DISSERTATIONES KINESIOLOGIAE UNIVERSITATIS TARTUENSIS
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THE EFFECT OF GLUCOCORTICOID MYOPATHY, UNLOADING AND RELOADING ON THE SKELETAL MUSCLE CONTRACTILE APPARATUS AND EXTRACELLULAR MATRIX

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Dissertation is accepted for the commencement of the degree of Doctor of Philosophy in Exercise and Sport Sciences (Functional Morphology) on 26 October 2007 by the Council of the Faculty of Exercise and Sport Sciences, University of Tartu, Tartu, Estonia.

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Commencement: 10 December 2007

Publication of this dissertation is granted by the University of Tartu

ISSN 1406–1058
ISBN 978–9949–11–763–5 (trükis)
ISBN 978–9949–11–764–2 (PDF)

Tartu Ülikooli Kirjastus
www.tyk.ut.ee
Tellimus nr 482

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LIST OF ORIGINAL PUBLICATIONS

The work is based on the following publications:

Riso E.-M., Ahtikoski A.M., Alev K., Kaasik P., Pehme A., Seene T. Relationship between extracellular matrix, contractile apparatus, muscle mass and strength in case of glucocorticoid myopathy. Journal of Steroid Biochemistry and Molecular Biology, 2007, in press.

Riso E.-M., Ahtikoski A.M., Takala T.E.S., Seene T. The effect of unloading and reloading on the extracellular matrix in skeletal muscle: changes in muscle strength and motor activity. Journal of Biology of Sport, 2007, accepted.

Ahtikoski A.M., Riso E.-M., Koskinen S.O.A., Risteli J., Takala T.E.S. Regulation of type IV collagen gene expression and degradation in fast and slow muscles during dexamethasone treatment and exercise. Pflügers Archive – European Journal of Physiology, 2004, 448: 123–130.

Riso E.-M., Ahtikoski A.M., Umnova M., Kaasik P., Alev K., Seene T., Takala T. Partial prevention of muscle atrophy in excessive level of glucocorticoids by exercise: effect on contractile proteins and extracellular matrix. The Baltic Journal of Laboratory Animal Science, 2003, 13: 5–12.

ABBREVIATIONS

3-MeHis	3-methylhistidine
BM	basement membrane
cDNA	complementary deoxyribonucleic acid
ECM	extracellular matrix
EDL	*extensor digitorum longus* muscle
FT	fast-twitch
GM	*gastrocnemius* muscle
kDa	kilodalton
LO	lysyl oxidase
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MyHC	myosin heavy chain
MyLC	myosin light chain
PLA	*plantaris* muscle
RNA	ribonucleic acid
SOL	*soleus* muscle
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
ST	slow-twitch
TIMP	tissue inhibitor of metalloproteinases

INTRODUCTION

Clinical practice has shown that excessive levels of glucocorticoids can promote tissue catabolism and atrophy by simultaneously accelerating the rate of protein breakdown and amino acid efflux and thus decreasing the rate of protein synthesis (Hickson *et al*., 1990). It is a well known fact that Cushing's syndrome and clinical treatment with glucocorticoids lead to a marked reduction in muscle weight, wasting of muscle, loss of strength, a selective atrophy of fast-twitch muscle fibres and changes in contractile proteins turnover (Seene, 1994). Exercise has been used to study the prevention of the muscle atrophy, associated with excessive levels of glucocorticoids. Four contractile activity model systems have given the supposed effect: endurance exercise, resistance exercise, functional overload after tenotomy of synergetic muscles and *in vitro* cell culture stimulation (Czerwinsky & Hickson, 1990). Our previous studies have shown that the short-lasting and more intensive exercise gives a better result: the decrease of muscle mass delays significantly (Seene, 1994).

In addition to the decrease of muscle mass, the glucocorticoid treatment influences the metabolism of collagen in skeletal muscle and tendons. Major collagens identified in skeletal muscle are types I and III in epi-, peri- and endomysium and types IV and V in the basement membranes surrounding individual muscle fibres, smooth muscle fibres of blood vessels, Schwann's cells and spindle capsule cells. The basement membranes are a complex protein network including type IV collagen, laminin, nidogen, proteoglycans and glycoproteins. Type IV collagen is a major component in basement membrane and therefore plays a critical role in the cellular arrangement in muscle tissue. Glucocorticoids are known to inhibit the synthesis of collagens by reducing mRNA expression of types I and III collagens and the activity of enzymes participating in the post-translational modification of collagens (Takala & Virtanen, 2000).

The decrease of physical activity, especially hypokinesia has a catabolic effect on both the contractile apparatus and extracellular matrix of the skeletal muscle. Inactivity causes the formation of muscle atrophy and the decrease of the synthesis of contractile proteins, resulting in the disturbance of the collagen metabolism (Takala & Virtanen, 2000).

Changing demands in muscle tissue, for example immobilization (Ahtikoski *et al*., 2003) and acute physical exercise (Koskinen *et al*., 2001), cause remodelling of the muscular extracellular matrix. In this remodelling, matrix metalloproteinases (MMPs) play a critical role. Glucocorticoids, as anti-inflammatory agents, decrease the levels of MMPs (Kylmäniemi *et al*., 1996; Morin *et al*., 1999; Xu *et al*., 2001).

Although the effect of glucocorticoids as well as hypokinesia on the skeletal muscle has been studied intensively during last 25 years, the peculiarities of the influence of the both factors on different skeletal muscles, their myofibrillar apparatus and the extracellular matrix are still not convincingly clarified.

REVIEW OF LITERATURE

1. Contractile apparatus of the skeletal muscle

1.1. Characteristics of skeletal muscle fibres

Muscle fibres, the contractile elements of which are actin and myosin filaments, can be classified by their metabolic, functional and morphological properties. Corresponding to metabolic enzyme histological analyses muscle fibres can be classified into three major fibre types: fast-twitch glycolytic, fast-twitch oxidative glycolytic and slow-twitch oxidative fibres. Histochemical staining of myosin according to its ATPase activity can be used for identification of muscle fibres of different types (Brooke & Kaiser, 1970). The basic skeletal muscle fibre types can be classified as slow fibre type I, and fast fibre type II with subtypes IIA, IIB and IIAB (Baldwin, 1984). The different fibre types are not strictly limited, but form a continuum between the slow and fast extremes (Thomason *et al*., 1986; Staron & Pette, 1987). Type IIX (D) muscle fibre is similar to that of type IIB according to myofibrillar actomyosin ATPase acid stability (Termin *et al*., 1989; Schiaffino *et al*., 1994).

The physiological and biochemical functions of skeletal muscle fibres provide a high degree of specialization. Type I fibres or slow-twitch fibres are innervated by frequently active motoneurons on low frequencies and have slow conduction velocities. They can sustain tension for longer periods than type II fibres.Aerobic energy production is typical to type I fibres because of the high activity of oxidative enzymes. Type II fibres fire at high frequencies and have fast conduction velocities, but can maintain tension for only short periods. Myosin ATPase activity is high in type II fibres. The subclasses, IIA and IIB, differ in energy metabolism, type IIB fibres having high glycolytic enzyme activity, whereas type IIB fibres also have high oxidative capacity. The intermediate type IIAB fibres share biochemical properties of type IIA and IIB fibres. The type IIX(D) fibres are rich in oxidative enzymes, their moto units are relatively resistant of fatigue, and they exist in muscles sustaining contractile activity (Termin *et al*., 1989; Schiaffino *et al*.,1994).

Nowadays skeletal muscle fibre types can be distinguished mainly according to their myosin heavy chain (MyHC) composition. Fibres expressing single MyHC profile are called pure fibres and the fibres expressing more than one MyHC isoform are termed hybrid fibres, respectively (Pette & Staron, 2001). Pure fibre types, for example, are type IIB, type IID/X, type IIA, and type I, express MyHC IIb, MyHC IId/x, MyHC IIa and MyHC Iβ, respectively, whereas hybrid fibres express more than one MyHC isoform. The percentage of hybrid fibres increases remarkably in transforming muscles (Pette & Staron 2001).

1.1.1. Skeletal muscle myosin

Myosin is the main contractile protein of striated muscle, which converts chemical energy to mechanical work. The native myosin contains one pair of heavy chains (MyHC) and two pairs of light chains (MyLC).

Nine distinct MyHC isoforms have been detected in mammalian muscles: MyHCβ/slow, MyHC IIa, MyHC IIx, MyHC IIb, MHC embryonic, MyHC neonatal, MyHCα, MyHC extraocular and MyHC masticatory. Only one slow (MyHCI) and two fast type MyHC isoforms (MyHC IIa and MyHC IIx) are expressed in human limb muscles. Although additional MyHC genes (MyHC Iα, MyHC IIb) are found in humans, these MyHC isoforms have not been detected in limb muscles on the protein level (Weiss *et al*., 1999).

Each MyHC is associated with a pair of MyLC. MyLC consists of essential light chain and regulatory light chain. Essential MyLC contains two fast (MyLC1f and MyLC3f) and one slow (MyLC1s) isoform. Regulatory part of MyLC includes both slow and fast type MyLC isoforms (MyLC2s, MyLC2f) (Bottinelli, 2001).

1.1.2. Factors influencing fibre type transitions

Various factors – development, innervation, increased and decreased neuromuscular activity, overloading and unloading, hormones, and aging – have been shown to influence the phenotypic expression of skeletal muscle fibres (Baldwin & Haddad, 2001, 2002). The factors decreasing neuromuscular activity or load result in slow-to-fast protein isoform and fibre type transitions, whereas those factors increasing neuromuscular activity or load cause transitions in the opposite direction (fast to slow) (Pette & Staron, 1997).

2. Extracellular matrix in skeletal muscle

The extracellular matrix (ECM) is formed by complex molecular networks, which determine the architecture of a tissue and regulate various biological processes (Aumailley & Gayraud, 1998). The functional significance of ECM in muscle pathologies and enhanced physical activity has to be clarified more precisely.

2.1. Functions and composition of extracellular matrix in skeletal muscle

The composition and function of ECM from the historical point of view has been shown that the intramuscular connective tissue, accounting for 1–10% of skeletal muscle, has multiple functions (Jaspers *et al*., 1999). It provides a basic mechanical support for vessels and nerves. The connective tissue ensures a passive elastic response of the muscle.

It is important to accept that both the tendon and the intramuscular connective tissue interact closely with the contractile elements of the skeletal muscle to transmit force (Sanes, 2003; Kjær, 2004). The force transmission from the muscle fibres is not only transformed to the tendon and the subsequent bone via the myotendinous junctions, but also via the lateral transmission between neighboring fibres and fascicles within the muscle (Knight *et al*., 1993). The tension developed in one part of the muscle can be transmitted via shear links to other parts of the muscle (Lieber & Friden, 2000). The perimysium is especially capable of transmitting tensile force (Trotter & Purslow, 1992).

As it was demonstrated already more than 30 years ago, muscular flexibility is partly provided by collagen. For this purpose the organization of fibrils and fibres is critical, because individual collagen molecules, fibrils, and fibres are intrinsically inextensible. The extensibility of collagen results from the straightening of curved fibrils and fibres (Viidik, 1973).

The ECM consists of a variety of substances, of which collagen fibrils and proteoglycans are the most widespread (Curwin *et al*., 1998). In addition to the proteoglycans, the hydrophylic ECM includes several other proteins such as noncollagen glycoproteins (Scott, 2001). The skeletal muscle ECM is organized in three levels: the epimysium surrounds the entire skeletal muscle, the perimysium surrounds muscle bundles consisting of a variable number of muscle cells, and the endomysium outlines the individual muscle fibres. The most abundant protein of the extracellular matrix is collagen, accounting for 20–25% of all protein in the whole body. At present, 21 different collagen types have been identified (Koch *et al*., 2001; Bosman & Stamenkovic, 2003).

2.2. Fibril forming collagen types in skeletal muscle

Type I and type III collagen are the most abundant fibril forming collagens in the skeletal muscle. Type I collagen dominates in the intramuscular collagen content – reported from 30% up to 90% of total collagen (Bailey, 2001; Bosman & Stamenkovic, 2003). The epimysium consists mainly of type I collagen with minor amounts of type III collagen (Light & Champion, 1984; Järvinen *et al*., 2002). Equal amounts of both collagen types are found in the perimysium. In the endomysium, type III collagen is the predominant form and only small amounts of type I collagen are found (Light & Champion, 1984; Järvinen *et al*., 2002). Slow muscles contain more type I collagen than type III collagen, the proportion of type III collagen is greater in fast muscles (Miller *et al.,* 2001).

Type I collagen is the major stress-tolerant fibrillar collagen in the muscle. It has a high tensile strength and limited elasticity and is thus well-suited for force transmission. Type III collagen, the other main fibrillar collagen, has a structure and arrangement similar to that of type I collagen, but it forms thinner and more

elastic fibres. The fibres of type III collagen can also form copolymers with those of type I collagen (Keene *et al*., 1987). Collagens I and III are fibril forming and serve as a supportive structure in the muscle tissue. They attach myocytes and muscle bundles to each other (Järvinen *et al*., 2002). Also nerves and capillaries are surrounded and attached to muscle by collagen (Järvinen *et al*., 2002). Type V collagen is also fibril forming and can be found in the endo- and perimysium in smaller amounts than the collagen types I and III (Light & Champion, 1984). Collagens III and V are known to copolymerize with type I collagen and they may have a role in collagen fibre diameter regulation (Birk *et al*., 1990; Fleischmayer *et al*., 1990; Birk & Mayne, 1997). Type V collagen is considered to form the core of the fibrils, and collagens I and III copolymerize around this core (Aumailley & Gayraud, 1998). Type II and XI collagens are also fibril forming and have been detected in the skeletal muscle only at mRNA level (Sandberg *et al*., 1993). Type V and XI collagens form heterotypic molecules and can be considered as a single kind of collagen (Prockop & Kivirikko, 1995). Fibril associated collagens with interrupted helix (FACIT) types XII and XIV are located only in the perimysium (Listrat *et al*., 2000). These FACIT collagens associate with the surface of interstitial collagen fibrils and possibly act as molecular bridges among or between fibrils and other components of the ECM (Wälchli *et al*., 1994). Although mRNAs of the other members of FACIT subfamily (IX, XVI, XIX, XXI) are detected in the skeletal muscle, the respective proteins have not been found (Lai & Chu, 1996; Bönnemann *et al*., 2000; Chou & Li, 2002).

2.3. Nonfibrillar collagens of skeletal muscle

Nonfibrillar collagens of the skeletal muscle are mainly located in the basement membranes.The basement membrane (BM) is a highly specialized sheet of the connective tissue surrounding individual muscle fibres, blood vessels, Schwann's cells and the spindle capsule cells. The components of the BM are the regulators of many biological activities such as cell growth, differentiation and migration which influence tissue development and repair (Erickson & Couchman, 2000). Integrins attach muscle cells to ECM and serve as the force-transmitters between ECM and the contracting components inside the muscle cells (Aumailley & Gayraud, 1998). They connect laminin to the cell membrane to form the inner layer of basement membrane (Aumailley & Gayraud, 1998).

Type IV collagen is a major component in the basement membrane and therefore plays a critical role in the cellular arrangement in the muscle tissue (Kühl *et al*., 1984). It is an integral component of basement membrane and forms a covalently stabilized polymer network around the muscle fibres (Yurchenko & O'Rear, 1994). Type IV collagen molecules form a mesh-like structure outside the laminin layer and give stability to the BM (Kühn, 1995).

Laminin and type IV collagen are connected to each other by nidogen-1 in the muscular basement membranes (Ries *et al*., 2001; Salmivirta *et al*., 2002). As a part of the flexible basement membrane, type IV collagen network is interconnected with other extracellular matrix compounds and sarcolemmal proteins, being consequently exposed to stretching effects during muscle contraction (Han *et al*., 1999a; Kovanen & Takala, 2001; Ahtikoski *et al*., 2004).

Type VI collagen interacts with type IV and type I collagens (Kuo *et al*., 1997), providing a link between the basement membranes and the surrounding matrix. Collagens XV and XVII belong to the multiplexin subfamily of nonfibrillar collagens (Erickson & Couchman, 2000) and are located in the basement membrane zone (Myers *et al*., 1996; Sasaki *et al*., 2000). Collagens XV and XVIII may have a role in stabilizing the muscle cells (Eklund *et al*., 2001). Type XIII collagen is the transmembrane protein (Hägg *et al*., 1998) which is capable of binding certain basement membrane proteins (Tu *et al*., 2002). It probably provides a link between the muscle cell and its basement membrane (Kvist *et al*., 2001). Type XIII collagen is concentrated in the myotendinous junctions (Hägg *et al*., 1998).

2.4. Collagen synthesis in the skeletal muscle

The synthesis of collagen is similar to other proteins, consisting of genetic transcription with messenger ribonucleic acid (mRNA) and ribosomal translation of the mRNA to prepro *α*-chains. In the skeletal muscle, collagens are expressed principially by fibroblasts, and their biosynthesis is characterized by the presence of an extensive number of co- and posttranslational modifications of the polypeptide chains (Prockop & Kivirikko, 1995; Trackman, 2005). Gross fractional synthesis rate for collagen is about 5% a day in the skeletal muscles of young adult rats (Mays *et al*., 1991), whereas the fractional synthesis rate for total protein is about 11–15% /day (Goldspink *et al*., 1986).

Collagen is a protein with three polypeptide chains where each chain contains at least one stretch of the repeating amino acid sequence $(Gly\text{-}X\text{-}Y)_n$ and X and Y can be any amino acid (often proline and hydroxyproline, respectively). Both fibrillar and non-fibrillar collagens consist of three long polypeptide chains, which may or may not be identical and combine together via their $(Gly\text{-}X\text{-}Y)_n$ sequencies to form a collagen triple helix. The molecular organization of different collagen types differs so that type I collagen is a heterotrimer of two identical α1(I)chains and one *α*2(I) chain, whereas type III collagen is a homotrimer with *α*1(III) chains. The repeating unique amino acid sequence Gly-X-Y, where the glycine is in every third position, has no interruptions in the fibril-forming collagen types, whereas a considerable number of interruptions occurs in the nonfibrillar collagens. The Gly-X-Y repeat unit gives requirements for coiling the three *α*-chains tightly around one

another. Proline and 4-hydroxyproline residues appear frequently at the X- and Y positions, respectively, and promote the stability of the triple-helix and the structure of collagen as a whole. The structure of type IV collagen genes is distinctly different from those of fibril forming collagens. The most common form of type IV collagen consists of two α1(IV) chains and one α2(IV) chain, although the combinations of α3(IV) and α4(IV) as well as α5(IV) and α6(IV) are found in some basement membranes (Prockop & Kivirikko, 1995).

2.4.1. Modifications of the polypeptide chains

An exception to the synthesis of other proteins is that collagen synthesis is characterized by an extensive number of co- and posttranslational modifications of the polypeptide chains. About three decades ago it was shown that the intracellular modifications of polypeptide chains involve hydroxylation and glycolysation reactions to form the procollagen. Hydroxylation of proline, the reaction catalyzed by prolyl 4-hydroxylase (P-4-H), influences the stability of the triple-helical structure of collagen (Berg & Prockop, 1973). The triple-helix formation of the pro-α-chains prevents any further hydroxylation. Intracellular events of collagen synthesis include also 3-hydroxylation of proline residues, hydroxylation of lysine residues and glycosylation of certain hydroxylysine residues of propeptides. The assay of prolyl-4-hydroxylase activity has been commonly used to estimate the changes in the rate of collagen synthesis (Takala *et al*., 1983; Kovanen *et al*., 1984). Fibrillar collagens are secreted as soluble procollagens, which are converted to collagen by the cleavage of C- and N terminal propeptides by procollagen N- and C-proteinases. Extracellular modifications in the collagen synthesis involve an ordered self-assembly for the formation of collagen fibrils and the crosslink formation to make the fibrils stable. The stabilization of the fibrils is provided by covalent cross-links generated by the conversion of some of the lysine and hydroxylysine residues to aldehyde derivates by lysyl oxidase (Laurent, 1987; Prockop & Kivirikko, 1995; Kadler *et al*., 1996). Lysyl oxidase (LO) is a key enzyme in the extracellular modification of collagen (Kagan & Li, 2002). LO, an amine oxidase expressed and secreted by fibrogenic cells, plays a critical role in the formation and repair of the extracellular matrix (ECM) by oxidizing lysine residues in elastin and collagen, thereby iniating the formation of covalent crosslinkages which stabilize these fibrous proteins (Kagan & Li, 2002). Type IV collagen molecules form their network with different processes. A tight meshwork is formed by irregularly branching lateral associations of the triple helical regions (Yurchenko & O'Rear, 1994).

2.5. Degradation of collagens

Degradation of collagen represents the obligatory step of a turnover and the remodelling of the connective tissue and during the mechanical loading of fibroblasts and extracellular matrix structures. Both intracellular and extracellular degrading pathways are present, using either lysosomal phagocytosis or ECM proteinases, respectively (Everts *et al.*, 1996; Gianelli *et al*., 2005). Collagens can be degraded prior to or after their secretion from the cell. Secreted collagen is degraded mainly by two different routes: proteolytic and phagocytotic. Proteolytic degradation occurs mainly through matrix metalloproteinase (MMP) activity. Macrophages remove ECM components, although also fibroblasts are able to the phagocytosis and degradation of collagen fibrils (Everts *et al*., 1996). Degradation is continued by specific proteinases and the collagen fragments are phagocytozed by cells and processed by lysosomal enzymes (Cimpean & Caloianu, 1997). About 26% of newly synthesized collagen is degraded per day in young adult rats (Mays *et al*., 1991). The most recently synthesized collagen seems to be more susceptible to degradation than mature collagen (Laurent, 1987).

2.5.1. Matrix metalloproteinases

Collagen degradation is initiated extracellularly by matrix metalloproteinases (MMPs), which are presented in tissues mostly as latent proMMPs (Asahi *et al*., 2000; Balbin *et al*., 2001). MMPs are a family of zinc-dependent proteolytic enzymes that function mainly in the ECM (Carmeli *et al*., 2004). The activation of specific matrix metalloproteinases has been implicated in degradative and atrophic changes in the ECM after muscle injury or in various myopathic conditions. These matrix metalloproteinases may cause structural and physiological alterations to the basal lamina and sarcolemma of myofibres, leading to uncontrolled influx and efflux of ions and subsequent myopathy (Kherif *et al*., 1999; Kieseier *et al*., 2001). Because of their ability to degrade ECM components, MMPs are considered to be important components in many biological and pathological processes (Sternlicht & Werb, 2001). They have regulatory roles in muscle growth and development and are also important in repair processes after traumatic injury or disuse myopathy (Reznick *et al*., 2003). MMPs are mainly produced from endotendon fibroblasts and intramuscular matrix fibroblasts (Ragoowansi *et al*., 2001), although some level of expression has been found to occur also in satellite cells (Balcerzak *et al*., 2001). MMPs are secreted or released in latent form and become activated in pericellular environments (Birkedal-Hansen, 1993; Trackman, 2005). The activities of MMPs are also under the control of enzyme tissue inhibitors of matrix metalloproteinases (TIMPs). Disturbances in the ratio of specific MMPs and their

inhibitors may be manifested by physiological dysfunction, resulting in clinical disorders (Carmeli *et al*., 2004).

Up till now, 24 different vertebrate MMPs have been identified, of which 23 have been found in humans. MMPs are usually divided according to their main substrate into collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs and others, although many of them have wide and overlapping substrate specificity (Visse & Nagase, 2003).

MMP-1, MMP-8, MMP-13 and MMP-18 are collagenases, which have the ability to cleave the native helical structure of collagens I, II and III. Cleavage products are then susceptible to the action of other MMPs (Cimpean & Caloianu, 1997; Li *et al*., 2000; Visse & Nagase, 2003).

Gelatinases MMP-2 and MMP-9 degrade denatured collagen, gelatin, native type IV, V and VII collagens as well as other ECM components (Wilhelm *et al*., 1989; Visse & Nagase, 2003). One of the most important MMPs associated with the function and dysfunction of the skeletal muscle appears to be MMP-2, also known as gelatinase A, or 72-kDa type IV collagenase. MMP-2, by regulating the integrity and composition of the ECM in skeletal muscle, plays essential role in myofibre proliferation and differentiation, the fibre healing after injury, and maintenance of the surrounding connective tissue (Matrisian, 1992). MMP-2 also digests fibrillar type I and II collagens (Patterson *et al*., 2001). MMP-2 and –9 are known to be overexpressed and present in higher amounts in patients with inflammatory myopathies ((Kieseier *et al*., 2001), which may increase ECM degradation and thus facilitate lymphocyte adhesion (Choi & Dalakas, 2000).

MMP-3 and MMP-10, or stromelysin-1 and –2, both digest ECM components and activate proMMP-1. The third stromelysin, MMP-11, differs from other stromelysins by its sequence and substrate specificity (Visse & Nagase, 2003).

Matrilysins- MMP-7 and MMP-26 are the smallest MMPs. MMP-7 can also process cell surface molecules (Visse & Nagase, 2003).

Six membrane-type MMPs (MT-MMPs) have been characterized. Except the MT4-MMP, they are all capable to activate proMMP2 (Hernandez-Barrantes *et al*., 2002; Visse & Nagase, 2003). For their pericellular fibrinolytic activity, MT-MMPs have an important role in angiogenesis (Sternlicht & Werb, 2001).

Six MMPs – MMP-12, MMP-19, MMP-20, MMP-22, MMP-23, MMP-28 are currently classified into the group of “other MMPs” (Shapiro *et al*., 1993; Visse & Nagase, 2003).

2.5.2. Inhibition of matrix metalloproteinases by tissue inhibitors of metalloproteinases

MMPs and tissue inhibitors of metalloproteinases (TIMPs) have an important role in the adaptive changes in the muscle in response to local developmental, physiological, surgical, and pathological conditions (Carmeli *et al*., 2004). TIMPs are the major cellular inhibitors of the MMP sub-family, exhibiting varying efficasy against different members, as well as different tissue expression patterns and modes of regulation (Baker *et al*., 2002; Visse & Nagase, 2003). Four mammalian TIMPs have been characterized and considered to regulate MMP activity during tissue remodelling (Greene *et al*., 1996; Stetler-Stevenson *et al*., 2001). All four TIMPs (TIMP-1, -2, -3 and -4) can inhibit all MMPs, except TIMP-1, which is a poor inhibitor of MMP-19 and most of the MT-MMPs (Baker *et al*., 2002). Although TIMP-2 inhibits MMP-2 in high concentrations, it has an important role in activating proMMP-2 in a complex with MT1-MMP, which demonstrates an integrated response of MMPs and TIMPs (Wang *et al*., 2000). In skeletal muscle, TIMP-1, TIMP-2 and TIMP-3 are expressed (Singh *et al*., 2000; Balcerzak *et al*., 2001). TIMP-4 appears to be cardiac-specific (Li *et al*., 2000) and has not been detected in the skeletal muscle.

The increased MMP activity and thus the enhanced degradation of collagen often parallels the stimulated activation of collagen synthesis. TIMPs are often activated together with MMPs in response to physical activity, indicating a simultaneous stimulation and the inhibition of degradation (Koskinen *et al*., 2002). MMP activity precedes TIMP activity and thus TIMP serves as the regulator of degradation termination to ensure a limited amount of degradation (Wang *et al*., 2000).

In addition to MMP-binding activities, TIMPs have many important biological functions. TIMPs can promote or inhibit cell growth, depending on the type of the cell and the inductor (Baker *et al*., 2002; Visse & Nagase, 2003).

3. Effect of glucocorticoids on the skeletal muscle

3.1. Effect of glucocorticoids on the skeletal muscle fibres

The administration of large doses of glucocorticoids causes muscle atrophy and the decrease of strength and initiates the selective atrophy of skeletal muscle fibres. The fast glycolytic muscle fibres are particularly sensitive to the catabolic effect of glucocorticoids (Seene *et al*., 2003).

It was shown that glucocorticoids increase the expression of myostatin, a negative regulator of skeletal muscle growth in polynucleated muscle fibres, expressing myosin heavy chain (MyHC) fast isoforms (Artaza *et al.*, 2002). In

glucocorticoid myopathic rats the turnover rate of MyHC was lower in the fast-twitch muscle fibres, myosin light chains (MyLCs) turned over more rapidly in all types of muscle fibres (Seene, 1994). The turnover rate of MyHC in myopathic rats is related to the changes in MyHC isoform pattern. In myopathic glycolytic (G) muscle fibres of rats relative content of MyHC IIB isoform decreased and IID increased (Kaasik *et al*., 2000). The ultrastructural studies showed the disarray of thick myofilaments in glycolytic muscle fibres of glucocorticoid treated rats and increased lysosomal activity in these fibres and in the satellite cells (Seene *et al*., 1988). There were only slight morphological changes in oxidative-glycolytic fibres and there were no structural changes in slow-twitch oxidative muscle fibres in glucocorticoid treated rats (Seene *et al*., 1988).

It was shown about two decades ago that MyHC are more sensitive to the action of alkaline proteinases than MyLC, at least under *in vitro* conditions (Seene & Alev, 1985; 1986). It was demonstrated twenty years ago that the glucocorticoid treatment increased the activity of alkaline proteinases in the skeletal muscle (Mayer *et al*., 1980; Seene & Viru, 1982). Alkaline proteinases are synthesized in mast cells (Seene, 1994). After degranulation the enzyme enters the muscle fibre. Upon administration of large doses of glucocorticoids there is an increase in the number of mast cells in the perivascular porous connective tissue of the muscle fibres (Seene, 1994). The granulation of mast cells is clearly expressed around the fast glycoclytic muscle fibres (Hussar *et al*., 1992). The lymph nodes are the sources of the muscle mast cells, which may be migrated from there. In glucocorticoid-caused myopathic muscles, where the alkaline proteinase activity is high, the glycolytic fibres separate from the adjacent ones, bend and lyse (Seene *et al*., 1988; Kaasik *et al*., 2000). Nowadays it is known that the myofibrils from the muscles of different metabolic and twitch characteristics have different sensibility to the action of proteinases. In fast-twitch muscles, where the oxidative potential is higher, the sensibility of MyHC IIB isoform to the alkaline proteinase activity is diminished (Seene *et al*., 2003).

3.2. Effect of glucocorticoids on the extracellular matrix

Glucocorticoids are potent inhibitors of collagen synthesis and are therefore used clinically in the therapy of fibrotic conditions of the liver, lung and skin (Schäcke *et al*., 2002). Already more than twenty years ago it was shown that glucocorticoids inhibit collagen synthesis by reducing mRNA levels of type I and III collagens (Graham *et al*., 1995), and activity of enzymes, participating in the posttranslational modifications of collagens (Oikarinen *et al*., 1986). Long-term corticosteroid therapy decreases collagen synthesis in the human skin, leading to skin atrophy (Oikarinen *et al.,*1986). Glucocorticoids inhibit

bone formation by suppressing osteoblast proliferation and activity (Schäcke *et al*., 2002). Dexamethasone also reduces the gene expression and secretion of type IV collagen from the cultured human fibrosarcoma cells (Oikarinen *et al*., 1987).

Glucocorticoids as anti-inflammatory agents decrease MMP levels in fibroblasts (Morin *et al*., 1999), macrophages (Shapiro *et al*., 1991), malignant cells (Kylmäniemi *et al*., 1996) and injured spinal cord (Xu *et al*., 2001). They also decrease TIMP-1 activity in macrophages (Shapiro *et al*., 1991).

3.3. Exercise and inhibition of glucocorticoid-induced muscle atrophy

Historically, the catabolic action of glucocorticoids on skeletal muscle has been found to depend on the functional activity of skeletal muscles (Goldberg, 1969; Seene & Viru, 1982). Four exercise and contractile activity model systems have been used to study the prevention of the muscle atrophy associated with excessive levels of glucocorticoids already several decades ago. These are functional overload (Goldberg & Goodman, 1969), resistance exercise (Gardiner *et al*., 1980), endurance exercise (Hickson & Davis, 1981) and *in vitro* cell culture stimulation (Chromiak & Vandenburgh, 1991). Functional overload involves usually tenotomy. The remaining synergistic muscle undergoes a compensatory overload and increases in mass. Resistance training consists of repetitions and sets by a specific muscle group. These forms of exercise can result in muscle enlargement, but the increased mass is not always an universal effect (Gardiner *et al.*, 1980). Endurance exercise does not increase muscle mass or strength to any great extent but develops aerobic energy-generating systems in skeletal muscle (Hickson & Davis, 1981; Hickson *et al*., 1984; Hickson *et al*., 1986). The intermittent repetitive mechanical stimulation of skeletal muscle cell cultures offers potential for examining atrophy inhibition by contractile activity in an *in vitro* model (Chromiak & Vandenburgh, 1991).

Already the first studies about the co-effect of physical activity and glucocorticoid action demonstrated that the effect of physical activity inducing a less pronounced catabolic action of corticosteroids seems to be caused by the elevation of anticatabolic activity of exercise (Seene & Viru, 1982). It has been found that only moderate exercise has an anticatabolic activity in skeletal muscles. Exhaustive exercise, on the contrary, augmented the catabolism in skeletal muscles (Seene & Viru, 1982; Fimbel *et al*., 1993).

4. Effect of inactivity on the skeletal muscle

It has been acknowledged for a long period that the inactivity of the skeletal muscle leads to the loss of muscle contractile proteins and strength. The weakening of the muscle is accompanied by the loss of the muscle mass and the reduction of the size of the muscle cell (Goldspink *et al*., 1986). The decrease in the protein synthesis and the increase in protein degradation appear both in the contractile apparatus and in the ECM. The skeletal muscle atrophy attributable to the muscular inactivity has significant adverse functional consequences, nevertheless the tight connections between the contractile machinery and the ECM are still unknown.

4.1. Effect of inactivity on the contractile apparatus of skeletal muscle

As the skeletal muscle is a highly plastic tissue, the conditions associated with the disuse are accompanied by adaptation. A period of time without weightbearing causes modifications of structure and the function of skeletal muscles, of which atrophy and a slow-to-fast transition are the most prominent. Many animal models such as the hind limb suspension, immobilization in shortened and lengthened position, spaceflight and denervation show that the removal of a mechanical load produces atrophy and contractile alterations, more evident in the slow muscle *soleus* than in fast muscles as *extensor digitorum longus* (Reiser *et al*., 1987; Gardetto *et al*., 1989). Besides spaceflight and bed rest experiments, long periods of muscle disuse in relation to a disease or traumatic injuries of the joints or of the bones are relatively common experience for patients (D'Antona *et al*., 2000). The inactivity causes only small increases in contractile speed and myofibrillar ATPase activity, and slight elevations in the percentage of the fast type MyHC isoforms in fast muscles, as compared to slow muscle (Degens & Alway, 2006).

4.1.1. Slow-to-fast transition

The different response can be explained, considering the fact that skeletal muscles in different parts of the body are subjected to different patterns of recruitment and activity. The anti-gravitational *soleus* muscle is recruited for prolonged periods at a moderate level of intensity, whereas the *extensor digitorum longus* muscle is less frequently recruited, performing short, high-force contractions (Desplanches *et al*., 1987a; Elder & McComas, 1987). It is commonly known that inactivity affects the functional and biochemical properties of antigravity muscles, causing a significant decrease in both contraction and relaxation times (Fitts *et al*., 1986; Elder & McComas, 1987)

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and a significant increase in the maximal shortening velocity (Diffee *et al*., 1993; Fitts *et al*., 1986) and myofibrillar adenosine triphosphatase activity (Diffee *et al*., 1993). The above-mentioned changes are considered to be a result of the gene expression, especially the genes involved in the fibre type transformation (Isfort *et al*., 2002; Stein *et al*., 2002; Wittwer *et al*., 2002). The coordinated changes in the gene expression are particularly apparent for myosin and consequently the disuse induces a slow-to-fast transition, as reflected by an increase in fast MyHC isoforms at the expense of slow MyHC in the *soleus* and a fast-to-faster MyHC shift in the *gastrocnemius* muscle (Thomason *et al*., 1987; Stevens *et al*., 2000; Talmadge, 2000). An increase in fast MyLC isoforms, an increased proportion of fast troponin subunits and hybrid fibres co-expressing fast and slow MyHC and MyLC appear during slow-to-fast transition in *soleus* muscle (Templeton *et al*., 1988; Ohira *et al*., 1992; Oishi, 1993; Stevens *et al*., 2002; Stevens *et al*., 2004). Several histochemical analyses have also suggested that the functional changes in immobilized muscles are due to an increase in fast-twitch IIa fibres (Desplanches *et al*., 1987a; Templeton *et al*., 1988). The increase in type IIa fibres has been suggested to be caused by the conversion from type I to type IIa fibres (Templeton *et al*., 1988; Oishi, 1993). In additon to above-mentioned facts, the increased sarcoplasmic reticulum calcium-ATPase activity and the preferential loss of thin filaments all contribute to faster contractile properties of the *soleus* muscle (Fisher *et al*., 1998; McDonald *et al*., 1994). The increased shortening velocity may be an attempt to compensate for the loss of power generating capacity during unloading caused by weakening (McDonald *et al*., 1994; Fitts *et al*., 2001).

Muscle disuse is often accompanied by increased fatigability, which is caused by the reduced oxidative capacity of disused muscles (Degens & Always, 2003; Ohira *et al*., 2002). Capillary loss and reduction in blood flow might contribute to the increased fatigability by an impaired supply of energy substrates and oxygen to the muscle (Kano *et al*., 2000).

4.1.2. Formation of muscle atrophy

While immobilization at shortened length induces atrophy, immobilization in lengthened position induces hypertrophy, which is largely attributable to addition of sarcomeres in the longitudinal direction (Goldspink *et al*., 1991). Immobilization in the shortened position, e.g. hindlimb suspension, induces preferential transcription of fast myosin heavy chain isoforms, reminiscent of the slow-to-fast transition observed in other models of disuse (Goldspink *et al*., 1991; Talmadge, 2000). The disuse atrophy is characterized by the loss of muscle mass and decrease of muscle diameter. In the case of muscle atrophy, there exist some noticeable changes in the muscle cell at the cellular level including sarcomere dissolution and endothelial degradation, a marked reduction in the number of mitochondrias (Mujika & Padilla, 2001), the accumulation of the

connective tissue, the elimination of apoptotic myonuclei and a decrease in capillary density (Smith *et al*., 2000). Selective susceptibility of fibre types to immobilization seems to exist, while the red muscle fibres show the greatest atrophy. The decreased synthesis of protein and increased protein degradation are distinctive to muscle atrophy. At least half of the total muscle protein is myofibrillar protein, and this fraction is lost at a faster rate than other muscle proteins during atrophy (Munoz, 1993). Three major proteolytic systems to skeletal mass protein loss are: the cytosolic calcium-dependent calpain system, the lysosomal proteases and the ATP-dependent ubiquitin-proteasome system, which work as partners during muscle proteolysis rather than one system being used exclusively during atrophy (Jackman & Kandarian, 2003). Recent advances in cellular biology show oxidative stress to be an important regulator of pathways leading to muscle atrophy during periods of disuse, increasing the expression of the key components of the proteasome proteolytic system. This proteolytic system is a prominent contributor to protein breakdown in skeletal muscle during periods of inactivity (Li *et al*., 2000).

4.2. Effect of inactivity on the extracellular matrix of skeletal muscle

The extracellular matrix (ECM) of connective tissues enables links to other tissues, and plays a key role in force transmission and tissue structure maintenance in tendons, ligaments, bone and muscle (Han *et al*., 1999a; Takala & Virtanen, 2000). ECM turnover is influenced by physical activity (Kjær *et al*., 2006). Immobilization causes a marked relative increase in the endo- and perimysial connective tissue (Józsa *et al*., 1990; Kannus *et al*., 1992; Järvinen *et al*., 2002), which results in changes of the mechanical properties of skeletal muscle.

4.2.1. Effect of inactivity on the synthesis of collagen

In contrast to physical loading, immobilization leads to the decrease in the enzyme activities of collagen biosynthesis, which suggests that the biosynthesis of the collagen network decreases as a result of reduced muscular and tendinous activity (Savolainen *et al*., 1988a; Savolainen *et al*., 1988b). The rate of the total collagen synthesis depends mostly on the overall protein balance of the tissue, but it seems to be positively affected by stretch in both muscle and tendon (Savolainen *et al*., 1988a; Savolainen *et al*., 1988b).

Collagen expression during immobilization has been shown to be at least partially downregulated at the pretranslational level (Ahtikoski *et al*., 2001). Although the relative amount of the connective tissue increases during immobilization, the gene expression of type I and III collagens decreases during the first three days of immobilization (Han *et al*., 1999b). The content of type

IV collagen was also reduced as a result of immobilization (Ahtikoski *et al*., 2003). The activities of prolyl 4-hydroxylase (P 4-H) and galactosylhydroxylysyl glucosyltransferase (GGT) decrease from the first three days of immobilization up to at least three weeks, suggesting decreased collagen biosynthesis during that time (Savolainen *et al*., 1987, 1988; Han *et al*., 1999b). The degradation of collagens has been found to be enhanced during immobilization, as the expression of both MMP-2 and MMP-9 increased after 30 days of immobilization (Reznick *et al*., 2003). The quantity of TIMP-1 was also increased after 30 days of immobilization (Reznick *et al*., 2003).

The collagen concentration increases when expressed both as a function of muscle dry weight or muscle cross-sectional area, but this increase in muscle collagen is primarily due to the muscle atrophy induced by immobilization (Miller *et al*., 2001).

4.2.2. Effect of inactivity on the structure of extracellular matrix

Several quantitative and qualitative changes in the intramuscular connective tissue contribute to the deteriorated function and biomechanical properties of the immobilized skeletal muscle (Järvinen *et al*., 2002).

Along with the increased amount of intramuscular connective tissue, the number of capillaries decreases dramatically (Józsa *et al*., 1990). Each capillary is surrounded by a dense layer of the connective tissue fibres, isolating the capillary from the adjacent muscle fibre, which disturbs the blood supply of the muscle fibres and further increases the muscle fibre atrophy (Kannus *et al*., 1992; Tyml & Mathieu-Costello, 2001).

During immobilization, the normal three-dimensional orientation of the collagen fibres is disrupted. The normal orientation constitutes of the fibres running parallel to the muscle fibres on their surface, preventing muscle cells from over-elongation and –contraction. In addition, thin perpendicular fibres connect adjacent muscle fibres to each other (Järvinen *et al*., 2002). As a result of decreased loading, the number of longitudinal fibres increases, the crimp angel of the collagen decreases and this diminishes the ability of the muscle to elongate (Järvinen *et al*., 2002) and because of that the skeletal muscle shows significantly decreased tensile strength (Järvinen *et al*., 1992). In addition to changes in collagen abundance, alterations in the degree of collagen cross-linking would have a profound effect on the mechanical properties of skeletal muscle, causing a decrease in muscle stiffness (Kovanen & Suominen, 1989).

4.3. Effect of reloading

The plasticity of muscle is apparent in the phenomena such as muscle atrophy caused by inactivity, and recovery from atrophy. When atrophic muscles once again become active, the muscle mass and the volume reportedly increase in a relatively short period of time, but the recovery of the muscle strength takes much longer (Pottle & Gosselin, 2000). The fact that the increases in the muscular strength lag behind those in the muscular mass suggests the presence of functionally immature muscle fibres during the recovery process following disuse atrophy (Itai *et al*., 2004). Several studies have shown that the increases in the muscle mass soon after reloading are attributable to oedema, and do not actually represent recovery (Itai *et al*., 2004).

Disuse muscle atrophy can be experimentally induced by suspending animals by their tails (Morey-Holton & Globus, 2002; Itai *et al*., 2004), immobilizing joints, severing tendons or conducting muscle denervation (Oishi *et al*., 2001). Muscle atrophy in tail suspension is caused by hindlimb unloading, conserving the functions of nerves and joints. Reloading is thus possible after tail suspension, and is suitable for investigating the recovery process following disuse muscle atrophy caused by sports injuries (Itai *et al*., 2004).

Concomitant to atrophy, a number of molecular events testify of a slow-to-fast transition of muscle properties (Thomason *et al*., 1987; Fitts *et al*., 2000; Baldwin & Haddad, 2001). The recovery of muscle properties effectively occurs on return to normal load (Thomason *et al*., 1987). It is also known that muscle fibre damage occurs during reloading, likely due to the inability of the muscle fibres to bear eccentric contractions and the consequent inflammation process (Bigard *et al*., 1997; Fitts *et al*., 2000; Kasper *et al*., 2002; Desaphy *et al*., 2005). Natural recovery seems to be most effective after reloading while several investigations show the delayed recovery of rats during running exercise (Bigard *et al.,* 1997; Lee *et al*., 2004).

UNSOLVED PROBLEMS

Although the effect of glucocorticoids and inactivity on the skeletal muscle has been studied over 25 years, their precise action on both the contractile apparatus and ECM has not been clarified. It is known that glucocorticoids reduce the protein synthesis among contractile proteins and collagen as well, but it is still unclear if the effect is similar in fast- and slow-twitch fibres and among fibrillar and non-fibrillar collagens.

We can only presume that the speed and amplitude of the effect of inactivity are not the same in endo-, epi- and perimysium. Therefore it can be also presumed that collagens types I, III and IV have different susceptibility to the inactivity. Although the effect of glucocorticoids on the synthesis and degradation of contractile proteins is significantly more pronounced in fast-twitch muscles, the peculiarities of the synthesis and degradation of collagens type I, III and IV in different types of muscles are still practically unknown.

Inactivity results in the slow-to-fast transition of slow muscle fibres. Unfortunately there exist no data about the dynamics of the synthesis and the degradation of fibrillar and non-fibrillar collagens during inactivity and following recovery.

The amplitude of changes in motor activity and muscle strength during the muscle atrophies with different genesis, connected with alterations in contractile apparatus and ECM, are still not clarified.

It is particularly interesting to get some knowledge about the adaptation process of the contractile apparatus and ECM during the reloading period, which has a significant practical value in the contemporary era of decreased physical activity and sedentary lifestyle.

AIMS OF THE STUDY

The purpose of the present study was to assess the effect of glucocorticoids and hypokinesia on the mechanism of atrophy development in fast-twitch and slow-twitch skeletal muscle fibres on the bases of changes in extracellular matrix and contractile apparatus and subsequent changes in motor activity and muscle strength.

The specific aims of the studies were as follows:

1. To investigate the atrophy in glucocorticoid-myopathic muscles and changes in the extracellular matrix – the specific level of mRNA for type I, III and IV collagen, lysyl oxidase, matrix metalloproteinases, tissue inhibitors of metalloproteinases and contractile proteins.
2. To examine the effect of exercise on the glucocorticoid-caused myopathic muscle, its extracellular matrix and contractile proteins.
3. To study the effect of unloading on the skeletal muscle contractile apparatus on the bases of changes of myosin heavy chain isoforms composition in the skeletal muscle and the effect of unloading on the extracellular matrix. To determine the specific mRNA level for type I, III and IV collagen, lysyl oxidase, matrix metalloproteinase-2, tissue inhibitors of metalloproteinases 1 and 2.
4. To examine the effect of reloading on the extracellular matrix of the skeletal muscle, estimating the specific level of mRNA for type I, III and IV collagen, lysyl oxidase, matrix metalloproteinase-2 and tissue inhibitors of metalloproteinase 1 and 2.

MATERIALS AND METHODS

1. Animals

The use of the animals was in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes and was controlled by the Committee of Laboratory Animal Science of the University of Tartu.

The used animals were 16–17 weeks old (at the beginning of experiment) Wistar and Sprague-Dawley rats (National Laboratory Animal Centre, Kuopio, Finland). All the animals were housed in identical conditions in polycarbonated type III cages, at 21ºC, two per cage at 12/12 h light/dark period. They received diet [SDS-RM1(C)3/8, Witham, Essex, UK] and water *ad libitum*. Rats were randomly divided into groups: the control group, dexamethasone treated, exercise+dexamethasone treated group, hindlimb suspended and hindlimb reloading group. The size of each group was 10 animals at the beginning of experiment.

2. Human subjects

Thirteen hypokinetic patients diagnosed with a medial meniscus lesion, as described by us earlier (Eller *et al*., 1999), volunteered to participate in this study. The study was approved by the Ethical Committee of the Medical Faculty, University of Tartu. The mean age of patients was 25 years, mean height 1.80 m for men and 1.68 m for women and mean weight 80 kg and 65 kg, respectively. Majority of subjects had participated in regular leisuretime sports activity. All patients were treated arthroscopically by resection of the ruptured part of the meniscus.

3. Dexamethasone treatment

Dexamethasone (Glucocortin-20, Interchemie, Holland) was diluted to 200 μ/ml with 0.15 M NaCl and administered intraperitoneally 100 μg/100 g b wt during 10 days. The control animals received appropriate amounts of 0.15 M NaCl.

4. Labelled amino acid infusion

L-[4.5 – ^{3}H] leucine (170 Ci/mmol) was infused to animals intraperitoneally for 6 h, 250 μCi/100 g bwt.

5. Hindlimb suspension procedure

The modification of the tail harness model of Morey-Holton and Globus (2002) was used to suspend the hindlimbs of rats. The animals were anesthetized, such that the tail was easily manipulated. The skin of the tail was thoroughly cleaned with alcohol and dried. The proximal part of the tail remained uncovered, thereby allowing normal thermoregulatory processes to occur. The portion of exposed tail maintained normal colour, indicating that the blood flow was not compromised. A swivel harness was attached on the tail with a strip of adhesive tape. The tape was checked daily and repaired, if necessary. The animal was suspended by the swivel harness from a hook above the suspension cage, allowing free 360º rotation. The height of the hook was adjusted so that only the front limbs were able to contact the cage. The size of cage was such that the animals could easily reach food and water without being able to touch the sides of cages with the hindlimbs. This form of suspension provides traction along the tail and does not cause any obvious lesions on the tail.

The rats were assigned to control, hindlimb-suspended for 1 and 3 weeks and hindlimb reloading group for 1 and 2 weeks. The size of each group was 10 animals. The reloading period consisted of free cage activity for two weeks after a 3-week hindlimb suspension.

6. Exercise training

After a brief 5-day acclimatization that consisted of treadmill running for 5–10 min per day the rats were subjected to run for 10 days. During the first five days the rats were running with the speed of 65 m/min for 30 s, after 90 s rest for 6 time per training. Starting from 6th day the running speed was 75 m/min and 120 s for rest between running sets. After 10 days of exercises the dexamethasone treatment began (during 10 days), and rats were running with the speed of 95 m/min for 10 s, 120 s rest between running sets.

7. Hypokinesia

The participants of the present experiment were diagnosed a medial meniscus lesion which resulted in a decreased mechanical load of the thigh muscles for 6 month averagely, as described by us earlier (Eller *et al*., 1999).

8. Muscle biopsies

The biopsy from the *vastus medialis* muscle of the knee injury patients was taken with a Bergström needle (Bergström, 1962), 6–8 cm proximal to the patella, before and 6 months after the knee arthroscopy. The muscle samples were immediately frozen in liquid nitrogen and stored at –70ºC until further analysis, as described by us earlier (Eller *et al*., 1999).

9. The measurement of strength and motor activity

The force and hindlimb grip strength were measured before and after the hindlimb suspension and after the recovery period with Grip Strength Meter 0167-004L (Columbus Instruments) and expressed as N per 100 g of bwt. The motor activity was measured by the screening of the amounts of ambulatory and total (stereotypic) movements by Opto-Varimex-Mini (Columbus Instruments). The ambulatory activity characterizes movemental activity, the total activity includes stereotypic (scratching, grooming, digging etc) non-ambulatory movements.

10. Tissue preparation

Twenty-four hours after the last experimental procedure, the animals were anesthetized by the intraperitoneal injection of ketamin (Calysol, *Gedeon Richter* A. O., Budapest, Hungary) 2.5 mg/100 g bwt and diazepam (*Lab Renaudin*, France) 2.5 mg/bwt and sacrified. *The gastrocnemius, plantaris, extensor digitorum longus* and *soleus* muscles were removed, trimmed clean of visible fat and connective tissue, weighed, frozen and stored in liquid nitrogen until further processing. Due to small size of *soleus* and *extensor digitorum longus* these muscles were pooled for mRNA analyses, so that one sample consisted of right and left muscle of the same animal.

11. Estimation of 3 Methylhistidine in skeletal muscle and urine

The 3 Methylhistidine (3-MeHis) in the skeletal muscle and urine was used as an indicator for contractile protein degradation. The muscle weight was taken to be 45% of body weight. To obtain sufficient purified actin and myosin for 3-MeHis content, pooled samples of muscle tissue from 10 rats were obtained. Myosin and actin were purified according to Haverberg *et al* (1974). Total

muscle protein, myofibrillar protein, actin and myosin for getting 3-MeHis was hydrolyzed in 6 M HCl (10 mg/ml acid) for 20 h at 110ºC in vacuum-sealed flasks. HCl was removed by evaporation, and the hydrolysate was dissolved in 0.2 M pyridine to achieve a concentration of 10–20 mg/ml. 3-MeHis pool excreted was expressed as percentage per day.

12. Protein assay

Total muscle protein and myofibrillar protein was assayed by using the technique described by Bradford (Bradford, 1976).

13. Separation of myosin heavy chains

Skeletal muscle myosin heavy chain (MyHC) proteins were separated on 12.5% acrylamide gel using standard SDS-PAGE technique described by Laemmli (1970).

14. Fractional synthesis rate of myosin heavy chains

MyHC fractional synthesis rate was calculated according to Sugden and Fuller, 1991. Ks = 100 x Sb/Sa x t, where Sa is the specific radioactivity of mixed protein and Sb is the specific protein bound radioactivity of MyHC; t is the incorporation time of radioactive label in days.

15. RNA isolation

For the total RNA isolation, muscle samples were homogenized with an Ultra-Turrax homogenizer in Trizol (Life Technologies, Painsley, Scotland, UK). Other steps were performed as described in the manufacturer's protocol (Life Technologies 1995). The purity and concentration of the total RNA were assessed spectrophotometrically. Northern blot was used for testing the specificity of cDNA probes, whereas the slot blot analysis was used for the quantification of the specific RNA amount.

15.1. mRNA analysis

For Northern blotting, 30 μg of total RNA was denatured in loading buffer, electrophoresed in a 1% agarose/formaldehyde gel, and transferred to a nylon membrane (GeneScreenPlus, Biotechnology Systems, Boston, USA) with a standard procedure (Chomczynski & Mackey, 1994). For slot blotting, 20 μg of total RNA was spotted on a nylon membrane using a vacuum filtration manifold (Minifold II; Schleicher and Schuell, Dassel, Germany) (Maniatis *et al*., 1982). All the membranes were incubated in 0.05 N NaOH for 5 min to bind the RNA to the membrane. Prehybridization was carried out in a solution containing 5 X SSC, 5 X Denhardt's solution, 50% formamide, ssDNA 100 μg/ml, 50 mM sodium phosphate pH 6.8, 10% dextran sulphate and 1% SDS for 2 hrs at 42ºC. The RNA-cDNA hybridization was performed for 24 hrs at 42ºC using the solution containing the same components as the prehybridization solution and [^{32}P] labeled cDNA probe labeled with a Ready-To-Go DNA Labeling Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The collagen probes were α12, a 2.4 kb human cDNA for proα1(I)-chain mRNA, E6, a 2.4 kb human cDNA for proα1(III)-chain mRNA and HT21, a 2.6 kb human cDNA for the α1(IV). The rat lysyl oxidase (EC 1.4.3.13) probe was a 0.6 kb product of an EcoRi digest of the p13L-0 clone. 1668 bp long cDNA was used for rat MMP-2 RNA. 0.6 kb *ECO* RV insert in pBlueprint II plasmid of mouse was used for TIMP-1 and 1.7-kb *ECO* RI insert in pBlueprint II plasmid of mouse for TIMP-2. After hybridization, the membranes were exposed to KodakX-Omat film (Eastman-Kodak, Rochester, NY, USA) at –70ºC. Attained signals were analysed using densitometry (Personel Densitometer SI, Molecular Dynamics, Sunnyvale, CA, USA). The signal obtained by hybridization with a 24 mer oligonucleotide for 18S ribosomal RNA was used to normalize RNA loading/transfer amount.

16. Separation of myosin heavy chain isoforms

Muscle pieces from the *vastus medialis* of knee injury patients were pulverized under liquid nitrogen, and crude extracts of myofibrillar proteins were prepared by homogenizing the muscle powder 1:7 (wt/vol) in the buffer containing: 0.3 M KCl, 0.1 M KH_2PO_4, 50 mM K_2HPO_4, 10 mM EDTA, pH 6.5 (Sugiura & Murakami, 1990). After extracting for 15 min at 0ºC, the homogenate was centrifuged at 11000 *g* for 10 min. The supernatant fraction was diluted at 1.1 (vol/vol) with glycerol and stored at –20ºC (Bär & Pette, 1988). Protein concentration was measured by the method of Lowry *et al* (1951). Aliquots containing 0.5 μg of protein in 10 μl were loaded on the gel after being incubated for 10 min at 65ºC in lysis buffer containing: 10% (vol/vol) glycerol, 5% (vol/vol) 2-mercaptoethanol, 2.3% SDS, 0.05% bromophenol blue, 60 mM

TRIS-HCl, pH 6.8 (Bär, Pette, 1988). MyHC isoforms were separated by 5–8% SDS-PAGE (Bär & Pette, 1988) using 1.0 mm thick gel (Sugiura & Murakami, 1990). Electrophoresis lasted for 24 h at 120 V. Gels were silver-stained by the method of Oakley (Oakley *et al*., 1980). Protein isoform bands were analysed densitometrically and the percentage distribution of the MyHC isoforms was evaluated.

17. Statistical analyses

Means and standard errors of means were calculated from the individual values by the standard procedures of Excel. The data were analysed by SAS, using the analysis of variance (ANOVA). The two-tailed independent *t*-test was used for comparison of two populations. Differences were considered significant at $p<0.05$.

RESULTS

1. Effect of dexamethasone treatment on contractile proteins

The comparison of the degree of dexamethasone-mediated atrophy of different skeletal muscles and a short-lasting, intensive exercise training atrophy prevention is given in Table 1. The rat muscles with a large percentage of fast-twitch fibres like *gastrocnemius, plantaris* and *extensor digitorum longus* undergo atrophy, while muscles that are predominantly slow-twitch like *soleus* are resistant to atrophy. The results obtained by us also indicated that our running programme exhibits the high degree of glucocorticoid resistance (Table 1).

Hindlimb grip strength decreased from 4.6 ± 0.4 N /100 g bwt to 3.4 ± 0.3 N 100 g bwt ($p<0.05$) during ten days of dexamethasone treatment.

Table 1. Changes in body weight and muscle weight during dexamethasone treatment

Group	Body weight (g)	*m.gastroc-nemius* (mg)	*m.plantaris* (mg)	*m. extensor digitorum longus* (mg)	*m. soleus* (mg)
Control 0 (n=10)	292±8.0	744,3±16.0	139.8±3.0	66.2±1.18	60.5±2.6
Control 10 (n=10)	334±8.8 **	900.2±20.7 ***	180.5±4.1 ***	87.0±2.3 ***	76.2±3.2 **
Dex 10 (n=9)	246±6.8 ***	639.7±14.1 ***	109.6±2.5 ***	46.8±1.3 ***	70.6±3.0
Dex+95m/min 10 (n=8)	266±7.4 ***	812.4±17.9 ** ###	121.2±2.7 *** ##	54.2±1.5 *** ##	74.1±3.1

0 – on the beginning of the experiment
10 – after10 days of dexamethasone administration
Dex – dexamethasone treated group
Dex+95 m/min – dexamethasone treated and exercised group
*** – $p < 0.001$ in comparison with control group
** – $p < 0.01$ in comparison with control group
– $p < 0.001$ in comparison with Dex group
– $p < 0.01$ in comparison with Dex group

2. Preventive effect of exercise during dexamethasone treatment

The total skeletal muscle weight decreased during dexamethasone treatment by ~30% and exercise training prevented this process by ~12% (Table 2). The fractional synthesis rate of MyHC decreased in fast-twitch but not in slow-twitch myopathic muscles (Figure 1). The exercise did not change significantly the fractional synthesis rate of MyHC in fast-twitch muscles neither the level of collagen I, III and IV mRNA. Myofibrillar protein content in skeletal muscle decreased by ~35%. Total 3-MeHis content in skeletal muscle during dexamethasone treatment decreased by ~31% (Table 2). The daily proportion of 3-MeHis pool, excreted during dexamethasone treatment, increased about 2.6 times and exercise training prevented this by ~27% (Table 2).

The exercise programme had more significant anticatabolic effect on the myosin filament level (~19%) than on the actin filament level (~11%) (Table 2).

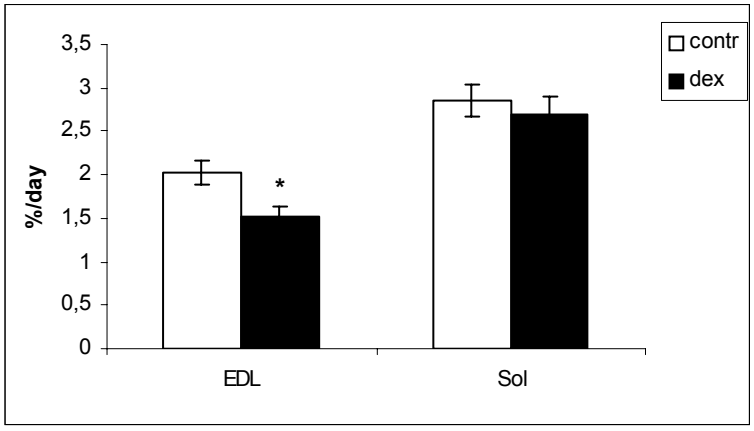

Figure 1. Changes in fractional synthesis rate of MyHC in FTand ST muscles of dexamethasone-treated (n = 9) animals
* – $p<0.05$ in comparison with control group (n = 10)

Table 2. Changes in proteins and 3-MeHis content in skeletal muscle and 3-MeHis excretion

	Control group n = 10	Dexamethasone treated group n = 9	Dexamethasone treated + exercise group n = 8
body weight (g)	334±8.8	246±6.8 ***	266±7.4 ***
muscle mass (g/rat)	150.3±7.9	103.32±7.21 ***	115.71±7.52 **
myofibrillar protein (g/rat)	19.8±1.18	13.04±0.78 ***	15.2±0.84 ***
myosin (g/rat)	11.8±0.71	7.87±0.69 ***	9.1±0.67
actin (g/rat)	5.94±0.3	3.81±0.21 ***	4.42±0.26
total 3-MeHis in actin (μmol/rat)	97.00	63.50	70.82
total 3-MeHis in myosin (μmol/rat)	21.52	15.87	18.90
total 3-MeHis in skeletal muscle (μmol/rat)	118.52±7.16	79.37±4.90 ***	89.72±5.60
daily excretion of 3-MeHis (μmol/rat)	2.51±0.12	5.29±0.26 ***	4.49±0.23 *** #
daily proportion of 3-MeHis pool excreted (%/day)	2.64±0.11	6.89±0.31 ***	5.00±0.22 *** ###

*** – $p < 0.001$ in comparison with control group
** – $p < 0.01$ in comparison with control group
– $p < 0.001$ in comparison with dexamethasone treated group
– $p < 0.05$ in comparison with dexamethasone treated group

3. Effect of dexamethasone treatment on the fibril- and network forming collagen specific mRNA

Both fibril- and network-forming collagen specific mRNA levels decreased during dexamethasone treatment in both FT and ST muscles (Figure 2).

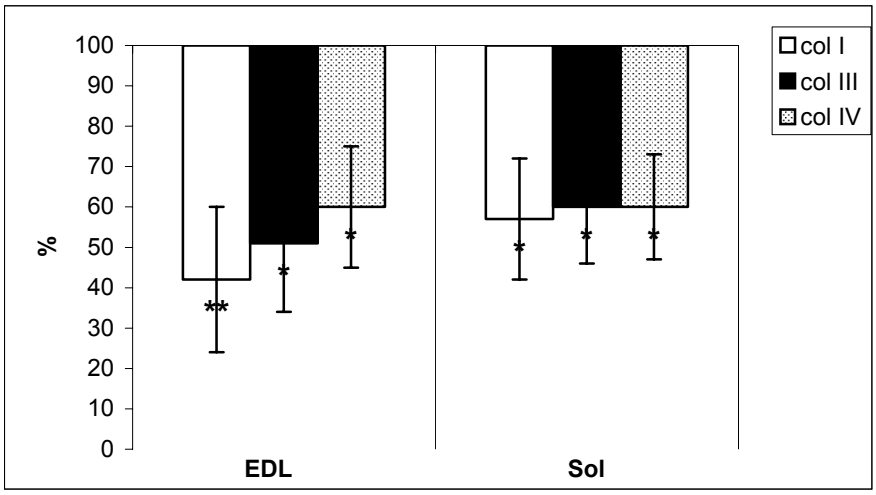

Figure 2. Changes in specific mRNA for type I, III and IV collagen in comparison with control group (100%, n = 10) in fast- and slow-twitch skeletal muscles after ten-day dexamethasone administration (n = 9)

EDL – *extensor digitorum longus* muscle
Sol – *soleus* muscle
col I – type I collagen mRNA (fibril-forming)
col III – type III collagen mRNA (fibril-forming)
col IV – type IV collagen mRNA (network-forming)
* – $p<0.05$ in comparison with control group
** –$p<0.01$ in comparison with control group

4. Effect of dexamethasone treatment on the specific mRNA level for MMP-2

Specific mRNA level for MMP-2 did not change significantly during dexamethasone treatment (Figure 3).

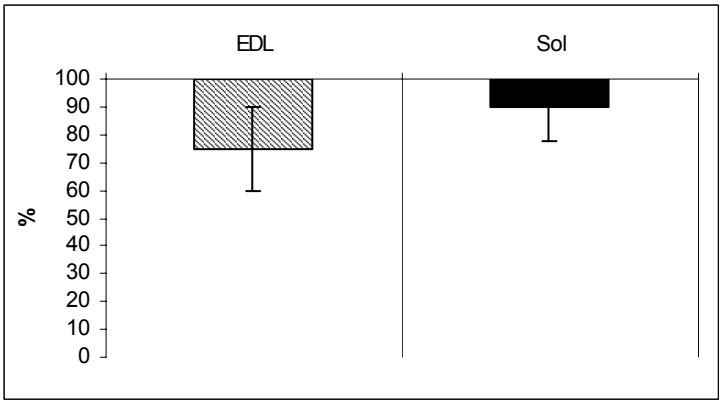

Figure 3. Changes in specific mRNA for MMP-2 in comparison with control group (100%, n = 10) in fast and slow-twitch skeletal muscles after ten-day dexamethasone treatment (n = 9)

EDL – *extensor digitorum longus* muscle
Sol – *soleus* muscle

5. Effect of unloading on the skeletal muscle

During the three weeks of hindlimb suspension the body mass did not increase as fast as in the control group (Figure 4.A). Starting from the first week of suspension till the end of the recovery, the body mass of hypokinetic animals was significantly lower than in the control group. The muscle mass decreased during the three weeks of suspension 36% ($p<0.05$) in Sol and 17% ($p<0.05$) in GM and 15% in Pla and 8% in EDL.

Dynamics of soleus muscle growth is demonstrated in the Figure 4.B.

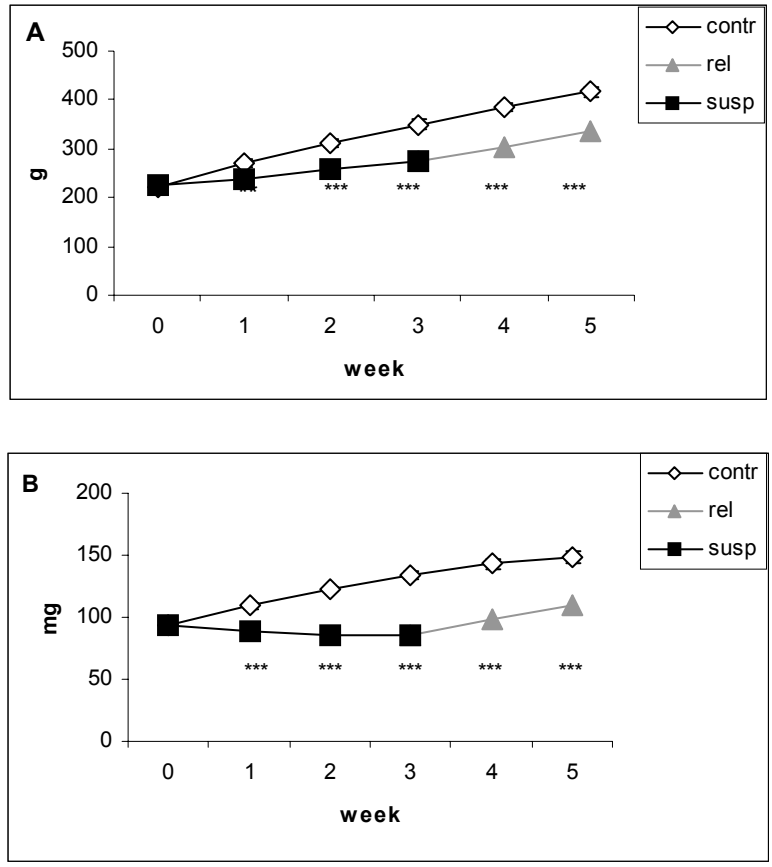

Figure 4. Dynamics of body weight (A) and muscle *soleus* mass (B) during suspension and reloading

0 – before suspension (n = 10)
3 week – end of suspension (n = 10)
5 week – end of reloading (n = 10)
Values are mean ± standard error
** – $p < 0.01$; *** – $p < 0.001$ in comparison with control group,
0 – control group; rel – reloading; susp – suspension

Motor activity decreased significantly during the three weeks of suspension and stayed on the same level even after one week of reloading (Figure 5.A). The muscle strength decreased gradually during three weeks of suspension and stayed on the same level in 1 week of reloading (Figure 5.B).

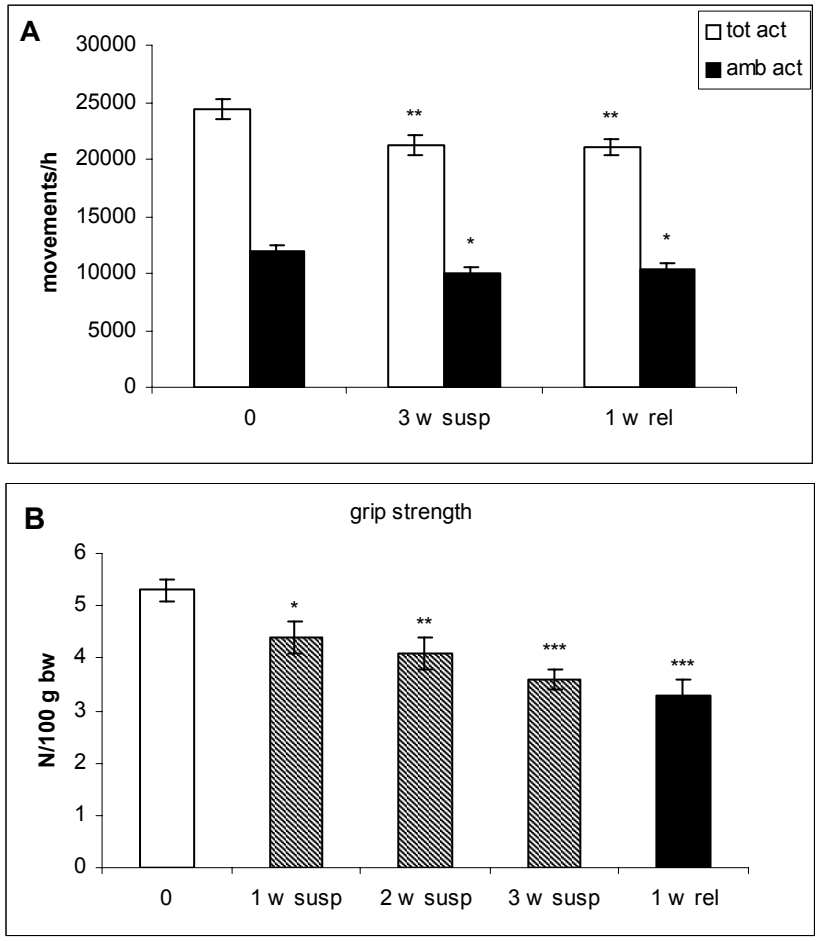

Figure 5. Changes in motor activity (A) and hindlimb grip strength (B) during 3 weeks of suspension and following 1 week reloading

Ambulatory activity characterizes movemental activity of animals, total activity includes stereotypic (scratching, groming, digging...) non-ambulatory movements. Grip strength of hindlimb was used for characterizarion of changes in muscle strength during 3 weeks suspension and 1-week reloading after suspension.
Values are mean ± standard error
* – $p< 0.05$; ** – $p< 0.01$; *** – $p<0.001$ in comparison with control group,
0 – control group (n = 10); susp – suspension; rel – reloading
tot act – total activity
amb act – ambulatory activity
3w susp – three weeks of hindlimb suspension (n = 10)
1w rel – one week reloading after hindlimb suspension (n = 10)

Specific mRNA level for type I collagen decreased during suspension period in Sol and GM muscles (Figure 6.A) and increased to the control group level during 2 weeks of recovery. mRNA level for type III collagen decreased during first week of suspension in GM (Figure 6.B). During the two weeks of reloading mRNA level for type III collagen increased in Pla and GM muscles (Figure 6.C). In GM muscle mRNA level for type IV collagen stayed on the low level during first week of reloading (Figure 6.C). mRNA level for lysyl oxidase (LO) decreased during the three weeks of suspension only in Sol muscle (Figure 6.D) and increased during reloading period in comparison with the control group in GM muscle (Figure 6.D). Matrix metalloproteinase 2 (MMP-2) mRNA level did not change significantly in the skeletal muscle during the reloading period in Sol and GM muscles (Table 3). mRNA level for tissue inhibitors of matrix metalloproteinases-1 (TIMP-1) decreased during the first week of suspension in Sol muscle and increased in GM muscle during 1 and 3 weeks of suspension (Table 3). mRNA level of TIMP-2 increased in GM muscle after 2 weeks of reloading (Table 3).

Table 3. Changes in matrix metalloproteinase 2, tissue inhibitors of metalloproteinases 1 and 2 mRNA in *soleus* (Sol), *extensor digitorum longus* (EDL), *plantaris* (Pla) and *gastrocnemius* (Gm) muscle during hindlimb suspension and following reloading

Group	n	muscle	MMP-2	TIMP-1	TIMP-2
hindlimb suspension 1 week	5	Sol	102±9.4	v69±7.6*	88±8.2
	5	EDL	97±8.9	94±8.7	103±9.8
	10	Pla	98±5.8	116±10.2	104±9.4
	10	Gm	108±6.1	130±11.0*	94±5.7
hindlimb suspension 3 weeks	5	Sol	119±10.6	120±10.9	95±9.7
	5	EDL	112±11.1	101±11.3	104±11.2
	10	Pla	96±6.8	124±7.4*	95±6.2
	10	Gm	114±7.1	131±8.1**	99±6.7
reloading after suspension 1 week	5	Sol	128±10.8*	105±10.3	107±10.1
	5	EDL	99±9.6	106±9.5	98±9.7
	10	Pla	115±7.4	103±6.9	104±7.1
	10	Gm	125±6.9*	108±6.6	110±6.8
reloading after suspension 2 weeks	5	Sol	149±16**	117±10.6	104±9.4
	5	EDL	116±12	112±10.4	101±10.2
	10	Pla	92±6.3	106±6.9	92±5.6
	10	Gm	110±5.7	109±6.1	119±7.3*

Values are mean ± standard error; * – $p<0.05$, ** – $p<0.01$ in comparison with control group

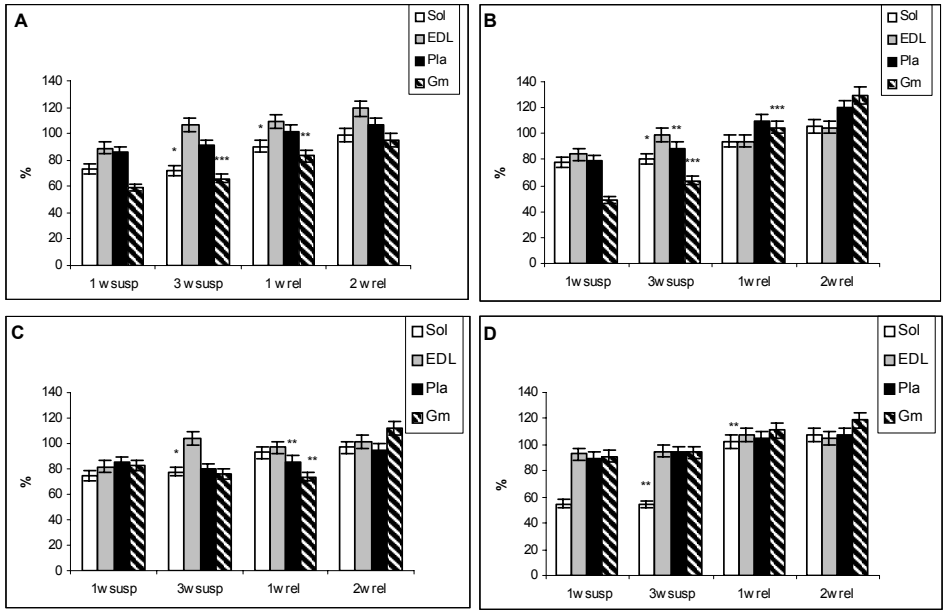

Figure 6. Specific muscular mRNA levels for type I(A), type III (B), type IV (C) and lysyl oxidase (D) in *soleus* (Sol), *extensor digitorum longus* (EDL), *plantaris* (Pla) and *gastrocnemius* (Gm) during suspension for 1 and 3 weeks and following reloading for 1 and 2 weeks

Values are mean ± standard error; n = 5 Sol, EDL; and n = 10 Pla, Gm.
* – $p<0.05$; ** – $p<0.01$; *** – $p<0.001$ in comparison with control group
Contr – 100% (n = 10)
1w susp – one week of hindlimb suspension (n = 10)
3w susp – three weeks of hindlimb suspension (n = 10)
1w rel – one week of reloading after hindlimb suspension (n = 10)
2w rel – two weeks of reloading after hindlimb suspension (n = 10)

6. Effect of hypokinesia on the MyHC composition of human *vastus medialis* muscle

The intact m. *vastus medialis* in our experiment contained 59.4 ± 2.0% MyHC I, or slow isoform, 24.3 ± 2.1% MyHC IIa isoform and 16.3 ± 1.3% MyHC IIX isoform. After the inactivity period, m.*vastus medialis* contained 48.0 ± 1.5% MyHC I isoform, 29.8 ± 1.81% MyHC IIa and 22.3 ± 2.1% IIx isoforms. The proportion of MyHC I isoform decreased from 59.4 to 48.0% ($p<0.05$) (Figure 7).

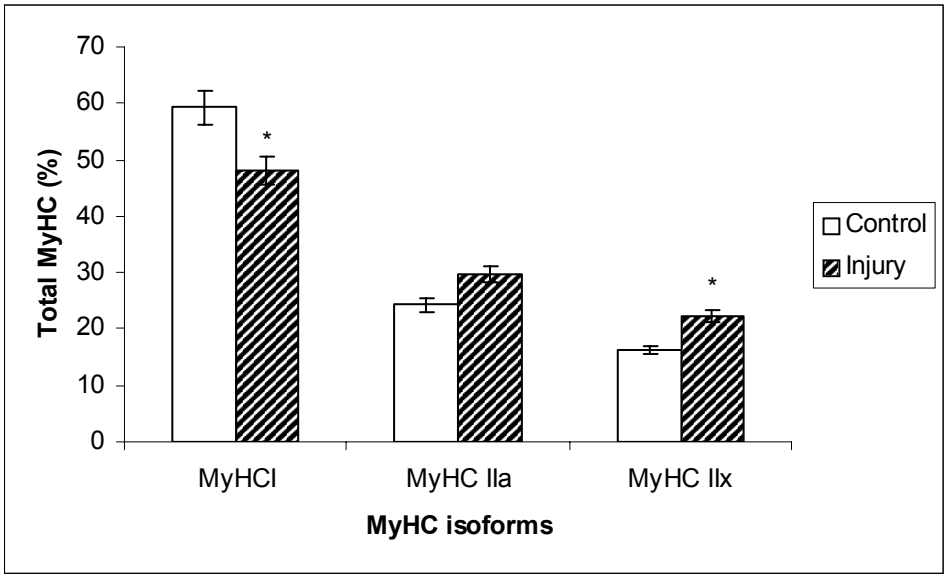

Figure 7. MyHC composition in the *m.vastus medialis* of the operated (n = 13) and the nonoperated (n = 13) extremity (mean ± standard error, * – $p<0.05$).

DISCUSSION

1. Effect of dexamethasone on the contractile apparatus

Glucocorticoid-mediated atrophy is selective for fast-twitch fibres and, particularly, for white regions of muscle (Goldberg & Goodman, 1969). The character of the exercise, by which fast-twitch glycolytic fibres are most frequently recruited, is the key issue in atrophy prevention. As it was shown previously, a short-lasting intensive treadmill running recruited fast-glycolytic muscle fibres (Kaasik *et al*., 1996; Seene & Alev, 1991). Our results show that the intensive, short-lasting running prevented significantly the fast-twitch muscle atrophy during glucocorticoid administration. The highest decrease was mentioned during dexamethasone administration in myofibrillar proteins. The comparison of the decrease of two main contractile proteins shows that the content of actin decreased somehow more than that of myosin, and the exercise prevented the catabolism of both proteins by ~16%. As it was shown previously on the ultrastructural level, the thin filaments are more resistant to any catabolic action of dexamethasone than thick filaments in glycolytic muscle fibres (Seene *et al*., 1999). The myofibrils of glycolytic fibres are thinner in dexamethasone-treated rats. This is caused by the splitting of myofibrils. In the oxidative-glycolytic fibres the destruction of thick myofilaments was remarkably less pronounced. Myofibrils were structurally normal in the dexamethasone-treated soleus muscle (Seene *et al*., 1999). Attempting to elucidate the potential anticatabolic effect of intensive exercise specifically on contractile proteins, we measured the loss of 3-MeHis from myosin and actin. Our results demonstrated that exercise prevented more the degradation of myosin than actin in skeletal muscle. Daily proportion of 3-MeHis pool excreted per day decreased as result of exercise training by ~28%, showing antiglucocorticoid action in skeletal muscle. This may be related with the steroid-receptor interaction in initiating hormone events, the ability of androgenic-anabolic steroids to exert antiglucocorticoid action by binding to the glucocorticoid receptor (Czerwinsky-Helms & Hickson, 1987). There is a possibility that androgenic-anabolic steroids can directly or indirectly inhibit glucocorticoid action at the gene level, as different steroid hormones are able to regulate the same set of genes (Beato, 1989). However the same mechanism could work in case of components of extracellular matrix as well, declyne in type I and type III procollagen expression in dexamethasone administration can be partially prevented by the intensive, short-lasting exercise training. It has been shown previously that the prevention of the decrease of type I collagen mRNA content in the skeletal muscle during the exercise is obviously related to anticatabolic effect of this exercise programme.

Our study shows that the intensive, short-lasting exercise training has antiglucocorticoid action in the skeletal muscle which is directly related to the

cellular contractile apparatus and the extracellular matrix. The recruitment of fast-twitch muscle fibres during glucocorticoid treatment seems to be functionally related to the anticatabolic effect in the musculoskeletal system.

2. Effect of dexamethasone on the extracellular matrix

The skeletal muscle weakness in case of glucocorticoid myopathy is caused by lesions of myofibrillar apparatus (Seene, 1994; Seene *et al.*, 2003) and by changes in neuromuscular synapses in fast-twitch (FT) muscle fibres (Seene *et al.*, 1988). The extracellular matrix outside of muscle fibres forms a dynamic meshwork which gives coherence and mechanical strength and functions as stress-tolerant system (Aumailley & Gayraud, 1998). This system distributes the forces of muscle contractions both in muscle and tendon. Extracellular matrix is also influenced by glucocorticoids (Oikarinen *et al.*, 1986; Hanson *et al*., 1997).

The excess of glucocorticoids has some similarities in action on the intracellular and extracellular compartments of skeletal muscle, like a decreased synthesis of proteins (Seene, 1994; Ahtikoski *et al.*, 2004). Down-regulation of collagen synthesis during dexamethasone administration shows that ECM components decrease (Ahtikoski *et al*., 2004). As shown by us previously (Seene *et al*., 2003), MyHC synthesis rate decreased only in FT muscles. In the present study the expression of collagen I, III and IV mRNA decreased in both FT and slow-twitch (ST) muscles. It seems that ten days of dexamethasone treatment influenced similarly fibril- and network-forming collagen expression in ST and FT muscles and differed at this point from contractile protein myosin synthesis which was depressed in FT muscles only.

The second principal difference between contractile proteins and ECM during the excess of glucocorticoids revealed in this study was the degradation rate of proteins. Dexamethasone increased the degradation of contractile proteins in skeletal muscle about 2.1 times but the expression of MMP-2 mRNA did simultaneously not change significantly, although the degradation of collagens occurs mainly through the MMP activity (Everts *et al.,* 1996). This is surprising as it was shown earlier that ST muscles contain about 50% more collagen than FT muscles (Kovanen *et al*., 1984).

The concentration of endomysial collagen is also higher around FT fibres (Kovanen *et al*., 1984), but as the downregulation of synthesis and unchanged degradation of type IV collagen are very similar with fibrillar collagen, it gives us reason to conclude that the mechanical stability in skeletal muscle fibres, ensured by collagen IV, does not differ between ST and FT fibres. The effect of excessive glucocorticoids on muscle weakness is applied through damaged contractile machinery of FT muscle fibres' intracellular compartment (Seene *et al*., 2003).

Our study shows that the excess of glucocorticoids has some similarities in its action on the intracellular and extracellular compartments of skeletal muscle like decrease in protein synthesis and collagen expression. The difference between the intracellular and the extracellular compartments appears in FT and ST fibres. The intracellular compartment of muscle reacts to the excess of glucocorticoids with decrease of synthesis only in FT muscles. In extracellular matrix the decrease of protein synthesis appears in both, FT and ST muscles. The second difference between the intracellular and the extracellular compartments is the reaction to the protein degradation. In the intracellular compartment of skeletal muscle, a significant degradation of contractile proteins is a typical effect of excessive glucocorticoids, while in the extracellular compartment the expression of the matrix metalloproteinases did not change significantly.

3. Effect of unloading on the contractile apparatus of skeletal muscle

3.1. Effect of hindlimb suspension and reloading

Unloading decreases selectively the mass of different muscles. One week spaceflight decreased Sol and GM muscle mass, but not the mass of EDL, and the decrease of myofibrillar proteins in ST muscles was significantly greater than the decrease of sarcoplasmic proteins (Fitts *et al*., 2000). The unloading has been shown to change muscle twitch characteristics. The decrease of myosin heavy chain (MyHC) I isoform expression and increase of MyHC IIb and IIx isoform expression in ST muscle improve abovementioned statement (Yamauchi *et al*., 2002).

The present study shows that the muscle mass of Sol and GM decreased significantly during the hindlimb suspension and increased during the two-week reloading. The muscle strength also decreased during hindlimb suspension, but this did not recover during reloading period so fast as did the muscle mass. The recovery of motor activity after the hindlimb suspension is as fast as the recovery of the muscle strength. It is propably related with the regeneration of the muscle structures from disuse atrophy (Itai *et al*., 2004). The two-week reloading period has shown that the SOL muscle metabolism can be restored (Desplanches *et al*., 1987b; Desaphy *et al*., 2005). Full recovery of ST muscle function via cross-sectional area and myonuclear domain size has been shown to need more time for restoration of neural and mechanical properties of muscle (Ohira *et al*., 2006).

3.2. Effect of decreased mechanical load on human *m. vastus medialis*

The results of this experiment demonstrate the effect of decreased mechanical load on the functional characteristics and MyHC composition in human m. *vastus medialis*. Prolonged periods of time spent with a diminished or no-weight bearing have a deleterious effect on skeletal muscle with the decreased protein synthesis, the loss of muscle mass and alterations of biochemical parameters (Edgerton *et al*., 1995). The main finding of our study confirmed that the proportion of slow MyHC isoforms decreased and the proportion of MyHC fast isoforms increased in consequence of altered functional conditions.

Clinical observations show that the atrophy of the skeletal muscle occurs as a result of immobilization and is caused by the changed functional conditions in the muscular system. Skeletal muscle function depends on the intact proprioceptive activity, motor innervation, mechanical load, and joint mobility. If one of these factors is altered, the muscle will undergo adaptation. As an increased muscular activity leads to the enhancement of the structures involved in contraction, inactivity or disuse is followed by the reduction of the muscle mass (Andersen *et al*., 1996).

Alterations of biochemical parameters and changes at the ultrastructural level of the contractile apparatus are considered to be characteristic of atrophied muscles. The effect of disuse on the skeletal muscle depends on the fibre type composition of the muscle. The degenerative changes in disused muscles at the ultrastructural level have been shown to be most severe in slow oxidative muscles (Itai *et al*., 2004). The antigravity muscles which maintain posture and which are permanently loaded consist mainly of slow oxidative muscle fibres. Lieber *et al*., (1988) suggested that the most vulnerable muscles were anti-gravity muscles crossing a single joint. M. *vastus medialis*, examined in our experiment, belongs to this group of muscles.

The changes observed in our study confirm the high susceptility of this muscle to the effects of inactivity due to disuse. M. *vastus medialis* consists mainly of slow oxidative and oxidative-glycolytic muscle fibres and the MyHC isoforms MyHC I and MyHC IIa, thus being very susceptible to the effects of disuse. The properties of muscle contraction which depend on the MyHC isoform composition decrease in atrophied skeletal muscle (Itai *et al*., 2004). Contractile activity can induce differential expression of myosin protein isoforms in skeletal muscle. MyHC composition has an important regulatory role in myosin ATPase activity and muscle fibre shortening velocity (Fauteck & Kandarian, 1995). A prolonged inactivity causes alterations in the MyHC composition. A decrease in the mechanical load stimulates the conversion of slow myosin in muscles of mixed fibre type composition, whereas a decrease in the weight-bearing load results in a decrease in slow myosin content (Thomason *et al*., 1987; Tsika *et al*., 1987; Pette & Staron, 2001).

Comparing the changes of proportion of MyHC isoforms in the contractile apparatus during unloading among human subjects and experimental animals, we can see that they are similar in their direction and amplitude. So we can conclude that the adaptation of the mammalian skeletal muscles to the unloading depends on the contractile characteristics of the muscle and is not dependent on the species of the mammal. As the certain connections exist between the contractile and metabolic characteristics of skeletal muscle it is understandable why the specific atrophy causes the decrease of the main function of the skeletal muscle.

4. Effect of unloading on the extracellular matrix

Fibrillar type I and III collagens are most abundant in the skeletal muscle epi- and perimyseum. Non-fibrillar type IV collagen is present only in basement membranes and has a critical role in the cellular arrangement of muscle tissue (Kühl *et al*., 1984; Light & Champion, 1984). There are differences in the collagen metabolism and the content between muscles. ST muscles contain 40–50% more collagen than FT muscles (Kovanen *et al*., 1984).

A reduced muscular activity decreases the collagen synthesis rate in the skeletal muscle, the immobilization downregulates the collagen synthesis at the pretranslational level, mainly among I and III collagens (Han *et al*., 1999b; Takala & Virtanen, 2000). Unloading also induces a shift in the relative proportion of collagen isoform type I to III (Miller *et al*., 2001). Decrease of collagen I mRNA level in Sol and GM muscle during the three-week hindlimb suspension in the present study shows that the fibrillar type I collagen is more sensitive to unloading and the effect is much more long-lasting than that of fibrillar type III collagen. This finding is in accordance with earlier findings where it was shown that hindlimb unloading induces reduction of collagen type I.

The reloading after the hindlimb suspension in the present study shows that collagen III mRNA level at the end of the second week is higher than in control group.

The present study reveals that non-fibrillar type IV collagen mRNA level is decreasing in both, ST and FT muscles during the three weeks of hindlimb suspension, but two weeks of reloading period is obviously not enough to restore the metabolic states of this collagen in the basal lamina of the muscle fibre. It was previously shown that the reorganization of the basement membrane compounds needs certain time (Koskinen *et al.*, 2001). As type IV collagen plays a role in the regenerative process on ECM, including the matrix-associated signals and the membrane-associated receptors that underline muscle fibre-matrix interactions, it shows how complicated is the evaluation of the functional significance of type IV collagen metabolism (Sanes, 2003).

Lysyl oxidase which plays an important role in the formation and regeneration of ECM by oxidizing lysine residues in elastin and collagen, initiates the formation of covalent cross-linkages which stabilize fibrous proteins (Kagan & Li, 2002). From this standpoint it is understandable that a significant decrease in LO mRNA level was registered in this study only in Sol muscle.

Matrix metalloproteinases are providing degradation of ECM compounds (Nagase & Woessner, 1999). MMP-2 level did not change significantly in the present study during three weeks of hindlimb suspension but increased in Sol muscle during two weeks of reloading and in GM muscle after one week reloading. TIMPs are proteins which inhibit ECM degradation (Gomez *et al*., 1997). In the present study, the mRNA level of TIMP-1 decreased in Sol muscle after one-week hindlimb suspension, but increased in GM muscle during three-week suspension. TIMP-2 mRNA level increased in GM muscle after two weeks reloading. As both intracellular (lysosomal phagocytosis) and extra-cellular degrading pathways (ECM proteinases) are present in the degradation of the skeletal muscle (Kjær, 2004) during the unloading and the following reloading, it is complicated to put all the role to the MMPs in this process.

The changes of fibrillar and non-fibrillar collagen, LO, MMPs and TIMPs on the pretranslational level are shown for the first time together with changes in muscle strength and motor activity during the three weeks of hindlimb suspension and following two week reloading. The biggest changes in the specific mRNA level of type I, III and IV collagen were registered in Sol and GM muscle, during the three weeks of unloading. mRNA level of LO decreased also in Sol muscle. A significant increase in mRNA level for MMP-2 was registered in Sol muscle during the reloading, showing that the reaction of MMP-2 on the pretranslational level is not fast in all muscles. Changes in TIMP-1 mRNA level during first week of hindlimb suspension were contradictory in Sol and GM muscle.

The present study demonstrates that the metabolism of fibrillar and non-fibrillar collagens in ECM plays a crucial role in exercise and sport, influencing the strength development through transmission of contractile force in skeletal muscle.

CONCLUSIONS

1. The glucocorticoid treatment caused atrophy and decrease in fractional synthesis rate of myosin heavy chains in fast-twitch skeletal muscles. The collagen synthesis rate during glucocorticoid treatment is independent of the kinetic properties of the skeletal muscle. The specific level of mRNA for collagens type I, III and IV, and matrix metalloproteinase-2 during the glucocorticoid myopathy does not differ in slow-twitch and fast-twitch muscle.
2. The simultaneous exercise training during the glucocorticoid myopathy diminished significantly the atrophy in fast-twitch skeletal muscles but does not prevent significantly the decrease of the myosin heavy chain fractional synthesis rate. The specific mRNA level for collagens type I, III and IV does not change significantly during simultaneous glucocorticoid administration and exercise.
3. Hypokinesia induced the slow-to-fast transition of myosin heavy chains in skeletal muscle. As a result of hypokinesia the extensive atrophy in slow-twitch muscles occured. The decrease of motor activity and muscle strength were accompanied by the muscle atrophy.

   The specific level of mRNA for all studied types of collagens decreases as a result of unloading in both slow-twitch and fast-twitch skeletal muscles. The decrease of the mRNA level of lysyl oxidase is most expressed in slow-twitch skeletal muscle. The specific level of matrix metalloproteinase-2 does not change in neither type of muscle, whereas the specific mRNA level for tissue inhibitor of metalloproteinase-1 increased in both slow-twitch and fast-twitch skeletal muscle.
4. 2-week reloading enhanced the specific level of mRNA for all studied types of collagens to the control level in both slow-twitch and fast-twitch skeletal muscles. The mRNA level for type III collagen exceeded the control level. Reloading increased the specific level of mRNA for matrix metalloproteinase-2 in fast-twitch skeletal muscles.

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SUMMARY IN ESTONIAN

Glükokortikoidse müopaatia, hüpokineesia ja koormuse mõju skeletilihase kontraktiilsele aparaadile ja rakuvälisele maatriksile

Töö eesmärgiks oli uurida glükokortikoidse müopaatia ja hüpokineesia mõju atroofia kujunemisele kiiretes ja aeglastes skeletilihastes, tuginedes muutustele rakuvälises maatriksis ja kontraktiilses aparaadis ning lihasjõus ja motoorses aktiivsuses.

Töös püstitati järgmised ülesanded: uurida glükokortikoidide poolt põhjustatud atroofia teket skeletilihastes ja sellega kaasnevaid fibrillaarse ning mittefibrillaarse kollageeni, lüsüül-oksidaasi ja maatriksi metalloproteinaaside spetsiifilise mRNA ning kontraktiilsete valkude sisalduse muutusi; võrrelda kehalise treeningu preventiivse toime ulatust erinevat tüüpi lihaste rakuvälisele maatriksile ja kontraktiilsetele valkudele glükokortikoidse müopaatia puhul; uurida hüpokineesia mõju skeletilihase kontraktiilsetele valkudele ja rakuvälisele maatriksile lähtudes müosiini raskete ahelate kompositsiooni muutustest ning fibrillaarse ja mittefibrilaarse kollageeni, lüsüül-oksidaasi, maatriksi metalloproteinaas-2 ja selle inhibiitorite mRNA taseme muutustest; analüüsida hüpokineesiale järgneval taastumisperioodil tekkivaid muutusi skeletilihase rakuvälises maatriksis.

Töö tulemused näitasid, et glükokortikoidide manustamisel kiiretes lihastes tekkiva atroofiaga kaasneb kontraktiilsete valkude sünteesi ja sisalduse langus. Samas ei sõltu kollageeni sünteesi intensiivsus glükokortikoidse müopaatia puhul lihaste kineetilistest omadustest, langedes nii aeglases kui kiires lihases. Hüpokineesia toimel tekib ulatuslik atroofia ainult aeglastes lihastes, kuid rakuvälise maatriksi komponentide süntees väheneb samal ajal nii aeglases kui kiires lihases. Lihasatroofiaga kaasnevad motoorse aktiivsuse ning lihasjõu vähenemine. Glükokortikoidide manustamine mõjutab I, III ja IV tüüpi kollageeni, kuid mitte maatriksi metalloproteinaas-2 mRNA taset nii aeglastes kui kiiretes lihastes. Kehaline treening samaaegselt glükokortikoidide manustamisega ei avalda märgatavat mõju I, III ja IV tüüpi kollageeni mRNA tasemele, kuid vähendab oluliselt atroofia teket ning valgusünteesi intensiivsust kiiretes lihastes.

Hüpokineesia tagajärjel väheneb mRNA hulk kõigis uuritud kollageeni tüüpides nii aeglases kui ka kiires lihases. Kollageeni sünteesi võtme-ensüümi lüsüül-oksidaasi mRNA tase langeb kõige enam aeglases lihases. Samal ajal ei muutunud üheski uuritud lihases maatriksi metalloproteinaas-2 mRNA hulk, kuid maatriksi metalloproteinaasi inhibiitori hulk suurenes oluliselt nii aeglases kui kiires lihases. Hüpokineesia põhjustas müosiini raskete ahelate isovormilise kompositsiooni muutuse, mis väljendus kiirete isovormide osakaalu suurene-

mises. Hüpokineesiale järgneval 2-nädalasel taastumisperioodil suurenes kõigi uuritud kollageenitüüpide mRNA tase jõudes kontrollrühma tasemele nii aeglases kui kiires lihases. Samal ajal suurenes ka maatriksi metalloproteinaas-2 mRNA tase kiires lihases.

ACKNOWLEDGEMENTS

This study was carried out at the Laboratory of Functional Morphology, University of Tartu and at the Department of Biology of Physical Activity, University of Jyväskylä. The work was partly supported by Estonian Science Foundation Grants N6501, 4611 and grant by CIMO.

I wish to thank all my colleagues, teachers and friends in the Institute of Exercise Biology and Physiotherapy and at the Department of Traumatology and Orthopaedics, University Hospital, University of Tartu and at the Department of Biology of Physical Activity, University of Jyväskylä.

I am very thankful to my supervisor Professor Teet Seene for his guidance and encouragement throughout this work.

I express my thanks to Priit Kaasik, PhD, who helped me through the difficult phases of this work.

It is a great pleasure to thank all my colleagues at the Laboratory of Functional Morphology: Karin Alev, PhD, for her goodwill and patience, Raivo Puhke, PhD, and Ando Pehme, PhD, for immense readiness to help and warm attitude, Ms Piret Pärsim for help in the preparation of manuscript and kind support.

I am grateful to all my Finnish colleagues and collaborators, especially: Professor Timo Takala, Vuokko Kovanen, PhD, Anne Ahtikoski, PhD and Satu Koskinen, PhD. I thank Ms Mervi Koistinen and Ms Aila Ollikainen for excellent technical support.

I would thank Aalo Eller, MD and Aivar Pintsaar, MD, for their collaboration in the clinical parts of this study.

I am thankful to Ms Tiiu Allik for revising the English language of my manuscript.

Finally, I wish to thank all my family. My warmest thanks belong to my husband Andrus and our children Katrin, Märt and Liisa for support and understanding during these years.

PUBLICATIONS

I

Riso E.-M., Ahtikoski A.M., Alev K., Kaasik P., Pehme A., Seene T.
Relationship between extracellular matrix, contractile apparatus, muscle mass and strength in case of glucocorticoid myopathy.
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II

Riso E.-M., Ahtikoski A.M., Takala T.E.S., Seene T.
The effect of unloading and reloading on the extracellular matrix in skeletal muscle: changes in muscle strength and motor activity.
Journal of Biology of Sport, 2007, accepted.

THE EFFECT OF UNLOADING AND RELOADING ON THE EXTRACELLULAR MATRIX IN SKELETAL MUSCLE: CHANGES IN MUSCLE STRENGTH AND MOTOR ACTIVITY

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Short title for pages: muscle unloading, reloading and extracellular matrix

Keywords: extracellular matrix, unloading, reloading, muscle strength, motor activity

ABSTRACT

During three weeks of hindlimb suspension muscle mass decreased 36% ($p<0.05$) in

Soleus (Sol) muscle, 17% ($p<0.05$) in Gastrocnemius (GM) and had tendences to decrease in plantaris (Pla) (15%) and in extensor digitorum longus (EDL) (8%) muscles. Hindlimb grip strength decreased gradually during three weeks of unloading. Specific mRNA level for type I collagen decreased during three weeks of unloading in Sol muscle by 28% ($p<0.05$) and in GM muscle by 34% ($p<0.05$). mRNA level for type III collagen decreased in Sol by 22%

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(p<0.05) and in GM by 51% (p<0,001). Non-fibrillar type IV collagen mRNA level decreased in both above-described muscles about 25% (p<0.05). Lysyl oxidase (LO) mRNA level decreased by 46% (p<0.05) during three weeks of unloading only in Sol muscle. Matrix metalloproteinase-2 (MMP-2) mRNA level increased during reloading period in Sol and GM muscles subsequently 28% (p<0.05) and 49% (p<0.001). During unloading the activity of tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2) in slow-twitch (ST) and fast-twitch (FT) muscles changed in different directions: during first week of suspension, their expression decreased in Sol muscle by 31% (p<0.05) and increased in Pla and GM muscle subsequently by 24% (p<0.05) and 31% (p<0.001). The pretranslational level of changes in fibrillar and non-fibrillar collagen, MMP-2, LO, TIMP-1 and TIMP-2 -are shown for first time together with changes in muscle strength and motor activity during unloading and reloading

Key words: extracellular matrix, unloading and reloading.

INTRODUCTION

Disuse causes muscle atrophy by decreased protein synthesis and increased degradation. Reduction in muscle cell size is accompanied with the enhanced volume of connective tissue (27). Impaired function of skeletal muscle occures not only due to atrophy, but also because of connective tissue, separating capillaries from fibers (11). Although the amount of connective tissue increases during disuse, the gene expression of fibrillar collagens in slow-twitch (ST) muscle decreases in the very beginning of disuse (9). Hindlimb unloading during 28 days did not cause significant changes in fibrillar collagen expression (21).

Lysyl oxidase and collagen types I, III and IV in skeletal muscle have been shown up-regulated rapidly at the pretranslational level after strenuous muscle activity (8),but the changes during unloading are still unclarified. Induction of the collagen chain synthesis and a lysyl oxidase (LO) initiated cross-linking of collagen in the extracellular matrix (ECM) is co-ordinated (4).

The effect of disuse on non-fibrillar type IV collagen is less studied. As type IV collagen represents only a small proportion of skeletal muscle collagens, measurement of total collagen synthesis does not reflect the changes in type IV collagen during unloading. It has been shown that unloading decreased collagen IV synthesis and degradation ratio in skeletal muscle but is strongly muscle specific (1).

Collagens' metabolism depends also from degradation prior to or after their secretion from the cell. Degradation may be proteolytic or phagocytotic. Matrix

metalloproteinases (MMPs) are the main components degrading extracellular matrix (ECM) components. MMP-1 has the ability to cleave the native helical structure of collagens I and III (30) and MMP-2 degrades denatured collagen IV and digests fibrillar type I collagen (26,30). Cytokines, growth factors and corticosteroids are known to induce or repress the transcription of MMP genes (13,20).

MMPs activity is inhibited by tissue inhibitors of metalloproteinases (TIMPs) which regulate MMP activity during tissue remodeling (7).

The purpose of this study was to assess the effect of three-week hindlimb suspension and following two-week reloading on the collagen I, III and IV expression, coordination of this process with lysyl oxidase, matrix metallo-proteinase-2 (MMP-2) and tissue inhibitors of metalloproteinases 1 and 2 (TIMP-1 and TIMP-2) on the pretranslational level. We also wanted to clarify relations between abovementioned changes and changes in muscle mass, muscle strength and motor activity.

We hypothesized that three-week hindlimb suspension decrease the expression of fibrillar and network-forming collagen and increase their degradation. We also hypothesize that reloading helps to recover the collagen expression and decrease their degradation rate.

MATERIALS AND METHODS

Use of the animals was in accordance with European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes and was controlled by the Committee of Laboratory Animal Science of the University of Tartu.

The used animals were 16 weeks old (at the beginning of experiment) Wistar rats (National Laboratory Animal Centre, Kuopio, Finland). All the animals were housed in identical conditions in polycarbonated type III cages, at 21°C. They received diet [SDS-RM1(C)3/8, Witham, Essex, UK] and water *ad libitum*.

The rats were assigned to control, hindlimb-suspended for 1 and 3 weeks and hindlimb reloading group for 1 and 2 weeks. The size of each group was 10 animals. The reloading period consisted of free cage activity for two weeks after 3-week hindlimb suspension.

Hindlimb suspension procedure

A modification of the tail harness model of Morey-Holton and Globus (22) was used to suspend the hindlimbs of rats. The animals were anesthetized, such that the tail was easily manipulated. The skin of the tail was thoroughly cleaned with alcohol and dried. The proximal part of the tail was wrapped with breathing tape so that half of the tail remained uncovered, thereby allowing normal

thermoregulatory processes to occur. The portion of exposed tail maintained normal color, indicating that blood flow was not compromised. A swivel harness was attached on the tail with a strip of adhesive tape. The tape was checked daily and repaired, if necessary. The animal was suspended by the swivel harness from a hook above the suspension cage, allowing free 360º rotation. The height of the hook was adjusted so that only the front limbs were able to contact the cage. The size of cage was such that the animals could easily reach food and water without being able to touch the sides of cages with the hindlimbs. This form of suspension provides traction along the tail and does not cause any obvious lesions on the tail.

The measurement of strength and motor activity

The force and hindlimb grip strength was measured before and after hindlimb suspension and after recovery period with Grip Strength Meter 0167-004L (Columbus Instruments) and expressed as N per 100g of bwt. Motor activity was measured by the screening of the amounts of ambulatory and total (stereotypic) movements by Opto-Varimex-Mini (Columbus Instruments). Ambulatory activity characterizes movemental activity, total activity includes stereotypic (scratching, grooming, digging etc) non-ambulatory movements.

Tissue preparation

Twenty-four hours after the last experimental procedure, the animals were anesthetized by intraperitoneal injection of ketamin (Calysol*, Gedeon Richter* A.O., Budapest, Hungary) 2.5 mg/100 g bwt and diazepam (*Lab Renaudin*, France) 2.5 and sacrified. *The gastrocnemius, plantaris, extensor digitorum longus* and *soleus* muscles were removed, trimmed clean of visible fat and connective tissue, weighed, frozen and stored in liquid nitrogen until further processing. Due to the small size of *soleus* and *extensor digitorum longus* these muscles were pooled for mRNA analyses, so that one sample consisted of right and left muscle of the same animal.

RNA isolation

For total RNA isolation, muscle samples were homogenized with an Ultra-Turrax homogenizer in Trizol (Life Technologies, Painsley, Scotland, UK). Other steps were performed as described in the manufacturer's protocol (Life Technologies 1995). The purity and concentration of total RNA was assessed spectrophotometrically. Northern blot analysis was used for testing the specificity of cDNA probes, whereas slot blot analysis was used for quantification of the specific RNA amount.

mRNA analyses

For Northern blotting, 30 μg of total RNA was denatured in loading buffer, electrophoresed in a 1 % agarose/formaldehyde gel, and transferred to a nylon

membrane (GeneScreen Plus, Biotechnology Systems, Boston, USA) with a standard procedure (2). For slot blotting, 20 μg of total RNA was spotted on a nylon membrane using a vacuum filtration manifold (Minifold II; Schleicher and Schuell, Dassel, Germany) (19). All the membranes were incubated in 0.05 N NaOH for 5 min to bind the RNA to the membrane. Prehybridization was carried out in a solution containing 5 X SSC, 5 X Denhardt's solution, 50% formamide, ssDNA 100 μg/ml, 50 mM sodium phosphate pH 6.8, 10% dextran sulphate and 1% SDS for 2 hrs at 42ºC. The RNA-cDNA hybridization was performed for 24 hrs at 42ºC using the solution containing the same components as the prehybridization solution and [^{32}P] labeled cDNA probe labeled with a Ready-To-Go-DNA Labeling Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The collagen probes were α12, a 2.4 kb human cDNA for proα1(I)-chain mRNA, E6, a 2.4 kb human cDNA for proα1 (III) –chain mRNA and HT21, a 2.6 kb human cDNA for the α1(IV). The rat lysyl oxidase (EC 1.4.3.13) probe was a 0.6 kb product of an EcoRI digest of the p13L-0 clone. 1668bp long cDNA was used for rat MMP-2 RNA. 0.6 kb *ECO* RV insert in pBluescript II plasmid of mouse was used for TIMP-1 and 1.7-kb *ECO* RI insert in pBluescript II plasmid of mouse for TIMP-2. After the hybridization, the membranes were exposed to KodakX-Omat film (Eastman-Kodak, Rochester, NY, USA) at –70ºC. Attained signals were analysed using densitometry (Personel Densitometer SI, Molecular Dynamics, Sunnyvale, CA, USA). The signal obtained by hybridization with a 24 mer oligonucleotide for 18S ribosomal RNA was used to normalize RNA loading/transfer amount.

Statistics

Means and standard errors of means were calculated from individual values by standard procedures of Excel. The data were analysed by SAS, using the analysis of variance (ANOVA). Differences were considered significant at $p<0.05$.

RESULTS

During three weeks of hindlimb suspension the body mass did not increased as fast as in control group (Fig 1. A). Starting from the first week of suspension till the end of the recovery body mass was significantly lower than in control group. Muscle mass decreased during three weeks of suspension 36% ($p<0.05$) in Sol and 17% ($p<0.05$) in GM and 15% in Pla and 8% in EDL. Dynamics of soleus muscle growth is demonstrated in the Fig 1.B.

Motor activity decreased significantly during 3 weeks of suspension and stayed on the same level even after one week of reloading (Fig 2.A). Muscle strength decreased gradually during three weeks of suspension and stayed on the same level in 1 week of reloading (Fig 2.B).

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Specific mRNA level for type I collagen decreased during suspension period in Sol and GM muscles (Fig 4A) and increased to the control group level during 2 weeks of recovery. mRNA level for type III collagen decreased during first week of suspension in Sol and Pla muscle and during one and three weeks of suspension in GM (FIG 3.B). During two weeks of reloading mRNA level for type III collagen increased in Pla and GM muscles in comparison with control group (Fig 3.B). mRNA level for type IV collagen decreased during first week of suspension in Sol muscle and during third week of suspension in Pla and GM muscles (Fig 3.C). In GM muscle mRNA level for type IV collagen stayed on the low level during first week of reloading (FIG 3.C). mRNA level for lysyl oxidase (LO) decreased during three weeks of suspension only in Sol muscle (Fig 3.D) and increased during reloading period in comparison with control group in GM muscle (Fig 3.D). Matrix metalloproteinase 2 (MMP-2) mRNA level did not change significantly in skeletal muscles during reloading period in Sol and GM muscles (Table 1). mRNA level for tissue inhibitors of matrix metalloproteinases-1 (TIMP-1) decreased during first week of suspension in Sol muscle and increased in GM muscle during 1 and 3 weeks of suspension (Table 1). mRNA level of TIMP-2 increased in GM muscle after 2 weeks of reloading (Table 1).

DISCUSSION

Fibrillar type I and III collagens are most abundant in skeletal muscle epi- and perimyseum. Non-fibrillar type IV collagen is present only in basement membranes and has a critical role in the cellular arrangement of muscle tissue (17,18). There are differences in collagen metabolism and content between muscles. Slow twitch (ST) muscles contain 40–50% more collagen than fast twitch (FT) muscles (16).

Reduced muscular activity decreases the collagen synthesis rate in skeletal muscle, immobilization downregulates the collagen synthesis at the pretranslational level, mainly among I and III collagens (9,29). Unloading decreases selectively the mass of different muscles. One week spaceflight decreased Sol and GM muscle mass, but not the mass of EDL, and decrease of myofibrillar proteins in ST muscles was significantly greater than decrease of sarcoplasmic proteins (5). Unloading has been shown to change muscle twitch characteristics. Decrease of myosin heavy chain (MyHC) I isoform expression and increase of MyHC IIb and IIx isoform expression in ST muscle improve abovementioned statement (31). Unloading also induces a shift in the relative proportion of collagen isoform type I to III (21).

The present study shows that muscle mass of Sol and GM decreased significantly during hindlimb suspension and increased during two-week reloading. Muscle strength also decreased during hindlimb suspension, but this

did not recovered during reloading period so fast as did muscle mass. Recovery of motor activity after hindlimb suspension is as fast as recovery of muscle strength. It is propably related with regeneration of muscle structures from disuse atrophy (10). Decrease of collagen I mRNA level in Sol and GM muscle during three-week hindlimb suspension in present study shows that fibrillar collagen type I is more sensitive to unloading and the effect is much more longlasting than that of fibrillar type III collagen. This finding is in accordance with earlier findings where it was shown that hindlimb unloading induces reduction of collagen type I and is the reason of the slow-to-fast myofiber transformation in Sol muscle (21). Reloading after hindlimb suspension in present study shows that collagen III mRNA level on the end of second week is higher than in control group. Two weeks of reloading have been shown to restore the Sol muscle metabolism (3). Full recovery of ST muscle function via cross-sectional area and myonuclear domain size has been shown need more time for restoration of neural and mechanical properties of muscle (25).

Present study shows that non-fibrillar type IV collagen mRNA level is decreasing in both, ST and FT muscles during three weeks of hindlimb suspension, but two weeks of reloading period is obviously not enough to restore the metabolic states of this collagen in muscle fiber basal lamina. It was previously shown that reorganization of basement membrane compounds needs certain time (15). As type IV collagen plays a role in regenerative process on extracellular matrix (ECM), including matrix-associated signals and membrane-associated receptors that underline muscle fiber-matrix interactions, it shows how complicated is the evaluation of the functional significance of type IV collagen metabolism (28).

Lysyl oxidase (LO) which plays an important role in the formation and regeneration of ECM by oxidizing lysine residues in elastin and collagen, initiates the formation of covalent crosslinkages which stabilize fibrous proteins (12). On this standpoint it is understandable that significant decrease in LO mRNA level was registered in this study only in Sol muscle.

Matrix metalloproteinases (MMPs) are providing degradation of ECM compounds (24). MMP-2 mRNA level did not change significantly in the present study during three weeks of hindlimb suspension but increased in Sol muscle during two weeks reloading and in GM muscle after one week reloading. Tissue inhibitors of matrix metalloproteinases (TIMPs) are proteins which inhibit ECM degradation (6). In the present study, the mRNA level of TIMP-1 decreased in Sol muscle after one-week hindlimb suspension, but increased in GM muscle during three-week suspension. TIMP-2 mRNA level increased in GM muscle, after two weeks reloading. As both intracellular (lysosomal phagocytosis) and extracellular degrading pathways (ECM proteinases) are present in degradation of skeletal muscle (14) during unloading and following reloading, it is complicated to put all the role to the MMPs in this process.

CONCLUSIONS

The changes of fibrillar and non-fibrillar collagen, LO, MMPs and TIMPs on the pretranslational level are shown for the first time together with changes in muscle strength and motor activity during three week of hindlimb suspension and following two week reloading. Biggest changes in specific mRNA level of type I, III and IV were registered in Sol and GM muscle, during three weeks of unloading. mRNA level of LO decreased also in Sol muscle. Significant increase in mRNA level for MMP-2 was registered in Sol muscle during reloading, showing that reaction of MMP-2 on the pretranslational level is not fast in all muscles. Changes in TIMP-1 mRNA level during first week of hindlimb suspension were contradictory in Sol and GM muscle.

The present study demonstrates that the metabolism of fibrillar and non-fibrillar collagens in ECM plays a crucial role in exercise and sport, influencing the strength development through transmission of contractile force in skeletal muscle.

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Table 1. Changes in matrix metalloproteinase 2, tissue inhibitors of metalloproteinases 1 and 2 mRNA in *soleus* (Sol), *extensor digitorum longus* (EDL), *plantaris* (Pla) and *gastrocnemius* (Gm) muscle during hindlimb suspension and following reloading

Group	n	muscle	MMP-2	TIMP-1	TIMP-2
hindlimb suspension 1 week	5	Sol	102 ± 9.4	69 ± 7.6*	88 ± 8.2
	5	EDL	97 ± 8.9	94 ± 8.7	103 ± 9.8
	10	Pla	98 ± 5.8	116 ± 10.2	104 ± 9.4
	10	Gm	108 ± 6.1	130 ± 11.0 *	94 ± 5.7
hindlimb suspension 3 weeks	5	Sol	119 ± 10.6	120 ± 10.9	95 ± 9.7
	5	EDL	112 ± 11.1	101 ± 11.3	104 ± 11.2
	10	Pla	96 ± 6.8	124 ± 7.4 *	95 ± 6.2
	10	Gm	114 ± 7.1	131 ± 8.1 **	99 ± 6.7
1 week reloading after suspension	5	Sol	128 ± 10.8 *	105 ± 10.3	107 ± 10.1
	5	EDL	99 ± 9.6	106 ± 9.5	98 ± 9.7
	10	Pla	115 ± 7.4	103 ± 6.9	104 ± 7.1
	10	Gm	125 ± 6.9 *	108 ± 6.6	110 ± 6.8
2 weeks reloading after suspension	5	Sol	149 ± 16 **	117 ± 10.6	104 ± 9.4
	5	EDL	116 ± 12	112 ± 10.4	101 ± 10.2
	10	Pla	92 ± 6.3	106 ± 6.9	92 ± 5.6
	10	Gm	110 ± 5.7	109 ± 6.1	119 ± 7.3 *

Values are mean ± standard error; * – $p<0.05$, ** – $p<0.01$ in comparison with control group (100%)

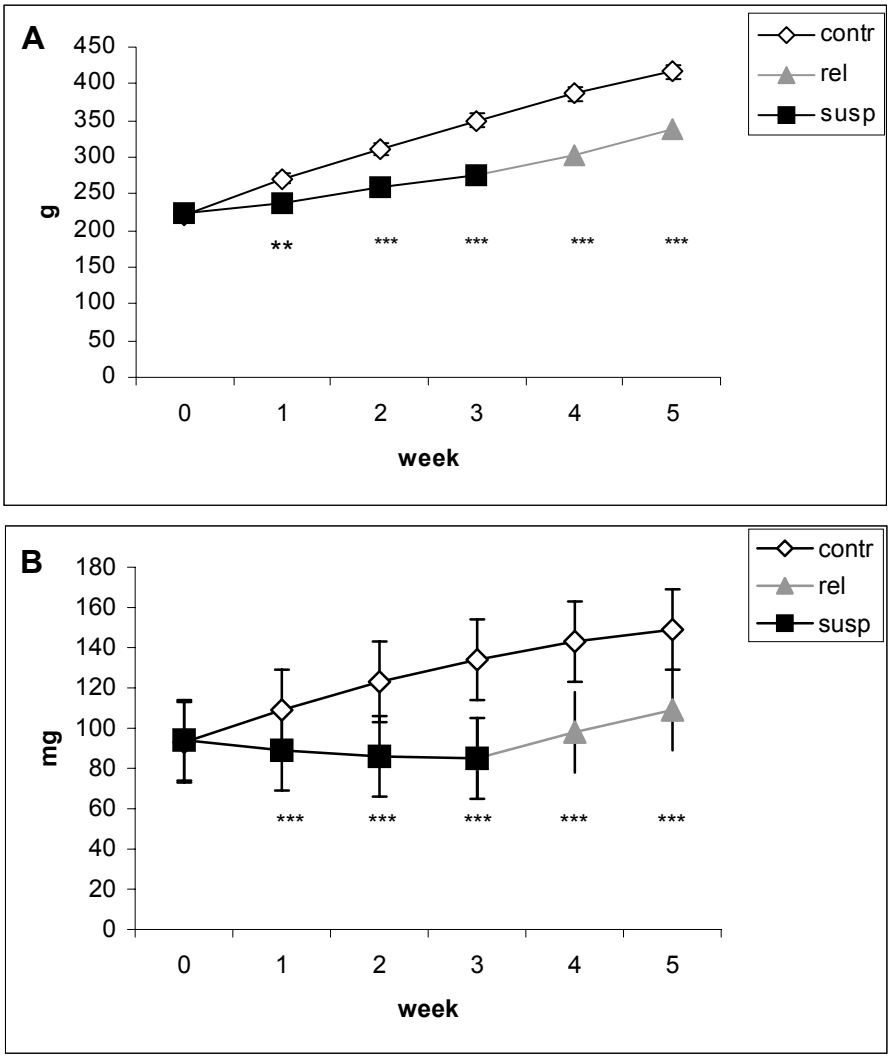

Figure 1. Dynamics of body weight (A) and muscle *soleus* mass (B) during suspension and reloading

0 week – before suspension
3 weeks – end of suspension
5 weeks – end of reloading
values are mean ± standard error
** – $p < 0.01$; *** – $p < 0.001$ in comparison with control group
0 week – control group
rel – reloading group
susp – suspension
n = 10 per group

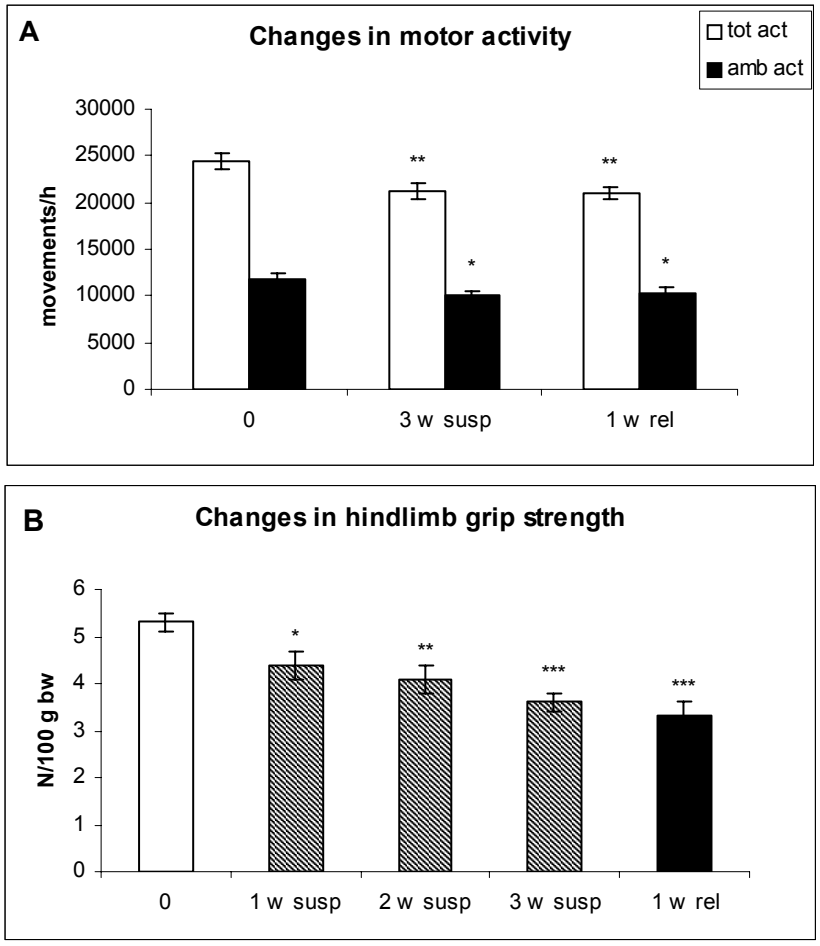

Figure 2. Changes in motor activity (A) and hindlimb grip strength (B) during 3 weeks immobilization and following 1 week reloading

Ambulatory activity characterizes movemental activity of animals, total activity includes stereotypic (scratching, grooming, digging…) non-ambulatory movements. Grip strength of hindlimb was used for characterization of changes in muscle strength during 3-weeks immobilization and 1-week recovery after immobilization
0 week – control group
rel – reloading group
susp – suspension
tot act – total activity
amb act – ambulatory activity
3w susp – three weeks of hindlimb suspension
1w rel – one week of reloading after hindlimb suspension
values are mean ± standard error
$p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$ in comparison with control group
n = 10 per group

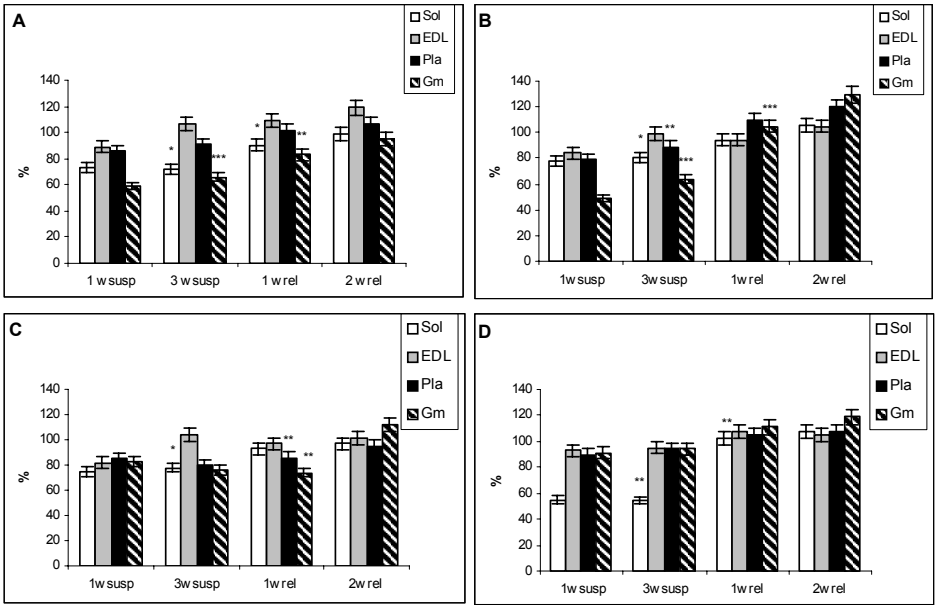

Figure 3. Specific muscular mRNA levels for type I (A), type III (B), type IV (C) and lysyl oxidase (D) in *soleus* (Sol), extensor *digitorum longus* (EDL), *plantaris* (Pla) and gastrocnemius (Gm) during suspension for 1 and 3 weeks and following reloading for 1 and 2 weeks

Control group – 100%
1w susp – one week of hindlimb suspension
3w susp – three weeks of hindlimb suspension
1w rel – one week of reloading after hindlimb suspension
2w rel – two weeks of reloading after hindlimb suspension
values are mean ± standard error; n = 5 Sol, EDL; and n = 10 Pla, Gm
* – $p<0.05$; ** – $p<0.01$; *** – $p<0.001$ in comparison with control group

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III

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Partial prevention of muscle atrophy in excessive level of glucocorticoids by exercise: effect on contractile proteins and extracellular matrix.
The Baltic Journal of Laboratory Animal Science, 2003, 13: 5–12.

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Erialaorganisatsioonid

Eesti Füsioloogia Seltsi liige
Eesti Katseloomateaduse Seltsi liige

Teadustegevus

Peamised uurimisvaldkonnad: glükokortikoidne müopaatia, hüpokineesia mõju skeletilihasele
Kokku on ilmunud 31 teaduspublikatsiooni, sh 8 artiklit rahvusvahelistes eel-refereeritavates ajakirjades ja kogumikes.

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