cells go through a series of proliferation stages as they differentiate into myoblasts and ultimately fuse with existing myofibers to repair a damaged muscle or provoking an increase in muscle size. Every proliferation stage is characterized by the expression of different markers. In the quiescent state satellite cells express CD34, Pax7 and the myogenic regulatory factor (MRF) Myf5 on the cell surface. Satellite cell activation is characterized by a burst of myogenic factors such as MRF, MyoD and myogenin (Zammit *et al* 2006). These nuclei are then incorporated into existing fibers where they may appear as central nuclei following damage and repair. However, some satellite cells do not differentiate but replace the activated satellite cells. In extreme cases, such as with Duchenne muscular dystrophy, this cycle of satellite cell renewal is ineffective, resulting in an imbalance between degradation and muscle repair.

With strength training the number of satellite cells can be increased (Kadi *et al* 1999,Kadi *et al* 2004). This may occur as early as 4 to 8 days after a single bout of maximal strength training (Crameri *et al* 2004). It seems that satellite cells may respond to a wide range of training intensities. Moreover, muscle fibers may also hypertrophy without increasing myonuclear number (Kadi *et al* 2004). This suggests that hypertrophy might occur solely by increasing protein synthesis up to the point where the addition of new myonuclei is required (Kadi *et al* 2005).

1.2.4 Fiber type switching

As mentioned above not every muscle has the same functional demands and therefore also has a different fiber type distribution. Studies of muscles from different athletes performing different speed and endurance exercises have highlighted the importance of the fiber type distribution in relationship to athletic performance, e.g. the *gastrocnemius* muscle of top sprint athletes is dominated by fast type II fibers and that of top endurance athletes by slow type I fibers (Costill *et al* 1976). The speed of movement, most easily characterized by the velocity at which peak power occurs (Vmax), is now known to be determined by the MHC isoform expressed (Bottinelli *et al* 1996). Moreover, this MHC pattern also determines the rate of force development (Harridge *et al* 1996).

Slow-to-fast transformations

Sprinting and weightlifting are sporting activities which require the generation of high power outputs in relatively short periods of time. Similarly, undertaking simple activities of daily living, such as rising from a chair or climbing onto a bus, also requires the generation of relatively high power outputs. Atrophy leads to loss of muscle mass (sarcopenia) due to a relatively greater loss of MHC-II than MHC-I isoforms (Klitgaard *et al* 1990) and this is of relevance particularly in elderly people. Although a switch towards MHC-II and particularly MHC-IIx isoforms would be of use for maximizing explosive power output, it occurs to a large extent only during atrophy. During muscle contraction fast MHC-II fibers are recruited after MHC-I. However, patterns of activity which recruit fast fibers first and which might up-regulate fast isoforms appear to have the opposite effect as they induce a down-regulation of MHC-IIx isoforms (Andersen *et al* 2000). The observation that MHC-IIx isoforms are down-regulated with increased activity has been found in a large number of studies. It is in fact only disuse, which has the effect of causing a slow-to-fast transformation in MHC expression. The reason for this phenomenon is not clear, but it has been suggested that eventually the MHC-IIx is the default form of this protein and the most efficient for storage (Goldspink *et al* 1991).

Fast-to-slow transformations

It has been known for many years that cross innervations of muscles containing predominantly fast-twitch fibers results in a gradual transformation of the fiber properties towards a slow fiber type (Pette and Vrbova 1985). In theory, this change in MHC isoform expression with chronic training goes through a phased process from MHC-IIx via MHC-IIa towards MHC-I. This indicates that the muscle senses and responds to its own activity pattern, which is presumably driven by adaptations in cellular calcium levels and metabolism. The result is an adaptation of the muscle fiber to meet the needs imposed by the exercise, which occurs normally within weeks or months (Pette and Staron 1997). Endurance athletes with a higher percentage of type I fibers are more efficient in performing an endurance-type exercise than those with a lower percentage of type I fibers (Coyle *et al* 1992). This is presumably due to the lower metabolic cost of contracting type I compared to type II fibers and they are therefore much more efficient. Additionally, by measuring ATPase activity during isometric contractions it was shown that the isometric tension cost of MHC-I fibers is less than half that of the type MHC-IIx fibers (Stienen *et al* 1996). These advantages of having more type I fibers when performing endurance exercise may finally lead to a fiber type switch. Each bout of exercise provokes an

adaptation in such a way that i) an mRNA response of specific genes is activated, ii) these genes are translated into new proteins and iii) these new proteins, e.g. more MHC I, can improve a specific function of the muscle (see Figure 6).

The family of myogenic transcription factors in general, and the members of the MRF family of transcription factors in particular, have been suggested to play an important role in the differentiation processes of skeletal muscle. MyoD mRNA has been shown to be the most prevalent regulator in fast glycolytic muscles, whereas myogenin mRNA has been shown to be the most prevalent regulator in slow oxidative muscles (Hughes *et al* 1993). These results lead to the suggestion that MyoD and myogenin control fast and slow fiber-type-specific gene expression, respectively (Harridge *et al* 2002,Vissing *et al* 2005).

1.2.5 Muscle plasticity

On a structural level skeletal muscle plasticity involves modifications of cellular (mitochondria, myofibrils, etc.) and extracellular structures (capillaries, nerves, connective tissue). Modulations of muscular proteins and to a lesser degree also of lipids are the molecular components of muscle malleability (Bruhn *et al* 1991,Booth and Baldwin 1996,Hoppeler and Weibel 1998). In particular, expressional changes of myofibrillar and metabolic proteins have been demonstrated to be involved in muscle plasticity (Booth and Thomason 1991,Schiaffino and Reggiani 1996). The change of proteins towards a new steady-state during an adaptive event can be controlled at many steps from DNA to the end-product. Changes in transcription, translational and post-translational events are all involved in the molecular regulation of the skeletal muscle phenotype (Fluck and Hoppeler 2003). Evidence for the essential role of transcription in loading induced skeletal muscle hypertrophy was presented already 40 years ago (Goldberg and Goodman 1969,Sobel and Kaufman 1970). These investigators showed the activity of RNA polymerase to be increased with hypertrophy. Treatment with an inhibitor of transcription was shown to prevent skeletal muscle hypertrophy. A decade ago, rapid transcriptional regulation of gene expression in human skeletal muscle during recovery from exercise was demonstrated by nuclear run-ons (Pilegaard *et al* 2000) and an increase in steady-state levels of specific metabolic mRNAs in human skeletal muscle where observed after endurance training (Puntschart *et al* 1995,Pilegaard *et al* 2000,Vogt *et al* 2001). The level of mRNA is determined by the rate of mRNA synthesis and the rate of mRNA degradation. It is well established that the half-life time of many mRNAs is intrinsically different and can fluctuate in response to nutrient levels, cytokines, hormones, temperature shifts and viral infections (Day and Tuite 1998). So far, only indirect evidence has been provided to

control the stability of RNA in muscle, indicating that an enhanced RNA stabilization may contribute to the enhanced mRNA level (Yan *et al* 1996).

Cellular and molecular adaptations in human and animal models for muscle plasticity are congruent when physiologically equivalent stimuli are compared. For example, a single bout of resistance training causes similar increases in protein synthesis in humans and in rats (Phillips *et al* 1999,Hernandez *et al* 2000). Involvement of the same biological processes, but to different extents, is noted in some skeletal muscle adaptations. Differences between human and rat models for load-induced muscle hypertrophy include the extent as well as the kinetics of cellular events such as the change in fiber diameter and the activation of satellite cells (Snow 1990,Kadi and Thornell 2000). These differences in response were tentatively explained by the higher relative severity of the stimuli in the rat versus the human model (Booth and Thomason 1991). Alternatively, we have to consider that observed differences in malleability and the time course of adaptations may simply reflect allometric scaling. A survey of the molecular responses of skeletal muscle to changes in contractile activity demonstrates that as a general rule the directional change of a given mRNA is in the same direction as the directional change of the protein during its adaptation (Booth and Baldwin 1996). When transcription and translation do not adapt in the same direction, this may be explained by high turnover rates or high basal concentrations of specific proteins (Andersen and Schiaffino 1997). An increased level of mRNA is assumed to be translated into protein and to cause an adaptation in protein concentration (Day and Tuite 1998). In the absence of a proportional increase in degradation, this would increase the total amount of protein. However, for highly abundant proteins or in the presence of increased protein degradation, increases in mRNA content may not be detected as net changes in protein concentration.

Post-translational modifications of proteins are a universal and efficient way to modulate the functional properties of enzymes and signaling pathways. Modifications of enzymes involved in metabolism and signaling have been reported as acute responses to several physiological stressors of skeletal muscle (Booth and Baldwin 1996). Moreover, modulation of translation efficiency has been noted to occur with muscle loading and increased contractile activity (Booth and Baldwin 1996). Several of these adaptations are short-lived and are down-regulated by degradation or recycling of the modified protein or by removal of the post-translational modification and are therfor important early regulators of muscle plasticity (Bergamini 1992,Sorkin and Waters 1993,Wilkinson 2000).

1.3 Molecular adaptations to training

The process of converting the signal generated during muscle contraction to a molecular event that promotes adaptation in a muscle fiber involves the up-regulation of primary and secondary messengers that initiate a cascade of events resulting in an activation and/or a repression of specific signaling pathways (Fluck and Hoppeler 2003,Fluck 2006). Before entering a specific discussion of muscle molecular plasticity it may be important to expand on some of the conceptual difficulties underlying current molecular exercise physiology. As mentioned above, any exercise carried out is characterized by a specific mixture of individual stressors to which muscle tissue is subjected during contraction. We can distinguish mechanical load, hormonal adjustment, neuronal activation and metabolic disturbance as the main identifiable stressors (Fluck and Hoppeler 2003). Each of these stressors is linked to several signaling pathways in muscle cells which carry information about the external conditions under which the muscle is activated. These signals have a dual purpose. They serve to reestablish myocellular homeostasis disrupted by muscle activity. However, they also serve to modify muscle tissue with the consequence of making muscle tissue more competent in dealing with similar stress in the future (Clarkson and Hubal 2002). A single training bout can therefore promote a response which is mainly characterized by one, or a combination of the stressors. In humans, muscle function and phenotype is related to the specific mode of muscle activation. Training is considered to be a repetitive stressful use of muscle tissue leading to characteristic muscle structural and functional modifications specific for the particular training mode with which the muscle has been stressed. Classically, we distinguish between endurance training (low load – high repetitive stimulus) and strength training (high load – low repetitive). These two training modalities represent the extremes of a continuum of exercise protocols of countless options differing in load, intensity, duration and frequency. Exercise training protocols consist of specific activation patterns of muscle repeated over days and weeks. In the strength training situation we typically find mechanical load to be the dominant stressor. In endurance training mechanical load is low and we find metabolic disturbance, neuronal activation as well as hormonal adjustments usually persisting over longer time periods. Depending on the exact nature of the training protocol there is an unlimited choice in the selection of the relevant training parameters load, intensity, duration and frequency. Moreover, it has been demonstrated that the muscle signaling response is different in the trained than in the untrained state (Fluck 2006) as well as it depends on the feeding before or after a training session (Tipton *et al* 1999).

1.3.1 Primary messengers

The complexity of the mechanisms that enable skeletal muscle cells to respond to contraction is complicated as there are numerous cellular candidates as potential primary messengers to transmit a signal. In addition, it is unlikely that these primary signaling messengers act in isolation. They probably result in a complex and multifaceted signal with redundancy and cross-talk. Nevertheless, there are numerous putative messengers emerging, including calcium flux, redox potential, phosphorylation state and mechanical stretch.

1.3.1.1 Calcium

Neuronal activation of skeletal muscle generates an action potential that results in Ca^{2+} release from the sarcoplasmic reticulum. After an action potential the transport of Ca^{2+} out of the sarcoplasm back to the sarcoplasmic reticulum is initiated. The rate and capacity of Ca^{2+} release and uptake is thus altered by contractile activity. Prolonged moderate exercise increases Ca^{2+} uptake by increasing the number of active pumps (Schertzer *et al* 2004). In contrast, high-intensity exercise generates a decrease in Ca^{2+} uptake and release (Matsunaga *et al* 2002). Interestingly, repeated bouts of exercise have been shown to induce minor perturbations in Ca^{2+} release and uptake and improved resistance to fatigue (Holloway *et al* 2005). These findings suggest that the amplitude and duration of the Ca^{2+} flux is regulated by the contractile stimulus. For example, endurance exercise likely results in extended periods of moderately elevated Ca^{2+}, while resistance exercise generates short cycles of high intracellular Ca^{2+} (Baar and Esser 1999). The specific Ca^{2+} responses also determine subsequent downstream events such as induction or repression of gene expression and protein synthesis (Chin 2005). Therefore, Ca^{2+} is an important early regulator in the specificity of short-term adaptive events to exercise.

To incorporate:

When the calcineurin pathway is inhibited for example, the number of fast fibers in the muscle is doubled (Chin *et al* 1998).

1.3.1.2 Redox potential

The redox potential is the tendency of a solution to either gain or lose electrons when it is subjected to change by introduction of a new chemical. The redox mechanism is primary determined as the ratio of oxidized and reduced buffering molecules, among them glutathione, NAD and FAD are the most important in skeletal muscle. The ratio of reduced to oxidized NAD is predominantly a result of the catabolic reactions occurring with the

glycolytic and lipolytic metabolism in the mitochondria (Smith and Reid 2006). The maintenance of the redox potential produces volatile reactive oxygen species (ROS). Because of the increase in demand for oxygen and activity of metabolic pathways, exercise represents a stimulus capable of generating elevated levels of ROS. This oxidative stress may also modulate exercise-induced signaling (Carrero *et al* 2000). Redox potential and resultant free-radical synthesis during and after exercise may regulate adaptive pathways in two ways. In the first instance, redox state may act as a primary messenger through a direct effect on transcriptional regulation and DNA binding specificity of transcription factors (Carrero *et al* 2000). In addition the redox state may act indirectly on the signaling machinery via its effect on the metabolism of mitochondria and a decrease in Ca^{2+} sensitivity (Smith and Reid 2006). These effects are a cause of the ROS acting on numerous elements of cellular function. Beside that, nitric oxide (NO) might have an important impact on the physiology of skeletal muscle during contraction (Kobzik *et al* 1994). In particular NO might help recovering from fatigue induced by endurance exercise (Kobayashi *et al* 2008). The potential of the redox mechanisms in acting as a primary messenger is much higher with endurance than with resistance exercise.

1.3.1.3 Phosphorylation

The intracellular free adenosine monophosphate (AMP) concentration is an important regulator of energy production and consumption during and shortly after exercise (Sakamoto and Goodyear 2002). Strong evidence exists to demonstrate an inverse relationship between AMP concentrations and contractile intensity and duration of exercise. Any cellular stress that inhibits ATP synthesis or accelerates ATP consumption and thus increases the AMP to ATP ratio initiates different molecular events (Hardie and Sakamoto 2006). As a primary messenger the phosphorylation state appears to control its downstream signaling via a potent secondary messenger, the 5'adenosine monophosphate activated protein kinase (AMPK) (Hardie and Sakamoto 2006). Therefore, phosphorylation potential and AMPK activation may ultimately regulate multiple cell signaling cascades, which may alter glucose uptake, fatty acid oxidation, hypertrophy and gene expression (Aschenbach *et al* 2004).

Additionally, the activation of a kinase via phosphorylation has great importance in the regulation of almost every signaling pathway. This will be expanded as an example in the chapter about the IGF signaling pathway.

1.3.1.4 Mechanical stretch

Mechanical stimuli directly modulate the function and form of a cell (Alenghat *et al* 2004). Currently, there is a lack of studies that integrate the accurate measurement of effects of mechanical stimuli in vivo on muscle signaling and function. However, the use of passive stretch on muscle in vitro and in situ demonstrates that mechanical stimuli induce numerous adaptive processes. Mechanical stress on skeletal muscle mediates an activation of the calcineurin, mitogen activated protein kinase (MAPK) and insulin-like growth factor (IGF) signaling cascades. Moreover, it has become apparent that muscle fibers distinguish between axial and transverse mechanical forces activating distinct signaling intermediates despite applying the same magnitude of mechanical stress (Kumar *et al* 2002). Similarly, a unique activation of signaling proteins when comparing uni- and multi-axial stretch was found (Hornberger *et al* 2005a). The rapid and differentiated mechano-chemical conversion induced by distinct models of mechanical stress strongly suggests the existence of mechano-transduction specificity. Therefore, the signaling events initiated by mechanical load with exercise (i.e. frequency and intensity of contraction) are likely to contribute to the specificity of exercise-induced adaptation and implicates mechanical stress and tension as a significant primary messenger.

As there is little knowledge on the situation in vivo, I think that my impact on this topic is of great importance. In the following chapter on mechano-transduction the methods I used to apply the mechanical stress and their consequences for the physiology of skeletal muscle will be introduced and discussed in more detail.

1.3.2 Secondary messengers

Following initiation of the primary signal, additional kinases and phosphatases are activated to mediate the induced signals. Numerous cascades exist and these pathways are regulated and connected at multiple levels producing a highly sensitive and complex signal-transduction network. This work will focus on the most important pathways and discuss key players involved in the regulation of muscle mass.

1.3.2.1 IGF-mediated signaling

IGF1 is among the best characterized muscle growth-promoting factors. In addition to circulating IGF1 local production of different IGF1 splicing products by skeletal muscle is much more important for load- and stretch-induced adaptations (Goldspink 1999). Increased IGF1 expression has been demonstrated following functional overload induced by elimination of synergistic muscles (McCall *et al* 2003). Muscle-specific overexpression

of an IGF1 isoform in normally active skeletal muscle results in muscle hypertrophy (Musaro *et al* 2001) and the growth of muscle mass matches with a physiological increase of muscle strength. The IGF signaling pathway is considered to be the main molecular regulator of muscle hypertrophy and atrophy (Glass 2005). Moreover, many of these components have additional roles for the regulation of glucose uptake, glycogen synthesis, cell growth and differentiation (Taniguchi *et al* 2006). The importance of IGF-1 in skeletal muscle has been demonstrated in a variety of models (Vandenburgh *et al* 1997,Musaro *et al* 1999,Chakravarthy *et al* 2000b,Rommel *et al* 2001,Stitt *et al* 2004,Latres *et al* 2005). Contractile activity of skeletal muscle stimulates the secretion of IGF-1, which acts as an autocrine and paracrine growth factor by binding to its membrane receptor and initiating a cascade of molecular events (Glass 2003,Glass 2005). The signaling pathways that links IGF1 to hypertrophy involves activation of Akt, mammalian target of rapamycin (mTOR), S6 Kinase (S6K), the eukaryotic initiation factor 4E (eIF4E) and eukaryotic elongation factor 2 (eEF2) and thus directly promotes protein synthesis.

Akt, also known as Protein Kinase B, is activated by IGF1 and insulin through the generation of phosphatidylinositol-3,4,5-triphosphates (PI3P) produced by PI3Kinase. PI3P recruit Akt to the plasma membrane by binding to its N-terminal domain. At the membrane, Akt is subsequently phosphorylated on separate residues by at least two distinct kinases, the 3'-phosphoinositide-dependent protein kinase-1 (PDK1) at threonine308 and the mammalian target of rapamycin (mTOR)-Rictor complex at serine473 (Sarbassov *et al* 2005b). Akt is a serine/threonine kinase and occurs in three different isoforms, two of which (Akt1 and Akt2) are primarily expressed in skeletal muscle (Nader 2005). Furthermore, the different Akt isoforms appear to have distinct functions: Akt1 has been associated with muscle hypertrophy, whereas Akt2 has been implicated in signaling towards an increase in glucose transport (Taniguchi *et al* 2006).
Akt has numerous molecular targets according its physiological functions, including those involved in protein synthesis, atrophy and glucose transport (Rommel *et al* 2001,Bodine *et al* 2001b,Inoki *et al* 2002,Stitt *et al* 2004,Bruss *et al* 2005,Cai *et al* 2006). There is strong evidence that the Akt-mTOR pathway mediates hypertrophy in skeletal muscle via activation of S6K and translation initiation and elongation factors (Rommel *et al* 2001,Bodine *et al* 2001b,Lai *et al* 2004,Nader *et al* 2005). Akt also directly phosphorylates TSC2, which would normally inhibit mTOR function and thus suppresses the inhibition of protein synthesis via AMPK (Rommel *et al* 2001,Inoki *et al* 2002,Hahn-Windgassen *et al* 2005,Cai *et al* 2006). Thus, in addition to mTOR phosphorylation at serine2448, Akt may

also indirectly enhance protein synthesis through inhibition of AMPK and tuberous sclerosis complex 2 (TSC2) signaling. Similarly, phosphorylation of the nuclear transcription factor FoxO by PI3K-Akt prevents transcription of atrophy genes responsible for degradation of contractile protein, thereby mediating a protective effect on skeletal muscle by down-regulating pathways of protein degradation (Rena *et al* 2002,Stitt *et al* 2004,Latres *et al* 2005).

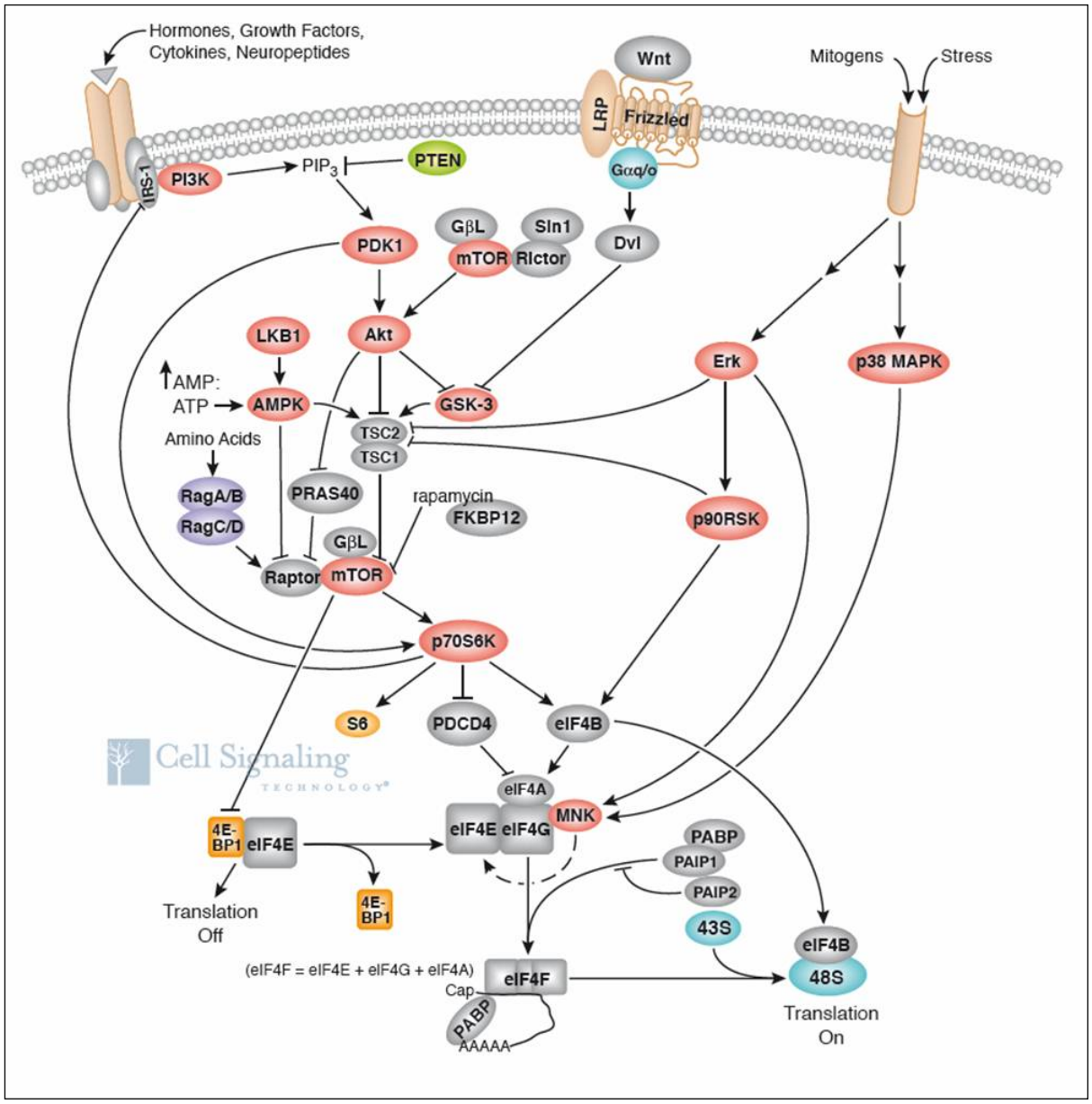

Figure 7: The IGF signaling pathway towards an activation of protein synthesis
Growth factors (such as IGF1), hormones, cytokines and neuropeptides can activate the IGF signaling pathway. Important mediators are PI3K, PDK1, Akt mTOR and S6K leading to an activation of eIF4 and promote translation initiation. Additionally, others stress stimuli may activate S6K via ERK. In skeletal muscle, the activation of the IGF signaling pathway promotes hypertrophy.
Source: Cell Signaling Technology, Inc.: http://www.cellsignal.com/pathways/translational-control.jsp

So far, the story seems quite straight forward, but different conflicting results bring the current understanding of the specific role and functions of Akt into question. Differences in the contractile stimulus such as load, intensity, duration and frequency may have contributed to the following conflicting findings. While some work has shown a rapid activation of Akt after exercise within the first hour of recovery (Sakamoto *et al* 2004,Creer *et al* 2005,Dreyer *et al* 2006,Wilson *et al* 2006,Leger *et al* 2006), a lack of Akt activation following exercise was also observed (Widegren *et al* 1998,Coffey *et al* 2005,Eliasson *et al* 2006,Fujita *et al* 2007,Terzis *et al* 2008,Deldicque *et al* 2008,Klossner *et al* 2009). In the latter studies an increase in protein synthesis is still observed and downstream factors such as S6K are activated in an Akt-independent manner. Thus, while there is strong evidence for Akt as a critical regulator of adaptation in skeletal muscle, defining and validating its role remains elusive. There might be other mediators that are capable in activating downstream targets of the IGF signaling pathway and induce increased protein synthesis without the involvement of Akt.

The mammalian target of Rapamycin (mTOR) is probably the most prominent activator of protein synthesis. Protein synthesis is conventionally divided into three main stages: initiation, elongation, and termination. Each involves a number of protein factors controlling the ribosome. Their regulation generally involves alterations in their phosphorylation. mTOR controls a number of components involved in the initiation and elongation stages of translation. In a number of cases, the rapid activation of protein synthesis by insulin, growth factors, or other growth-promoting agonists is inhibited by rapamycin, implying that mTOR signaling is involved in stimulating the translational machinery. mTOR signaling can be activated by hormones and growth factors such as insulin. Additionally, mTOR signaling is also regulated by amino acids, primarily leucine and by the cellular energy status. Being positioned downstream of IGF1, Akt and AMPK and upstream of S6K and different translation initiation and elongation factors, mTOR is a potent mediator and regulator of all these signaling events.

Protein complexes involving mTOR are indeed capable of sensing diverse signals and produce a multitude of responses including mRNA translation, ribosomal biogenesis and nutrient metabolism (Sarbassov *et al* 2005a). The two mTOR protein complexes are composed of mTOR with either a rapamycin-sensitive raptor or a rapamycin-insensitive rictor protein (Kim *et al* 2003,Sarbassov *et al* 2005b). The mTOR-raptor complex is a positive regulator of cell growth, while mTOR-rictor has a key role in Akt activation and actin cytoskeleton regulation (Sarbassov *et al* 2004,Park *et al* 2005,Wang *et al*

2005,Sarbassov *et al* 2006). In addition to Akt, direct upstream regulators of mTOR-raptor include the Ras homologue enriched in brain (Rheb) G protein and TSC2, which is in turn phosphorylated by Akt (Tee *et al* 2002,Manning and Cantley 2003,Garami *et al* 2003). Primary downstream targets of mTOR-raptor include p70 ribosomal protein S6K, 4E-BP1 and eIF4B, which links mTOR with translation and increased cell size (Ohanna *et al* 2005,Ali and Sabatini 2005,Ruvinsky and Meyuhas 2006).

The involvement of mTOR in regulating muscle mass is quite evident, since overloaded muscle has been shown to increase mTOR phosphorylation and total protein content (Reynolds *et al* 2002,Thomson and Gordon 2006) and resistance training did as well (Bolster *et al* 2003b,Dreyer *et al* 2006,Leger *et al* 2006). Also intermittent high-frequency electrical stimulation induced significant increases in mTOR phosphorylation in a number of different muscle groups (Parkington *et al* 2003,Parkington *et al* 2004,Atherton *et al* 2005). Conversely, no change in mTOR phosphorylation was seen following sustained low-frequency electrical stimulation (Atherton *et al* 2005). This suggests a tension-specific contractile response of mTOR activation and further points to the involvement of additional signaling molecules possibly involved in the regulation of muscle protein synthesis that might be activated in parallel, in addition of instead of mTOR (and Akt).

S6K is a well defined effector of Akt and mTOR signaling and is directly implicated in the control of protein synthesis (Bolster *et al* 2003a,Ruvinsky and Meyuhas 2006). Mammalian cells express two S6K isoforms (S6K1 and 2), and the S6K1 isoform subsequently has a cytosolic (p70S6K) and a nuclear (p85S6K) complex (Ruvinsky and Meyuhas 2006). In the hypertrophy response of skeletal muscle p70S6K has the most important effect in the regulation of protein synthesis and is therefore mostly referred to in the following. S6K appears to function downstream of mTOR-raptor and regulates cell size in skeletal muscle (Shima *et al* 1998,Ohanna *et al* 2005). S6K exerts its effect through multiple substrate targets and has been implicated in orchestrating the regulation of numerous cellular functions (Ruvinsky and Meyuhas 2006). Numerous studies support the idea of a fundamental role of S6K in skeletal muscle hypertrophy (Baar and Esser 1999,Nader and Esser 2001,Bodine *et al* 2001b,Karlsson *et al* 2004,Atherton *et al* 2005). These results highlight the important role for S6K in skeletal muscle hypertrophy processes. Results of other studies reveal that the exercise-induced S6K activation occurs with resistance but not endurance exercise (Nader and Esser 2001,Coffey *et al* 2005,Atherton *et al* 2005,Kubica *et al* 2005). Indeed, recent work showing increased S6K phosphorylation with stretch-activated mechano-transduction in skeletal muscle suggests that eccentric loading

may be critical for S6K activation hypertrophy (Hornberger *et al* 2005a,Hornberger *et al* 2005b,Spangenburg and McBride 2006). In human skeletal muscle an up-regulation of S6K has also been observed only following an acute bout of resistance training (Coffey *et al* 2005,Eliasson *et al* 2006,Dreyer *et al* 2006,Koopman *et al* 2006). The long-term regulation of hypertrophy and other cellular processes by S6K is less clear, as this kinase may promote reciprocal effects on protein synthesis and repress IGF signaling via a negative feedback loop through insulin receptor substrate 1 phosphorylation (Ruvinsky and Meyuhas 2006).

The S6 kinases are activated by phosphorylation at multiple sites. Phosphorylation of S6K on Serine411 and the tandem Threonine421/Serine424 relieves the phospho-transfer activity from auto-inhibition prior to a full activation of the enzyme (Pullen and Thomas 1997). S6K phosphorylation on Threonine421/Serine424 has been shown to correlate with gains in muscle mass in different animal models (i.e. stretch and resistance exercise) for muscle hypertrophy (Baar and Esser 1999,Bodine *et al* 2001b,Reynolds *et al* 2002,Thomson and Gordon 2006,Spangenburg *et al* 2008). The assessed sites control biochemical function of S6K and their enhanced phosphorylation is believed to stimulate protein synthesis (Kimball *et al* 1998,Sale *et al* 1999,Gingras *et al* 2001). One of the last events of S6K activation is the phosphorylation at Threonine389, which occurs directly via the mTOR-raptor complex (Alessi *et al* 1997,Pullen *et al* 1998). This phosphorylation is required for the subsequent phosphorylation by PDK1 at a threonine229, laying in the activation loop of the catalytic domain and leading to the complete activation of S6K (Avruch *et al* 2001). S6K can then activate other substrates involved in protein synthesis, such as eIF4B. The phosphorylation of this protein by S6K is likely to lead to more efficient initiation (Wang *et al* 2001,Raught *et al* 2004). The physiological role of phosphorylation of the main target of S6K, the ribosomal protein S6 which is a component of the small (40S) ribosomal unit, is still quite unclear, but this phosphorylation of S6 seems to increase cell growth and induce cell proliferation (Ruvinsky and Meyuhas 2006).

Eukaryotic initiation factors mediate key steps in translation initiation, such as the recruitment of the mRNA to the small (40S) ribosome subunit (eIF4 group of factors) (Gingras *et al* 1999). Additionally, they regulate the recruitment of the initiator methionyl-tRNA that recognizes the start codon at the beginning of the coding region. This scanning process, during which the preinitiation complex (including the 40S subunit and Met-tRNAi) inspects the 5'-untranslated region (5'-UTR) of the mRNA for a suitable start codon is the main step in translation initiation. Therefore, eIF4E binds to the 5'-cap structure of the

mRNA and additionally binds protein partners such as eIF4G and eIF4A. eIF4E also binds small phospho-proteins termed 4E binding proteins (4E-BPs), which regulate its activation. There are three 4E-BPs in mammals, with 4E-BP1 being by far the best characterized. These proteins bind to the same region of eIF4E as eIF4G does, so binding of 4E-BPs to eIF4E prevents eIF4E from binding eIF4G and engaging in active translation initiation complexes (Gingras *et al* 1999). The association of 4E-BP1 with eIF4E is regulated by phosphorylation of 4E-BP1. In skeletal muscle, phosphorylation of 4E-BP1 is associated with muscle hypertrophy (Bolster *et al* 2003a) and is believed to occur via prior mTOR-raptor activation (Richter and Sonenberg 2005,Sarbassov *et al* 2005a). Thus, effects of 4E-BP1 phosphorylation in skeletal muscle are comparable to those of S6K (Bodine *et al* 2001b,Atherton *et al* 2005,Kubica *et al* 2005,Thomson and Gordon 2006).

Taken together, the results of the investigations discussed provide evidence for the resistance training-induced increase in protein synthesis via thee whole or components of the IGF signaling pathway. But this may also include other kinases acting directly on mTOR, S6K or one of their downstream signaling molecules. Furthermore, endurance training promotes an Akt-mediated glucose transport, but not hypertrophy signaling and appears to have a significant negative effect on the translational machinery. The activation of S6K correlates best with the gains in muscle mass induced by mechanical stimuli (Baar and Esser 1999,Nader and Esser 2001,Burry *et al* 2007) and this activation also occurs when the Akt-mTOR pathway is not activated (Eliasson *et al* 2006,Fujita *et al* 2007,Terzis *et al* 2008,Deldicque *et al* 2008). All these findings indicate that possibly Akt-mTOR but certainly S6K regulate protein synthesis in skeletal muscle (see Figure 7 and Figure 19).

1.3.2.2 AMPK-mediated signaling

The role of AMPK in skeletal muscle was introduced before and seems to be the critical sensor of the energy state and thus may inhibit energy consuming processes such as protein synthesis during and shortly after exercise (Hardie and Sakamoto 2006,Jorgensen *et al* 2006). AMPK is directly activated by AMP and consequently is sensitive to changes in cellular AMP to ATP ratios (Aschenbach *et al* 2004). Acute activation of AMPK in response to cellular energy depletion (e.g. skeletal muscle contraction) initiates conservation and generation of ATP (Aschenbach *et al* 2004). AMPK is implicated in enhancing ATP production by stimulating insulin-independent glucose uptake (Musi *et al* 2001,Nakano *et al* 2006) and increasing fat oxidation (Kaushik *et al* 2001,Lee *et al* 2006). AMPK has been linked to the control of gene expression by activating transcription factors associated with

mitochondrial fatty acid oxidation and with the inhibition of protein synthesis by inhibiting components of the IGF signaling pathway (Bolster *et al* 2002,Jorgensen *et al* 2006).

1.3.2.3 CaM kinase signaling

The Ca2+-calmodulin-dependent serine/threonine kinases (CaMK) detect and respond to calcium (Chin 2005). From the specific kinases of the CaMK family CaMKII and IV are expressed in skeletal muscle (Wu *et al* 2002,Rose and Hargreaves 2003). CaMKII and IV have been linked with activation of gene expression of contractile and mitochondrial proteins, respectively (Fluck *et al* 2000). CaMKII activity has been shown to increase with stretch overload and wheel running animal models (Fluck *et al* 2000) and with cycling exercise in humans (Rose and Hargreaves 2003). Indeed, it appears that CaMKII is the predominant CaMK in response to endurance exercise and is rapidly up-regulated after commencing exercise in an intensity-dependent manner (Rose *et al* 2006). It has been suggested that the downstream effects of CaMK may be mediated through nuclear factor of activated T cells (NFAT) signaling (Chin 2005). Prolonged intracellular calcium fluxes have been shown to increase calcineurin de-phosphorylation and activation of the transcriptional promoter NFAT (Michel *et al* 2004). Calcineurin has been implicated in several adaptive responses inducing muscle fiber growth and regeneration and it appears to act as a co-regulator of muscle hypertrophy with IGF and also contributes to differentiation of satellite cells during skeletal muscle regeneration and myogenic proliferation (Dunn *et al* 1999,Sakuma *et al* 2003). The use of calcium/calmodulin inhibitors suppresses growth of overloaded muscle, while calcineurin overexpression reduces atrophy of disused muscle (Dunn *et al* 1999). In addition, calcineurin is involved in fiber-type plasticity and fast-to-slow phenotype transformation (Naya *et al* 2000,Michel *et al* 2004,Parsons *et al* 2004). These two different calcineurin-regulated pathways (i.e. the hypertrophic versus the oxidative phenotype) appear to be paradoxical, but they may represent important alternating adaptive responses that are specific for the intensity and duration of the contractile activity performed.

1.3.2.4 Cytokine signaling

Cytokines are small polypeptides released at the site of an inflammation in response to numerous factors, including exercise-induced muscle damage, and used in cellular communication (Glass 2005). Several cytokines have been implicated in initiating protein degradation and suppression of protein synthesis following injury in skeletal muscle, most notably tumour necrosis factor alpha (TNFα). Elevated TNFα concentration in skeletal

muscle generates an increase in ubiquitin-mediated protein degradation and an inhibition of the IGF signaling pathway through a decrease in IGF-1 and IGF binding protein gene expression (Lang *et al* 2006). The increase in free ubiquitin protein and ubiquitin gene expression after damaging exercise highlight the role of TNFα with regard to elevated muscle proteolysis. This effect has been linked to muscle atrophy (Li *et al* 2005).

Eccentric exercise and heavy resistance training would be expected to generate acute increases in TNFα concentration. Indeed, there is a significant increase in circulating systemic TNFα after muscle-damaging eccentric resistance training and marathon running (Ostrowski *et al* 1999). An increase in TNFα mRNA abundance after downhill running was also observed, indicating an acute inflammation associated with muscle damage (Hamada *et al* 2004). As less damaging exercise protocols provoke only little changes in TNFα activation, it appears that exercise is capable of generating pro- and anti-inflammatory effects on skeletal muscle.

1.3.2.5 Hormonal signaling

Hormonal signaling is a potent stimulus of inducing the IGF signaling pathway (see Figure 7). Beside the already introduced effects of insulin, I would like to shortly mention the effects of testosterone and myostatin on skeletal muscle.

The androgen testosterone is a steroid hormone synthesized from cholesterol in the testes of males and the ovaries of females. Testosterone is the basis for the anabolic steroid family of banned substances and it is illegally used in strength sport and body building. Males have approximately 10-fold higher circulating values than females and this contributes to the increased muscle mass in males at the onset of puberty. Strength training acutely increases testosterone levels and the number of androgen receptors and thus influences muscle growth (Bamman *et al* 2001). The combination of testosterone uptake with strength training increases lean body mass, quadriceps CSA and strength parameters significantly more than the exercise alone (Bhasin *et al* 1996). Furthermore, fiber size and strength increased in a dose-dependent manner. Even the administration of testosterone to elderly men (so that their circulating levels were equivalent to those of young males) increased muscle strength and protein synthesis significantly (Urban *et al* 1995). This increase in protein synthesis was postulated to be mediated through the IGF signaling pathway, as the mRNA concentration of IGF-I was elevated.

Myostatin is a growth factor that limits muscle tissue growth, i.e. higher concentrations cause the individual to have less developed muscles. The myostatin protein is produced primarily in skeletal muscle cells, circulating in the blood and lymph and acting on muscle

tissue, apparently by slowing down the development of muscle stem cells. It was identified as a negative regulator of muscle mass in Belgian Blue and Piedmontese breeds of cattle (McPherron *et al* 1997,Lee and McPherron 1999). These animals have a markedly hypertrophied phenotype associated with a mutation in their myostatin gene (McPherron *et al* 1997). Hypertrophy induced by a mutation of myostatin was also observed in humans (Schuelke *et al* 2004). In contrast to IGF-I and testosterone, which stimulate protein synthesis, myostatin is a negative regulator of muscle growth and signals via Smad transcription factors. It controls cell cycle progression in a manner opposite to that of IGF signaling, by inhibiting satellite cell proliferation. Human exercise studies have shown that myostatin is down-regulated with strength-training exercise (Roth *et al* 2003,Kim *et al* 2005). Therefore, the potential therapeutic benefits of increasing muscle mass through myostatin inhibition are interesting. However, there is evidence that the quality of muscle, in terms of its function, may be poor, as it was found that although muscles of myostatin knockout mice are twice as large, they have a significantly lower specific force (Amthor *et al* 2007).

1.3.3 Genetic and molecular responses to training

The following sections highlight the most important molecular and genetic adaptations with resistance and endurance exercise. This aims to illustrate the most important signaling proteins that have been shown to activate numerous immediate early genes, transcription factors and promoting the rate of transcription and translation of target mRNAs.

1.3.3.1 Resistance exercise

Repeated bouts of heavy resistance exercise results in increased muscle cross-sectional area and altered neural recruitment patterns (Hakkinen 1989). Increased cross-sectional area (i.e. hypertrophy) following resistance training occurs when the rate of protein synthesis is greater than protein degradation (Chesley *et al* 1992,Phillips *et al* 1997). Fundamentally, the hypertrophy response to overload is qualitatively and quantitatively controlled via the production of cellular proteins and the addition of new muscle cells. Adaptation to resistance training includes increased protein synthesis via regulatory changes in transcriptional and translational mechanisms, and in the production of muscle cells which are added to existing myofibers or combine and form new contractile filaments, each providing additional contractile machinery with which to generate force (Bolster *et al* 2003a,Rennie *et al* 2004).

Hypertrophy

Regulation of protein synthesis is controlled by phosphorylation events altering translation initiation, elongation and termination and the cellular ribosome content, which determines the synthesis of protein per mRNA (Farrell *et al* 2000,Wang *et al* 2001,Richter and Sonenberg 2005). Modulation of translation initiation is a particularly important regulatory site for global protein synthesis in response to a resistance exercise stimulus and is the rate limiting step and therefore the most frequent target for translational control (Richter and Sonenberg 2005). Given its ability to ultimately enhance protein synthesis through translation initiation, IGF-1 and IGF-binding protein gene expression following an exercise stimulus has been the focus of extensive investigation. As introduced above, IGF-1-mediated protein synthesis primarily involves PI3K-Akt-mTOR signaling (Shen *et al* 2005,Vary 2006). IGF-1 has also been shown to enhance satellite cell recruitment, proliferation and life span (Chakravarthy *et al* 2000a,Chakravarthy *et al* 2000b). Thus, IGF-1 appears capable of inducing hypertrophy via an enhanced gene expression, increased ribosomal-mediated translation and satellite cell activation. This strongly implicates IGF-1 as a potent multifactorial regulator of hypertrophy. Importantly, activation of protein synthesis via phosphorylation of S6K and 4E-BP1, both being most proximal to the actual translational machinery, seems to be most effective (Klossner *et al* 2009).

Activation and differentiation of non-specialized satellite cells into new muscle cells is an additional mechanism that contributes to compensatory hypertrophy. Eccentric contraction during resistance exercise is capable of inducing substantial damage to contractile and structural components of skeletal muscle (Macpherson *et al* 1997). Primary regulators of satellite cell activation include the myogenic regulatory factor (MRF) family of transcription factors and cell cycle kinases, which provoke the transition from satellite cell quiescence to activation, proliferation and differentiation (Zammit *et al* 2006). The best characterized of MRFs are the myogenic differentiation factor (MyoD) and myogenin (MyoG). Satellite cell gene expression from functionally overloaded skeletal muscle reveals that both MyoD and MyoG are expressed during the hypertrophy process and activated satellite cells can significantly contribute to muscle growth (Ishido *et al* 2004,Petrella *et al* 2006). A single bout of contractile activity is sufficient to increase MyoD and MyoG mRNA in skeletal muscle (Adams *et al* 1999,Haddad and Adams 2002,Bickel *et al* 2005,Vissing *et al* 2005,Yang *et al* 2005). Moreover, this response does not appear to be attenuated with long-term resistance training, which induced MyoD and MyoG mRNA responses equal to a single resistance training bout (Kosek *et al* 2006). This implicates the MRFs in contributing

to the myogenic program and the resultant compensatory hypertrophy response with resistance training.

Atrophy

Skeletal muscle atrophy is characterized by a decrease in structural and contractile protein content and fiber diameter (Kandarian and Jackman 2006). Moreover, while hypertrophy pathways may suppress the activity of some mediators of protein breakdown, atrophy is not simply the reversal of hypertrophy, but comprises also unique mechanisms in a series of pathways regulating proteolysis (Glass 2005). Atrophy occurs when protein degradation exceeds protein synthesis and is dominant in conditions such as inactivity, aging and disease (Reid 2005).

The current understanding of skeletal muscle atrophy involves at least three systems in the regulation of proteolysis: calpain, caspase and cathepsin. The calcium-dependent protease calpain and the proteolytic caspase have been proposed to mediate skeletal muscle myofibrillar disassembly and cleavage of actin and myosin proteins, respectively (Du *et al* 2004). Similarly, cathepsin, a proteolytic enzyme involved in the lysosomal proteolysis, is involved in the degradation of membrane proteins such as receptors, channels and transporters. While the initial fragmentation of structural and contractile protein via calpain and caspase or via the lysosomal pathways with cathepsin is required to enable degradation, the destruction of the protein fragments appears to be coordinated by a common system (Bodine *et al* 2001a). The destruction is primarily carried out by the ATP-dependent ubiquitin proteasome pathway, a process involving the interaction of multiple enzymes regulating 'ubiquitin-tagging' of proteins for destruction by the proteasome (Kandarian and Jackman 2006). The muscle-specific atrophy F box (MAFBx, also known as atrogin-1) and muscle ring finger (MuRF) proteins are important ubiquitin ligase proteins that are up-regulated during skeletal muscle atrophy (Gomes *et al* 2001,Bodine *et al* 2001a). The evidence supporting their proposed role in muscle atrophy is compelling, as an increase in gene expression of these proteins has been systematically induced in different atrophy models (Bodine *et al* 2001a,Jones *et al* 2004). However, the molecular proteins that initiate MAFBx and MuRF gene expression are still largely unknown. Principal candidates implicated in MAFBx and MuRF activation include the forkhead (FoxO) transcription factors and TNFα (Glass 2005). Both of them are negatively regulated by members of the IGF signaling pathway.

1.3.3.2 Endurance exercise

Endurance training causes both central and peripheral adaptations. It alters the neural recruitment pattern and causes adaptations in the substrate supply and oxidation of them (Hoppeler and Fluck 2003). Briefly, endurance adaptation results in increased muscle glycogen stores and glycogen sparing at submaximal workloads via increased fat oxidation, enhanced lactate kinetics and morphological alterations, including greater type I fiber proportions per muscle area and increased capillarity and mitochondrial density. Moreover, repeated bouts of endurance exercise result in altered expression of a multiplicity of gene products, resulting in an altered muscle phenotype with improved resistance to fatigue (Fluck and Hoppeler 2003,Irrcher *et al* 2003). Mitochondria are the main subcellular structures that determine the oxidative capacity and fatigue resistance to prolonged contractile activity in skeletal muscle (Hoppeler and Fluck 2003).

Mitochondrial Biogenesis

The new formation of mitochondria is a highly regulated and complex process that appears to require the coordinated expression of a large number of genes (Goffart and Wiesner 2003). Mitochondrial biogenesis requires both the nuclear and mitochondrial genomes. Thus, an important aspect of mitochondrial biogenesis is the import machinery regulating the transport of nuclear encoded precursor proteins into the organelle (Irrcher *et al* 2003). However, expression of genes promoting mitochondrial biogenesis is predominantly controlled by the global principles of gene regulation, that is, transcription initiation and interaction at the gene promoter (Goffart and Wiesner 2003). Therefore, transcription factors and transcriptional co-activators represent critical regulators of mitochondrial biogenesis. Peroxisome proliferator receptor gamma co-activator-1alpha (PGC-1α) is an important regulator of mitochondrial content in skeletal muscle due to its apparent co-activation of multiple mitochondrial transcription factors (Hood *et al* 2006). Indeed, PGC-1α is the founding member of a family of transcriptional co-activators that has been proposed as a potential 'master regulator' of mitochondrial biogenesis (Irrcher *et al* 2003). The biogenesis and maintenance of mitochondrial architecture is controlled by altered rates of mitochondrial protein fusion and fission (Santel and Fuller 2001), a role for which mitofusin has been strongly implicated (Santel *et al* 2003). PGC-1α mediates as a regulatory pathway involving mitofusin, as this pathway has been shown to be up-regulated following endurance exercise (Soriano *et al* 2006). PGC-1α is the co-activator of the peroxisome proliferator activated receptor (PPAR) family (Oberkofler *et al* 2002). The three PPAR subtypes appear to regulate lipid homeostasis via expression of genes involved in

mitochondrial fatty acid oxidation (Finck and Kelly 2006). The physiological significance of increased PGC-1α-PPAR activated gene expression with endurance training is an enhanced capacity for fat utilization during prolonged exercise and may also be related to fast-to-slow fiber type conversion (Wang *et al* 2004).

Collectively, the results from these studies not only implicate PGC-1α in the regulation of aerobic metabolism, but also mitochondrial architecture and fast-to-slow fiber type transformation.

Metabolic Gene Expression

In addition to mitochondrial biogenesis, increased gene expression of metabolic proteins following endurance exercise contributes to promoting an improved endurance phenotype (Mahoney and Tarnopolsky 2005). These include genes encoding enzymes and transporters involved in carbohydrate and fat metabolism such as hexokinase, lipoprotein lipase and carnitine palmitoyl transferase (Pilegaard *et al* 2000,Tunstall *et al* 2002). Endurance exercise has been shown to increase the mRNA abundance and transcription of a variety of metabolic genes in the recovery of an exercise (Pilegaard *et al* 2000,Tunstall *et al* 2002,Yang *et al* 2005,Pilegaard *et al* 2005). This up-regulation of metabolic genes following exercise appears to peak in the initial hours of recovery and generally returns to resting levels within 24 hours (Yang *et al* 2005,Pilegaard *et al* 2005,Schmutz *et al* 2006). It has been postulated that the cumulative effect of this transient up-regulation with repeated bouts of exercise may be an underlying mechanism for exercise-induced adaptation with endurance training (Pilegaard *et al* 2000,Fluck and Hoppeler 2003,Hoppeler *et al* 2007).

1.4 Mechano-transduction

Mechanical factors exert a key influence on the phenotype of striated muscle (Loughna *et al* 1990). This control is well illustrated by the reversible alterations in size and contractile composition of lower limb muscle. These muscle groups undergo remarkable atrophy when they are relieved from gravitational mechanical loading for a prolonged period by bed rest or microgravity (VandenBorne *et al* 1998,Narici *et al* 2003). Conversely, pronounced fiber hypertrophy is observed with physiological regimes that functionally overload muscle groups (VandenBorne *et al* 1998,Fluck and Hoppeler 2003). There is limited knowledge of the critical molecular steps underlying the integration of mechanical signals towards downstream gene expression and protein synthesis in intact tissue. Culture studies indicate that both a direct path of mechano-transduction via the hard-wired cytoskeleton and mechanically induced chemical signaling transmit mechanical forces and convert them to intracellular responses (Chiquet and Flück 2001,Ingber 2006). With regard to the latter mechano-chemical signaling, sensory sites along the cytoskeleton-extracellular matrix axis and the plasma membrane have been proposed as points of conversion from mechanical signals into intracellular second messengers. Thereby the activation of a phosphorylation cascade subsequent to the induction of an upstream phosphotransfer enzyme (kinase) appears as the frequent theme for signal propagation towards the control of mRNA stability and translation (Sadoshima and Izumo 1997,Chiquet and Flück 2001,Proud 2007).

The integrin-associated focal adhesion kinase (FAK) is a mechano-sensitive signaling molecule (Durieux *et al* 2007,Klossner *et al* 2009) which is positioned upstream of S6K (Malik and Parsons 1996,Gan *et al* 2006) and it is therefore a candidate key player in the control of mechano-transduction. FAK localizes to sarcolemmal focal adhesion sites which play an important role in the conversion of mechanical stress to myocellular hypertrophy signaling (Fluck *et al* 2002,Samarel 2005,Romer *et al* 2006,Ingber 2006). A possible functional coupling between the activation of S6K and FAK is suggested by the observation that the phosphotransfer activity and phosphorylation status of both kinases correlate with protein synthesis and load-dependent increases in muscle mass (Cary and Guan 1999,Fluck *et al* 2002,Ingber 2006). A FAK-dependent and Akt-independent activation of S6K was recently shown *in vivo* (Klossner *et al* 2009) suggesting that in mechano-transduction an FAK-mediated pathway towards an increase in protein synthesis might be involved.

1.4.1 Focal adhesion site

Focal adhesion (FA) sites provide important characteristics to coordinate and mediate the sensing of an extracellular mechanical force towards an intracellular chemical signal. Mechano-transduction starts with cell adhesion to specific ligands in the extracellular matrix (ECM) environment via transmembrane integrin receptors. In skeletal muscle new ECM contacts have to be established after each muscle contraction between individual muscle fibers and thus initiate the formation of FA sites. Additional intergrins and the associated cytoskeletal proteins are recruited and activated in order to sense the extracellular signal. Further signal propagation involves the ordered interaction and aggregation of more than 50 diverse proteins to form a FA site (Dueber *et al* 2004). Basically, four major factors influence the assembly rate, size, constituency, signaling repertoire and the functional impact of FAs. These are (i) the physical and biochemical properties of the ECM, (ii) integrin activation, (iii) the contraction state of the cytoskeleton and (iv) the specific cellular and tissue milieu in which these events occur (Zamir and Geiger 2001,Hynes 2002).

1.4.2 Cell-matrix adhesion

The first studies on cell adhesion to the ECM recognized that these sites begin as small aggregates of fibronectin and the cytoskeleton-associated protein vinculin (Singer 1979). These nascent adhesive structures were later termed focal complexes and shown to contain FAK, talin and paxillin (Depasquale and Izzard 1991,Nobes and Hall 1995). They were shown to be induced by the Rho family members Rac and cdc42, but not by Rho itself. The maturation of focal complexes into FA is dependent on interactions with the actin cytoskeleton and tension of the actin-myosin network (Ren *et al* 2000). Since then, plenty of other proteins have been identified to be involved in FA and might react to mechanical stimulation of integrins in different cell types and under different conditions.

1.4.3 Integrin

Integrins are a family of transmembrane adhesion receptors. They contain an extracellular ligand-binding region, a further extracellular segment, a transmembrane domain and a cytoplasmic tail. A total number of 18 α-chains and 8 associated ß-chains form noncovalently bound heterodimers. Specificity in integrin signaling is made possible by the particular α and ß chains that form the heterodimeric pair and the distinct binding interactions of the cytoplasmic tails with the actin cytoskeleton and with signaling molecules of the FA site. In skeletal muscle, FA are mostly associated with ß1-integrins.

The strength and stability of cell attachments to the ECM are regulated to an important part by the clustering of integrins to form cell-matrix adhesions. Increased density of integrin receptor molecules at these sites increases the affiliation for adhesion. Concomitant with clustering, integrins must be switched "on" by conformational modification of extracellular ECM-binding domains that increase the specific binding activity of individual integrin molecules (Schwartz *et al* 1995).

Integrin signaling may occurs via a large array of intracellular second massagers including calcium channels, PIP3, phospholipase-C, tyrosine and serine/threonine kinases, phosphatases, Rho family GTP-binding proteins, mitogen-activated protein (MAP) kinases, and cyclin D1 (Schwartz *et al* 1995,Chen *et al* 1999,Zamir and Geiger 2001). Reciprocal communication between integrins and proteins that regulate the actin cytoskeleton is an important feature of FA signaling. Thus, Rho activation promotes FA growth, whereas integrin activation has direct effects on Rho activity via Src (Arthur *et al* 2002) and FAK (Ren *et al* 2000,Zhai *et al* 2003).

1.4.4 Focal Adhesion Kinase

As mentioned, FA sites contain a rich diversity of proteins that direct cell fate, shape and (loco)motion (Zamir and Geiger 2001,Hynes 2002,Brown and Turner 2004). Among them, vinculin is selectively activated and regulated by binding to talin (Izard *et al* 2004), actinin forms a signaling complex with the different kinases (Ronty *et al* 2005) and paxillin integrates diverse inputs including tyrosine kinases and Rho family regulators (Brown and Turner 2004). The nonreceptor tyrosine kinases Src and FAK have the greatest impact on mechano-transduction (Frisch *et al* 1996,Flück *et al* 1999,Gordon *et al* 2001,Ilic *et al* 2004). Src and FAK are each activated by auto-phosphorylation on specific tyrosine residues (Y419 and Y397 respectively) and are dependent on localization to and activation of FA sites (Schaller 2001,Katz *et al* 2003). Auto-activated FAK recruits Src by binding to its Src-homology type 2 (SH2) domain and the proline-rich sequences in FAK are potential binding sites for the SH3 domain of Src (Schaller 2001). Both, the activity and the interaction of Src and FAK are controlled by their subcellular localization.

FAK may regulate key cellular processes influencing cell fate (Frisch *et al* 1996,Gilmore *et al* 2000). FAK was shown to influence protein synthesis (Kurenova *et al* 2004), regulate the cell cycle (Golubovskaya *et al* 2005), induce apoptosis (Lieman *et al* 2005), provoke cell mobility (Lee *et al* 2002), tissue invasion (Wu *et al* 2005) and many more. Although cell survival, mobility and invasion may be complementary programs, the number and variety of cellular processes and binding interactions in which FAK is involved suggests

that FAK serves as an important cellular signaling "switch". The ablation of the corresponding FAK gene is lethal (Ilic *et al* 2004).

In skeletal muscle, FAK seems to have a key role in adhesion-dependent signaling. FAK activation relates to the downstream induction of two main signal transduction cascades, i.e. the PI3K-Akt-mTOR-S6K and the Rho-Rock-SRF-dependent pathways (Malik and Parsons 1996,Cary and Guan 1999,Gerthoffer and Gunst 2001). Therefore, FAK emerges as a putative key player in mechano-transduction relating the typically increased protein synthesis rate with muscle loading (Durieux *et al* 2007,Klossner *et al* 2009,Durieux *et al* 2009).

1.4.5 Mechanical signaling

The association of activated FAK signaling with muscle remodeling after mechanical stimulation is likely, while the one of titin, another cytoskeletal-associated phosphotransfer system possibly involved in the early steps of mechano-transduction, is not established yet. The titin kinase domain has been proposed to sense mechanical load via conformational changes (Lange *et al* 2005). Until recently, titin has been considered to be a simple molecular spring controlling the length of the sarcomeres. But titin might also serve to integrate longitudinal forces together with contractile forces to promote muscle gene expression (Linke 2008).

The role of FAK in mechano-sensing was first demonstrated in culture experiments (Fluck *et al* 2002). The phosphotransfer activity of FAK and downstream signaling molecules is activated within minutes after the deformation of integrins by mechanical forces (Ingber 2006). In striated muscle, FAK is enriched at the myotendinous junction, which is involved in the main mode of force transmission (Huijing 1999). Investigations of load-dependent muscle plasticity demonstrate that the phosphotransfer activity of FAK is controlled by the degree of muscle loading. This is visualized by an augmentation of FAK tyrosine phosphorylation after functional overload (Gordon *et al* 2001,Klossner *et al* 2009). It was further shown that the sarcolemmal localization of FAK corresponds to the degree of fiber recruitment (Fluck *et al* 2002). Collectively, the results imply that both expression and posttranslational mechanisms co-operate to regulate FAK activity by mechanical loading. This suggests further that FAK may be part of the regulatory loop that governs the fate of frequently recruited fibers. Therefore, focal adhesions evolve as a highly organized functionally entity, capable of sensing a mechanical stimulus, transuding it towards the cytoskeleton and translating it into a chemical signal (Strosberg 2001,Durieux *et al* 2007).

2 Methods

In this section I would like to shortly introduce the methods used of the three main studies during my PhD. I applied a mechanical stimulus to skeletal muscle of humans, mice and rats in order to provoke a hypertrophy response. All these methods were not aimed at detecting hypertrophy on the physiological level, i.e. actual gain in muscle mass and muscle strength, but aimed at investigating the molecular events on the transcriptional and translational level shortly after a single intervention.

2.1 Eccentric exercise

As introduced with the force-velocity relationship, the greatest forces can be generated during eccentric (lengthening) contractions. Thus, muscle damage occurs after an unaccustomed eccentric exercise. As eccentric contractions require lower levels of voluntary activation by the nervous system to achieve a given muscle force and as muscle consumes less oxygen and energy at a given muscle tension when contracting eccentrically (Bigland and Lippold 1954), this training might be particularly suitable for elderly and weak individuals. Muscle contracting eccentrically shows a greater resistance to fatigue (decline in force) during repeated contractions. On the other hand, eccentric contractions may result in muscle soreness and muscle swelling. Also immediate strength loss and increased levels of muscle protein in the blood such as creatine kinase (CK) and myoglobin are observed in the first few days after the exercise. Additionally, after eccentric contraction muscle soreness and muscle pain occurs, generally termed Delayed Onset Muscle Soreness (DOMS).

In the eccentric study, six untrained male humans performed an single eccentric exercise on a custom built eccentric-bike (e-bike) (Vogt *et al* 2003) at 35% of their concentric Pmax for 15 minutes. This represents a mild eccentric exercise. Biopsies were taken within the first 24 hours of recovery and analyzed using microarray technology to investigate the mRNA levels of specific genes involved in muscle regeneration. It was our aim to characterize the specific changes of the muscle transcriptome to a single mild eccentric stimulus, which was previously shown to increase muscle cross-sectional area and muscle strength, when applied repetitively over longer time periods (Lastayo *et al* 2000). I hypothesized that eccentric exercise would result in a broad up-regulation of transcripts relevant for the processes initiating muscle growth and improved strength generation within the first 24 h post-exercise. Furthermore I wanted to compare the gene expression profile after the eccentric exercise with the profile after concentric endurance exercise.

2.2 Hindlimb suspension and reloading

Hindlimb suspension, pioneered by Emily M. Morey in 1979 (Morey 1979,Morey-Holton and Globus 2002) is an established rat and mouse model for atrophy inducing muscular and systemic changes seen in humans as a consequence of muscle disuse (Thomason and Booth 1990,Dapp *et al* 2004). Hindlimb suspension affects postural muscles of the hindlimb. On the physiological level, hindlimb suspension has been recognized to increase muscle shortening velocity while decreasing peak tension (Thomason and Booth 1990). These functional adaptations go along with a loss in muscle mass and mean fiber area within days (Thomason and Booth 1990). Additionally, reduction in the capillary-to-fiber ratio (Desplanches *et al* 1987a) and the fiber phenotype, i.e. a shift toward fast fibers (Thomason and Booth 1990,Desaphy *et al* 2005), are observed. Consequently, an up-regulation in the expression of genes involved in glycolysis, protein turnover and growth arrest as well as an attenuation of cell proliferation and genes involved in fat metabolism have been noted in rat *soleus* muscle with prolonged hindlimb suspension (Wittwer *et al* 2002,Stevenson *et al* 2003).

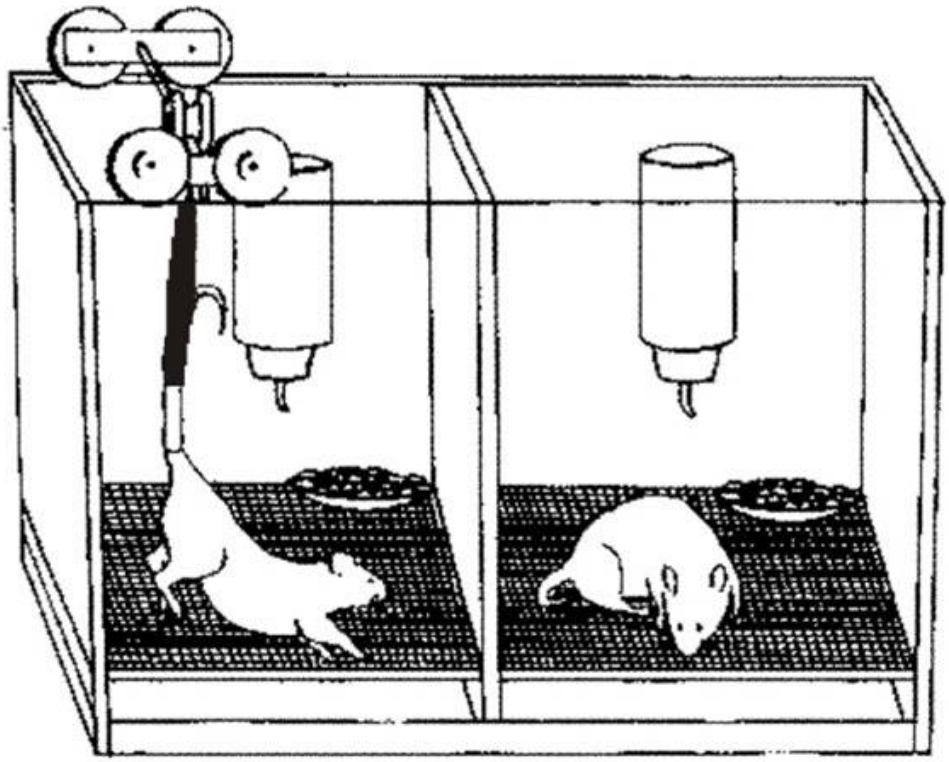

Figure 8: Hindlimb suspension. Tail suspension of rats used for studying effects of simulated microgravity on skeletal muscles of the hindlimbs (Morey 1979).

Subsequent reloading of the hindlimbs by resuming normal cage activity is known to induce hypertrophy and to produce muscle fiber damage. This leads to initiation of muscle fiber regeneration (Flück *et al* 2003) resulting in the recovery of muscle structures, i.e.

mean fiber area, fiber type composition, capillary-to-fiber ratio and functions toward normal levels within days to weeks (Desplanches *et al* 1987b,Krippendorf and Riley 1993,Flück *et al* 2003,Itai *et al* 2004,Desaphy *et al* 2005).

I used the weak mechanical stimulus of reloading of previously unloaded and atrophied muscle to investigate the early signaling events leading to the expected regain in muscle mass. Therefore, mice were subjected to 7 days of hindlimb unloading by tail suspension and subsequently reloaded for up to 24 hours. I hypothesized that mechano-signaling between FAK and S6K is the missing molecular connection between mechanical muscle stimulation and activation of muscle protein synthesis (Baar and Esser 1999,Kimball *et al* 2004,Gan *et al* 2006). To this end, I tested whether muscle fiber-targeted FAK overexpression (Durieux *et al* 2002,Durieux *et al* 2004) would enhance activation of S6K in a load-dependent manner (Kimball *et al* 1998,Sale *et al* 1999,Gingras *et al* 2001). I was interested in elucidating the time course and relationship of the early FAK activation (Gordon *et al* 2001) to the putative downstream phosphorylation of S6K and explored whether this pathway distinguishes to regulatory activation of S6K by Akt-mTOR and downstream phosphorylation of key translation factors eIF4E-BP1, eEF2 (Baar and Esser 1999,Gingras *et al* 2001,Baar *et al* 2006).

2.3 Tenotomy

Tenotomy is a surgical act which involves the dissection of a muscle tendon. A muscle is ablated in order to functionally and mechanically overloads its agonist. With the ablation of the *gastrocnemius* and *plantaris* muscle, the subjacent *soleus* muscle has to function for the whole muscle group during normal cage activities and is heavily mechanically stressed. Within a few days, an activation of the protein synthesis machinery (Spangenburg *et al* 2008) and subsequent muscle hypertrophy (Lesch *et al* 1968) can be observed. Therefore, the ablation of a synergist muscle seems to be a potent inducer of muscle hypertrophy and a suitable model to study early signaling events in the hypertrophy response.

Although most research on muscle hypertrophy has focused on the responses of muscle cells to high mechanical loading, only few studies investigated the influence and potential disturbance of inflammatory cells. In tenotomy experiments it was shown that neutrophils and specially macrophages accumulate in skeletal muscle following increased mechanical loading and they were potentially involved in the hypertrophy response (DiPasquale and Koh 2007). Much remains to be learned about the role of inflammatory cells in muscle hypertrophy, including the molecular signals involved in calling neutrophils and

macrophages to skeletal muscle as well as those that regulate their function in muscle. Although it was demonstrated that macrophages produce growth promoting factors during muscle hypertrophy (DiPasquale and Koh 2007), the full range of functional activities involved in muscle hypertrophy remains to be determined.

In order to verify our results from the reloading experiments combined with the overexpression of FAK, I aimed to apply this much stronger mechanical stimulus to skeletal muscle. I wanted to test whether an activation of FAK and S6K occurs also with these extreme mechanical forces. Therefore, rats were anaesthetized with isoflurane inhalation and were maintained under anesthesia during the surgical procedure. An incision was made along the dorsal plane of both hindlimbs. A bilateral tenotomy was then performed by sectioning the distal tendon of the medial and lateral *gastrocnemius* muscles as well as of the *plantaris* muscle, i.e. the functional synergists of the *soleus* muscle were cut. The sectioned tendons were shortened in order to prevent reattachment. For different time durations up to 1 day of recovery the rats were placed back in their home cages and remained there until harvesting. I further included mock control animals, which were anesthetized and the *soleus* tendon was liberated without cutting it. Unoperated age-matched control rats were kept in standard cages for the same duration.

3 Results

On the following pages and in the appendix the results of my 3 main projects are summarized. An overview of all the projects I have been involved during my PhD is given at the beginning in section B.
As the manuscripts of the eccentric study and the hindlimb suspension are already accepted, I have attached them in the appendix without giving any further comments or showing additional results.

3.1 Eccentric Exercise

The manuscript has been published in Pflügers Archive and is attached at the end of this thesis in appendix A.

3.2 FAK overexpression and hindlimb suspension

The manuscript has been published in the European Journal of Applied Physiology and is attached at the end of this thesis in appendix B.

3.3 Mechano-transduction with tenotomy

The results or the tenotomy experiments are not published yet and are therefore shortly summarized in this section.

3.3.1 Animal and muscle weight

A total number of 4 animals were harvested for each condition. Therefore, totally 8 *soleus* muscles could subsequently be introduced in the analysis of signaling events for each time point. The experimental procedure of tenotomy induced a significant loss of body weight of 5 g within 6 hours (Figure 9). This loss may be explained by increased expulsion as a result of the anesthesia and reduced cage activity in the first few hours after surgery. This loss in body weight was recovered after 24 hours when rats began to feed and drink normally (personal observations).
Tenotomy to the *gastrocnemius* and *plantaris* muscle induced a significant gain in *soleus* muscle to body weight ratio after 24 hours of tenotomy of 16%.

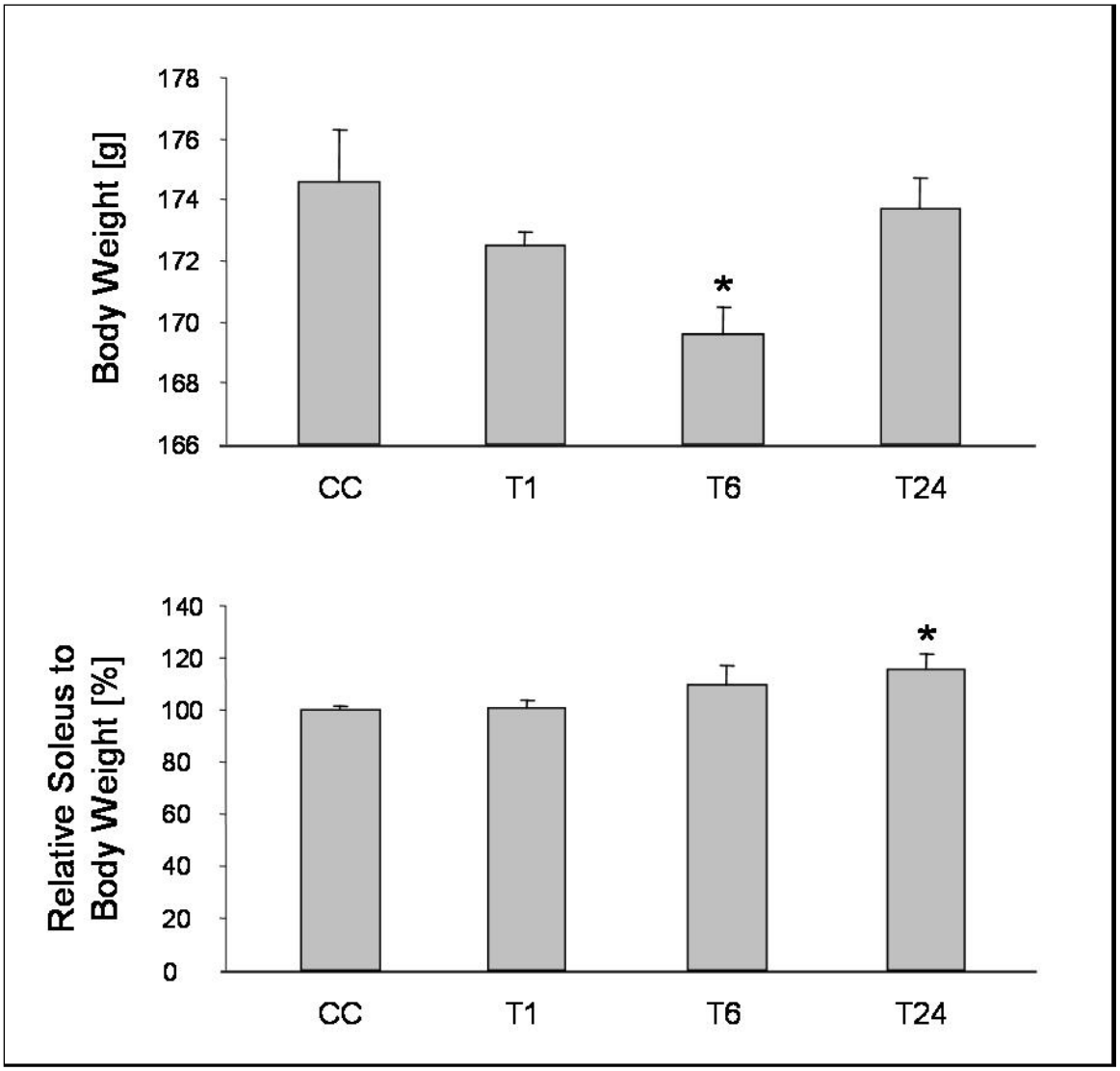

Figure 9: Body weight and *soleus* to body weight ratio of female Wistar rats kept as cage controls (CC) and with different durations of tenotomy (T1: tenotomy of 1 hour, T6: tenotomy of 6 hours; T24: tenotomy of 24 hours). *: $p<0.05$ versus CC

3.3.2 Activation of signaling towards increased protein synthesis

S6K

Activation of S6K was measured via phosphorylation at Threonine389 (Figure 10) and the dual phosphorylation site Threonine421and Serine424 (Figure 11) after 1 and 24 hours of tenotomy in the overloaded *soleus* muscle. The significant increases in the phosphorylation status for both regulatory sites indicate a duplicate and time-dependent activation via upstream kinases. The protein level of S6K remained stable (Figure 12).

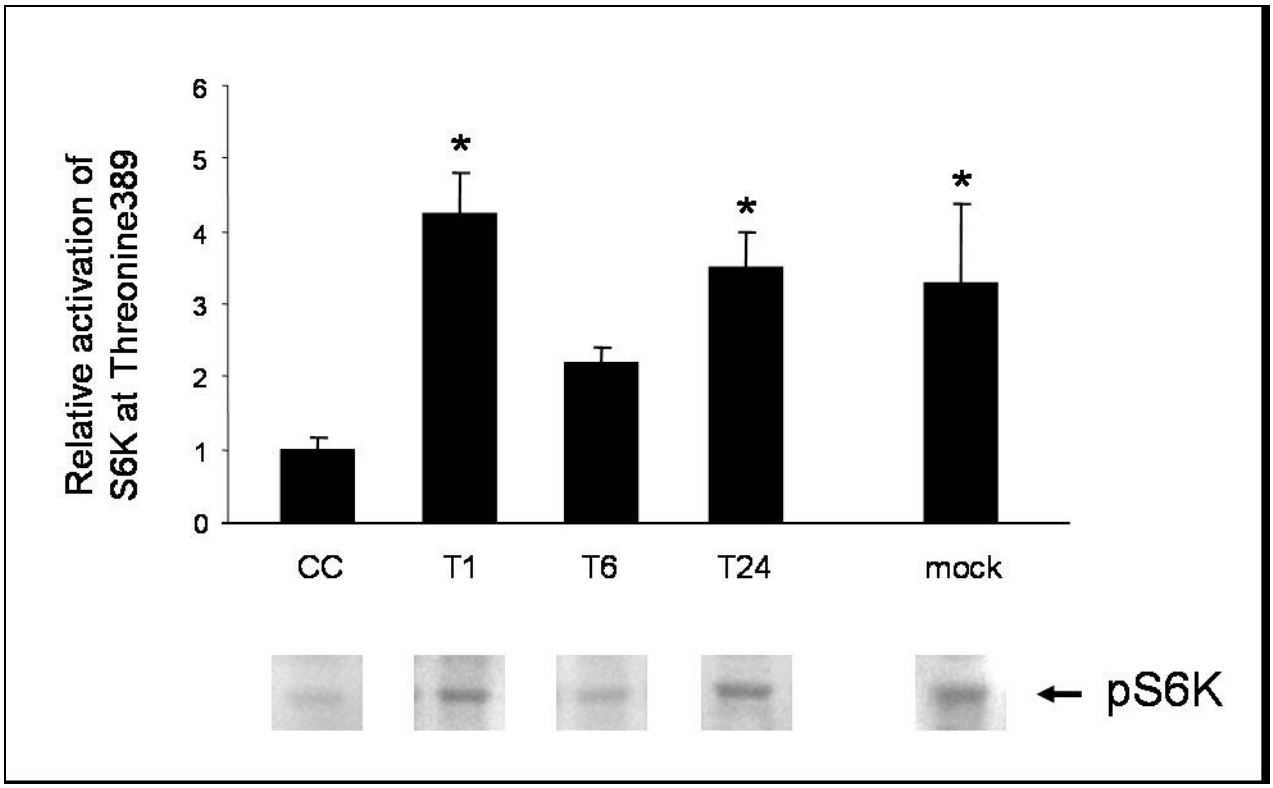

Figure 10: S6K activation at Threonine389 after tenotomy. CC: cage control; T1: tenotomy of 1 hour, T6: tenotomy of 6 hours; T24: tenotomy of 24 hours. *: $p<0.05$ versus CC

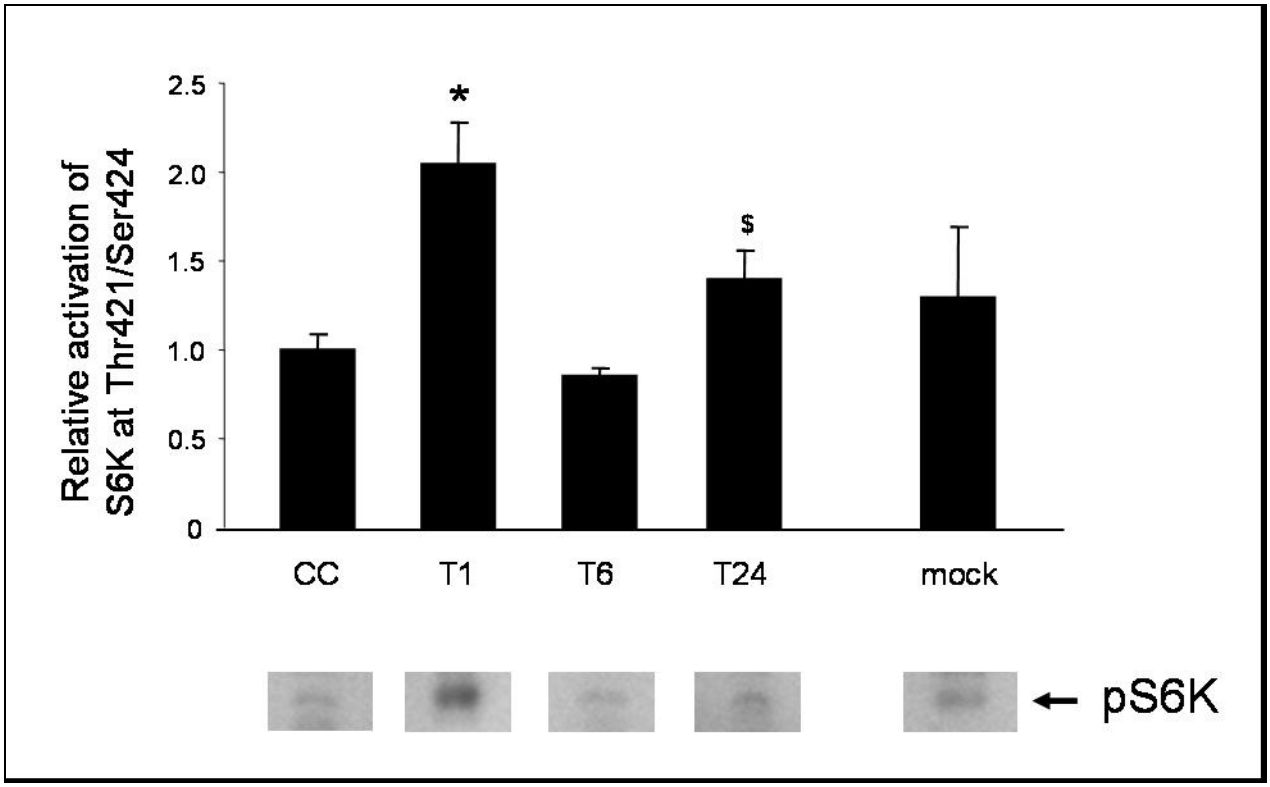

Figure 11: S6K activation at Threonine421and Serine424 after tenotomy. CC: cage control; T1: tenotomy of 1 hour, T6: tenotomy of 6 hours; T24: tenotomy of 24 hours. *: $p<0.05$, $: $0.1<p<0.05$ versus CC

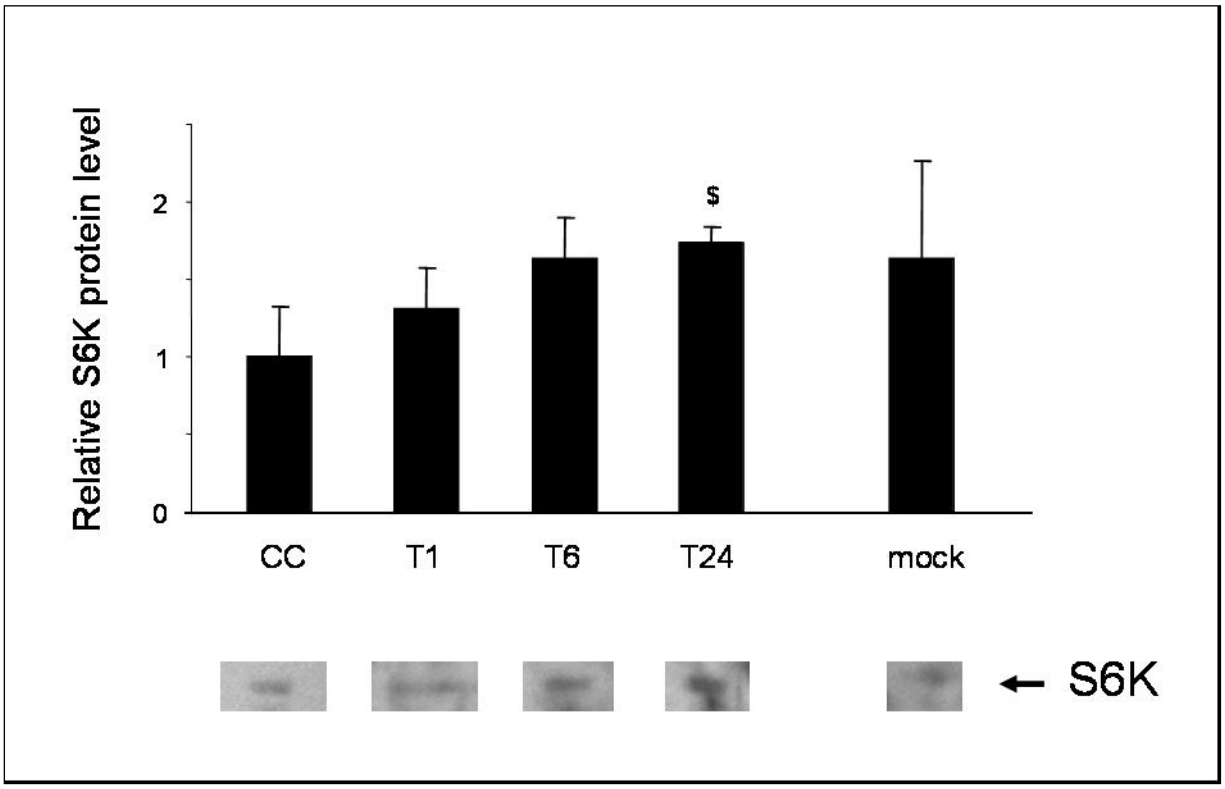

Figure 12: S6K protein level after tenotomy. CC: cage control; T1: tenotomy of 1 hour, T6: tenotomy of 6 hours; T24: tenotomy of 24 hours. $: $0.1<p<0.05$ versus CC

Akt

Analysis of the phosphorylation status of Akt at its major activation site Serine473 revealed a significant increase after 1 hour of tenotomy (Figure 13). No further activation was observed suggesting an involvement of Akt mediated signaling towards increased protein synthesis only shortly after the high mechanical stress of tenotomy. The early activation after one hour of tenotomy was observed although the protein level was decreased at that time point (Figure 14).

This early activation peak of Akt after 1 hour might explain the first and rapid activation of S6K at both phosphorylation sites.

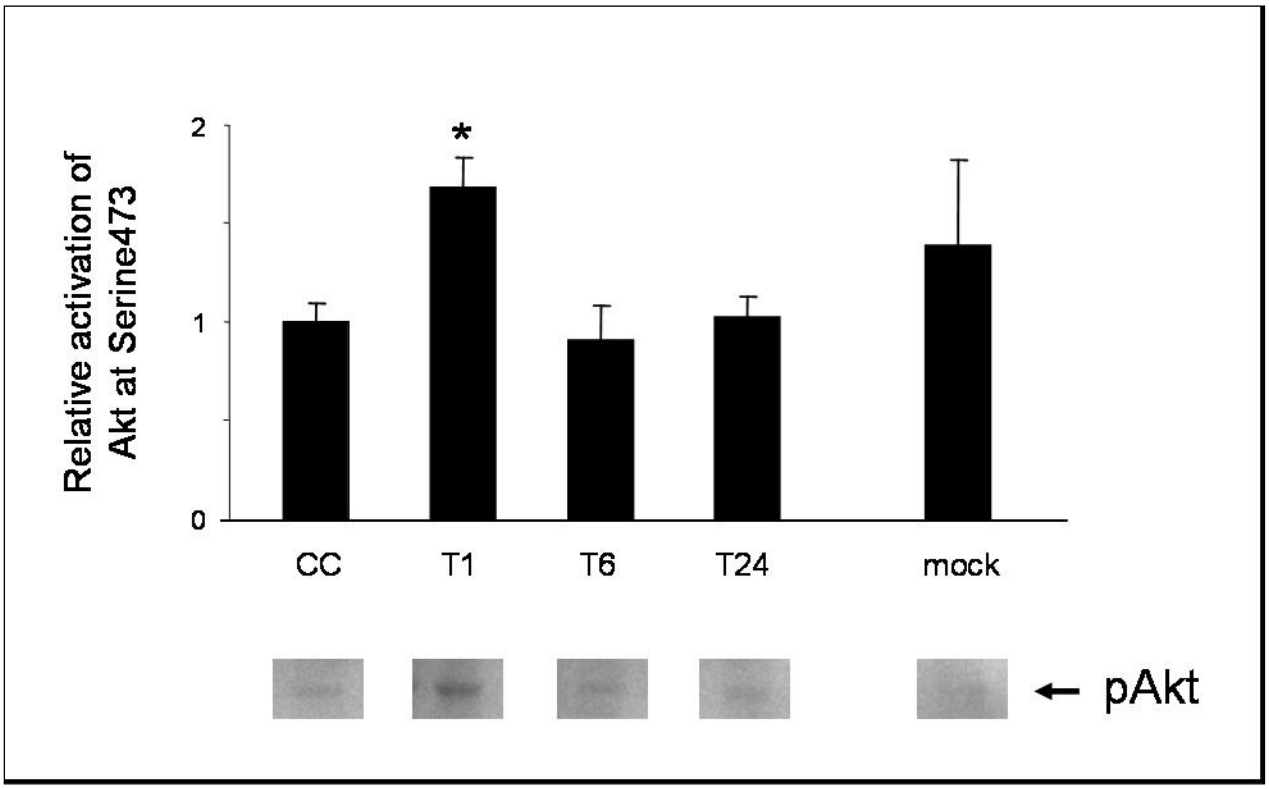

Figure 13: Akt activation at Serine473 after tenotomy. CC: cage control; T1: tenotomy of 1 hour, T6: tenotomy of 6 hours; T24: tenotomy of 24 hours. *: $p<0.05$ versus CC

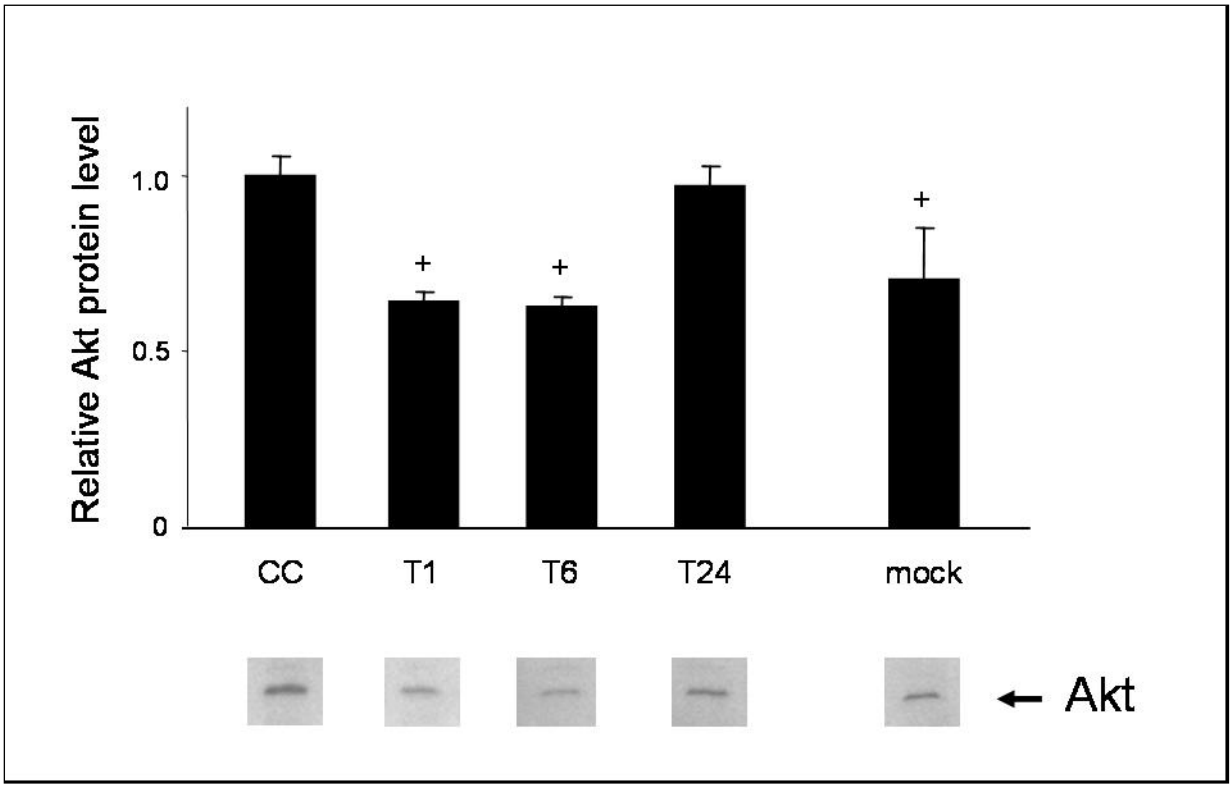

Figure 14: Akt protein level after tenotomy. CC: cage control; T1: tenotomy of 1 hour, T6: tenotomy of 6 hours; T24: tenotomy of 24 hours. +: $p<0.05$ down-regulated versus CC

FAK

FAK was activated at its auto-phosphorylation site Tyrosine397 only 24 hours after the tenotomy (Figure 15). It was accompanied by the activation of FAK protein (Figure 16). This increase in FAK-mediated signaling may explain the second activation peak of S6K after 24 hours of mechanical stimulation. This might confirm the involvement of FAK in mechano-transduction via influencing the signaling cascade towards increased protein synthesis in skeletal muscle.

The early decrease in phosphorylation of FAK after 1 hour might indicate that FAK-mediated signaling is possibly only activated constantly, when occurring in combination with muscle contraction and not with extreme mechanical stretch alone.

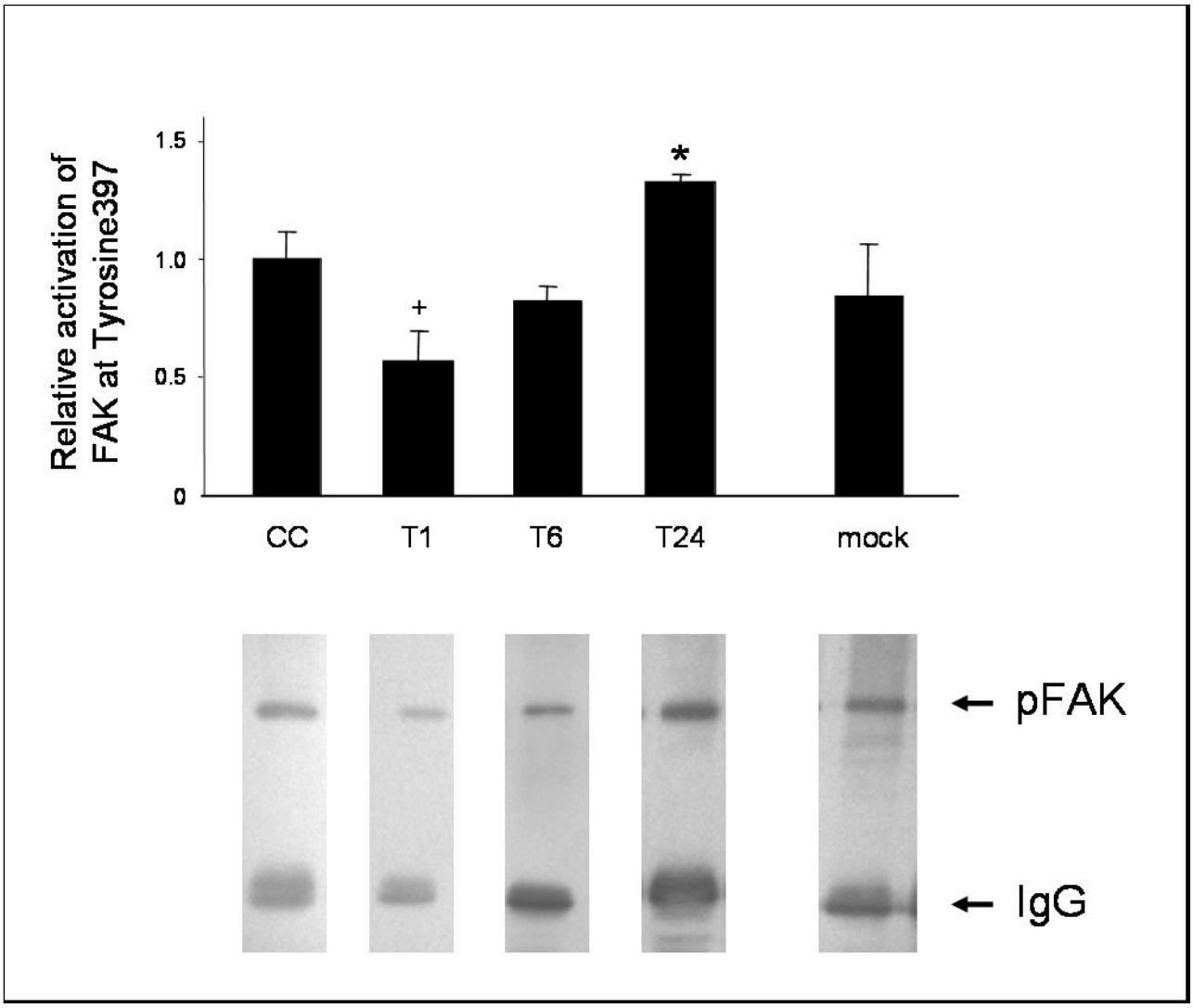

Figure 15: FAK activation at Tyrosine397 after tenotomy. CC: cage control; T1: tenotomy of 1 hour, T6: tenotomy of 6 hours; T24: tenotomy of 24 hours. *: $p<0.05$ up-regulated, +: $p<0.05$ down-regulated versus CC

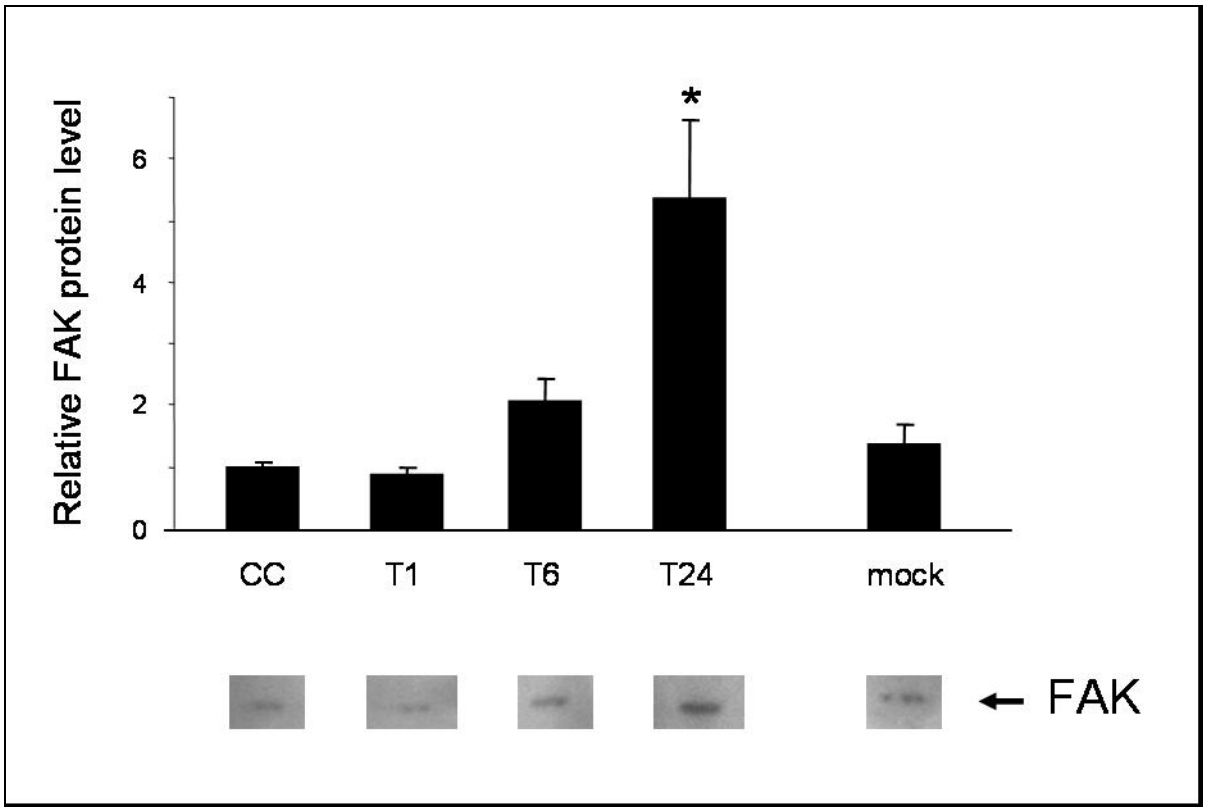

Figure 16: FAK protein level after tenotomy. CC: cage control; T1: tenotomy of 1 hour, T6: tenotomy of 6 hours; T24: tenotomy of 24 hours. *: $p<0.05$ up versus CC

eIF4E-BP1

The increase in protein synthesis was visualized by measuring the activation level of eIF4E-BP1. The early and consistent phosphorylation of eIF4E-BP1 at Serine65 and Threonine70 between 1 and 24 hours (Figure 17) indicates the enduring activation of translation initiation and partly explains the significant increase in *soleus* muscle mass.

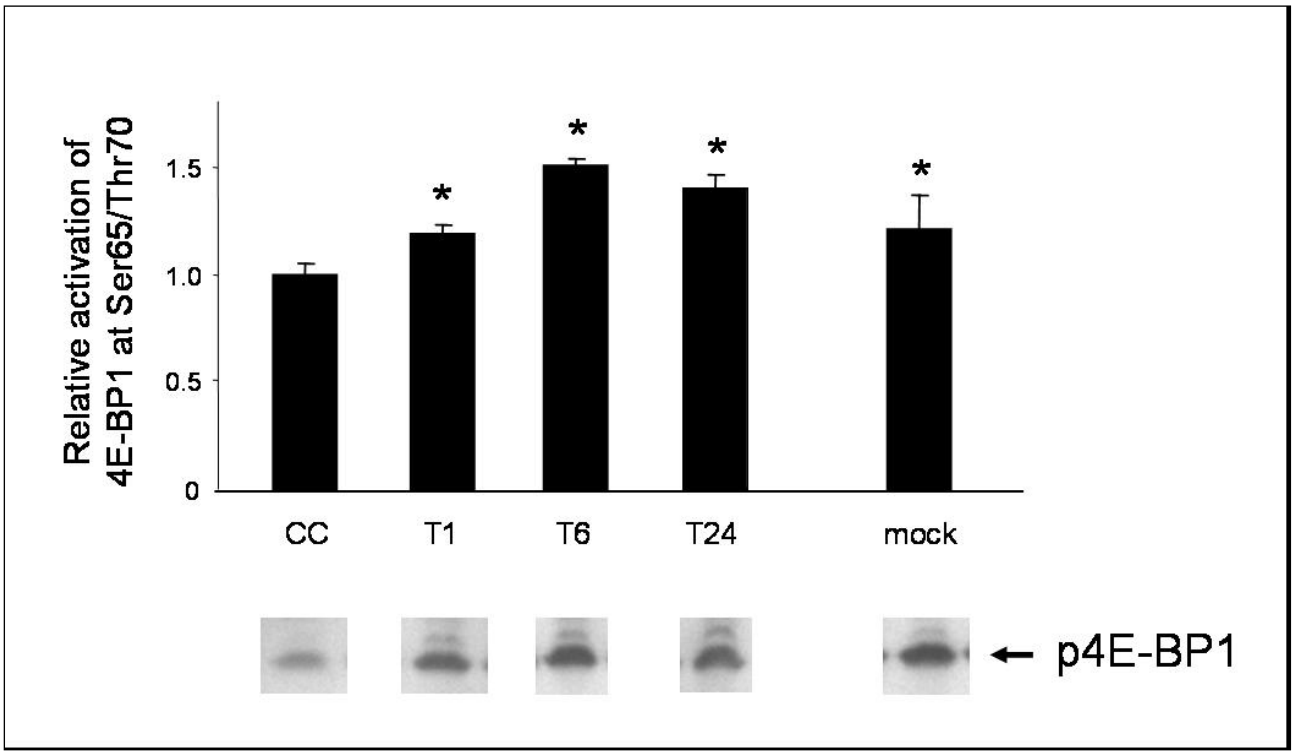

Figure 17: Enduring activation of eIF4E-BP1 at Serine65 and Threonine70 after tenotomy. CC: cage control; T1: tenotomy of 1 hour, T6: tenotomy of 6 hours; T24: tenotomy of 24 hours. *: $p<0.05$ versus CC

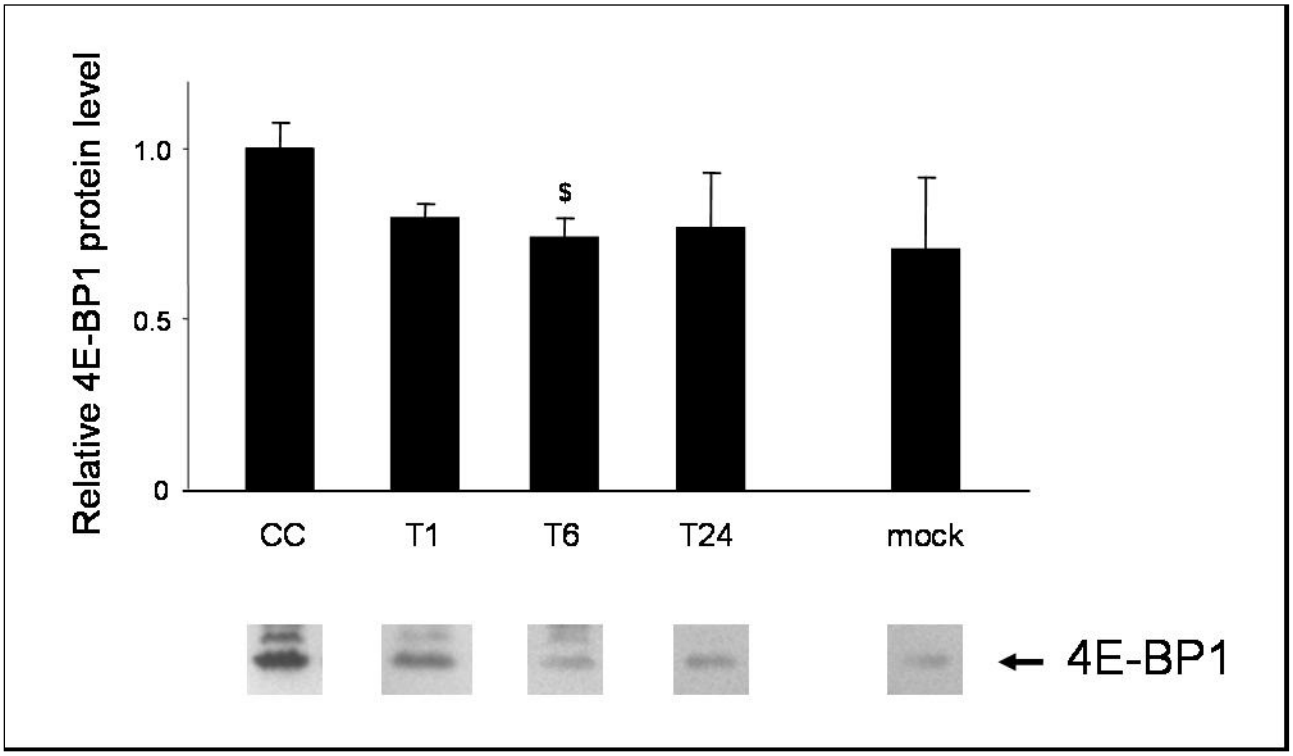

Figure 18: eIF4E-BP1 protein level after tenotomy. CC: cage control; T1: tenotomy of 1 hour, T6: tenotomy of 6 hours; T24: tenotomy of 24 hours. $: $0.1<p<0.05$ down-regulated versus CC

3.3.3 Summary

The hypertrophy signal towards S6K and increased protein synthesis seems to be mediated via Akt-signaling in the first hour of intense mechanical stress after tenotomy. 24 hours post-operation, when the mechanical stress is less extreme and animals move more frequently, the S6K signaling seems to be mediated via FAK activation. At this time point the observed increase in translation initiation seems to be Akt-independent.

These observations suggest that FAK is only involved in mechano-transduction towards an increased protein synthesis, which results in muscle hypertrophy, when the muscle actually contracts and thus the FA site is activated.

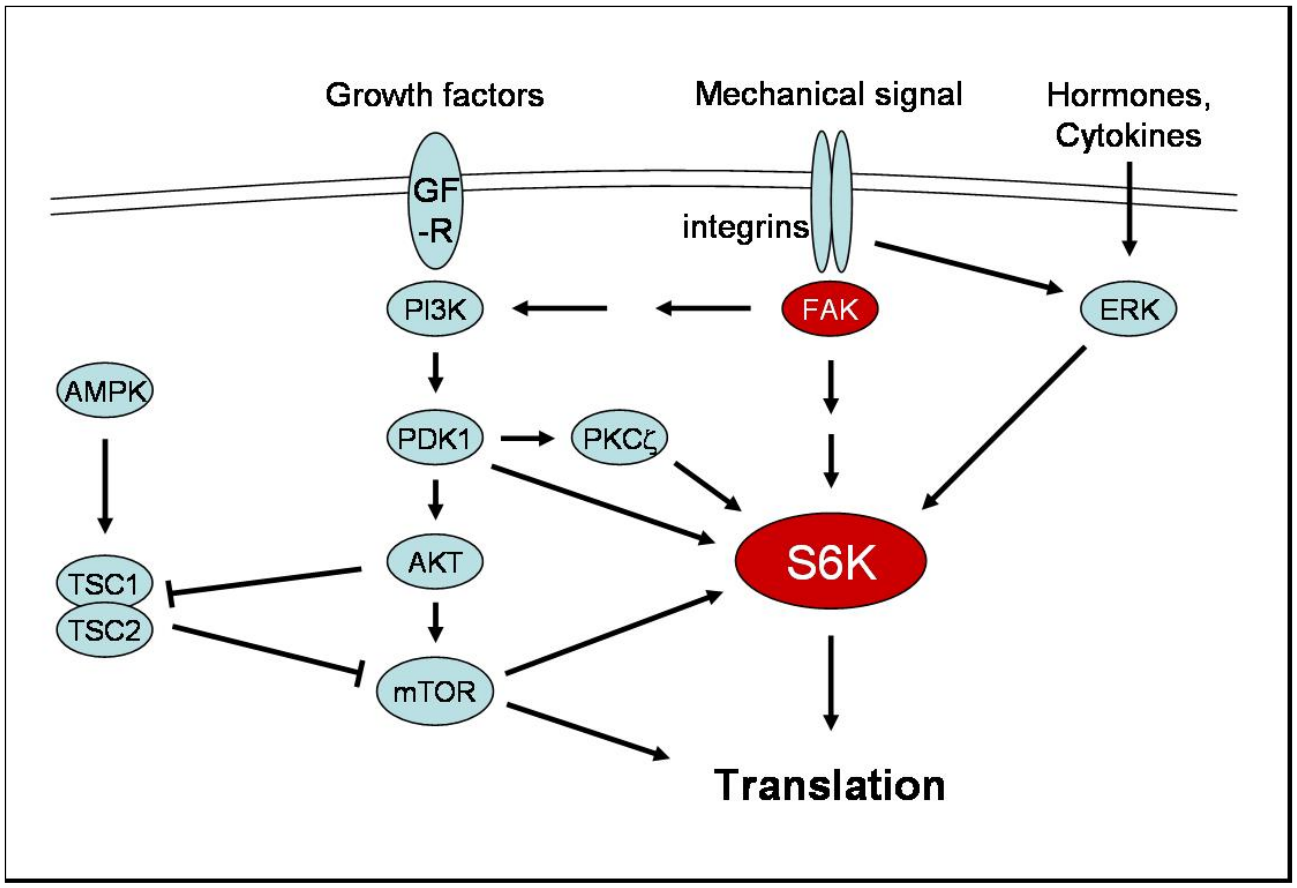

Figure 19: Summarizing and simplified scheme on how mechanical and other signals may promote protein synthesis. The highlighted FAK and S6K are the most important regulators of protein synthesis in the transduction of a mechanical stress.

4 Discussion

4.1 Major findings

In the human eccentric study, I investigated the temporal response of muscle gene expression to a single bout of mild eccentric ergometer exercise. I had to revise the hypothesis that I might identify a major up-regulation of transcripts relevant for processes supporting muscle growth within the first 24 hours of recovery. Instead, I found that the mRNAs of almost all important muscle regulatory gene transcripts are significantly down-regulated and take close to (or more than) 24 hours to revert to pre-exercise values. This study demonstrates for the first time that mild eccentric exercise has a molecular signature distinctly different from intensive concentric exercise as well as from maximal eccentric exercise. This basic difference between the molecular response of the two training regimes lead to the question of whether the observed muscular adaptations in terms of muscle growth and muscle strength after eccentric exercise are mediated primarily through an early adaptation in the protein synthesis machinery.

I therefore used the mouse model of hindlimb suspension to check, whether a mechanical stimulus activates protein synthesis via the mechano-transducer FAK. Combining the mechanical stimulus of reloading of atrophied mice muscle and the overexpression of FAK I identified FAK as an upstream element of the mechano-regulated pathway for activation of S6K. The further finding of a disconnection of this pathway that impacts on protein synthesis from the commonly involved Akt-mTOR signal had a major impact on the control of muscle mass in physiological situations. These finding expanded our view of understanding muscle biology in response to hypertrophic stimuli and established FAK as an important upstream element in mechano-transduction (Favier *et al* 2008).

In the third major part of my PhD I investigated this activation of FAK and S6K in a rat model, where the mechanical stress was much greater. Applying tenotomy to the *gastrocnemius* and *plantaris* muscle and thus, heavily overloading the subjacent *soleus* muscle induced a gain in muscle weight within 24 hours. The hypertrophy signal towards S6K and increased protein synthesis was mediated via Akt in the first hour of intense mechanical stress. After 24 hours when the mechanical stress is less extreme and animals move more frequently, the S6K signaling is mediated via FAK in an Akt-independent manner. These observations suggest that FAK seems only to be involved in mechano-transduction to increase protein synthesis, resulting in hypertrophy, when the muscle actually contracts and the FA site is activated.

4.2 Limitations

4.2.1 Technical limitations

Considering the finding of the gene expression study after an eccentric exercise, one has to be aware of the fact that the observed general down-regulation of mRNA levels after exercise can be either the result of no transcription or of active mRNA degradation. Degradation is mostly coupled to prior translation (Sachs 1993). This was not investigated due to limited muscle samples from the human study. The only possible support of this may be the fact that UBC is more than twofold up-regulated and that this may cause an enhanced tagging of proteins for proteolytic degradation by the ubiquitin pathway. It is thus possible that enhanced protein degradation could also include degradation of muscle-specific mRNAs (Gardrat *et al* 1999,Cascone and Schwartz 2001).

Technical considerations on the experimental approach of hypertrophy stimulation by reloading indicate the contribution of other biological variables than muscle loading. The previously reported damage response of transfected muscle portions by the gene transfer may influence the observed results (Gehl *et al* 1999,Durieux *et al* 2002,Durieux *et al* 2004). In our setting this bias was controlled by comparing the signaling events with empty transfection controls in contralateral muscle leg. This paired design allowed the identification of statistically significant effects of FAK-transfection on S6K-signaling. This is remarkable taking into account the relatively low percentage of actually transfected muscle fibers (Durieux *et al* 2002,Durieux *et al* 2004). Additionally, the responsiveness of TA muscle to hindlimb suspension is relatively low compared with other hindlimb muscles (Carlson *et al* 1999,Dapp *et al* 2004). These considerations highlight the resolution of our approach for exposing muscle signaling. Nevertheless, the effect of the damage response of the gene electrotransfer was omitted in the tenotomy model, where the critical limitations were the amount and duration of cage activity and consequently the muscle contraction within the first hours after the surgery. A reasonable quantification of cage activity was not possible. I observed that within the first 10 minutes after awakening from anesthesia, the animal was moved around the cage considerably more than normal and stressed the muscle excessively for about 3-5 minutes. The activity decreased afterwards and was limited to some exploratory walks around the cage within the first 6 hours. With night-activity, the behavior of these rats was almost normal on the other morning. This is supported by the fact that body weight loss was stopped and the animals apparently increased food and water uptake. My observations of cage activity after 24 hours suggest that the animals have recovered well and had almost returned to their normal activity level. This may have influenced our results such that the augmentation of the FAK

phosphorylation, which is presumably coupled to muscle contraction, was observed only after 24 hours.

4.2.2 Specificity of exercise

The response of skeletal muscle to training depends very much on the actual stimulus that is applied. With muscle showing a great plasticity, the potential for adaptation is large. Strength training provokes a completely different phenotype than endurance exercise and at a first glance, the molecular mechanism underlying these adaptations are diverse and not balanced. With endurance exercise muscle reacting primarly via the up-regulation of specific mRNAs coding for metabolic and mitochondrial proteins, i.e. with an increase in transcription, the induction of the translation machinery seems to be the primary effect of strength exercise. Nevertheless, there are several conserved mechanisms which lay at the heart of these adaptations. They show us that although endurance and strength training lead to diverse phenotypes, the central molecular key players are the same, but used in different ways and to different degrees.

A possible mechanism regulating training specificity involves the elongation phase of translation mediated by eukaryotic elongation factors, which represent a rate-limiting step in protein synthesis. A key component of this translational machinery is eukaryotic elongation factor 2 (eEF2), which mediates translocation of the ribosome along the mRNA. eEF2 is phosphorylated and inactivated by eEF2K in response to stimuli that increase energy demand or reduce energy supply (Browne and Proud 2002). Moreover, the activation of eEF2K appears to be regulated upstream via calmodulin and AMPK-mediated signaling, which are kinases activated in response to endurance exercise (Ryazanov 1987,Horman *et al* 2002). It is suggested that inhibition of eEF2 activity by endurance exercise results in a decrease in translation elongation and protein synthesis (Atherton *et al* 2005,Rose *et al* 2005). Phosphorylation by S6K inactivates eEF2K, while mTOR has also been shown to phosphorylate eEF2K, decreasing kinase activity (Wang *et al* 2001,Browne and Proud 2004). Increased mTOR and S6K activity following resistance training would be expected to promote hypertrophy in part via increased eEF2 activity. In support of this contention, a resistance-like stimulus has been demonstrated to decrease eEF2 phosphorylation, likely enhancing elongation and protein synthesis (Atherton *et al* 2005). Nonetheless, contrasting regulation of eEF2-mediated elongation by endurance and resistance training may represent a point of divergence for control of protein synthesis.

Additionally, the FoxO transcription factor has been implicated in promoting mRNA abundance of genes involved in processes as varied as mitochondrial biogenesis and myofibrillar protein degradation (Goffart and Wiesner 2003,Glass 2005). FoxO functions on the promoter regions and initiates transcription of a number of genes, including PGC-1α and MAFBx. Nuclear abundance and the activity of FoxO is regulated by Akt (Bodine *et al* 2001b,Sandri 2008). When Akt phosphorylates FoxO, it translocates from the nucleus to the cytosol and is prevented from promoting transcription. Akt activation following resistance exercise would likely result in phosphorylation of FoxO and subsequent inhibition of ubiquitin ligase gene expression. While these events would be expected to promote hypertrophy, the concomitant down-regulation of PGC-1α gene expression has been observed (Southgate *et al* 2005). Equally, endurance exercise is associated with increased PGC-1α gene expression and mitochondrial biogenesis, promoting an oxidative phenotype (Irrcher *et al* 2003). However, the nuclear location of FoxO with endurance exercise may suppress net protein synthesis due to increased activity of ubiquitin gene expression and subsequent protein degradation. Therefore, altered regulation of FoxO activity with contrasting modes of exercise may generate contradictory gene expression profiles, ultimately reducing the specificity of adaptation.

The most compelling mechanism proposed to mediate the specificity of training and subsequent interference effect with concurrent training may be the AMPK-Akt 'master-switch' hypothesis (Atherton *et al* 2005). In a rodent model in which muscle fibers were electrically stimulated for prolonged periods at low frequency (to mimic endurance training) or for short periods with high frequency (to mimic resistance training), a reciprocal relationship in the activation of AMPK and Akt pathways in response to these divergent stimuli was observed. Specifically, after low-frequency stimulation they observed increased AMPK-TSC2 activity, PGC-1α gene expression, and an inhibition of mTOR-mediated translation initiation. Conversely, after high-frequency stimulation there was increased Akt-mediated hypertrophy signaling concomitant with a decrease in AMPK and suppression of TSC2 activity. Based on these findings they proposed that the AMPK and Akt signaling may represent divergent pathways that, when activated, direct skeletal muscle adaptation to either an oxidative or hypertrophic phenotype.

The current literature provides a number of possible mechanisms to explain the specificity of training adaptation in response to strength and endurance exercise. Indeed, it appears that divergent adaptive phenotypes are induced via the complex interactions of numerous common signaling and gene expression pathways, highlighting the challenge of interpreting observed adaptation to exercise. Regardless, alternating endurance and

strength type of exercise likely reduces the capacity for the simultaneous acquisition of hypertrophy and/or mitochondrial training induced adaptation responses compared with single mode training.

4.3 The control of muscle mass

4.3.1 Muscle hypertrophy

Skeletal muscle hypertrophy occurs following a mechanical stimulation, e.g. after repeated bouts of resistance or of eccentric exercise. While each individual exercise is necessary it is not sufficient to produce hypertrophy by itself. This indicates that following an acute exercise, there is a transient alteration within the muscle that when repeated over a longer time period, produces skeletal muscle hypertrophy. Therefore, in order to understand what drives the increase in muscle mass we need to understand what happens immediately following a single bout of resistance or eccentric exercise. The most important acute response to resistance or eccentric exercise is an increase in the rate of protein synthesis.

In humans, a bout of high resistance exercise increases the fractional rate of protein synthesis 50% after 4 hours and 115% by 24 hours (Chesley *et al* 1992). In some studies this increase in protein synthesis is observed to maintain up to 48 hours before returning to control levels (Phillips *et al* 1997). Theoretically, this increase might be due to an activated transcription and translation. However, in the eccentric study I showed that this is not the case for most important muscle transcripts (Klossner *et al* 2007). Others showed no change in the RNA content of the muscle at either 4 or 24 hours following a single bout of resistance exercise (Chesley *et al* 1992). This suggests that the immediate changes in protein synthesis are the result of an increase in the amount of protein synthesized per molecule of mRNA and not an increase in total mRNA, thus, it is the efficiency of translation that is increased following loading. An immediate increase in the rate of protein synthesis following an acute bout of resistance exercise has also been shown in a number of animal models, where the initial response to either concentric and eccentric strength exercise was a 25–50% increase in protein synthesis (Wong and Booth 1990a,Wong and Booth 1990b). This increase in protein synthesis was still observed 12 –17 hours after the exercise bout, it was concluded that increases in RNA do not have a primary role in increased protein synthesis after a single bout of resistance exercise (Wong and Booth 1990b).

As noted in the introduction the IGF signaling pathway seems to be a prominent activator of protein synthesis and therefore a good target of investigations. Indeed skeletal muscle IGF-1 and IGF-binding protein mRNA and protein content increase in response to

contractile activity in a variety of overload models (Adams *et al* 1999,Hameed *et al* 2003,Spangenburg and McBride 2006). However, the impact of IGF-1 on hypertrophy is questionable, as e.g. IGF-1 mRNA levels were reported to increase (Bamman *et al* 2001,Petrella *et al* 2006), decrease (Psilander *et al* 2003,Bickel *et al* 2005) and remain unchanged (Hameed *et al* 2003,Bickel *et al* 2003) in response to resistance exercise. Differences in exercise mode, individual variability and the unknown time course of expression for the IGF-1 response may provide some explanation for these divergent findings. Nevertheless, the IGF-1 genotype appears to enhance the strength response to resistance exercise in humans, as the IGF-1 promoter polymorphism has been associated with greater strength gains following resistance training (Kostek *et al* 2005). Therefore, despite the incomplete clarity in the human data, the available investigations implicates that IGF signaling is involved in exercise-induced muscle hypertrophy in response to resistance training, although this pathway may be activated via different mediators depending on the mode of contraction, duration and intensity of the exercise stimulus (Favier *et al* 2008). Thus, S6K, being most proximal to the translation machinery in this pathway, is probably the best target to study hypertrophy.

4.3.2 Control of protein synthesis

To determine which step of protein synthesis is affected in response to resistance exercise, polysome profiling was performed to show that there was an increase in the association of mRNA with ribosomes suggesting that the rate of initiation of protein synthesis had increased more than the rate of elongation and termination (Baar and Esser 1999). This stimulation of translation initiation may be promoted through phosphorylation and thus activation of S6K and eIF4E-BP1.

Initiation can largely be separated into two regulated steps. The binding of the initiator tRNA to the 40S ribosomal subunit to built a pre-initiation complex, which is regulated by eIF2 and the successive cap-dependent binding of mRNA to the preinitiation complex which is regulated by eIF4E and its repressor 4E-BP (Gingras *et al* 2001,Sonenberg and Dever 2003,Richter and Sonenberg 2005). Both of these processes can be controlled following loading by the components of the IGF signaling pathway. The mTOR-raptor complex has been widely studied for its role in controlling protein synthesis through phosphorylation of its downstream targets the translational inhibitor 4E-BP1 and the S6K. Phosphorylation of S6K by mTOR-raptor and Akt changes the conformation of this protein making it constitutively active for further signaling.

Resistance exercise is known to induce a transient increase in the phosphorylation of mTOR (Parkington *et al* 2003), Akt (Nader and Esser 2001), 4E-BP1 (Bolster *et al* 2003b), S6K (Baar and Esser 1999), as well as the activity of eIF2 (Kubica *et al* 2005). The increase in S6K phosphorylation 6 h after a single bout of resistance exercise correlates with the increase in muscle mass following 6 weeks of training, suggesting that it may play an important role in regulating muscle mass (Baar and Esser 1999). In support of this hypothesis, the blocking of mTOR activity with the bacterial antibiotic rapamycin, blocks the activation of S6K, the increase in eIF2 activity and the increase in muscle mass following overload (Bodine *et al* 2001b,Kubica *et al* 2005). Consequently, muscle mass in S6K knockout mice is decreased (Ohanna *et al* 2005). This shows that components of the IGF signaling pathway have a direct influence on the hypertrophic response by regulating the rate of translation initiation.

Along with increasing the rate of initiation, another way to promote the rate of protein synthesis is to increase the number of ribosomes within muscle following increased loading. The number of ribosomes in a cell plays a fundamental role in growth regulation because it affects the amount of protein being synthesized per mRNA molecule. The cellular content of ribosomes is mainly regulated by an increase in their biosynthesis, which requires the coordinated synthesis of approximately 80 ribosomal proteins and four RNA species in addition to several hundred accessory enzymes. It has been reported that ribosome biogenesis during skeletal muscle hypertrophy is indeed regulated (Nader *et al* 2005). It is thought to occur via a cell cycle mechanism that is dependent on mTOR signaling as these effects could be blocked with rapamycin treatment.

As well as increasing protein synthesis, resistance exercise increases the rate of protein degradation. The importance of the increase in degradation can be seen in the correlation between the fractional synthesis rate and the fractional breakdown rate in muscle following loading (Phillips *et al* 1997). As the rate of protein breakdown increases there is a concomitant rise in protein synthesis suggesting that there might be a molecular link between the two processes. However, the association is not always seen. Eating a meal rich in essential amino acids can decrease the effects of resistance exercise on protein degradation whilst at the same time increasing the rate of protein synthesis (Tipton *et al* 1999). Taking in a carbohydrate or mixed amino acids meal decreases the rate of degradation, possibly by decreasing circulating corticosteroids, without affecting the rate of synthesis. Therefore, it is only in the fed state that the net protein balance becomes positive allowing the muscle to grow (Tipton *et al* 1999).

Since exercise stimuli always represent a mixture of metabolic, hormonal, neural and mechanical stimuli, single contributions are hard to discriminate from each other. Endurance exercise has a pronounced metabolic component, whereas the metabolic component is insignificant in pure eccentric exercise, which is primarly a mechanical stressor. Given the fact, that sensors or integrators of metabolic (AMPK) and mechanical (FAK) stimuli are competitive, a continuum of different adaptations depending on the magnitude of single contributing stimuli might be expected. Moreover, the actual status of muscle cells potently contributes to the final response. For example the energy status of muscle tissue (i.e. glycogen depleted) influences the activation of the main energy sensor AMPK which is an inhibitor of the mTOR/S6K stimulated protein synthesis. Strength exercise with either filled or depleted glycogen stores leads to a distinct immediate response pattern with regard to the molecular signaling events.

5 Outlook

The enormous and well-described plasticity of muscle tissue and the possibility to easily collect biopsies makes muscle a very interesting organ to study gene regulatory phenomena in humans. With the advent of technology to monitor thousands of genes and signaling events at once, skeletal muscle has a great potential to study and understand adaptations to different physiological stimuli *in vivo*. With the appropriate molecular techniques it has been demonstrated in the past years that rapid changes in mRNA occur with exercise in humans and rodent species (Neufer and Dohm 1993,Puntschart *et al* 1998,Pilegaard *et al* 2000,Fluck *et al* 2005). Recent expression profile data demonstrate that transcriptional adaptations in muscle due to changes in loading involve adaptations of genes in several functional categories (Fluck *et al* 2008,Durieux *et al* 2009).

For endurance type exercise, expressional changes of genes belonging to functional categories indicate that expression of a battery of genes is controlled by master transcriptional regulators. Signaling pathways involving the nuclear-encoded transcription factors NRF-1, TFAM, AP-1, PPAR and AMPK, HIF-1α, and myogenic regulatory factors may be implicated in transmitting and integrating physiological stress into transcriptional adaptations of metabolic and contractile genes. These changes are matched to structural and functional adaptations and enzyme activity. Nuclear reprogramming is recognized as an important event in muscle plasticity and may be related to the adaptations in the myosin type, protein turnover, and the maintanance of the cytoplasma-to-myonucleus ratio.

In the case of strength exercise, the main immediate response is not regulated via alterations in the transcription but in the translation machinery. Currently signaling pathways that control skeletal muscle protein synthesis are intensivly investigated. The mTOR signaling plays a very important role in this process, although the way that muscle protein synthesis is regulated mainly under physiological conditions in whole systems such as tissues or organisms still remains to be fully unraveled. Identification of new key molecules or novel functions of already known parameters that play a major role in cellular signaling cascades and interact with different pathways are the basis for further expanding our current understanding about skeletal muscle adaptability and will provide new insights regarding muscle therapeutic strategies or exercise training recommendations. The discovery of FAK as an important mediator of mechanical stress and the identification of FAK being involved in hypertrophy signaling may open new doors to understand muscle physiology. Particularly, it is well established that different stimuli affect and activate the available signaling pathways to different extents and in different modes. Therefore, the

findings of my PhD projects enlarge the current understanding of hypertrophy signaling in response to mechanical stimulation and identify FAK as an important stimulator of S6K activation and downstream signaling towards increased protein synthesis.

Future experiments are required to better understand the acute regulatory phenomena occurring as a consequence of a single exercise and to relate them to the adaptations with chronic exercise. Acute regulatory phenomena need to be linked to the steady state changes in mRNA levels of structural genes ultimately responsible for the structural and functional modifications occurring with repeated exercise. Future approaches will make use of high-throughput technology such as protein and DNA arrays to identify the characteristic molecular adaptations in animal and human models. This will help unravel functions of the identified pathways and expand it with new connections to other processes, such as the activation of FA sites during mechano-transduction. Although most of these pathways were and will be established in model organisms, they have yet to be verified in human studies. This will facilitate to interprete results from model organisms and help in translating them into human situations. Understanding the molecular mechanisms of muscle plasticity therefore is of importance for the understanding of the development of diseases such as obesity, hypertension, muscular dystrophy, myasthenia, atrophy and so forth and will give insight into these important clinical processes (Booth *et al* 2002).

6 Acknowledgements

As sportsmen I have always been interested in strategies to improve human muscle performance. Although different training methods are advertised to be the best for improving strength or endurance (or both), the cellular mechanisms and the molecular pathways underlying the remarkable plasticity of skeletal muscle are still poorly understood. It was this impulse of understanding muscle performance that motivated me to work in this field. The integrative approach of the group of Prof Hans Hoppeler in studying skeletal muscle plasticity simultaneously on the structural, functional and molecular level with a mixture of applied and basic research was so fascinating to me that I joined his group in 2005 for my Master thesis and then stayed for my PhD project.

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Appendix

A) Muscle transcriptome adaptation with mild eccentric ergometer exercise
Klossner S et al. Pflügers Archive 2007

B) Mechano-transduction to muscle protein synthesis is modulated by FAK
Klossner S et al. Eur J Appl Physiol 2009

C) Biologically relevant sex differences for fitness-related parameters in active octogenarians
Lötscher F et al. Eur J Appl Physiol 2007

D) Gene expression in working skeletal muscle
Hoppeler et al. Hypoxia and the Circulation 2007

E) Training in hypoxia and its effects on skeletal muscle tissue
Hoppeler et al. Scand J Med Sci Sports 2008

F) Mechano-regulated Tenascin-C orchestrates muscle repair
Flück et al. PNAS 2008

G) Focal adhesion kinase is a load-dependent governor of the slow contractile and oxidative muscle phenotype
Durieux et al. J Physiology 2009

H) Different response to eccentric and concentric training in older men and women
Mueller et al. Eur J Appl Physiol 2009

I) CV and publication list

J) Declaration of originality

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SKELETAL MUSCLE

Muscle transcriptome adaptations with mild eccentric ergometer exercise

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Abstract The muscle has a wide range of possibilities to adapt its phenotype. Repetitive submaximal concentric exercise (i.e., shortening contractions) mainly leads to adaptations of muscle oxidative metabolism and endurance while eccentric exercise (i.e., lengthening contractions) results in muscle growth and gain of muscle strength. Modified gene expression is believed to mediate these exercise-specific muscle adjustments. In the present study, early alterations of the gene expression signature were monitored by a muscle-specific microarray. Transcript profiling was performed on muscle biopsies of vastus lateralis obtained from six male subjects before and in a 24-h time course after a single bout of mild eccentric ergometer exercise. The eccentric exercise consisted of 15 min of eccentric cycling at 50% of the individual maximal concentric power output leading to muscle soreness (5.9 on a 0–10 visual analogue scale) and limited muscle damage (1.7-fold elevated creatine kinase activity). Muscle impairment was highlighted by a transient reduction in jumping height after the eccentric exercise. On the gene expression level, we observed a general early downregulation of detected transcripts, followed by a slow recovery close to the control values within the first 24 h post exercise. Only very few regulatory factors were increased. This expression signature is different from the signature of a previously published metabolic response after an intensive endurance-type concentric exercise as well as after maximal eccentric exercise. This is the first description of the time course of changes in gene expression as a consequence of a mild eccentric stimulus.

Keywords Human · Skeletal muscle · Eccentric exercise · Gene expression · Microarray · Muscle damage

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Introduction

Exercise induces phenotypical adaptations in skeletal muscle, which critically depends on specific mechanical, metabolic, and hormonal responses elicited by the stimulus. This is illustrated by a different response of mitochondria and contractile elements in muscles after long-term low-load high-repetitive "endurance-type" exercise [13] vs high-load low-repetitive "strength-type" exercise [28, 44].

We have shown that endurance training causes improvements in oxidative metabolic characteristics including enhanced capillary and myocellular lipid metabolism as well as changes in glycogen metabolism [37]. Recent research demonstrates that the mechanisms responsible for the adaptive processes are reflected by modifications in gene expression. In particular, it was shown that ribonucleic acid (RNA) concentrations of mitochondrial transcripts are increased approximately in proportion to the gain in mitochondrial volume [33]. The idea of a dominant control of muscle phenotype through an increase in muscle gene expression was supported by the group of Pilegaard [31], who performed nuclear run-on assays after concentric endurance type exercise. These experiments furnished

direct evidence of the transcriptional control of RNA concentrations in training studies. Further evidence came from Hood et al. [18], who elucidated the mechanisms by which mitochondrial biogenesis is regulated and integrated in an exercise setting [31]. Looking at the time course of the transcript response to a single concentric exercise bout, Schmutz et al. [37] could demonstrate that a majority of muscle transcripts were upregulated after 8 h of recovery, with most transcript levels returning to baseline values after 24 h [37].

In strength training, the dominant phenotypical adaptation of the skeletal muscle tissue consists of an increase in myofibrils and associated proteins. This is classically achieved by high-load muscle contractions [36]. More recently, it was shown that considerable gains in muscle strength and fiber cross-sectional area can also be achieved by chronic eccentric exercise (medium-load high-repetitive negative work=mild eccentric ergometer exercise) [17, 20, 25]. This finding is of great practical implication, as chronic eccentric exercise could be used as an important mean to increase muscle performance and muscle mass in situations where high-load exercise may not be tolerated [27, 38]. The mild eccentric exercise analyzed in this study circumvents muscle damage and pain while maintaining the mechanical stress of the eccentric muscle contractions and its potential gain in muscle strength. Currently, there are few data on the molecular mechanisms underlying the phenotypical adaptations to eccentric exercise. Studies using low-repetitive high-load eccentric exercise indicate that gene transcripts involved in extracellular remodeling and the inflammatory response are strongly increased up to 24 h postexercise [7]. The same group showed a direct relation between the mechanical load and the activation of the inflammatory response [8]. These results indicate that high mechanical stress experienced by muscles during heavy eccentric exercise leads to a specific molecular response, different to that after concentric exercise but similar in time course.

It was our aim to characterize the specific changes of the muscle transcriptome to a single mild eccentric stimulus, which was previously shown to increase muscle cross-sectional area and muscle strength, when applied repetitively over longer time periods [25]. We hypothesized that eccentric exercise would result in a broad upregulation of transcripts relevant for the processes initiating muscle growth and improved strength generation within the first 24 h postexercise.

Materials and methods

Subjects and training intervention

This study was conducted with permission of the Ethics Committee of Bern, Switzerland, in compliance with the Helsinki Convention for Research on human subjects. Six untrained male subjects gave their written consent to participate in the study. They were recruited to perform a single bout of chronic negative work (eccentric exercise) on a custom-built eccentric-bike (e-bike [27, 38]). The e-bike is driven by a 5-hp motor that regulates pedal revolutions per minute and torque. The subject had to perform eccentric muscle contractions by resisting the motor, such that the applied eccentric torque developed by the leg was equal to a given target load displayed on a computer monitor. Anthropometric parameters such as age, height, weight, lean body mass, and body mass index (BMI) were determined at the outset of the study. The subjects performed a VO_{2max} test estimating their maximal concentric power output (P_{max}). Two weeks later, they performed a single eccentric ergometer exercise bout on the e-bike at 50% of their individual P_{max} for 15 min (see Fig. 1).

Functional analysis

The creatine kinase (CK) activity in the plasma was measured before the eccentric ergometer exercise and after 3, 8, 24, 48, and 96 h of recovery. The subjects also indicated their muscle soreness on a 0–10 visual analogue scale (VAS) [5] at 3, 8, 24, 48, 72, and 96 h postexercise.

Muscle strength was assessed by counter movement and squat jumps before and 1 and 4 days after the eccentric ergometer exercise. A one-dimensional force platform and associated software (Quattro Jump®, Kistler, Switzerland) was used to analyze jump parameters.

Muscle sampling

Before the eccentric exercise bout and at 1, 3, 8, and 24 h postexercise, fine-needle biopsies (14 gauge, single-use needles; Medilink, Pressagona, Switzerland) were taken from the vastus lateralis muscle [21] (Fig. 1). Our previous study of similar design but with concentric exercise [37] suggested that a gene transcript response to a single bout of

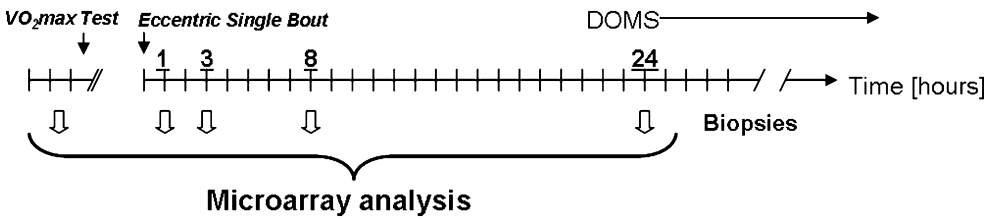

Fig. 1 Experimental protocol timeline. Muscle biopsies are taken before and after a single bout of mild eccentric ergometer exercise. Delayed onset of muscle soreness (*DOMS*) peaks between 24 and 48 h of recovery from an eccentric single bout

exercise is completed within 24 h. We therefore stopped sampling muscle tissue after 24 h. Muscle samples were immediately frozen in isopentane cooled by liquid nitrogen and stored in liquid nitrogen until analysis.

Microarray analysis

Total RNA was isolated from 25 μm cryosections of the muscle biopsies as described previously [41]. The amount of isolated RNA was determined with the Ribo Green assay (Juro Supply, Lucerne, Switzerland). Subsequently, microarray experiments were carried out using custom-designed low-density Atlas complementary deoxyribonucleic acid (cDNA) expression arrays (BD Biosciences, Allschwil, Switzerland) as described [9]. The array held 231 double-spotted probes of human cDNAs associated with particular aspects of skeletal muscle function. Additionally, cDNA probes for an internal reference, 18S ribosomal RNA (rRNA), were included on the array. Batches of five samples (all five time points of one individual) were processed simultaneously. ^{32}P Deoxyadenosine triphosphate (dATP)-labeled cDNA was generated from 1.2 μg of total RNA by using the 231 gene-specific primers supplied. Probe synthesis for the measurement of the internal 18S rRNA reference was carried out in parallel. Total RNA (0.3 μg) of each sample was, respectively, run for the generation of ^{32}P dATP-labeled cDNA with a specific primer for 18S rRNA. Arrays were hybridized with a mix of total cDNA and 18S cDNA diluted 1:1800. After 7 days of exposure, a phosphor imager (Molecular Dynamics, Sunnyvale, CA) was used to detect signal intensities.

Array evaluation

A template was created using the AIDA Array Metrix software (Raytest Schweiz AG, Urdorf, Switzerland). The raw signals, given as the sum of pixel intensities, were determined from the average signal intensities of the two corresponding dots. This mode calculates the average pixel intensity in a ring around each spot. The background intensity was estimated from 54 dots on each array. Transcripts were considered "detected" when the corresponding signal intensity was 30% above background in at least four of the six possible filter hybridizations for one time point. Microarray and detection procedures were identical to those used by Schmutz et al. [37].

Statistical analysis

Raw data were background-corrected and logarithmized to the base of 2. Standardization was performed by subtracting the 18S rRNA from the value of each transcript. Standardization to 18S rRNA values was chosen because rRNA represents a major portion of the total RNA (i.e. ~27%) [12, 24].

To determine whether a signal of a particular messenger RNA (mRNA) was significantly ($p \leq 0.05$) different or showed a tendency ($0.05 < p \leq 0.1$) throughout the time course, each detected gene was tested with the Friedman analysis of variance (ANOVA; Statistica 6.1; StatSoft [Europe], Hamburg, Germany). The nonparametric Friedman ANOVA was used to account for the paired design of the sampling. To identify significant differences between before and after the single exercise bout, the paired Wilcoxon test was applied ($p \leq 0.05$). No adjustments were made for multiple testing. The results were validated with the L2 permutation regression analysis whereby expressional changes were identified as outliers to a linear regression line in scatter plots. Raw values of all detected gene transcripts were included in this analysis. This approach is justified by the robust-linear relationship between RNA expression levels from different individuals [14]. While the Friedman ANOVA is based on a paired comparison of the value of each time point to the prebiopsy value, the L2 regression is a compound analysis of all time points in a single regression.

The physiological variables of this study (CK activity, evaluation of muscle soreness [VAS] and jump height in the squat and counter movement jump [CMJ]) were analyzed using a nonparametric ANOVA with a Wilcoxon post-hoc test. Significance was accepted for $p \leq 0.05$ for all variables.

Results

Physiology

Subjects were of age 22±2.6 years (mean±SD), height 176.8±6.7 cm, weight 69.9±12.3 kg, lean body mass 61.5±7.4 kg, BMI 22.3±3.0 kg/m^2, VO_{2max} 45.1±6.0 ml min^{-1} kg^{-1}, and P_{max} 260±31 W/kg. After the eccentric ergometer exercise bout, all six individuals showed a significant reduction in the jumping height of the CMJ (−6.4%) 24 h postexercise but recovered to normal levels after 96 h (+8.8% higher than value after 24 h). The decrease in squat jump height did not reach the level of significance 24 h after the eccentric exercise bout (−2.4%) but was significantly higher after 96 h compared to the value after 24 h (+5.4%), indicating a similar trend as seen for the CMJ (Table 1).

CK activity was significantly increased until day 2 after the eccentric exercise (see Table 1). However, CK values were not altered to the same extent as observed after more intense eccentric exercise [8]. A VAS for muscle pain showed a strong induction of delayed onset of muscle soreness (DOMS) that peaked between 24 and 48 h and stayed elevated until day 4 (see Table 1).

Table 1 Physiological results

Time point (h)	0	3	8	24	48	72	96
SJ (height in cm)	41±5.9			40.5±4.4			42.7±5.1[a]
SJ (P_{max} in W/kg)	50.2±3.2			47.4±2.9			51.0±3.1[a]
CMJ (height in cm)	43.5±3.6			40.7±3.1[a]			44.3±1.7[a]
CMJ (P_{max} in W/kg)	48.6±2.8			46.7±2.4[a]			49.7±2.3[a]
CK in blood (U/l)	91.5±26.5	122.1±21.8[a]	150.6±47.7[a]	152.0±58.1	128.8±34.4[a]		82.4±14.2
VAS (0,10)	0.3±0.2	1.5±1.0[a]	2.7±2.9[a]	5.9±1.4	5.6±4.2	3.0±1.2	1.5±1.1

Mean of height, P_{max} of squat jump (SJ), and counter movement jump (CMJ) ± standard error are given. Characteristics of DOMS: Creatine kinase (CK) activity and visual analogue scale (VAS). Significantly changed values from one time point to the other are marked with a superscripted "a"

Microarray analysis

Microarray analysis of 231 gene transcripts yielded a total of 147 detected gene transcripts. The time course of these, when analyzed together, showed a significant downregulation (Fig. 2). From the 147 detected gene transcripts, 80 turned out to have significantly changed throughout the time course (Friedman ANOVA). Out of these 80 transcripts, 58 were significantly changed at least at one time point throughout recovery (Wilcoxon test). Only seven of these were upregulated, while 51 were downregulated (Table 2). The downregulation of most of these transcripts was apparent already after 1 h postexercise and lasted over the entire observation period of 24 h. Expression levels of many early downregulated mRNAs were significant higher at 24 than 1 h postexercise (see Table 2). This is indicative for the recovery of the overall gene transcript levels (Fig. 2). A few regulatory factors were upregulated. Among these, we found an early induction of mRNAs for c-jun and ubiquitin C (UBC) 1 h post exercise. Later, between 3 and 24 h postexercise, mRNA levels for myogenic factor 6 (MYF6), cyclin-dependent kinase inhibitor 1A (p21), tubulin alpha 1 (TUBA1), insulin-like growth factor binding protein 4 (IGFBP4), and interleukin 6 receptor (IL6R) were enhanced. All changes observed in transcript levels were validated, using the permutation-based L2 regression analysis (see supplemental Table 2). A close match between the two statistical analyses was found.

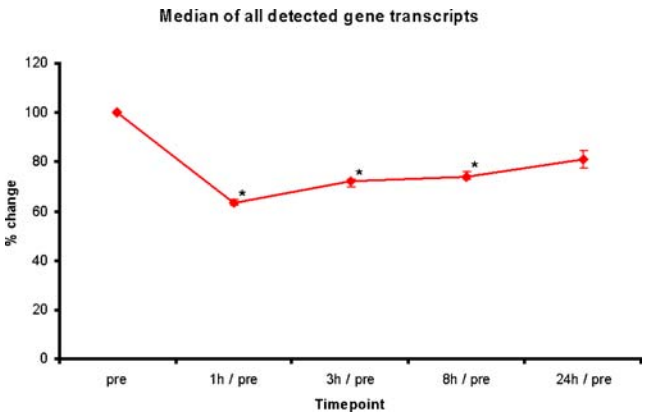

Fig. 2 The overall gene response. Median values and 95% confidence intervals of all detected gene transcripts, relative ratio (in percent) of the different time points (1, 3, 8, and 24 h postexercise) to the prebiopsy. Friedman ANOVA shows a significant effect of time (p= 0.013), *Asterisk*, significant downregulation compared to previous value (p<0.05)

Discussion

Study limitations

An important limitation of this study is the relatively low number of subjects (n=6) that could be included. It therefore seemed justified to use a relatively "coarse-grained" statistical approach (the Friedman ANOVA) to describe the dominant effects of the eccentric training intervention. This was done in particular with regard to the companion study on concentric exercise where the same number of subjects performed a concentric exercise and biopsies were analyzed with the identical molecular and statistical procedures. To have a more detailed view of potential changes related to our experimental intervention, we carried out a more sensitive analysis (L2 regression analysis with corrections for false discover rate) in addition to the Friedman ANOVA [12]. We have previously used this technique to identify muscle transcript level changes in a microarray study of a rat soleus muscle subjected to hindlimb suspension and subsequent reloading [14, 41]. The L2 regression analysis detects 90 transcripts that are significantly altered throughout the time course. This is a higher number than with the nonparametric Friedman ANOVA (58 transcripts). It shows a broadly similar signature of transcript level responses as the Friedman ANOVA but with a potentially higher resolving power. We find that the L2 regression analysis confirms and extends the Friedman ANOVA results and may allow for a finer grain view of the transcriptional events.

Table 2 Effect of an eccentric single bout on the gene transcript signature

	Category	Gene	GenBank ID	p F-Anova	1h / pre	3h / pre	8h / pre	24 h / pre	24 h / 1 h
myogenic regulation	cell cycle	MEF2B	X63380	0.015	0.75	0.44	0.62	0.66	0.88
		MYF4	X17651	0.026	0.52	0.85	1.10	1.61	3.11
		MYF6	X52011	0.078	1.18	2.18	1.60	1.81	1.54
		SRF	J03161	0.056	0.64	0.55	0.54	0.53	0.82
	proliferation	CD34	M81104	0.002	0.36			0.55	1.54
	hormonal	IGFBP4	M62403	0.026				2.90	
		IGFBP5	M65062	0.003	0.63	0.92	0.56	0.47	0.75
		IGF2	M29645	0.005	0.67	1.03	0.80	0.60	0.89
		IL6R	M20566	0.097			3.09		
		LGALS1	J04456	0.07	0.64	0.73	0.68	0.62	0.97
		p21	L25610	0.006	0.51	0.91	2.99	0.97	1.92
sarcomere	myofiber	MYH2/MyHC IIA	AF111784	0.005	0.51	0.74	0.61	0.56	1.10
		MYH4/MyHC IIB	AF111783	0.066	0.59	0.66	0.74	0.81	1.37
		MYH7/MyHCb	M58018	0.01	0.62	0.94	0.69	0.49	0.80
		DES	U59167	0.005	0.51	0.74	0.70	0.60	1.17
		Titin	X69490	0.029	0.57	0.98	0.71	0.99	1.73
interstitial remodeling	capillary	ADORA1	S56143	0.024	0.82	0.71	1.10	1.37	1.67
		ANG	M11567	0.048	0.34			0.42	1.22
	cytoskeleton	CCT1	X52882	0.036	0.91	0.54	0.83	1.24	1.36
		TUBA1	K00558	0.004	0.68	0.72	1.03	1.41	2.08
	degradation	*MMP 8*	J05556	0.082	0.69	0.38	0.51	0.58	0.85
		MMP11	X57766	0.053	0.62	0.57	0.78	1.24	2.00
		MMP14	D26512	0.002	0.56	0.45	0.40	0.49	0.87
		MMP15	Z48482	0.032	0.69	0.40	0.63	0.68	0.98
		PLAT	M15518	0.014	0.69	0.51	0.62	0.68	0.99
		UBC	M26880	0.048	2.04	1.25	2.15	3.22	1.58
	ECM	COL1A1	K01228	0.023	0.34	0.33	0.37	0.49	1.43
		TNC	X78565	0.008	0.56	0.56	1.00	1.47	2.66
		VWF	M10321	0.038	0.63	0.64	0.61	0.88	1.40
cell regulation	proliferation	ADMR	BC034761	0.01	0.38	0.16	0.17	0.21	0.57
		FGFR1	M37722	0.012	0.47	0.40	0.74	1.20	2.54
		FGFR4	L03840	0.032	0.34	0.29	0.28	0.29	0.85
		ITGB1	X07979	0.027	0.35	0.21	0.40	0.41	1.16
		LAMR1	U43901	0.045	0.84	0.98	0.97	1.53	1.82
	transcription	HIF1b	M69238	0.002	0.56	0.43	0.54	0.67	1.19
		EPAS1	U81984	0.066	0.48	0.57	0.65	0.73	1.53
		PPARA	L02932	0.004	0.80	0.51	0.47	0.79	0.99
		PPARG	L40904	0.097	0.87	0.66	0.72	1.29	1.48
	signaling	c-jun	J04111	0.003	2.85	0.74	0.66	0.68	0.24
		DMPK	L19268	0.003	0.45			1.17	2.60
		ITGA8	L36531	0.019	0.79	0.72	0.57	1.15	1.47
		VCAM1	X53051	0.063	0.73	0.48	0.84	0.76	1.04
metabolism	beta oxidation	ACADVL	D43682	0.019	0.61	0.69	0.70	0.66	1.07
		CPTI	D87812	0.004	0.54	0.62	0.46	0.41	0.76
		DCI	L24774	0.011	0.58	0.67	0.55	0.52	0.90
		ECH1	U16660	0.097	0.72	0.64	0.56	0.51	0.70
		ECHS1	D13900	0.038				0.33	
		HADHB	D16481	0.07	0.53	0.52	0.59	0.89	1.67
	CHO metabolism	GLUT4	M20747	0.006	0.50				
		SCP2	M75883	0.016	0.77	0.61		0.73	0.96
	detoxification	*GPX5*	AJ005277	0.078	0.78	0.68	0.56	0.64	0.82
	glycolysis	ALDOA	M11560	0.021	0.74	0.96	0.60	0.58	0.79
	O_2storage	MB	M14603	0.029	0.90	0.94	0.77	0.78	0.87
	redox	*SOD1*	M13267	0.074	0.82	0.74	0.74	1.03	1.26
		SOD3	J02947	0.009	0.67	0.62	0.64	0.81	1.21
	respiration	CA3	M29458	0.015	0.86	1.27	0.70	0.66	1.23
		COX5B	M19961	0.082	1.10	1.17	0.94	0.90	0.82
		UCP3	AF011449	0.003	0.71	0.68	0.55	0.65	0.91

Median values of 18S standardized transcript levels relative to the prebiopsy and the 1-h biopsy, respectively, are indicated. When the field is blank, the gene is not detected. Normal gene name: significant changes throughout the time course; Italic gene name: tendency in the time course. Significant downregulated gene transcripts ($p \leq 0.05$) are indicated in gray and significant upregulated gene transcripts in black

mRNA decline after mild eccentric exercise

The prominent finding of this study is a gene response characterized by a general decline of muscle-specific mRNA concentrations right after the exercise, followed by a recovery of transcript levels close to control values within 24 h.

There are two basic mechanisms that can influence mRNA levels: transcription, which is generally under positive control, and mRNA degradation, which is related to prior translation of the mRNAs [35]. The fast and substantial suppression of most mRNA levels could thus be explained by a lack of de novo synthesis of mRNAs and by an increased degradation (see also discussion of UBC regulation below). An early decline in mRNA concentrations at 1 h has been observed before [37], but mRNA concentrations of metabolic factors were subsequently increased 8 h postexercise. The observed low mRNA concentrations at 8 h in the present study seem to be a consequence of a different time course of transcription-related accretion and translation-related degradation of mRNA. Both concentric and eccentric resistance exercises lead to an increase in muscle protein synthesis 3 h after exercise bouts [30]. There is evidence suggesting a protection of mitochondrial RNAs from degradation after high-repetitive low-load contractions [42]. In the eccentric experiment, mRNA concentrations remained low at a time (8 h), when there was an expected enhancement of mRNA transcription [7].

Upregulated gene transcripts

The upregulation of a few gene transcripts points to the activation of distinct biological processes are as follows.

Myogenesis Several of the observed cell-regulatory RNAs relate to myogenesis. The immediate upregulation of c-jun and UBC represents its de novo RNA synthesis, which was shown to occur in many cell types after stress [22]. Upregulation of c-jun induces a partial activation of the cell cycle (G1) via the c-jun NH_2-terminal kinase pathway. This finding relates to the transient elevation of the myogenic factor MYF6 (also called MRF4) mRNA after 3 h (see Table 2) and the temporary increase in this mRNA 2–4 h after single-resistance exercise [43]. The transcripts for other myogenic master regulators, MYOD1 and myogenin, were not altered in our study (Table 2 and supplemental Table 2). We therefore view this signature of response as a partial activation of the myogenic pathways. The upregulation of IGFBP4 and downregulation of IGFBP5 expression reproduces the effect of muscle loading in rodents [1, 9]. This conserved response indicates that the muscle hypertrophy-associated IGF-1 system [3, 32] is subjected to a complex regulation in higher vertebrates. This finding coincides with the upregulation of the cyclin-dependent kinase inhibitor p21. Changes in the p21 mRNA after resistance exercise are coregulated with IGFBP4 mRNA changes and related to cytoskeletal remodeling with muscle differentiation, which is indicated by the upregulation of TUBA1 mRNA 24 h after the eccentric exercise [4, 39]. Our observation of an early rise in UBC mRNA is compatible with other studies, which showed an increase in UBC mRNA and protein levels after eccentric exercise [40]. These authors suggested that these changes were responsible for the observed increase in muscle proteolysis, as this was also indicated in another eccentric study with high exercise intensity [11]. The more than twofold upregulation of UBC mRNA in the current study points to enhanced tagging of proteins for proteolytic degradation by the proteolytic ubiquitin pathway. It is possible that enhanced protein degradation could also include degradation of muscle-specific mRNAs and explain our results [6, 16].

Damage The low increase in CK activity points to a moderate damage response to the eccentric ergometer exercise. This may suggest a low level of cell infiltration. We do, however, not observe a mRNA response of interleukin (IL) 6 and Tenascin-C, both markers of muscle damage [14, 29]. This relates to the grading of the inflammation response with respect to muscle damage and elevated CK values after mild and hard eccentric protocols [8]. Instead, we see an upregulation of the mRNA for the IL6R. This puzzling observation implies that altered expression of IL6 receptor has to be considered for interpretations on damage-induced IL6 signaling after eccentric contractions [23].

Gene signature comparisons

In a previous study, we investigated the gene expression signature after concentric exercise with an identical microarray technology and statistical analysis (Friedmann ANOVA) [37]. Although this concentric exercise was performed at different duration and intensity, it allows us to compare these exercises with minimal uncertainties introduced by the technical procedures. Moreover, the initial load of the two training sets, when applied and adapted over a longer time period, would result in a specific and measurable training response [25, 37]. This comparison is interesting because eccentric contractions require less motor unit activation and consume less oxygen and energy for a given muscle force than concentric contractions [27]. They therefore represent training modalities with a different application profile. Schmutz et al. [37] showed that concentric exercise induces an upregulation of several metabolic pathways including glycolysis, beta-oxidation, respiration, the Krebs cycle, and detoxification, after an

initial (1 h) decline in mRNA levels [37]. This upregulation was not observed in the current study (see supplemental Table 2). It thus seems that the metabolic stimulus of the eccentric exercise was not sufficient to induce upregulation of these pathways involved in aerobic energy generation and mitochondrial biogenesis. This finding is consistent with our findings showing mRNA for cytochrome c oxidase subunit IV to be significantly downregulated after eccentric and upregulated after concentric type of exercise in stable coronary artery disease patients [44]. We only found evidence for a slight mitochondrial reaction. The L2 regression analysis detects weakly upregulated mRNA levels of cytochrome c oxidase subunit 5b, cytochrome c oxidase 1, and cytochrome c.

Our observations also contrast with the findings from Chen et al. [7], who investigated the gene response to maximal low-repetitive eccentric exercise in humans with an Affymetrix Human Genome microarray. This investigation found no consistent downregulation of any gene transcript but showed a limited increase in expression of gene transcripts involved in the inflammatory response, e.g., IL1 receptor and Tenascin-C. Eccentric exercise is also associated with exercise-induced muscle damage, proteolysis [11], and increased serum levels of IL6 [8, 29] and CK [8]. This supports the idea that maximal eccentric exercise leads to mechanical damage of myofibers and the stimulation of an inflammatory response in a load-dependent manner. Because we did not observe markers of muscle damage, we suggest that the mechanical load was not high enough to activate a more pronounced inflammatory response.

Events underlying muscle hypertrophy

The molecular observations in the eccentrically challenged vastus lateralis muscle are astonishing with regard to the observed hypertrophy response to the same type of exercise, when carried out repetitively. It was shown that 8 weeks of mild eccentric exercise lead to an increase in capillary-to-fiber ratio of 47% and fiber cross-sectional area of 52% [25].

Based on the results of the Friedman ANOVA, we have to revise the hypothesis of a general upregulation of transcripts relevant to muscle growth. The early selective upregulation of c-jun and MYF6 suggests a mechano-dependent activation of some aspects of myogenesis. A load-dependent activation of jun pathway in situ [26] and c-jun expression in myonuclei and interstitium has been seen before with running exercise with a high component of eccentric loading [34]. This notion of an activated myogenesis was also corroborated by the results of the L2 regression analysis, which identified enhanced amount of myogenic factor MYF4 and desmin mRNA. High-load single bout of eccentric exercise was shown to increase mRNA levels of the myogenic factors MYF6, MYOD, and myogenin in humans [43]. This statistical test indicating enhanced message for the ribosomal proteins (RPS9 and RPS29) and 18S and 28S ribosomal RNAs after 8 and 24 h (see supplemental Table 2) also provided circumstantial evidence for activation of protein translation. This finding points to the key role of muscle loading as a trigger for protein synthesis [19] and the suggested enhanced ribosome number after a 2-month period of eccentric training [15]. The latter observations may be indicative of an elevated translation capacity after eccentric exercise and thus eventually explain part of the observed decline in mRNA levels. In support of this, it was found that eccentric contractions are more effective than concentric contractions in stimulating protein synthesis [2, 10]. Thus, we conclude that there is some molecular evidence for a transcriptional basis of the elevated protein synthesis with eccentric ergometer exercise, which critically depends on the applied mechanical load.

Conclusion

In this study, we investigated the temporal response of muscle gene expression to a single bout of mild eccentric ergometer exercise. We had to revise our hypothesis that we could identify a major upregulation of transcripts relevant for processes supporting muscle growth within the first 24 h of recovery. Instead, we found that the mRNAs of almost all important muscle regulatory gene transcripts are significantly downregulated and take close to (or more than) 24 h to revert to pre-exercise values. This study demonstrates for the first time that mild eccentric exercise has a molecular signature distinctly different from *intensive* concentric exercise as well as from *maximal* eccentric exercise.

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ORIGINAL ARTICLE

Mechano-transduction to muscle protein synthesis is modulated by FAK

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Abstract We examined the involvement of focal adhesion kinase (FAK) in mechano-regulated signalling to protein synthesis by combining muscle-targeted transgenesis with a physiological model for un- and reloading of hindlimbs. Transfections of mouse tibialis anterior muscle with a FAK expression construct increased FAK protein 1.6-fold versus empty transfection in the contralateral leg and elevated FAK concentration at the sarcolemma. Altered activation status of phosphotransfer enzymes and downstream translation factors showed that FAK overexpression was functionally important. FAK auto-phosphorylation on Y397 was enhanced between 1 and 6 h of reloading and preceded the activation of p70S6K after 24 h of reloading. Akt and translation initiation factors 4E-BP1 and 2A, which reside up- or downstream of p70S6K, respectively, showed no FAK-modulated regulation. The findings identify FAK as an upstream element of the mechano-sensory pathway of p70S6K activation whose Akt-independent regulation intervenes in control of muscle mass by mechanical stimuli in humans.

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Introduction

Striated muscle demonstrates a pronounced load-dependent phenotype (Fluck and Hoppeler 2003). This is illustrated by the dramatic reduction in mass of anti-gravitational muscles with unloading and the induction of hypertrophy with subsequent reloading. Biochemical investigations point out that modulated protein synthesis is a main mechanism underlying the early regulation in protein turnover with altered muscle loading (Rennie 2007).

Protein phosphorylation is a central theme in the regulation of protein synthesis (Chiquet and Flück 2002; Hunter 2000). Studies focusing on the molecular control of muscle size identified a number of signalling pathways that control protein synthesis via the phosphorylation of translation factors at critical amino acids, thus regulating their activity. It is known that the phospho-transfer enzymes (kinases) mTOR (mammalian target of rapamycin) and Akt are organized in a pathway that enhances translational efficiency (Bodine et al. 2001; Deldicque et al. 2008; Pallafacchina et al. 2002). This occurs via downstream activation of 70 kDa ribosomal S6 kinase (p70S6K) and critical translation initiation factors (e.g. eIF4E-BP1, eEF2) (Gingras et al. 2001; Sandri 2008). The physiological regulation of this signalling pathway supports the view that protein synthesis is controlled by muscle activity via enhanced loading (Sandri 2008). In this respect, the activation of p70S6K correlates best with the gains in muscle mass induced by mechanical stimuli (Baar and Esser 1999; Burry et al. 2007; Nader and Esser 2001). This activation occurs when the Akt-mTOR pathway is not activated (Deldicque et al. 2008;

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Eliasson et al. 2006; Fujita et al. 2007; Terzis et al. 2008). This indicates that Akt-mTOR and p70S6K independently regulate protein synthesis. The mechano-responsive pathway which connects to p70S6K activation is not known.

The integrin-associated focal adhesion kinase (FAK) is a mechano-sensitive signalling molecule (Durieux et al. 2007), which is positioned upstream of p70S6K in cell culture (Gan et al. 2006; Malik and Parsons 1996). FAK localises to sarcolemmal focal adhesion sites, which play an important role in the conversion of mechanical stress to myocellular hypertrophy signalling (Fluck et al. 2002; Ingber 2006; Romer et al. 2006; Samarel 2005). A possible functional coupling between the activation of p70S6K and FAK is suggested by the observation that the phosphotransfer activity and phosphorylation status of both kinases correlate with protein synthesis and load-dependent increases in muscle mass (Cary and Guan 1999; Fluck et al. 2002; Ingber 2006).

We hypothesized that mechano-signalling between FAK and p70S6K is the missing molecular connection between mechanical muscle stimulation and activation of muscle protein synthesis (Baar and Esser 1999; Gan et al. 2006; Kimball et al. 2004). Towards this end, we tested whether muscle fibre-targeted FAK overexpression (Durieux et al. 2002, 2004) would enhance activation of p70S6K in tibialis anterior (TA) muscle of mice in a load-dependent manner (Gingras et al. 2001; Kimball et al. 1998; Sale et al. 1999). This was assessed in a physiological model which allows prolonged muscle unloading and subsequent reloading (Dapp et al. 2004). We were interested in elucidating the time course and relationship of the early FAK activation (Gordon et al. 2001) to the putative downstream phosphorylation of p70S6K and explored whether this pathway distinguishes to regulatory activation of p70S6K by Akt-mTOR and downstream phosphorylation of key translation factors eIF4E-BP1, eEF2 (Baar et al. 2006; Baar and Esser 1999; Gingras et al. 2001) (Fig. 1).

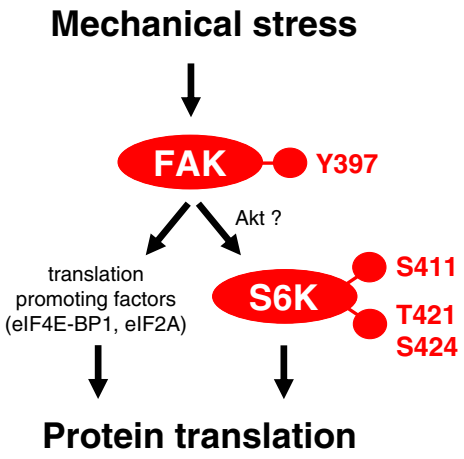

Fig. 1 Model summarizing the working hypothesis on FAK-mediated signalling towards enhanced protein translation via S6K and other translation-promoting factors

Methods

Materials

Cytomegalovirus (CMV) promoter-driven expression plasmid encoding chicken FAK gene (pCMV-FAK) or an empty plasmid (pCMV) were isolated under endotoxin-free conditions at Plasmid Factory GmbH (Bielefeld, Germany, www.plasmidfactory.de). Fluorescent-compatible mounting medium was from DAKO (DAKO, Carpinteria, CA, USA). Bicinchoninic acid assay reagents and protein A Sepharose were from Sigma (Sigma-Aldrich, St. Louis, MO, USA). The deployed antibodies against the signalling molecules in focus and the verified phosphorylation sites involved in their regulation are summarized in Table 1. Peroxidase-conjugated secondary antibodies goat anti-rabbit IgG and goat anti-mouse whole IgG were obtained from ICN Biomedicals GMBH (Germany). Alexa Fluor® 488-conjugated goat-anti-rabbit IgG antibody was from Molecular probes (Invitrogen Ltd, Paisley, UK).

Super Signal West Femto Kit and Kodak XAR5 films were from Pierce (Perbio Science, Lausanne, Switzerland) and Sigma (Buchs, Switzerland), respectively.

Animals

The animal protocol was approved by the Animal Protection Commission of the Canton Berne, Switzerland. The 6-month-old male mice 129/SVEV weighed 35.4 ± 0.7 g ($n = 17$) before the intervention. They were housed individually in a temperature-controlled room (21°C) with a 12:12 h light–dark cycle. Animals were allowed food and water ad libitum.

Gene electro transfer

Intramuscular gene transfer was achieved via injection of plasmid DNA and subsequent electric pulse delivery in both legs basically as previously described (Durieux et al. 2002; Durieux et al. 2004; Pallafacchina et al. 2002). The mice were individually anesthetized with isoflurane and the lower limbs were shaved. Twenty-five microgram of expression plasmid in 25 µl physiological saline solution (0.9% NaCl) was injected with a sterile 100 µl syringe into the tibialis anterior (TA) muscle. After 5 min of incubation, electric pulses (2 trains of 100 pulses of 100 µs each at 50 mA) were delivered at four different locations in the belly portion using the GET42 pulser with needle electrodes (E.I.P. Electronique et Informatique du Pilat, Jonzieux, France). This technique allows to maintain the overexpression for more than 1 week (Fluck et al. 2008). Typically, mice recovered rapidly from this procedure and began to move freely 2 h after the intervention.

Table 1 Name, function, epitope and source of all primary antibodies used to identify FAK-dependent signalling in this study

Antibody	Function	Epitope	Source
FAK	Tyrosine kinase	N-terminal (A-17)	Santa Cruz, CA, USA
FAK	Tyrosine kinase	C-terminal (Lulu)	Gift from A. Ziemiecki (Flück et al. 1999)
pFAK-Y397	Major auto-phosphorylation and activation site	Phospho-Tyrosine 397 (Y397)	Santa Cruz, CA, USA
pFAK-Y397	Major auto-phosphorylation and activation site	Phospho-Tyrosine 397 (Y397)	BioSource Europe (Nivelles, Belgium)
p70S6K	Key regulator of mRNA translation	p70S6K (C-18)	Santa Cruz, CA, USA
p-p70S6K-S411	Pre-activation of p70S6K	Phospho-Serine 411 (S411)	Santa Cruz, CA, USA
p-p70S6K-T421/S424	Pre-activation of p70S6K (a Serine/Threonine kinase)	Phospho-Threonine 421 and Serine 424 (T421/S424)	Santa Cruz, CA, USA
p-eIF2alpha-S52	Regulation of translation initiation	Phospho-Serine 52 (S52)	BioSource Europe, Nivelles, Belgium
p-4E-BP1-T37/46	Regulation of translation initiation	Phospho-Threonine 37 and 46 (T37/46)	Cell Signaling Technology, Danvers, MA, USA
p-Akt-T308	Activation of signal transduction	Phospho-Threonine 308 (T308)	Santa Cruz, CA, USA
p-Akt-S473	Activation of signal transduction	Phospho-Serine 473 (S473)	Cell Signaling Technology, Danvers, MA, USA
p-ERK	Activation of signal transduction	Phospho-Threonine 204 (T204)	Santa Cruz, CA, USA

Modulation of muscle loading

Animals were subjected to either of five different loading conditions basically as previously described (Dapp et al. 2004; Fluck et al. 2005). Two days after the gene electrotransfer, animals were subjected to 7 days of hindlimb unloading (HU) by tail suspension or kept as cage controls (CC). Subsequently, a set of suspended animals was subjected to reloading for 1 h (R1), 6 h (R6) or 24 h (R24). Cage activity was encouraged in the first hour of reloading by tipping the finger into the suspension cage. TA muscles were harvested from anesthetized animals, rapidly weighed, frozen in nitrogen-cooled isopentane and stored at −70°C for subsequent analysis.

Confocal microscopy

The subcellular localization of FAK was detected on cryosections as previously described (Flück et al. 1999; Fluck et al. 2002), but with the modification that fluorescent-labelled secondary antibodies were used. The deployed primary FAK antibody A-17 was applied at a 1:100 dilution in 0.3% BSA/PBS, reacted with 200-fold diluted Alexa488-conjugated anti-rabbit IgG and embedded in fluorescent-compatible mounting medium. Fluorescence and digital phase contrasts were analyzed with a Leica TCS SP5 confocal microscope (Leica Microsystem CMS, Mylton Keynes, UK). The subcellular localization of FAK was analyzed on cryosections of cage control, of 6 h and of 24 h reloaded animals combined together. Sarcolemmal and sarcoplasmic FAK staining was quantified with the colocalization macro of Leica Application Suite. Signals were calculated from the mean of integrated channel intensity of the sarcoplasmic and sarcolemmal region of each fibre along six parallel "optical slices" at 10 µm distance. Six fibres were quantified per treatment. Raw data were exported for statistical analysis.

Immunoblotting

SDS-PAGE, western blotting and immunodetection were performed with specific antibodies (Table 1) as previously described (Flück et al. 1999; Gordon et al. 2001). Standardized amounts of protein (20 µg) were loaded per well. Signal was recorded with enhanced chemiluminescence by using the Super Signal West Femto Kit and Kodak XAR5 films. The signal intensity of the specific protein band was determined using the line and band density mode in the Quantity One 1-D analysis software 4.6.1 (Bio-Rad, Life Science Research, Hercules, CA, USA).

FAK phosphorylation on Y397 was determined after immunoprecipitation. One milligram of soluble protein in 750 µl RIPA buffer was isolated and combined with 1 µl pFAK serum from BioSource and 10 µl p-FAK from Santa Cruz. Five milligram Protein A Sepharose (Sigma) was added and incubated with shaking at 4°C for 2 h. After incubation of antibodies with the protein sample over night, the immunocomplexes were precipitated by centrifugation

for 10 min (10,000*g*, 4°C), washed twice in 1 ml RIPA and resuspended in SDS loading buffer for separation by 7.5% SDS-PAGE. Proteins were subsequently subjected to immunoblotting for FAK protein with antibody "Lulu" (Table 1).

In vitro S6 kinase activity assay

Phosphotransfer activity of p70S6K was evaluated in vitro. S6 kinase phosphorylation was initiated by the addition of 75 µg protein homogenate to 45 µl preheated phosphorylation mixture including S6K substrate peptide (RRRLRRLRA) at 30°C basically as described (Akimoto et al. 1998; Napoli et al. 1998). The reaction was stopped after 5 min by spotting 20 µl on a Whatman P81 filter and by washing in 75 mM H_3PO_4 and acetone (Fluck et al. 2000). Quantification of incorporated 32P was performed by liquid scintillation counting. Two technical replicas were measured from each sample. Background values (radioactivity background: same reaction without homogenate; homogenate background: same reaction without substrate peptide) were subtracted.

Statistics

For immunoblotting, samples from contralateral muscle pairs (i.e. pCMV-FAK and pCMV-transfected TA) were separated in adjacent lanes of the SDS-PAGE gel and a reference sample was run in all gels. Data were related to the reference sample. Statistical analysis was carried out with Statistica 6.1 (StatSoft Europe, Hamburg, Germany). The expression and phosphorylation level in pCMV-transfected muscles, as well as body and muscle weight, were evaluated with a one-factor analysis of variance (ANOVA). Differences between pCMV-transfected and pCMV-FAK muscle pairs were analyzed with a Friedman-ANOVA with repeated measurements. Subsequently, a Fisher post hoc test was carried out to localize the effect. Linear regression analysis was carried out with Pearson correlation. A p value of 0.05 was selected as the significance level for all tests. Values are given as means ± standard error (SE).

Results

Interaction of electrotransfer and muscle loading

TA muscle pairs of adult mice were subjected to gene electrotransfer. The right TA muscle was transfected with constitutive-active plasmid for chicken FAK, i.e. pCMV-FAK. The contralateral TA was transfected with an empty pCMV plasmid. Subsequently, animals were subjected to the different loading protocols. 7 days of unloading produced the expected drop in body weight, which did not recover within 24 h of reloading (Table 2). The ratio of the TA muscle to the body weight was unchanged and not affected by the unloading and reloading for both transfections.

FAK overexpression

The expression level and localization of FAK protein in transfected TA muscle was analyzed by immunoblotting and immunofluorescence. Figure 2a and b depict FAK-immunolocalization after pCMV-FAK transfection in a single positive fibre. Figure 2c shows the mosaic pattern of FAK localisation after pCMV-FAK transfection at a lower magnification. The amount of FAK localized in the sarcolemma was threefold higher than the amount of FAK in the sarcoplasm, regardless of the muscle loading (Fig. 2d). A proportion of fibres in empty-transfected muscle demonstrated FAK-immunostaining, but staining intensity was substantially more pronounced in pCMV-FAK transfected muscle (data not shown). Gene electrotransfer also caused muscle fibre damage in the transfected region, which was comparable for both conditions of transfection (data not shown). Qualitative immunoblotting identified a 1.6-fold increase in FAK protein levels in cage controls 9 days after electrotransfer (Fig. 3a). The FAK protein was detected as a single band at 125 kDa in empty and chicken FAK transfected mouse TA muscles (Fig. 3b).

Effect of muscle loading on FAK overexpression and activity

The difference in FAK expression was load-dependent. FAK overexpression in pCMV-FAK transfected muscle

Table 2 Alterations in body weight and tibialis anterior (TA) muscle-to-body weight ratio with the intervention

	HU7	R1	R6	R24	CC
Body weight versus pre intervention (%)	−14	−15	−11	−12	−3*
SE (%)	3	2	4	1	2
TA/body weight pCMV [mg/g]	1.11	1.29	1.19	1.17	1.21
SE	0.09	0.05	0.01	0.17	0.07
TA/body weight pCMV-FAK (mg/g)	1.25	1.37	1.11	1.30	1.18
SE	0.01	0.09	0.04	0.07	0.04

Effect of time of reloading on the body weight was analyzed with a one-factor ANOVA, the effect of time of reloading and transfection on TA-to-body weight on contralateral muscle pairs was verified with an ANOVA for repeated measures. The effect was localized with a Fisher-LSD post hoc test (*). Body weight was significantly higher in cage control (CC) animals than at all other time points (7 days of unloading and 0, 1, 6 and 24 h of reloading)

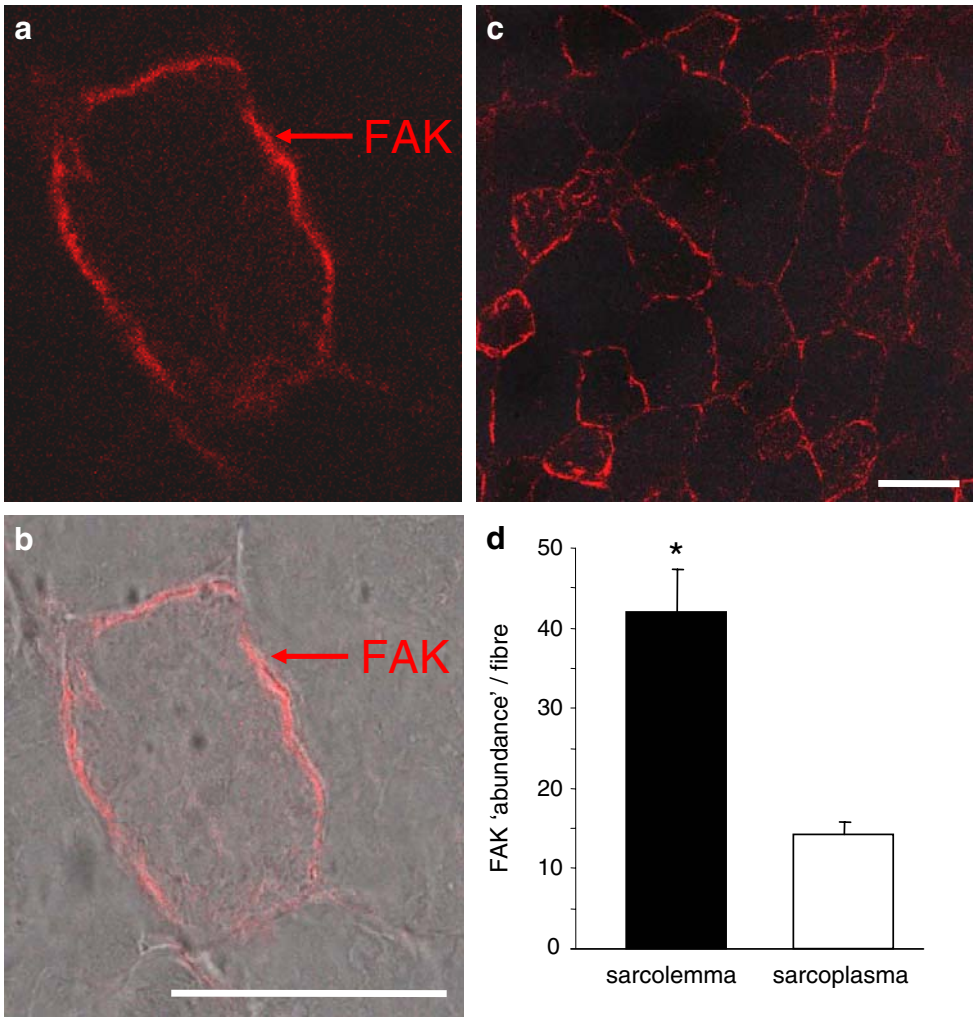

Fig. 2 FAK staining in muscle cross-sections. **a**, **b** Representation of a FAK-positive fibre in a cross-section from a TA muscle transfected with pCMV-FAK plasmid without (**a**) and with differential interference contrast (**b**). **c** Lower magnification picture of FAK immunostaining after pCMV-FAK plasmid transfection. The majority of exogenous FAK protein within the FAK positive fibres was located near the plasma membrane (sarcolemma). *Bar* denotes 50 µm. **d** Mean ± SE of FAK abundance per fibre (% of total pixel count) associated with the sarcolemma and the sarcoplasma

was lost with 7 days of unloading but was re-established within 6 h of reloading (Fig. 3a). This increase in FAK protein with reloading was preceded by enhanced phosphorylation of FAK on the major activation site Y397 after 1 h of reloading (Fig. 3c, d). This tyrosine phosphorylation with reloading was transient and lost after 24 h of reloading.

Load-dependent p70S6K-signalling

We tested whether the added mechanical stress of reloading in combination with FAK-overexpression would activate p70S6K in muscle deconditioned by unloading. Unloading did not bring about significant differences in p70S6K amount, phosphorylation and phosphotransfer activity between pCMV-FAK- and pCMV transfected TA muscles (Fig. 4a–d). Subsequent reloading altered the phosphorylation status of p70S6K in deconditioned TA muscle both qualitatively and quantitatively: 6 h after the first ground contact of hindlimbs, p70S6K was increasingly phosphorylated on the dual site T421/S424 in pCMV-FAK transfected muscle versus their contralateral controls. This response peaked after 24 h of reloading (Fig. 4a, b). Phosphorylation on S411 showed a near trend for FAK-transfection mediated elevation 6 h after reloading ($p = 0.10$). Functionally important regulation of p70S6K by loading was emphasized by a significant 3.8-fold enhanced p70S6K-phosphotransfer activity in pCMV-FAK- versus pCMV transfected muscle after 24 h of reloading (Fig. 4c). p70S6K protein levels were not affected by reloading between FAK overexpressing and contralateral control muscle (Fig. 4d). p70S6K phosphorylation status of the verified sites pS411 and pT421/S424 and phosphotransfer activity of p70S6K during the reloading response were significantly correlated for both transfection conditions (i.e. pS411: $r = 0.73$; pT421/S424: $r = 0.60$).

To test whether this activation of p70S6K was induced by an activation of Akt, we analyzed the phosphorylation status of Akt on its major phosphorylation sites T308 (Fig. 5a) and S473 (Fig. 5b). No activation of Akt was detected throughout the intervention.

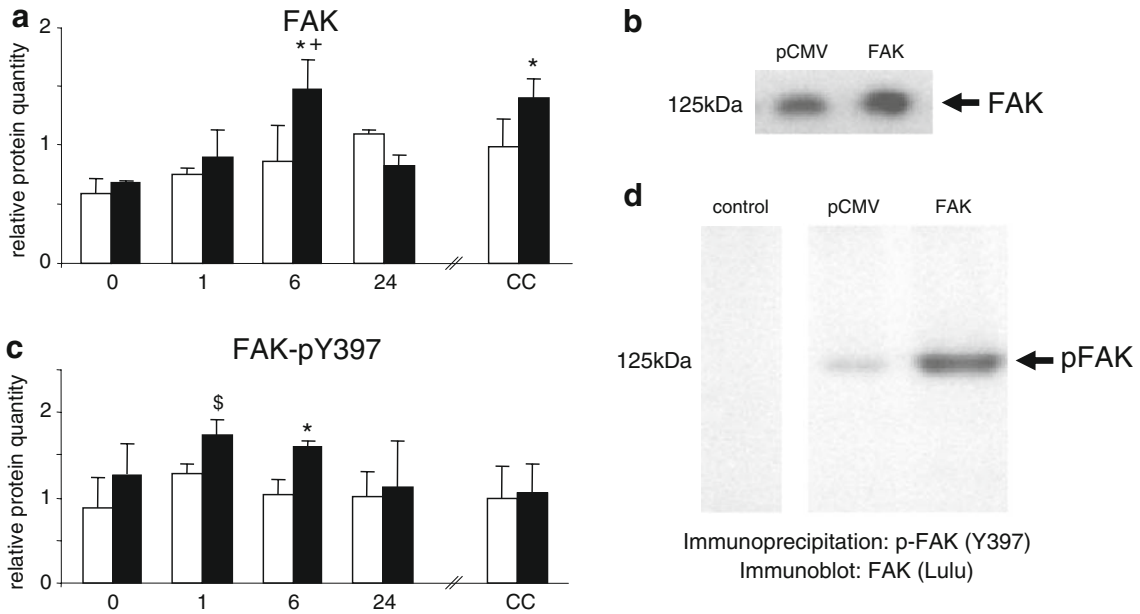

Fig. 3 Assembly showing FAK protein and tyrosine 397 phosphorylation levels in mouse TA muscle in function of gene transfer and hindlimb un- and reloading. **a** Mean ± SE of FAK signal relative to pCMV-empty transfected CC value in muscles from the different loading interventions: *white bars* pCMV-empty plasmid in left TA muscle; *black bars* pCMV-FAK plasmid in TA right muscle. *CC* cage control, 9 days after transfection; *0*: 7 days of hindlimb unloading and no reloading; *1*: 1 h of reloading; *6*: 6 h of reloading, *24*: 24 h of reloading. **b** Representative immunoblot showing FAK in cage control muscle 9 days after transfection. **c** Mean ± SE of FAK relative to pCMV-empty transfected CC value of FAK phosphorylation on Y397 in cage controls and with un- and reloading. **d** Representative immunoblot detecting FAK phosphorylation on Y397 in immunoprecipitates in function of gene transfer and hindlimb un- and reloading. Note the absence of FAK protein in the negative control reaction where no antibody was added to the precipitation. Friedman-ANOVA: $+p < 0.05$ versus time point 0; $*p < 0.05$ versus contralateral control; \$ $0.05 < p < 0.10$ versus contralateral control

FAK-signalling to translation factors

The verification of two key translation initiation factors identified no significant effect of FAK-transfection. Neither phosphorylation on the key regulatory sites T37/T46 in the eukaryotic translation initiation factor 4E binding protein 1 (eIF4E-BP1) nor the activating site S52 in the eukaryotic initiation factor 2 alpha (eIF2A) were significantly affected by the introduction of FAK (Fig. 6a, b). Reloading per se increased; however, S52 phosphorylation of eIF2A after one hour of reloading in both transfection conditions. The total protein content of eIF2 was unchanged (data not shown). eIF2A pS52 was negatively correlated to p70S6K pT421/S424 and eIF4E-BP1 pT37/T46 phosphorylation.

Discussion

Mechano-transduction: from FAK to an activation of protein synthesis

Our molecular analysis demonstrates that the experimental enhancement of FAK-signalling transduces a mechanically imposed stimulus to the delayed activation of p70S6K-signalling. The temporal relationships between FAK and p70S6K-phosphorylation and phosphotransfer activity establish that the load-modulated signalling pathway of translation control in muscle is modulated by FAK. The time-course and specificity of p70S6K phosphorylation allows important regulatory conclusions on the pathway connecting FAK to p70S6K activation. The FAK-modulated phosphorylation on S411 and the dual phosphorylation T421 and S424 of p70S6K points to the involvement of serine/threonine kinases since FAK activity explicitly targets tyrosine residues. The measured p70S6K phosphorylation sites are targeted by numerous kinases, including PI3K (Dardevet et al. 1996; Thomas and Hall 1997), Akt (Hemmings 1997), PDK1 (Chan et al. 1999), mTOR (Isotani et al. 1999) and PKC (Laser et al. 1998), which could mediate the identified connections of FAK and p70S6K activation in vivo (Baar et al. 2006; Baar and Esser 1999; Gan et al. 2006).

In this context we further tested differences in phosphorylation of the serine/threonine-protein kinases Akt at T308 and S473 (Fig. 5), as wells as extracellular signal-regulated kinase (ERK) at T204 (results not shown), without being able to detect any changes neither in the FAK overexpression nor in the empty transfected muscles. These observations support the view that an activation of

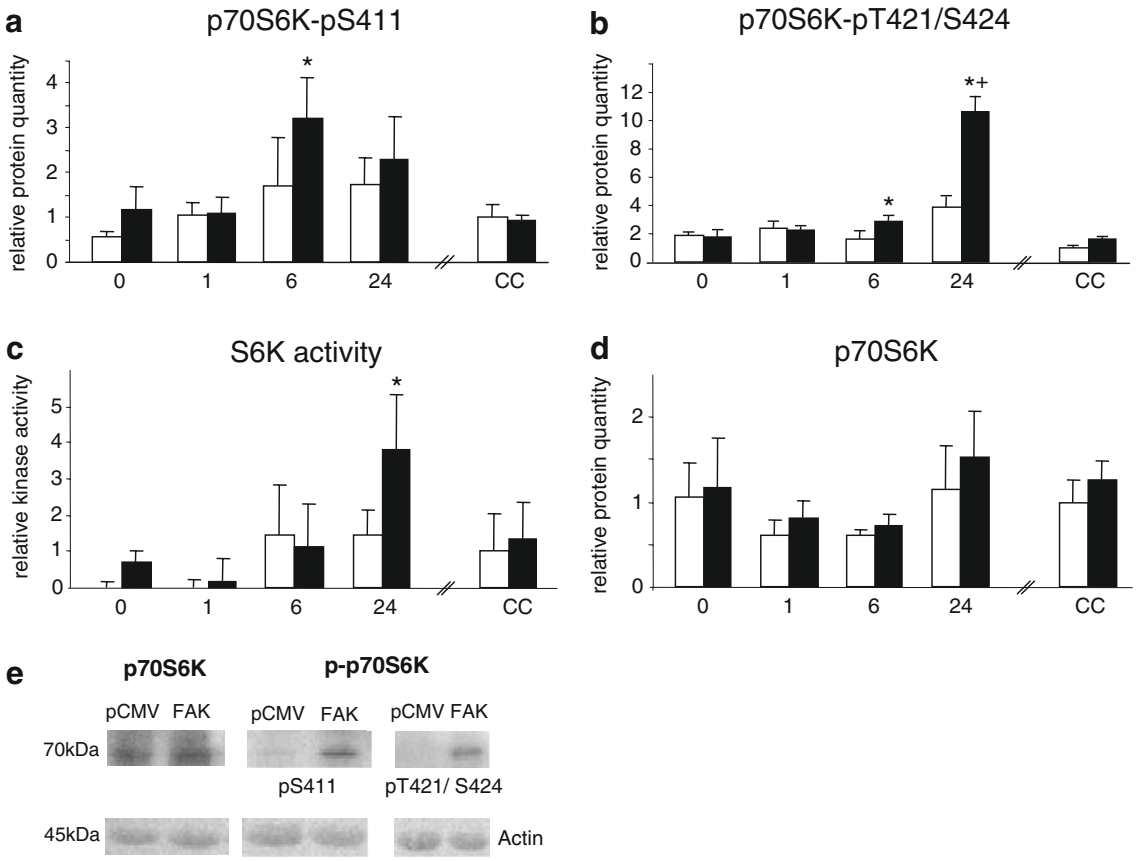

Fig. 4 Effect of FAK overexpression on p70S6K phosphorylation on S411 (**a**) and on T421/S424 (**b**), S6K in vitro kinase activity (**c**) and p70S6K protein level (**d**). Data are presented relative to the pCMV-empty transfected CC value. *White* and *black bars* denote mean ± SE of signal in pCMV-transfected and pCMV-FAK-transfected muscle, respectively. Friedman-ANOVA: $*p < 0.05$ versus contralateral control; $+p < 0.05$ time point 0. **e** Representative panels visualizing the quantified p70S6K signals in western blots as shown in **a**–**d**

Akt (and possibly mTOR) primarily responds to insulin- and feeding-dependent signals to muscle mass. It seems that Akt and mTOR are resistive to mechanical stimuli in the fasted state (Deldicque et al. 2008; Sandri 2008). Rather, our results confirm the transient downregulation of Akt activation state shortly after a mechanical stimulus (Fig. 5b; Deldicque et al. 2008). The activation of p70S6K to mechanical stimulation in humans is also apparent when the activation status of Akt or mTOR is not altered (Deldicque et al. 2008; Eliasson et al. 2006; Fujita et al. 2007; Terzis et al. 2008). These observations and our results suggest that p70S6K activation by mechanical signals is Akt-independent.

The observations recapitulate the reported role of p70S6K phosphorylation for protein synthesis in intact muscle. For instance, p70S6K phosphorylation on T421 and S424 has been shown to correlate with gains in muscle mass in different animal models (i.e. stretch and resistance exercise) for muscle hypertrophy (Baar and Esser 1999; Bodine et al. 2001; Reynolds et al. 2002; Spangenburg et al. 2008; Thomson and Gordon 2006). The assessed sites control biochemical function of p70S6K and their enhanced phosphorylation is believed to stimulate protein synthesis in vitro (Gingras et al. 2001; Kimball et al. 1998; Sale et al. 1999). Phosphorylation of p70S6K on S411 and the tandem T421/S424 relieves the phospho-transfer activity from autoinhibition prior to a full activation of the enzyme (Pullen and Thomas 1997). The correlation of phosphorylation at the latter tandem sites in FAK-transfected muscles supports the notion of a functional implication of FAK-stimulated p70S6K activation in translation control in vivo.

The findings also corroborate earlier suggestions on a role of FAK in protein synthesis (Baar and Esser 1999; Kimball et al. 2004) and cell size regulation (Ingber 2006). Our present results now imply a functional contribution of FAK in modulating the load-induced hypertrophy response of muscle due to p70S6K-mediated induction of protein translation. They therefore expand the recently published summarizing scheme about signalling during muscle hypertrophy (Sandri 2008).

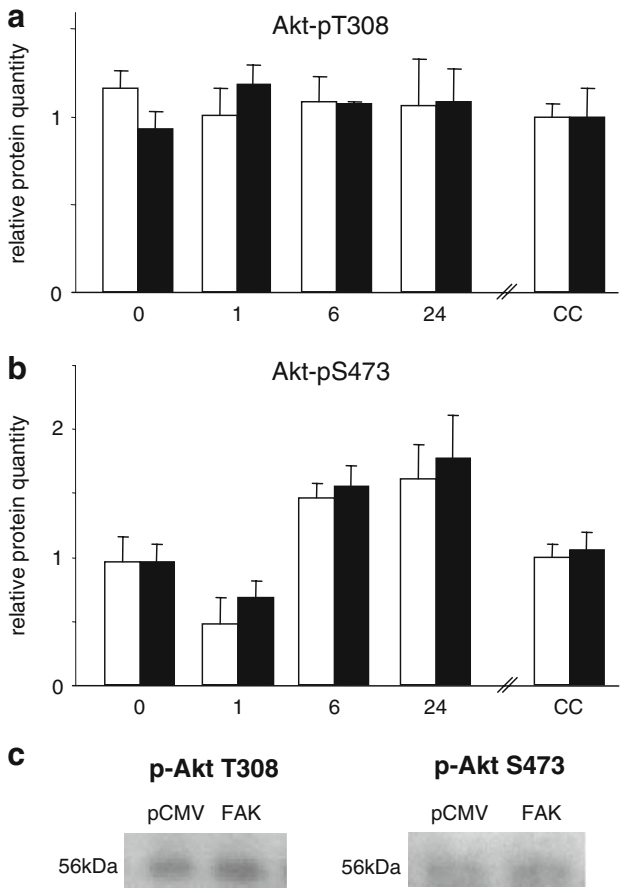

Fig. 5 Effect of FAK overexpression on Akt phosphorylation on T308 (**a**) and S473 (**b**). Representative detection of the protein in immunoblots of phosphorylated Akt at T308 and S473 are shown in (**c**). Data are presented relative to the pCMV-empty transfected CC value. *White* and *black bars* denote mean ± SE of signal in pCMV-transfected and pCMV-FAK-transfected muscle, respectively

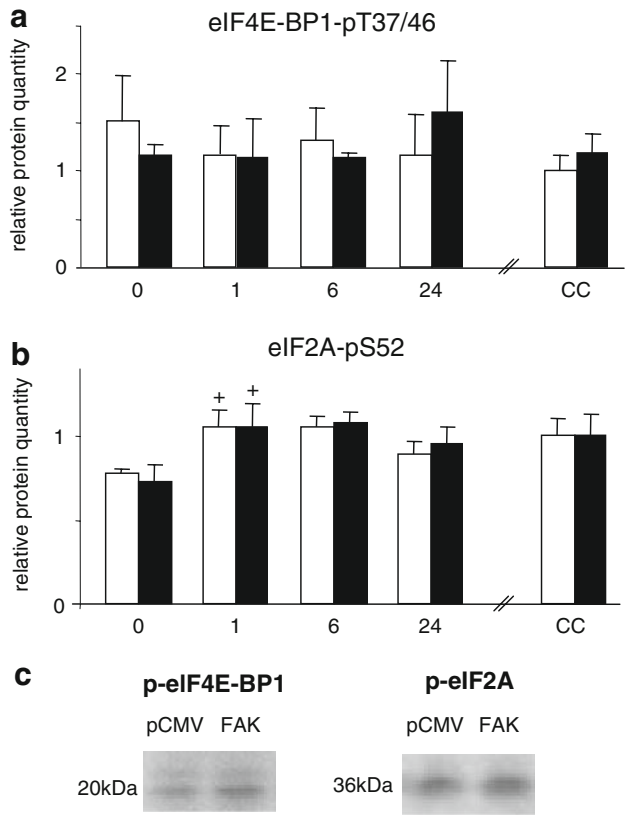

Fig. 6 Effect of FAK overexpression on eIF4E-BP1 phosphorylation on T37/46 (**a**) and eIF2A on S52 (**b**). Data are presented relative to the pCMV-empty transfected CC value. *White* and *black bars* denote mean ± SE of signal in pCMV-transfected and pCMV-FAK-transfected muscle, respectively. Friedman-ANOVA: $+p < 0.05$ versus time point 0. **c** shows representative detection of proteins in immunoblots

Study limitations

Technical considerations on the deployed experimental approach indicate the important contribution of biological variables other than FAK and muscle loading to the measured FAK-mediated signalling. Foremost this is presented by the reported damage response of transfected muscle portions by the selected methodology of gene transfer (Durieux et al. 2002, 2004; Gehl et al. 1999). In our setting, this bias was controlled by comparing the net effect of transfection in contralateral muscle pairs between FAK-producing and empty expression plasmid. This paired design allowed the identification of statistically significant effects of FAK-transfection on p70S6K-signalling in transfected TA muscles which were mechano-modulated throughout time. This is considerable taking into account the incomplete percentage of muscle fibre transfection (Durieux et al. 2002, 2004), the moderate responsiveness of TA muscle to hindlimb unloading compared to other leg muscle groups (Carlson et al. 1999; Dapp et al. 2004) and the restrictions imposed by the relatively low number of animals per experimental group. This highlights the resolution power of our approach for exposing muscle signalling.

Specificity of FAK-mediated mechano-signalling

The measured control of FAK protein and tyrosine phosphorylation levels in transfected muscles implies an important physiological modulation of FAK function by muscle loading. This regulation of Y397 phosphorylation and the amount of FAK between pCMV-FAK- and pCMV transfected muscle differed with regard to the "effective" time of loading. For instance, total level of Y397 phosphorylation was transiently enhanced by reloading without a change in FAK protein. In cage controls, no difference in Y397 phosphorylation was, however, between FAK-transfected and empty-transfected TA muscle when total FAK protein levels were elevated. We suggest that the elevated FAK activation within the first hours of reloading reflects the possibly

higher mechanical impact of normal cage activity on mechano-transduction in TA muscle after a period of unloading.

Conclusion

Our investigation on the mechano-biology of striated muscle identifies FAK as an upstream element of the mechano-regulated pathway for activation of ribosomal S6 kinase. The disconnection of this pathway towards protein translation from the commonly invoked Akt-mTOR signal has major bearing for control of muscle mass in physiological situations.

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ORIGINAL ARTICLE

Biologically relevant sex differences for fitness-related parameters in active octogenarians

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Abstract The number of elderly people is growing in western populations, but only few maximal performance data exist for people >75 years, in particular for European octogenarians. This study was performed to characterize maximal performance of 55 independently living subjects (32 women, 81.1 ± 3.4 years; 23 men, 81.7 ± 2.9 years) with a focus on sex differences. Maximal performance was determined in a ramp test to exhaustion on a bicycle ergometer with ergospirometry, electrocardiogram and blood lactate measurements. Maximal isometric extension strength of the legs (MEL) was measured on a force platform in a seated position. Body composition was quantified by X-ray absorptiometry. In >25% of the subjects, serious cardiac abnormalities were detected during the ramp test with men more frequently being affected than women. Maximal oxygen consumption and power output were 18.2 ± 3.2 versus 25.9 ± 5.9 ml min^{-1} kg^{-1} and 66 ± 12 versus 138 ± 40 W for women versus men, with a significant sex difference for both parameters. Men outperformed women for MEL with 19.0 ± 3.8 versus 13.6 ± 3.3 N kg^{-1}. Concomitantly, we found a higher proportion of whole body fat in women (32.1 ± 6.2%) compared to men (20.5 ± 4.4%). Our study extends previously available maximal performance data for endurance and strength to independently living European octogenarians. As all sex-related differences were still apparent after normalization to lean body mass, it is concluded that it is essential to differentiate between female and male subjects when considering maximal performance parameters in the oldest segment of our population.

Keywords Elderly · Exercise · Maximal performance · Maximal oxygen consumption

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Introduction

The portion of elderly in all western communities is increasing and thus the characterization of their physical work capacity becomes increasingly important. Physical work capacity is known to decline with age (Farazdaghi and Wohlfart 2001). According to the American Heart Association, i.e., maximum values of maximal oxygen consumption (V_{O_2max}) occur between the ages of 15 and 30 years and decrease with an average decline of 6–12% per decade, in both sedentary and athletic populations (Fletcher et al. 2001; Rogers et al. 1990; Wiswell et al. 2001). Incremental exercise tests are commonly used to quantify the function of the cardio-respiratory system and to diagnose its diseases. In elderly, these tests are likely to be performed as ramp tests with continuous increase of the load on bicycle ergometers because testing to exhaustion on treadmills may become more and more difficult for frail elderly (Cicoira et al. 2001). Several studies present reference values for exercise tests for all age classes (Farazdaghi and Wohlfart 2001; Buskirk and Hodgson 1987; Nordenfelt et al. 1985; Wohlfart and Farazdaghi 2003). But values for subjects in the old age (>75 years) are generally extrapolated

from data points of younger subjects, taking body weight, height, age and maximal workload into account. To our knowledge, only very few actually measured data exist for this age category (Evans et al. 2005; Malbut et al. 2002; Ehsani et al. 2003) whereby only few studies have emphasized the importance of total body composition, e.g., with inclusion of dual X-ray absorptiometry (DXA) measurements (Neder et al. 1999). In many studies, people with regular intake of medication or with a medical history of cardiac, respiratory or neuromuscular diseases were excluded (Farazdaghi and Wohlfart 2001; Nordenfelt et al. 1985, 1999; Wohlfart and Farazdaghi 2003; Simar et al. 2005). This approach can lead to a sample population not representing an average population of people of this age in which regular intake of medication is frequently observed. Recently published studies with European people in most advanced age have been conducted with a very small population (N = 10–17) (Farazdaghi and Wohlfart 2001; Wohlfart and Farazdaghi 2003; Simar et al. 2005) but sex differences were neglected (Simar et al. 2005). The aim of this study was to characterize the maximal physical work capacity (endurance and strength) and body composition in a segment of independently living female and male elderly people (>75 years) with a focus on sex differences.

Methods

Subjects and study design

The data presented were recorded from active subjects in stable health condition, willing to enter the Swiss National Foundation Program 53 ''Musculoskeletal health and chronic pain''. The subjects were recruited from the University of the Third Age (all >60 years) in Bern, Switzerland, and from local exercise groups for elderly. The study was approved by the local ethical committee and subjects provided written consent to participate in this study.

Initially, 55 independently living voluntary subjects (32 women, 23 men) between 75 and 89 years with stable medication, able to reach the test facilities unaided were included in the study. Subjects with severe diseases, i.e., neuromuscular diseases, myocardial infarction and/or severe hip or knee arthrosis, showing a significant negative impact on physical exercise were excluded from participation. All the subjects were asked to continue usual medication over the test period. In a clinical examination, a physician recorded the anthropometric data, took a medical history and a complete physical status. Whole body composition (lean and fat tissue mass) was determined using DXA (QDR-4500A, Hologic Inc., Bedford, USA).

Exercise testing

Timed Up & Go test and Berg balance scale

The Timed Up & Go (TUG) (Shumway-Cook et al. 2000) and the Berg balance scale (BBS) (Steffen et al. 2002; Berg et al. 1992) are age-specific tests for community-dwelling elderly people to assess general mobility and the risk for falling. For both tests, verbal instruction was given to the subjects prior to the different tasks whereas during the task no additional encouragement was administered. For the TUG, the time was measured, which was needed for getting up from an armchair, walking safely a distance of 3 m, returning to the chair and sitting down again (Shumway-Cook et al. 2000). After one test trial, the better of two trials was taken for the analysis. For the BBS, 14 items (interview combined with execution of balance, coordination and strength tasks) were scored on a scale of 0–4 (Berg et al. 1992).

Ramp test to exhaustion

A continuous ramp test to exhaustion was performed on an electromagnetically braked bicycle ergometer (Ergoline 800S, Ergoline GmbH, Bitz, Germany). The test started with a period of rest followed by a 2 min warm-up without load. The initial exercise load of 20 W was increased in a linear ramp pattern with 5 W every 20–60 s, dependent on the subject's individual fitness level, such that the total test duration would be 6–12 min (Fletcher et al. 2001). The subjects were asked to continuously pedal until exhaustion, maintaining constant revolutions-per-minute >45. Gas exchange parameters and ventilatory variables were recorded breath-by-breath (Oxicon alpha, Jäger GmbH, Würzburg, Germany). A 12-lead electrocardiogram (CardioSoft, GE, Houston, Texas, USA) was recorded in lying position before and during the ramp test sitting on the ergometer. Systolic and diastolic blood pressure and blood lactate levels (Lactate Pro, Axon Lab AG, Baden, Switzerland) were taken at rest and at the end of the test. Systolic and diastolic blood pressure and rating of perceived exertion (BORG, scale 6–20) were additionally recorded every 2 min (Borg 1982). By applying rigorous standards for BORG, lactate and RER at the end of the ramp test (see Table 1) we ascertained that the incremental exercise tests were performed to exhaustion.

Maximal isometric strength

Maximal isometric extension strength of the legs (MEL) was measured by pushing against a force platform (Quattro Jump®, Kistler Instrumente AG, Winterthur, Switzerland) in a sitting position on a chair (Fig. 1). This setup with a closed chain measurement of muscle extension strength of the legs was chosen to minimize the stress produced in the knee joint by a single joint measurement, i.e., an isometric assessment of quadriceps strength as well as to avoid strain imposed on the vertical column such as with a subject strapped into a leg press. The subjects were positioned on the chair so that the lower limb joint angles (foot, knee and hip) were at 90° and they were fixed in this position with a seatbelt. They were asked to push maximally against the force platform (hip and knee extension) and to maintain the contraction for about 3–4 s. Force data from 3 to 4 trials were recorded with a resolution of 500 Hz. The highest average force over a 1-s period was assigned as the subject's MEL.

Data analysis

Data are presented as mean ± standard deviation. Sex-grouped data were compared using Student's *t* test. The level for significance was set at $P < 0.05$.

For comparison of our measured data with predicted values for this age group, anthropometric data of our subjects were used in the following formulas proposed by the respective authors:

Neder et al. (1999):

$$V_{O_2max}\ \text{men} = -24.3 \times \text{age} + 12.5 \times \text{body mass} + 9.8 \times \text{height} + 702$$

$$V_{O_2max}\ \text{women} = -13.7 \times \text{age} + 7.5 \times \text{body mass} + 7.4 \times \text{height} + 372$$

$$P_{max}\ \text{men} = -1.78 \times \text{age} + 0.65 \times \text{body mass} + 1.36 \times \text{height} - 45.4$$

$$P_{max}\ \text{women} = -1.19 \times \text{age} + 0.96 \times \text{height} + 28.1$$

Wohlfart and Farazdaghi (2003):

$$P_{max}\ \text{men} = [244.6 \times (\text{height}/100) - 92.1]/[1 + e^{0.038(\text{age}-77.3)}]$$

$$P_{max}\ \text{women} = [137.7 \times (\text{height}/100) - 23.1]/[1 + e^{0.064(\text{age}-75.9)}]$$

Paterson et al. (1999):

$$V_{O_2max}\ \text{men} = (-0.31 \times \text{age}) + 44.23$$

$$V_{O_2max}\ \text{women} = (-0.25 \times \text{age}) + 36.63$$

Myers et al. (2002):

$$V_{O_2max}\ \text{men} = [18.4 - (0.16 \times \text{age})] \times 3.5$$

with V_{O_2max} (ml min^{-1}), P_{max} (W), body mass (kg), height (cm) and age (years).

Results

The data of all female and male subjects for anthropometry, assessment of fitness, mobility level and risk for falling (TUG and BBS), and maximal work capacity (performance and strength) are reported in Table 1.

Anthropometric data

The subjects' overall mean values for age, weight and height were 81.3 ± 3.8 years, 69.2 ± 11.5 kg and 166 ± 10 cm, respectively. Men were significantly heavier (+8.0 kg) and taller (+15.0 cm) than women. No significant difference was observed for the body mass index (BMI) whereas DXA results showed that females had a significantly higher proportion of fat tissue than male subjects, with a remarkable difference of +59%.

The different categories of drugs taken by the subjects are listed in Table 2. No significant difference between the sexes was observed for the number of medication products taken by the subjects. Most frequently consumed drugs were acetyl-salicylic acids ($n = 22$) and diuretics ($n = 13$) whereas beta-blockers were taken by 8 of the 55 subjects. Less than 10% (5 out of 55) did not take any drugs on a regular basis.

Assessment of general shape and risk for falling

Mean duration in the TUG was 8.2 ± 1.6 s and mean score in the BBS was 53.5 ± 3.1 pts with no significant difference between females and males.

Ramp test

Only 3 out of 55 subjects (2 females, 1 male) completed the ramp test with BORG <15 and lactate level <3.0 mM or a respiratory exchange ratio (RER) ≤1.0. As a consequence, their tests were classified as submaximal and therefore excluded from the analyses. For the remaining subjects ($n = 52$), mean exercise duration was 8:51 ± 1:34 min and in the end of the ramp test mean BORG, lactate level and RER were 16.0 ± 2.0, 4.9 ± 1.7 and 1.17 ± 0.08 mM, respectively. Mean values for V_{O_2max}, P_{max}, maximal

Table 1 Anthropometric and functional data for females and males

	Women		Men		*P* value
	Mean ± SD	Range	Mean ± SD	Range	
Anthropometric data	*n* = 32		*n* = 23		
Age (years)	81.1 ± 3.4	75–89	81.7 ± 2.9	76–87	0.50
Body mass (kg)	65.8 ± 11.5	49–97	73.8 ± 9.7*	60–98	<0.01
Height (cm)	160.0 ± 6.1	146–175	175.0 ± 6.9*	161–187	<0.01
BMI (kg m^{-2})	25.8 ± 4.8	18.4–38.9	24.1 ± 2.6	19.2–28.0	0.12
DXA measurement	*n* = 30		*n* = 20		
Lean body mass (kg)	41.4 ± 4.1	34.1–51.5	54.7 ± 6.1*	44.8–68.6	<0.01
Fat mass (%)	32.4 ± 6.2	15.1–44.8	20.4 ± 4.4*	13.4–28.9	<0.01
Timed Up & Go (TUG)	*n* = 32		*n* = 23		
TUG (s)	8.3 ± 1.6	5.7–12.1	8.0 ± 1.7	6.3–12.2	0.50
Berg balance scale (BBS)	*n* = 29		*n* = 16		
BBS (pts; 0–56)	53.6 ± 1.7	49–56	53.4 ± 4.7	36–56	0.80
Ramp test to exhaustion	*n* = 30		*n* = 22		
P_{max} (W)	66 ± 12	50–95	138 ± 40*	70–210	<0.01
V_{O_2max} (ml min^{-1} kg^{-1})	18.2 ± 3.2	10.7–25.4	25.9 ± 5.9*	15.2–34.8	<0.01
Max. heart rate (min^{-1})	135 ± 22	85–166	144 ± 14	111–166	0.08
Max. oxygen pulse (ml $beat^{-1}$)	9.0 ± 1.2	6.6–12.2	13.2 ± 2.8*	8.3–18.4	<0.01
Max.ventilation (l min^{-1})	47.5 ± 8.5	32.2–63.4	78.9 ± 19.1*	43.9–128.2	<0.01
Max. respiratory exchange ratio	1.15 ± 0.09	1.01–1.36	1.19 ± 0.07	1.05–1.32	0.05
Max. systolic blood pressure (mmHg)	173 ± 21	125–210	178 ± 17	140–210	0.36
Max. lactate level (mmol l^{-1})	4.7 ± 1.6	2.0–8.2	5.2 ± 1.9	2.9–9.7	0.34
Max. BORG (6–20)	15.9 ± 2.2	12.5–19	16.1 ± 1.7	13–19	0.68
Maximal isometric strength (MEL)	*n* = 31		*n* = 21		
MEL (N kg^{-1})	13.6 ± 3.3	8.72–22.1	19.0 ± 3.8*	11.3–26.2	<0.01

Student's *t* test was applied for detection of significant sex differences between females and males (*) with a level of significance of $P < 0.05$

heart rate and maximal systolic blood pressure were 21.5 ± 5.9 ml min^{-1} kg^{-1}, 96 ± 45 W, 139 ± 19 min^{-1} and 175 ± 19 mmHg, respectively. Men significantly outperformed women for V_{O_2max} (+42%) and P_{max} (+109%). V_{O_2max} normalized to whole body lean mass was still significantly higher ($P < 0.01$; +17%) in men (33.7 ± 6.9 ml min^{-1} kg^{-1} lean mass) than in women (28.9 ± 4.1 ml min^{-1} kg^{-1} lean mass). Likewise, a significant sex dependence was apparent for oxygen pulse and ventilation whereas no significant differences were observed for maximal heart rate, systolic blood pressure, end lactate level and RER (see Table 1 for overview).

ECG measurements

During the maximal exercise tests, cardiac abnormalities (signs of myocardial ischemia and/or arrhythmias) were observed in 14 subjects (five occurred in women and nine in men). Three major cardiac abnormalities with signs for serious myocardial ischemia (st segment depression and/or ventricular arrhythmias) were observed in men. After the ramp test, all subjects presenting cardiac abnormalities were sent to a cardiologist for further examination.

Maximal isometric strength

Mean MEL was 15.8 ± 4.4 N kg^{-1} being significantly higher (+40%) in men than in women (see Table 1). When MEL was normalized to lean body mass the sex dependent difference in force production was persistent ($P < 0.05$).

Discussion

The data presented in this study describes a population of elderly women and men tested to determine maximal strength and endurance as well as general mobility and balance. Females and males >75 years of age were included in the study when the medical condition allowed for safe testing and when medications were taken on a long term bases. Less than 10% of our subjects received no medication. In the analysis, we specifically focused on performance differences between female and male subjects. The study was conducted with a relatively large population of 55 active and independently living subjects (32 women and 23 men), considerably more than in other studies with subjects of similar age [Simar et al. (2005): $N = 17$].

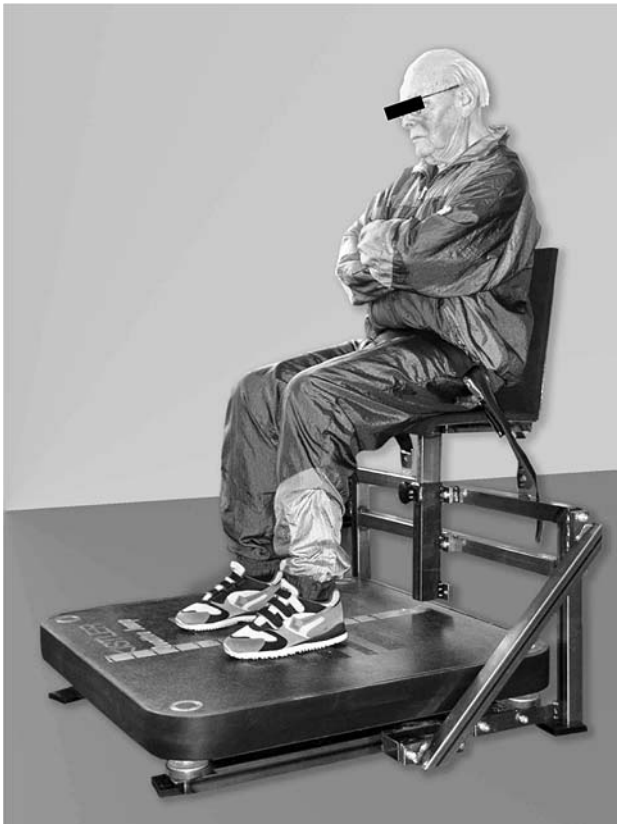

Fig. 1 Maximal isometric extension strength of the legs (MEL). Measurement of MEL (hip and knee extension) on a force platform (Quattro Jump®, Kistler Instrumente AG, Switzerland) in a seated position at 90° for the lower limb joint angles (foot, knee and hip)

Table 2 Frequency count of medication taken by the subjects on a regular basis

	Women, n = 32		Men, n = 23	
	n	%	n	%
Beta blockers	6	19	2	9
Statins	5	16	5	22
Diuretics	8	25	5	22
Ca^{2+} channel blockers	6	19	2	9
ACE inhibitors	5	16	3	13
Angiotensin II antagonists	8	25	4	17
Coumarins	2	6	4	17
ASA	12	38	10	43

ASA acetyl-salicylic acid

Our results show significant differences between male and female subjects for body composition (proportion of fat) and for maximal work capacity. Men significantly outperformed women in V_{O_2max}, P_{max} and MEL. A presentation of maximal physical performance values (Simar et al. 2005) without differentiation between male and female subjects in this age group is therefore of limited value. It is important to note that this differentiation between male and female subjects still remains relevant after normalization to lean body mass estimated by DXA measurement, for V_{O_2max} (+17%), P_{max} (+58%) and MEL (+19%). The sex-specific differences are noteworthy as in younger subjects sex-specific differences for V_{O_2max} are known to disappear with normalization to lean body mass (Uth 2005; Vinet et al. 2003; Washburn and Seals 1984). Vinet et al. (2003) stated that differences for V_{O_2max} between male and female children are due to differences in body composition. As this was not observed in the present study population our data suggest that differences for maximal performance parameters between male and female subjects become accentuated in old age. This notion is supported by Johnson et al. (2000) who suggested that differences for V_{O_2max} between elderly male and female subjects after normalization to fat-free mass might be due to ‘‘factors related to cellular aerobic capacity’’ or ‘‘cultural differences such as levels of habitual exercise’’. It therefore appears that differentiation between sex (biological differences) and gender (sociocultural differences) (Torgrimson and Minson 2005) becomes increasingly important with old age as these differences could accumulate over a longer lifetime period. However, it is currently unclear whether the observed physiological differences are due to genetically determined factors or are behaviorally induced.

Our current data result in an extension of existing maximal performance values (Farazdaghi and Wohlfart 2001; Nordenfelt et al. 1985; Wohlfart and Farazdaghi 2003; Astrand 1960; Shvartz and Reibold 1990; Neder et al. 2001) to females and males >75 years (see Fig. 2). The data demonstrate a continuing age-dependent decrease for maximal performance variables like V_{O_2max} and P_{max}, in particular for women. V_{O_2max} values for male subjects were found to be somewhat higher than expected from a linear extrapolation of the known age-dependent decrease at lower age (Fig. 2). In male subjects, the high levels of V_{O_2max} may be a reflection of the active lifestyle of the study population with several male subjects being actively mountaineering at the age of >80 years.

Our measured mean values for V_{O_2max} were slightly higher in both females and males compared with values calculated with current formulas for prediction of maximal performance (Neder et al. 1999; Paterson et al. 1999; Myers et al. 2002) (Fig. 3). Above-average performance values of our study population (130% of predicted exercise capacity in both men and women) are as well supported by the comparison to values from

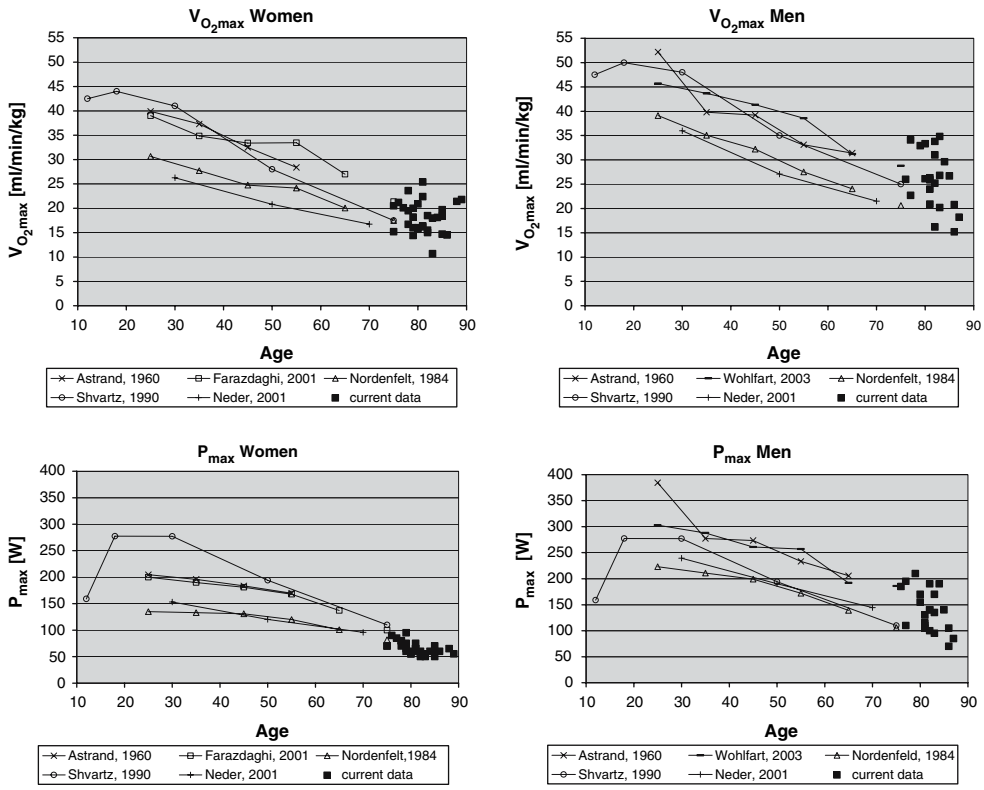

Fig. 2 Maximal performance values. Maximal performance values for females and males extending reference values for younger subjects from published work (Farazdaghi and Wohlfahrt 2001; Nordenfelt et al. 1985; Wohlfahrt and Farazdaghi 2003; Astrand 1960; Shvartz and Reibold 1990; Neder et al. 2001). P_{max}, maximal power output; V_{O_2max}, maximal oxygen consumption

nomograms for women (Gulati et al. 2005) and men (Morris et al. 1993) assessed in large clinical studies investigating >1,000 patients free from apparent heart disease.

This phenomenon is not observed for P_{max} achieved in the exhaustive incremental exercise test, which was not increased compared with predicted values neither for females nor for males. This seems best explained by differences in ramp protocols applied in the cited studies (Neder et al. 1999; Paterson et al. 1999; Myers et al. 2002) while P_{max} values are likely to vary with different ramp protocols (i.e., steeper or shallower); V_{O_2max} seems to be relatively protocol-independent as long as the total test duration remains in the range of 6–12 min (Fletcher et al. 2001).

Aging goes along with fading of physical abilities, increased occurrence of health deficits and decreased performance. Thus, testing of maximal performance parameters in elderly is potentially more risky than in younger populations. It therefore seems mandatory to adjust testing conditions (e.g., cycling instead of running and fitness level related ramp test protocols) and to take all necessary precautions for emergencies (clinical environment with ECG control and resuscitation readiness). However, under these conditions safe testing seems to be possible (for an overview, see Fletcher et al. 2001). As a consequence of the risks involved with testing old people, submaximal performance tests are often preferred (Witham and McMurdo 2003). Submaximal data can be sufficient to provide advice for exercise training of elderly. However, only testing to exhaustion yields useful V_{O_2max} values, a strong prognostic factor for the risk of death among patients with or without cardiovascular disease (Cicoira et al. 2001; Myers et al. 2002). Furthermore, V_{O_2max} allows a comparison of elderly-specific maximal performance data with data from younger subjects yielding quantification of the age-dependent decrease of human maximal performance parameters. As our study shows, testing to exhaustion is feasible and safe. However, the occurrence of minor to severe cardiac abnormalities for more than 25% of our elderly subjects during the ramp test to exhaustion high lightens the necessity for safety precautions and the availability of well trained medical personal during all testing procedure.

The sex difference for V_{O_2max} and P_{max} was confirmed as well for the measures of MEL. In particular, the low mean value for MEL (13.6 N kg^{-1}) in female subjects with ~20% of the values being below the acceleration of gravity was impressive. It has to be considered however, that with our method for measuring MEL (devised to minimize stress on joints and vertebral column) only the vertical component of the force applied to the force platform (at an angle of approx. 45°) is assessed (see Fig. 1). Compared to

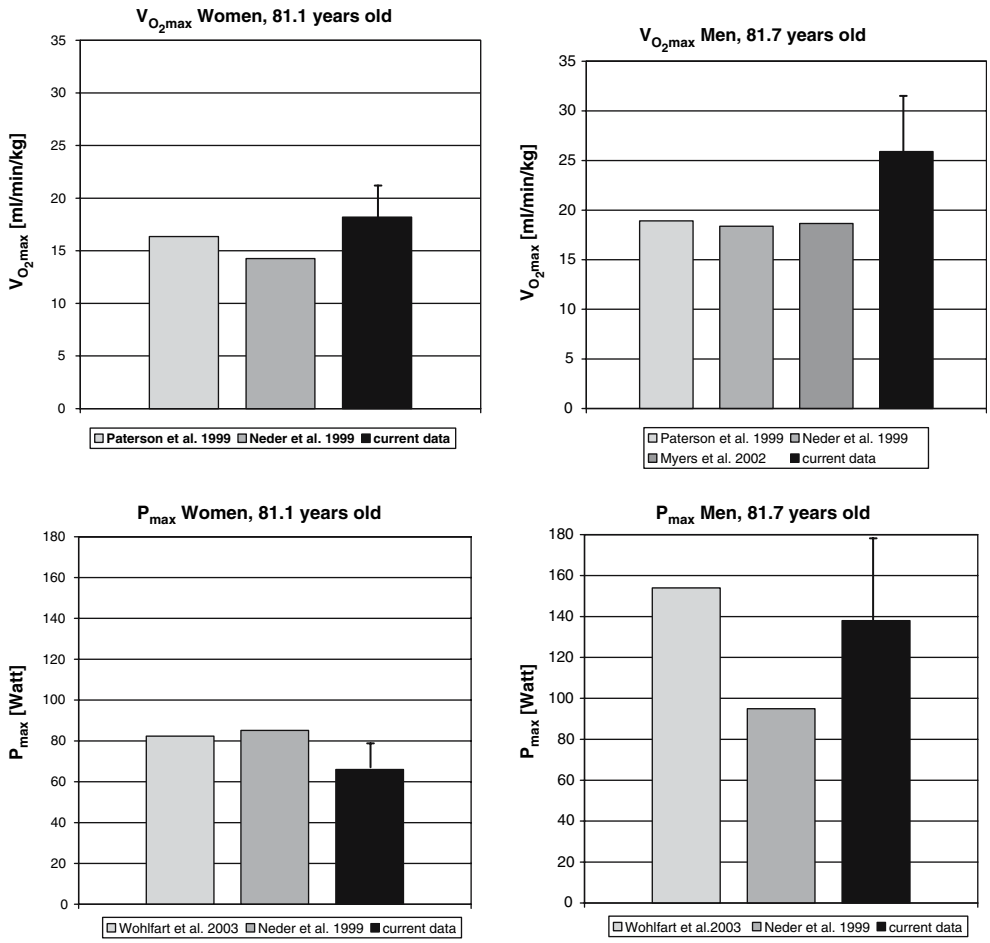

Fig. 3 Comparison of maximal performance values Comparison of the current data with predicted values using formulas from earlier studies (Paterson et al. 1999; Neder et al. 1999; Wohlfahrt and Farazdaghi 2003; Myers et al. 2002). P_{max}, maximal power output; V_{O_2max}, maximal oxygen consumption

standard tests of MEL, we would therefore expect our values to be smaller by some 30%. This approach was found to be safe and showed an excellent short and long-term test re-test reliability ($r^2 = 0.85$).

We are aware of limitations of the study design in which we recruited independently living subjects (independently living, physically active and open-minded for new experiences). The estimated parameters are hence superior to an average elderly population of >75 years. This assumption is confirmed by the high values, reached by our subjects in standard tests to determine the risk for falling (BBS, TUG) in community-dwelling populations. Except 1 out of 55 subjects, who scored only 36 points in the BBS and who needed 13.0 s in the TUG, all other subjects had score levels without any significant risk for falling (Shumway-Cook et al. 2000; Berg et al. 1992). However, the inclusion of subjects with regular medication intake may well represent an average population of independently living women and men of that age.

We found no significant impact of medication (i.e., beta-blockers, ACE inhibitors and statins) on maximal physical performance levels of the subjects, which could be expected according to previous studies (Teixeira et al. 1992; Kaiser 1984). This was evident as no significant difference for V_{O_2max} between groups with a certain medication and the control group (without medication) was recorded (data not shown). This is according to what was reported by Taniguchi et al. (2003) and Phillips et al. (2004).

In conclusion, this study presents maximal performance values of a relatively large population of active and independent elderly Swiss subjects >75 years. A comparison with current predictions for maximal performance values for this age group shows that the data are above average for the population because of the good age-appropriate shape of our subjects. We could show that differences between female and male subjects for physiological performance parameters (V_{O_2max}, P_{max}, MEL) are sex- and/or gender-specific and should be considered, e.g., when setting up training guidelines. In particular, this seems relevant for people >75 years, where men still record higher values than women even after normalization to lean body mass.

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Chapter 21

GENE EXPRESSION IN WORKING SKELETAL MUSCLE

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Abstract: A number of molecular tools enable us to study the mechanisms of muscle plasticity. Ideally, this research is conducted in view of the structural and functional consequences of the exercise-induced changes in gene expression. Muscle cells are able to detect mechanical, metabolic, neuronal and hormonal signals which are transduced over multiple pathways to the muscle genome. Exercise activates many signaling cascades - the individual characteristic of the stress leading to a specific response of a network of signaling pathways. Signaling typically results in the transcription of multiple early genes among those of the well known fos and jun family, as well as many other transcription factors. These bind to the promoter regions of downstream genes initiating the structural response of muscle tissue. While signaling is a matter of minutes, early genes are activated over hours leading to a second wave of transcript adjustments of structure genes that can then be effective over days. Repeated exercise sessions thus lead to a concerted accretion of mRNAs which upon translation results in a corresponding protein accretion. On the structural level, the protein accretion manifests itself for instance as an increase in mitochondrial volume upon endurance training or an increase in myofibrillar proteins upon strength training. A single exercise stimulus carries a molecular signature which is typical both for the type of stimulus (i.e. endurance vs. strength) as well as the actual condition of muscle tissue (i.e. untrained vs. trained). Likewise, it is clearly possible to distinguish a molecular signature of an expressional adaptation when hypoxic stress is added to a regular endurance exercise protocol in well-trained endurance athletes. It therefore seems feasible to use molecular tools to judge the properties of an exercise stimulus much earlier and at a finer level than is possible with conventional functional or structural techniques.

Key Words: Exercise, molecular, morphology, mitochondria

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PHENOTYPIC PLASTICITY OF MUSCLE STRUCTURE AND FUNCTION

Human exercise performance capacity varies widely. While most healthy young male subjects are able to pedal a bicycle ergometer at 200 Watts for 15 to 20 minutes the best of human athletes can maintain just over 500 Watts for one hour (24). This stunning feat is the consequence both of athletic endowment (genetically determined) as well as years of highly specific exercise training. The extraordinary performance of endurance athletes, such as cyclists, is due to many concerted adaptations of the pathway for oxygen from lungs to skeletal muscle mitochondria (17). On the level of skeletal muscle tissue, we see in highly trained endurance athletes a much larger capillarity (2.2 capillaries per muscle fiber, than in untrained subjects of similar age (1.1 C/F; (38) Likewise, the mitochondrial content of trained skeletal muscle fibers also differs by more than two-fold between world class athletes (11.4 percent of the muscle fiber volume, Vvmito) and untrained subjects (4-5 percent Vvmito; (14) . Interestingly, a similar two-fold difference can be found between athletes and sedentary subjects with regard to their intramyocellular lipid content (IMCL; (14). Not only do we find remarkable structural differences between muscle samples of athletes and sedentary subjects in cross-sectional studies, it is amply demonstrated that skeletal muscle tissue can rapidly change its oxidative capacity when a training regimen is implemented in previously sedentary subjects. Typically, mitochondria and capillaries can increase by approximately 30% with 6 weeks of endurance exercise training (15). Endurance training can be characterized as “low- load, high-repetitive” exercise. In a typical one- hour training session, muscles are loaded up to 5000 times with as little as 10 to 15% of their maximal voluntary contraction force (MVC). Strength training by contrast is typically “high-load, low-repetitive”. A single muscle, or rather a functional muscle group, may experience only 10 to 12 contractions per training session, but those contractions are near maximal, very close to MVC. In power-lifting we find even larger differences between trained and untrained individuals as seen in endurance exercise. While most of us are able to clear some 80kg on a bench-press, the current world record for this exercise

is 457 kg (Scot Mendelson). Likewise, improvements in muscle strength with appropriate training occur over similar time courses and with similar gains both structural and functional as those seen with endurance exercise training (28; 33).

In the context of the current review we would like to exploit the fact that both endurance and strength training protocols have been modified with the aim to optimize training stimuli. In endurance exercise, hypoxia (equivalent to altitudes of 1800 to 4000m) during training sessions has been used with the rationale that local muscle tissue hypoxia is an important signal for metabolic muscle adaptations and that systemic hypoxia should increase this signal (16). In strength training emphasis has been on the use of eccentric contractions (i.e. activation of muscles during lengthening) to enhance the effect of training regimens (9; 34). Eccentric contractions have a number of particular properties. At higher given angular velocities, peak torque in eccentric contractions can exceed peak torque in concentric contractions by more than two-fold (7), putting muscles at risk of damage and leading to delayed onset muscle soreness (DOMS; (21). At similar force developments eccentric contractions are performed with substantially lower electromyographic activities (indicating the recruitment of fewer motor units) and hence have up to four-fold lower energy requirement (2). The lower energy requirements of eccentric contractions result in massively reduced physiological responses of the cardiovascular system to a given mechanical exercise load. This interesting feature of eccentric exercise has been exploited in exercise paradigms for people with cardiovascular limitations in order to maximize mechanical stress on muscle tissue at low metabolic costs (23). The clinical relevance of this property of eccentric exercise stems from the observation that repeated continuous mild eccentric exercise results in gains in muscle force and cross-sectional area similar to those seen after classical high-load concentric strength training (19).

The purpose of the present review is to analyze the classical training paradigms of endurance and strength training as well as their modifications discussed above with regard to their molecular signature. We suggest that gene-expressional analyses should allow for a very detailed evaluation of the effect of training interventions, much finer than functional tests or structural analyses. Current functional laboratory tests for ath-

letes estimate relevant performance variables with error margins of a few percent, while differences of race times between the winner and the runner-up are typically at least one order of magnitude smaller. It is thus currently not possible to identify winners with lab tests. This analogy is not to say that molecular tools will eventually allow us to identify winners. The analogy indicates however, that functional tests currently used as "gold standard" are very coarse global assessment tools that carry virtually no information as to the fine biological adjustments that enable an organism or subject to perform the particular function in question.

THE MOLECULAR BASIS OF MUSCLE PLASTICITY

The adoption of molecular techniques for the study of muscle adaptation in the early 1990s gave access to studies aimed at delineating the mechanisms underlying muscle plasticity. From animal work using chronic electrical stimulation it appeared that pre-translational events had a major share in activity-related changes in enzyme activities (see (4). A particular challenge of early work in this area was to develop technical protocols that worked for small (mg) samples of muscle tissue obtained through biopsies and capable of reliably ascertaining differences in RNA concentrations of less than two-fold. We used quantitative PCR to look at biopsies (vastus lateralis muscle) of highly trained endurance runners and sedentary controls differing 2-fold in VO^2max and 1.9-fold in total muscle mitochondrial volume (26). We found the expression of all six investigated RNAs coding for enzymes of oxidative phosphorylation to be increased in direct proportion to the higher mitochondrial content of the muscle. Our data furthermore suggested pretranslational mechanisms to be responsible for the increase in nuclear encoded mitochondrial transcripts. By contrast, mitochondrially encoded RNAs were found to be increased as a consequence of an increase in mitochondrial DNA. A recent review of Hood (13) a key figure in research on mitochondrial biogenesis, confirms these early findings and presents the current view of the coordination of transcription of two genomes, synthesis of proteins and lipids as well as the assembly of multisubunit protein complexes which is characterized morphologically as "increase

in mitochondrial volume". With an elegant experiment using nuclear run-ons on human muscle biopsies at various time points after exercise Pilegaard (25) provided the direct evidence that a transient increase in transcription rate was followed by an increase in mRNA of several metabolic genes. They also showed that mRNA concentrations remained elevated beyond 22 hours when exercise was carried out repeatedly over several days. This was taken to suggest that transient increases in transcription during recovery from repeated exercise result in a gradual accumulation of mRNA, thus representing the basic mechanism of muscle cellular adaptation to increased contractile activity.

Having established that an important mechanism of muscle plasticity is the accretion of mRNA through repeated transient increases of transcription of metabolic and presumably of structure genes – it remains to be elucidated how the muscle cell senses the specificities of exercise related phenomena and uses the resulting signals to control gene expression. From common sense functional considerations it seems clear that a muscle cell must be able to sense a number of different stimuli. General molecular principles indicate that these initial queues are then transmitted over signaling cascades resulting in activation of a number of transcription factors. In turn, these act on early response genes (such as fos or jun and many others) that influence downstream targets. Alternatively, some transcription factors may directly affect downstream "structure" genes. We have proposed that under conditions of exercise muscle cells are subjected to at least four different important stressors: mechanical load, metabolic disturbances, neuronal activation and hormonal alterations (10). gives a coarse overview of known key factors that modulate the response to these stressors in muscle cells and their relationship. Note that with any type of exercise all stressors will always be active to some degree; however, depending on the type of exercise individual stressors will predominate. In strength-type exercise the dominant stressor is mechanical load while in endurance exercise metabolic disturbance is more important.

Mechanical load is thought to act primarily through integrins and integrin-associated signaling pathways, (see (5). Integrins serve as the link between extracellular matrix and cytoskeleton and are therefore critical to sensing of external mechanical events. In this context the formation of focal adhesion complexes is critical for the cellular trans-

duction of a mechanical signal such as stretch of skeletal muscle fiber (11; 27).

Metabolic disturbances such as shifts in pH, temperature, oxygen tension and energy status within the muscle cell are key feature of muscle work. A central role in metabolic sensing has been assigned to the AMP activated kinase, AMPK, (12). AMPK is implicated in regulation of substrate metabolism and mitochondrial biogenesis, and via inhibition of the TOR pathway in muscle impedes protein metabolism and hence muscle hypertrophy (3).

Neuronal activation is a prerequisite for normal muscle contraction activity. Fluctuations in intramyocellular Ca^{2+} levels are decoded for amplitude and frequency and linked to gene expression by Calmodulin dependent Kinases (CaMK). While CaMKII seems to be involved in regulating oxidative enzyme expression, mitochondrial biogenesis and fiber type specific myofibrillar protein expression, the role of CaMKIV remains more elusive (6).

Hormonal alterations both local and systemic are inevitable consequences of any type of exercise activity. Androgens, growth hormone, IGF-I and its splice variants, Insulin and Vitamin D positively affect muscle growth/volume mostly through their activation of satellite cells. By contrast, satellite cell activation is repressed by myostatin, glucocorticoids, TNF and IL-1 and IL-6 (30).

While typical training paradigms for strength and endurance are well established – and the functional and structural outcome of these training protocols is well defined and relatively stereotyped; the molecular machinery that intervenes between the stimulus and the outcome is of bewildering complexity. There is massive crosstalk between the different pathways, many factors are sensitive in the time domain and important players might not yet be discovered. There is considerable more research needed to understand training phenomena in muscle on the mechanistic level – and much more when training is seen as a system function of an organism with other organs such as the heart, the brain, the kidney and the liver contributing significantly to the overall training response.

THE MOLECULAR SIGNATURE OF TRAINING IN HYPOXIA

As indicated above we have been interested in the effect of hypoxia on working muscle with the aim of modifying a metabolic stressor presumed to be important for muscle adaptations in endurance type exercise. While the advantage of "training high – living low" in terms of improvement of athletic performance capacity (at altitude or at sea level) has remained disputed (1; 20) this type of training has been shown to have some specific physiological effects which are coherent with molecular changes observed at the muscle tissue level.Vogt et al (36) using quantitative PCR have shown the steady state levels of mRNA for hypoxia inducible factor 1 (HIF-1α) to be increased in subjects trained for six weeks in normobaric hypoxia (aequivalent to an altitude of 3850m) when compared to subjects working at similar relative workloads in normoxia. This important finding of an up-regulation of the key transcription factor HIF-1α with training in hypoxia has been replicated by (37) who found HIF-1α mRNA to be up-regulated in 9 well trained endurance athletes who had a short hypoxia stress added to their regular training schedule. While it has been difficult to demonstrate clear-cut improvements in accepted physiological descriptors of aerobic performance capacity such as VO2max (8) ; the addition of hypoxia to endurance exercise stress leaves a prominent signature on a number of genes involved in key regulatory functions of hypoxia adaptation (37). Key transcripts of carbohydrate metabolism (Glut-4,glucose transporter 4 ; PFKm, 6-phosphfructokinase muscle type), mitochondrial biogenesis (PGC1α, peroxisome proliferator activated receptor; Tfam, mitochondrial transcription factor 1) and mitochondrial metabolism (CS, citrate synthase; COX-1, cytochrome oxidase subunit 1; COX-4, cytochrome oxidase subunit 4) as well as myoglobin mRNA are significantly up-regulated when training is carried out with an extra hypoxia stress. This is broadly compatible with observed functional and/or structural observations after training augmented by hypoxia (22; 31; 32; 36). Interestingly, Zoll et al (37) could demonstrate a significant correlation between the up-regulation of transcripts involved in pH regulation (CA3, carbonic anhydrase 3 and MCT-1, monocarboxylate transporter 1) and the time subjects could run at VO2max. It is further suggestive that the observed

increase in transcripts of factors mitigating oxidative stress (MnSOD, manganese superoxide disnutase and Cu/ZnSOD cytoplasmic coppere/zinc dismutase) is related to the capacity of intermittent hypoxia training to perform respiratory function under low oxygen tension.

The observation of a complex and specific molecular response to the addition of a hypoxia stress to an endurance training protocol is intriguing and promising. We suggest that other tissues challenged by a training intervention such as the heart, the brain, the liver and the kidney may also react specifically to hypoxia. These changes remain to be determined but may be an important part of a hypoxia-modified global training response. It is further evident that not all changes that are invoked by hypoxia are necessarily beneficial for all athletes under all circumstances. Hypoxia favors glucose metabolism; this may be detrimental for athletes that could profit from developing their potential for fat oxidation (35).

TIME COURSE OF TRANSCRIPTOME CHANGES

In the previous paragraphs we have looked at the specific signature that a training intervention in hypoxia leaves on the steady state expression levels of performance relevant muscle genes. We now look at the way specific transcript levels change over time after a single bout of exercise. The rationale here is that we expect transcript levels to change in a typical fashion over a 24 hour period between exercise bouts in response to the specifics of the stress that the muscle tissue was exposed to during exercise. We have looked at the time course of the muscle transcriptome changes using a custom made microarray containing 229 transcripts of interest for muscle plasticity (29). We took biopsies of untrained subjects before exercise as well as 1, 8 and 24 hours after a 30 min near exhaustive exercise bout on a bicycle ergometer. From 112 detected transcripts we found 23 transcripts to be significantly up-regulated while 3 were significantly down-regulated. The overall response of the transcriptome reveals a pronounced and significant drop of transcript levels at 1 hour post-exercise, a significant up-regulation over pre-test values at 8 hours post-exercise and a drop towards

pre-test values at 24 hours post-exercise (Fig. 2; (29). After the initial exercise bout, subjects were endurance trained for 6 weeks (30 min/day; 5 days/week) before being subjected to a second exercise challenge and biopsy procedure, whereby the exercise load was adjusted to the training induced improvement in power output. Overall we found pre-test (steady state) concentrations of transcripts to be significantly increased. In the trained state the response to a single similar exercise challenge was massively attenuated. Instead of 20 transcripts being significantly up-regulated 8 hours post-exercise in untrained condition, only 2 were significantly up at 8 hours (29). More recently we have studied the time course of the transcriptome response over 24 hours following a mild bout of 15 min of eccentric work in previously untrained subjects (18) Since it has been documented that this type of exercise can lead to a considerable gain in muscle strength and in muscle fiber cross-sectional area, we expected to find an up-regulation of transcripts relevant for muscle growth. This was not the case. As indicated in Fig. 2, we saw an initial drop in transcript levels similar to that seen after concentric exercise. However, there was no evident immediate up-regulation thereafter and the general transcript level took 24 hours to return to close to pre-test values (Fig 2). With both training interventions the stimulus provoked an early down-regulation of transcription. This seems to be a normal reaction of tissue that has a high demand of energy and shuts down dispensable cellular reactions that use energy. For translational events it is quite well established that AMP-kinase, a sensor of intracellular energy levels is activated and inhibits translational events (12).

Taken together, the data from time course studies indicate that specific training protocols not only provoke specific changes of the transcriptional profile – but that these changes follow a discrete time course which is found to be different for different training interventions. Transcriptional profiling in a time series may therefore be a tool to optimize the timing of training interventions. In the case of eccentric exercise the current data suggests that the muscle takes more than 24 hours to recover and react to a preceding exercise bout.

CONCLUSIONS

Over the last ten years, molecular tools have started to provide insight into the expressional changes underlying muscle adaptational phenomena. The initial course of signaling events and their complex interactions have remained elusive; however the ensuing transcriptional response of muscle tissue starts to emerge and is seen to be specific in term of its character and of its time course. Exercise scientists using gene expressional screens on muscle tissue demonstrate a very detailed picture of the muscle tissue response. This will eventually enable them to tailor training regimes much more specifically in view of desired functional goals and test predicted functional outcomes with more selective and specific tests than those that are currently in use. This road seems particularly promising when exercise is used in clinical settings.

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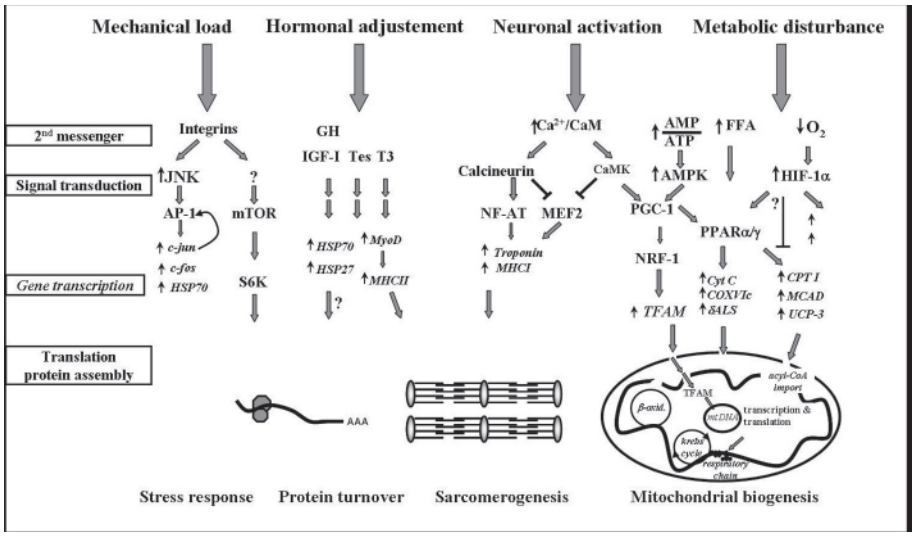

Figure 1. Schematic representation of influence of main stressors of muscle tissue on gene expression in skeletal muscle tissue. Only selected pathways are presented. During exercise all stressors are active – but differ in importance depending on the quality of the exercise stimulus (adapted from (10).

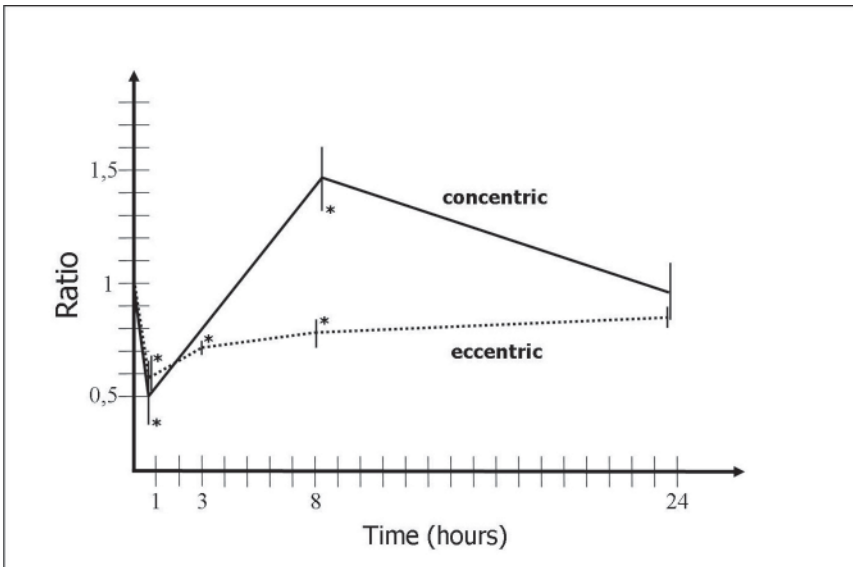

Figure 2. Time course of changes of the muscle transcriptome after a single bout of concentric or eccentric exercise. Exercise was carried out by previously untrained subjects at an intensity and duration typical on an initial training bout in the respective training proto col. Values for concentric exercise represent mean of 112 detected transcripts, values for eccentric exercise represent mean of 147 detected transcripts. The custom made microarray contained 220 transcripts. (Asterix indicates significantly different to pre-exercise value18; 29).

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Training in hypoxia and its effects on skeletal muscle tissue

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It is well established that local muscle tissue hypoxia is an important consequence and possibly a relevant adaptive signal of endurance exercise training in humans. It has been reasoned that it might be advantageous to increase this exercise stimulus by working in hypoxia. However, as long-term exposure to severe hypoxia has been shown to be detrimental to muscle tissue, experimental protocols were developed that expose subjects to hypoxia only for the duration of the exercise session and allow recovery in normoxia (live low–train high or hypoxic training). This overview reports data from 27 controlled studies using some implementation of hypoxic training paradigms. Hypoxia exposure varied between 2300 and 5700 m and training duration ranged from 10 days to 8 weeks. A similar number of studies was carried out on untrained and on trained subjects. Muscle structural, biochemical and molecular findings point to a specific role of hypoxia in endurance training. However, based on the available data on global estimates of performance capacity such as maximal oxygen uptake (V_{O_2}max) and maximal power output (P_{max}), hypoxia as a supplement to training is not consistently found to be of advantage for performance at sea level. There is some evidence mainly from studies on untrained subjects for an advantage of hypoxic training for performance at altitude. Live low–train high may be considered when altitude acclimatization is not an option.

Hypothesis

Environmental hypoxia influences the working conditions of skeletal muscle tissue such that myoglobin oxygen saturation and thus intramyocellular oxygen partial pressure is significantly lower in hypoxia at rest and under all submaximal working conditions up to V_{O_2}max (Richardson et al., 1995, 2006). It is contended that hypoxic conditions in muscle tissue lead to specific signaling events that result in consistent changes of the muscle phenotype that are relevant for muscle performance for athletes under certain conditions. This contention is suggested by ample evidence that hypoxia induces hypoxia-inducible factors (HIF). HIF-mediated signaling impacts the expression of a large number of genes (Semenza et al., 2006), many of those with a functional significance in skeletal muscle tissue (Hoppeler & Flück, 2002).

This review focuses on studies in which training sessions were carried out either in hypobaric or normobaric hypoxia while subjects were under normoxic conditions for the remainder of the time (live low–train high or hypoxic training).

Overview

The use of hypoxic conditions for athletic preparation is most often motivated by the expected gains in aerobic performance capacity related to the increase in hemoglobin (Hb) and thus oxygen-carrying capacity of the blood. This training modality (live high–train low), its potentials and limitations are dealt with in the accompanying papers (Richalet, 2008; Schmidt & Prommer, 2008; Stray-Gundersen & Levine, 2008).

The main incentive of using hypoxic conditions solely for the time period of an individual training session while spending the remainder of the day in normoxia (hypoxic training) stems from the observation that permanent exposure to severe hypoxia (i.e., residency at altitudes around 5000 m and higher = Everest base camp) leads to a considerable deterioration of skeletal muscle tissue. There is general agreement that extended exposure to real or simulated altitude over the time periods of typical mountaineering expeditions to the Himalayas (5–6 weeks) leads to a loss of muscle volume of the order of 10% to 15%, with a concomitant decrease in muscle fiber size of 20% to 25% but with no change in muscle fiber-type distribution (Green et al., 1989; Hoppeler et al., 1990; MacDougall et al., 1991). In mountaineers, it was further shown that muscle oxidative capacity characterized by muscle mitochondrial volume density is decreased by as much as 20% with a corresponding decrease of enzyme activities of the citric acid cycle, fatty acid oxidation, ketone body utilization and

respiratory chain (Howald et al., 1990). The latter study also showed enzymes of glycolysis to be increased post expedition. The same studies further found muscle capillary to fiber ratio to be essentially unchanged with altitude exposure (Hoppeler et al., 1990; MacDougall et al., 1991). However, due to the loss in fiber size, capillary density was increased after hypoxia exposure, leading to a better supply with oxygen of the remaining smaller quantity of muscle mitochondria (Hoppeler et al., 1990). Further indication of muscle deterioration or muscle fiber damage after long-term altitude exposure is furnished by the observation of a near tripling of the volume fraction of lipofuscin in muscle fibers in mountaineers after return to sea level (Martinelli et al., 1990). Lipid peroxidation of mitochondrial membranes as a consequence of oxidative stress in hypoxia has been proposed to be at the origin of lipofuscin accumulations (Hoppeler et al., 2003). However, the interaction of oxidative stress with mitochondria is complex and involves also damage to DNA and proteins as well as apoptosis (Lee & Wei, 2007). In the context of hypoxia induced damage and loss of mitochondria it is of interest to note that in biopsies of Sherpas, which are believed to have evolutionary adaptations to living and working in hypoxia, the increase in muscle lipofuscin after prolonged high-altitude exposure cannot be demonstrated (Gelfi et al., 2004). This study found antioxidant enzymes (such as gluthatione-S-transferase) to be massively upregulated in this high-altitude population, presumably preventing accumulation of lipofuscin.

As muscle faces serious oxygen deprivation during exercise, it has generally been assumed that hypoxia could be an important signal contributing to the typical muscular responses to endurance exercise training. Endurance exercise increases muscle oxidative capacity and muscle capillary supply and it was suggested that this response should be more pronounced under hypoxic conditions (Hochachka et al., 1982). In view of the unexpected fact of a sizeable muscle loss and mitochondrial deterioration with prolonged exposure to high altitude, it was reasoned that permanent sojourn in hypoxia might be detrimental to muscle tissue possibly due to a hypoxia-dependent decrease in muscle protein synthesis (Desplanches et al., 1993). This contention led to experiments and training programs that consisted in applying hypoxia only during all or a limited number of training sessions, thus maximizing the hypoxia stimulus on muscle during contraction; while subjects remained under normoxia for the rest of the time with optimal conditions for muscle recovery.

Tables 1 and 2 report key functional data from studies in which exercise sessions were carried out in hypoxia (subjects recovered at normoxia for the remainder of the time) and that included a control training group in normoxia. Table 1 reports data on nine studies that were performed on trained subjects while Table 2 reports data on 12 studies on untrained subjects. Studies are arranged in chronological order. In addition to the tables, key findings of these studies are reported below in the text – with the aim of reaching an overall assessment of the hypoxic vs normoxic training benefits taking into account the widely varying conditions under which these studies were carried out.

In one of the earlier studies on hypoxic training, the same subjects were sequentially trained in normoxia and hypoxia (Davies & Sargeant, 1974). This study (not reported in Table 2 because of lack of a proper control group) reports the effect of single leg exercise training at a constant 75% of V_{O_2}max load ($n = 4$, 3×30 min at a fraction of inspired oxygen (FiO_2) of 0.12, equivalent to an altitude of approximately 3850 m, over 5 weeks). The study finds no significant additional effect of training in hypoxia for all submaximal or maximal variables measured but notes a larger difference between normoxic and hypoxic single leg V_{O_2}max after training in hypoxia. The authors are the first to stress one of the key problems of all studies comparing exercise in normoxia and hypoxia – namely the fact that hypoxia reduces V_{O_2}max and limits power output. Hence, studies in which exercise is carried out at the same absolute workload in normoxia and hypoxia differ in their relative workload under given conditions, with the hypoxic condition imposing an identical mechanical load but a larger metabolic "stress" on muscle tissue. It must be noted, however, that with increasing altitude it may ultimately become impossible to continue to work at the same absolute intensity under normoxic and hypoxic conditions. If training is carried out at the same relative work intensity, then by necessity the hypoxic group will experience a smaller mechanical load on muscle tissue. This said, it becomes apparent that it is virtually impossible to compare exercise conditions for studies using different training paradigms at different training altitudes.

A common feature of virtually all studies reported in Tables 1 and 2 is that hypoxia exposure for the time of exercise sessions alone is not sufficient to induce changes in hematologic parameters. Hematocrit and Hb concentrations remain in general unchanged with live low–train high. Only two studies report changes in hematocrit and Hb concentrations (Meeuwsen et al., 2001; Hendriksen & Meeuwsen, 2003) likely related to uncontrolled changes in plasma volume. Likewise, among all studies reported in Tables 1 and 2, there are no trends in changes in maximal end of exercise lactates or end of exercise heart rates between hypoxic and normoxic training

Table 1. Synopsis on studies with trained subjects

Study	Training	Conditions	$V_{O_2}max$		W_{max}		Other functional aspects of study	Additional variables studied and summary statements
			N	H	N	H		
Terrados et al. (1988)	Cyclists 4–5 × 100–150 min 3–4 weeks	N = sea level $n = 4$ H = 574 torr ≈ 2300 m $n = 4$	−1.2%NS +3.4%NS	NA NA	+6.7%NS +12.2%S	+3.6%NS +10.6%NS	Sign. larger increase in total work performed in H	No changes in fibertype composition, capillarity but sign. larger, decrease in PFK and LD in H; no difference in CS, HAD or CK.
Bailey et al. (2000a)	Trained runners same volume of training at same relative intensity estimated by HR double blind	N = PiO_2 150 mmHg $n = 18$ H = PiO_2 115 mmHg $n = 14$	+5%NS +15%S	NA NA	NA NA	NA NA	Additional group in permanent Hypoxia showed increased illness and depressed glutamine levels	Hypoxic training has beneficial effects while continuous hypoxia has potential deleterious consequences
Meeuwsen et al. (2001)and Hendriksen & Meeuwsen, (2003)	Triathletes cycling 10 × 120 min at same relative Intensity 60–70% hr reserve +cross-over study with same modalities, but different n	N = sea level $n = 7$ H = 75.2kPa ≈ 2500 m $n = 7$ Same altitudes N: $n = 8$ H: $n = 8$	+3.5%NS +8.3%S 1.5%NS +2.6%NS	NA NA NA NA	+3.0%NS +9.1%S +1.8%NS +5.5%S	NA NA NA NA	In practically all measured parameters (mean power, peak power, time to peak) hypoxia group increased more than normoxia group	Hypoxic training can increase aerobic and anaerobic energy systems in triatheletes. Hb and Hk increased sign. 2 days after training Cross-over study shows generally smaller changes in all variables; small advantage of hypoxic training for anaerobic power
(Truijens et al., (2003)	Trained swimmers Swimming 12.5 min of high intensity bouts added 3x/week for 5 weeks	N = FiO_2 20.9% $n = 8$ H = $FiO_2$15.3% ≈ 2500 m $n = 8$	+5.6%S +3.8%S	NA NA	NA NA	NA NA	Free style time trials over 100 m and 400 m were significantly improved with no difference between groups	No additive effect of high intensity hypoxia work
Ventura et al. (2003)	Trained cyclists cycling 3 × 30 min, 6 weeks Same relative intensity in addition to normal training	N = 560 m $n = 5$ H = $FiO_2$13% 3200 m $n = 7$	−0.6%NS +0.6%NS	−2.7%NS −5.0%NS	−3.6%NS +0.8%NS	−7.6%S −1.9%NS	Testing in hypoxia showed increase in capillary blood oxygen saturation for hypoxic training only	No additive effect of high intensity hypoxic training; symptoms of overreaching
Roels et al. (2005)	Trained cyclists Cycling 2 weekly high intensity sessions added to normal training 7 weeks	N = $PiO_2$160 mmHg $n = 11$ H = PiO_2 100 mmHg ≈ 3000 m $n = 11$	+4.9%NS NA	NA +5.9%NS	+2.7%NS NA	NA +0.7%NS	Additional training group that did warm up and recovery (not training) in hypoxia improved VO_{2max}	Intense training periods improved performance only in first 4 weeks. No additional advantage for hypoxic training
Morton and Cable (2005)	Team sport player Cycling 3 × 30 min (10 × 1 min at 80%VO_{2max}, 10 × 2 min at 50%VO_{2max}) 4 weeks	N = FiO_2 20.9% $n = 8$ H = $FiO_2$15% ≈ 2750 m $n = 8$	+8.0%S +7.2%S	NA NA	+17.8%S +15.5%S	NA NA	Power output at 4 mMol lactate, and peak and mean power in Wingate increased in both groups	No advantage for hypoxic training for sea level performance

Table 1. (continued)

Study	Training	Conditions	V_{O_2}max		W_{max}		Other functional aspects of study	Additional variables studied and summary statements
			N	H	N	H		
Dufour et al. (2006) and Ponsot et al. (2006) and Zoll et al. (2006)	Trained runners Running normal training protocol (five sessions per week) plus 2 times per week at VT2 during 6 weeks	N = FiO_2 20.9% $n = 9$ H = $FiO_2$14.5% ≈ 3000 m $n = 9$	+1.2%NS +5.3%S	NA NA	NA NA	NA NA	Time to exhaustion at speed eliciting VO_{2max} was only increased (+35%s) in hypoxic training group	Hypoxic training has small advantage over normoxic training at high intensities. Hypoxic training improves mitochondrial function increasing respiratory control and coupling between ATP demand and supply. Hypoxic training increases steady state mRNA levels of transcripts of oxygen signalling, oxidative metabolism, CHO metabolism, oxidative stress and pH regulation
Roels et al. (2007)	Endurance athletes 2 interval, 3 endurance sessions for 60–90 min during 3 weeks Same relative workload 60% VO_{2max}	N = PiO_2 160 mmHg $n = 8$ H = $PiO_2$100 mmHg ≈ 3000 m $n = 10$	+5.0NS − 0.3NS	NA NA	+7.2%S +6.6%S	NA NA		Maximal ADP-stimulated mitochondrial respiration was changed in H only for some substrate combinations.

H, hypoxia; N, normoxia; *n*, number of subjects in the training group; VO_{2max}, maximal oxygen uptake; W_{max}, highest work intensity; NS, not significant; S, significant; NA, not analyzed; PFK, phosphofructo kinase; LD, lactate dehydrogenase; CS, citrate synthase; HAD, 3-hydroxyl coenzyme A dehydrogenase; CK, creatine kinase; HVR, hypoxic ventilatory response; GH, growth hormone; Epo, erythropoietin; C/F, capillary to fiber ratio; HR, heart rate; BP, blood pressure; RPP, rate pressure product; SDH, succinate dehydrogenase; Hb, hemoglobin; Hk, hematokrit; HIF1α, hypoxia inducible factor 1α; VEGF, vascular endothelial growth factor; mRNA, messenger ribonucleic acid; HSP70, heat shock protein 70; VT2, ventilatory threshold 2; ATP, adenosine triphosphate; ADP, adenosine diphosphate; CHO, carbohydrate.

Table 2. Synopsis of studies on untrained subjects

Study	Training	Conditions	$V_{O_2}max$		W_{max}		Other functional aspects of study	Additional variables studied and summary statements
			N	H	N	H		
Roskamm et al. (1969)	Untrained Bicycle ergometer 6 × 30 min 4 weeks Same relative intensity of heart rate	N = 260 m $n = 6$ H1 = 2250 m $n = 6$ H2 = 3450 m $n = 6$	+6.4%S +17.5%S +10.0%S	+4.4%S +7.5%S +15.0%S	NA NA NA	NA NA NA	Maximum ventilation increased in H not in N group Sign higher improvement over all test of H2 group compared with N group	Greater increases of VO_{2max} of both H groups; increased capacity for energy mobilization from aerobic pathways post exercise
(Terrados et al., 1990	Untrained single leg training 3–4 × 30 min 4 weeks Same absolute workload ≈ 65% VO_{2max}	N = sea level $n = 10$ H = 572 bar ≈ 2300 m $n = 10$	NA NA	NA NA	NA NA	NA NA	Time to fatigue +342% sign Time to fatigue +413% sign more than N	Significantly larger increase in CS and increase in myoglobin in H; LD decreased significantly in H; no change in fiber types, fiber size or capillarity
Benoit et al. (1992)	Untrained bicycle training 6 × 60 min 3 weeks Same relative workload 80% VO_{2max}	N = 500 m $n = 9$ H = $FiO_2$10% 5400 m $n = 9$	+9.3%S +6.7%S	−0.2%NS +4.8%NS	NA NA	NA NA	HVR: no change HVR: +54%s	Adaptation of HVR in H group and maintenance of N vs H VO_{2max} is seen as pre-acclimatization to hypoxia
Levine et al. (1992) and Engfred et al. (1994)	Untrained bicycle training 5 × 45 min 5 weeks Hypoxic group trained either at same relative or abs. workload 70% VO_{2max}	N = sea level $n = 7$ H = 560 torr 2500 m $n = 14$	+13.7%S +12.1%S	NA NA	NA NA	NA NA	HVR: no change HVR: +41.4%s	Adaptation of HVR in H group only Training induced changes in hormones noradrenaline, adrenaline, GH, glucagon, insulin and Epo similar in both groups
(Desplanches et al., 1993	Untrained Bicycle training 6 × 120 min 3 weeks Same relative workload 75% VO_{2max}	N = 500 m $n = 5$ H = $FiO_2$10% 5700 m $n = 5$	+0.5%NS +6.4%NS	+1.9%NS +11.0%S	NA NA	NA NA	Additional control group that trained at same absolute workload as H (approximately 50% normoxic VO_{2max}) showed no structural or functional adaptations	Significant increase in mitochondrial volume densities in both groups; sign. increase in fiber size and C/F ratio in H group only
Melissa et al. (1997) and Green et al. (1999)	Untrained Single leg ergometer 3 × 30 min 8 weeks Same relative workload 75% VO_{2max}	N = ambient air $n = 10$ H = $FiO_2$13.5% ≈ 3200 m $n = 10$	+13%S +11%S	NA NA	NA NA	NA NA	Time to fatigue at 95% VO_{2max} unilateral cycling +400%s in Normoxic and +510% sign. longer in hypoxic trained leg	CS+51%in N, +71% in H sign more in H SDH and PFK sign. increased in both leg. Mito volume density, capillaries, fiber types and fiber size not sign. larger Na+-K+-ATPase increased significantly with N and decreased with H
Emonson et al. (1997)	Untrained cycle ergometer	N = 90 m $n = 9$	+8.7S +15.7S	NA NA	NA NA	NA NA		H and N groups have similar improvements in functional

Table 2. (continued)

Study	Training	Conditions	V_{O_2}max		W_{max}		Other functional aspects of study	Additional variables studied and summary statements
			N	H	N	H		
	3 × 45 min 5 weeks Same relative workload 70% VO_{2max}	H = 554 torr ≈ 2500 m $n = 9$					Endurance time increased by 66.2% in N and by 77.3% in H	variables, no advantage of H training
Katayama et al. (1999)	Untrained cycle ergometer 5 × 30 min 2 weeks Same relative workload 70% VO_{2max}	N = sea level $n = 7$ H = 432 torr 4500 m $n = 7$	+5.0S +7.1S	NA NA	NA NA	NA NA	HVR decreased sign. in N group and increased not sign. in H group	All functional variables reverted to pretraining levels after 2 weeks of detraining
Bailey et al. (2000)	Physical active Cycling 3 × 20–30 min 4 weeks Same relative workload 70–85% HR_{max} Double blind	N = FiO_2 20.9% $n = 14$ H = $FiO_2$16% 2500 m $n = 18$	+4.0%NS +13.5%S	NA NA	+7.0%S +4.6%S	NA NA NA NA	Decrease in BPmax and RPP were observed in hypoxic group only; Homocysteine only in H group sign. improved, other cardiovascular risk factors were similarly improved in both groups	Hypoxic training has additive beneficial effects for cardiovasc risk factors
Masuda et al. (2001)	Untrained cycling 60 min every second day for 8 weeks Same relative intensity 70% VO_{2max}	N = sea level $n = 7$ H = 560 torr ≈ 2500 m $n = 7$	+16.6%S +12.4%S	NA NA	NA NA	NA NA		No significant changes in muscle fiber composition after H or N; CS activity and C/F ratio significantly increased in H and N group. No additive advantage of hypoxic training
Geiser et al. (2001) and Vogt et al. (2001)	Untrained cycling 5 × 30 min 6 weeks Same relative intensity 80% VO_{2max}	N = 600 m $n = 8$ H = $FiO_2$12% ≈ 3850 m $n = 7$	+9.5%S +11.1%S	+3.4%S +7.2%S	+13.2%S +11.3%S	+9.5%S +14.3%S	Hypoxia group increased VO_{2max} significantly more than normoxic group	Volume density of mitochondria and capillary length sign. more increased in H group. Increase in HIF-1α, myoglobin and VEGF mRNA concentrations significant after hypoxic training only. mRNA for oxidative enzymes and HSP70 increased after both training modalities
Bakkman et al. (2007)	Untrained one leg training 4 × 30 min 4 weeks Same relative workload 65% VO_{2max}	N = sea level $n = 8$ H = 526 mmHg ≈ 3000 m $n = 8$	NA NA	NA NA	+34%S +34%S	NA NA		CS increase with normoxic (21%) but not with hypoxic training (+5%); max ADP stimulation rate increased only after N; altitude training may be disadvantageous

conditions. These variables are therefore neither reported nor discussed. Further note that it is virtually impossible to blind subjects to hypoxic conditions, when severe hypoxia is applied during exercise sessions, except for special settings (Truijens et al., 2003). This is an important limitation for the design of hypoxic training studies as both the experimenter and the subjects are aware of the specifics of the experimental conditions. Depending on the design and the expectations of a study, this may have an influence on outcome variables.

As early as 1969, Roskamm et al. (1969) studied three groups of untrained subjects ($n = 6$ each) training on bicycle ergometers 6×30 min for 4 weeks at altitudes of 260, 2250 and 3450 m simulated in a hypobaric chamber. V_{O_2}max increased in all groups, but it increased more in the hypoxic training groups when tested in normoxia or hypoxia. The group training at 3450 m increased V_{O_2}max most when tested at the training altitude. Terrados et al. (1988) trained competitive cyclists in a hypobaric chamber at an altitude of 2300 m and found integrated work capacity significantly more increased after hypobaric training. In a subsequent study (Terrados et al., 1990), using single leg training in untrained subjects, they found a larger time to fatigue, muscle citrate synthase (CS) enzyme activity and myoglobin concentration in the hypoxia-trained leg. Benoit et al. (1992) exposed untrained subjects to training in normobaric hypoxia (5700 m) for 3 weeks and noted an improved maximal exercise capacity and improved hypoxic ventilatory drive in hypoxia-trained subjects. This was seen as an advantageous pre-acclimatization effect. Levine et al. (1992) and Engfred et al. (1994) exposed untrained subjects to 2500 m altitude at the same absolute and relative workload (70% of V_{O_2}max) for 5 weeks. V_{O_2}max was similarly increased by 12% in all groups while hypoxic ventilatory drive was increased in the hypoxia groups only. Hormonal changes with training were similar in all groups. Desplanches et al. (1993) trained untrained subjects for 3 weeks at increasing altitudes up to 5700 m. Hypoxic V_{O_2}max increased significantly only in the hypoxia training group while structural differences (increase in mitochondria) were similar in both groups. Melissa et al. (1997) and Green et al. (1999) trained untrained subjects with unilateral moderate hypoxia training and found that V_{O_2}max and time to fatigue similarly increased with both training modalities. Mitochondrial volume density, fiber size and capillarity were unchanged with both training modalities. The increase in succinate dehydrogenase and phospho-fructo-kinase activities was not significantly different among groups, while CS activity was more increased in the hypoxic training group. Emonson et al. (1997) had untrained subjects train in a hypobaric chamber under conditions equivalent to an altitude of 2500 m. He found that hypobaric hypoxia during endurance training had no synergistic effect on the degree of improvement in sea-level V_{O_2}max or endurance time. Katayama et al. (1999) trained untrained subjects on the ergometer for 2 weeks and found a similar increase in V_{O_2}max. Hypoxic ventilatory response (HVR) decreased significantly in the normoxia group and increased non significantly in the hypoxic group. Bailey et al. (2000a, b) had trained runners do additional training bouts in a double blind approach either in normoxia or normobaric hypoxia at a FiO_2 of 15%. Only hypoxia-trained athletes improved their V_{O_2}max significantly under these conditions. Exposing untrained subjects to cycling training at an inspired FiO_2 of 16% resulted in a significant increase in V_{O_2}max only in subjects training in hypoxia. Hypoxic training was further found to result in additive beneficial effects in selected cardiovascular risk factors. Masuda et al. (2001) trained untrained subjects under hypobaric conditions (2500 m; 60 min every second day for 8 weeks) at the same relative intensity of 70% of V_{O_2}max. He found similar increases in V_{O_2}max in normoxia and hypoxia. Likewise, there was no added benefit of hypoxic training for muscle CS activity or capillarity. Meeuwsen et al. (2001) and Hendriksen and Meeuwsen (2003) published a study and a subsequent cross-over study in which competitive triathletes were exposed to 10 consecutive days of training in hypobaric hypoxia (2500 m) continuously for 120 min at moderate intensity (60–70% of heart rate reserve). The first study showed better improvements with hypoxic than with normoxic training both for aerobic and anaerobic performance variables. The cross-over study showed generally smaller changes with a lesser advantage for hypoxic training. Geiser et al. (2001) and Vogt et al. (2001) had untrained subjects train at normobaric hypoxia (3850 m) or in normoxia (five times 30 min) over 6 weeks. Hypoxic training showed generally greater improvements in V_{O_2}max and P_{max}. Mitochondria and capillaries as well as concentrations of mRNA of oxidative enzymes were increased under both training modalities. mRNA concentrations of HIF-1α, myoglobin and VEGF were increased after hypoxic training only. Truijens et al. (2003) exposed trained swimmers to high-intensity interval training in hypoxia or normoxia added to their normal (normoxic) training program. No additive effect of hypoxic training bouts was found. Ventura et al. (2003) exposed trained cyclists to 6 weeks of three added high-intensity work bouts (three times per week for 30 min) and found no increase in V_{O_2}max or any other performance variable for either training group. The only significant difference was that the hypoxic group increased capillary oxygen saturation when exercising in

hypoxia. The lack of adaptation of functional variables with both training groups was seen as a consequence of training overload. Roels et al. (2005) added two high-intensity interval sessions per week for 7 weeks to the normal training program of competitive cyclists and triathletes. Athletes improved performance – but only during the first 4 weeks of the training intervention, with no significant differences between the training modalities. Morton and Cable (2005) exposed moderately trained team players to 4 weeks of hypoxic training (2750 m, 30 min, three times per week). Training consisted in 10 intervals of 1 min @ 80% V_{O_2}max, followed by 2 min @ 50% V_{O_2}max. Training-induced changes were measured in normoxia. They found no additive effects of hypoxic training. Dufour et al. (2006), Ponsot et al. (2006) and Zoll et al. (2006) exposed competitive runners to a twice-weekly added hypoxic exercise bout for 6 weeks. They found V_{O_2}max and time to exhaustion significantly more increased in athletes who received an additional hypoxic training stimulus. In isolated mitochondria, they demonstrated a tighter coupling of ATP demand and supply. A gene expressional analysis indicated significant increases in a number of genes relevant for oxidative metabolism, CHO metabolism, oxidative stress and pH regulation (see Fig. 1). None of the gene entities analyzed showed significant regulation with added normoxic training. Bakkman et al. (2007) used 4 weeks of unilateral leg exercise in hypobaric hypoxia (3000 m) in untrained subjects and found larger increases of CS and maximal ADP stimulation rates in normoxic but not in hypoxic trained legs. He concluded that it might be disadvantageous to train in hypoxia. Roels et al. (2007) trained untrained subjects for 3 weeks and found minimal functional improvements both in the normoxic and in the hypoxic training group, with no advantage for hypoxic training. By contrast, maximal ADP stimulation rate of mitochondria of the hypoxic training group was increased for some substrate combinations.

Taking a coarse grain view on all studies summarized in Tables 1 and 2, the somewhat deceptive conclusion must be drawn that no clear-cut picture emerges. The hypothesis of a distinct functional phenotype associated with live low–train high must be rejected. However, there are a number of qualifying remarks that one should consider before "throwing out the baby with the bath." It is apparent that the conditions under which hypoxic training were carried out varied considerably among interventions. The total duration of intervention varied between 10 days and 8 weeks, exposure altitude varied between 2300 and 5700 m (both normobaric and hypobaric exposures) and exercise intensity during hypoxic exercise bouts ranged from 50% to 80% of V_{O_2}max. A similar number of studies was carried out with untrained subjects (12) and with trained subjects (9); a few studies used competitive athletes. Moreover, most studies involved whole body work (running or cycling) but certain studies were performed using a unilateral leg exercise model – in which, by necessity, the "normoxic control" leg was also exposed to hypoxia during the hypoxic exercise session of the contralateral leg. From the nine studies on trained subjects listed in Table 1, four studies show some additive functional improvement with hypoxic training while from the twelve studies on untrained subjects reported in Table 2 nine showed some additional benefit of hypoxic training. One single study (Bakkman et al., 2007) reports similar functional results (maximal power output+34% for both training modalities) but shows CS and maximal ADP stimulation rate only to be increased with normoxic training. This is the only study that cautions against the use of hypoxic training as potentially harmful for the training processes.

There is no obvious pattern among the studies reported in Tables 1 and 2 as to the differential effect of the severity of hypoxia or the duration of hypoxia exposure. In untrained subjects, the main stimulus seems to be that of adding exercise to a previously sedentary lifestyle. With regard to the functional results, it looks as if it is of minor importance whether exercise is carried in normoxia or hypoxia. All studies on untrained subjects used exercise training at the same relative intensity in normoxia and hypoxia, except for Terrados et al. (1990); Levine et al., (1992); Engfred et al., (1994). While Terrados showed a larger improvement with exercising at the same absolute workload in hypoxia than in normoxia, Levine and Engfred showed similar results for training in normoxia and hypoxia regardless of whether exercise was carried out at the same relative or absolute workload.

The functional outcome of the studies on trained subjects is also ambiguous. Four of these studies report some functional advantage of added hypoxic training sessions while the remainder of the studies do not find additive advantages for live low–train high. A closer look at the studies with trained subjects indicates that it may be of importance that the added hypoxic training sessions do not overstress the athletes. The added hypoxic training bouts in these studies are therefore only a small fraction of the total (normoxic) training load of the athletes studied. In view of the widely varying protocols used in all studies analyzed in this review, no recommendations can be made as to which altitude or exposure time and exercise intensity should be chosen.

There is a lack of studies that tested subjects under normoxic as well as hypoxic conditions. Only five studies tested performance variables in a hypoxic environment. In view of the fact that certain disciplines have important competitions at altitudes in

excess of 2000 m (i.e. soccer, cross country skiing, mountain biking), this is unfortunate. From these five studies, four reported generally larger gains for some performance variables estimated at altitude for those subjects who trained at altitude. Because of the generally small number of subjects in these studies, not all of these differences reach the level of statistical significance. In one study that failed to show an improvement at altitude after hypoxic training (Ventura et al., 2003), it was noted that the added hypoxia work may have been too stressful for the athletes and that athletes progressed into a state of documented overreaching. Despite the lack of functional improvement with either training paradigm, this study reported a significantly increased oxygen saturation in hypoxia during exercise after hypoxic training. Additional circumstantial support for a potential benefit of hypoxic training for competition at altitude comes from three studies that estimated HVR after hypoxic training (Benoit et al., 1992; Levine et al., 1992; Katayama et al., 1999). All three studies found that HVR positively influenced only after hypoxic training and remained unchanged or decreased after normoxic training.

In sum, then, there is limited evidence for an advantage of hypoxic training for competition at altitude. However, the currently available data are not sufficient to make specific recommendations with regard to any particular training protocol that should be followed. More studies estimating performance variables in hypoxia after hypoxic training are therefore clearly warranted to delineate potential benefits of hypoxic training for competition at altitude.

Mechanisms

The rationale of using normobaric or hypobaric hypoxia during exercise sessions is to increase the "metabolic" stress on skeletal muscle tissue. Hypoxic training protocols carried out with the hope of achieving adaptational results beyond what can be achieved under normoxia; i.e. the intention behind all studies reported in Tables 1 and 2 was to increase the metabolic disturbance and thus potentially the adaptational stimulus in particular on muscle tissue - but also with regard to overall athletic performance. When judging now the effect of hypoxic training on muscle tissue, we have to turn to the nine studies in Tables 1 and 2 that have data on muscle biopsies. We have to keep in mind that any exercise intervention leads to a number of perturbations, both local and global, which act together to achieve a particular adaptational result. We have shown how the four major stressors in exercise, mechanical load, metabolic disturbance, neuronal activation and hormonal changes, act through specific but interlinked signaling pathways to modify gene expression in muscle tissue with exercise (Flück & Hoppeler 2003; Hoppeler et al., 2007). An observed specific training response is never the sole outcome of a single perturbation but represents the integrated pattern of signaling that has occurred over all four major stress pathways. In this context, it is also relevant to note that an adaptational response not only depends on the stressors but also on the condition of subjects before the training intervention. We have shown that the response of the muscle transcriptome to a single training load is attenuated in trained subjects (Schmutz et al., 2006) as well as changed qualitatively (Wittwer et al., 2004). In view of the different training protocols used and the different training background of subjects in the hypoxic training studies discussed in this overview, it is hardly surprising to find such a disparity in outcomes.

Concentrating now on hypoxia, perceived to magnify the metabolic stress in muscle tissue, we note that the transcription factor, HIF-1α, is seen as a key player of hypoxia response in most tissues (Semenza et al., 2006). HIF-1α is stabilized under hypoxic conditions and functions as a master regulator of many genes, notably of erythropoiesis, angiogenesis, pH regulation and glycolysis (Lee et al., 2004). Ameln et al. (2005) presented evidence that exercise activates HIF-1α and downstream function in muscle. Likewise, Lundby et al. (2006) have shown with one-legged exercise in normoxia that HIF-1α and HIF-2α are transiently increased after single bouts of endurance exercise and that this response is blunted after training. These findings were taken to support an active role of of HIF-1α in regulating the gene response to exercise. Vogt et al. (2001) and Zoll et al. (2006) have shown that steady-state mRNA concentrations of HIF-1α were increased after hypoxic but not after normoxic training (Fig. 1). Taken together, this evidence is taken to suggest that the more pronounced transcriptional response of genes to hypoxic training noted by both of these authors is importantly – but not exclusively – related to increased levels of HIF-1α (message) under hypoxic training conditions.

Recent evidence from experiments with muscle-specific HIF-1α knock-out mice is in apparent contrast to the findings of HIF-1α involvement in exercise adaptations in muscle (Mason et al., 2007). These authors find that only wild-type mice adapt to endurance exercise with the classical adaptational features such as an increased mitochondrial and capillary density. HIF-1α knock-outs exhibit spontaneously elevated capillary densities and oxidative enzyme activities presumably caused by a constitutively activated AMP-activated protein kinase. These authors suggest that HIF-1α signaling is therefore not essential for the muscular response to endurance

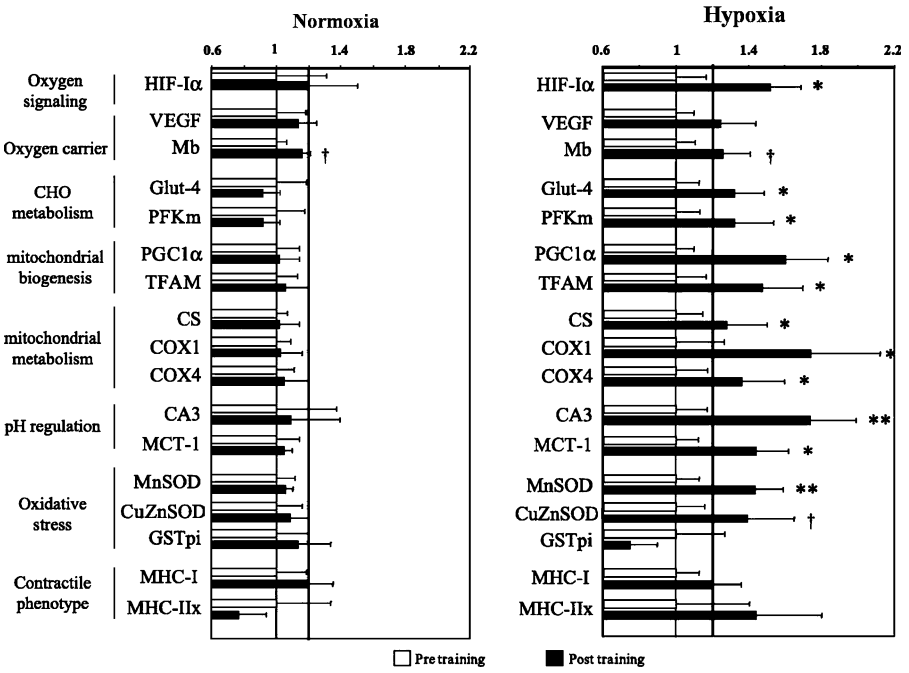

Fig. 1. Changes in steady state levels of gene expression over a 6 weeks training period with added normoxic or hypoxic training in endurance athletes. Values are means ± SE. $*P<0.05$, $**P<0.01$, and $+P<0.10$ vs before training; adapted from Zoll et al. (2006).

training and that high levels of HIF-1α might have a strong negative effect on mitochondrial adaptation. The controversial findings on the role of HIF-1α signaling cannot currently be resolved and need more experimental work. However, the data presented by Mason et al. (2007) need not be in conflict with the more conventional views on the role of HIF-1α in muscle adaptation and may just be related to the time course of HIF-1α expression. Permanent severe hypoxia during sojourn at Everest base camp is well documented to lead to mitochondrial depression and may be due to permanently elevated HIF-1α levels (Hoppeler et al., 1990; Howald et al., 1990; MacDougall et al., 1991). By contrast, the HIF-1α expression of exercise in hypoxia is short lived, with a significant peak of mRNA expression detectable only at 6 h post-exercise (Lundby et al., 2006). This is in contrast to the HIF-1α knock-outs of Mason et al. (2007) that have permanently and completely eliminated HIF-1α signaling and may thus permanently activate the AMPK pathway leading to an "exercise phenotype." The importance of the HIF-1α pathway for adaptions to hypoxia is further supported by experiments involving HIF-1α+/− heterozygotes, which show both impaired and delayed responses when exposed to hypoxia (Yu et al., 1999). A more detailed analysis is therefore needed before final conclusions on the role of HIF-1α in muscle exercise and hypoxia adaptations can be drawn. In future studies, we will also have to consider that there are many more players than HIF-1α that orchestrate oxygen-related adaptational processes in tissues (Chandel & Budinger, 2007).

The complex and comprehensive pattern of gene expressional changes observed by Vogt et al. (2001) and Zoll et al. (2006) (see Fig. 1) induced with supplemental training in hypoxia but not normoxia suggests that muscle tissue specifically responds to hypoxia. Whether and to what degree these gene expressional changes are translated into significant changes of protein concentrations ultimately responsible for observable structural or functional phenotypes remains open. It is quite conceivable that the global functional parameters V_{O_2}max and P_{max} are simply markers too coarse to detect more subtle changes that might still be functionally relevant to high-level athletes.

Conclusions

Looking at the global functional outcome of all studies, it can be stated that a functional benefit for competition at sea level cannot be expected with a training intervention using hypoxic training as the sole training modality (in untrained subjects) or using hypoxic training as an adjunct to normoxic training in athletes. However, it must also be said that there is only one out of 20 studies that cautions against the use of hypoxic training. Using hypoxia in athletes should therefore not be encouraged – but should not actively be discouraged either. It looks as if under some conditions, certain athletes can profit from hypoxic training bouts. The final decision on whether to use hypoxia as a training aid should therefore be left with the trainer and athlete depending on individual preference and availability of the necessary training equipment or environment. The different type of stress that muscle tissue experiences when working in hypoxia as documented by structural, biochemical and molecular studies may be an option to vary training stress in athletes with a long training history.

Only six studies measured functional performance variables under hypoxic conditions. Again the results are not clear cut. There is a tendency for functional differences to be larger for the hypoxically trained subjects when tested under hypoxic conditions. It is also of note that all three studies that tested the HVR show that HVR increased in the hypoxic training group only. One could therefore tentatively conclude that athletes who prepare for competition at altitude, not having access to altitude in preparation of the competition (acclimatization), may profit from hypoxic training sessions. This position is adopted in view of the fact that hypoxic training under these conditions does not seem to be detrimental, provided care is taken that athletes are not overloaded. Additional research into live low–train high should therefore be designed such that performance estimates are also obtained under hypoxic conditions.

Recommendations

Live low–train high has not been shown to be superior to training in normoxia for competition at sea level. It is left to trainers and athletes whether the additional effort, expenses and the potential risk of overtraining warrants considering hypoxic training as an option to vary training stress in athletes with a long-term training history.

There is some evidence mainly from untrained subjects that hypoxic training may be of advantage for competition at altitude. Hypoxic training may be considered when other options of acclimatization to altitude are not available. However, based on the limited evidence with athletes live low–train high cannot be recommended.

The available data are insufficient to recommend specific training protocols in case hypoxic training is considered as a training adjunct in athletes.

Key words: hypoxia, normoxia, VO_{2max}, molecular mechanism, HIF1-α.

Conflicts of interest: The authors have declared that they have no conflict of interest.

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Mechano-regulated Tenascin-C orchestrates muscle repair

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Tenascin-C (TNC) is a mechano-regulated, morphogenic, extracellular matrix protein that is associated with tissue remodeling. The physiological role of TNC remains unclear because transgenic mice engineered for a TNC deficiency, via a defect in TNC secretion, show no major pathologies. We hypothesized that TNC-deficient mice would demonstrate defects in the repair of damaged leg muscles, which would be of functional significance because this tissue is subjected to frequent cycles of mechanical damage and regeneration. TNC-deficient mice demonstrated a blunted expression of the large TNC isoform and a selective atrophy of fast-muscle fibers associated with a defective, fast myogenic expression response to a damaging mechanical challenge. Transcript profiling mapped a set of de-adhesion, angiogenesis, and wound healing regulators as TNC expression targets in striated muscle. Expression of these regulators correlated with the residual expression of a damage-related 200-kDa protein, which resembled the small TNC isoform. Somatic knockin of TNC in fast-muscle fibers confirmed the activation of a complex expression program of interstitial and slow myofiber repair by myofiber-derived TNC. The results presented here show that a TNC-orchestrated molecular pathway integrates muscle repair into the load-dependent control of the striated muscle phenotype.

damage | expression | extracellular | gene therapy | myogenesis

Tenascin-C (TNC) is an extracellular matrix protein that assembles from differently spliced isoforms (1, 2). TNC is expressed only in actively remodeling musculoskeletal tissue, subject to high mechanical stress (3). This expression is load-dependent and reversible (4–6). Microdamage may contribute to the mechano-regulation of TNC expression (6).

TNC exerts a strong anabolic and proliferative effect on interstitial and myogenic cells in culture (1, 7–10). This is mediated by TNC's de-adhesive property that relieves the growth inhibition of substrate attachment (8). The transition to an intermediate adhesive state may facilitate the expression of genes specific for tissue repair and adaptation (8). This view is supported by the *de novo* accumulation of TNC in muscle connective tissue after damaging muscle loading and the correlation of ectopic TNC protein with the growth-related gene expression during muscle fiber regeneration (5, 11). These observations suggest that TNC-mediated de-adhesion contributes to cell repair in mechano-sensitive tissues.

The functional role of TNC in tissue morphogenesis remains unclear, mainly because transgenic mice engineered for TNC deficiency show only subtle phenotypic defects (10, 12). The pathologies in transgenic mouse lines include reduced neovascularization and cell migration in injured muscle tissue and mechanically stressed corneal wounds (1, 7, 8, 10, 13, 14). The aberrations in TNC-deficient mice could be somewhat masked by the permissive expression of an abnormal TNC variant (15). This ambiguity may relate to the transgenic strategy of abolishing the production of extracellular TNC protein by disrupting the N-terminal signal sequence for protein export (12, 16). This genetic manipulation may leave downstream translation initiation sites intact for the production of shortened TNC variants (15, 16). Proteins can exit the cytoplasm of cells residing in mechanically stressed tissues by diffusion after plasma membrane disruption (17). The implications of such a mechanism for TNC in tissue repair and the minor phenotype of transgenic mice with deficient TNC secretion are not understood.

We have adopted a multilevel approach that monitors damage-related changes in muscles of both TNC-deficient and TNC knockin mice. Leg muscles are suitable for this approach because they are amenable to physiological modulation of their mechanical activity (18), and they are accessible to somatic transgenesis (19). The pathways of TNC action were identified by monitoring transcript expression of muscle-relevant gene ontologies (GOs) in antigravitational muscle. We focused on deregulated gene expression reflected in the differences between the mechano-responsiveness of transcript levels in the soleus muscle in WT and transgenic littermates (18, 20), bearing in mind the possible production of an aberrant TNC variant with muscle damage. The control of selected TNC-dependent gene products was verified ad hoc with muscle fiber-targeted somatic knockin experiments.

Results

TNC Isoform Expression Distinguishes Muscle from Noncontractile Tissues. In WT mice, leg muscles variably expressed the small 200-kDa TNC isoform (Fig. 1*A*), whereas in lung, brain, and skin, the large 250-kDa TNC predominated (Fig. 1*B*). TNC expression was blunted in the noncontractile tissues of transgenic littermates. However, a 200-kDa TNC-immunoreactive band remained detectable at a 10-fold lower level in the muscle tissue of TNC-deficient mice (Fig. 1*B*).

TNC-Deficient Mice Demonstrate Fast-Muscle Fiber Atrophy. One-year-old, TNC-deficient mice demonstrated reduced mass of the pure fast-type muscles, tibialis anterior and extensor digitorum longus (Fig. 2*A*). At this age, no genotypic difference was seen in the mixed slow/fast musculi soleus. Quantitative microscopic analysis demonstrated a selective reduction of mean cross-sectional area (CSA) for fast-type muscle fibers in the extensor digitorum longus and soleus muscles of TNC-deficient mice (Fig. 2*B*). The musculi solei of TNC-deficient mice showed a significant slowing of muscle contractions (Table 1).

Author contributions: M.F. designed research; M.F., S.K., A.-C.D., and M.-N.G. performed research; S.I.M., J.C.S., and M.-N.G. contributed new reagents/analytic tools; M.F. analyzed data; and M.F. and M.-N.G. wrote the paper.

Conflict of interest statement: We report a potential conflict of interest related to the preparation of a provisional patent application.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession nos. GSE8549, GSE8550, GSE8551, and GSE8552).

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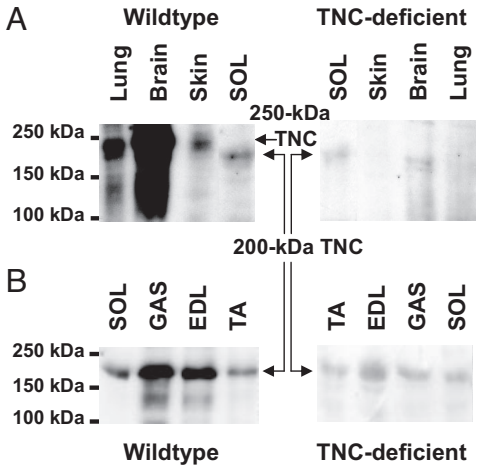

Fig. 1. Preserved small TNC isoform expression in TNC-deficient mice. (*A*) TNC expression in noncontractile tissues. (*B*) Different leg muscles of WT and TNC-deficient mice. Arrows indicate the large 250-kDa and the small 200-kDa TNC isoforms, detected by monoclonal rat antibody mTN12 against mouse TNC. SOL, musculi soleus; GAS, musculi gastrocnemius; EDL, musculi extensor digitorum longus; TA, musculi tibialis anterior.

Atrophy of fast soleus muscle fibers in TNC-deficient mice was progressive and became evident at the whole muscle level after two years of age (Fig. 2 *A* and *B*).

Transcript Adjustments with TNC Deficiency. The contribution of expressional reprogramming to fast-fiber atrophy in the soleus muscle was evaluated. Transcript profiling of muscle-relevant factors identified general up-regulated mRNA levels in musculi solei of one-year-old, TNC-deficient cage controls ($P = 4 \times 10^{-15}$). The major theme was the up-regulation of transcripts for GOs associated with the myofiber compartment, adhesion, and angiogenesis [Table 2 and supporting information (SI) Table S1], including factors associated with slow fibers. At two years of age, a majority of genotypic differences in muscle mRNAs were preserved except for those associated with myofibers.

TNC-Related, Mechano-Responsiveness of Muscle Gene Expression. The soleus muscles of TNC-deficient and WT mice were mechanically challenged by reloading after 7 d of deconditioning by hind limb suspension. The mechanical stimulus selectively induced damage of soleus muscle fibers in the TNC-deficient animals (Fig. 3*A*).

In the 1 d reloading response of one-year-old mice, 155 transcripts showed a significant TNC genotype dependency (Table S2). Multicorrelation testing identified two main clusters of coregulated mRNA levels (Fig. 3*B*). Within the cluster of coincidentally up-regulated RNAs, discrete GOs assigned to de-adhesion, angiogenesis, and wound healing were enriched (Table S3). Conversely, factors associated with myofibers were concentrated in the cluster of down-regulated RNAs. The main exceptions were three up-regulated myogenic regulators, myogenin (myoG), serum response factor (SRF), and myocyte enhancer factor-1 (MEF2A).

The comparison with cage controls revealed that reloading inverted the transcript expression ratios between genotypes ($P = 1 \times 10^{-12}$) (Table S3) except for GOs relating to myofibers. This "mirror effect" correlated with the expression of TNC mRNA, which was selectively elevated in musculi solei of TNC-deficient mice after reloading (mean $r^2 = 0.92$) (Fig. 3*C*).

Table 1. Genotype effect on contraction in soleus muscle

Parameter	WT	TNC-deficient
Force single twitch, mN	0.8 ± 0.4 (6)	0.9 ± 0.2 (6)
Tetanic force, mN	11.4 ± 4.2 (3)	9.2 ± 1.7 (6)
Time-to-peak, ms	31.8 ± 3.7 (4)	36.4 ± 1.9 (7)*
Contraction duration, ms	24.7 ± 2.8 (5)	29.2 ± 1.6 (7)*
Half relaxation time, ms	20.3 ± 0.5 (4)	31.8 ± 3.6 (6)*
Fatigue, s	57.3 ± 12.9 (3)	52.0 ± 5.7 (5)
Tibia length, mm	19.8 ± 0.6 (4)	20.7 ± 0.3 (7)*

Mean ± SE of contractile parameters in musculi solei of one-year-old littermates. Numbers in parentheses indicate the biological replicates. *, $P < 0.01$ between genotypes. ANOVA with honestly significant difference posthoc test.

Proof-of-Concept for the Myocellular TNC-Signaling Pathway. Muscle fiber-targeted overexpression of the chicken TNC homologue (chTNC) in TNC-deficient mice was carried out to validate TNC-mediated expression control at the protein level. The pure fast-type muscle tibialis anterior was studied. We looked for key regulatory factors with deregulated transcript expression in TNC-deficient mice relative to fast-type muscle fibers. The master regulators of myogenesis in slow- and fast-muscle fibers, myoG, and myogenic differentiation 1 (myoD) (21), and the proliferation regulator cyclin A (22) met these criteria (Fig. 3*C*).

The exogenously introduced 190-kDa chTNC was exclusively overexpressed in the right tibialis anterior muscle after transfection with a constitutively active expression plasmid but was not overexpressed in empty vector transfected left controls. Expression was maximal 2 d after transfection and maintained for 1 week (Fig. 4 *A* and *B*). Quantitative immunoblotting of muscle pairs identified a transient increase of cyclin A and myoG protein levels at 2 d (but not 1 d) after knockin (Fig. 4 *C* and *E*). MyoD protein levels were not significantly affected by TNC overexpression (Fig. 4*D*).

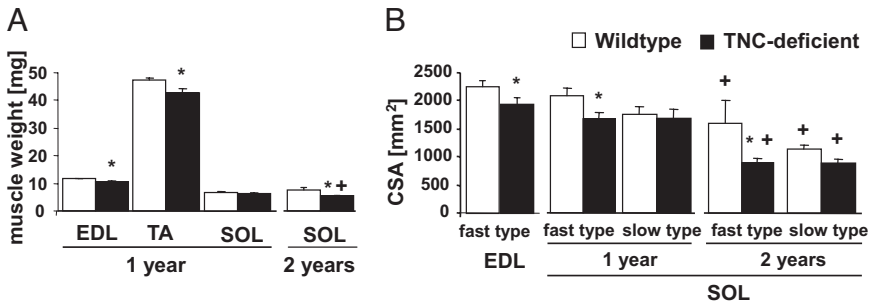

Fig. 2. Fast-fiber atrophy in TNC-deficient mice. Mean ± SE of the mass (*A*) and CSA (*B*) of fiber types in fast (EDL, TA) and mixed slow/fast muscle (SOL) of WT and TNC-deficient mice at 1 and 2 years of age. *, $P < 0.05$ between genotypes of same age; +, $P < 0.05$ vs. same genotype at 1 year of age [two-factor ANOVA (genotype × age) with the posthoc test of Fisher].

Table 2. Shifted transcript expression with TNC deficiency

Gene ontology	Gene counts	*P*
Novel TNC targets	45	<0.001
Adhesion	12	<0.001
Angiogenesis	14	<0.001
Proliferation	12	<0.001
Myofiber-associated	23	<0.001
ECM–sarcomere axes	5	<0.01
Myogenesis	9	<0.01

Enriched GOs which demonstrated unidirectional increases in transcript levels between soleus muscles of one-year-old TNC-deficient vs. WT littermates.

Induced Expression of the Small 200-kDa TNC-Related Protein in Injured Muscle. Expression of the 200-kDa TNC-immunoreactive protein (Figs. 4*A* and 5*A*) and muscle damage (data not shown) were readily detectable on both muscle sides of electrotransfected musculi tibialis anterior in TNC-deficient mice. In WT mice, a 3-fold up-regulation of both the small and the large TNC isoform was evident after electrotransfer (Fig. 5*B*). Expression of the small TNC-immunoreactive protein was selectively induced at the periphery of ≈10% of soleus muscle fibers in TNC-deficient mice after reloading (Fig. 5*C* and Fig. S1).

Discussion

The role of TNC in regenerative processes has been a riddle because transgenic mice with targeted ablation of TNC secretion were found to have no obvious phenotype (10, 12, 23). Our findings shed light on this matter. We find abnormal myogenesis and atrophy of fast-differentiated myofibers of locomotor muscles in the original TNC-deficient mouse strain of Faessler *et al.* (12). This pathology was related to the blunted expression of the large TNC isoform in TNC-deficient mice and to the unexpected expression of a TNC-related protein upon muscle fiber damage.

Assumption Bias. Our experiments led us to suspect the possible production of an atypical TNC protein in the transgenic mouse line under study (12, 15). Doubt arose with immunoblotting experiments that demonstrated induced expression of a 200-kDa protein with muscle reloading and electropulsing of two different leg muscles (Fig. 5). Based on its antigenicity and size, this protein was indistinguishable from the small TNC isoform. In TNC-deficient mice, functional similarity of this protein to the small TNC isoform is suggested by the inversion of genotype differences of transcript expression in cage controls after reloading when expression of the 200-kDa TNC protein was elevated (Figs. 3*B* and 5*C*). This notion is corroborated by the corresponding selective enhancement of TNC mRNA levels in TNC-deficient mice with reloading (Fig. 3*C*). Western blot experiments excluded any contribution from the related, and similarly sized, Tenascin-W isoform (Fig. S2).

We reasoned that the expression of a TNC-related protein in muscle tissue is the consequence of an alternative in-frame start codon in the modified TNC gene sequence and protein release from damaged cells via a secretion-independent mechanism (17). DNA sequencing of the modified TNC gene identified an in-frame start codon shortly after the ablated signal peptide. The context of this start codon meets the consensus requirements for translation initiation (Fig. S3). The resulting protein would not be easily distinguishable from the processed muscle-specific 200-kDa TNC isoform (Figs. 1*A* and 5*B*) because the anticipated cleavage site of the TNC signal peptide during secretion is only a few amino acids away from the alternative start codon (Fig. S3). Damage of the sarcolemma, with reloading of deconditioned soleus muscle and/or electropulsing (20, 24), would allow the release of a TNC variant from muscle fibers. This conclusion is compatible with the observation that the expression of the large 250-kDa TNC isoform, which relies on active secretion from interstitial cells (2), is blunted in leg muscles of the transgenic line (Fig. 5 *A* and *B*).

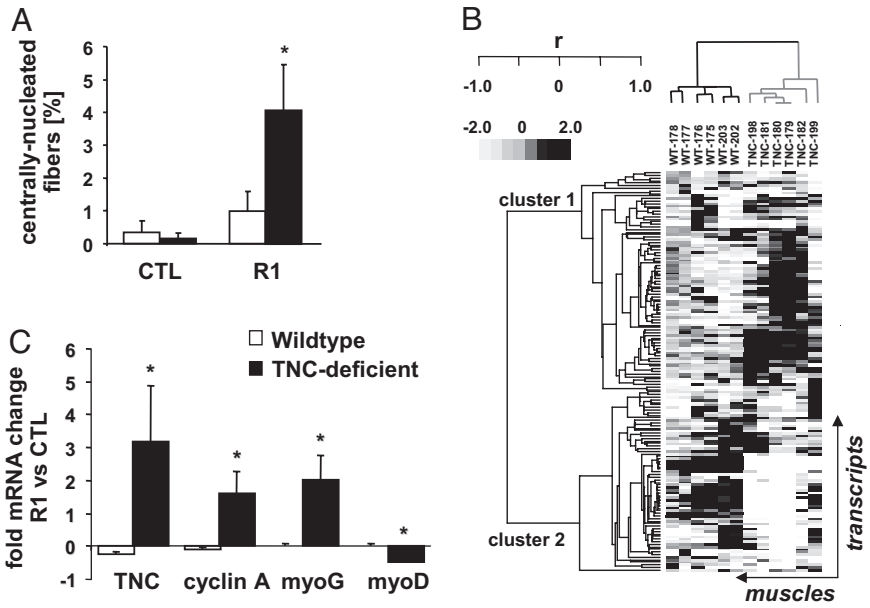

Fig. 3. Genotype differences in the reloading response. (*A*) Mean + SE percentage of damaged soleus muscle fibers in cage control (CTL) and 1 d reloaded (R1) WT and TNC-deficient mice. *, $P < 0.05$ vs. cage controls by using two-factor ANOVA (genotype × age) with the posthoc test of Fisher. (*B*) Centered hierarchical cluster analysis visualizing the global reloading response of the 155 altered gene transcripts in soleus muscle between the two genotypes. The expression ratio (R1 vs. CTL) of muscle transcripts in each animal is visualized by a gradient between black (up-regulated) and white color (down-regulated). (*Top*) Codes of the individual WT and TNC-deficient mice. (*Bottom*) Two clusters with coregulated up- and down-regulation of transcripts are identified. (*C*) Mean ± SE of reloading-induced TNC, cyclin A, myoG, and myoD mRNA level alterations in the soleus muscle of WT and TNC-deficient mice. *, $P < 0.05$ vs. WT by using SAM software.

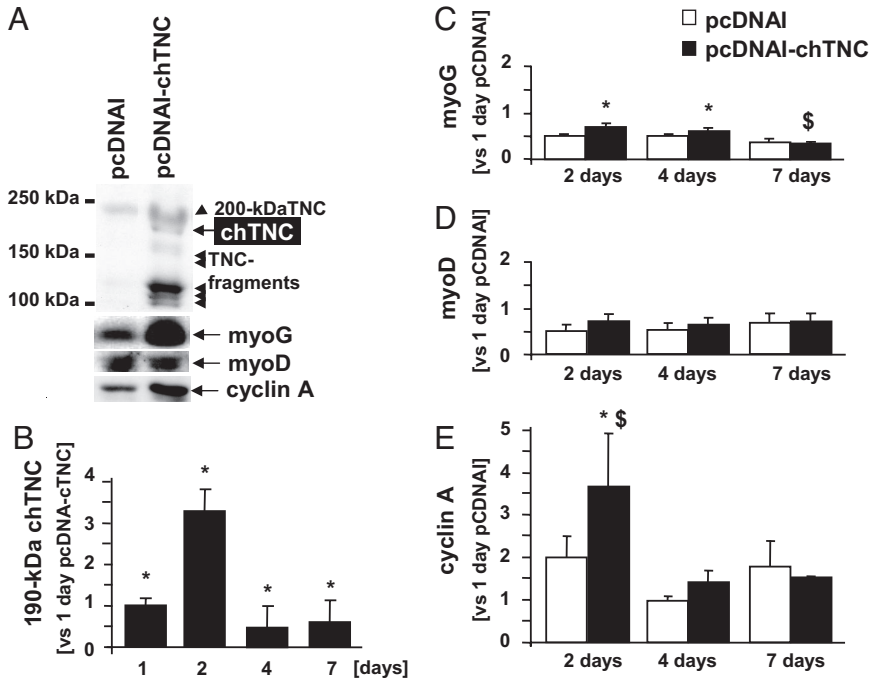

Fig. 4. Proof-of-concept on TNC-dependent expression control. Time course of protein level adjustments of selected TNC targets in TA muscle of TNC-deficient mice after TNC knockin. Right muscles were transfected with expression plasmid for chicken TNC (pcDNAI-chTNC). Left muscles were transfected with empty vector pcDNAI as intra-animal controls. (*A*) Representative immunoblots after 2 d of overexpression. (*Top*) 190-kDa chTNC (arrow) and chTNC fragments (arrowhead) detected with monoclonal antibody Tn20. The mouse 200-kDa TNC isoform (tilted arrowhead) is detected as well. (*Bottom*) MyoG, myoD, and cyclin A protein. (*B–E*) Mean ± SE of protein levels of 190-kDa chTNC (*B*), myoG (*C*), myoD (*D*), and cyclin A protein (*E*) in the transfected muscles. *, $P < 0.05$ vs. empty plasmid transfected left muscle; $, $P < 0.05$ vs. 1 d transfection with same plasmid (paired Wilcoxon test, one-tailed).

TNC-Dependent Muscle Phenotype. Our multilevel approach identified a discrete shift of transcript expression in the mixed soleus muscle of TNC-deficient mice toward the characteristics of slow fibers (Table 2 and Table S1). This was accompanied by correspondingly reduced fast-fiber volume and slowed contraction (Table 1). We also noted that the reduction in fast-fiber, CSA in the belly portion of the soleus muscle was not matched by the differences in muscle mass in one-year-old TNC-deficient mice (Fig. 2). Together with the observation on the elongated tibial bone, which defines soleus muscle length (Table 1), this unmatched reduction indicates a complex role for TNC in determining the architecture of the musculoskeletal system. Until now, this complex role has been overlooked (23).

Mapping of TNC-Expression Targets. The marked TNC genotype-specific up-regulation of gene messages on reloading of the soleus muscle identified a series of targets for TNC signaling in skeletal muscle (Table S3). The concurrent up-regulation of mRNAs for wound healing, de-adhesion, and angiogenesis, along with the regulators of myogenesis (myoG, SRF, and MEF2A), provided direct evidence for regulation of both interstitial and myogenic processes by TNC. The molecular analysis of muscle fiber-targeted overexpression of the chTNC confirmed this association. It identified the TNC-modulated up-regulation of the master regulator of slow-type myogenesis, myoG, and the regulator of interstitial cell proliferation, cyclin A (Fig. 4 *C* and *E*) (21, 22). This regulation opposes the down-regulation of the governor of fast-muscle gene expression, myoD (21).

Damage-Induced Coordination of Myocellular and Interstitial Repair via TNC Isoforms. Muscle loading induces a pleiotropic response (25). The functional implications of this complex process are largely unknown. Our results imply that a damage-inducible TNC pathway coordinates the myocellular and interstitial response to mechanical fiber damage. The observations connect the regulation of small and large TNC isoforms to the differential control of slow- and fast-type myogenesis and cell proliferation. The up-regulation of the small TNC-related protein after muscle fiber damage (by reloading and somatic transgenesis) relates to the promotion of the slow myogenic program via myoG- and cyclin A-activated cell proliferation. Conversely, the production of the putative secreted large TNC isoform (which is absent in TNC-deficient mice) is associated with enhanced transcript expression of the fast-type myogenic factor, myoD. These observations are compatible with the idea that a blunted, fast myogenic program explains the deterioration of the fast-type characteristics of leg muscles in TNC-deficient mice.

Mechanically Induced TNC Production in Muscle and Repair. Expression of the de-adhesive TNC protein is believed to be a requirement for repair of mechanically stressed cells. De-adhesion allows relief from strain (3, 8). We observed that TNC-dependent RNA control factors are involved in de-adhesion, myogenesis, and wound healing after the mechanical challenge of reloading. This observation indicates that de-adhesion occurs in striated muscle tissue (Table S3). The TNC-modulated control of major regulators of cell proliferation and myofiber differentiation, myoG and cyclin A, provides important insight. The observed time course of TNC-promoted up-regulation (Fig. 4 *C* and *E*) mirrors the retarded cell recruitment after myocardial injury in TNC-deficient mice (13). This indicates that damage-induced TNC production governs the pace of muscle fiber repair by modulating interstitial and myogenic cell activation.

Cycles of microdamage and repair may contribute to the basal muscle turnover of skeletal muscles (26). Our observations imply a role of load-regulated TNC up-regulation in this damage-repair cycle. This is indicated by the increased susceptibility of muscle to mechanical damage in TNC-deficient mice (Fig. 3*A*)

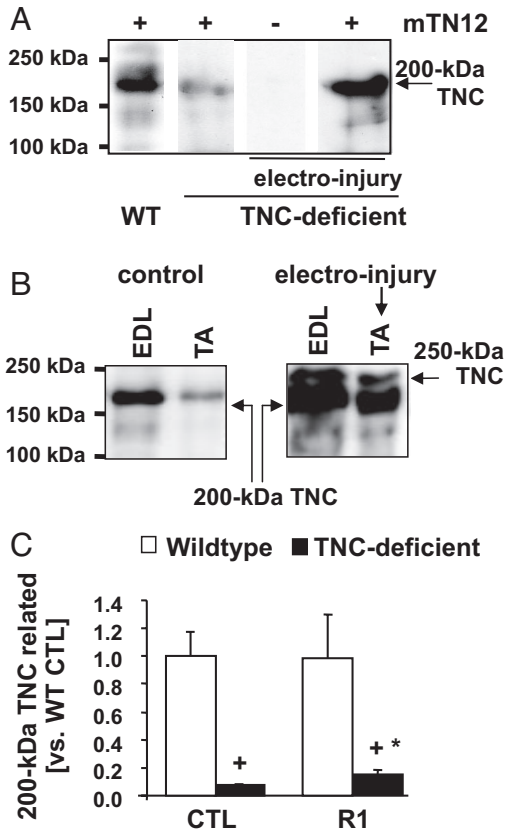

Fig. 5. TNC-related protein expression in TNC-deficient mice after muscle damage. (*A* and *B*) Immunoblots visualizing expression of the 200-kDa and 250-kDa TNC isoforms (*A*) in muscle as a function of empty vector electrotransfer in TNC-deficient and WT mice (*B*). A negative control omitting the first antibody mTN12 and a 10-fold underexposed positive reaction with homogenate from a WT muscle are included in *A*. (*C*) Mean ± SE of 200-kDa TNC levels in SOL muscle of control WT, reloaded WT, and TNC-deficient mice. +, $P < 0.05$ vs. WT; *, $P < 0.05$ vs. CTL for same the genotype (two-factor ANOVA with posthoc test of Fisher).

and the atrophy of fast fiber during the mouse lifespan (Fig. 2). Interestingly, fast-type muscle fibers show preferential vulnerability to reloading damage in rodents (27) and ectopic TNC staining with atrophy and age-induced atrophy in humans (sarcopenia) (28). These arguments point to deregulated TNC expression as a possible cofactor for the etiology of sarcopenia in humans.

Conclusions. TNC is part of a pleiotropic pathway that protects fast-muscle fiber mass from the deleterious consequences of mechanically induced microdamage. This insight into the biomechanical control of the muscle phenotype is relevant for reducing or healing musculoskeletal injuries.

Materials and Methods

Bio-Reagents. Endotoxin-free plasmids for the CMV-driven expression of the 190-kDa chicken TNC isoform, pcDNAI-chTNC (9), and empty vector (pcDNAI), were isolated according to industrial standards at Plasmidfactory (Bielefeld, Germany). Established monoclonal antibodies from rat (mTN12) and mouse hybridomas (TN20) were used to detect TNC, myosin heavy chain, and myoG (11, 29). Commercial antibodies were deployed to detect the other proteins including cyclin A (BD Transduction Laboratories), myoD, and myoG (Santa Cruz Biotechnology). HRP-coupled secondary antibodies were from Sigma-Aldrich and ICN.

Animals. Male TNC-deficient mice of the 129/SV strain, with the targeted insertion of a β-lactamase cassette in the *Nco*I site of exon 2 of the TNC gene (12), were used for the study. Animals were derived from the original strain and back crossed with WT 129/SV mice. Genotype was determined with PCR on tail DNA (7). For details see Fig. S1.

Cage Controls and Reloading of the Soleus Muscle. WT and TNC-deficient mice were acclimatized to housing in single cages for 1 week before they were assigned to the reloading group (R1) or the cage control group (CTL). Hind limb muscles were deconditioned for 7 d by unloading, subsequent reloading, and harvesting of muscle pairs (18). Unloading reproduced the reported reduction in whole-body mass and soleus muscle mass. All procedures were approved by the Animal Protection Commission of the Canton Bern, Switzerland.

Aging. Animals were housed in standard cages in cohorts with regular chow and water ad libitum. One week before the experiments, the mice were acclimatized to single cages.

Muscle-Targeted TNC Knock-In. Overexpression experiments with the CMV-driven plasmid were carried out in a paired design: empty pcDNAI plasmid was electrotransferred into the left tibialis anterior muscle of TNC-deficient mice, and pcDNAI-chTNC was transfected into the contralateral right muscle. Electrotransfer was carried out with modifications as described (19). In brief, 30 μg of plasmid in 30 μl of 0.9% NaCl was injected into the central portion of the muscle and electropulses (3 trains of 100 pulses of 100 μs each, at 50 mA) were delivered with needle electrodes by using a GET42 electropulser (Electronique Informatique du Pilat). Transfected muscle portions were collected after 1, 2, 4, and 7 d (18). In additional experiments, tibialis anterior and extensor digitorum longus muscles were harvested 7 d after transfection with empty plasmid.

Muscle Fiber Structure. Composition and mean CSA of slow- and fast-type muscle fibers and muscle fiber damage were determined with standard morphometry on hematoxylin-stained cross-sections from the muscle belly portion after immunostaining for fast- and slow-type myosin heavy chains (11, 18). On average, 224 muscle fibers were counted per section.

***In Situ* Testing of Muscle Contractility.** Contractile characteristics of isolated soleus muscles were determined by using the method of Andrade *et al.* (30) with modifications, by using a muscle tester operated by a PowerLab system (ADInstruments). Single twitch and maximal tetanic contractions were evoked at optimal length by stimulation at 1 Hz for 0.4 ms and 60 Hz for 4 s, respectively, with 10 V from an Ion Optix Myopacer (IonOptix). Fatigue was determined from a drop below 50% of the original force of repeated tetanic contractions every 4 s.

Transcript Profiling. Microarray experiments were carried out on total RNA by using a validated, custom-designed ATLAS™ cDNA nylon filter holding cDNA probes for 222 muscle-relevant mRNAs (18). The curation of transcripts to a GO was based on the information available through the electronic literature (http://www.expasy.org/sprot/ and http://www.ncbi.nlm.nih.gov/sites/entrez). Data sets and platform design were deposited under accession codes GSE8551, GSE8549, GSE8550, and GSE8552 at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo).

Normalized microarray data were analyzed for differentially expressed RNAs by using Significance Analysis of Microarrays (SAM) software (18). Genotype differences in the mechano-responsiveness (the reloading response) of transcript expression were evaluated by using the R1 vs. CTL ratio of significantly affected transcripts after centering to the mean of cage controls. The global pattern of the reloading response was visualized with hierarchical cluster analysis of median-centered R1 vs. CTL ratios (11). Global themes of coregulation were assessed by a sign test verifying the enrichment of codirectional transcript level alterations in a given GO between genotypes and/or with reloading (Microsoft Excel). Deregulated transcripts were identified from a shifted or inverted reloading response (R1 vs. CTL) between TNC-deficient and WT mice compared with cage controls.

Immunoblotting. Sample preparation, SDS/PAGE, and quantitative immunoblotting was carried out as described (5), except that ultra-sensitive ECL was used (Supersignal-Femto, Pierce).

Statistics. Individual data were assembled in Microsoft Excel. Probability-based statistical tests were carried out with Statistica (StatSoft). Statistical significance was assumed at $P < 0.05$. Trends were assumed at $0.05 \leq P < 0.10$.

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Focal adhesion kinase is a load-dependent governor of the slow contractile and oxidative muscle phenotype

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Striated muscle exhibits a pronounced structural–functional plasticity in response to chronic alterations in loading. We assessed the implication of focal adhesion kinase (FAK) signalling in mechano-regulated differentiation of slow-oxidative muscle. Load-dependent consequences of FAK signal modulation were identified using a multi-level approach after electrotransfer of rat soleus muscle with FAK-expression plasmid *vs.* empty plasmid-transfected contralateral controls. Muscle fibre-targeted over-expression of FAK in anti-gravitational muscle for 9 days up-regulated transcript levels of gene ontologies underpinning mitochondrial metabolism and contraction in the transfected belly portion. Concomitantly, mRNA expression of the major fast-type myosin heavy chain (MHC) isoform, MHC2A, was reduced. The promotion of the slow-oxidative expression programme by FAK was abolished after co-expression of the FAK inhibitor FAK-related non-kinase (FRNK). Elevated protein content of MHC1 (+9%) and proteins of mitochondrial respiration (+165–610%) with FAK overexpression demonstrated the translation of transcript differentiation in targeted muscle fibres towards a slow-oxidative muscle phenotype. Coincidentally MHC2A protein was reduced by 50% due to protection of muscle from de-differentiation with electrotransfer. Fibre cross section in FAK-transfected muscle was elevated by 6%. The FAK-modulated muscle transcriptome was load-dependent and regulated in correspondence to tyrosine 397 phosphorylation of FAK. In the context of overload, the FAK-induced gene expression became manifest at the level of contraction by a slow transformation and the re-establishment of normal muscle force from the lowered levels with transfection. These results highlight the analytic power of a systematic somatic transgene approach by mapping a role of FAK in the dominant mechano-regulation of muscular motor performance via control of gene expression.

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Abbreviations CSA, cross-sectional area; FAK, focal adhesion kinase; FRNK, FAK-related non-kinase; MHC, myosin heavy chain; GO, gene ontology; SAM, statistical analysis for microarrays.

Striated muscle exhibits a pronounced phenotypic plasticity in response to work-related stimuli (Loughna *et al.* 1990; Pette & Staron, 1990; Booth & Thomason, 1991; Fluck & Hoppeler, 2003). This malleability is exemplified by specific adjustments of muscle force *versus* fatigue resistance subsequent to strength as opposed to endurance training. Muscle conditioning by functional demand is dramatically visualized in the prolonged reductions in weight-bearing during bed-rest, when the force and metabolic capacity in anti-gravitational muscles are reduced (Desplanches *et al.* 1987; Fluck & Hoppeler, 2003). These deteriorations are reversible and the muscles recover with subsequent elevations in muscle loading and activity-induced energy consumption (Desplanches *et al.* 1987).

Muscle plasticity has its foundation in the capacity of the individual muscle cells, the muscle fibres, to remodel their contractile and metabolic makeup in response to neuronal, mechanical, metabolic and hormonal stimuli (Loughna *et al.* 1990; Pette & Staron 1990; Booth & Thomason, 1991;

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Fluck & Hoppeler, 2003; Dapp *et al.* 2006). Accordingly, the transformation of muscle fibres underlies the shift from a slow-oxidative to an atrophic, fast-glycolytic phenotype with unloading, and its reversion upon muscle loading (Desplanches *et al.* 1987; Fluck & Hoppeler, 2003).

Distinct signalling pathways have been implied in the control of muscle form and function (Chin *et al.* 1998; Pallafacchina *et al.* 2002; Wu *et al.* 2002; Puigserver & Spiegelman, 2003; Koulmann & Bigard, 2006; Sandri, 2008). The consensus is that AKT–mTOR–S6K signalling and calcium/calmodulin signalling separately integrate the hormone- and nerve-dependent control of muscle size and contractile proteins, respectively. In regards to metabolic perturbations, signalling pathways connecting the transcriptional regulators peroxisome proliferator-activated receptor γ coactivator (PGC)-1α and hypoxia-inducible factor (HIF)-1α appear to be central for the molecular regulation of muscle metabolism via the control of gene regulation (Dapp *et al.* 2006; Koulmann & Bigard 2006; Sandri, 2008). Currently, there is a distinct lack of understanding about the implication of signalling mechanisms in the conditioning of muscle structure and function by mechanical stimuli. Concerted control of gene ontology (GO) expression, which instructs the contractile and metabolic adjustments of muscle function with unloading and reloading (Stevenson *et al.* 2003; Fluck *et al.* 2005), implies the existence of a load-dependent master pathway. The upstream elements of this mechano-sensory pathway for muscle transcript differentiation, and the downstream consequences for contractile and metabolic protein expression and muscle function, are not known.

Sarcolemmal focal adhesion complexes (costameres) are key elements for the transmission of contraction force from muscle fibres to tendons and adjacent fibres (reviewed in Huijing 1999; Fluck *et al.* 2002; Bloch & Gonzalez-Serratos, 2003; Samarel, 2005; Quach & Rando, 2006; Durieux *et al.* 2007) and constitute potential sites for the conversion of contraction forces into mechano-sensitive signalling within the myocellular compartment (Huijing 1999; Bloch & Gonzalez-Serratos, 2003; Fonseca *et al.* 2005; Samarel, 2005; Quach & Rando, 2006). This idea is supported by the load-dependent post-translational regulation of the associated protein tyrosine kinase, focal adhesion kinase (FAK). Tyrosine phosphorylation of integrin-bound FAK at residue 397 (pY397) reflects the mechano-chemical coupling between mechanical stimulation of integrins and activation of intracellular signal transduction (Shyy & Chien, 1997; Parsons, 2003). Sarcolemmal FAK concentration corresponds to the degree of muscle fibre recruitment for contraction (Fluck *et al.* 2002). It is particularly high in slow-oxidative muscle (Gordon *et al.* 2001), suggesting an increasing biological relevance of FAK for mechano-regulation in this muscle type and the assembling muscle fibres. The activation of FAK is known to promote the growth and differentiation of cardiac and skeletal muscle cells in culture via a mechanism involving the translocation of FAK to costameres (reviewed in (Sastry *et al.* 1999; Pham *et al.* 2000; Kovacic-Milivojevic *et al.* 2001; Fonseca *et al.* 2005; Quach & Rando, 2006). The functional implication of focal adhesion signalling for the mechano-regulation of skeletal muscle function with regard to gene expression has not been addressed experimentally (Bloch & Gonzalez-Serratos, 2003; Durieux *et al.* 2007).

We hypothesized that myocellular FAK is a load-dependent switch controlling the expression programme underlying the structural–functional differentiation of the contractile machinery and energy metabolism in slow-oxidative muscle. To address this research question we deployed gene electrotransfer because this technology allows the overexpression of native molecules in striated muscle (Durieux *et al.* 2002) under the inclusion of inter-animal specificity controls. We monitored muscle transcript expression, structure and function after somatic overexpression of a FAK homologue and its competitor, FAK-related non-kinase (FRNK), in anti-gravitational muscle, which has a physiologically altered loading state (Gordon *et al.* 2001; Fluck *et al.* 2005). The effect of FAK overexpression was evaluated from the paired comparison to empty-transfected muscles in contralateral limbs. The inference of somatic gene transfer was assessed *vs.* non-transfected muscles of matched controls for the investigated loading conditions.

Methods

Ethical approval

The experiments were performed at the Universities of Berne (Switzerland), Lyon (France), and Pavia (Italy) with the permission of the local Animal Care Committee of the Canton of Berne (Switzerland) and following the recommendations provided by the European Convention for the protection of Vertebrate Animals used for Experimental and Scientific purposes (Strasbourg, 18.III.1986).

Experimental design

Three-month-old female pathogen-free Wistar rats (Charles River Laboratory, L'Arbresles, France) were used for this study. Focal adhesion signalling in soleus muscle was manipulated by overexpression of a FAK homologue via gene electrotransfer of expression constructs and by different loading protocols: hindlimb unloading–reloading or tenotomy (Fig. 1). A paired design was adopted to allow for intra-animal comparisons of the effects of FAK signal modulation on muscle and

the interaction with muscle loading. Equal amounts of FAK construct pCMV-FAK and empty control plasmid pCMV were electro-transferred into the right and left soleus muscle, respectively. Another experimental group co-expressed FAK and FRNK in the left soleus muscle whereas contralateral muscles were double-transfected with FAK construct pCMV-FAK and empty pCMV plasmid. At least six biological replicas were analysed per experimental condition. In addition, the data were compared to published results on the effect of a hindlimb unloading–reloading protocol on non-transfected soleus muscle (Fluck *et al.* 2005).

Plasmid construction

Cytomegalovirus (CMV) promoter-driven plasmids for the constitutive overexpression of chicken FAK (pCMV-FAK) and FRNK homologues (pCMV-FRNK) were a gift from Tony Parsons (University of Virginia, Charlottesville, USA). The amino acid sequences are highly conserved between the chicken and rat FAK homologues (92%) with all major regulatory sites present. Empty pCMV plasmid was constructed by the excision of the DNA insert from pCMV-FRNK via *Bam*HI restriction digestion and ligation. Plasmids were sent to plasmidfactory (Bielefeld, Germany, www.plasmidfactory.de) for propagation and isolation of endotoxin-free DNA.

Somatic transgenesis

Gene electrotransfer was carried out as previously described (Durieux *et al.* 2002) and optimized for the soleus muscle. Rats were anaesthetized by intra-peritoneal injection of sodium pentobarbital (60 mg (kg body

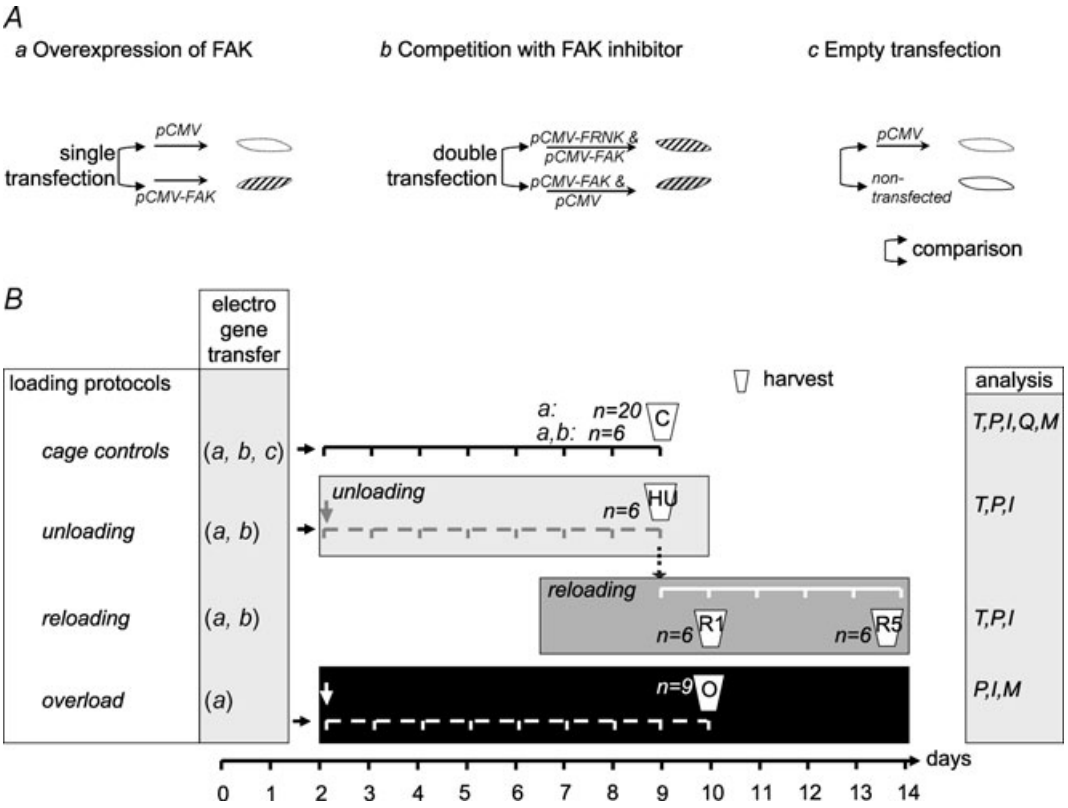

Figure 1. Experimental design
Sketch depicting the comparisons used to test the interaction effect of FAK overexpression (*A*) and loading (*B*) on molecular, cellular and functional variables of rat soleus muscle. *A*, drawings visualizing the approach to quantify the effect of FAK overexpression via (*a*) intra-animal gene transfer with pCMV-FAK and empty plasmid pCMV in contralateral (CTL-CTL) soleus muscles, (*b*) intra-animal competition experiments deploying co-transfection of FAK construct with empty plasmid pCMV or pCMV-FRNK construct (encoding the FAK inhibitor FRNK) in contralateral muscles, and (*c*) inter-animal assessment of the effect of transfection with empty plasmid *vs.* non-transfected muscle. *B*, summary of the loading protocols imposed on non-transfected and transfected muscles and time-points of sampling. The procedures included unloading-reloading and overload via bilateral tenotomy and normal cage activity. The number of biological replicas per transfection and loading protocol is indicated. The specific analyses being performed are indicated in abbreviation and detailed in Methods: T, transcript profiling; P, protein biochemistry; I, immunostaining; Q, quantitative confocal microscopy; M, myography.

weight)$^{-1}$, Sanofi, France). The depth of anaesthesia during the intervention was checked by verifying the absence of muscle reflexes to pinching the digits with fine forceps and by monitoring the respiratory rhythm. Hindlimbs were shaved and cleaned with Betadine (Viatris, France) and soleus muscle was surgically exposed by a lateral split of the connective tissue sheet between the gastrocnemius and tibialis anterior muscles. Endotoxin-free plasmid in 0.9% NaCl solution (50 μg, 70 μl) was injected in the deep and superficial region of the belly portion of the soleus muscle and three trains of 80 100 μs pulses, each at 100 mA, were delivered using needle electrodes with the GET42 generator (E.I.P. Electronique et Informatique du Pilat, Jonzieux, France). The skin and fascia were closed with sutures and the animals transferred to single cages.

Muscle loading

The unloading of the soleus muscles by 7 day hindlimb suspension was performed as previously described (Fluck *et al.* 2005) at the Université Lyon 1 (France) and started 2 days after transfection. Subsequent reloading was provoked by allowing the animals to return to normal cage activity for 1 or 5 days.

For the tenotomy experiments, animals were anaesthetized 2 days after gene electrotransfer by intra-peritoneal injection of sodium pentobarbital 40 mg kg^{-1}. An incision was made with a scalpel to the superficial gastrocnemius portion of the Achilles tendon of both limbs. The site was secured with stitches and the rats were allowed to re-establish in a quiet environment while signs of pain or distress were carefully monitored. Rats recovered rapidly and started to walk 2 h after the procedure. Correct healing was checked during the following days and favoured by topical application of Vulnamin (Errekappa Euroterapici, Milan, Italy). Subsequently animals were allowed free cage activity for 8 days. Gene electrotransfer was carried out at the University of Berne under anaesthesia with 2% isoflurane (Rhodia, France). The freshly transfected animals were transported to the University of Pavia (Italy) for tenotomy. Cage control groups were analysed 7–9 days after gene electrotransfer.

At the end of the respective protocol, the rats were weighed, and the m. solei of both hindlimbs were harvested under pentobarbital anaesthesia. Killing of the anaesthetized animals was carried out by dislocation of the cervical vertebrae and rapid exsanguination. For the molecular analysis, the transfected belly portion was rapidly dissected and frozen in melting-isopentane. Muscles for the contractile measures were excised from extracted legs into oxygenated Krebs solution (95% O_2, 5% CO_2). For single fibre analysis, muscles were stored for up to 3 weeks at −20°C in a 1 : 1 (v/v) mixture of skinning solution (150 mM potassium propionate, 5 mM KH_2PO_4, 5 mM magnesium acetate, 3 mM Na_2ATP, 5 mM EGTA, pCa 9.0 and glycerol including 20 μM and 10 μM of the protease inhibitors leupeptin and E-64, respectively, and fibres chemical skinned as described (Rossi *et al.* 2001; D'Antona *et al.* 2006).

Transcript profiling

RNA isolation and microarray analysis for 1185 transcripts (ATLAS TM rat 1.2 cDNA array, BD Clontech, Basel, Switzerland) was carried out basically as described (Fluck *et al.* 2005). In brief, total mRNA was extracted from cryosections of the transfected soleus portions of contralateral muscle pairs with the RNA mini-kit (Qiagen, Basel, Switzerland) after homogenization with a rotor-stator homogenizer (Polytron PT1200, Kinematica, Lucerne, Switzerland) and digestion with proteinase K for 1.5 h at 45°C (600 mAU ml^{-1}; Qiagen). RNA concentration was quantified with ribogreen (Molecular Probes, Eugene, OR, USA) and equal RNA amounts (2.5 μg) were subjected to reverse-transcription under standardized conditions. The reactions were carried out at 37°C using a specific set of primers for the cDNAs spotted on the array, nucleotide mix with calibrated radio-labelled [α-^{32}P]dATP (3000 Ci mmol^{-1}, 10 μCi μl^{-1}, Hartmann Analytic, Braunschweig, Germany) and initiated with the addition of 200 units of Superscript II reverse transcriptase (Invitrogen). The radiolabelled cDNA was purified by column chromatography (ChromaSpin, supplied with the ATLAS TM kit) and subjected to array filter hybridization in ExpressHyb solution overnight at 68°C. The mean labelling efficiency of target cDNAs from pCMV-FAK and pCMV-transfected samples was $4.1 \pm 0.8 \times 10^6$ c.p.m. and $4.3 \pm 0.3 \times 10^6$ c.p.m. per μg total RNA, respectively. Subsequently, the filters were washed and exposed for 6 days to detect cDNA signals with a Phosphorimager no. 425E running under ImageQuant v. 3.3 (Molecular Dynamics, Sunnyvale, California, USA). The signals corresponding to the individual cDNA spots were identified and quantified as the sum of pixels using the AIDA Array Easy software (Raytest Schweiz A.G., Urdorf, Switzerland). The integrated signal and local background values were estimated by the 'local dot ring' mode.

Six biological replicates were run separately for all conditions of transfection and loading, except for the unloading experiments where two pairs of the six samples were pooled for reverse transcription. Significantly altered transcripts were identified from raw signals with statistical analysis for microarrays (SAM) for a two class paired design (Dapp *et al.* 2004). False discovery rate corresponded to the automatically computed value for the selected T-statistics. Expression

ratios were calculated from the mean of pCMV-FAK *vs.* pCMV-transfected muscle pairs. Subsequently, the enrichment of the co-directional level changes of altered transcripts within a GO, i.e. up- or down, was analysed as described (Flück *et al.* 2008). The grouping of transcripts into muscle-relevant GOs was based on the information available for the microarray platform and the electronic literature (http://www.expasy.org/sprot/ and http://www.ncbi.nlm.nih.gov/sites/entrez). Changes in the RNA of sarcomere proteins, MHC1, 2A and 2X were separately assessed in cage control and 1-day-reloaded muscle using the reverse-transcriptase PCR against 28S RNA (Fluck *et al.* 2005) and combined with the microarray data. The PCR primers were MHC1: 5′-CAGCCTACCTCATGGGACTGA-3′, 5′-TGACATACTCGTTGCCCACTTT-3′; MHC2A: 5′-AGAATGACAACTCTTCACGATTTGG-3′, 5′-GGCGGATAGCACGAGATTTC-3′; MHC2X: 5′-GGCCAGGGTCCGTGAACT-3′, 5′-GCTTCAACATTGCGCTTCTG-3′ (Microsynth, Balgach, Switzerland). *P*-values were visualized via Cluster and Treeview (http://rana.lbl.gov/EisenSoftware.htm) and assembled with CorelDraw X3 (Corel Corporation) and Powerpoint (Microsoft Office for Windows XP). Data series were deposited under provisional accession codes GSE12743, GSE12744, GSE12745, GSE12746 and GSE12747 at Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo).

Protein biochemistry

Sample preparation in RIPA buffer, protein detection, and quantification by immunoblotting were carried out as previously described (Fluck *et al.* 1999, 2002). Equal loading (25 μg total protein) per lane was verified with Ponceau S staining of the nitrocellulose blot before immunodetection. The FAK-pY397-specific antibody was from BioSource International and the C-terminal FAK serum 'Lulu' was a gift of Dr Andrew Ziemiecki (University of Berne) (Fluck *et al.* 1999). Monoclonal antibodies against components of complex I–V of the mitochondrial respiratory chain were from Molecular probes (Molecular Probes/Invitrogen Ltd, Paisley, UK). Antibodies against type 1 and all type 2 MHC isoforms, and horseradish peroxidase-conjugated secondary antibodies were applied as described previously to visualize MHC expression (Fluck *et al.* 2005). The content of different MHC isoforms, MHC1, MHC2A, MHC2X and MHC2B, in the belly part of the muscle was quantified by additional highly resolving SDS-PAGE electrophoresis (D'Antona *et al.* 2006). The signal for each MHC was normalized to the total MHC signal per sample lane. The respective MHC signal per sample was determined from the mean of three technical replicas per sample. A Wilcoxon test was applied to test the effect of pCMV-FAK plasmid gene electrotransfer and the paired pCMV-transfected control on protein expression in the homogenate.

Immunostaining and morphometry

Microscopic measurements were carried out after the reaction of muscle cross-sections with polyclonal rabbit antibody A-17 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) against the FAK N-terminus and detection with a horseradish peroxidase-labelled secondary antibody (Fluck *et al.* 2002).

The percentage of fibres showing FAK overexpression was determined from visual fields of stained sections using a microscopic station (Leitz DMRBE, Vienna, Austria) running under Analysis 5.0 software (Olympus Soft Imaging Solutions GmbH, www.olympus-sis.com). Sections were visualized with a PL Fluotar 20× 0.50 or PL Fluotar 40× 0.70 objective and digitally recorded with a digital camera (Color view, Soft imaging system) through a 0.63× objective. The frequency distribution and mean of fibre cross-sectional area (CSA) in the targeted muscle portion was estimated by the circumference method from the recorded picture. For the measure of CSA in dependence of FAK localization, fibres were classified into those with exclusive sarcolemmal staining or those exhibiting staining of both the sarcoplasm and sarcolemma. The numbers were pooled for muscles with the same treatment.

Quantitative confocal microscopy

The analysis for co-expression of FAK and FAK-modulated factors in muscle fibres was carried out with a Leica TCS SP5 confocal microscope on a DMI6000 stage powered by Argon laser and He–Ne lasers (Leica Microsystem CMS, Mylton Keynes, UK). In brief, cryosections were reacted with a 1 : 100 dilution of rabbit FAK antibody A-17 (Santa Cruz) in 0.3% BSA in phosphate-buffered saline (PBS) as described (Fluck *et al.* 2002) but with the modification that a 1 : 200 dilution of a second primary antibody from mouse was added to detect MHC1 or MHC2 (Fluck *et al.* 2005) or subunits of complex I–V of mitochondrial respiration (Molecular Probes/Invitrogen). Sections were reacted with fluorescent-labelled secondary antibodies (Alexa488-conjugated anti-rabbit IgG, and Alexa555-conjugated anti-mouse IgG, Molecular Probes/Invitrogen) and embedded in fluorescence-compatible mounting medium (DAKO, Glostrup, Denmark). Quantification of the signal for FAK and the co-detected protein was carried out on digital images from the FAK-transfected region of double-stained sections. Fluorescence was excited at 458 nm, 476 nm and 488 nm with sampling in channels between 510–533 nm (Alexa 488) and 593–614 nm (Alexa 555). Four to five

images were recorded from different visual fields for each section with a 10× objective (HCX PL APO CS 10.0 × 0.40 DRY UV) using the specific channel settings. Resolution was set to 521 pixels × 512 pixels (8 bit) with a scan speed of 400 Hz and a pinhole of 53 μm. Four to ten muscle fibres were selected from each image. Mean signal intensity per selected fibre was determined in separate channels with the intensity quantification tool of Leica Application Suite version 2.0.0. For each fibre, pixel intensity (in bits) was sampled in a systematic manner across 2–5 parallel optical slices at 5–10 μm distance. The raw data for the recording in the channel for FAK-staining (Alexa488) and the staining for the FAK-modulated factor (Alexa555) were exported as csv-format into Microsoft Excel. The signal intensity per fibre was calculated for each channel from the mean of integrated channel intensities per sampled pixels along the optical slices. Fibres were classified in FAK-positive and FAK-negative fibres based on the intensity of the Alexa488 channel. The cut-off for FAK staining was declared at a mean intensity of 50 bits pixels^{-1} in line with the optic discrimination on the screen. The fibre signals from the different fields from one muscle cryosection were related to the mean of pixel signals of FAK-negative fibres. Thereby normalized data were pooled to reveal the relative myocellular expression per FAK-positive and FAK-negative fibres. Statistical analysis was carried out with Wilcoxon's test.

FAK expression in single fibres of pCMV-FAK-transfected soleus muscle was evaluated after collection of the chemically skinned fibres on microscope slides by immunofluorescence using the A-17 antibody.

Myography

The contractile parameters in freshly isolated soleus muscle were evaluated at 25°C as previously described (Rossi *et al.* 2001; D'Antona *et al.* 2006).

Model considerations

We chose a somatic option for transgenesis due to the proven efficiency of electrotransfer for skeletal muscle (Durieux *et al.* 2002). This approach, unlike a complex germline approach, allows us to include a specificity control via the co-overexpression of the FAK inhibitor FRNK, which circumvents lethal effects of FAK's elimination (Ilic *et al.* 1995) or the labour and cost-intensive generation of conditional or muscle-specific knock-out for FAK or its activation site (Booth *et al.* 1998). A sizeable increase in FAK levels in muscle fibres within the pCMV-FAK transfected muscle portion by up to 40-fold indicates the effectiveness and myocellular specificity of our somatic approach. Due to the selective targeting of exogenous protein expression in the belly portion, only an approximate 4% of the total fibre mass was transfected, i.e. 20% of the muscle cross-section injected with plasmid times 20% of fibres transfected. Therefore, we limited the analysis of molecular consequences to the belly portion, which was targeted by the transfection, and followed the alterations at the cellular level by microscopy.

The contrast with non-transfected muscle implied that adjustments due to FAK overexpression occur in the context of de-differentiation of slow-type soleus muscle to a hybrid slow/fast type during the regeneration of muscle after gene transfer (Fig. 2*A* and online Supplemental Material Fig. S2*A*; Rizzuto *et al.* 1999; Durieux *et al.* 2002; Fluck & Hoppeler, 2003). We therefore adopted a paired approach that allowed subtracting the combined influence of surgery and transfection via quantitative intra-animal comparisons (Fig. 1). A high correlation ($r^2 \geq 0.98$) for mean transcript level differences between empty transfection of soleus muscle and non-transfected muscle in the cage control, unloaded and 1-day-reloaded groups indicated that transfection-related background is largely comparable for the studied samples.

Statistics

A paired design was adopted to test the effect of FAK overexpression in muscle compared to empty-transfected contralateral controls. Protein level changes were calculated from the expression ratio between contralateral muscle pairs. Probability-based statistical tests (Wilcoxon's Test, χ^2 test) were performed with StatSoft v. 6 (Statistica, Inc., Tulsa, OK, USA www.statsoft.com) as indicated in the respective Methods paragraph and figure legends. Statistical significance was assumed at $P < 0.05$, with $0.05 \leq P < 0.10$ being considered a trend.

Results

Muscle fibre targeted FAK overexpression

Electrotransfer of the constitutively active expression plasmid for FAK, pCMV-FAK, into soleus muscle induced the appearance of FAK-immunoreactive fibres in the transfected portion (Fig. 2*A*). The FAK protein localized exclusively to muscle fibres and was detected in both the sarcolemma and the sarcoplasm (Fig. 2*A* and *B*). Contralateral muscles being transfected with empty pCMV plasmid did not demonstrate notable FAK immunostaining. Eight days after electrotransfer, 18% of the total cross-sectional area (CSA) in the targeted portion corresponded to fibres overexpressing FAK. The total FAK protein content in the targeted portion was increased 2.6-fold between the pCMV-FAK and pCMV-transfected soleus muscle pair (Fig. 2*C*).

FAK modulates the muscle transcriptome

We analysed FAK-dependent muscle gene expression in rats housed under normal cage activity by comparing differences in transcript levels between six contralateral soleus muscles being transfected with empty plasmid or FAK construct (Fig. 1). Transcript profiling identified a general upregulation of mRNAs in soleus muscle after 8 days of FAK overexpression *vs.* empty transfected contralateral controls. This involved muscle-relevant GOs associated with energy metabolism, control of contraction and protein turnover (Table 1). The majority of RNA changes were less than 50% (see online Supplemental Material, Table S1).

Co-expression of the FAK competitor FRNK in pCMV-FAK-transfected soleus muscles (Figs 1 and 2*D*) resulted in the down-regulation of the expression of FAK-regulated GOs related to mitochondrial oxidative metabolism, voltage-gated ion channels, adhesion and cell regulation compared to transfection with FAK alone in the contralateral muscle (Table 1). Within the many FRNK affected transcripts, the expression of two factors, ATP4B and CAMK2B, was altered above 1.5-fold with FAK overexpression.

Translation of FAK-regulated muscle gene expression

Key components of the GOs underpinning the energy metabolic and contractile muscle phenotype and for which transcripts were regulated by FAK were verified at the protein level in paired transfection experiments of cage control animals (Fig. 1). This included the mitochondrial components cytochrome c oxidase subunit 1 and 4 (COX1 and COX4), the factor ATP5A1 of oxidative phosphorylation, myosin heavy chain 1 (MHC1), and MHC2 isoforms MHC2A and MHC2X. The measures in the transfected muscle portion confirmed the FAK-dependent transcript level alterations of COX4 and MHC2A at the protein level and demonstrated a

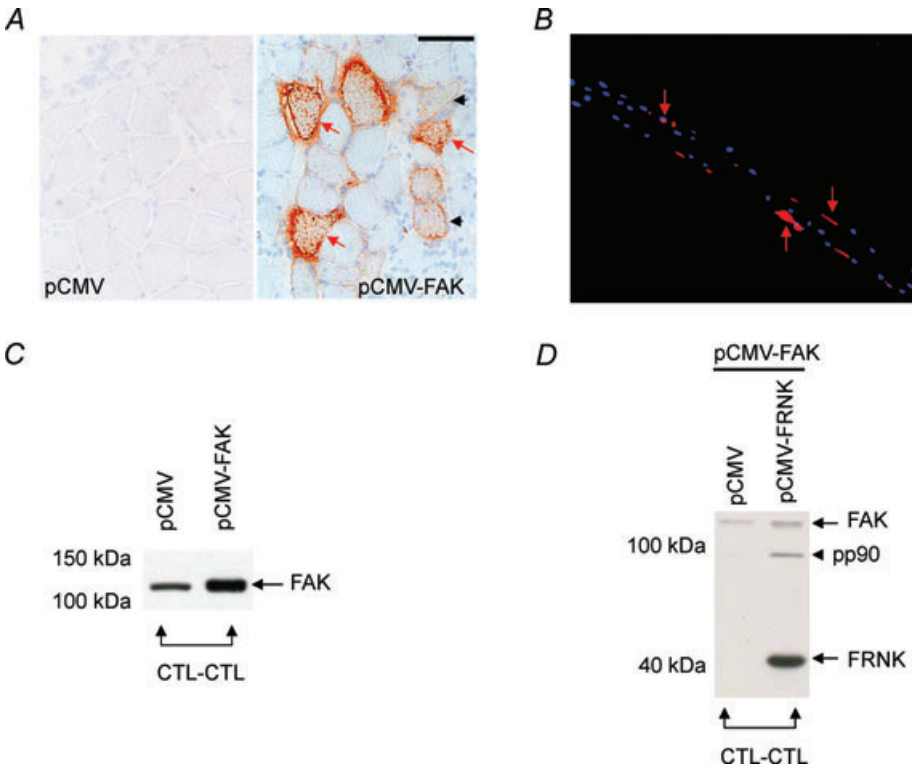

Figure 2. Muscle fibre targeted FAK overexpression
A, immunostaining of FAK protein (orange, red arrow) in cross-sections of a soleus muscle pair after gene electro-transfer. The right soleus muscle was transfected with FAK-expression construct (pCMV-FAK) and the contralateral (left) muscle was transfected with empty plasmid (pCMV). Nuclei appear in blue. Regenerating fibres are indicated with arrowheads. *B*, FAK immunostaining of an isolated muscle fibre from pCMV-FAK-transfected muscle. The bar denotes 50 μm. *C*, immunoblot visualizing FAK protein levels in homogenate from pCMV-FAK-transfected muscle compared to its empty-transfected contralateral control (CTL-CTL). *D*, immunoblot visualizing the induced expression of the endogenous competitor FRNK in soleus muscle after double transfection with FAK and FRNK expression construct compared to CTL-CTL muscle being transfected with FAK construct and empty pCMV plasmid. The positions of the FAK and FRNK proteins, and a 90 kDa FAK-gene product, pp90, are indicated (40).

Table 1. FAK-dependent gene ontologies

	Effect FAK		Effect FRNK	
Gene ontology	Trend	*P*	Trend	*P*
Energy				
Mitochondria	↑ 38	<0.001	↓ 31	<0.001
Mitochondrial oxidative metabolism	↑ 50	<0.001	↓ 46	<0.001
Carbohydrate metabolism	↑ 7	0.029	– 6	0.300
Contraction				
Voltage-gated ion channels	↑ 21	<0.001	↓ 35	<0.001
Adhesion	↑ 15	<0.001	↓ 19	0.002
Cytoskeleton/mobility	↑ 8	0.014	– 7	1.000
Sarcomere	– 1	1.000	– 1	1.000
Protein turnover				
Protein synthesis	– 5	0.100	– 3	1.000
Proteases	↑ 29	<0.001	– 10	0.476
Proteasome	↑ 11	0.002	– 5	1.000
Cell regulation				
Intracellular signalling	↑ 151	<0.001	↓ 87	<0.001
Extracellular signalling	↑ 30	<0.001	↓ 44	<0.001
G-protein	↑ 16	<0.001	↓ 44	<0.001
All	↑ 594	<0.001	↓ 729	<0.001

GOs which transcripts showed unidirectional level alterations upon FAK overexpression in rat soleus muscle *vs.* contralateral controls as revealed by SAM for a two class paired design. $n = 6$ per biological replica. The trend of significant alterations per GO is indicated with arrows (↑, up; ↓, down, –, no effect) alongside the number of affected transcripts. Specificity of the FAK effect was controlled by the co-expression of FAK with its inhibitor FRNK. For a comprehensive list of the affected transcripts see Supplemental Material Table S1.

tentative increase in ATP5A1 after FAK overexpression *vs.* empty transfected contralateral controls (Fig. 3*B*). The 50% decrease in fast type MHC2A occurred concomitantly with a 9% elevation of slow type MHC1 content. Additional measures identified sizable elevations in protein content for subunits of complex I (NDUFA9) and complex III (UQCRC1) of the mitochondrial respiratory chain (Fig. 3*B*). The increase in MHC1 protein and combined subunits of mitochondrial respiration in pCMV-FAK transfected muscle was confined to FAK-overexpressing fibres (Fig. 3*C*). Total MHC2 protein content was not different between FAK-overexpressing and non-overexpressing fibres of pCMV-FAK transfected muscle. The number of hybrid slow/fast type fibres was reduced in pCMV-FAK transfected muscle *vs.* its empty-transfection control (Supplemental Material, Fig. S2). The alterations of sarcomeric and mitochondrial proteins after FAK overexpression in cage controls did not translate into functional changes of muscle contraction *ex vivo* (Fig. S2 and Table S2).

A comparison with non-transfected muscle showed that the high content of hybrid slow/fast type fibres in cage controls was due to de-differentiation of slow type soleus muscle fibres with electrotransfer (Fig. 2*A* and Fig. S2).

FAK overexpression elevates fibre size

A trend of increased muscle weight with FAK overexpression was observed for the six soleus muscle pairs of cage controls being subjected to transcript profiling. There was a significant 12% increase when 14 muscles were pooled (Table S3). Microscopic analysis revealed a shift towards larger fibre callipers in pCMV-FAK-transfected muscle compared to the empty-transfected muscle (Fig. 4*A* and *C*). The mean CSA of muscle fibres was 6% higher in FAK-overexpressing muscle (Fig. 4*B*).

Muscle loading modulates FAK-dependent muscle gene expression

We tested whether altered muscle loading modifies the FAK-dependent muscle transcriptome. Towards this end we deployed paired transfection experiments in combination with a protocol for muscle unloading and reloading (Fig. 1). The FAK protein level remained elevated in pCMV-FAK-transfected soleus muscle *versus* pCMV-transfected contralateral control, after 7 days of unloading and 1 day of subsequent reloading (Fig. 5*A*). Unloading resulted in a general suppression of RNA levels

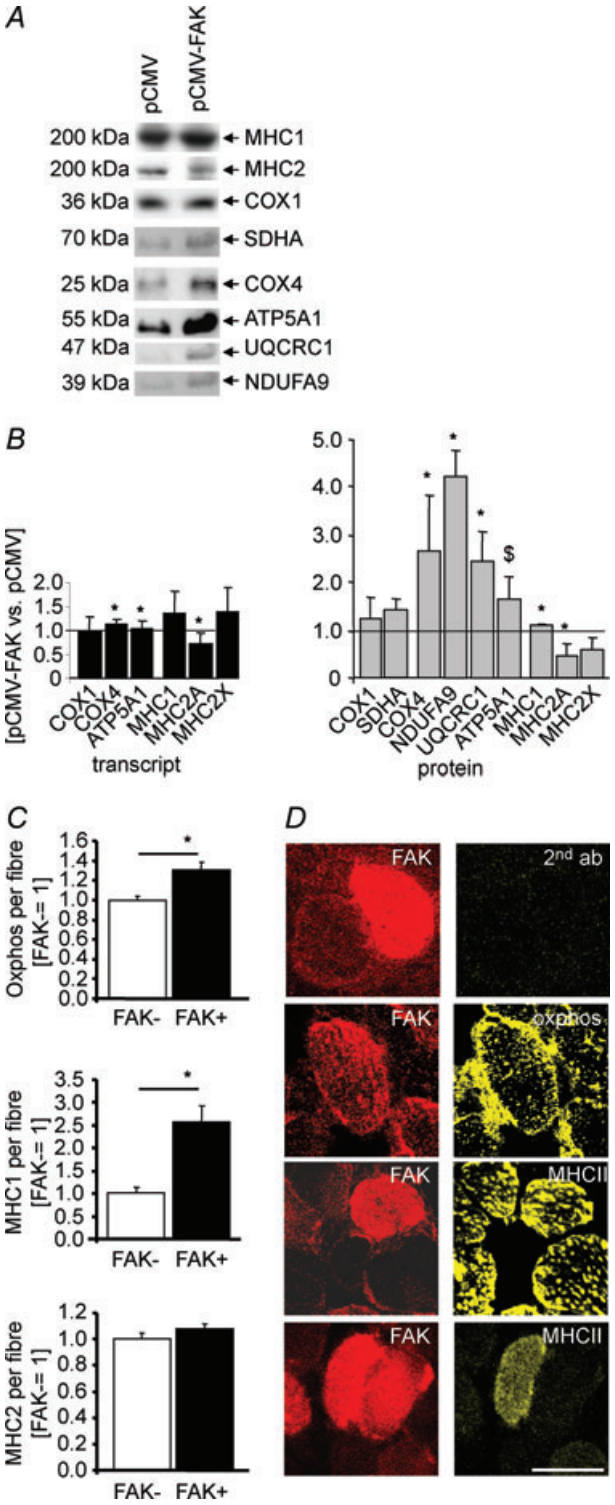

Figure 3. Expression of selected gene products in FAK-modulated GOs

A, immunoblots visualizing the detection of selected mitochondrial proteins of oxidative phosphorylation (oxphos), i.e. complex I (NDUFA9), complex II (SDHA), complex III (UQCRC1), complex IV (COX1 and COX4) and complex V (ATP5A1), and sarcomeric proteins MHC1 and combined MHC2 isoforms in pCMV and pCMV-FAK transfected muscle pairs. *B*, mean ± S.E.M. of the expression ratio of transcripts and corresponding protein of the selected factors between pCMV-FAK-transfected soleus muscle and empty-transfected CTL-CTL. $n = 6$ per transfection. $^*P < 0.05$ *vs.* CTL-CTL, SAM for a two class paired design of microarray data (COX1, COX4, ATP5A1) or a sign-test for PCR data (MHC1, MHC2A, MHC2X), respectively. The line of identity is indicated. *C*, mean ± S.E.M. of the relative myocellular expression of FAK-modulated proteins in FAK overexpressing (FAK+) and negative fibres (FAK–) of pCMV-FAK-transfected soleus muscle of cage controls. Fifty-eight FAK+ and 74 FAK– fibres were analysed on average from four different muscles. $^*P < 0.05$, Wilcoxon's test. *D*, double panels presenting examples of confocal microscopic images of co-expression of FAK and the respectively FAK-modulated factor (i.e. MHCI, MHCII and combined oxphos subunits). A panel with a control reaction omitting the use of primary mouse antibody (2nd ab) is included as well. The bar denotes 50 μm.

in the muscle overexpressing FAK *vs.* empty contralateral controls (Fig. 5*B*). Reloading reversed this trend; several GOs with a FAK-dependent reduction of transcript levels during unloading demonstrated up-regulated expression with reloading. The comparison to empty-transfected muscle identified that the effects of FAK overexpression resulted from a promotion of the load-dependent expression changes of GOs associated with mitochondria, oxidative metabolism, adhesion, protein turnover and signalling (Fig. 5*A* and *B*). The major exception to this general trend was the GO for voltage-gated ion channels.

Post-translational regulation of FAK by muscle loading

The load-modulated adjustments of the muscle transcriptome due to FAK overexpression corresponded to the post-translational regulation of FAK; FAK-pY397 content was higher in pCMV-FAK-transfected soleus muscle compared to contralateral muscle from cage controls. The ratio of phosphorylated FAK content decreased with unloading and was tentatively elevated after 1 day of reloading (Fig. 6*A* and *B*). Correspondingly, immunoreactivity against the FAK N-terminus was reversibly modified by muscle unloading and reloading (Fig. 6*C* and *D*).

Load-dependent effect of FAK on fibre size

The differences in MHC and COX protein levels in FAK overexpressing soleus muscle from empty-transfected contralateral controls were lost with unloading (data not shown). As well, the tendency for FAK-promoted muscle growth disappeared with unloading and was only transiently re-established with reloading (Table S3). The differences in muscle weight with 1 day of reloading were associated with FAK localization. Exclusive sarcolemmal FAK localization in 1-day-reloaded muscle coincided with a 16% increase in mean fibre CSA compared to fibres with exclusive sarcoplasmic FAK expression (Fig. 6*E* and *F*).

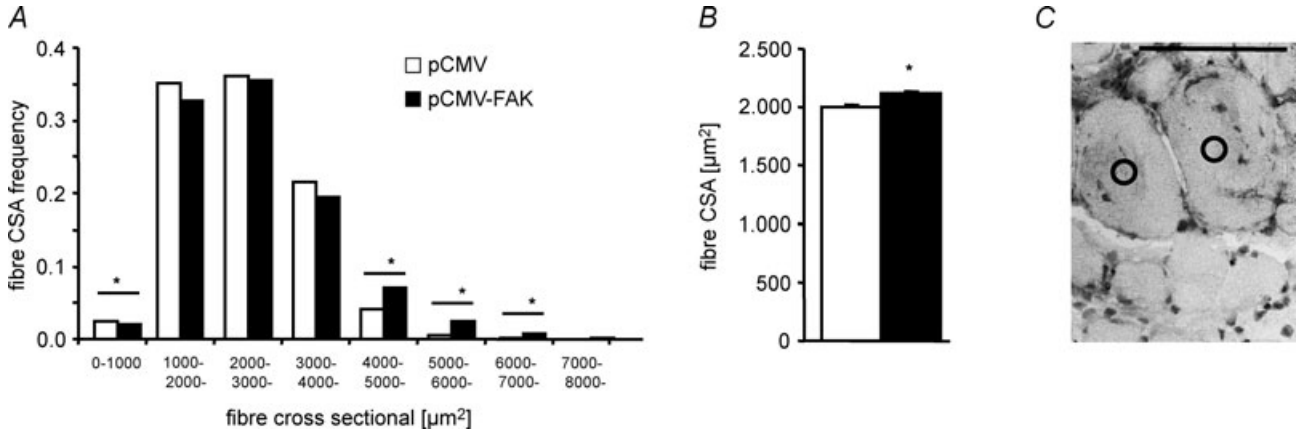

Figure 4. FAK overexpression and fibre size
A, bar graph of the cross-sectional area (CSA) of soleus muscle fibres as a function of FAK overexpression in cage controls. $^*P < 0.05$ between comparisons (χ^2 test). *B*, mean ± S.E.M. of fibre CSA in FAK-overexpressing muscle and its contralateral control. $^*P < 0.05$ *vs.* cage control (Wilcoxon's test). On average, 608 fibres were counted from six muscles per transfection. *C*, representation of two FAK overexpressing fibres with large callipers (circled) in pCMV-FAK transfected muscle. The bar denotes 100 μm.

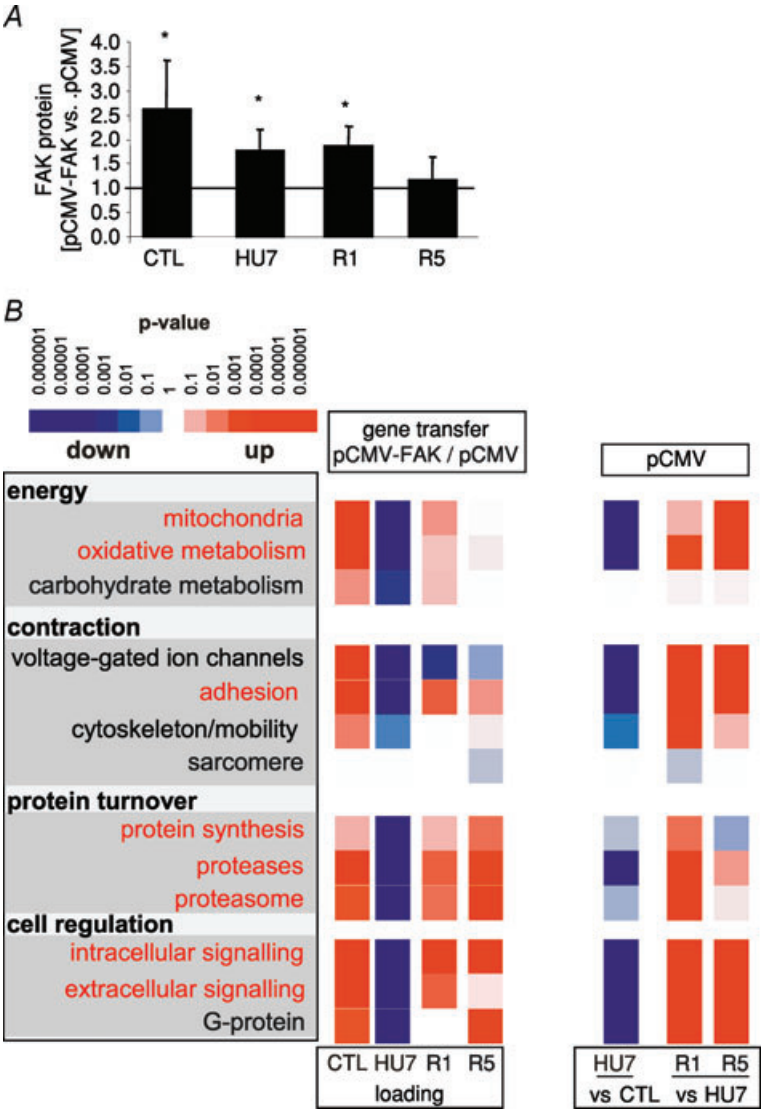

Figure 5. Global control of the FAK-dependent muscle transcriptome by muscle loading
A, FAK protein ratio (mean ± S.E.M.) between pCMV-FAK-transfected soleus muscle and empty-transfected CTL-CTL from cage control rats and rats after unloading and reloading. $^*P < 0.05$ *vs.* CTL-CTL (sign test). $n = 5$–10 per intervention. The line of identity is indicated. *B*, gene ontology (GO) map visualizing RNA expression changes per muscle-relevant GO with altered loading in pCMV-FAK *vs.* pCMV transfected muscle (left) and in empty-transfected muscle (right). The significance level of co-directional transcript level alterations per GO for each condition is indicated by colour coding. Names in red denote those GOs which were co-directionally affected between both comparisons. CTL, cage control; HU7, 7 days of unloading; R1, 1 day reloaded; R5, 5 days reloaded. $n = 6$ per intervention except for HU7 where $n = 4$.

Functional overload promotes slow contractile muscle characteristics in muscle overexpressing FAK

We tested whether enhanced muscle loading in the context of FAK overexpression would provoke a functional manifestation of FAK-modulated molecular adjustments. We reasoned that overload of the soleus muscle by tenotomy (Gordon *et al.* 2001) would be a suitable alternative to avoid FAK overexpression fading during the prolonged experimental duration of the unloading–reloading model (Fig. 5*A*).

The results confirmed the reduction of fast MHC2A composition and elevated MHC1 content by FAK overexpression (Fig. S3). A concomitant slowing of muscle contraction and relaxation was evident in the FAK-transfected muscles (Fig. 7). Fatigue resistance and soleus mass were not altered by FAK overexpression during overload (Table S2). The specific force of tetanic contractions was increased 2-fold in pCMV-FAK-transfected m. solei reaching the levels in non-transfected controls (Fig. 7 and Fig. S2C).

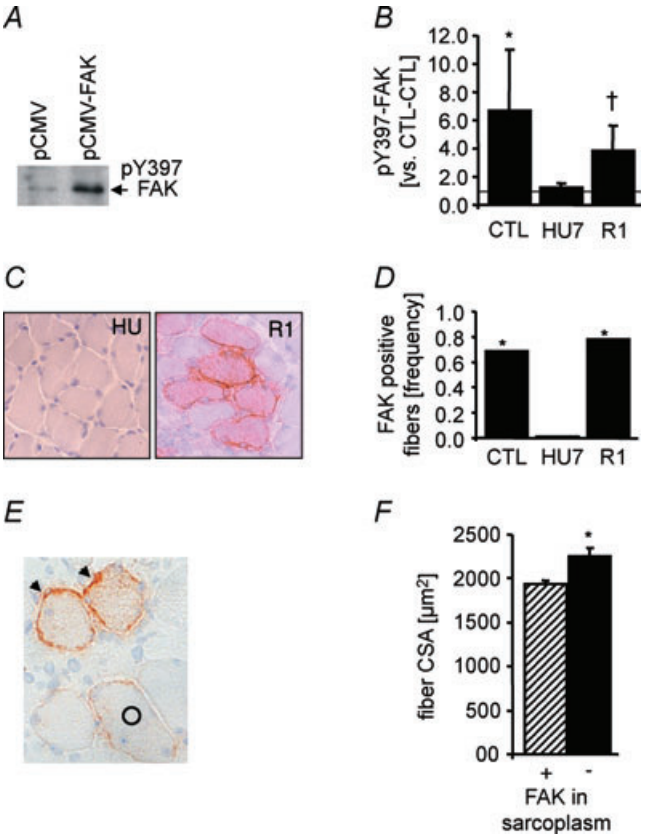

Figure 6. Load-modulated post-translational regulation of FAK
A, representative immunoblot detecting phosphorylated FAK (FAK-pY397) levels in pCMV-FAK-transfected soleus muscle and pCMV-transfected CTL-CTL from a cage control rat. *B*, FAK-pY397 ratio (mean ± S.E.M.) between pCMV-FAK-transfected soleus muscle and empty-transfected CTL-CTL as a function of unloading and reloading. $n = 3$–9 per time point. $^*P < 0.05$ *vs.* CTL-CTL; $\dagger 0.05 \leq P < 0.10$ *vs.* CTL-CTL (Wilcoxon's test). Representative immunostaining (*C*) and frequency (*D*) of N-terminal FAK immunoreactivity (orange) in cross-sections of FAK-overexpressing soleus muscle of cage controls and after unloading and 1 day of reloading. $^*P < 0.05$ between comparisons (χ^2 test). *E*, representative picture depicting muscle fibres with exclusive FAK staining at the sarcolemma (arrowhead) or with additional staining in the sarcoplasm (circle). *F*, mean ± S.E.M. of CSA in FAK-overexpressing muscle fibres as a function of sarcoplasmic FAK abundance. On average, 67 FAK-positive fibres were counted from each of six biological replicas per comparison. $^*P < 0.05$ *vs.* comparison (Wilcoxon's test).

Discussion

Several biochemical pathways have been proposed for the remodelling of the contractile and metabolic properties

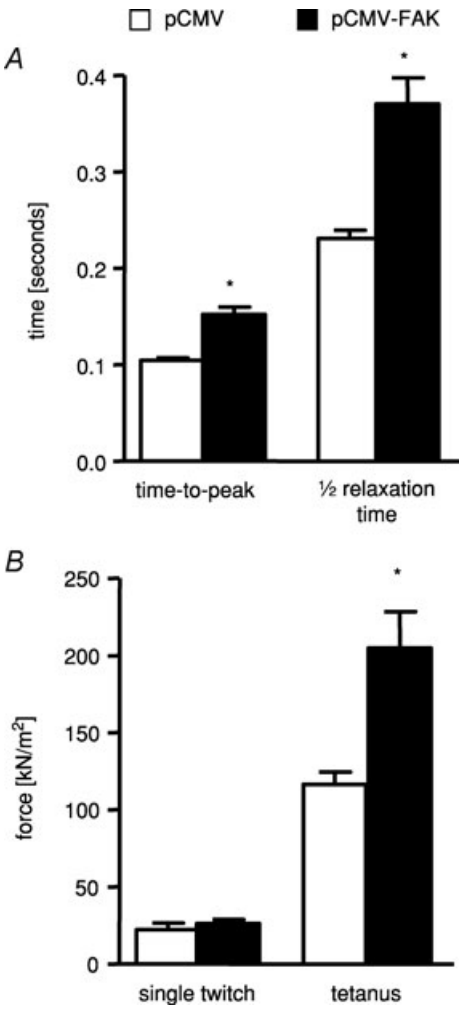

Figure 7. Load-dependent shift to a slow type muscle phenotype after FAK overexpression
Mean ± S.E. of contractile properties in pCMV-FAK- and pCMV-transfected soleus muscle after 8 days of overloading. *A*, time-to-peak and half relaxation time; *B*, force of a single twitch and tetanic contractions as quantified by myography. $N = 4$–7 per measure. $^*P < 0.05$ *vs.* CTL-CTL (Wilcoxon's test).

of striated muscle in response to use-related stimuli (Pallafacchina *et al.* 2002; Fluck & Hoppeler, 2003; Koulmann & Bigard 2006; Sandri, 2008). The functional implication of possible signalling routes for muscle conditioning by physiological stimuli is not understood. Thus, our muscle-targeted transgenic investigation focused on FAK, which complies with an active role in mechano-transduction within striated muscle (Fluck *et al.* 1999, 2002; Gordon *et al.* 2001; Quach & Rando, 2006) through post-transcriptional regulation and localization to sites of mechano-sensation. The multi-level approach analysing the consequences of muscle fibre-targeted overexpression of a FAK homologue delineated that the load-dependent functional differentiation of slow contractile features and fibre-growth in oxidative rat muscle is regulated by a FAK-mediated pathway to gene expression.

A main strength of this approach was that myocellular focal adhesion signalling was probed via a 'native' FAK homologue in the exceptionally load-dependent soleus muscle. We reasoned that the exogenous production of a native rather than a constitutively active FAK protein (Sastry *et al.* 1999) in muscle fibres would allow for the exposure of the physiologically motivated post-translational regulation of this signalling molecule. By adopting a paired transfection approach of intra-animal comparisons we took account for the inference caused by surgery and somatic gene transfer.

FAK promotes differentiation of slow-oxidative muscle characteristics

The significant drop in the number of hybrid slow/fast fibre types and fast MHC2A expression (Fig. 3*B* and Fig. S2A) in FAK transfected muscle of cage controls implies that FAK overexpression protects fibres from de-differentiation induced by electrotransfer. Microscopic examination indicates that this promotion of slow-type characteristics is not limited to the FAK overexpressing fibres but also involves reduced fast MHC2A expression in fibres which do show low FAK levels (Fig. 3*C*). The concurrent increase in slow MHC1 with FAK overexpression in the transfected muscle portion of cage controls was related to elevated protein content of key factors of mitochondrial respiration and corresponding alterations of the encoding transcripts (Fig. 3*B*). This observation extends previous findings in cardiomyocytes on a role of FAK in sarcomere organization (Kovacic-Milivojevic *et al.* 2001) to imply the differential control of both slow and fast myosin turnover and mitochondrial biogenesis by FAK. The concentration changes of major elements of muscle make-up in the transfected fibre population (Fig. 3*C* and *D*) is astonishing given that gene transfer with plasmid for a native (i.e. not constitutively active) FAK molecule was carried out only 9 days earlier. The present novel finding provides evidence that FAK is part of a myocellular pathway which mediates the expression of slow-oxidative muscle fibre characteristics.

The FAK-driven expressional adjustments of the metabolic and contractile phenotype occurred concomitantly with transcript up-regulation of both proteolytic and synthetic aspects of protein turnover and a trend for an increase in fibre cross-sectional area (Table 1 and Figs 4*A* and 5*B*). This observation with FAK overexpression in the slow tonic muscle under investigation relates to the elevated FAK content in muscle fibres with a high degree of load-bearing fibre activity (Fluck *et al.* 1999, 2002; Fluck & Hoppeler, 2003; Evans *et al.* 2008). This association of FAK with characteristics of the frequently recruited muscle is supported by the general reduction of FAK-induced transcript levels in soleus muscle after co-expression of the FAK inhibitor FRNK (Table 1; Taylor *et al.* 2001; Mansour *et al.* 2004; Quach & Rando, 2006). These considerations identify FAK as a broadly effective facilitator of the transcriptome programme, which promotes the activity-dependent re-establishment of the normal phenotype of anti-gravitational muscle after gene transfer.

Mechano-regulated pathway of slow-oxidative muscle differentiation

The load-dependent signature of FAK-dependent GOs complies with our general hypothesis that FAK is a major myocellular transducer of mechanical signals towards gene expression. Several of the combined FAK- and load-regulated transcript families (Fig. 5*B*) are associated with particularly developed features in soleus muscle such as mitochondrial metabolism, adhesion and protein turnover (Habets *et al.* 1999; Fluck & Hoppeler, 2003). The similarity in load-dependent control of these GOs by FAK overexpression with the effect of unloading and reloading in non-transfected and empty-transfected muscle (Fig. 5*A* and *B*; Stevenson *et al.* 2003; Fluck *et al.* 2005) supports the mechano-regulated expression of major transcripts in soleus muscle by FAK.

The contention of a functional implication of FAK in mechano-regulated muscle differentiation is supported by the reestablishment of specific tetanic force in FAK-transfected and overloaded muscle to normal levels (compare Fig. 7 and Fig. S2). This observation was paralleled by the load-dependent prolongation of half-relaxation time of muscle contraction in FAK-transfected muscle. Conversely, the upregulation of mitochondrial factors after FAK overexpression did not translate into alterations in the functional proxy of energy metabolism, such as fatigue, in either control or overloaded muscle (Table S2). The inspection of mitochondrial

proteins indicates that this lack of adaptation relates to a dissonance in the up-regulation of nuclear and mitochondrially encoded factors of respiration. For instance, the nuclear-encoded NDFUA9, UQCRC1 and COX4 (and tentatively ATP5A1) show sizable elevations while this is not observed for the mitochondrially encoded proteins COX1 and SDHA (Fig. 3*B*). This suggests a lack of coordination during mitochondrial biogenesis after FAK overexpression due to the absence of elevations in energy consumption in our model (Desplanches *et al.* 1987; Fluck & Hoppeler, 2003). Collectively this indicates selectivity in FAK's involvement in the expressional regulation of contractile *versus* energetic aspects of the muscle phenotype.

Chemical mechano-transduction via FAK

The probing with non-constitutively active FAK revealed that the FAK modulated expression control corresponds to phosphorylation of FAK at Y397 (compare Figs 5*B* and 6*B*). This post-translational modification induces a conformation change in the FAK molecule which promotes the binding of signalling factors to FAK (Shyy & Chien, 1997; Parsons, 2003). Support for such a scenario was provided by the correspondence of elevated phospho-Y397 content and induced FAK immunoreactivity in muscle fibres when probed with an N-terminal antibody (Fig. 6*C* and *D*). Elevated pY397 content of FAK corresponds to the translocation of FAK from a myofibrillar pool to the sarcolemma during hypertrophy of cardiac cells in culture (Fonseca *et al.* 2005). Similarly, FAK activation close to the sarcolemma is related to control of myofibrillogenesis (Fluck *et al.* 1999, 2002; Gordon *et al.* 2001; Quach & Rando, 2006). Our findings in fully developed tissue on the association of elevated CSA of muscle fibres with enhanced sarcolemmal FAK localization (Fig. 6*F*) is compatible with a mechanism whereby FAK shuttles between a myofibre-associated and a sarcolemmal pool during radial growth of muscle fibres.

Fibre hypertrophy after FAK overexpression corresponded to the load-dependent up-regulation of transcripts being associated with protein synthesis (Fig. 5*B*). We and others have shown recently that tyrosine phosphorylation of FAK is an upstream event of the mechano-induced activation of the regulator of protein synthesis and ribosomal biogenesis, p70S6K, in rodent muscle (Jastrzebski *et al.* 2007; Klossner *et al.* 2009). These considerations suggest that mechano-regulated expression control of protein turnover via post-translational modification of FAK underpins FAK's load-dependent association with fibre hypertrophy and muscle protein synthesis (Gordon *et al.* 2001; de Boer *et al.* 2007). Mechano-regulated phosphorylation of FAK at Y397 evolves as upstream event of the physiological expression pathway that governs the size and differentiation of slow oxidative muscle fibres by muscle loading.

Conclusions

The findings identify focal adhesion kinase (FAK) translocation to the sarcolemma as an upstream signalling element of load-dependent contractile differentiation and growth in slow-oxidative muscle. Corresponding adjustments between molecules, muscle fibres and function point out that FAK is part of the signalling pathway that governs the mechano-regulation and repair of striated muscle.

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ORIGINAL ARTICLE

Different response to eccentric and concentric training in older men and women

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Abstract Sarcopenia is the age-related loss of muscle mass and strength and has been associated with an increased risk of falling and the development of metabolic diseases. Various training protocols, nutritional and hormonal interventions have been proposed to prevent sarcopenia. This study explores the potential of continuous eccentric exercise to retard age-related loss of muscle mass and function. Elderly men and women (80.6 ± 3.5 years) were randomized to one of three training interventions demanding a training effort of two sessions weekly for 12 weeks: cognitive training (CT; $n = 16$), conventional resistance training (RET; $n = 23$) and eccentric ergometer training (EET; $n = 23$). Subjects were tested for functional parameters and body composition. Biopsies were collected from *M. vastus lateralis* before and after the intervention for the assessment of fiber size and composition. Maximal isometric leg extension strength (MEL: +8.4 ± 1.7%) and eccentric muscle coordination (COORD: −43 ± 4%) were significantly improved with EET but not with RET (MEL: +2.3 ± 2.0%; COORD: −13 ± 3%) and CT (MEL: −2.3 ± 2.5%; COORD: −12 ± 5%), respectively. We observed a loss of body fat (−5.0 ± 1.1%) and thigh fat (−6.9 ± 1.5%) in EET subjects only. Relative thigh lean mass increased with EET (+2.5 ± 0.6%) and RET (+2.0 ± 0.3%) and correlated negatively with type IIX/type II muscle fiber ratios. It was concluded that both RET and EET are beneficial for the elderly with regard to muscle functional and structural improvements but differ in their spectrum of effects. A training frequency of only two sessions per week seems to be the lower limit for a training stimulus to reveal measurable benefits.

Keywords Eccentric · Elderly · Strength · Fat · Coordination · Fibertyping

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Introduction

Sarcopenia is a condition of muscle tissue characterized by the loss of muscle fibers and fiber atrophy (Doherty 2003; Lexell 1995) accompanied by increased infiltration of non-contractile components such as connective tissue and fat (Overend et al. 1993). These structural changes along with impaired neuronal functions result in loss of muscle strength (Skelton et al. 1994). The mechanisms underlying muscle atrophy and loss of innervation are not fully understood. Reduced physical activity, decline in anabolic hormone levels (dehydroepiandrosterone, testosterone, growth hormone) concomitant with a chronic low-grade inflammation (increased tumor necrosis factor α and cortisol serum levels) contribute to the loss of muscle mass (Doherty 2003; Vandervoort 2002). Improvement of leg strength by means of strength training is a broadly applied strategy to reduce the risk of falling, since these two parameters seem causally associated (Perry et al. 2007; Shigematsu et al. 2006). Heavy resistance training can successfully be applied in the elderly (Hruda et al. 2003; Wieser and Haber 2007). However, it can result in significant cardiovascular as well as substantial mechanical stress on single joints

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(Hungerford and Barry 1979; Kaufman et al. 1991). On the other hand, endurance ergometer training, carried out in a closed muscle chain at high-angular velocities, has a broad application, improving body composition and insulin sensitivity (Hersey et al. 1994), but with minor benefits for strength and muscle mass, as mechanical stress on muscles remains low in endurance cycling.

It is well documented that strength training in the elderly results in substantial strength gain and muscle hypertrophy (Kryger and Andersen 2007). A recent meta-analysis by Roig et al. (2008) evaluated 20 studies comparing eccentric and concentric strength training and concluded that subjects profited more from eccentric than concentric training with regard to strength gain and muscle hypertrophy. These studies compared maximal eccentric training protocols applied to young adults and trained athletes and draw attention to the prospects of eccentric exercise modalities. In view of potential benefits of eccentric exercise we explored a training protocol of continuous eccentric exercise and compared it to established conventional strength training protocols. The eccentric exercise training (EET) consisted of resisting the pedal movement of a motor driven recumbent ergometer. As the energy cost of eccentric work is approximately four times less than that of concentric work of a comparable external load (Lastayo et al. 1999) large torques can be exerted at a manageable metabolic cost. The ensuing low stress on the cardiovascular system is particularly important for elderly, since they are characterized by a reduced aerobic capacity (Lotscher et al. 2007). The objective of using EET thus was to achieve a high mechanical load on muscle tissue with a restricted aerobic demand. Similar to concentric ergometer training, EET is executed in a closed muscle chain at relatively high-angular velocities, minimizing peak forces on single joints (Ericson and Nisell 1986, 1987). Due to these favourable features, eccentric exercise has been applied to people with a limited tolerance for conventional strength training (LaStayo et al. 2003), chronic obstructive pulmonary disease (Rooyackers et al. 2003) and coronary disease (Steiner et al. 2004). As a drawback, eccentric exercise is potentially associated with delayed onset muscle soreness (DOMS) due to muscle tissue damage (Friden et al. 1983). In the present study, muscle damage was avoided by carefully increasing muscle load over repeated exercise sessions beginning with very low eccentric loads.

It has been suggested that strength gain in the elderly is more related to neural mechanisms and less to muscle hypertrophy (Moritani and deVries 1980). Using an intensive exercise protocol, Kryger and Andersen (2007) still reported a significant increase of the type IIA fiber area and an impressive 37% strength gain in subjects, aged 85 and older, after a 12 week training period with three heavy resistance training sessions (45 min each) per week, indicating the maintenance of muscular plasticity on a structural as well as on a functional level even in very old people.

The aim of this study was to investigate EET as an alternative to conventional resistance training for the elderly to increase leg strength and leg muscle mass. This was done in a setting in which subjects were asked to perform only two sessions/week to maximize adherence. Based on the study of LaStayo et al. (Lastayo et al. 2002), we hypothesized that EET would be more effective in increasing muscle strength and mass than conventional resistance training. We further expected larger improvements of muscle strength in females than in males due to their initial lower fitness level (Lotscher et al. 2007). We did not expect any changes in muscle fiber type composition as the imposed training regime (2×20 min effective training time per week for 12 weeks) seemed to be insufficient to produce fiber type changes.

Materials and methods

Subjects and study design

A total of 62 subjects (71–89, average 80.6 years) with stable medication and health conditions were included in the study. Subjects with severe neuromuscular disease, instable coronary disease or severe hip or knee arthritis were excluded (Lotscher et al. 2007). The study was part of the National Foundation Program 53 ''Musculoskeletal health and chronic pain'' and carried out in accordance with the guidelines and the approval #190/04 of the "Kantonale Ethische Kommission".

The training period lasted for 12 weeks and comprised two guided training sessions (45 min each) per week. In order to familiarize the subjects with the functional tests they were sham tested 2 weeks before the real testing procedure. Subjects from whom biopsies were collected were randomized to one of the physical intervention groups and all others were randomly distributed as follows:

1. Cognitive training (CT) consisted of computer-guided cognitive training. The subjects (10 women, 6 men) did not perform any physical training and served as a control to account for the influence of social aspects of the training sessions.
2. Conventional resistance training (RET) was performed by 23 subjects (13 women, 10 men). RET was carried out in a gym and comprised four exercises for the lower extremity (leg press, knee extension, leg curl, hip extension). The sessions consisted of a 10-min warm-up with cardiovascular activation and gymnastics, 20 min training and 10 min cool-down with stretching. For the first six sessions the individual loads were set very low to familiarize subjects with the exercises.

Exercises included three sets with ten repetitions and loads were gradually increased during this time. The subsequent sessions consisted of one warm up set and two sets with eight to ten repetitions. If subjects were able to do ten repetitions or more, the load was increased in the next session. The load was not increased if people suffered from DOMS as indicated by scores ≥3 on a visual analog scale (VAS) (Langley and Sheppeard 1985) or when rating of the perceived exertion (RPE) of the whole training session was >13 according to BORG (Borg et al. 1987). VAS ranges from 0 to 10, where 0 is no soreness and 10 is the highest perceived muscle soreness. DOMS ratings of subjects were in the interval between 0 and 4.

3. Eccentric ergometer training (EET) was carried out by 23 subjects (13 women, 10 men) on a custom-built motor-driven ergometer (Meyer et al. 2003). The trainings started with a 10-min warm-up on a conventional ergometer with minimal loads (females 10 W, males 20 W) and closed with 10 min cool-down with stretching, while the actual EET lasted 20 min. The initial load on the eccentric bike was set very low (females 30 W, males 50 W). Initially, subjects exercised for only 5 min to prevent severe DOMS. During the first sessions the training duration was gradually increased in 5-min steps until it reached 20 min, before the imposed load was ramped. Load was ramped in consecutive sessions by 20% of the individual maximal power output achieved in the initial ergometer ramp test to exhaustion (Lotscher et al. 2007). Contraindications to increase the workload were the same as those for RET (DOMS; RPE).

Some of the subjects had to be partly or entirely excluded from physical tests due to illness such as *herpes zooster* (1 woman EET), *appendicitis* (1 woman CT), *osteoporosis* (1 woman CT), progressive *morbus Alzheimer* (1 woman CT, 1 woman RET) or injuries and persisting joint pain (1 man CT, 1 woman RET, 1 man RET, 1 man EET). Other subjects were excluded because they were unable to complete the required test (MEL: 1 women CT, 1 man RET, 1 woman EET) or because they were unable to follow the training protocol (1 woman EET was not able to dose the eccentric ergometer). Compliance in the sessions was secured by coaches (at least one coach per two subjects). Subjects attended on average 89 ± 2% of the training sessions.

Subjects were specifically instructed to continue their usual diet. However, no written reports on the dietary regime were obtained.

Specific training loads

Changes of training loads were assessed by the comparison of the loads after the 3 weeks of habituation to those at the very end of the intervention for RET and EET. For RET the average loads of the four exercises were compared.

Timed up & go and Berg balance scale

The Berg balance scale (BBS) (Berg et al. 1992) and the timed up & go (TUG) (Shumway-Cook et al. 2000) are functional tests designed for elderly people in order to assess their risk of falling (Lotscher et al. 2007). These tests were carried out at the beginning and at the end of the training period.

Body composition, muscle biopsies

Whole body composition (lean and fat tissue mass) was determined by dual energy X-ray absorptiometry (DEXA) (QDR-4500A, Hologic Inc., Bedford, USA). Thigh was defined as the part from *tuber ischiadicum* to the distal end of the femur, whereas the leg included the distal section of the limb below the *tuber ischiadicum*. Fat and lean values of thighs and legs include right and left extremities.

Biopsies were taken from 27 subjects (RET: 6 women, 7 men; EET: 7 women, 7 men) using the Bergström technique (Bergstrom 1975) from the mid thigh position of the M. *vastus lateralis* before and after the 12-week training period in a resting state, 48–72 h after the last exercise bout. Pre- and post-biopsies were collected from the same leg with the incision approximately 2 cm apart. For ethical reasons muscle biopsies were taken from subjects of the physical intervention groups only. Muscle samples were immediately frozen in liquid nitrogen cooled isopentane and stored in liquid nitrogen until required for further analysis.

Histochemistry

Selective myofibrillar ATPase inactivation and subsequent staining was processed at 12 µm cryostat cross-sections with preincubation at pH 4.5 and 10.5 as described by Billeter et al. (1980). Fibers were classified as type I, type IIA and type IIX. The type II fiber population consists of the sum of the type IIA type IIX fibers. On average, 351 fibers were counted per biopsy. Reliability of the technique and the technician was assessed by the test–retest method with 10 randomly chosen biopsies. Pearson product–moment correlation coefficient was 0.95. For the estimation of fiber type specific cross sectional areas a 30 × 30 µm grid was overlaid and points on fibers were counted. This procedure was applied in areas that appeared reasonably cross-sectioned (80 fibers per biopsy on average).

Maximal isometric extension of the legs (MEL)

Strength testing was performed as described by Lötscher et al. (2007). Subjects were fixed in a sitting position

(90° angle; ankle-knee-hip) on a force platform (Quattro Jump, Kistler Instrumente AG, Winterthur, Switzerland). They were verbally encouraged to push maximally against the platform for about 4 s. The force was permanently recorded with a resolution of 500 Hz. The best trial out of three was evaluated by determining the highest mean force over a one-second period. Normalization to the subject's body mass resulted in relative MEL.

Eccentric coordination

Estimation of eccentric coordination was carried out on the eccentric ergometer (Meyer et al. 2003). This parameter estimates a subject's ability to adjust the power of braking the pedals to the eccentric target load [W]. The appropriate load is self-monitored on a screen where the actual and the target load are graphically displayed in "real time" (Fig. 1). The deviation of actual from target load is estimated by the Root Mean Square (RMS; indicated by the hatched area in Fig. 1). The target load in the testing was set to 30 W for females and 50 W for males; subjects were tested over a 5-min period. In the EET group the post-test for eccentric coordination was carried out at the individual training load of each subjects' last training session (Table 1, 2).

Data analysis

Data are presented as mean ± SE. Interaction of training modality (CT, RET, EET) on functional parameters such as TUG, BBS, MEL and eccentric coordination was verified with an analysis of variance (ANOVA) for repeated measures and Tukey's Honest Significant Difference (HSD) post hoc test. For the ANOVA, the level of significance was set to $P < 0.05$ and marked with * and ** for $P < 0.01$, respectively. *P*-values in the figures are from Tukey's HSD and indicate the probability that differences between pre and post-intervention occur randomly. Analysis of sex specific improvements in MEL was analyzed in the eccentric group only using ANOVA with repeated measures. For the comparison of pre-post differences between groups we applied a Kruskal–Wallis ANOVA and verified significant results ($P < 0.05$) with Mann–Whitney *U* post hoc testing

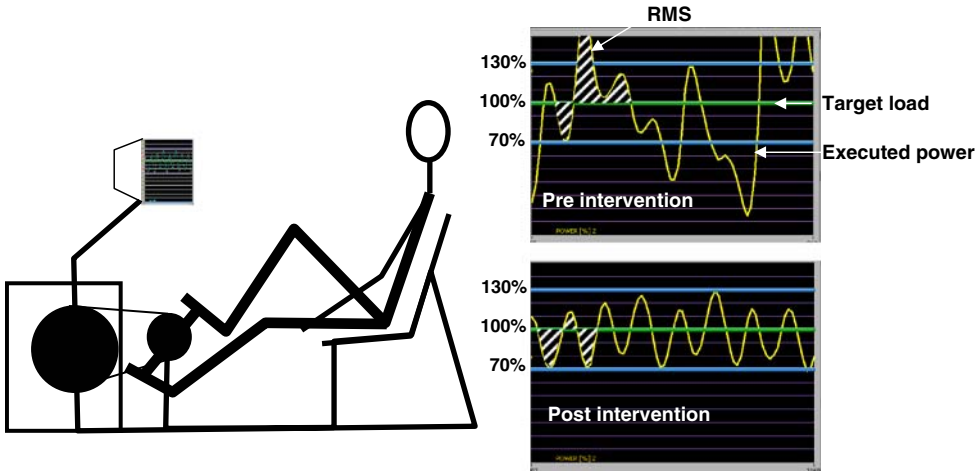

Fig. 1 Schematic set-up of the eccentric ergometer and evaluation of eccentric coordination. Root mean square (*RMS*) represents the hatched area comprised by the target load and the executed power. The smaller the RMS, the better the eccentric coordination. Representative illustrations of pre- and post-test graphs from EET subjects. Note the difference of the match of the executed power and the target load in the post-test

Table 1 Anthropometric characteristics of the study subjects

	Women (36)	Men (26)	CT (16)	RET (23)	EET (23)
Age (years)	80.4 ± 0.6	80.9 ± 0.6	81.8 ± 0.8	80.1 ± 0.8	80.3 ± 0.7
Height (cm)	161 ± 1	176 ± 1	166 ± 3	167 ± 2	168 ± 2
VO_{2max} (ml/min)	1210 ± 40	1940 ± 90	1380 ± 150	1640 ± 110	1500 ± 80
Body mass (kg)	65.5 ± 1.9	71.1 ± 1.7	70.5 ± 2.8	67.7 ± 2.6	66.1 ± 1.8
BMI (kg/m^2)	25.3 ± 0.7	23.1 ± 0.6	25.6 ± 0.9	24.3 ± 1.0	23.5 ± 0.6
Lean (kg)	42.7 ± 1	53.6 ± 1.3	48.4 ± 2.5	47.3 ± 1.7	46.5 ± 1.6
Fat (kg)	21.0 ± 1.3	15.4 ± 1.1	18.9 ± 1.3	19.1 ± 2.0	17.6 ± 1.3
Fat rel (%)	31.7 ± 1.1	21.2 ± 1.3	29.3 ± 2.1	26.7 ± 1.9	26.5 ± 1.6

Parameters are displayed as mean values ± SE. VO_{2max} = maximal oxygen uptake, BMI = body mass index, Lean = whole body lean content, Fat = whole body fat content, Fat rel = relative whole body fat content. Mean values did not differ among groups (ANOVA) but among sex (all except age) according to a two tailed student's *t* test ($P < 0.05$)

Table 2 Estimation of subjects' fiber type composition: parameters are displayed as mean values ± SE

	EET		RET	
	Pre	Post	Pre	Post
Number (%)				
Type I	55.2 ± 3.3	57.5 ± 2.2	52.3 ± 3.7	54.3 ± 3.9
Type IIA	34.4 ± 2.0	35.6 ± 1.9	32.7 ± 1.6	31.4 ± 2.4
Type IIX	10.3 ± 2.0	6.9 ± 1.6	15.1 ± 4.2	14.2 ± 4.9
Area (%)				
Type I	62.9 ± 2.7	63.1 ± 2.5	56.6 ± 3.3	56.5 ± 3.4
Type IIA	31.6 ± 2.2	32.6 ± 2.3	34 ± 1.7	35.2 ± 2.0
Type IIX	5.5 ± 1.2	4.2 ± 1.1	9.3 ± 3.3	8.4 ± 3.7
Area (μm^2)				
Type I	4,250 ± 210	4,392 ± 200	4,050 ± 240	4,030 ± 270
Type IIA	3,890 ± 340	3,570 ± 290	3,990 ± 490	4,430 ± 490
Type IIX	2,320 ± 230	2,220 ± 210	2,070 ± 200	1,950 ± 160

Top panel displays individual fiber numbers in percentage, middle panel displays the cross sectional areas of the same fiber population in percentage of all fibers and the last panel shows the estimated average cross sectional area in square micrometers

(M–W *U* test). The sex difference in eccentric coordination was verified with a two tailed student's *t* test. The level of significance was set to $P < 0.05$. Coefficients of correlation were calculated using Pearson Product Moment Correlation for pooled pre- and post-training datasets. All statistical analyses were carried out with the Statistica software package 6.1 (StatSoft (Europe) GmbH, Hamburg, Germany).

Results

Training specific improvements

Statistically significant improvements of the training loads could be recorded for RET and for EET subjects. RET subjects improved leg extension on average by 84.8% from 30.2 ± 2.2 to 55.8 ± 3.7 kg, while EET subjects increased average training load from 69.6 ± 4.3 to 314.8 ± 27.0 W (+352%). The increased training loads in EET overestimate the training progress since the subjects were ramped carefully to avoid muscle injury.

Timed up & go (TUG), Berg balance scale (BBS)

Subjects improved significantly in TUG from 7.37 ± 0.16 to 6.88 ± 0.16 s (−6.7 ± 0.2%) independent of training modality (EET −7.5 ± 0.2%; RET −7.3 ± 0.2%; CT −4.9 ± 0.5%). No significant improvements could be recorded for BBS (EET +1.7 ± 0.3%; RET +0.7 ± 0.3%; CT +0.7 ± 0.4%) since the study-subjects had on average already achieved 53.8 of maximal 56 points in the pre-test.

Body composition

The EET group experienced a reduction in whole body fat (−5.0 ± 1.1%) and thigh fat content (−6.9 ± 1.5%) not observed in RET (body: −0.6 ± 1.0%, thigh: −2.7 ± 0.9%) and CT (body: +1.4 ± 1.2%, thigh: +0.6 ± 1.9%). Subjects' relative thigh muscle mass increased significantly with EET (+2.5 ± 0.6%) and RET (+2.0 ± 0.3%) but not with CT (+0.4 ± 0.4%) (Fig. 2). Pre-Post differences between RET and EET were verified for body ($P = 0.002$) and thigh fat ($P = 0.03$) by a Mann–Whitney *U* test (M–W *U* test).

Muscle fiber types

A significant reduction of the type IIX/type II ratio was recorded for EET subjects (−22 ± 14%) but not for RET subjects (−8 ± 14%) (Fig. 3a). Type IIX/type II fiber ratio correlated significantly ($P < 0.01$) with body composition parameters: positively with body fat ($R = 0.75$) and thigh fat ($R = 0.70$) and negatively with relative thigh lean mass ($R = 0.69$). Biopsies from women displayed a significantly higher type IIX/type II fiber ratio than biopsy samples from men (women: 29 ± 5%; men: 19 ± 3%; two-tailed students *t* test, $P < 0.05$). Pre-post differences between RET and EET were not significant ($P = 0.2$; M–W *U* test).

Maximal isometric extension strength of the legs (MEL)

The EET group significantly improved MEL (+7.5 ± 1.7%), whereas no significant strength changes were noticed for RET (+2.3 ± 2.0%) and CT (−2.3 ± 2.5%). Improvements of EET subjects were even more pronounced when MEL was normalized to body mass (+8.4 ± 1.7%) (Fig. 4). However, pre-post differences in MEL were not significant between EET and RET ($P = 0.1$; M–W *U* test). Focusing on EET subject's relative MEL, exclusively women improved significantly (from 13.7 ± 2.8 to 14.4 ± 2.8 N/kg; +13.8 ± 2.5%), whereas in men the observed increase was not significant (i.e. from 16.9 ± 3.5 to 17.2 ± 3.6 N/kg; +4.8 ± 1.4%). The sex specific analysis was verified with a two tailed student's *t* test with a level of significance offset at 5%.

Eccentric coordination

The ability to match instantaneous muscle torque to eccentric target load was improved significantly by EET subjects (−43 ± 6% RMS) but not by RET (−13 ± 3%) and CT subjects (−12 ± 5%) (Fig. 5). The initial inferior coordination performance of women compared to men (Pre: women: 79.6 ± 7.3 RMS; men: 55.4 ± 6.7 RMS) was lost following EET (Post: women: 24.9 ± 1.0 RMS; men: 22.9 ± 2.3 RMS). Pre-Post differences between RET and EET were significant ($P = 0.02$) according to M–W *U* test.

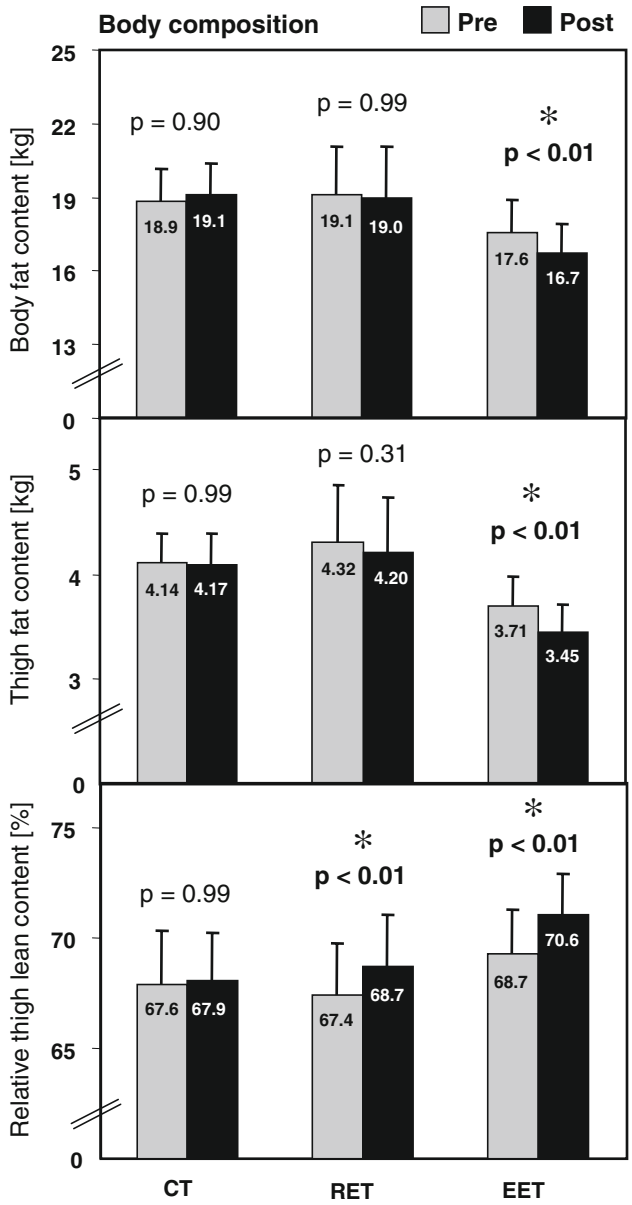

Fig. 2 Whole body fat, thigh fat and relative thigh lean content as assessed by dual energy X-ray absorptiometry. *Bars* (*grey* pre; *black* post) represent mean contents ± SE in kg (fat) and % (lean) of CT ($n = 14$), RET ($n = 21$) and EET subjects ($n = 19$). (ANOVA with repeated measures; $*P < 0.05$; indicated P-values of Tukey's HSD Post hoc test)

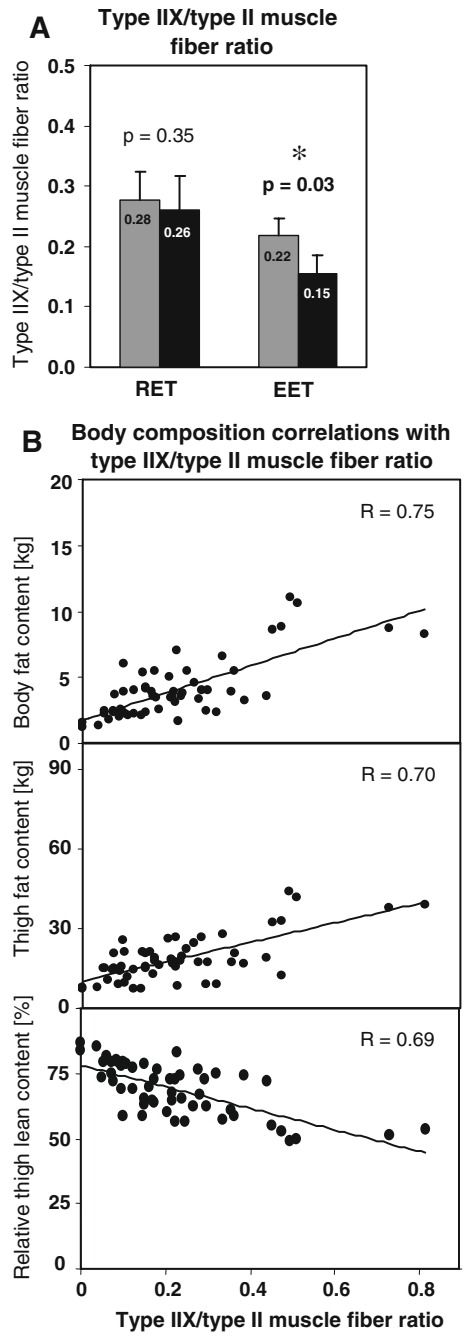

Fig. 3 **a** Ratio of type IIX/type II muscle fiber content of EET ($n = 14$) and RET ($n = 13$). *Bars* represent means of pre- (*grey*) and post-values (*black*) ± SE. (Indicated P-values of one tailed paired student's t test; $*P < 0.05$). **b** Highly significant ($P < 0.01$) correlation of fibertype composition with body fat, thigh fat and relative thigh lean content. Data points consist of individual pre- or post-values of body composition parameters with the corresponding type IIX/type II muscle fiber ratio evaluated from muscle biopsies ($n = 56$)

Discussion

This study shows eccentric ergometer exercise and resistance training to be well tolerated by elderly. Despite a low training frequency of just two sessions per week we observed a moderately positive outcome. EET improved leg muscle strength, body composition and eccentric muscle coordination in elderly. The positive effects of RET were similar in magnitude but mostly statistically not significant. The fact that strength gain in our study was measured on a training independent device underlines the task specific improvements in most other studies which don't necessarily reflect the "useful" benefits.

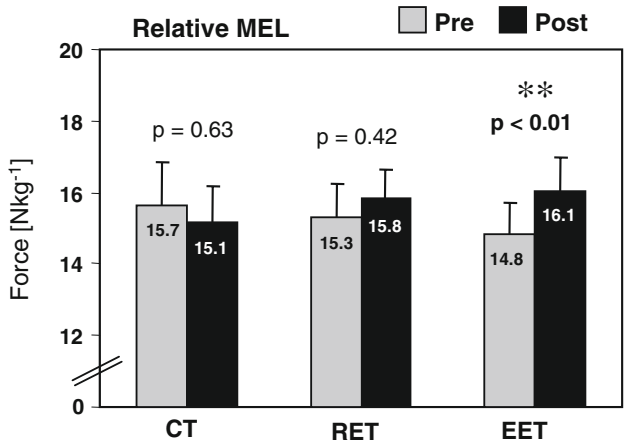

Fig. 4 Maximal isometric extension strength of the legs (*MEL*) normalized to body weight. *Bars* (*grey* pre; *black* post) represent mean leg force in N kg^{-1} of CT ($n = 13$), RET ($n = 18$) and EET subjects ($n = 19$) ± SE. (ANOVA with repeated measures; $^{**}P < 0.01$; indicated *P*-values of Tukey's HSD Post hoc test)

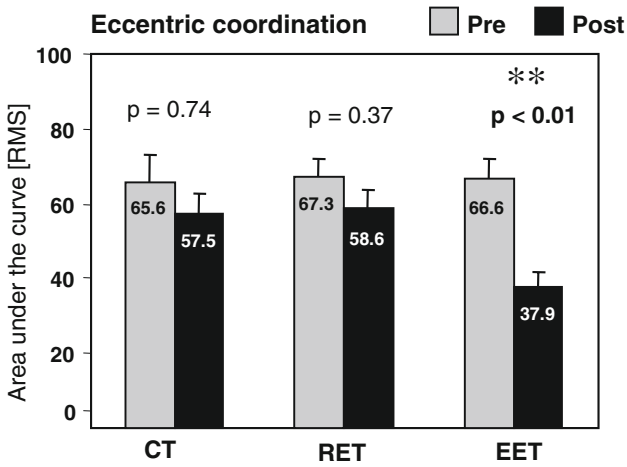

Fig. 5 Eccentric muscle work load as assessed on the eccentric ergometer. *Bars* represent root mean square (*RMS*) of CT ($n = 14$), RET ($n = 19$) and EET ($n = 19$) ± SE. (ANOVA with repeated measures; $^{**}P < 0.01$; indicated *P*-values of Tukey's HSD Post hoc test)

A major limitation of our study was the low training frequency of our subjects which was chosen to maximize adherence for independently living volunteers, characterized by an active lifestyle and concomitant duties and obligations. Together with the exceptionally good physical condition of our subjects, functional tests such as TUG and BBS were not sensitive enough to detect differences. The modest increase in strength (as measured by MEL) seen in our study when compared to strength increases reported in other studies with elderly subjects (Hauer et al. 2001; Kryger and Andersen 2007) has several reasons: (1) our subjects were already in exceptional physical condition. (2) We used a low training frequency with just two sessions of 20 min specific training per week. We believe this to be a realistic sustainable training program for this population. (3) Our force measurement setting was devised to mimic an every day situation and thus independent from the training procedure for both training modalities. The randomisation procedure had to be adapted because not all of our subjects agreed to get biopsied. Due to medical reasons (i.e. use of anticoagulants) we could not biopsy some of the subjects. Subjects who fulfilled biopsy criteria were then randomized to one of the physical intervention groups (RET, EET). We did not collect biopsies from CT subjects as this would have represented an unwarranted risk. Since there were no significant initial functional differences between the groups we think it unlikely that the de facto stratified randomisation influences our results.

A major concern in this study was to avoid the negative consequences of eccentric exercise, consisting of muscle damage i.e. DOMS (Friden et al. 1983). To this end, we chose very low initial training loads (30 and 50 W for women and men, respectively) and short training times (5 min). Reported discomfort after eccentric exercise was thus, between 0 and 1 and never exceeded a value of 4 on a VAS scale of 0–10. The prerequisite for safe eccentric exercise is to tailor and monitor eccentric load individually. This was achieved with a computer-based visual feedback system in which subjects were matched the instantaneous training load to a target load displayed on a computer screen (Fig. 1). Matching of the training load to a target load turned out to be a demanding coordination task which required coaching and some practice and turned out to be feasible for all except one subject. The software for our eccentric ergometer allows for quantitative assessing the deviation of the eccentric performance from the required load (eccentric coordination; see Fig. 1). Not surprisingly, only EET-trained subjects were able to improve eccentric coordination (by 43%) significantly over the entire training period. The initial difference in coordination between better performing males than females was lost after training, as women improved their eccentric coordination more than men. It is currently difficult to assess the relevance of the massive improvement of eccentric coordination as defined in our setting. Matching eccentric performance to a target load is a complex task involving the integration of visual feedback with motor control. In a previous study using the same eccentric ergometer on world cup level alpine skiers, a positive correlation was found between the eccentric coordination and success in ski slalom races (Vogt et al. 2003). Whether improvements in eccentric coordination results in a lowered risk of falling needs further evaluation.

In the current study, the risk of falling was assessed by standard tests such as TUG and the BBS. All our subjects ranked in the lowest risk category at the outset of the study. For TUG, subjects performing the task in less than 14 s belong to the category of people with no increased risk of

falling. The subjects in our study performed this task in the pre-test in 7.4 s. For TUG we still found a significant 7% improvement, independent of training modality. Other studies (Hauer et al. 2001; Kryger and Andersen 2007; Lastayo et al. 2002) in which frail or reconvalescent elderly subjects were trained showed improvements of TUG and leg strength in the order of 50%. We see the failure of the standard risk assessment tools TUG and BBS, to demonstrate large improvements as a consequence of the good physical condition of our subjects at the outset of the study (Lotscher et al. 2007).

Leg strength was assessed in this study by measuring Leg strength developed in a restrained sitting position (MEL). Relative MEL increased significantly by 8.4% in subjects only after eccentric training. We believe that the small but significant improvement in MEL in EET subjects is biologically relevant. Small gains in maximal performance may cause larger improvements in submaximal performance as a consequence of the non-linear relationship of power versus time or maximal versus repeated activity (Dufour et al. 2006; Wilkie 1985). Maximal performance testing has the advantage of yielding reliable results (Schroeder et al. 2007), while submaximal performance is difficult to assess reliably, but it is more relevant in order to characterize practical benefits for the subjects.

The assessment of body composition showed an unexpected but significant 5% decrease in body fat content along with a significant 6.9% decrease in thigh fat content in EET subjects only. Both RET and EET subjects showed a significant increase in relative thigh muscle mass after training. This relative increase was due to an increase in thigh muscle mass (more pronounced with RET), combined with a decrease in thigh fat mass (significant with EET). From these estimates we assume that the observed increase in leg extension strength in EET (reported above) cannot entirely be attributed to a structural change of muscle tissue, but seems to be a consequence of functional (i.e. neural) improvements. It is difficult to assess the potential benefit of a 5% decrease in body fat content in an elderly population with EET; however, a simultaneous gain of muscle mass with a decrease of body fat counteracts the sarcopenia that usually develops with age.

The muscle fiber type analysis showed two major results: a significant correlation between the type IIX/type II ratio and body composition and a decrease of the type IIX/type II ratio exclusively with EET. The type IIX/type II ratio describes the fraction of the type IIX fibers from the type II fiber pool. It can be assumed that the lower typeIIX/type II ratio of leaner subjects is related to physical activity and lifestyle. It has previously been described that physical activity in elderly leads to a decrease of type IIX fibers (representing the most anaerobic muscle fiber type) in favor of more aerobic type IIA fibers (Herbison et al. 1982). Since types I and IIA muscle fibers possess a larger oxidative capacity than type IIX fibers (Herbison et al. 1982) they are better able to couple ATP regeneration with fatty acid and carbohydrate catabolism. In fact, Kriketos et al. (1996) showed a significant positive correlation of relative body fat content with type IIX fibers as well as negative correlations with oxidative enzyme activity (citrate synthase, hexokinase) and insulin sensitivity. This result is in accordance with the limited capacity of type IIX fibers to utilize fatty acids as substrates for ATP regeneration due to their relatively small mitochondrial density (Gueguen et al. 2005). The observed changes of the type IIX/type II ratio with individual changes in body composition parameters indicates the maintenance of plasticity of muscle fibers and body composition as influenced by physical training into old age even at low exercise frequencies.

The higher proportion of type IIX fibers in women may reflect their initial lower fitness possibly due to their less active lifestyle. Due to their greater potential for improvements (strength, coordination) we find women to profit more from training (MEL, eccentric coordination) than men. It has been suggested that muscle fiber composition is implicated in the correlation of body fat content and risk for non-insulin-dependent diabetes mellitus (NIDDM) (Jensen et al. 2007). This view is compatible with our findings, as we find a positive influence of physical training (EET) on fiber composition and potentially reducing the risk of developing age-dependent metabolic diseases such as NIDDM.

In conclusion, at the low training frequency of our study EET was similarly successful as RET in improving muscle functional and structural parameters analyzed in this study. EET significantly improved MEL, body composition and eccentric coordination. EET further showed the persistence of muscular plasticity in elderly as evidenced by a decrease of the type IIX/type II muscle fiber ratio. The latter was found to correlate with body composition in all the subjects studied. These findings suggest that eccentric exercise modalities, given their low metabolic costs, merit further evaluation with regard to their potential to improve muscle motor and metabolic functionality in elderly.

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Curriculum vitae

of Klossner Stephan

Particulars

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Situation

30.05.1981	Birth in Thune
1988 – 1997	Primar and Secondary School, Reichenbach im Kandertal
1997 – 2001	Grammar School, Interlaken
2001 – 2005	Studies in Biology, University of Berne
2004 – 2005	Diploma work, Institute of Anatomy, University of Berne
2005 – 2007	Grammar School Teacher formation, University of Berne
2006	50% engagement as Assistant III in the congress organization for the ECSS2006, Institute of Anatomy, University of Berne (8 month)
2006 – 2009	Dissertation on effects of mechano-transduction on translation in skeletal muscle, Institute of Anatomy, University of Berne

Degrees

2001	Matura, Type D
2005	Biologist, diploma direction: Cell biology
2007	Teacher in Biology, grammar school (Sekundarstufe II)

About me

Hobbies:	Sport in general, particularly: - Football (Official Referee in the Challenge League) - Jogging, Skiing, Biking, Squash, Inline bladeing Traveling

International Congresses

2005	USGEB, Zürich, CH	Poster
2005	DISS, Lausanne, CH	Poster and Posterpresentation
2006	ECSS, Lausanne, CH	Congress Organizer
2007	USGEB, Basel, CH	Poster
2007	Omics, Ascona, CH	Poster and Posterpresentation
2007	DKF, Berne, CH	Poster
2008	USGEB, Lausanne, CH	Poster
2008	ECSS, Estoril, Portugal	Oral presentation
2008	JSPFSM, Beppu, Japan	Oral presentation
2008	SGSM, Fribourg, CH	Oral presentation
2009	USGEB, Interlaken, CH	Poster

Awards

2005 Special award of the committee, DISS, Lausanne, CH
for the work: Klossner, Däpp, Schmutz, Vogt, Hoppeler, Flück
"The gene response to eccentric exercise in human skeletal muscle"
Prize money: CHF 1000.-

2008 Young Investigator Award", 5th plyace, Annual Congress of the European College of Sport Science 2008, Estoril, Portugal
for the work: Klossner, Durieux, Freysennet, Flück
"FAK transmits mechanical stress towards increased protein synthesis in skeletal muscle"
Prize money: Euro 500.-

2008 Invitation to the Annual Congress of Japanese Society of Physical Fitness and Sports Medicine in Beppu, Japan
Prize money: CHF 4000.-

Publications

As First Author:

Klossner S, Däpp C, Schmutz S, Vogt M, Hoppeler H, Flück M.
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Klossner S, Hoppeler H, Flück M.
Tenotomy activates FAK and S6K in rat soleus muscle
Manuscript in preparation

As Co-Author:

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Manuscript in preparation

Hoppeler H, Lurman G, Mueller M, Klossner S, Baum O.
Molecular mechanisms of muscle plasticity with acute and chronic exercise
Manuscript in preparation for the "Handbook of Physiology"

Declaration of Originality

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I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.
All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.
I am aware that in case of non-compliance, the Senate is entitled to divest me of the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 20, of 17 December 1997.

Berne, 19th of June 2009 Signature

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