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To my parents Kaarina and Kalevi Virtanen

ABSTRACT

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Effects of physical activity and experimental diabetes on carbonic anhydrase III and markers of collagen synthesis in skeletal muscle and serum
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The adaptations of skeletal muscle carbonic anhydrase III (CA III) and collagen and tendon collagen expression to immobilization and subsequent remobilization were studied. The adaptation of skeletal muscle collagen was also studied during denervation and subsequent reinnervation process. Collagen synthesis was evaluated by the activities of prolyl 4-hydroxylase (PH) and galactosyl-hydroxylysyl glucosyltransferase (GGT), and collagen content by the concentration of hydroxyproline (Hyp). The effects of streptozotocin-induced diabetes and endurance training were studied on CA III expression at mRNA and protein levels. In addition, the effects of concentric exercise were studied on the release of muscle proteins and markers of collagen synthesis. The specific PH and GGT activities decreased significantly after one week of immobilization in rat soleus muscle immobilized in shortened position, whereas in tibialis anterior muscle immobilized in stretched position, the activities increased. The degree of muscle stretch during immobilization seemed to be an important regulator of collagen expression in muscle. During the follow-up of remobilization, the activities of PH and GGT returned towards the control levels. In human vastus medialis muscle, total CA III decreased to the same extent as the muscle cross-sectional area during immobilization for six weeks. Denervation brought with it an increased level of collagen biosynthesis and Hyp concentration in rat skeletal muscle. During the follow-up of reinnervation, both the PH and GGT activities and the Hyp concentration decreased to the control level. Both streptozotocin-induced diabetes and endurance training increased CA III expression in rat skeletal muscle independently. The finding may represent an adaptation of skeletal muscle cells to increased oxidative stress, which is known to be caused by both treatments. A single bout of heavy concentric exercise caused protein leakage from human muscles and most probably the activation of type I collagen secretion from collagen synthetizing cells which seemed to depend on the strain and damage of the musculoskeletal system.

Key words: Carbonic anhydrase III, collagen, denervation, immobilization, exercise, skeletal muscle, streptozotocin-induced diabetes

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ABBREVIATIONS

ATPase	adenosine triphosphatase
CA	carbonic anhydrase
CA III	carbonic anhydrase III
cDNA	complementary deoxyribonucleic acid
CK	creatine kinase
CS	citrate synthase
CT	computed tomography
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDL	extensor digitorum longus muscle
EMG	electromyogram
Eu	europium
GGT	galactosylhydroxylysyl glucosyltransferase
Gly	glycine
GM	gastrocnemius muscle
Hyp	hydroxyproline
KDa	kilodalton
LDH	lactic dehydrogenase
LEC	liver endothelial cells
Mb	myoglobin
MHC	myosin heavy chain
MW	molecular weight
mRNA	messenger ribonucleic acid
MVC	maximal voluntary contraction
NS	nonsignificant
PGK	phosphoglycerate kinase
PH	prolyl 4-hydroxylase
Pro(I)-C-P	carboxyterminal propeptide of type I procollagen
Pro(I)-N-P	aminoterminal propeptide of type I procollagen
Pro(III)-N-P	aminoterminal propeptide of type III procollagen
RF	rectus femoris muscle
RIA	radioimmunoassay
RNA	ribonucleic acid
S	serum
Sm	samarium
SD	standard deviation
SE	standard error
TA	tibialis anterior muscle
TGF-β	transforming growth factor β
TGF-β1	transforming growth factor β1
TnI	troponin I
TnT	troponin T
VL	vastus lateralis muscle

LIST OF ORIGINAL ARTICLES

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

I Virtanen P, Väänänen HK, Pasanen L, Lähde S, Puranen J and Takala TES 1991. Effect of immobilization on carbonic anhydrase III andmyoglobin content in human leg muscle. Acta Physiol Scand 142: 303-306.
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II Virtanen P, Vuori J, Väänänen K, Han X-Y, Wang W and Takala TES. Effects of streptozotocin-induced diabetes, training and their combination on the expression of carbonic anhydrase III in rat skeletal muscle. Submitted for publication.

III Karpakka J, Virtanen P, Väänänen K, Orava S and Takala TES 1991. Collagen synthesis in rat skeletal muscle during immobilization and remobilization. J Appl Physiol 70(4): 1775-1780.
https://doi.org/10.1152/jappl.1991.70.4.1775

IV Karpakka J, Väänänen K, Virtanen P, Savolainen J, Orava S and Takala TES 1991. The effects of remobilization and exercise on collagen biosynthesis in rat tendon. Acta Physiol Scand 139: 139-145.
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V Virtanen P, Tolonen U, Savolainen J, Takala TES 1992. Effect of reinnervation on collagen biosynthesis in rat skeletal muscle. J Appl Physiol 72(6): 2069-2074.
https://doi.org/10.1152/jappl.1992.72.6.2069

VI Virtanen P, Viitasalo JT, Vuori J, Väänänen K, Takala TES 1993. Effect of concentric exercise on serum muscle and collagen markers. J Appl Physiol 75(3): 1272-1277.
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CONTENTS

1 INTRODUCTION

Skeletal muscle is the most abundant tissue in the body, and is responsible for all bodily movements. The adaptability of skeletal muscle to changes in physical activity is remarkable (Saltin and Gollnick 1983). However, both very small and very extensive physical loading may lead to undesirable changes in muscles and tendons. Proper muscular function depends on intact proprioceptive activity, motor innervation, mechanical load, ability to perform the stretch-shortening cycle, and mobility of the joints. These factors may influence each other, and if one of them is altered muscular adaptation will occur.

Injuries incurred in sports are frequently located at the limbs. Ruptured ligaments and tendons or fractured bones and dislocations of joints require surgery or conservative treatment and subsequently a period of immobilization. Prolonged immobilization of a limb is associated with so-called disuse atrophy (e.g. Wolf et al. 1971, Ingemann-Hansen and Halkjaer-Kristensen 1980). Loss of innervation causes the inhibition of active voluntary contractions of the affected muscle and also results in muscle atrophy (e.g. Jaweed et al. 1975, Niederle and Mayr 1978). Restoration from atrophy is slow, and does not always lead to a full recovery.

Some muscles of the lower extremities are active even when standing. The soleus muscle of the leg is such an antigravity muscle which is mainly composed of type I fibers. Type I fibers are responsible for force production at lower intensities of physical activity. As the intensity is increased type IIa fibers and after that type IIx and finally type IIb fibers are recruited. Type II fibers are also activated when low intensity activity lasts longer. Gradually, adaptive changes in type I fibers occur and type II fibers change in a more oxidative direction. Carbonic anhydrase III (CA III) is one of the major soluble proteins in skeletal muscle. Its concentration is highest in type I fibers, lower in type IIa and lowest or even absent in type IIb fibers (Väänänen et al. 1982, 1985, Jeffery et al. 1987). The physiological function of CA III has not been fully resolved, but evidence has gathered recently suggesting a role as an antioxidant.

Collagen is the major protein of the muscular connective tissue matrix (e.g. Duance et al. 1977, Mayne and Sanderson 1985). The amount of collagen in muscle, tendons and ligaments and its physical properties point to the fact that changes in collagen biosynthesis play a major role in the adaptation of the musculoskeletal system to changes in physical activity.

The purpose of the present study was to examine the adaptive responses of CA III expression and collagen biosynthesis to variations in physical activity, the response of CA III to streptozotocin-induced diabetes and endurance training, the response of collagen to denervation and subsequent reinnervation process, and the effects of concentric exercise on the release of muscle proteins and markers of collagen synthesis.

2 REVIEW OF THE LITERATURE

2.1 Effects of physical activity on skeletal muscle fiber types

Muscle mass is known to respond to alterations in activity patterns. Increased work demands lead to compensatory growth, while loss of activity induces tissue atrophy. It has been shown that the loss of normal weight bearing function, in addition to a reduction in contractile activity, is of major importance in facilitating the wasting of skeletal muscle and also the attenuation of muscle growth (Alford et al. 1987, Babij and Booth 1988). Different experimental models have been used to assess the effects of the loss of normal activity in skeletal muscle. Methods designed to produce a state of muscle inactivity without interrupting the nerve supply include e.g. hind limb immobilization, hind limb suspension and tenotomy (Musacchia et al. 1988, Thomason and Booth 1990). Space flights are also known to cause a marked weight loss in mammalian skeletal muscle as a result of exposure to microgravity (Martin 1988, Desplanches 1997), the effects of which have been simulated by different hypokinesia models (Josza et al. 1988, Roy et al. 1996). Denervation, which involves cutting the motor nerve supply to the tissue, is an animal model that is often used (e.g. Herbison et al. 1973, Gundersen and Merlie 1994). In addition to these models, for instance experimental diabetes results in loss of protein, particularly from muscle, by affecting nitrogen metabolism (Manchester 1970). This is due to lack of insulin, which leads to a decrease in the protein synthesis rate (Pain and Garlick 1974).

Prolonged immobilization of a limb is associated with disuse atrophy of the casted muscles, but the extent of this atrophy differs between muscles and fiber types. During rat hind limb immobilization, the muscles involved in anti-gravity or postural work are more liable to the disuse effect. The ankle joint plantar flexors, and of those especially the soleus and gastrocnemius muscles, are sensitive to disuse (Herbison et al. 1978), whereas the dorsal flexors such as tibialis anterior and extensor digitorum longus (EDL) are less responsive (Musacchia et al. 1988). Type I fibers are known to atrophy more than type II fibers (Edgerton et al. 1975). Thus in soleus, which is mainly composed of type I

fibers, the atrophy is more pronounced than in gastrocnemius or plantaris, which consist mostly of type II fibers (Ariano et al. 1973). Muscle stretch during immobilization is an important determinant of the muscular protein balance. Immobilization of the muscle in shortened position produces a rapid loss of muscle mass, whereas a chronically lengthened position has been shown to either attenuate or prevent muscle atrophy, and in some cases to produce hypertrophy (Hurme et al. 1990, Booth et al. 1996). Denervation is known to cause considerable muscle atrophy, but the fiber types are affected differentially in the slow and fast muscles: type I fibers of rat soleus atrophy as much or slightly more than type II fibers, whereas the type II fibers of EDL, tibialis anterior and plantaris atrophy significantly more than the type I fibers during the first weeks of denervation (Jaweed et al. 1975, Niederle and Mayr 1978, Henning and Lomo 1987, Lu et al. 1997). Lu et al. (1997) found that in EDL and tibialis anterior, type I fibers atrophy more slowly than type II fibers, and more pronounced atrophy in type I fibers is seen only after denervation periods of several months. In mouse soleus muscle, which normally contains slightly more slow than fast twitch fibers, denervation followed by self-reinnervation was shown to cause a significant increase in type I fiber content (Foehring et al. 1986, Bishop and Milton 1998).

Regardless of the techniques adopted to induce muscle disuse in animals, the consequent atrophy is associated with reductions in total protein content without decrements in protein concentration in general (Musacchia 1988). Individual proteins however, can exhibit significant reductions in concentration, e.g. myofibrillar proteins appear to be particularly susceptible to disuse effects (Herbison et al. 1979, Jaspers et al. 1985, Booth and Criswell 1997). Disuse atrophy during immobilization features both slower protein synthesis and faster protein degradation (Witzmann et al. 1982, Jaspers and Tischler 1984, Goldspink and Lewis 1985, Booth and Criswell 1997). Alterations in protein turnover following denervation or tenotomy are effected primarily by marked enhancement of protein breakdown with a smaller impact on protein synthesis (Musacchia et al. 1988). Aside from the potential limitations imposed by inadequate levels of precursors or a reduced capacity for energy production, depressed rates of protein synthesis in disused muscles can ultimately be traced to the translational and transcriptional levels.

In addition to muscle weight and fiber size, muscle atrophy may be accompanied by many functional and biochemical properties. One of the most evident responses to atrophy is loss of strength in the affected muscles (Appell 1990). Also changes in myosin isoforms, oxidative enzyme content and glycolytic enzymes have been demonstrated (Appell 1990, Thomason and Booth 1990). In rat skeletal muscle atrophy is generally accompanied by a shift in the contractile and enzymatic profiles of a slow-twitch oxidative muscle toward that of a fast-twitch glycolytic muscle (Booth and Kelso 1973a, Laurila et al. 1991, Kannus et al. 1998, Laurila et al., submitted). The slow muscle starts transcribing the fast myosin heavy chain (MHC) gene (Goldspink et al. 1992). During remobilization the contraction time returns to the control level earlier than muscle strength or biochemical properties (Booth and Seider 1979,

Witzmann et al. 1982). As stated in the previous chapter, immobilization of a fast-twitch muscle like the tibialis anterior or plantaris in a stretched position may either attenuate or prevent muscle atrophy. A fiber-type-shift from type IIb to IIa has been observed, however (Cotter et al. 1988, Loughna et al. 1990, Laurila et al. 1991), although the electrical activity is not changed (Cotter et al. 1988). Hind limb suspension (Ishihara et al. 1997) and hypokinesia (Marsh 1992, Roy et al. 1996) cause a I-to-IIa fiber-type-shift in the rat soleus and gastrocnemius.

Also in humans plasticity of the skeletal muscle phenotype can result in response to changes in innervation patterns (Calvo et al. 1996, Burnham et al. 1997). Denervation may be caused by spinal cord injury, in which the upper motoneuron is affected while the lower motoneuron is intact. The effect of denervation on different fiber types in humans seems to resemble that in the rat, in that during the early phases of denervation, type II muscle fibers seem to be mostly affected (Lotta et al. 1991, Lu et al. 1997). During the later course also the oxidative type I fibers are affected (Lotta et al. 1991, Lu et al. 1997), resulting in a predominance of type II, more specifically type IIb fiber (Lotta et al. 1991). At least in the rat, myosin expression is changed after denervation so that fiber type IIa re-expresses embryonic and neonatal myosin (Schiaffino et al. 1987). Specific electrical stimulation has also been shown to induce a fiber type shift in both rat and human muscle (Howald 1982, Staron et al. 1987, Kirschbaum et al. 1989, Martin et al. 1992, Greve et al. 1993, Andersen et al. 1996). The shift is usually from type IIb to type IIa (Greve et al. 1993, Andersen et al. 1996), or an increase in the relative proportion of type I fibers (Staron et al. 1987, Kirschbaum et al. 1989, Martin et al. 1992).

After immobilization, the recovery of muscle weight lasts 2-4 times longer than the period of immobilization (Goldspink 1977, Laurila et al., submitted). Following crush denervation, the nerve conduction reappears about 3 weeks after the neuronal damage, indicating that functional reinnervation takes place 2 to 3 weeks after crushing of the nerve (Herbison et al. 1973). Muscle weight also starts to increase after 2 to 3 weeks, so that the slow muscles increase in weight and fiber diameter a little earlier than the fast muscles (Jaweed et al. 1975). After transection of the nerve, a deficit in the power output of the reinnervated rat muscles has been observed as much as four months later (Yoshimura et al. 1999).

According to clinical studies, in humans third-degree sprains (complete ruptures) of knee ligaments, whether treated conservatively or operatively, may result in 10-35% strength and power deficits of the quadriceps and hamstring muscles when evaluated several years after the injury (Kannus et al. 1992). Long-lasting deficits can also be observed in the electrical activity of the atrophied muscles. According to Luthi et al. (1989) six months of supervised retraining could not fully restore the strength and endurance characteristics of the immobilized muscles. The volume densities of the mitochondria, as determined from muscle biopsies, were also below normal. It has been suggested that the atrophying effects of immobilization can be prevented by preimmobilization training, early controlled mobilization, optimal positioning

of the immobilized joint, muscular training during immobilization, early weight bearing, exercise with the non-immobilized extremity, and electrical stimulation (Kannus et al. 1992). Early motion and loading of injured tissues is not without risks, however, because excessive or premature loading and motion of repair tissue can inhibit or stop healing (Buckwalter 1996).

2.2 Carbonic anhydrase III

2.2.1 General

Carbonic anhydrase (CA) was first discovered in high concentrations in erythrocytes in the early 1930s (Meldrum and Roughton 1933) and has been intensively investigated since then. CA is a ubiquitous metalloenzyme found in all animals and photosynthesizing organisms examined for its presence, as well as in some non-photosynthetic bacteria (Lindskog 1997). A relatively new discovery is the existence of three evolutionarily unrelated CA families (Hewett-Emmett and Tashian 1996). All known CAs from the animal kingdom belong to the same family. So far, nine isoenzymes have been found in mammals: CA I-VII, CA IX and CA XII (Table 1), and they are widely distributed both among species and among tissues, cell types and organelles (e.g. Dodgson et al. 1991, Sly and Hu 1995, Hewett-Emmett and Tashian 1996, Tureci et al. 1998). In addition, three catalytically inactive CA-related proteins have been characterized: CA VIII, CA X and CA XI (Sly and Hu 1995). CAs catalyze the reversible hydration of carbon dioxide: $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$. The isoenzymes differ in their hydratase activities, with CA II having the highest activity, by virtue of which it is ranked among the most efficient enzymes known, and CA III having a considerably lower activity than isoenzymes I, II, IV, V and VI. In addition to the hydratase activity, CAs can act as an esterase and catalyze the hydration of various aldehydes and pyruvic acid (Pocker and Meany 1965, Pocker and Sarkanen 1978). Some of the CA genes are expressed in nearly all tissues, whereas others are more limited in their distribution. In many tissues the functions of CAs are well described; the isoenzymes are involved in the transport of CO_2 between metabolizing tissue and the lungs, in many secretory processes, in ion transport and in the provision of bicarbonate and hydrogen ions for fatty acid synthesis, gluconeogenesis, ureagenesis and excitation-contraction coupling (Tashian and Hewett-Emmett 1984, Wetzel et al. 1990).

TABLE 1 Classification of carbonic anhydrase (CA) isozymes.

Isoenzyme	MW	Activity	Cellular location	Tissue distribution
CA I	29 kDa	low	cytoplasm	widespread
CA II	29 kDa	high	cytoplasm	widespread
CA III	29 kDa	low	cytoplasm	skeletal muscle, fat cells, liver
CA IV	35-68 kDa	high	cell membrane	widespread
CA V	34 kDa	high	michondria	widespread
CA VI	36-42 kDa	high	secreted	parotid salivary gland
CA VI I	29 kDa	?	cytoplasm	salivary gland
CA IX	54, 58 kDa	present	plasma membrane/ nucleus	neoplastic cells and tissues
CA XII	39 kDa	present	cell membrane	human renal cell carcinoma

Collected from references occurring in the text.

Until the late 1970s it was thought that skeletal muscle did not contain CA, but then Holmes (1977) found a low activity carbonic anhydrase (CA III) in red skeletal muscle. CA III is now known to be present in large amounts in animal and human skeletal muscle and in animal liver and adipose tissue (Holmes 1977, Register et al. 1978, Carter et al. 1979, Shiels et al. 1984, Spicer et al. 1990). In muscle it is highly concentrated in the type I fibers, comprising about 8% of the cytoplasmic protein (Carter et al. 1991). CA III is also found in small amounts in type IIa fibers, but is basically undetectable in type IIb fibres (Jeffery et al. 1987, Fremont et al. 1988). CA III expression has also been found in human erythrocytes, uterus, bladder and lung (Jeffery et al. 1980, Carter et al. 1984), human smooth muscle and myoepithelial cells (Väänänen and Autio-Harmainen 1987) and in the epithelium of rodent salivary gland ducts, colon, bronchi and the male genital tract (Spicer et al. 1990). Ultrastructural studies have shown that CA III is diffusely distributed through the muscle fiber sarcoplasm (Väänänen et al. 1985, Fremont et al. 1988). In addition to the cytoplasm, traces of CA III have been found in rat liver cell nuclei (Dodgson et al. 1993).

2.2.2 Function of CA III in skeletal muscle

It has been suggested that CA III may facilitate the transport of metabolic CO_2 inside muscle cells (e.g. Gros and Dodgson 1988). However, during the past few years evidence has gathered suggesting other possible functions. CA III has several characteristics that distinguish it from the other carbonic anhydrase isoenzymes. Firstly, it has a very low specific activity as a CO_2 hydratase, only about 1% of that of CA II (Engberg et al. 1985). In spite of the low activity, a well-defined binding site for bicarbonate is retained (Eriksson and Liljas 1993). It has been speculated that this binding site may predispose CA III to oxidative modification (Cabiscol and Levine 1995), because the bicarbonate/carbonate system has been shown to accelerate the oxidation of amino acids, peptides and proteins as compared with other buffer systems (e.g. Stadtman et al. 1990). Thus

the reaction that is catalyzed by CA III may lead to its own oxidative modification (Cabiscol and Levine 1995).

A second characteristic that distinguishes CA III from the other isoenzymes is that it has inherent phosphatase activity (Koester et al. 1981). This activity is regulated by reversible glutathiolation, in which a disulphide link is formed between two of CA III's cystein residues and glutathione (Cabiscol and Levine 1996). The glutathiolation of CA III is known to increase during oxidative stress (Rokutan et al. 1991, Chai et al. 1994a,b, Lii et al. 1994). Recent experiments with CA III overexpressing cells have confirmed that these cells have a lower steady-state level of oxygen radicals and are more resistant to H_2O_2-induced apoptosis than their parent cells (Räisänen et al. 1999). This antioxidant function could point to an important cellular role of CA III, especially in the oxidative type I muscle fibers, since exercise is known to increase free radical formation in skeletal muscle (Frankiewicz-Jozko et al. 1996). On the other hand, endurance training increases antioxidant defence protein expression, especially in highly oxidative muscles (Sen 1995).

Other factors besides exercise that are known to induce oxidative stress include ageing and diabetes mellitus (Starke-Reed and Oliver 1989, Asayama et al. 1994, Sardesai 1995, Giugliano et al. 1996, Laaksonen et al. 1996). Muscular atrophy *per se* has also been suggested to be implicated in oxidative stress (Kondo and Itokawa 1994). It was observed that while the absolute amount of CA III in male rat liver decreased during ageing, the specific CO_2 hydratase and esterase activities changed only to a minor degree (Cabiscol and Levine 1995). In contrast, the phosphatase activity was almost completely lost by 18 months of age, due to the considerable increase in the extent of CA III glutathiolation with age (Cabiscol and Levine 1996). High levels of the thiolated forms of CA III were also detected in skeletal muscle.

Other possible physiological functions for CA III have also been discussed. According to Fremont et al. (1987), there is no correlation between the oxidative capacity of a given muscle fiber type and its CA III activity and content, whereas a highly significant negative correlation exists between the level of CA III activity and the activities of various glycolytic enzymes (Baldwin et al. 1973). Also, when rats are rendered hypo- or hyperthyroid, several changes occur at the energy metabolism level that are coupled with a rapid increase or decrease in CA III expression, respectively (Cote et al. 1997). It was proposed on the basis of examinations of glucose and glycogen metabolism under conditions of CA III inhibition that CA III could have a down-regulatory effect on the glycogenolytic and/or glycolytic rate in muscle (Cote et al. 1993). Later, Cote et al. (1997) found no influence of CA III inhibition on muscle glycolytic flux during a muscle fatigue protocol.

2.2.3 Effects of immobilization, remobilization and denervation on CA III expression in skeletal muscle

Immobilization of muscles in shortened and lengthened positions affects CA III expression in different ways, and these effects are muscle-specific. When a fast

muscle such as the tibialis anterior or plantaris is immobilized in lengthened position the protein and mRNA concentrations of CA III are increased (Laurila et al. 1991, Brownson and Loughna 1996). At the same time, a fiber type shift from type IIb to IIa was observed by Laurila et al. (1991), which is in accordance with the reports by Cotter et al. (1988) and Loughna et al. (1990) of a fiber type shift in rat fast muscles after immobilization. Reversal of the elevation in CA III concentration is slow, the control level not being reached even after a remobilization period of nine weeks (Laurila et al., submitted). In slow muscle the changes in CA III expression caused by immobilization are smaller. When soleus was immobilized in shortened position for 3 weeks, a fiber type shift from type I to type IIa and increased variation in the intensity of CA III immunostaining were observed (Laurila et al. 1991). The changes were not reversed during a nine-week remobilization period (Laurila et al., submitted). CA III mRNA diminished in soleus muscle immobilized in shortened position for 2 days (Brownson and Loughna 1996). Immobilization in lengthened position caused first an increase in CA III mRNA (after 2 days) and subsequently a decrease (after 5 days). Denervation has also been shown to increase CA III expression at both the protein and mRNA level in the type II fibers of rat skeletal muscle (Wistrand et al. 1987, Carter et al. 1988).

2.2.4 Other factors affecting CA III expression in skeletal muscle

In addition to neural stimuli, the amount of CA III in skeletal muscle is responsive to hormonal stimuli and electric stimulation. During development, in both rat and human the major increase in CA III expression occurs at a stage when foetal polyneuronal innervation has regressed and the specific fiber/nerve impulse patterns are being established (Jeffery et al. 1980). During ageing, the concentration of CA III increases slowly in rat fast muscle reaching its maximum level at 26 months, whereas in slow muscle the concentration is declining at this time (Jeffery et al. 1988). Thyroidectomy leads to a considerable increase in the number of CA III -positive fibers in rat muscle (Jeffery et al. 1987). These fibers have been classified as belonging to the C-fibers, which are intermediate between types IIa and IIb (Jeffery et al. 1987). Steroids and other sex hormones are not known to affect muscle CA III, but in rodent liver, CA III is induced by testosterone (Carter et al. 1984). Chronic low-frequency stimulation induces CA III and slow myosin heavy chain (MHC) in rabbit fast muscle (Gros and Dodgson 1988) but not in that of the rat (Jeffery et al. 1990). According to Jeffery et al. (1990), CA III expression is connected with slow MHC expression.

2.3 Connective tissue in skeletal muscle and tendon

2.3.1 Structure and synthesis of collagen in skeletal muscle and tendon

Multicellular organisms are formed by specialized cells which are assembled in tissues. The extracellular matrix (ECM) outside the cells is a complex and dynamic meshwork containing collagens, noncollagenous glycoproteins and proteoglycans. ECM supports the cellular elements and maintains the structural integrity of multicellular organisms. It helps cells to bind together and regulates various cellular processes, such as cell growth, proliferation, differentiation, migration and adhesion. Collagen is the most abundant protein of the ECM, constituting 20-25% of all protein in the body (Waterlow et al. 1978). Up to the present, 19 distinct collagen types have been found in vertebrates (Prockop and Kivirikko 1995). They are usually divided into two subgroups, fibril-forming and nonfibril-forming collagens on the basis of their supramolecular structures. In skeletal muscle (Table 2), collagen is mainly present in three fibrillar forms, collagen types I, III and V, and one nonfibril-forming collagen, type IV of basement membranes (Duance et al. 1977, Bailey et al. 1979). Of these, types I and III are the most abundant. In addition, collagen types II, XI, XIII, XIV, XV, and XVIII have been found in skeletal muscle and type VI in cardiac muscle (Sandberg et al. 1989 and 1993, Bashey et al. 1992, Wälchli et al. 1994, Kivirikko et al. 1995, Saarela et al. 1998). Tendon consists almost entirely of type I collagen fibrils, and type I collagen accounts also for most of the organic matrix of bones (Davison 1982).

TABLE 2 Collagen types found in skeletal muscle.

Collagen type	Localization
Fibrillar:	
I	endo-, peri- and epimysium
II	human fetal tissues
III	endo-, peri- and epimysium
V	endomysium
Non-fibrillar:	
IV	basement membrane, endomysium
XI	human fetal tissue
XIII	human fetal endomysial, mesenchymal cells
XIV	chicken embryonic tissue
XV	human fetal and adult tissues, endomysium
XVIII	human fetal and adult tissues, epi- and perimysium

Collected from references occurring in the text.

The network of the ECM in skeletal muscle is divided into three subdivisions (Leeson and Leeson 1981):

(i) endomysium, which surrounds each individual muscle fiber,
(ii) perimysium, which surrounds several muscle fibers, forming muscle bundles, and interconnects epimysium by collagenous septa,
(iii) epimysium, which surrounds the entire muscle.

Collagen types I and III are present in epimysium and perimysium, with type I dominating in both layers. In endomysium, all the major skeletal muscle collagen types have been found (Foidart et al. 1981, Light and Champion 1984). It has been shown that slow-twitch muscles contain more collagen than fast-twitch muscles (Garcia-Bunuel and Garcia-Bunuel 1967, Kovanen et al. 1980, 1984a), and that the concentration of endomysial collagen is higher around slow than fast skeletal muscle fibers in rats (Kovanen et al. 1984a).

In skeletal muscle, collagen is produced principally by fibroblasts on the membrane-bound ribosomes of the rough endoplasmic reticulum. Collagen biosynthesis is characterized by the presence of an extensive number of co- and post-translational modifications of the polypeptide chains, which contribute to the quality and stability of the collagen molecule (Kivirikko and Myllylä 1982b). The polypeptide chains form triple-helical procollagen molecules, which are secreted into the extracellular space by exocytosis. Procollagens contain aminoterminal and carboxyterminal extension peptides at the respective ends of the collagen molecule, and after secretion, the amino-propeptides are cleaved off by procollagen N-proteinase and the carboxyl-propeptides by procollagen C-proteinase. After this, the collagens self-assemble into fibrils or other supramolecular structures.

The three polypeptide chains which form the triple-helical structure are called α-chains. The molecular organization of the different collagen types differs in that type I collagen is a heterotrimer of two identical α1(I) chains and one α2(I) chain, while type III collagen is a homotrimer of α1(III) chains. The most common form of type IV collagen consists of two α1(IV) chains and one α2(IV) chain, but other forms also exist. The α-chains are composed of repeating amino acid sequences Gly-X-Y, in which the glycine residue in every third position enables the three α-chains to coil around one another. Proline and 4-hydroxyproline (Hyp) residues appear frequently at the X- and Y-positions, respectively, and promote the formation of intermolecular cross-links (Vuorio and de Crombrugghe 1990). The stability and quality of the collagen molecule is largely based on its intra- and intermolecular cross-links. Hyp as a part of the polypeptide chain is an almost unique feature of collagen, and its assay is therefore suitable for evaluating collagen content (Kivirikko and Myllylä 1982a). The formation of Hyp is catalyzed by prolyl 4-hydroxylase (PH). The levels of PH activity generally increase and decrease with rates of collagen biosynthesis, and thus assays of the enzyme activity have been used to estimate changes in the rate of collagen biosynthesis in many experimental and physiological conditions (e.g. Turto et al. 1974, Kivirikko and Myllylä 1982b, Takala et al.

1983, 1991, Myllylä et al. 1986, Savolainen et al. 1987, 1988a, 1988b, Karpakka et al. 1990, 1992, Han et al. 1995, Kääpä et al. 1995). Also changes in the activity of galactosylhydroxylysyl glucosyltransferase (GGT), another posttranslational enzyme of collagen synthesis, reflect changes in collagen biosynthesis rate (e.g. Kivirikko and Myllylä 1982b, Takala et al. 1983, 1991, Savolainen et al. 1987, 1988a, 1988b, Karpakka et al. 1990, 1992, Han et al. 1995, Kääpä et al. 1995). While the amount of Hyp is quite constant, the degree of glycosylations can vary considerably (Kivirikko and Myllylä 1982a).

Muscle is attached to its tendon at the end of the fibers, where the collagen fibrils of muscle fuse or interdigitate with the collagen fibrils of tendon (Davison 1982). The type I collagen fibrils of tendon run in parallel, to form a tough, rather inelastic structure representing 60-85% of the total dry weight of tendon (Tipton et al. 1975, Kirkendall and Garrett 1997). The rest of the substance consists mostly of proteoglycans, and about 2% is noncollagenous proteins, mainly elastin.

2.3.2 Functions of collagen in skeletal muscle and tendon

The connective tissue network of skeletal muscle has a dynamic role during muscle differentiation and normal muscle growth, and it serves as a supportive structure in skeletal muscle and tendon (Duance et al. 1977, Bailey et al. 1979, Kühl et al. 1984, Mayne and Sanderson 1985). Collagen forms the linkages between the muscle and its associated collagenous tissues such as tendon or fascia, and is also the fibrillar component of the cell-to-cell connections both between individual muscle cells and between the muscle cells and neighbouring small blood vessels and nerves. It gives coherence and mechanical strength and also functions as an elastic, stress-tolerant system as well as distributes the forces of muscular contractions in both muscle and tendon. Its tensile strength is based on intra- and intermolecular crosslinks, the orientation and density of the fibrils and fibers. In addition, collagen, like the other ECM compounds, conforms to the micro-environment around the individual muscle fibers and participates in the growth of the cells and tissue regeneration after damage (Chiquet et al. 1996).

2.3.3 Effects of immobilization on collagen in skeletal muscle and tendon

The metabolism of muscular and tendinous collagen and the connective tissue network are known to respond to altered levels of physical activity. Cast immobilization of the rat hind limb leads to a decrease in the enzyme activities of collagen biosynthesis in both skeletal muscle and tendon (Savolainen et al. 1988a,b), suggesting that the biosynthesis of the collagen network decreases as a consequence of reduced muscular and tendinous activity. The rate of total collagen synthesis depends mostly on the overall protein balance of the tissue (Turto et al. 1974), but it seems to be promoted by stretch in both muscle and tendon (Savolainen et al. 1988a,b) and by low resistance exercise (Karpakka et al. 1990) and running training in muscle (Kovanen 1989). According to

Savolainen et al. (1988a) there is a difference between the plantar flexors and dorsal flexors of the rat hind limb in response to stretch during immobilization. In plantar flexors, especially the soleus, the collagen biosynthesis seems to be more dependent on muscle stretch and loading than in dorsal flexors. Changes in the total collagen content of muscle, measured in terms of hydroxyproline content, are usually small or absent during immobilization lasting for a few weeks, which probably reflects the slow turn-over of collagen (Savolainen et al. 1988a). Also, increased collagen content has been observed in muscle after immobilization (Jozsa et al. 1988). In tendon, findings on the effects of immobilization to collagen content vary (Klein et al. 1982, Savolainen et al. 1988b, Vailas et al. 1989).

Collagen expression during immobilization has been shown to be at least partially down-regulated at the pretranslational level (Han et al. 1999b). The mRNA for the α-subunit of PH was decreased already after one day's immobilization and the mRNAs for collagen types I and III were decreased after three days of immobilization. Ahtikoski et al. (1997) observed down-regulation of the expression of type I collagen and its posttranslational modificators in the soleus immobilized in shortened position, but the effect was partly prevented by stretch.

2.3.4 Effects of denervation on collagen in skeletal muscle

The responses in muscular collagen biosynthesis differ between the denervation and disuse forms of atrophy. Denervation atrophy is associated with an increase in the activities of PH and GGT and muscular collagen concentration (Savolainen et al. 1988b). When the denervated rat hind limb muscles were immobilized no profound length-associated changes were observed in the PH, GGT and hydroxyproline responses of the muscles. However, the denervated muscles immobilized in shortened position atrophied more than the lengthened ones (Savolainen et al. 1988b). Thus the denervated muscle can also adapt to a chronically stretched or slackened position by increasing or reducing the number of sarcomeres in its fibers (Tabary et al. 1981). Consequently, denervation seems to "uncouple" the regulation of the adaptive responses of muscular collagen biosynthesis from the atrophy process taking place in the muscle as a whole (Savolainen et al. 1988b).

2.3.5 Effects of increased physical activity on collagen in skeletal muscle and tendon

Skeletal muscle is known to respond to increased loading caused by endurance training (Kovanen et al. 1980, 1987, Takala et al. 1983, Zimmerman et al. 1993), acute exercise (Myllylä et al. 1986) or experimental compensatory hypertrophy (Turto et al. 1974, Williams and Goldspink 1981) by increased collagen synthesis and/or accumulation in the muscle. In tendon, neither a single exercise bout nor endurance training has been shown to increase collagen synthetizing enzyme activities or collagen content (Tipton et al. 1975, Woo et al. 1980, 1981,

Viidik 1986). Strenuous exercise, especially acute weight bearing exercise that contains eccentric components, is known to cause muscle damage (Armstrong et al. 1983). During endurance training, elevated activities of beta-glucuronidase, a marker of muscle injury, have been observed (Vihko and Salminen 1986). On the other hand, up-regulation of collagen synthesis may also occur without any evidence of muscle damage (Han et al. 1999a). Acceleration of collagen biosynthesis after exercise may thus reflect both physiological adaptation and repair of the damage, which is known to be more pronounced in the red part of the muscle (predominantly type I fibers) than in the white part (predominantly type II fibers) (Myllylä et al. 1986). Exercise training before exhaustive running has been shown to partially prevent the increase in collagen synthetizing enzymes (Myllylä et al. 1986). On the other hand, swimming training before immobilization slows down the decrease in collagen synthetizing enzymes caused by disuse and even accelerates collagen synthesis (Karpakka et al. 1990). It is known that the specific activities of PH and GGT, and also the Hyp concentration, are greater in the tonically activated antigravity soleus muscle than in the tibialis anterior muscle, which is not tonically active (Kovanen et al. 1984, Savolainen et al. 1987). As during swimming the muscle contractions are concentric, lacking weight bearing and eccentric components, concentric contractile activity *per se* may be one stimulus for collagen biosynthesis.

2.4 Effects of experimental diabetes on skeletal muscle

Insulin is known to play an extensive role in the regulation of skeletal muscle protein turnover (Grizard et al. 1999). It increases amino acid uptake into muscle cells and accelerates both translation and transcription (Miers and Barrett 1998). Insulin also inhibits the breakdown of muscle protein (Charlton and Nair 1998). In diabetes mellitus the insulin action on protein synthesis and degradation is decreased due to the lack of insulin, and a catabolic state is produced if insulin is not replaced. Streptozotocin-induced diabetes is a widely used experimental model of diabetes mellitus in rats. It is accompanied by muscle atrophy and reduction in the weight gain of the animal (e.g. Harpur 1980, Moynihan and Ennis 1990, Kainulainen et al. 1994). According to Kimball and Jefferson (1991) the inhibition of protein synthesis in the rat muscles composed primarily of slow-twitch fibers (e.g. soleus), can be accounted for entirely by a decrease in the amount of RNA during experimental diabetes, while in muscles of mixed fiber composition (e.g. gastrocnemius), it is associated with an impairment of peptide-chain initiation.

Diabetes is also known to cause delayed wound healing, toughened skin (Buckingham et al. 1984) and decreased bone formation (Goodman et al. 1984) as well as decreased solubility of collagen, increased tensile strength and increased non-enzymatic glycation (Brennan 1989). The effects of diabetes on collagen synthesis have been studied in many organs and tissues, e.g. bone

(Harvey and Nakamoto 1988), myocardium (Reddi 1988), cartilage (Spanheimer 1992), skin (Fullana et al. 1993) and periodontal tissue (Oliver and Tervonen 1994). Han et al. (1995) found that streptozotocin-induced diabetes reduced the rate of collagen synthesis, which exceeded the negative total protein balance in the skeletal muscle of the rat. Physical training had an increasing effect on muscular collagen synthesis in non-diabetic rats, but was unable to prevent the decreasing effect of diabetes on collagen synthesis.

During the past few years evidence concerning the connection between diabetes mellitus and increased oxidative stress has accumulated (Asayama et al. 1994, Sardesai et al. 1995, Giugliano et al. 1996, Laaksonen et al. 1996). Laaksonen et al. (1996) found that exercise caused greater oxidative stress in insulin dependent diabetic patients than in healthy subjects. Moynihan and Ennis (1990) studied the effect of streptozotocin-induced diabetes on CA III in rats. They observed that diabetes resulted in a decrease in CA III activity in the male rat liver. In soleus muscle the CA III activity was slightly increased, but not statistically significantly.

Streptozotocin-induced diabetes is also known to reduce oxidative enzyme activity in rat skeletal muscle (Ianuzzo et al. 1974, Ianuzzo and Armstrong 1976, Goodyear et al. 1988, Kainulainen et al. 1994) in a manner that is both dose dependent and fiber type specific (Ianuzzo and Armstrong 1976, Kainulainen et al. 1994).

2.5 Effects of exercise on serum muscle and collagen markers

Strenuous physical exercise that results in injury to the skeletal muscle also causes protein leakage from the muscle cells (Armstrong et al. 1983, Armstrong 1986). In addition to direct muscle studies, serum activities or concentrations of certain muscle-specific proteins have been widely used to verify skeletal muscle damage and to estimate its magnitude (Armstrong et al. 1983, Janssen et al. 1989). The damage increases with both the intensity and duration of the exercise bout, intensity having the greater influence (Tiidus and Ianuzzo 1983). Eccentric contractions are known to cause more damage than concentric ones (Armstrong et al. 1983).

Serum measurements of muscle proteins such as the sarcoplasmic enzymes creatine kinase (CK) and its isoenzymes, and lactic dehydrogenase (LDH) and its isoenzymes, as well as nonenzymatic markers such as myoglobin (Mb), have commonly been used to monitor protein leakage from muscular cells (e.g. Armstrong 1986). Later, serum concentrations of contractile proteins such as troponin T (TnT) (Gerhardt et al. 1991, Mair et al. 1991, Katus et al. 1992, Löfberg et al. 1995), troponin I (TnI) (Sorichter et al. 1997), and myosin (Mair et al. 1992, Melin et al. 1997) have been studied. TnT has in some studies been advocated as a marker for myocardial damage (Bhayana and Henderson 1995), but in other studies it has been shown to be increased in the serum of patients with skeletal muscle damage (Gerhardt et al. 1991, Mair et al. 1991, 1992, Katus et al. 1992,

Löfberg et al. 1995, Melin et al. 1997). CA III is a marker protein concentrated in type I muscle fibers (Väänänen et al. 1985, Zheng et al. 1992). It has been shown to be a more sensitive serum marker of muscle damage than CK or LDH (Väänänen et al. 1986b, 1988) and approximately as sensitive as Mb (Takala et al. 1989a, Väänänen et al. 1990). Increased serum CA III concentrations have been observed after various forms of running and cross-country skiing exercises (Takala et al. 1989a, Takala et al. 1989b) and in connection with neuromuscular diseases (Väänänen et al. 1988). It has also been questioned whether the serum activities or concentrations of muscle proteins are adequate indicators of muscle damage (Van der Meulen 1991, Komulainen et al. 1995). In the study of Komulainen et al. (1995) serum CK activities were increased without concomitant muscle damage and, on the other hand, muscle damage occurred without a statistically significant increase in serum CK activity (Komulainen et al. 1995).

Elevated serum markers of collagen biosynthetizing enzymes have been observed in neuromuscular, hepatic and dermatological diseases (Kuutti-Savolainen 1979, Kuutti-Savolainen et al. 1979, Myllylä et al. 1982) and after physical exercise (Takala et al. 1986, Takala et al. 1989b). The activity of serum GGT correlates with the activities of GGT and PH in muscle and liver biopsy specimens (Kuutti-Savolainen et al. 1979, Myllylä et al. 1982), which suggests that increased serum GGT reflects accelerated collagen synthesis. Hyp is not recycled for the synthesis of new collagen, and its serum and urine concentrations are thus useful biochemical markers of collagen turnover (Kivirikko et al. 1967, Adams and Frank 1980, Cundy et al. 1983).

During the conversion of procollagen to collagen (in the formation of new collagen), the propeptides of the procollagens split off in a stoichiometric manner, and are released into the extracellular fluid (Kivirikko and Myllylä 1982a). Serum measurements of the carboxyterminal propeptide of type I procollagen (Pro(I)-C-P), the aminoterminal propeptide of type I procollagen (Pro(I)-N-P) and the aminoterminal propeptide of type III procollagen (Pro(III)-N-P) have been used to assess collagen synthesis in various pathological states (Lammi et al. 1997, 1999, Tapanainen et al. 1997, Crowley et al. 1998, Hunzelmann et al. 1998, Santala et al. 1998, Toivonen et al. 1998). Elevated serum concentrations of Pro(III)-N-P have been observed in connection with diseases affecting muscular tissue and resulting in increased collagen formation (Myllylä et al. 1982). Serum Pro(III)-N-P shows a high correlation with serum GGT and with muscular PH and GGT (Myllylä et al. 1982). Prolonged heavy exercise has also been shown to cause an increase in serum Pro(III)-N-P and GGT (Takala et al. 1986). Serum concentrations of Pro(I)-C-P and Pro(I)-N-P have been shown to correlate with the rate of bone formation (Risteli et al. 1991, Risteli and Risteli 1997, Toivonen et al. 1998). Type I collagen degradation can be specifically detected by analysing either cross-linked carboxy- or aminoterminal telopeptides or the cross-links themselves liberated during the degradation process (Risteli and Risteli 1997, Liesegang et al. 1998, Toivonen et al. 1998).

3 PURPOSE OF THE STUDY

The aims of the present study were:

1. To study the adaptive changes in the expression of CA III in skeletal muscle to immobilization (I)

2. To study the effects of streptozotocin-induced diabetes, endurance training and their combination on the expression of CA III at mRNA and protein levels in skeletal muscle (II)

3. To study the adaptive changes of collagen biosynthesis in skeletal muscle to variations in physical activity (III, IV), denervation and the subsequent reinnervation process (V) and the changes in tendon collagen biosynthesis upon variations in physical activity (IV)

4. To study the effect of an acute bout of high intensity concentric exercise on serum CA III and collagen marker proteins (VI)

4 MATERIALS AND METHODS

4.1 Animals

Adult male Sprague-Dawley rats from the stock of the Department of Physiology, University of Oulu (studies III, IV and V) and from the stock of Laboratory Animal Unit, University of Tampere (study II), were used. The rats were housed under normal cage conditions (10:14 h light-dark cycle in studies III, IV and V; and 12:12 h light-dark cycle in study II) and maintained on a standard rodent diet (Astra-Ewos, Sweden) and water *ad libitum*. The experimental manipulations were approved by the Committee of Animal Experimentation in the University of Oulu (studies III, IV and V) or by the Institution of Animal Care and Use at the University of Tampere (study II). Casting (studies III and IV) and denervation (study V) were performed under neuroleptic anaesthesia (fentanyl citrate 0.315 mg, fluanisone 10 $mg \cdot ml^{-1}$; Hypnorm®, intramuscularly). In the casted rats the dose was 1 $mg \cdot kg^{-1}$ and in the denervated rats it was 1.3 $mg \cdot kg^{-1}$. In study V, the rats were also given Hypnorm® 0.3 $mg \cdot kg^{-1}$ after the operation for pain relief, and 0.5 $mg \cdot kg^{-1}$ before the electrophysiological measurements. The anaesthesia agent was used similarly in all rats. In study II, the animals were made insulin deficient by injecting intraperitoneally 65 $mg \cdot kg^{-1}$ streptozotocin dissolved in 0.05 M citrate buffer. Controls were injected with the citrate buffer only. The rats were randomized into different experimental groups at the beginning of the experiments. In studies II, IV and V 8-10 animals per group were used. In study III, the immobilized groups consisted of 7-10 animals and the number of controls per group was 6-8. For electrophysiological measurements (study V), 15 rats were used.

4.2 Human subjects

In study I, 14 patients, 13 males and one female, with rupture of the anterior cruciate ligament were studied before and 6 weeks after postoperative leg immobilization following knee ligament reconstruction. The nature, purpose and potential risks of the study were explained to the subjects before they gave their voluntary informed consent to participate. The study protocol was approved by the ethical committee of Oulu University Hospital.

In study VI, 9 male students in physical education with a sports training background volunteered as subjects. During the experiment they were healthy having no musculoskeletal injuries. They were not allowed to use any medication or alcohol for three days before the study. A written informed consent was obtained in all cases.

4.3 Immobilization and remobilization

In study I, half of the patients were immobilized with conventional cast so that the knee joint was fixed at 20° flexion. The other half used functional derotation-braces allowing a motion of 30-70°. The immobilization time was six weeks.

In studies III and IV, the right hindlimb of the rat was immobilized with plaster of Paris, so that the ankle joint was in a full plantar flexion (150-160º angle). Thus, the soleus muscle and Achilles tendon were immobilized in the shortened position, and tibialis anterior muscle and tendon in the lengthened position. Immobilization periods of three days (III), one week (III, IV) and six weeks (III) were used. The casts were changed weekly. During remobilization (III, IV), the rats were allowed to move freely in their cages. Remobilization times of 3, 7 and 14 days (III, IV) were used.

4.4 Denervation and reinnervation

Denervation of the crural muscles of the rat right leg (study V) was produced by crushing the sciatic nerve in the gluteal region with small forceps. The controls were sham-operated. Postoperatively, the animals were allowed to move freely in their cages. Time periods of 19, 26, 40 and 61 days were allowed for spontaneous reinnervation process to occur.

4.5 Streptozotocin-induced diabetes and exercise

In study II, the rats were made insulin deficient by streptozotocin-injections (Kainulainen et al. 1994). Induction of diabetes was confirmed 3 days later from urinary glucose levels. Blood glucose and plasma insulin levels were also measured after the experimental period (see the assays section). After this, the rats were subjected to running on a motor-driven treadmill. The animals trained five days a week for 12-16 weeks (Table 1 in study II). Running time and speed as well as the inclination of the tracks were gradually increased during the first seven weeks of the training. The total running distance was 61-93 km per animal.

4.6 Other exercise studies

In study IV, a single bout of exercise was used. The rats ran on a motor-driven treadmill for 3-5 hours. The speed was 16 $m \cdot min^{-1}$ and the inclination of the tracks 8° uphill. The rats ran until exhaustion, to the point after which they refused to run. Only those rats that ran a minimum of 3 h, were included (76% of the rats). After every hour, there was a 15-min pause during which the rats could freely eat and drink. The average running distance was 3648 m (2880-4800 m). After the exertion, the rats lived for 2, 5 or 10 days under normal cage conditions, as did the sedentary controls.

In study VI, after warming-up the subjects performed three countermovement jumps on a Kistler force-platform with a 20 s time-interval (Viitasalo and Bosco 1982). The force-time signal was used for determination of the average eccentric and concentric ground reaction forces, total contact time, and flight time. The countermovement jumps were followed by three isometric maximal bilateral lower limb extensions on a dynamometer with a 20 s time-interval. Maximal strength was measured in a sitting position with hip, knee, and ankle angles of 120°, 107°, and 90°, respectively. Then a fatigue loading of 50 maximal concentric successive bilateral lower limb extensions was performed on the dynamometer, which limited the knee motion to an angle range of 60-170°. This was followed by three isometric extensions and three countermovement jumps like before the fatigue loading.

4.7 Electrophysiology

In study V, the amplitude and the conduction time (latency) of the nerve-stimulated response and the electromyogram (EMG) were measured from the muscles of the operated leg 3, 7, 14, 19, 21, 28, 35, 42 and 63 days after the nerve crush. Two monopolar needle electrodes were placed on the sciatic nerve for

stimulation and, for recording, a concentric needle electrode was inserted in the muscle studied.

In study VI, the EMG activity of the subject's muscles was recorded by bipolar miniature surface electrodes. The EMG signals were presented for the eccentric and concentric parts of the vertical jumps for the isometric maximal strength as maximal voluntary contraction (MVC) as well as for each concentric fatigue contraction.

4.8 Blood samples and analyses

In study II, the rats were anaesthetized and aortic blood samples were taken, after which the animals were killed. Blood glucose concentration was measured by a commercial kit (Merck GmbH, Germany) and plasma insulin concentration by Rat Insulin RIA (Novo Nordisk a/s, Denmark).

In study VI, venous blood samples of the subjects were taken before and immediately after the exercise as well as 1 and 2 h and 1, 2, 3 and 4 days after the exercise. Blood lactate was analyzed by an electrochemical enzymatic method (Lactate analyzer 640, Roche) from a sample taken from the fingertip immediately after the exercise. Serum was separated by centrifugation (twice for 10 min at 3000 g) after 30 min of clotting in an ice bath. Serum samples were stored at -20°C.

4.9 Muscle samples

The rats were killed by decapitation (II, III, IV, V). The gastrocnemius (II, V), soleus (III, V), tibialis anterior (III, V), rectus femoris (II) and vastus lateralis (II) muscles and the Achilles (IV) and tibialis anterior (IV) tendons were excised. The muscles were dissected, saving the epimysium, and weighed. The samples for biochemical analyses were frozen in liquid nitrogen and stored at -70°C until analyzed.

In the human studies I and VI, muscle biopsies were obtained. In study I, biopsies from vastus medialis of the operated leg were taken before and 6 weeks after the surgery. In study IV, biopsies were obtained from vastus lateralis 2 weeks after the exercise. The biopsies were frozen in liquid nitrogen (I) or in isopentane precooled with liquid nitrogen (VI) and stored at -70°C (I) or -80°C (VI).

4.10 Biochemical assays

4.10.1 Tissue preparation

The muscle (I, II, III, V) and tendon (IV) samples were separately homogenized with an Ultra-Turrax homogenizer in two (muscle) or six (tendon) bursts of 5 s in a cold solution containing 0.2 M NaCl, 0.1% (w/v; muscle) or 0.5% (w/v; tendon) Triton X-100, 0.01% (w/v) soybean trypsin inhibitor, 0.1 M glycine, 50 μM dithiotreitol and 0.2 M Tris-HCl buffer, pH adjusted to 7.5 at 4 °C (6-10%, w/v). The homogenates were centrifuged at 15000 (muscle) or 11000 (tendon) × g for 30 (muscle) or 20 (tendon) min at 4 °C. The supernatants were taken for the assays of CA III concentration (I, II), myoglobin concentration (I) and the enzyme activities of PH (III, IV, V), GGT (III, IV, V), CS (II) and PGK (II) as well as protein concentrations (I-V). Pellets were used for Hyp analysis (III, IV, V). The biochemical values are given per gram of soluble (supernatant) protein.

For total RNA isolation (II), individual frozen weighed muscle samples were homogenized with Ultra-Turrax in 1 ml of Trizol reagent (Gibco, USA) per 50-100 mg of tissue. The steps of the isolation were performed according to the manufacturer's instructions.

4.10.2 CA III and myoglobin concentrations

In study I, radioimmunoassay was used to measure CA III concentration in human muscle as previously described (Väänänen et al. 1986a). In study II, CA III concentration in rat muscle was measured with time-resolved fluoroimmunoassay in which Eu^{3+} -labelled rat CA III was used as antigen. The method was essentially the same as that described for human serum (Vuori et al. 1991). Myoglobin concentration (I) was analyzed radioimmunologically using commercial kit (Cis, France).

4.10.3 Northern and slot blot analysis

In study II, for the RNA Northern and slot blot analyses, the total RNAs were transferred to a nylon or to a nitrocellulose filter, respectively, following the standard procedures (Chomczynski and Mackey 1994). The complementary DNA (cDNA) probe for CA III (Kelly et al. 1988) was labelled with commercial random primer [α-^{32}P] dCTP labelling kit (Pharmacia, Sweden). The membranes were prehybridized for 1 h after which the radioactive probe was added, and then hybridized for 24 h. After hybridization the filters were washed and exposed to X-ray films at -70°C. The autoradiographs generated by the Northern and slot blots were scanned with a densitometer (Molecular Dynamics, USA). The hybridization signals for CA III mRNA were normalized to that of 18S ribosomal RNA for each sample to correct for potential differences in loading and/or transfer.

4.10.4 PH and GGT enzyme activities, and Hyp concentration

The assay of PH (III, IV, V) was based on measurement of the labelled Hyp formed from the peptide-bound prolyl residues of the unhydroxylated labelled procollagen substrate (Kivirikko and Myllylä 1982a). GGT activity was assayed by determining the amount of radioactive glucosylgalactosylhydroxylysine formed in a heat-denatured gelatinized calf skin collagen substrate, as described by Myllylä et al. (1975). Hyp content was measured according to the method of Kivirikko et al. (1967) after hydrolysis for 16 hours in 6 M HCl at 120 °C.

4.10.5 Citrate synthase and phosphoglycerate kinase enzyme activities and protein contents

In study II, the activity of citrate synthase (CS) was determined according to Srere (1969) and the activity of phosphoglycerate kinase (PGK) according to Bucher (1955). Protein contents were assayed by the method of Bradford (1976) in studies I-V. The ratio of supernatant protein to muscle wet weight was constant throughout the experiments.

4.10.6 Serum analyses

Serum CA III and myoglobin (VI) were measured by a dual-label time-resolved fluoroimmunoassay (Vuori et al. 1991) based on competition between Eu^{3+} - or Sm^{3+} -labeled antigen and the sample antigen for polyclonal rabbit antibodies. Creatine kinase was measured according to the Recommendations of The Scandinavian Enzyme Committee (1978). The concentrations of Pro(I)-C-P and Pro(III)-N-P were determined with the use of commercial radioimmunoassay kits from Farmos Diagnostica (Oulunsalo, Finland) using polyclonal antibodies. GGT was assayed by the method of Myllylä et al. (1975) as modified for serum samples (Anttinen 1977). This method is based on the transfer of (^{14}C)glucose from UDP-(^{14}C)glucose (Radiochemical Centre, Amersham, UK) to galactosylhydroxylysyl residues in denatured collagen substrate, and an assay of radioactivity in the (^{14}C)glucosylgalactosylhydroxylysine liberated by alkaline hydrolysis and purified further by a brief ion-exchange chromatography. Hyp was measured according to Kivirikko et al. (1967). The assays of serum testosterone and cortisol were performed by radioimmunoassays using reagent kits from Farmos Diagnostica (Turku and Oulunsalo, Finland).

4.11 Enzyme histochemistry

In study VI, serial transverse cryostat sections of muscle were stained for adenocine triphosphatase (Padykula and Herman 1955) for identification of type I and type II muscle fibers and for calculation of the percentage distribution of the fiber types (Väänänen et al. 1985).

4.12 Computed tomography

In study I, the atrophy of the human muscle was monitored by computed tomography (Somatom 2, Siemens, Germany). The cross-sectional area of the medial part of m. quadriceps femoris consisting of m. vastus medialis and a part of m. intermedius (Fig. 1 in study I) was estimated immediately after the knee ligament reconstruction operation and six weeks later.

4.13 Statistical analyses

Statistical evaluation of the results was performed using one-way (V) or two-way (II) analysis of variance or the randomized block design of variance analysis with repeated-measures statistical procedure (VI). Comparisons between two specific groups were determined by Student's t-test (I, III, V), by Wilcoxon rank sum test (II) or signed-ranks test (IV, VI). Correlation was analyzed with Spearman's rank correlation test (VI).

5 RESULTS

5.1 Effects of immobilization (I, III, IV) and subsequent remobilization (III, IV) on skeletal muscle and tendon

5.1.1 Weight parameters and estimation of muscle atrophy by CT (I, III, IV)

The body weights of the casted rats (III, IV) were 2% (NS), 6% ($p<0.05$) and 12% ($p<0.001$) lower than those of their respective controls after 3, 7 and 42 days of immobilization, respectively. The wet weight of the soleus muscle immobilized in shortened position was 22% ($p<0.001$), 40-42% ($p<0.001$) and 63% ($p<0.001$) below the control and contralateral values after 3, 7 and 42 days of immobilization, respectively. The weight of the tibialis anterior muscle immobilized in lengthened position was 12-18% less ($p<0.001$) than that of the control and contralateral values after 7 days of immobilization.

After 1 week of immobilization and subsequent 3 days of remobilization (III, IV) the body weight was still 8% ($p<0.05$) below the control level but after 7 and 14 days of remobilization (III, IV) there was no significant difference compared with the controls. The weights of soleus and tibialis anterior were still 12-15% ($p<0.01$) and 7-8% ($p<0.01$), respectively, below the control and contralateral values after 14 days of remobilization. In study I, immobilization of knee joint either with cast or with brace for 6 weeks caused a 38% decrease ($p<0.001$) in the cross-sectional area of the medial part of m. quadriceps femoris.

5.1.2 CA III and myoglobin concentration in skeletal muscle (I)

The specific concentrations of CA III and myoblobin (Fig. 1) remained unaltered after the immobilization of knee joint for 6 weeks, but the concentrations per the cross-sectional area of the medial part of m. quadriceps femoris decreased by 37% (CA III) and 31% (myoglobin).

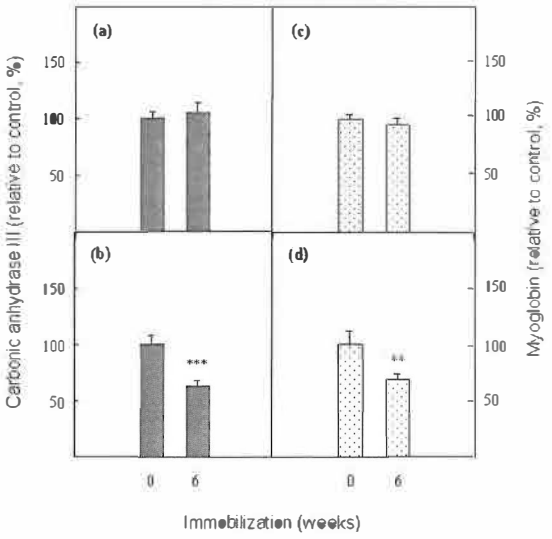

FIGURE 1 Human leg carbonic anhydrase III (CA III) (left) (a) specific concentration and (b) concentration multiplied with muscle cross-sectional area; and myoglobin (right) (c) specific concentration and (d) concentration multiplied with muscle cross-sectional area, expressed as % of control before and 6 weeks after postoperative immobilization in vastus medialis muscle in 13 patients. The results are means, SE. Statistical significance: **p <0.01, ***p<0.001.

5.1.3 PH and GGT enzyme activities, and Hyp concentration in skeletal muscle (III)

The specific activity of PH in the shortened soleus muscle (Fig. 2) decreased by 47-53% ($p<0.01$) after 1 week of immobilization compared with the contralateral side and the control group. After 3 days of remobilization, the PH activity increased 32% ($p<0.05$) above the control values and remained thereafter above the controls. The specific activity of PH in the lengthened tibialis anterior muscle (Fig. 3) increased by 44% ($p<0.05$) after the immobilization. It decreased gradually during remobilization and was on the control level after 7 days of remobilization.

The specific activity of GGT in the shortened soleus (Fig. 2) was 23% ($p<0.001$) below the control values after 1 week of immobilization. It increased rapidly during the early phases of remobilization. The activity was 42% ($p<0.001$) and 135 ($p<0.001$) above the control values after 3 and 7 days of remobilization, respectively, but reached the control level after 2 weeks. In the lengthened tibialis anterior, the GGT specific activity (Fig. 3) was increased by 84% ($p<0.001$) after the immobilization and stayed above the control level during the remobilization period of 14 days.

The specific concentration of Hyp in supernatant (soluble collagen) appeared to decrease after immobilization in the shortened soleus muscle (Fig. 2) by 12-18% compared with the contralateral and control groups, but the

changes were not significant. After 3 days of remobilization, the concentration was increased being 15-22% ($p<0.05$) above the contralateral and contol levels. After 2 weeks of remobilization, the concentration was 17% ($p<0.05$) above the contralateral level. The total soluble collagen content was significantly decreased (27%, $p<0.01$) in soleus after immobilization, but reached the control level after 3 days remobilization. In the lengthened tibialis anterior muscle, the soluble collagen concentration (Fig. 3) was 17% ($p<0.05$) above the control level after immobilization, but thereafter it was on the control level. The total soluble collagen content was slightly and transiently below the controls during the remobilization period.

The Hyp concentration in pellet (insoluble collagen) was 41-47% ($p<0.001$) above the contralateral and control levels in the soleus muscle after immobilization. It decreased gradually reaching the control level after 7 days of remobilization. There was no change in the total insoluble Hyp content in soleus. The insoluble Hyp concentration in the tibialis anterior remained unchanged. In the total insoluble Hyp of tibialis anterior, a slight transient decrease was observed during the remobilization when compared to the control group, but not when compared to the contralateral values. The concentration of the insoluble collagen was approximately ten times the concentration of the soluble collagen.

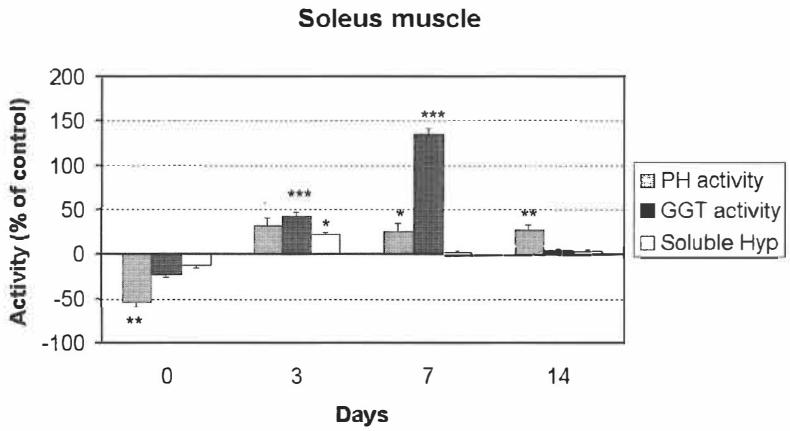

FIGURE 2 Effects of 7 days of immobilization (0 days) combined with 3, 7 or 14 days of remobilization on the specific activities of prolyl 4-hydroxylase (PH), galactosylhydroxylysyl glucosyltransferase (GGT) and soluble hydroxyproline (Hyp) concentration in rat soleus muscle. The differences in activities and concentrations from controls, which represent the zero level, are expressed as percentages. The results are means±SE. Statistical significance: *p (vs. control) <0.05, **p<0.01, ***p<0.001.

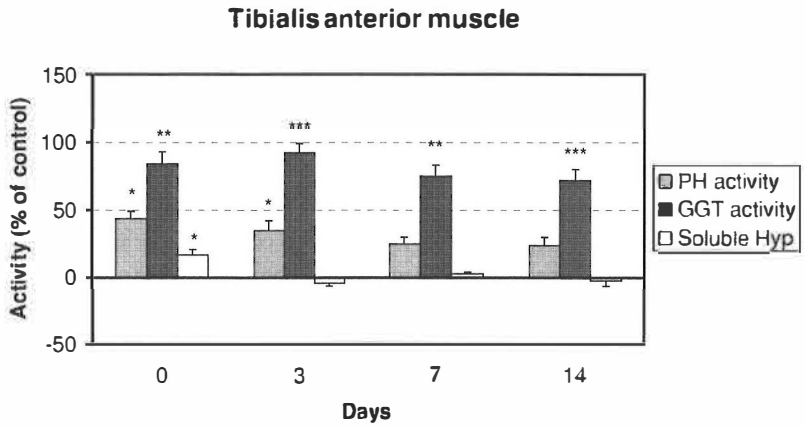

FIGURE 3 Effects of 7 days of immobilization (0 days) combined with 3, 7 or 14 days of remobilization on the specific activities of prolyl 4-hydroxylase (PH), galactosylhydroxylysyl glucosyltransferase (GGT) and soluble hydroxyproline (Hyp) concentration in rat tibialis anterior muscle. The activities and concentrations are expressed as percentages of control, which is equal to the zero level. The results are means±SE. Statistical significance: *p (vs. control)<0.05, **p<0.01,***p<0.001.

5.1.4 PH and GGT enzyme activities, and Hyp concentration in tendon (IV)

The specific activity of PH in the Achilles tendon immobilized in shortened position was decreased by 23-25% ($p<0.05$) after 1 week of immobilization compared to the contralateral and control levels. During remobilization, the activity increased gradually being 46% ($p<0.01$) above the control level after 14 days. The activity in the lengthened tibialis anterior tendon was decreased by 54% ($p<0.01$) after the immobilization. During remobilization, the activity increased gradually reaching the control level after 7 days.

The specific GGT activity in the shortened Achilles tendon was decreased by 23% ($p<0.05$) after 1 week of immobilization compared with the contralateral level. The activity increased rapidly being 53% ($p<0.01$) above the control values after 3 days of remobilization. After this, the activity decreased gradually being 30% ($p<0.05$) and 18% (NS) above the control and contralateral activities, respectively, after 7 days of remobilization. After 14 days, the value reached the control level. In the lengthened tibialis anterior -tendon, the specific GGT activity was 28% ($p<0.05$) below the contol values after the immobilization. The activity increased rapidly being 55-60% ($p<0.001$) above the controls after 3 and 7 days of remobilization. After 14 days, the activity was still 24% ($p<0.05$) above the control values.

There were no significant changes in the Hyp concentration in either of the tendons after the 1 week's immobilization period. In tibialis anterior tendon, a slight and transient decrease in the concentration was observed during

remobilization. Similar results were obtained also when the Hyp concentration was calculated per soluble protein.

5.2 Effects of denervation and reinnervation on rat skeletal muscle (V)

5.2.1 Weight parameters

The body weights of the denervated rats increased evenly and did not differ from the controls at any time point. The wet weights of the gastrocnemius, soleus and tibialis anterior muscles were 52, 46 and 57% below the control values ($p<0.001$), respectively, 19 days after the nerve crush. After 26 days, the weights were 49, 31 and 48% below the controls ($p<0.001$), after 40 days, 33 ($p<0.001$), 22 ($p<0.01$) and 35% ($p<0.01$) and after 61 days, 19 ($p<0.001$), 16 ($p<0.01$) and 19% ($p<0.01$) below the control values, respectively.

5.2.2 Electrophysiology

Nerve conduction to the muscles was strongly decreased after the nerve crush. The electrical responses did not differ whether stimulated proximal or distal to the site of crush. In gastrocnemius and soleus muscles the amplitudes of the responses were 80% lower than the control values 3 days after the nerve crush. In gastrocnemius, the amplitude started to increase strongly 14 days after the crush and had reached the control level when measured 63 days postcrush. In soleus, the increase began after 19 days reaching the control value 63 days postcrush. In tibialis anterior the amplitude of the response was 93% lower than the control value till 14 days postcrush, increased thereafter and reached the controls 63 days after the crush.

The conduction latencies were prolonged after the operation. They returned slowly, but did not reach the control values during the time the measurements were done. The latencies of tibialis anterior muscle were more delayed than those of gastrocnemius or soleus muscle during the period. Fibrillations and positive monophasic potentials were seen during the early phases of reinnervation in all three muscles. They diminished slowly and were lost 21 days after the crush in gastrocnemius and 28 days after it in soleus and tibialis anterior muscles.

5.2.3 PH and GGT enzyme activities, and Hyp concentration

The enzyme activities are expressed per amount of collagen, but the difference in the relative changes to the activities per soluble protein which was used in the study of Savolainen et al. (1988b) is very small, which was verified by using both calculations here (not shown). Specific PH activity in the gastrocnemius (Fig. 4) was 196% higher ($p<0.001$) than in the controls 19 days after the nerve

crush and decreased towards the control levels during reinnervation. The activity was 43% ($p<0.05$) above controls after 40 days, and reached the control level after 61 days. In soleus (Fig. 5) and tibialis anterior (Fig. 6), the PH activity, when measured per the amount of collagen, did not differ significantly from the controls. When calculated per muscle soluble protein, however, the PH activity of tibialis anterior was elevated: 70% ($p<0.01$), 77% ($p<0.001$) and 97% ($p<0.05$) above the controls after 19, 26 and 40 days, respectively.

Specific GGT activity was above the control levels in denervated gastrocnemius (Fig. 4) and soleus (Fig. 5) and decreased gradually during reinnervation. In gastrocnemius the activity was 126% ($p<0.001$) above the control level 19 days after the nerve crush. In soleus the activities were 91% ($p<0.001$) and 65% ($p<0.001$) above the controls after 19 and 26 days, respectively. In tibialis anterior (Fig. 6) the activity was below the control level 19 days after the onset of denervation being 92% ($p<0.05$) lower than the control values.

Hyp concentration in gastrocnemius (Fig. 4) was elevated during reinnervation: 113% ($p<0.05$) and 37% ($p<0.05$) above the control level 26 and 61 days after the nerve crush, respectively. In soleus (Fig. 5), the concentration did not differ from the controls. In tibialis anterior (Fig. 6), the concentration was above controls till 40 days after the crushing of the nerve and reached the control value after 61 days.

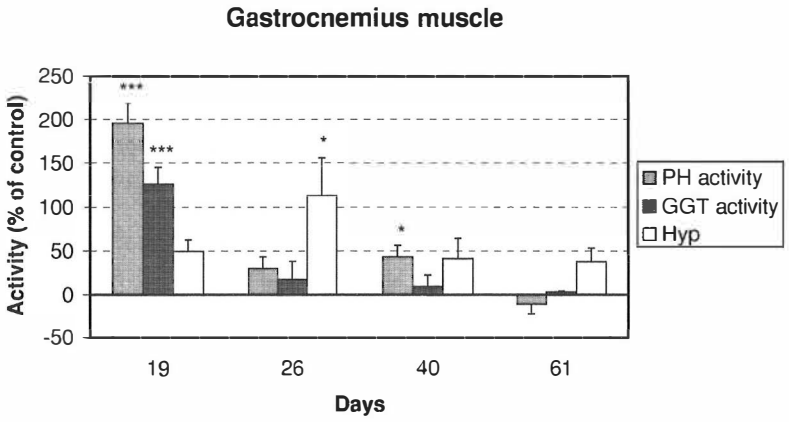

FIGURE 4 Effects of reinnervation 19, 26, 40 and 61 days after crush denervation of sciatic nerve on prolyl 4-hydroxylase (PH), galactosylhydroxylysyl glucosyltransferase (GGT) and hydroxyproline (Hyp) concentration in rat gastrocnemius muscle. The activities and concentrations are expressed as percentages of control, which is equal to the zero level. The results are means±SE. Statistical significance: *p (vs. control) <0.05, ***p <0.001.

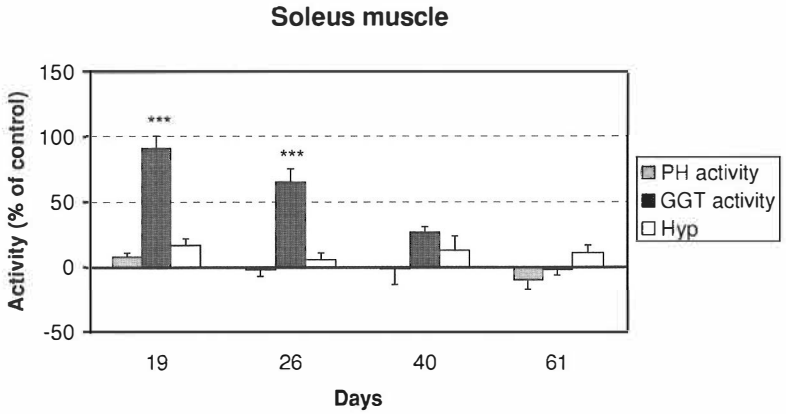

FIGURE 5 Effects of reinnervation 19, 26, 40 and 61 days after crush denervation of sciatic nerve on prolyl 4-hydroxylase (PH), galactosylhydroxylysyl glucosyltransferase (GGT) and hydroxyproline (Hyp) concentration in rat soleus muscle. The activities and concentrations are expressed as percentages of control, which is equal to the zero level. The results are means±SE. Statistical significance: ***p (vs. control)<0.001.

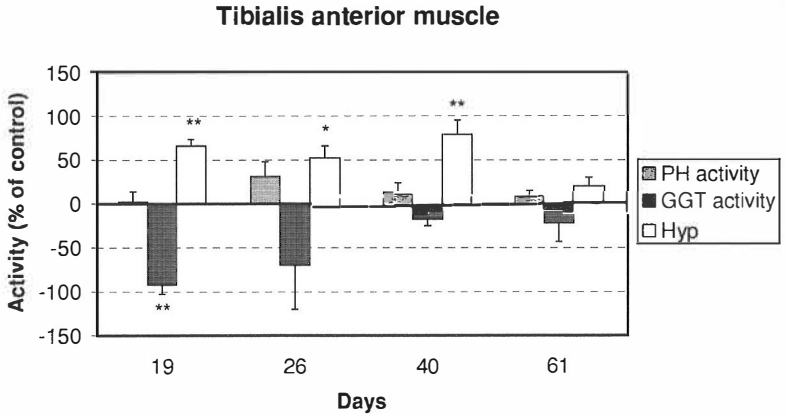

FIGURE 6 Effects of reinnervation 19, 26, 40 and 61 days after crush denervation of sciatic nerve on prolyl 4-hydroxylase (PH), galactosylhydroxylysyl glucosyltransferase (GGT) and hydroxyproline (Hyp) concentration in rat tibialis anterior muscle. The activities and concentrations are expressed as percentages of control, which is equal to the zero level. The results are means±SE. Statistical significance: *p (vs. control) <0.05, **p<0.01.

5.3 Effects of streptozotocin-induced diabetes, training and their combination on rat skeletal muscle (II)

5.3.1 Weight parameters

The body weights of the diabetic sedentary and diabetic trained groups were 37% and 30%, respectively, lower than the body weights of the respective control groups ($p<0.001$). The weights of the trained group appeared to be slightly (8%) below those of the sedentary controls ($p<0.05$), but no significant difference in the body weights was found between the diabetic trained and diabetic sedentary animals. The weights of the gastrocnemius, vastus lateralis and rectus femoris muscles were lower in the diabetic groups than in the non-diabetic animals ($p<0.001$). The absolute muscle mass was 49-51% ($p<0.001$) lower in the sedentary diabetic group than in the sedentary controls. In gastrocnemius, training increased the muscle weight in diabetic animals ($p<0.05$).

5.3.2 CA III expression at protein and mRNA levels

In diabetic rats, the specific CA III concentration (Fig. 7a) was elevated by 73% ($p<0.01$), 417% ($p<0.05$) and 131% (NS) compared with the controls in rectus femoris, vastus lateralis and gastrocnemius muscles, respectively. Training also increased the CA III concentration in all muscles, but the increase was significant only in vastus lateralis (240%, $p<0.05$). In the diabetic trained rats, the concentration was above the control level in all muscles, the increase being significant in rectus femoris (88%, $p<0.01$). At the mRNA level (Fig. 7b), the effect of diabetes on CA III expression was qualitatively similar to the effect at the protein level in gastrocnemius and vastus lateralis muscles being significant in gastrocnemius. In diabetic sedentary rats, CA III mRNA was elevated by 88% ($p<0.01$) and in diabetic trained rats by 70% ($p<0.01$) in gastrocnemius.

5.3.3 Citrate synthase and phosphoglycerate kinase

Training increased the specific citrate synthase activity by 131% ($p<0.01$) and 138% ($p<0.01$) in vastus lateralis and gastrocnemius muscles, respectively. In the trained diabetic rats (DT), the activity was 126% ($p<0.05$) and 174% ($p<0.05$) higher than in controls; and 133% ($p<0.05$) and 240% ($p<0.05$) higher than in the sedentary diabetic rats in vastus lateralis and gastrocnemius, respectively.

The specific phosphoglycerate kinase activity was slightly increased (by 20%, $p<0.05$) in the vastus lateralis muscle of the diabetic rats. Training decreased the activity by 17% ($p<0.05$) in rectus femoris. In the trained diabetic group, the activity was decreased by 16% ($p<0.05$) and 11% ($p<0.01$) in rectus femoris and vastus lateralis muscles, respectively. In vastus lateralis the activity was still lower in the diabetic trained group than in the trained group ($p<0.05$).

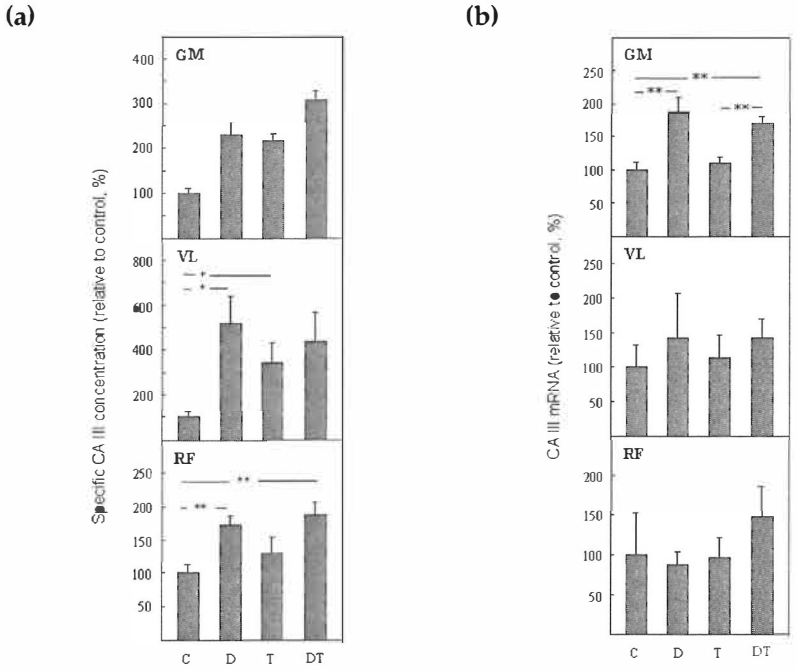

FIGURE 7 The effects of diabetes, physical training and their combination on (a) the specific carbonic anhydrase III (CA III) concentration and (b) the CA III mRNA, expressed as % of control in the gastrocnemius (GM), vastus lateralis (VL) and rectus femoris (RF) muscles of rat. The results are means, SE from 8-10 animals; (C) control, (D) diabetic, (T) trained, (DT) diabetic trained; *p<0.05, **p<0.01.

5.4 Effects of a single bout of exhaustive exercise on collagen synthesis in skeletal muscle and tendon (IV)

A single bout of treadmill running for 3-5 h caused an increase of 52% ($p<0.05$) in the specific activity of PH in the soleus muscle on the second day after the exercise. The activity remained elevated also thereafter until the 20th day after the exercise, but the differences from the initial value were not statistically significant. Changes in the specific activity of PH in the tibialis anterior muscle were not significant. There were also no significant changes in the concentration of Hyp in the soleus and tibialis anterior muscles. In Achilles tendon the specific PH and GGT activities as well as Hyp concentration were constant throughout the experiment.

5.5 Effects of concentric exercise on serum muscle and collagen markers (VI)

The area of type II fibers in the vastus lateralis muscle of the subjects was 56±5% (mean±SE). The isometric maximal strength decreased 12.8% ($p<0.01$) and concentric strength 9.1% ($p<0.05$) due to the 50 fatigue contractions. No statistically significant changes were seen in the EMG parameters or in the vertical jumping height and its ground reaction forces or in contact time between the pre- and post-loading measurements. The serum testosterone concentration was elevated by 17% ($p<0.01$) immediately after the exercise, but decreased to the initial level in 1 hour. There was no change in the cortisol concentration.

Serum CA III (S-CA III) increased after the exercise being 57% ($p<0.05$) above the initial value after 2 hours, and started to decrease thereafter. S-Mb increased markedly after the loading being 138% ($p<0.01$) and 143% ($p<0.01$) above the initial value 1 and 2 hours after the exercise, respectively. The initial value was reached after 1 day. The ΔS-CA III/ΔS-Mb -ratio, when calculated for the maximal increase 2 hours after the exercise, was 0.53±0.11. S-CK increased after the exercise being 9% ($p<0.01$), 15% ($p<0.01$) and 21% ($p<0.01$) above the initial level 0, 1 and 2 hours after the exercise, respectively. The value started to decrease after 3 days.

S-Pro(I)-C-P initial value decreased by 5% ($p<0.05$) 1 hour after the exercise. A day afterwards the value started to increase, being 5% ($p<0.05$) and 11% above the initial value, 2 and 4 days after the exercise, respectively. In S-Pro(III)-N-P, no significant changes due to the exercise were seen. S-GGT was elevated immediately and 3 days after the exercise by 17% ($p<0.05$) and 24% ($p<0.05$), respectively. In S-Hyp no significant changes were seen.

Correlations between the various parameters were calculated. The incease in S-Mb showed a correlation to the fiber type II % ($p<0.05$), and S-CK ($p<0.05$) 2 hours after the exercise, and to the maximal decrease in S-Pro(I)-C-P ($p<0.001$) 1 hour after the exercise. Also the change in S-Mb correlated to the decrease in S-Pro(I)-C-P ($p<0.01$). The increase in S-Pro(I)-C-P 2 days after the exercise correlated to the increase in S-CK immediately ($p<0.01$) and 1 hour after the exercise ($p<0.01$). The serum lactic acid concentration showed a negative correlation to the change in maximal isometric force and in flying time of the jumps ($p<0.05$).

6 DISCUSSION

6.1 Effects of immobilization and subsequent remobilization on skeletal muscle and tendon

6.1.1 Weight parameters

Cast immobilization in itself induces muscle atrophy without causing any actual nerve lesions. Immobilization-induced disuse atrophy is known to involve both slower protein synthesis and faster protein degradation (Goldspink 1977, Appell 1990) and is known to vary between muscles (Booth and Kelso 1973b, Herbison et al. 1978, Ingemann-Hansen and Halkjaer-Kristensen 1980). Here, the responses to cast- immobilization and brace in human vastus medialis muscle (I), and the responses to cast-immobilization and subsequent remobilization in rat soleus and tibialis anterior muscles (III) were followed. The atrophy of the vastus medialis was estimated from the cross-sectional area of the medial part of the quadriceps femoris muscle. Since there exists no apparent septum between the vastus medialis and intermedius muscles in a CT scan, a straight line was continued from the septum between the vastus medialis and rectus femoris muscles to the femoral bone in the cross-sectional image of the thigh. This means that the measured cross-sectional area may, in addition to vastus medialis, include part of the intermedius muscle, but this is the only way to determine the atrophy of vastus medialis from an image. Significant atrophy was evident. To eliminate the effect of the concomitant oedema after surgery, the contralateral thigh was taken to represent the zero point results. In principle, the effect of alteration in water content cannot be excluded when determining atrophy from the wet weights of the muscles either. In study III, the soleus immobilized in shortened position was observed to atrophy more than the tibialis anterior immobilized in lengthened position. This is in agreement with previous findings (Savolainen et al. 1987, 1988a, 1988b, Laurila et al. 1991, Laurila et al., submitted) and is due both to the great

sensitivity of the antigravity muscles, and of the crural muscles especially soleus, to disuse and to the immobilization position. Muscle stretch is an important determinant of the muscular protein balance. Immobilization in shortened position leads to a rapid loss of muscle mass whereas immobilization in lengthened position either attenuates or prevents muscle atrophy. In some cases the lengthened position may even produce hypertrophy (Booth et al. 1996). In this study the weight of the immobilized tibialis anterior muscle was observed to decrease but not as much as that of the soleus which is in accordance with previous studies (Savolainen et al. 1987, 1988a, 1988b, Laurila et al. 1991, Laurila et al, submitted). The body weights of the casted rats were also slightly decreased, which may be caused by stress, as also noted earlier (Savolainen et al. 1987, Laurila et al. 1991, Laurila et al., submitted). It is possible that increased levels of glucocorticoids may contribute to the negative protein balance and muscle atrophy during immobilization.

During remobilization, the body weights of the rats reached the control level after one week. The same recovery time has also been found to apply to an immobilization period of three weeks (Laurila et al., submitted). Muscle weights are relatively slow to recover, the time required being dependent on the duration of immobilization. The weights of the soleus and tibialis anterior did not reach the control level after a two weeks follow-up in this study. After three weeks of immobilization, the muscle weights have been shown to remain below the control level even after a nine-week follow-up period (Laurila et al., submitted).

6.1.2 CA III expression

The CA III concentration did not change during immobilization in the human vastus medialis muscle, the superficial part of which consists mainly of type II fibers (56% according to Johnson et al. 1973). The degree of stretch has been shown to affect the expression of CA III during immobilization. In rat, the concentration of CA III was increased significantly in the fast tibialis anterior muscle (95-99% type II fibers according to Armstrong and Laughlin 1983, Armstrong and Phelps 1984) immobilized in stretched position for three weeks (Laurila et al. 1991, Laurila et al., submitted). This is due to an increase in the number of CA III -positive fibers. According to ATP-ase staining, a fiber type shift from type IIb to IIa occurred in the tibialis anterior (Laurila et al. 1991, Laurila et al., submitted). Also, CA III mRNA has been reported to increase in rat fast plantaris muscle (91-93% type II fibers according to Armstrong and Laughlin 1983, Armstrong and Phelps 1984) when immobilized in stretched position (Brownson and Loughna 1996). Reversal of the immobilization-induced change seems to be slow; in the study of Laurila et al. (submitted), the elevated level of CA III was maintained throughout the nine-week remobilization period. The effects of denervation on fast muscle CA III seem to be very similar to those of immobilization. CA III mRNA is also increased in the tibialis anterior and extensor digitorum longus, another fast-type muscle, after denervation (Carter et al. 1988). According to Jeffery et al. (1990), CA III expression is coordinated with slow myosin heavy chain (MHC) expression.

Chronic low frequency nerve stimulation of fast muscle induces the expression of both slow MHC (Staron et al. 1987) and CA III (Gros and Dodgson 1988).

Type II fibers may shift towards a less differentiated state during immobilization (Laurila et al., submitted). It has been shown that in the late prenatal and early postnatal stages all rat muscle fibers contain about equal amounts of CA III (Laurila et al. 1989). It seems that CA III is constitutively expressed in skeletal muscle, and that in adult fast muscle the CA III expression is suppressed by phasic nerve stimulation. An alteration in motoneuron activity, caused by denervation, chronic stimulation or stretch during immobilization, may release the CA III expression in type II fibers. As the normal distribution of CA III expression in fiber types is restored very slowly during remobilization, other regulatory factors in addition to phasic nerve stimulation may be required to suppress CA III. Some neurotrophic substance(s) present in sciatic nerve extract, as suggested by Milot et al. (1994), may be involved in the down-regulation of CA III.

In slow muscle the changes in CA III immobilized in shortened position, have been shown to be small (Laurila et al. 1991, Laurila et al., submitted). A fiber type shift from type I to type IIa has been observed (Laurila et al. 1991, Laurila et al., submitted). This is in accordance with the literature stating that a decrease in the number of type I fibers occurs during disuse atrophy (Appell 1990). It has been assumed that atrophy and fiber degeneration reflect a secondary reaction to inactivity and its attendant impaired circulation, which particularly affect type I fibers (Appell 1990). In CA III mRNA expression, the change is again in the same direction as in the corresponding protein, i.e. diminishing (Brownson and Loughna 1996).

The myoglobin concentration in this study did not change during immobilization, which differs from the earlier results of Jansson et al. (1988). In that study the concentration in biopsies taken from the lateral portion of the quadriceps femoris increased together with a drop in oxidative enzyme activity. The physiological significance of that result remains indistinct unless the functional significance of increased myoglobin content is to facilitate oxygen extraction, since blood flow in the leg may be reduced during immobilization (Appell 1990). At all events, as both muscle thickness and the concentration of the proteins may change differentially in various parts of the muscle, caution should be exercised when making general conclusions concerning the whole muscle.

6.1.3 Collagen synthesis

Also in collagen biosynthesis, as in CA III expression, the changes caused by immobilization are dependent on the muscle concerned and/or the position the muscle is immobilized in. Specific PH and GGT activities decreased significantly after one week of immobilization in the soleus immobilized in shortened position, whereas in the tibialis anterior immobilized in stretched position, the activities were increased. This is in accordance with previous observations (Savolainen et al. 1988a, 1988b). The down-regulation takes place at the pretranslational level. According to Han et al. (1999b) immobilization

causes a decrease in the mRNA for the PH α-subunit, followed by a decrease in PH activity and mRNA levels for collagen types I and III. Tension is known to be an important positive regulator of collagen biosynthesis (Savolainen et al. 1988a, 1988b) which is also reflected in the fact that the specific activities of PH and GGT, as well as Hyp concentration, are greater in the antigravity soleus muscle, consisting mainly of type I fibers, than in the dorsiflexor tibialis anterior, which is mainly composed of type II fibers and is not tonically active (Kovanen et al. 1984a, Savolainen et al. 1987). Ahtikoski et al. (1997) observed down-regulation of the expression of type I collagen and its posttranslational modificators in the soleus immobilized in shortened position, but the effect was partly prevented by stretch. All this fits with the concept, that force transmitting regulates collagen expression in muscle.

In the tibialis anterior tendon, which was immobilized in lengthened position, a decrease in the specific activities of PH and GGT was observed. This is in accordance with reports in the literature of decreased specific activites in the immobilized tibialis anterior and Achilles tendons (Savolainen et al. 1988b). According to Savolainen et al. (1988b) immobilization in shortened position causes greater decreases in PH and GGT specific activities than immobilization in lengthened position, which may even prevent the decrease.

The adaptation of muscular connective tissue to immobilization is very rapid, the changes in collagen synthetizing enzymes appearing after only three days of immobilization when decreased PH and GGT activities were observed in the soleus muscle. Han et al. (1999b) found a decrease in the mRNA for PH α-subunit in gastrocnemius and plantaris muscles after immobilization for one day and a decrease in PH activity after immobilization for three days. In the present work the PH and GGT activities were also decreased after immobilization for six weeks. The decrease is in accordance with the observed decrease in the concentration of newly synthetized soluble Hyp. The concentration of soluble Hyp was in accordance with PH and GGT activities also in the lengthened tibialis anterior after one week's immobilization; they were all increased. Also during remobilization, the soluble Hyp concentration followed the PH and GGT activities. The changes in soluble Hyp, any more than those in PH and GGT, need not reflect alterations in the rate of collagen deposition, however, because a high proportion of newly synthetized soluble collagen is degraded (Laurent 1987). The concentration of the insoluble Hyp in soleus muscle, in contrast to the soluble Hyp, increased during immobilization. This is obviously caused by a greater net degradation of noncollagenous proteins, as the total muscular Hyp content was unchanged. This is in accordance with other observations of unchanged or slightly decreased total collagen content in muscles after immobilization for one to several weeks (Savolainen et al. 1987, 1988b, Karpakka et al. 1990, Han et al. 1999b). These results are in conformity with the slow rate of collagen synthesis in rat muscles, which is 1.3% /day (Reeds et al. 1980). On the other hand, also increased total muscular collagen contents have been observed in morphometric studies (Jozsa et al. 1990, Lapier et al. 1995, Kannus et al. 1998). A study of the long-term effects of forced muscle contractions on the expression of the basement

membrane type IV collagen points to an increase in the collagen concentration and its degradation capacity (Takala et al. 1998).

After removal of the cast, the activities of PH and GGT increased above the control level in soleus in the early phases of remobilization, whereas in tibialis anterior they slowly decreased towards the control levels. After immobilization in shortened position, the cage activity causes to the connective tissue of the soleus both increased static tension, due to the antigravity function of the muscle, and increased transmittance of dynamic forces. In tibialis anterior muscle, which was immobilized in lengthened position, removal of the cast probably resulted in a decrease in the permanent tension, because tibialis anterior is a phasic muscle and is not tonically activated. Remobilization also resulted in a more rapid re-growth of the atrophied soleus than of the atrophied tibialis anterior. According to the literature, rapid muscular growth is accompanied by a high rate of collagen biosynthesis (Turto et al. 1974, Kovanen et al. 1987). In the tibialis anterior, the activities of PH and GGT did not follow the overall protein balance of the muscle during immobilization in the lengthened position and subsequent remobilization. It seems that in tibialis anterior immobilized in lengthened position tension may be a more important acute regulator of collagen biosynthesis than the overall muscular protein balance. No changes in total muscular collagen content were seen in the soleus, probably due to the slow turnover of collagen (Reeds et al. 1980). During immobilization, the changes in the activity of PH were more pronounced than those in GGT, as also stated before (Savolainen et al. 1987, 1988a). However, during remobilization, the situation was the opposite, the changes in GGT activity being greater in both muscles. The same also applied to the tibialis anterior and Achilles tendons. This suggests that collagen glycosylation may be more accelerated than collagen hydroxylation during remobilization, which may promote the cross-linking of collagen (Kivirikko and Myllylä 1982a) and may thus yield collagen fibrils with enhanced stability. According to Kellett (1986), damaged tendons contain a large proportion of type III collagen which is deficient in the number of cross-linkages between and within the tropocollagen subunits. Kannus et al. (1992) suspect that remobilized tendons will not achieve all the biochemical and biomechanical properties of healthy tendons, and that there may be an increased risk of tendinitis and tendon ruptures during postimmobilization activity.

6.2 Effects of denervation and reinnervation process on rat muscle collagen

In this study the effects of denervation and the subsequent reinnervation process were studied in the soleus, tibialis anterior and gastrocnemius muscles of the rat. Both immobilization and denervation are known to cause atrophy in hind limb muscles and fibers, but in different ways. During immobilization, it is mostly the type I fibers of ankle joint plantarflexors that atrophy, whereas the

fibers of the dorsiflexors, being mostly of type II, are less responsive to disuse (Edgerton et al. 1975, Savolainen et al. 1987). Following denervation, however, both fiber types atrophy but they are affected differentially in the slow and fast muscles (Jaweed et al. 1975, Niederle and Mayr 1978, Lu et al. 1997). The type I fibers of the rat soleus atrophy as much or slightly more than the type II fibers, whereas the type II fibers of fast type muscles, such as tibialis anterior and gastrocnemius, atrophy significantly more than the type I fibers during the first weeks of denervation atrophy. In this study, denervation was produced by crushing the nerve, which, according to the electrophysiological measurements, resulted in a commencement of the reinnervation process about three weeks after the crush. The time point is in accordance with observations in the literature, where the start of reinnervation has been evaluated from the increase in muscle wet weight (Herbison et al. 1973) or in fiber diameter (Jaweed et al. 1975). In this study, it is possible that the weight of the soleus had started to increase before the first measurement, made 19 days after crushing of the nerve.

During disuse atrophy process without neural damage, the overall protein balance is an important regulator of collagen biosynthesis in skeletal muscle as stated earlier. The activities of the collagen synthetizing enzymes PH and GGT decrease, reflecting the adaptation of muscular collagen biosynthesis to diminished activity or loading of the musculotendinous system. During denervation, however, the activities of the collagen-synthetizing enzymes were elevated despite rapid muscular atrophy in this as in an earlier study where denervation was caused by transection of the sciatic nerve (Savolainen et al. 1988b). When denervated muscles have been immobilized, length-associated changes have been observed in the atrophy process of the muscle the shortened muscles atrophying more than the lengthened ones, but not in the PH, GGT and Hyp responses (Savolainen et al. 1988b). Thus, denervation seems to "uncouple" collagen synthesis from the overall muscular protein synthesis. Accelerated muscular growth normally increases collagen synthesis (Turto et al. 1974, Kovanen et al. 1987) as discussed in the previous chapter. During reinnervation, on the contrary, the rate of collagen synthesis decreases despite muscular growth according to the present study.

The fact that changes in PH activity are generally more pronounced than those in GGT activity in reflecting changes in collagen biosynthesis did not hold good in all the denervated muscles here. In the soleus only GGT was elevated, which is in accordance with the previous denervation experiments of Savolainen et al. (1988b). Greater changes in GGT than in PH activity were also seen during accelerated collagen biosynthesis in the remobilization of muscle and tendon. On the other hand, according to Sawai (1982) the content of glycosylated hydroxylysine decreases during denervation although total muscular collagen increases. The Hyp concentration was elevated during the first weeks of reinnervation, but total muscular Hyp was not, which is in accordance with the previous nerve transection study (Savolainen et al. 1988b). Thus the increase in collagen concentration caused by denervation seems to have occurred only in relation to noncollagenous proteins. It is also possible that the elevated biosynthesis may be associated with increased degradation of the old or the newly synthetized collagen, which may lead to qualitative

changes in the intramuscular collagen (Savolainen et al. 1988b). Increases in type I and III collagens in denervated rat skeletal muscles have been observed earlier by immunofluorescence techniques (Salonen et al. 1985). In a recent study, accelerated type IV collagen turnover in the skeletal muscle of patients with spinal cord injuries, was observed (Koskinen et al., submitted).

6.3 Regulation of collagen metabolism

In this study the biochemistry of the adaptive responses of the muscular and tendinous collagen network was studied. The mechanisms for the changes in collagen metabolism in skeletal muscle and tendons are largely unknown. From studies in other tissues it is known that the expression of collagen and other ECM components is regulated by different hormones and growth factors, like transforming growth factor β (TGF-β), and mechanical stretch (Kähäri et al. 1988, Penttinen et al. 1988, McAnulty et al. 1991, Peltonen et al. 1991, Feres-Filho et al. 1995, Alevizopoulos and Mermod 1997). Transforming growth factor β1 (TGF-β1) bioactivity is known to regulate both the synthesis and degradation of ECM components (Massague 1990). It increases the synthesis of e.g. collagen and fibronectin and of protease inhibitors such as tissue inhibitor of metalloprotease. TGF-β1 also alters the expression of the integrin class of cell adhesion receptors (Heino 1989, Ignotz 1989). Transmembrane integrins bind collagen and may transmit tension or relaxation signals to the intracellular phosphorylation cascades which govern the gene expression of cells. Han et al. (1999a) recently found increased TGF-β1 mRNA in the soleus and quadriceps femoris muscles of rats after a single bout of running exercise. Thus TGF-β1 may be involved in the repair of muscle damage. The gene expression of PH has also been found to be up-regulated by TGF-β1 in cultured fibroblasts isolated from rat skeletal muscle (Han et al., unpublished).

6.4 Effects of streptozotocin-induced diabetes, training and their combination on rat muscle CA III expression

In this study, the effects of streptozotocin-induced diabetes, training and their combination on CA III expression at the protein and mRNA levels were studied in the gastrocnemius, vastus lateralis and rectus femoris muscles of the rat. Streptozotocin-induced diabetes caused a pronounced decrease in the body and muscle mass of the rats. Diabetes is known to affect nitrogen metabolism, resulting in loss of protein, particularly from muscle (e.g. Manchester 1970, Grizard et al. 1999). This is due to a lack of insulin, which causes the protein synthesis rate to decrease (Pain and Garlick 1974) and the breakdown to increase (Charlton and Nair 1998). There was also a slight decrease in the body weight gain of the trained non-diabetic rats, which is in accordance with reports in the

literature (Harpur 1980, Moynihan and Ennis 1990). The muscle mass of the trained animals remained at the control level, and in the gastrocnemius of diabetic animals training seemed to partially prevent the decrease in the muscle mass. The exercise used here was probably less strenuous than in some earlier studies, in which reductions in muscle weight after long-term endurance training have been demonstrated (Fitts and Holloszy 1977, Kovanen et al. 1984b, 1987). According to Fitts and Widrick (1996), endurance exercise, when maintained over prolonged periods, may induce atrophy in type I and IIa muscle fibers.

Training caused a considerable increase in the specific activity of citrate synthase in the gastrocnemius and vastus lateralis muscles, indicating that the training was efficient enough to increase the oxidative capacity of these muscles. Streptozotocin-induced diabetes, on the other hand, is known to reduce oxidative enzyme activity in rat skeletal muscle (Ianuzzo et al. 1974, Ianuzzo and Armstrong 1976, Goodyear et al. 1988), an effect that is known to be both dose-dependent and fiber type-specific (Ianuzzo and Armstrong 1976, Kainulainen et al. 1994). In the present study, the three muscles assayed consist mainly of type II fibers, the gastrocnemius differing from the other two in that its red part contains more type I fibers than that of the others (Armstrong and Laughlin 1983, Armstrong and Phelps 1984). Here the citrate synthase activity was decreased in gastrocnemius. The glycolytic pathway marker enzyme, phosphoglycerate kinase, was decreased in the trained vastus lateralis and rectus femoris, as observed previously in endurance trained rats (e.g. Holloszy 1975, Harpur 1980).

Both streptozotocin-induced diabetes and aerobic running training independently increased CA III expression in rat skeletal muscle. The specific CA III concentration was markedly elevated in the vastus lateralis muscle of both the diabetic and the trained rats, while slighter changes were observed in the rectus femoris and gastrocnemius muscles. Changes in the mRNA level were qualitatively similar to those in the protein level, which suggests the regulation to be at the pretranslational level. Both treatments are known to increase oxidative stress, thus the present finding of CA III expression may represent an adaptation of skeletal muscle cells to increased oxidative stress. According to the literature, protein S-thiolation/dethiolation is an early cellular response to oxidative stress (Rokutan et al. 1991, Lii et al. 1994, Chai et al. 1994a,b). It has been demostrated that the glutathiolation of CA III, which occurs *in vivo* and increases during ageing, reversibly regulates its tyrosine phosphatase activity (Cabiscol and Levine 1996). Since this activity is dependent on the cellular redox state, CA III may well have a role in intracellular signalling, particularly in response to oxidative stress. In a recent study, the effect of overexpression of CA III on cellular response to oxidative stress was studied (Räisänen et al. 1999). The results suggested that CA III functions as an oxyradical scavenger and thus protects cells from oxidative damage. The results presented in this study agree with the literature cited in suggesting a role for CA III in an oxidizing environment.

6.5 Effects of concentric exercise on serum muscle and collagen markers

Most exercise types contain both concentric and eccentric as well as weight bearing components, so their effects on musculoskeletal strain and exercise-induced damage cannot be distinguished. Therefore, in this study, the strain and damage of the musculoskeletal system was measured in a purely concentric exercise model by examining the specific effects of this exercise type on muscle cells and collagen serum markers.

It was observed that the single bout of heavy concentric exercise was strenuous enough to cause protein leakage from muscles, and probably also from the collagen synthetizing cells of the connective tissue. Both the nonspecific muscle markers S-Mb and S-CK and the muscle fiber type I specific S-CA III increased in response to the exercise. CA III was found to be almost as sensitive a marker of exercise-induced leakage from muscle cells as S-Mb, and CK the least sensitive of the three, which is in accordance with previous findings (Takala et al. 1989a). The concentrations of S-CA III and S-Mb peaked simultaneously 2 h after the exercise, as also observed previously (Takala et al. 1989a). S-Mb and S-CA III returned to the reference level more rapidly than did S-CK, which is in accordance with their shorter half-life (Väänänen et al. 1986). The collagen marker S-GGT increased during the exercise, as observed previously after prolonged exercise (Takala et al. 1986, Takala et al. 1989b). The rapid increase is more likely to reflect leakage from collagen synthesizing fibroblasts or osteoblasts than increases in tissue GGT activity and collagen synthesis, which are known to occur a few days later (Myllylä et al. 1986, Takala et al. 1986). The rate of protein release from muscle and other tissues may also depend on lymphatic flow, which has been shown to be increased during exercise (Havas et al. 1997).

The exercise also affected type I collagen production. The serum marker of type I collagen synthesis, S-Pro(I)-C-P, decreased at first, but started to increase two days after the exercise. The site of the altered collagen production cannot be identified with the test used here, but tissue production in the bones is probably reflected, as most type I collagen is found there (Hassager et al. 1991). Since the decrease in S-Pro(I)-C-P correlated with the increase in S-Mb, the strain and damage imposed on the musculoskeletal system could be one reason for the transient decrease in the rate of type I collagen production. The later increase in S-Pro(I)-C-P could reflect increased type I collagen synthesis as an adaptive and/or reparative process in the bones after the exercise. Exercise is known to have a positive effect on bone formation (Lanyon 1987). In rodent muscles, reparative increase in the rate of total collagen synthesis has been observed after a single bout of treadmill running in mice (Myllylä et al. 1986). In addition, increased gene expression for collagens I and III and the enzymes responsible for their posttranslational modifications has been observed after a single bout of strenuous running exercise in rats (Han et al. 1999a). In the present study, there was no increase in the S-Pro(III)-N-P concentration suggesting unchanged type III collagen synthesis (Simon et al. 1988). This is in accordance with previous

investigations (Takala et al. 1986, Takala et al. 1989), and suggests that S-Pro(III)-N-P increases only after very strenuous physical exercise, probably reflecting a repair process. The metabolic fate of the circulating connective tissue -derived marker proteins also contributes to the results. Thus the serum levels of the markers can also reflect changes in the degradation rates of the proteins (Smedsrod 1988, Smedsrod et al. 1990).

7 PRIMARY FINDINGS AND CONCLUSIONS

In the present study, the adaptations of skeletal muscle CA III isoenzyme and collagen, and tendon collagen expression were studied during immobilization and subsequent recovery when remobilization was allowed. The adaptation of skeletal muscle collagen expression was also studied during denervation and the subsequent reinnervation process. The effects of streptozotocin-induced diabetes, training and their combination were studied on CA III expression at the mRNA and protein levels. In addition, the effects of concentric exercise were studied on the release of muscle proteins and markers of collagen synthesis.

In addition to the overall muscular protein balance, the degree of muscle stretch during immobilization seemed to be an important regulator of collagen expression in muscle. The specific PH and GGT activities were significantly decreased after one week of immobilization in the soleus muscle immobilized in shortened position, whereas the activities were increased in the tibialis anterior muscle immobilized in stretched position. The adaptation of muscular connective tissue to disuse was relatively rapid, as significant changes in PH and GGT activities were seen already after three days of immobilization in the antigravity soleus muscle. After removal of the cast, the activities of PH and GGT increased above the control level in soleus in the early phases of remobilization, whereas in tibialis anterior they slowly decreased towards the control levels. In tibialis anterior, the activities of PH and GGT did not follow the overall protein balance of the muscle during immobilization in lengthened position and subsequent remobilization, thus tension seemed to be a more important acute regulator of collagen biosynthesis than the overall muscular protein balance. During immobilization, the changes in the activity of PH were more pronounced than those in GGT, whereas during remobilization the situation was the opposite, the changes in the GGT activity being greater in both muscles studied, and also in the tendons. This suggests that during remobilization collagen glycosylation may increase more markedly than collagen hydroxylation, which may in turn be connected with the promotion of the cross-linking of collagen.

In human muscle the specific CA III concentration was unaltered during immobilization, while total CA III decreased to the same extent as the muscle cross-sectional area. This suggests that the net breakdown of CA III occurs at the same rate as the average net degradation of mixed muscle proteins.

Contrary to the situation in disuse atrophy, denervation atrophy was associated with an increased level of collagen biosynthesis. Denervation seems to "uncouple" the regulation of the adaptive responses of muscular collagen biosynthesis from the atrophy process of the muscle as a whole. During the follow-up of the reinnervation process (19 to 61 days from the start of denervation), both the PH and GGT enzyme activities and the Hyp concentration decreased to the control level in spite of accelerated muscular growth. During the reinnervation process, as also during remobilization, the collagen glycosylation capacity increased more markedly than the hydroxylation capacity.

Both streptozotocin-induced diabetes and endurance training on a treadmill independently increased CA III expression at protein and mRNA levels in rat skeletal muscle. Both treatments are known to increase oxidative stress. The present finding that the gene expression of CA III is increased in response to streptozotocin-induced diabetes and endurance training may represent an adaptation of skeletal muscle cells to increased oxidative stress.

A single bout of heavy concentric exercise was shown to cause protein leakage from muscles; serum CA III, Mb and CK were all increased. Changes in the serum markers of collagen synthesis suggest an initial decrease and subsequent increase in type I collagen secretion from the collagen synthetizing cells. The activation of type I collagen secretion seems to depend on the strain and damage of the musculoskeletal system and may represent increased type I collagen synthesis as an adaptive and/or reparative process after the exercise.

The present findings indicate that changes in physical activity and muscle innervation are associated with adaptive responses in the metabolism of the muscular collagen network. The finding that muscular CA III expression is increased in response to experimental diabetes and endurance training may represent an adaptation of skeletal muscle cells to oxidative stress.

8 YHTEENVETO

Tässä tutkimuksessa selvitettiin luustolihaksen CA III:n ja kollageenien ekspressiota sekä kollageenisynteesiä jänteessä immobilisaation ja sen jälkeisen remobilisaation aikana. Lihaskudoksen kollageenisynteesin adaptaatiota tutkittiin myös denervaation ja reinnervaation yhteydessä. Lisäksi selvitettiin kokeellisen streptozotocin-indusoidun diabeteksen ja kestävyysharjoittelun vaikutuksia lihaskudoksen CA III:n ekspressioon sekä korkeaintensiteettisen konsentrisen lihastyön vaikutusta lihassyistä vapautuneiden ja kollageenisynteesin markkeriproteiinien esiintymiseen seerumissa.

Lihaksen atrofian lisäksi lihaksen venytystilan todettiin vaikuttavan lihaksen kollageenisynteesiin immobilisaation aikana. Kollageenia syntetisoivien PH- ja GGT-entsyymien aktiivisuudet laskivat lyhentyneeseen asentoon immobilisoidussa rotan soleus-lihaksessa, kun taas näiden entsyymien aktiivisuudet lisääntyivät venytystilaan immobilisoidussa tibialis anterior -lihaksessa. Kollageenisynteesin adaptaatio immobilisaatioon oli suhteellisen nopea; muutoksia PH- ja GGT-entsyymien aktiivisuuksissa havaittiin jo kolmen päivän immobilisaation jälkeen soleus-lihaksessa. Kipsin poiston jälkeisen remobilisaation aikana PH- ja GGT-aktiivisuudet palautuivat vähitellen lähtötasoilleen. Kollageenin synteesissä tapah-tuneet muutokset eivät vaikuttaneet lihaksen kokonaiskollageenin määrään. Akillesjänteessä ja tibialis anterior -jänteessä kollageenisynteesi laski immobilisaation aikana palautuakseen kontrollitasolle kipsin poiston jälkeisen seurannan aikana. Yksittäinen raskas harjoittelukerta puolestaan lisäsi kollageenisynteesiä soleus-lihaksessa, mutta ei akillesjänteessä. Immobilisaation aikana muutokset PH:n aktiivisuudessa olivat suurempia kuin muutokset GGT:n aktiivisuudessa, kun taas immobilisaation jälkeisen liikkeelläolon yhteydessä muutokset olivat suurempia GGT-aktiivisuudessa. Näyttää siltä, että remobilisaation aikana kollageenin glykosylaatiokapasiteetti lisääntyy voimakkaammin kuin kollageenin hydroksylaatiokapasiteetti. CA III -pitoisuus oli ihmisen vastus medialis -lihaksessa lähtötasolla kuuden viikon immobilisaation jälkeen. Lihasatrofiasta johtuen CA III:n kokonaismäärä kuitenkin väheni; nettohajoaminen tapahtui yhtä nopeasti kuin lihaksen totaaliproteiinin väheneminen.

Denervaatioon liittyi lihaksen atrofian lisäksi lisääntynyt kollageenisynteesi ja Hyp-pitoisuus. Reinnervaation seuranta-aikana sekä PH- ja GGT-entsyymiaktiivisuudet että lihaksen Hyp-pitoisuus laskivat kontrollitasolle samanaikaisesti lihaksen painon lisääntymisen kanssa. Lihaksen kollageenisynteesin vasteet immobilisaatioon ja denervaatioon ovat siis erilaisia huolimatta molempiin käsittelyihin liittyvästä lihasatrofiasta. Lihaksen normaali hermotus näyttäisi hillitsevän kollageenisynteesiä lihaksessa. Myöskin reinnervaation samoin kuin remobilisaation aikana kollageenin glykosylaatiokapasiteetti lisääntyi enemmän kuin hydroksylaatiokapasiteetti.

Sekä kokeellisen streptozotocin-indusoidun diabeteksen että kestävyystyyppisen juoksuharjoittelun yhteydessä CA III:n pitoisuus lisääntyi rotan lihaskudoksessa. Lihasharjoittelun ja diabeteksen tiedetään lisäävän vapaiden happiradikaalien muodostusta, ja havainto voi liittyä lihassyiden adaptaatioon lisääntyneeseen oksidatiiviseen stressiin.

Voimakkaan konsentrisen lihastyön havaittiin nostavan ihmisen seerumissa lihassyistä vapautuvien proteiinien kuten CA III:n, myoglobiinin ja kreatiinikinaasin pitoisuuksia. Myös kollageenisynteesin markkeriproteiinien pitoisuudet lisääntyivät. Tyypin I kollageenin synteesin markkerin vapautuminen seerumiin näyttää riippuvan rasituksen tukikudoksissa aiheuttaman vaurion laajuudesta ja voi olla seurausta lisääntyneestä tyyppi I kollageenin synteesistä edustaen adaptiivista ja/tai vaurioita korjaavaa prosessia.

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I

Effect of immobilization on carbonic anhydrase III and myoglobin content in human leg muscle

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II

Effects of streptozotocin-induced diabetes and training on carbonic anhydrase III in rat skeletal muscle

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III

Collagen synthesis in rat skeletal muscle during immobilization and remobilization

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IV

The effects of remobilization and exercise on collagen biosynthesis in rat tendon

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V

Effect of reinnervation on collagen synthesis in rat skeletal muscle

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VI

Effect of concentric exercise on serum muscle and collagen markers

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