Cross transfer effects after unilateral muscle overuse

An experimental animal study about alterations in the morphology and the tachykinin system of muscles

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“To learn without thinking, one will be lost in his learning. To think without learning, one will be imperilled”

Confucius

To my family
ABBREVIATIONS

ACE  Angiotensin-converting enzyme
ACH  Acetylcholine
AChE Acetylcholinesterase
AP   Alkaline-phosphatase
ATP  Adenosine triphosphate
C    Captopril
CAF  Capillaries around fibers
CAFA Capillaries around fibers related to cross sectional area
CD   Capillary density
CGRP Calcitonin gene-related peptide
CSA  Cross-sectional area
CV   Coefficient of variation
E/EMS Exercise/electrical stimulation
EIA  Enzyme immunoassay
EMG  Electromyography
H&E  Haematoxylin & eosin
IHC  Immunohistochemistry
ISH  In situ hybridization
LDV  Large dense-cored vesicles
mAb  Monoclonal antibody
mATPase Myofibrillar adenosine triphosphate
MyHC Myosin heavy chain
NEP  Neutral endopeptidase
NK-1R Neurokinin-1 receptor
PBS  Phosphate buffered saline
SP   Substance P
Th   DL-Thiorphan
VD   Vessel density
ABSTRACT

Unilateral exercise can produce certain contralateral strength effects. Deleterious events can be cross-transferred as well, as illustrated by a strict symmetry in some chronic inflammatory diseases. To date, knowledge on the effects of marked overuse of skeletal muscles is limited, and there is largely no information if unilateral overuse affects the contralateral muscles. In view of this, the present study was undertaken to test the hypothesis that unilateral muscle overuse causes alterations in tissue structure and the tachykinin system, with a focus on substance P (SP), not only in the exercised muscles, but also in the contralateral muscles. SP is a well-known neuromodulator that is known to be proinflammatory.

An experimental rabbit model with unilateral muscle overuse of the soleus and gastrocnemius muscles caused by exercise via electrical muscle stimulation (E/EMS) was used. In total, 40 rabbits were randomly divided into seven groups of which two groups served as controls. The rabbits were anaesthetized and then set on a “kicking machine” to perform exercise via EMS for 2h every second day. Experimental periods for groups 1-3 were 1, 3 and 6w, respectively, whereas groups 4-6 were exercised for 1w but also subjected to injections in the peritendinous tissue with SP, NaCL, Captopril (C), an ACE inhibitor, and DL-Thiorphan (Th) which inhibits the activity of neural endopeptidase. One group was not subjected to the experiment at all. The day after the last session of E/EMS, the soleus muscle and the gastrocnemius muscle from both legs were collected for analysis. Alterations in muscle structure and the tachykinin system were analyzed with enzyme and immunohistochemical techniques, in situ hybridization and EIA methods.

After 1w of E/EMS, focal areas of the exercised muscles contained a mild infiltration of inflammatory cells (myositis) and small morphological changes. After 3 and 6w of E/EMS, distinct myositis and muscle changes were bilaterally present in focal areas of both muscles. The structural changes, which mainly were observed in myositis areas, consisted of increased fiber size variability, split fibers, internal myonuclei, necrotic fibers, fibrosis, fat infiltration, and small fibers containing developmental MyHCs. Bilateral morphological changes, such as loss of axons, were also observed in nerves. In addition, expressions of tachykinin and the SP-preferred receptor, the neurokinin-1 (NK-1R), were bilaterally upregulated in nerve structures and blood vessel walls. Infiltrating white blood cells exhibited tachykinin—like and NK-1R immunoreactivity. NK-1R immunoreactions were also found in necrotic and regenerating muscle fibers.

The concentration of tachykinin (SP) was significantly increased in both soleus and gastrocnemius muscles after E/EMS. There was a significant correlation between the two sides in concentration of tachykinin and in the intensity of tachykinin—like immunoreaction in blood vessel walls. The muscle fiber size and capillary supply of fibers were bilaterally decreased after 3w of EMS. The myositis areas contained an increased number of vessels with a larger size than capillaries, while areas with increased amount of connective tissue contained a very low number of capillaries. A bilateral fiber type shift against a lower proportion of slow MyHCI fibers and higher proportion of fast MyHCII fibers was observed in both muscles. The local injections of C+Th and SP+C+Th led to marked structural changes in the muscle tissue and marked NK-1R and tachykinin—like immunoreactivity in the myositis areas and increased tachykinin concentration in the tissue.

In conclusion, the repetitive unilateral muscle overuse caused by E/EMS led overtime to muscle injury and myositis. The affected areas contained both degenerative and regenerative alterations in the muscle tissue and nerves, and an upregulation of the tachykinin system. Most interestingly, the changes not only occurred in the exercised side, but also in the homologous contralateral muscles. The tachykinin system appears to be an important factor in the processes of crossover effects.
LIST OF PUBLICATIONS

This thesis is based on the following original papers. They will be referred to by their roman numerals.

I. **Song Y, Forsgren S, Yu J, Lorentzon R, Stål P**

II. **Song Y, Stål P, Yu J, Forsgren S**
    Marked effects of tachykinin in myositis both in the experimental side and contralaterally: Studies on NK-1 receptor expressions in an animal model. ISRN Inflammation 2013;Doi:10.1155/907821

III. **Song Y, Stål P, Yu J, Forsgren S**
    Bilateral increase in expression and concentration of tachykinin in a unilateral rabbit muscle overuse model that leads to myositis. BMC Musculoskeletal Disorders 2013;14:134

IV. **Song Y, Forsgren S, Liu JX, Yu J, Stål P**
    Unilateral muscle overuse causes bilateral changes in muscle fiber composition and vascular supply (Manuscript)

V. **Song Y, Stål P, Lorentzon R, Backman C, Yu J, Forsgren S**
    Experimental studies favour that tachykinins are involved in the processes of myositis and muscle derangement in an overuse animal model (Manuscript)
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BACKGROUND

There is a wide range of examples in the literature showing that alterations or interventions in one side of the locomotor system in the body can be transferred to the other side. The cross-transfer effects vary from adaptations of muscle performance to alterations in gene expression, inflammation and tissue remodeling. Previous studies have for example shown that unilateral strength training increases strength not only in the trained muscle but also to some extent in the homologous muscle of the contralateral limb (Zhou, 2000; Carroll et al., 2006; Mann et al., 2011). This effect has been reported for different muscles and can occur by training that is accomplished by voluntary efforts as well as by electrical muscle stimulation (EMS). However, there are also reports that deleterious events can be cross-transferred as well. There is thus a strictly symmetrical distribution of inflammation in some chronic inflammatory diseases (Shenker et al., 2003; Kelly et al., 2007) and a presence of a mirror image of the nervous system for pain (Sluka et al., 2001; Twining et al., 2004).

Observations show that the nervous system may have a role in inflammation. Denervation of joints leads to regression of established rheumatoid arthritis (RA) and protection from RA (Glick and Buchan, 1962). Furthermore, painful Achilles tendinopathy frequently develops bilaterally (Shalabi, 2004) and recent findings indicate that there are bilateral cross-transfer effects after unilateral treatment for this condition (Alfredson et al., 2012). A neurological explanation to the symmetry in inflammation might be that neuropetides that have important roles in propagating inflammation, such as the tachykinin substance P, are involved in the cross-transfer effects (Coderre and Melzack, 1985; Aloisi et al., 1993). At present, no information is available clarifying whether unilateral overuse leading to muscle damage is accompanied by morphological alterations and inflammation (myositis) in the homologous contralateral muscles and whether the tachykinin system is involved in the process.
INTRODUCTION

Contralateral strength effects

The first report on cross-transfer effects in limb muscles was published in 1894. The authors of this report described an increase in voluntary force generating capacity of the opposite untrained limb after unilateral resistance training (Scripture et al., 1894). Since then, cross-transfer strength effects have been extensively examined. Recent compilation of published data on contralateral effects of unilateral strength training has estimated that strength gain will improve in the untrained limb by approximately 8% (Munn et al., 2004; Munn et al., 2005; Carroll et al., 2006). The contralateral effects have been observed for both small and large limb muscles, in both dominant and non-dominant limbs, and in response to different types of training (static, dynamic) (Panzer et al., 2011). The contralateral effect is neither gender nor age specific (Zhou, 2000). It has also been demonstrated that exercise induced via EMS resulted in greater contralateral effects than that induced by voluntary contraction training (Hortobágyi et al., 1999; Lee and Carroll, 2007; Bezerra et al., 2009). Hortobágyi and collaborators (1999) demonstrated that six weeks of unilateral exercise induced by EMS led to 104% strength gain in the contralateral limb, but a similar period of voluntary exercise only led to 34% strength gain. It is well established that symptoms of exercise-induced damage will be reduced following a repeated bout of eccentric exercise (Nosaka and Clarkson, 1995; Brown et al., 1997). This protective effect of a prior bout of eccentric exercise can be transferred to the contralateral limb (Howatson and van Someren, 2007; Starbuck and Eston, 2012).

Contralateral effects in the muscle tissue

Several studies have demonstrated that there is no increase in circumference or cross-sectional area in the contralateral untrained limbs after unilateral exercise, indicating that the increased contraction force in the contralateral muscles is not correlated to muscle fiber hypertrophy (Houston et al., 1983; Cabric and Appell, 1987; Ploutz et al., 1994; Bemben and Murphy, 2001). Since currently there is no evidence that morphological changes are coupled to the increased force in the contralateral limbs, most authors suggest that the major mechanism of cross-transfer effects has to reside in the nervous system.

Only a few studies have examined whether there are morphological or biochemical changes in the contralateral muscles after unilateral exercise, allowing that there still is a possibility that some morphological adaptations could occur in the contralateral muscles after unilateral exercise. In a study of biopsies after dynamic strength training, Houston and collaborators (Houston et al., 1983) observed strength gain in both legs and fiber area changes in the exercised leg after 10w of exercise, but no
changes in fiber type composition or in metabolic capacity in either of the legs. A single session of unilateral EMS of the rat soleus muscle upregulated expression of genes related to metabolism and oxidative stress in both the stimulated and the contralateral limbs (Pimenta et al., 2009). It has been demonstrated that vascular changes and strength gains occurred in untrained contralateral forearm after 6w of unilateral grip training. The study showed that maximal strength, endurance and peak blood flow increased not only in the trained limb, but also in the untrained limb (Yasuda and Miyamura, 1983). Unilateral chronic low frequency EMS has also been shown to cause increased interstitial proliferation and capillary swelling in both ipsilateral and contralateral muscles (Hudlicka et al., 2003). Since there still is limited information about morphological or biochemical changes in the contralateral muscles after unilateral exercise, there is a possibility that some adaptations could occur in the contralateral muscles after unilateral exercise.

**Contralateral effects related to the nervous system**

The nervous system exhibits a high degree of symmetry, which necessitates transmedian communication to integrate actions for e.g. the locomotor system (Koltzenburg et al., 1999). Electromyography (EMG) recordings have demonstrated that there is some nerve activity in the contralateral muscles during unilateral exercise (Gregg et al., 1957; Panin et al., 1961; Devine et al., 1981). A symmetrical behavior of the peripheral nervous system has also been shown in a wide range of conditions where unilateral interventions produced bilateral effects. Contralateral changes are thus reported in the motor and autonomic as well as the sensory branches of the peripheral nervous system following unilateral nerve injury (Koltzenburg et al., 1999). Rotshenker and Tal (Rotshenker and Tal, 1985) reported that unilateral section of a motor nerve caused degeneration of neuromuscular junctions in the contralateral muscles and robust signs of sprouting at the junctions of these otherwise intact muscles. Contralateral motor neuron changes have been demonstrated in the facial (Pearson et al., 1988), hypoglossal (Streit et al., 1989) and sciatic (Hughes and Smith, 1989; Piehl et al., 1991; Booth and Brown, 1993) nerves following unilateral nerve injury. Peripheral nerve lesions of sensory neurons caused sprouting of sympathetic nerve fibers on the contralateral side, the alterations being restricted to the homonymous segments of the spinal cord (Chung et al., 1993; McLachlan et al., 1993). Moreover, unilateral axotomy of sensory afferents led to changes in the content of neuropeptides in the corresponding nerves in the opposite side (Kolston et al., 1991). It has also been suggested that neuronal mechanisms of symmetrical upregulation of neuropeptides are involved the symmetrical distribution of pain and inflammation in certain chronic inflammatory diseases (Shenker et al., 2003; Kelly et al., 2007). It is supported by the findings that injections in the skin of one side caused an upregulation of substance P and CGRP in the contralateral homonymous sensory neurons (Amann et al., 1996; Donnerer et al., 1996). Furthermore, experimental studies have demonstrated that injury to one limb produced swelling and hyperalgesia in the contralateral limb (Denko and Petricevic,
Kidd and co-workers (Kidd et al., 1995) found a vascular and cellular response in the contralateral normal knee following experimental induction of a strict unilateral monoarthritis in the opposite side. All these findings indicate that the contralateral effects can be related to a cross-transfer activity of the nervous system.

**Skeletal muscle**

**Muscle structure**

The skeletal muscle is composed of numerous multinucleated densely packed muscle fibers that are surrounded by a thin layer of connective tissue (Figure 1). The main components of the muscle fibers are the myofibrils and the mitochondria, where the myofibril is the actual force generator and the mitochondria is concerned with the energy supply of the fibers. The myofibrils are composed of series of sarcomeres, the functional unit of the myofibril. The sarcomere consists of thick filaments that are mainly composed of myosin, and thin filaments, that are mainly composed of actin, troponin and tropomyosin. Interaction between these filaments is the basic mechanism for muscle contraction.

Sarcomeric myosin, the major contractile component, is an important protein that powers muscle contraction and determines the contractile speed. Each myosin molecule consists of two myosin heavy chains (MyHC) and four light chains that exist in multiple isoforms, i.e proteins with slight variations in their amino acid composition (Schiaffino and Reggiani, 1994). Myosin converts free energy derived from the hydrolysis of ATP to mechanical work and the speed at which ATP can be hydrolyzed determines the speed of contraction. Slow myosin predominates in muscles with slow contracting properties, fast myosin in muscles with fast contracting properties. The MyHC composition in a muscle is regarded as the best marker of the functional heterogeneity among muscle fibers (Larsson and Moss, 1993).
**Muscle fiber types**

Skeletal muscle fibers can be divided into different fiber types by enzyme and immunohistochemical (IHC) techniques. Enzyme histochemistry has been generally used to classify muscle fibers on the basis of myofibrillar ATPase (mATPase) reactions at alkaline and acid pH. The fibers are classified into type I fibers and type II fibers with subtypes IIA, IIB and IIC (Brooke and Kaiser, 1970). Type I fibers have a weak mATPase staining activity at alkaline pH and a strong staining at acid pH. This fiber type has high mitochondrial oxidative capacity and low glycolytic activity; it is resistant to fatigue and belongs to slow twitch motor units that have relatively long contraction time. Type II fibers have strong mATPase staining at alkaline pH and weak to strong staining at acid pH. Type IIA fibers have intermediate mitochondrial oxidative and glycolytic activity and belong to fast twitch motor units that contract fast and are relatively resistant to fatigue. Type IIB fibers have high glycolytic activity and low oxidative capacity, are fatigable and belong to fast twitch motor units. Type IIC fibers have characteristics in between type I and type II fibers (Dubowitz, 2007).

The generation of monoclonal antibodies (mAbs) led to advances in characterization of fiber types in relation to different MyHC isoforms. By the use of the IHC technique the MyHC composition of individual fibers could be distinguished. According to the principles for the major MyHC isoforms in adult mammalian muscles, four MyHC isoforms have been identified, which are correlated to the myosin ATPase-based classification system. The slow twitch MyHC isoform I/β-cardiac is present in type I fibers. The fast-twitch myosin, MyHCIIa, is present in type IIA fibers, and MyHCIIx and/or MyHCIIb is present in type IIB fibers (Pette and Staron, 1990; Schiaffino and Reggiani, 1994; Lucas et al., 2000). The slow MyHC and fast MyHCIIa and MyHCIIx isoforms have been found in all mammalian species studied whereas there is a large inter-species variability in the expression of MyHCIIb. The use of MyHC mAbs makes it possible to distinguish fibers expressing a single MyHC isoform or as hybrid fibers containing two or more mixed isoforms. Type IIC is a hybrid fiber that contains a mixture of slow MyHC and fast MyHCII isoforms. Based on the predominant MyHC isoforms in the fiber, the fibers can be classified into the following MyHC fiber types; MyHC, MyHC+II, MyHCIIa, MyHCIIa+IIx, MyHCIIx, MyHCIIb and MyHCIIx+IIb. Collectively the pure and hybrid fibers form a continuum from slow to fast.

**Muscle capillarization**

Capillaries are the smallest blood vessels. They are parts of the microcirculation which connect arterioles and venules, and which enable the exchange of water, oxygen, carbon dioxide and many other nutrients as well as waste chemical substances between the blood and the surrounding tissues. A network of parallel and cross-anastomosing capillaries surrounds all muscle fibers. The dimension of this network is the major determinant for oxygen delivery to the muscle cell and is therefore important for muscle performance. However, the blood flow and oxygen
delivery to a muscle depends also on an adequate vascular function and intact auto-regulation. The extent of the capillary network in a muscle is normally well adapted to match its composition of different fiber phenotypes and the size of the fibers (Cebasek, 2005). Thus, large muscle fibers are surrounded by more capillaries than small fibers and slow contracting fibers containing MyHCI have generally higher oxidative mitochondrial capacity and are supplied by more capillaries than fast contracting fibers containing MyHCII, especially fast fibers containing MyHCIIx/IIb (Ranvier, 1874; Hudlicka, 1991).

The triceps surae muscle in rabbits

The rabbit triceps surae muscle is composed of two major muscles. The gastrocnemius muscle lies superficially with its two heads (lateral and medial) attaching to the base of the femur directly above the knee. It attaches to the calcaneal bone, whereby a calcaneal tendon complex (Achilles tendon) is formed. The gastrocnemius muscle is responsible for flexing the knee and plantarflexing the foot. The deep mass of the triceps surae muscle consists of the soleus muscle with its head attaching the superior posterior area of the tibia. This muscle exerts only a plantar flexor movement for the ankle. The soleus muscle attaches to the fibers of the calcaneal tendon complex. In addition to these two main muscles, a thin muscle, flexor digitorum superficialis, is located in between the soleus and gastrocnemius muscles. The muscle wraps around the calcaneus and continues underneath the foot.

![Figure 2](image-url) Lateral view of the triceps surae muscle in rabbit. It consists of the two heads of the gastrocnemius muscle and the soleus muscle. A thin muscle, flexor digitorum superficialis, is located in between the soleus and gastrocnemius muscles. The muscle wraps around the calcaneus and continues underneath the foot.

The fiber type proportion in the triceps surae muscle has been studied in a wide range of species such as man (Gollnick et al., 1974), cat (Burke et al., 1971) and dog (Armstrong et al., 1982). These studies show that most mammals in general have
between 70-100% slow fibers in the soleus muscle and between 49-96% fast fibers in the gastrocnemius muscle. The rabbit soleus muscle has been reported to contain 96% slow fibers (Peter et al., 1972), whereas the fiber composition in the rabbit gastrocnemius muscle has not been reported.

**Nerve supply of muscle**

The nerves supplying a muscle are composed of motor, sensory and non-myelinated efferent autonomic nerves. Large myelinated alpha motor efferents supply the extrafusal fibers in the muscle. Smaller myelinated gamma efferents supply intrafusal fibers of muscle spindles, sensory proprioceptors in the muscle that provide information on the body position and that refine and control muscle contraction. The fine non-myelinated autonomic efferents and the myelinated and non-myelinated sensory afferents are associated with vasoconstriction or vasodilatation of blood vessels and the latter lead to pain signaling and vascular effects leading to neurogenic inflammation in pathological situations (Furness et al., 1984).

**The motor unit**

A motor unit is made up of a motor neuron and the muscle fibers innervated by its axon. When a motor-unit is activated, all fibers in the motor unit contract and produce force. Groups of motor units work together to coordinate the contractions of a single muscle. All muscle fibers in a motor unit will be of the same fiber type. Muscles that require precision and fine movement control usually have many motor units with a small number of fibers in each unit. There is an orderly recruitment of motor units. Small, slowly contracting, fatigue resistant motor units, are first recruited and produce small forces. With increasing force demands, large, fast-contracting fatigable motor units join in. Slower motor units are thereby more frequently used than faster ones.

**The neuromuscular junction**

The neuromuscular junction connects the motor neuron to the muscle fiber via synapses. The electrical impulse is transferred by release of acetylcholine (ACh), a neurotransmitter molecule, which diffuses from the synapse of the motor nerve to the receptor on the plasma membrane of the muscle fiber. The binding of ACh to the receptor results in a depolarization of the membrane causing a cascade of events that eventually results in muscle contraction. Studies have shown that changes in the activity of the motor unit affect the pre-synaptic and post-synaptic relationship of the neuromuscular junction. Exercise training affects pre-synaptic nerve terminal branching by increasing its length and complexity, but elicits no modifications in post-synaptic structure. On the other hand, muscle unloading exclusively impacts the post-synaptic endplate by diminishing its size without affecting pre-synaptic nerve terminal branching (Deschenes et al., 2006).
Muscle plasticity

The skeletal muscles have an adaptive potential to modify their composition of muscle fiber phenotypes and the fiber size in response to altered patterns of activity (Pette and Staron, 1997; Scott et al., 2001). This adaptive responsiveness has been termed “muscle plasticity” and reflects the ability of a muscle cell to alter either the quantity of protein and/or the type of protein isoform in response to changed use or inactivity (Baldwin and Haddad, 2002). A given protein can be replaced by another protein, which is better suited for a specific physiological or pathological state. For example, aerobic exercises trigger the expression of slow MyHC, whereas weight-lifting training is known to induce fiber type transition from fast to less fast fiber types (Abernethy et al., 1994; Schiaffino and Reggiani, 2011). Fiber type transformation from slow to fast fibers are normally not seen after exercise. On the other hand, a decreased use of skeletal muscle in response to microgravity exposure, long term complete inactivity, spinal cord injury or detraining (decreased muscle use from a previously high activity level) lead to a conversion of muscle fiber types in a slow to fast direction (Pette and Staron, 1997; Schiaffino and Reggiani, 2011). It is well established that the neural impulse pattern to a muscle is the major cause for changes in muscle fiber phenotype composition (Hoh, 1975; Pette and Vrbova, 1985). By using tonic low frequency stimuli, a fast to slow twitch transition has experimentally been induced in muscles, whereas phasic high frequency EMS has been shown to cause a slow to fast twitch transition (Hamalainen and Pette, 1996).

Physical training usually results in fiber hypertrophy, i.e increased myofibrillar protein synthesis and increased oxidative capacity of the fibers. Strength training can result in activation of myogenic stem cells, i.e. precursor cells and satellite cells. Satellite cells fuse with existing myofibrils and contribute to increased numbers of myonuclei and hypertrophy of muscle fibers (Eriksson et al., 2006). Physical training can also lead to extension of the capillary network by angiogenesis (Fitts and Widrick, 1996). Endurance training (Myatt et al., 2011) and long-term EMS (Brown et al., 1976) are effective processes to increase the vascular network, thereby improving the exchange properties between blood and muscle tissue. In contrast, inactivity and denervation of muscles often lead to a degeneration of the capillary network (Lu et al., 1997; Borisov et al., 2000; Dedkov et al., 2002).

Skeletal muscle injury

Muscle damage has been observed after various types of muscle overuse, especially marked eccentric contractions. EMS-evoked exercise has been reported to result in a significantly higher degree of muscle injury compared with voluntary exercise (Crameri et al., 2007; Black and McCully, 2008; Nosaka et al., 2011). The injury is generally characterized by changes in fiber morphology and an increased amount of connective tissue, as well as muscle fiber degeneration, necrosis and inflammation (Hikida et al., 1983; O’Reilly et al., 1987; Friden et al., 1989; Biral et al., 2000; Crameri et al., 2007; Jubeau et al., 2008; Hansen et al., 2009; Nosaka et al., 2011).
Despite the type of background to muscle damage, the consequent injury and repair processes are in principle similar. After injury, the muscle typically undergoes stages of degeneration, inflammation, regeneration and fibrosis (Tidball, 1995), while the extent of each stage depends on the severity of the damage and the outcome of the repair. The repair process can activate regenerative mechanisms related to myogenic factors and satellite cells, which fuse with injured myofibers (Hurme and Kalimo, 1992; Malerba et al., 2009). In cases of injury of the basal lamina, a connective tissue scar is formed from fibrin and fibronectin. The fibrinous scar tissue strengthens the muscle during contractions as the muscle heals. However, if there is continued injury to the muscle and excessive proliferation of fibroblasts, a dense fibrous tissue may form which interferes with the repair process and contributes to incomplete functional recovery (Stauber, 2004).

**Muscle inflammation (myositis)**

Myositis is a general term for muscle inflammation that can be caused by injury, infection or autoimmune disease. Very vigorous exercise of an untrained muscle can result in muscle damage that is followed by inflammatory cell infiltration. The inflammatory process is a part of the complex biological response to harmful stimuli. The initial inflammatory cells to the site of injury are polymorphonuclear leukocytes, which are eventually replaced by monocytes within hours after the injury. Over time these cells transform into macrophages, which phagocytose and remove the necrotic tissue (Chazaud et al., 2009). Macrophages, along with fibroblasts and the extracellular matrix, also produce growth factors, cytokines, and chemokines (McCroskery et al., 2005; Toumi et al., 2006) that can be involved in the following regenerative process including satellite cell activation.

There is also evidence that the nervous system can be involved in inflammatory processes. Inflammation that arises from local release from afferent neurons of inflammatory mediators such as the neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) is classified as neurogenic inflammation (Holzer, 1992). Neurogenic inflammation is a trigger or a mechanism by which several pathological conditions or other conditions can spread in the body.

**The tachykinin system**

**Tachykinins**

The tachykinin peptide family is one of the largest families of neuropeptides. It is mainly constituted by substance P (SP), neurokinin A and neurokinin B, but also the more recently described endokinins and hemokinins (Hokfelt et al., 2001; Page, 2004; Ogawa et al., 2012; Satake et al., 2012). Among these peptides, SP is the most well known and most studied. However, also other tachykinin peptides have been verified. These includes a new tachykinin gene-related peptide named EK-2 (Page,
The genes that produce tachykinins encode precursor proteins that are named preprotachykinins. Tachykinins share the C-terminal sequence Phe-X-Gly-Leu-Met-NH2 but have distinct N-terminal sequences (Frisch et al., 2010). As with other neuropeptides they are initially produced as pro-peptides which are then spliced in order to become active neuropeptides. An axonal transport occurs, the peptides being stored in large dense-core vesicles (LDVs) (Hokfelt et al., 2001). There are three known mammalian tachykinin receptors termed neurokinin-1 receptor (NK-1R), neurokinin-2 receptor (NK-2R) and neurokinin-3 receptor (NK-3R) (Harrison and Geppetti, 2001). They mainly bind substance P, neurokinin A, and neurokinin B, respectively.

**Substance P (SP)**

SP is an 11 amino acid long peptide encoded by the preprotachykinin-A gene (TAC1). It has a molecular weight of 1,348 g/mol (Harrison and Geppetti, 2001). The preprotachykinin-A gene (TAC1) is known to become spliced into four different forms of mRNA known as α, β, γ and δ forms. The α and δ forms code for the synthesis of SP whilst the other forms encode for SP and other tachykinins in addition (Bhatia et al., 2003). SP was the first tachykinin to be discovered (Von Euler and Gaddum, 1931). It was identified as a tissue extract that caused intestinal contraction in vitro. Subsequently, SP has been widely studied as a neurotransmitter of somatic sensory primary afferent nerve fibers (Cuello et al., 1978; Skrabanek and Powell, 1980).

The origin of SP is the sensory neuronal cell bodies (McCarthy and Lawson, 1989). It is transported in LDVs to the axonal terminal endings (Wharton et al., 1988). It is mostly present in small-diameter (A-delta and C-fiber) neurons (McCarthy and Lawson, 1989), in which terminal parts the final enzymatic processing is performed (Brimijoin et al., 1980). It is subsequently released from the peripheral terminals. That occurs for the skin, muscle, joints, intestine and airways and various other structures. SP is, however, not only released peripherally but also centrally (Olgart et al., 1977; White and Helme, 1985; Klein et al., 1992). The dorsal root ganglion neurons containing SP thus project to the substantia gelatinosa of the spinal cord.

Released SP does not only exert its effects locally but can diffuse in the extracellular space for distances larger than the synaptic cleft and reach NK-1Rs at some distances from the synaptic site (Li et al., 2000). SP is not only produced in neurons but also in non-neuronal cells, an aspect which will be considered below. A number of enzymes such as neutral endopeptidase (NEP) and angiotensin converting enzyme (ACE) are involved in the metabolism/cleavage of SP within the periphery (Harrison and Geppetti, 2001).

**SP in non-neuronal cells**

SP expression has been observed in non-neuronal cells, such as epithelial cells, endothelial cells, macrophages and cells in endocrine tumours (Watanabe et al., 2004).
2002; Castro et al., 2005). That also includes the cells in the urothelium in the bladder (Birder et al., 2010) and human fibroblasts (Bae et al., 2002). SP mRNA expression has been observed in the tendon cells of human tendons (Andersson et al., 2008). There may be an induction of tachykinin production in cells in airway epithelia in response to viral infection (Stewart et al., 2008).

SP expression in the endothelium of blood vessels has not only been shown by the use of immunohistochemical techniques but also via immune electron microscopy (Loesch and Burnstock, 1988; Milner et al., 1989). A presence of SP has been found for the endothelium of airway blood vessels in rabbits (Larsen et al., 2006) and in the smooth muscle of vessels walls of the human placenta (Munoz et al., 2010b).

Of particular interest is the fact that white blood cells including macrophages (Bost et al., 1992), eosinophils (Aliakbari et al., 1987) and lymphocytes (Lai et al., 1998) have been shown to produce SP. It is suggested that tachykinin produced in non-neuronal cells, such as white blood cells, is of particular importance in pathological conditions (Erin and Ulusoy, 2009).

The diverse functions of SP

Involvement in pain perception

SP is an important element in pain perception. SP thus modulates pain sensitivity by activating the NK-1 receptor. SP is released into the dorsal horn of the spinal cord following intense peripheral stimulation promoting central hyperexcitability and increased pain sensitivity (Laird et al., 1993; De Felipe et al., 1998). It coexists with the excitatory neurotransmitter glutamate in primary pain afferents (De Felipe et al., 1998).

Effects on vessels

In addition to the occurrence of orthodromic inputs to the spinal cord and brain from the periphery, action potentials in afferent neurons can be transmitted antidromically back down to the periphery. The released SP from the terminals of the nerve endings of these neurons acts on vascular endothelial and smooth muscle cells. SP is hereby producing vasodilation and increased capillary permeability, leading to plasma extravasation and edema (Lembeck and Holzer, 1979; Figini et al., 1997). SP can furthermore induce angiogenesis, and directly induce new blood vessels from pre-existing vessels by affecting endothelial cells (Ziche et al., 1990; Wiedermann et al., 1996; Ziche et al., 1997).

Proinflammatory effects

Tachykinins such as SP are on the whole considered to have proinflammatory effects (Bhatia et al., 1998; O’Connor et al., 2004; Wu et al., 2007). SP is thus contributing to focal inflammatory infiltrations (Sauer et al., 2001; Hong et al., 2009). The release of SP can lead to a recruiting of granulocytes through the induction of cell migration and adhesion, and direct transmigration across the vasculature into the site of
affected tissue (Kohara et al., 2010). In parallel with angiogenesis, SP can induce enhanced recruitment of granulocytes, macrophages, B lymphocytes, and circulating endothelial progenitor cells that have been confirmed to have angiogenic potentials (Angeli et al., 2006; Dirkx et al., 2006; Tazziyman et al., 2009; Shrestha et al., 2010). The density of SP immunoreactive nerve endings in a peripheral tissue is known to increase significantly under the influence of persistent inflammation (Reinert et al., 1998). Tachykinins such as SP have been considered to be of great importance in various inflammatory diseases. That includes inflammatory bowel diseases (Renzi et al., 2000), rheumatoid arthritis and osteoarthritis (Inoue et al., 2001).

**In relation to healing and other events**

SP is known to be involved in wound healing (Nakamura et al., 2003; Nishida, 2005), including healing of the skin (Scott et al., 2008b). Recently, SP has been reported to be a messenger of injury and a mobilizer of CD29+ stromal-like cells in order to participate in wound healing after injury of the rabbit eye (Hong et al., 2009). In that study, the local release of SP was found to achieve the recruitment of circulating cells from the blood (Hong et al., 2009). SP is also considered to have protective roles. NK-1R mediated functions can thus have protective roles in acute hyperoxic lung injury (Dib et al., 2009). On the other hand, SP can also be involved in destructive events (Sio et al., 2008).

In addition, SP is also involved in bone marrow fibrosis (Rameshwar et al., 2001) and proliferation (Al-Sarraj and Thiel, 2002), including for tumor cells. SP has been found to be involved in remodelling and growth-related events for tendons (Burssens et al., 2005b; Andersson et al., 2011; Backman et al., 2011; Carlsson et al., 2011). SP is on the whole known to have autocrine/paracrine effects (Munoz et al., 2010b). It is thus previously shown that SP acting via NK-1R has autocrine/paracrine effects for blood vessel walls (Milner et al., 1989), as well for other cell types such as tumor cells (Munoz et al., 2010a). SP is also associated with mood disorders and anxiety (Ebner and Singewald, 2006) and nausea/emesis (Hesketh, 2001).

**The NK-1 receptor**

The preferred endogeneous receptor for SP is the neurokinin 1 receptor (NK-1R), which is a G protein coupled receptor found in various structures such as neurons, brainstem, vascular endothelial cells, muscle, the gastrointestinal tract, the genitourinary tract, pulmonary tissue, thyroid gland and different types of immune cells (Takeda et al., 1991; Saria, 1999; Almeida et al., 2004; Datar et al., 2004; Satake and Kawada, 2006). However, SP can also bind to the NK-2R and NK-3R. In studies in our research group, expression of NK-1R has been shown for blood vessel walls and tendon cells (Forsgren et al., 2005; Andersson et al., 2008; Backman et al., 2011) and in the epithelium of the human colon (Jonsson et al., 2005).

The NK-1Rs can be divided into at least two isoforms (Kage et al., 1993; Shimizu et al., 2001). They can also be classified into classic and septime-sensitive forms, the
latter being a truncated form of the receptor missing the intracellular C-terminus (Torrens et al., 2000). The binding affinity to the truncated form is thus 10 times lower (Douglas and Leeman, 2011). The binding of SP to the NK-1R leads to various effects that not least relates to transmission of stress signals and pain, but also contraction of smooth muscles, vessel reactions and inflammatory modifications (Ohkubo and Nakanishi, 1991; Quartara and Altamura, 2006). The NK-1R plays an important role in the modulation of the accumulation of white blood cells that occurs in inflammatory processes (Cao et al., 2000) (c.f. above). The NK-1R is considered to be an important proinflammatory mediator in many inflammatory conditions including asthma, immune-complex-mediated lung injury, experimental arthritis, and inflammatory bowel disease (Bhatia et al., 2000; Bhatia et al., 2001; Bhatia, 2002; Lau et al., 2005) as well as myositis (Hoheisel et al., 1998; Reinert et al., 1998) and to play a key role in damaging processes (Lau et al., 2005). However, NK-1R mediated functions have protective roles in situations like acute hyperoxic lung injury (Dib et al., 2009). Overexpression of the NK-1R has actually been reported in situations with tissue repair and healing events e.g. during gastric wound healing in rodents (Schmassmann et al., 2004).

The tachykinin system in skeletal muscle

There is comparatively little information on the tachykinin system for muscle tissue. What is known is that SP-containing nerve fibers are related to blood vessels in the tenuissimus muscle of rabbits (Ohlen et al., 1988) and that there is sensory innervation involving SP in the origin of the extensor carpi radialis muscle of man (Ljung et al., 1999). The concentration of SP and various other signal substances obtained via microdialysis was found to be higher in lysates from myofascial trigger points of the trapezius muscle than in lysates from normal trapezius muscle (Shah et al., 2008). Functionally, tachykinins are shown to be involved in local reflex modulation of striated muscle contractions in the rat esophagus via effects on NK-1 receptors (Shiina et al., 2006). It was experimentally shown that craniofacial muscle inflammation leads to an increase in the number of SP- and CGRP-immunopositive ganglion neurons that innervate the inflamed muscle (Ambalavanar et al., 2006). It was also shown that injection of Freund's adjuvant into the gastrocnemius muscle of rats is followed by an increase in SP-innervation (Reinert et al., 1998) and that there is relationship between neuronal activity and the pattern of SP-immunoreactivity in the rat spinal cord during muscle inflammation (Hoheisel et al., 1998).

Why study the tachykinin system?

It is apparent that there is limited information concerning the tachykinin system for skeletal muscle. There are thus no studies available that have evaluated the possibility that there is a local production of tachykinins in infiltrating white blood cells in muscle inflammation, nor if these cells exhibit the NK-1R. It is also unknown if nerve proliferation (nerve sprouting) occurs for markedly affected and inflamed areas of muscle tissue and if tachykinins to some extent are related to the processes of muscle
fiber necrosis and regeneration. Furthermore, there have been no studies which describe the possibility that the tachykinin system not only is involved in situations with marked muscle injury and inflammation of the exercised muscle, but also in the homologous non-exercised muscles.
AIMS OF THE STUDY

To test the hypothesis that pronounced unilateral muscle overuse causes structural tissue alterations and changes in the tachykinin system not only in the exercised muscle, but also in the contralateral muscles, the aim of his thesis was to study:

• Morphological alterations in the muscle tissue
• Muscle fiber composition and capillary supply of the fibers
• Morphological alterations in the nerves within the muscle tissue
• Levels and distribution patterns of tachykinin
• Distribution patterns of the NK-1 receptor
• Effects of increased tachykinin levels via local injections of inhibitors of enzymes that degrade substance P

For all parameters above, comparison was made between the experimental and non-experimental sides of both the soleus and gastrocnemius muscles.
MATERIALS AND METHODS

Animals

A total of 40 female New Zealand white rabbits with a weight of approximately 4 kg, aged from 6-9 months, were used in this project. The animals were divided into seven groups (see Table 1). Eighteen animals were first subjected to an exercise protocol leading to marked muscle overuse on their right leg every second day, for a total period of 1, 3 and 6 weeks, whilst six of the animals constituted a control group and that was not subjected to any exercise.

Additionally, in order to achieve muscle inflammation and to examine for the special importance of the tachykinin system, the muscle overuse was combined with injection treatment for 16 animals divided into three groups (see Table 1). The injections were given directly after each of the exercise regimens. The substances injected were Captopril (Cap) (C4042, Sigma), DL-Thiorphan (DL) (T6031, Sigma), substance P (SP) (S6883, Sigma) and NaCl. Captopril is an angiotensin-converting enzyme inhibitor (ACE inhibitor) and DL-Thiorphan is a neutral endopeptidase inhibitor (NEP inhibitor). The substances were delivered in the loose connective tissue around the Achilles tendon, i.e. in the paratenon region, of the experimental side, in combination with 1w of exercise.

All animals were anaesthetized throughout the experiment by intramuscular injections of fentanylfluanison (0.2–0.3 ml/kg) and diazepam (0.2 ml/kg; 5 mg/ml). In order to maintain anaesthesia, fentanylfluanison (0.1 ml/kg) was further injected every 30–45 min during the experimental procedure. To minimize pain after the experiment, buprenorphine (0.01–0.05 mg/kg), was given subcutaneously.

Table 1. Summary of groups of animals analyzed.

<table>
<thead>
<tr>
<th>Group</th>
<th>Exercise length</th>
<th>Injection</th>
<th>Number of animals</th>
<th>Papers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No</td>
<td>No</td>
<td>6</td>
<td>I II III IV</td>
</tr>
<tr>
<td>2</td>
<td>1 week</td>
<td>No</td>
<td>6</td>
<td>I II III IV</td>
</tr>
<tr>
<td>3</td>
<td>1 week</td>
<td>NaCl</td>
<td>5</td>
<td>V</td>
</tr>
<tr>
<td>4</td>
<td>1 week</td>
<td>SP+Cap+DL</td>
<td>5</td>
<td>V</td>
</tr>
<tr>
<td>5</td>
<td>1 week</td>
<td>Cap+DL</td>
<td>6</td>
<td>V</td>
</tr>
<tr>
<td>6</td>
<td>3 weeks</td>
<td>No</td>
<td>6</td>
<td>I II III IV</td>
</tr>
<tr>
<td>7</td>
<td>6 weeks</td>
<td>No</td>
<td>6</td>
<td>I II III IV</td>
</tr>
</tbody>
</table>

Experimental design

An experimental model involving a kicking machine, which was originally designed by Backman and collaborators (Backman et al., 1990), was used with some
modifications (Andersson et al., 2011) (Figure 3). Passive flexions/extensions of the ankle joint were produced by a pneumatic piston attached to the right leg, whilst the left leg was not attached to the kicking machine. The movement was set to 9.5 cm, giving a range of motion in the ankle of 55-65°, of which 20-25° was dorsiflexion and 35-40° was plantarflexion. A band was tied around the pelvis of the rabbit to restrict moments. Muscle contraction was furthermore induced during the plantar flexion phase by electrical stimulation via surface electrodes (pediatric electrode 40426A, Hewlett Packard, Andover, MA, USA) placed approximately 2 cm apart over the surface of the right triceps surae muscle. A microswitch synchronized the stimulation unit (Disa stimulator Type 14E 10; Disa Elektronik A/S, Herlev, Denmark). A single impulse with a duration of 0.2 ms was delivered 85 ms after the initiation of the plantar flexion at an amplitude of 35-50 V. The electrical stimulus was controlled by an oscillograph. The movement frequency was 150 movements per minute (2.5 Hz). The experiment was performed for 2 h every second day and the total length of the experiment period was 1 w (with or without injections), 3 and 6 w respectively. Before and after the exercise, the animals were kept in ordinary cages allowing good freedom of movement and they had free access to food and water.

Collection of muscle samples, fixation and sectioning

The animals were sacrificed using an overdose of pentobarbital (the day after the last exercise). The triceps surae muscle was dissected out from both experimental and contralateral sides. The tissue samples were immediately taken to the laboratory, and samples corresponding to the distal parts of the soleus and gastrocnemius muscles were further processed for microscopic analyses (morphology, enzyme and immunohistochemistry and in situ hybridization) and for EIA. Muscle specimens of an approximate size of 5 x 10 mm were processed in three different ways. One type of the specimens from each muscle was directly mounted in an OCT compound (Tissue Tek®, Miles Laboratories, Naperville, IL, USA) on a thin cardboard and rapidly frozen in liquid propane (around 30 sec) chilled with liquid nitrogen. A second type of specimens was immediately fixed in 4% formaldehyde in 0.1 M phosphate buffer, pH 7.0, for 24 hours at 4°C. After overnight washing at 4°C in Tyrode’s solution containing 10% sucrose, these chemically fixed samples were mounted and frozen as described above. Both the chemically fixed and the unfixed specimens were stored at -80°C until sectioning for microscopic analyses. A third type of specimen was also
prepared. These samples weighed approximately 30 mg and were after being weighed directly put in liquid nitrogen for later EIA analyses. Cross-sections of specimens used for enzyme and immunohistochemical staining, 5-8 µm thick, were cut in a cryostat microtome (Leica Microsystems CM 3000, Heidelberg, Germany) at −20°C and mounted on glass slides (pre-coated with crome-alun gelatine, left to dry, and stored at 4°C). The use of chemically fixed or unfixed specimens for immunohistochemical antibody staining is given in Table 2. Postfixation of sections of unfixed tissue samples (2% paraformaldehyde for 10 min) was performed when comparison between unfixed and fixed samples was needed. Sections of all specimens (chemically fixed as well as unfixed) were processed for morphology including detection of degenerative and regenerative processes and inflammation, using staining with haematoxylin & eosin (H&E). In addition, series of 10 µm thick sections were cut from fixed specimens (but under certain procedures, see further below) and mounted on slides (Menzel-Gläser, Braunschweig, Germany) for in situ hybridization to detect tachykinin (SP) and NK-1R mRNA reactions.

**Staining for morphology**

Muscle cross-sections were stained with haematoxylin & eosin (H&E) for demonstration of basic morphology according to the following procedure:

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Air dried sections put onto slides</td>
</tr>
<tr>
<td>2</td>
<td>Sections placed in Harris' haematoxylin for 2.5 min</td>
</tr>
<tr>
<td>3</td>
<td>Rinsing in distilled water</td>
</tr>
<tr>
<td>4</td>
<td>Dipping in 0.1% acetic acid for 15 seconds</td>
</tr>
<tr>
<td>5</td>
<td>Rinsing in tap water (around 37°C) for 4 min</td>
</tr>
<tr>
<td>6</td>
<td>Staining with 1% eosin for 1 min</td>
</tr>
<tr>
<td>7</td>
<td>Dehydration in ethanol 3 x 2 min</td>
</tr>
<tr>
<td>8</td>
<td>Clearing, and mounting</td>
</tr>
</tbody>
</table>

**Immunohistochemistry**

Immunohistochemical stainings were performed using previously established techniques at the laboratory. In order to enhance specific immunofluorescence reactions for certain antibodies (Hansson and Forsgren, 1995), sections of fixed tissue were pretreated with acid potassium permanganate for 2 min as an initial step. The sections of fixed and unfixed frozen specimens were dried for 10 minutes, and initially rinsed in 0.01 M phosphate buffered saline (PBS), pH 7.2, containing 0.1% sodium azide 3x5 min. They were then incubated for 20 min in a 1% solution of Triton X-100 (Kebo lab, Stockholm) in PBS as preservative, and rinsed in PBS 3x5 min. After this procedure, the sections were incubated in 5% rabbit normal serum (Dako, Copenhagen, Denmark) or donkey normal serum (Jackson ImmunoResearch, West Grove, PA, USA) in PBS for 15 min. The sections were thereafter incubated with previously characterized primary antibodies (Table 2) for 60 min at 37°C.
Table 2. Primary antibodies used for immunohistochemistry.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Code</th>
<th>Source</th>
<th>Raised in</th>
<th>Tissue</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tachykinin (SP)</td>
<td>Sc-14104</td>
<td>Santa Cruz Biotechnology, CA, USA</td>
<td>Goat</td>
<td>Fixed</td>
<td>III, V</td>
</tr>
<tr>
<td>SP</td>
<td>8450-0505</td>
<td>Biogenesis, Poole, UK</td>
<td>Rat</td>
<td>Fixed</td>
<td>II, III, V</td>
</tr>
<tr>
<td>NK-1R</td>
<td>Sc5220</td>
<td>Santa Cruz Biotechnology, CA, USA</td>
<td>Goat</td>
<td>Fixed</td>
<td>II, V</td>
</tr>
<tr>
<td>CD68</td>
<td>Mo814</td>
<td>DAKOCytomation, Glostrup, Denmark</td>
<td>Mouse</td>
<td>Fixed</td>
<td>I, II, III</td>
</tr>
<tr>
<td>T cell/neutrophil marker</td>
<td>MCA805G</td>
<td>AbD Serotec, Oxford, UK</td>
<td>Mouse</td>
<td>Fixed</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Eosinophil peroxidase</td>
<td>MAB1087</td>
<td>Chemicon, Temecula, CA, USA</td>
<td>Mouse</td>
<td>Fixed</td>
<td>I, II, III</td>
</tr>
<tr>
<td>BetaIII-tubulin</td>
<td>T8660</td>
<td>Sigma-Aldrich, New York, NY, USA</td>
<td>Mouse</td>
<td>Fixed</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>S-100beta</td>
<td>S2532</td>
<td>Sigma-Aldrich, New York, NY, USA</td>
<td>Mouse</td>
<td>Fixed</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>Desmin</td>
<td>M0760</td>
<td>DAKOCytomation, Glostrup, Denmark</td>
<td>Mouse</td>
<td>Fixed, unfixed</td>
<td>I, II, IV</td>
</tr>
<tr>
<td>CD31</td>
<td>M0823</td>
<td>DAKOCytomation, Glostrup, Denmark</td>
<td>Mouse</td>
<td>Fixed, unfixed</td>
<td>III, IV, V</td>
</tr>
<tr>
<td>AChE</td>
<td>MAB303</td>
<td>Chemicon, Temecula, CA, USA</td>
<td>Mouse</td>
<td>Unfixed</td>
<td>I</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>1891</td>
<td>Chemicon, Temecula, CA, USA</td>
<td>Mouse</td>
<td>Unfixed</td>
<td>I, IV</td>
</tr>
<tr>
<td>MyHC-embryonic</td>
<td>F1.652</td>
<td>Developmental studies, Hybridoma Bank, IA, USA</td>
<td>Mouse</td>
<td>Unfixed</td>
<td>I, IV</td>
</tr>
<tr>
<td>MyHC-neonatal/fetal</td>
<td>NCL-MHCn</td>
<td>Novocastra Laboratories Ltd, Newcastle, UK</td>
<td>Mouse</td>
<td>Unfixed</td>
<td>I</td>
</tr>
<tr>
<td>Laminin</td>
<td>PC128</td>
<td>Binding Site Group, Birmingham, UK</td>
<td>Sheep</td>
<td>Unfixed</td>
<td>IV</td>
</tr>
<tr>
<td>Laminin a2</td>
<td>NCL-Merosin</td>
<td>Novocastra Laboratories Ltd, Newcastle, UK</td>
<td>Mouse</td>
<td>Unfixed</td>
<td>I</td>
</tr>
<tr>
<td>MyHCl</td>
<td>A4.951</td>
<td>Developmental studies, Hybridoma Bank, IA, USA</td>
<td>Mouse</td>
<td>Unfixed</td>
<td>IV</td>
</tr>
<tr>
<td>MyHClIa</td>
<td>A4.74</td>
<td>Developmental studies, Hybridoma Bank, IA, USA</td>
<td>Mouse</td>
<td>Unfixed</td>
<td>IV</td>
</tr>
<tr>
<td>MyHCl, MyHClIa</td>
<td>N2.261</td>
<td>Developmental studies, Hybridoma Bank, IA, USA</td>
<td>Mouse</td>
<td>Unfixed</td>
<td>IV</td>
</tr>
</tbody>
</table>

Immunofluorescence staining (IFS)

After incubation with specific antiserum, and after washes in PBS or PBS with BSA 3x5 min, another incubation in normal serum followed for 3x5 min, after which the sections were incubated with secondary antiserum (Table 3) for 30 min at 37°C. The sections were thereafter washed in PBS or PBS with BSA 3x5 min and then mounted by mounting medium.

Bovine serum albumin (BSA) was extensively used for blocking non-specific binding of antibodies and for stabilizing antibodies and other proteins during freeze drying and in diluted solutions. However, BSA contains bovine IgG which interferes with anti-goat IgG (as well as anti-sheep IgG) secondary antibodies. Therefore, BSA was not used for the procedures for anti-goat or anti-sheep staining.
Table 3. Secondary antibodies used for immunofluorescence staining.

<table>
<thead>
<tr>
<th>Secondary ab</th>
<th>Code</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRITC conjugated Rabbit Anti-Mouse</td>
<td>R0276</td>
<td>DakoCytomation, Glostrup, Denmark</td>
</tr>
<tr>
<td>Alexa 568 Donkey Anti-Goat</td>
<td>A11057</td>
<td>Invitrogen, Carlsbad, CA, USA</td>
</tr>
<tr>
<td>FITC-conjugated AffiniPure Donkey Anti-Goat</td>
<td>705-095-147</td>
<td>Jackson ImmunoResearch, West Grove, PA, USA</td>
</tr>
<tr>
<td>TRITC-conjugated AffiniPure Donkey Anti-Rat</td>
<td>712-025-150</td>
<td>Jackson ImmunoResearch, West Grove, PA, USA</td>
</tr>
<tr>
<td>Rhodamine Red-X AffiniPure Donkey Anti-Sheep</td>
<td>713-295-003</td>
<td>Jackson ImmunoResearch, West Grove, PA, USA</td>
</tr>
<tr>
<td>TRITC-conjugated AffiniPure Donkey Anti-Mouse</td>
<td>715-295-150</td>
<td>Jackson ImmunoResearch, West Grove, PA, USA</td>
</tr>
<tr>
<td>Alexa Fluor ®488 Goat Anti-Mouse</td>
<td>A11029</td>
<td>Invitrogen, Carlsbad, CA, USA</td>
</tr>
<tr>
<td>FITC-conjugated AffiniPure Donkey Anti-Mouse</td>
<td>715-095-15</td>
<td>Jackson ImmunoResearch, West Grove, PA, USA</td>
</tr>
</tbody>
</table>

IFS double stainings

The first antibodies used were anti-tachykinin (sc-14104) or anti-NK-1R (sc5220) goat antibodies. The initial procedures followed the methods described above, the sections thereafter being incubated with secondary antiserum (FITC-conjugated AffiniPure donkey anti-goat) (Table 3) for 30 min at 37°C. The sections were washed in PBS 3x5 min. After the procedures for tachykinin or NK-1R immunolabelling were finished, the section was incubated in 5% rabbit normal serum in PBS with BSA (Dako, Copenhagen, Denmark) or donkey normal serum (Jackson ImmunoResearch, West Grove, PA) in PBS for 15 min. The sections were then incubated with the mouse or rat monoclonal antibody for 60 min at 37°C. After incubation with this primary antiserum and 4x2.5 min washes in PBS, a new incubation in normal rabbit or donkey serum followed, after which the sections were incubated in rabbit anti-mouse immunoglobulins/TRITC (R0276), donkey anti-mouse immunoglobulins/TRITC or TRITC-conjugated AffiniPure donkey anti-rat IgG for 30 min at 37°C.

In double staining for CD31 (M0823)/tachykinin, the mAb CD31 was initially used and thereafter the tachykinin antibody (sc-14104). The CD31 staining was visualized with FITC-conjugated anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) and Alexa Donkey anti-goat (Invitrogen, Carlsbad, CA, USA) was used for visualization of tachykinin.

The sections were finally washed in PBS for 4x2.5 min and were then mounted in Vectashield Mounting Medium (H-1000) or Mounting Medium with DAPI (H-1500) in order to identify nuclei. Examination was carried out in a Zeiss Axioscope 2 plus microscope equipped with an Olympus DP70 digital camera.
Peroxidase-antiperoxidase (PAP) staining

Indirect PAP technique (Sternberger, 1979) was performed to visualize the antibody stainings of MyHC isoforms and CD31 positive capillaries in muscle (Table 2). After the incubation with primary MyHC or CD31 antibodies in 4°C over the night (c.f. above) the sections were washed in 0.01M PBS for 15 min, incubated with 5% normal rabbit serum for 15 min, followed by incubation with 2% rabbit anti-mouse antibody for 30 min in room temperature. After washing in 0.01M PBS for 15 min, the sections were incubated with 1% mouse peroxidase-antiperoxidase (Dakopatts, Glostrup, Denmark) for 30 min and then washed in 0.01M PBS for 15 min. The peroxidase binding was revealed by applying a solution containing diaminobenzidine and hydrogen peroxidase (H$_2$O$_2$) for 10 min. Finally, the sections were rinsed with running water for 5 min, dehydrated in graded concentration of ethanol, followed by xylene treatment and mounted with DPX (BDH, Limited Poole, England). For more details of the laboratory procedures, see Stål and Lindman (Stål and Lindman, 2000), Liu et al. (Liu et al., 2002) and Österlund et al (Österlund et al., 2013).

PAP double staining

Double staining of the muscle cross-sections with indirect PAP technique was performed with mAb M0823 against CD31 and polyclonal Ab Pc128 against laminin. Each of the five different mAbs against adult and developmental MyHCs isoforms (mAbs N2.261, A4.74, A4951, F1.652, NCL-MHCn) was double-stained with mAb NCL-merosin against laminin α-2 (Table 2). After incubation overnight with the two primary antibodies, the procedure followed as above.

Control stainings

For control for staining specificity, polyclonal antibodies (against tachykinin/NK-1R) were preabsorbed with corresponding antigen (peptide) to eliminate the binding of the antibody to the protein in the tissue (Table 4). The preabsorption was made at 4°C overnight. Ordinary staining was performed in parallel. Other control stainings conformed to staining when the primary antibodies were excluded (PBS or PBS/BSA).

Table 4. Peptides used for preabsorbtions.

<table>
<thead>
<tr>
<th>Preabsorption peptide</th>
<th>Code</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substance P</td>
<td>S6883</td>
<td>Sigma, St Louis, MO, USA</td>
</tr>
<tr>
<td>Substance P</td>
<td>14104P</td>
<td>Santa Cruz, CA, USA</td>
</tr>
<tr>
<td>NK-1R</td>
<td>S5220P</td>
<td>Santa Cruz, CA, USA</td>
</tr>
</tbody>
</table>
Enzyme histochemistry

To distinguish classical fiber types by enzyme histochemistry, serial sections were stained for myofibrillar ATPase (EC 3.6.1.3) after preincubations at pH 10.3, 9.4, 4.6 and 4.3 (Dubowitz, 2007).

In situ hybridization

Digoxigenin (DIG)-hyperlabeled oligonucleotide probes (ssDNA) were used for detection of the NK-1R and tachykinin (SP) mRNA, the probe sequences being as shown in Table 5. Concerning NK-1R (TACR1) a DIG-hyperlabeled oligonucleotide triple probe cocktail was used.

Table 5. Antisense probes (ssDNA) used for detection of the NK-1R and tachykinin (SP) mRNA.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Code</th>
<th>Source</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP</td>
<td>GD1001</td>
<td>GeneDetect, New Zealand</td>
<td>CCGTTGCCCATTAATCCAAAGAAGCTGAGGCTTG</td>
</tr>
<tr>
<td></td>
<td>-CS</td>
<td></td>
<td>GGTCTCCG</td>
</tr>
<tr>
<td>NK-1R</td>
<td>GD1001</td>
<td>GeneDetect, New Zealand</td>
<td>1:GGCTGCAGAACTGGTTAGACTCAGAGGTGTTGGTG</td>
</tr>
<tr>
<td>(TACR1)</td>
<td>-CS</td>
<td></td>
<td>GAGATGTTGGGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2:TGGAGCTTTCGTAGCAGTTGGCTGCTGGAGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AGGAGGCCGTTGGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3:TGACCACCTTGGCGCTTGGCGAGACTTGCTCGTGGT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AGCCGTCAGAG</td>
</tr>
</tbody>
</table>

The corresponding sense DIG-hyperlabeled ssDNA probes were used as negative controls and a β-actin probe (GD5000-OP, GeneDetect, New Zealand) was used as positive control probe. In situ hybridization was performed according to an established protocol (Panoskaltsis-Mortari and Bucy, 1995) using alkaline-phosphatase (AP) labeled anti-DIG antibody for detection, with a few modifications (Danielson et al., 2007; Scott et al., 2008a). Ten µm thick fresh sections were cut by a cryostat with a knife washed in 70% EtOH in DEPC-H2O and mounted onto Super Frost Plus slides (nr.041200, Menzel Gläser). Concerning the further procedures, see Papers II, III. In brief, the sections were fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, diluted in DEPC-H2O) and mounted onto Super Frost Plus slides (nr.041200, Menzel Gläser). Concerning the further procedures, see Papers II, III. In brief, the sections were fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, diluted in DEPC-H2O) (the PFA solution was first passed through a 0.45 µm sterile filter) for 1h at room temperature (RT). The sections were then incubated in 0.2 M HCl at RT for 8 min to inhibit endogenous alkaline phosphatase activity. Thereafter acetylation by incubating for 15 min at RT in a mixture of 195 ml DEPC-H2O, 2.7 ml tiethanolamine, 0.355 ml HCl and 0.5 ml acetic anhydride was made (acetic anhydride was added after the slides had been placed in the slide holder). The slides were then washed in 2x SSC. 15 µl of the hybridization solution and 1 µl of the antisense probe (50ng/µl diluted in TE-buffer) were put into one 1.5 ml eppendorf tube, denaturated for 5 min at 80°C and the sections were thereafter placed on ice.
The slides were covered with a siliconized cover slip, sealed with nail polish and incubated overnight at 56°C. The sections were finally mounted in Pertex mounting medium.

**Enzyme immunoassay (EIA)**

**Tissue homogenisation**

Tissue samples from all experimental groups were directly after being weighed frozen in liquid nitrogen. The weights were approximately 30 mg in each case. Before the EIA procedure, the samples were mechanically homogenised, by using Precellys 24 tissue homogenizer (Bertin Technologies, Saint Quentin en Yvelines Cedex, France), in a prepared 100mM TRIS-HCl buffer, pH 7.0, containing 1M NaCl, 2% BSA, 4mM EDTA, 0.2% Triton X-100 (pH 7), 0.02% Na-azide and the protease inhibitors Pepstatin A (0.1µg/ml), Aprotinin (5µg/ml), Antipain (0.5µg/ml), Benzamidin (167µg/ml) and PMSF (5.2µg/ml). All protease inhibitors were purchased from Sigma-Aldrich, Germany. Tissue and buffer were mixed in a 1:20 relation and the procedure was performed on ice. Directly after the homogenisation procedure, the tissue samples were centrifuged in +4°C, 13,000g for 15 min. The supernatant was then transferred to a new Eppendorf tube and stored at -80°C.

**EIA procedure**

The concentration of tachykinin (SP) was measured by commercially available enzyme immunoassay SP kits (Catalog No: EK-061-05) (Phoenix Pharmaceuticals, Burlingame, CA, USA). All assays were performed according to the manufacturer’s instructions. In order to obtain comparable results between different plates, reference samples were included in the various analyses in order to avoid deviation from procedures. The concentration of tachykinin was normalized to the weights of the tissue samples, i.e. the values are expressed as pg/mg tissue.

**Muscle fiber classification**

Based on the immunohistological staining pattern for the different MyHC mAbs, the fibers were classified as fibers containing pure MyHCI, MyHCIIa, MyHCIIx/IIb isoforms (as we could not distinguish between MyHCIIx and MyHCIIb with the mAbs used in this study, fibers containing these two isoforms were classified as one fiber type in this study, MyHCIIx/b) or as hybrid fibers co-expressing MyHCI and MyHCIIa (MyHCI+IIa) or MyHCIIa and MyHCIIx/b (MyHCIIa+IIx/b). The basis for classification is shown in Figure 4. For control and comparison, the muscle fibers were enzyme-histochemically typed according to their staining intensities for myofibrillar ATPase (mATPase) after alkaline and acid preincubations (c.f above). For detailed description of classical mATPase fiber type classification, see (Dubowitz, 2007).
Morphometric analyses

Three to six randomly chosen areas of the immunohistochemically stained muscle cross-sections for demonstration of the different MyHC mAbs were scanned in a light microscope (Zeiss Axiophot, Carl Zeiss, Oberkochen, Germany) equipped with an MTICCD 72 video camera (DAGE-MTI, Michigan City, USA). All measurements were performed with an image analysis program (Image-Plus, Media Cybernetics, Silver Spring, MD). The fibers were classified concerning fiber phenotypes based on their MyHC isoform composition (c.f above) and the proportion of different types was estimated. The fiber area was measured by tracing the circumferences of each fiber along the periphery of the basement membrane stained for laminin α2 mAb and the numbers of capillaries were counted around each individual fiber and on the whole chosen muscle areas. Capillarization of muscle fibers and atrophied fibers in affected (myositis) areas were not included in the measurements as they could highly bias the calculation. In total, 11,676 fibers were included for the calculation of fiber area and capillary supply of each fiber (average 139 per section, range 124-197) and 70,056 capillaries were included in the calculation of capillary density. A single investigator, who was blind regarding which leg or group the muscle samples was taken from, determined all morphological analyses.

Estimation of morphological changes in muscles

In order to quantify the magnitude of muscle changes and inflammatory cell infiltration in each muscle of the different experimental (1, 3 and 6w) and control groups, the degrees of alterations in each sample were scored according to the criteria given (Table 6) (paper I).
Table 6. The principal basis for scoring of morphological changes in the muscles.

<table>
<thead>
<tr>
<th>Score</th>
<th>Variability in Fiber size</th>
<th>Fibrosis</th>
<th>Fibers with internal nuclei (%)</th>
<th>Fiber splitting (fibers/mm²)</th>
<th>Necrotic fibers (fibers/mm²)</th>
<th>Inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal</td>
<td>None</td>
<td>&lt; 2.5</td>
<td>&lt;0.025</td>
<td>&lt;0.025</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>Small</td>
<td>Mild</td>
<td>2.5-10</td>
<td>0.025-0.1</td>
<td>0.025-0.1</td>
<td>Mild</td>
</tr>
<tr>
<td>2</td>
<td>Moderate</td>
<td>Moderate</td>
<td>10-20</td>
<td>0.1-0.4</td>
<td>0.1-0.4</td>
<td>Moderate</td>
</tr>
<tr>
<td>3</td>
<td>Large</td>
<td>Marked</td>
<td>&gt;20</td>
<td>&gt;0.4</td>
<td>&gt;0.4</td>
<td>Severe</td>
</tr>
</tbody>
</table>

In addition, evaluations of the magnitude of overall myositis and morphological changes were made for paper V based on knowledge that was obtained from the previous morphological scoring in Paper I. The pattern of scoring used in this case was 0=no changes; morphology resembling that seen in normal muscle tissue, 1=mild degree of changes, 2=moderate degree of changes, 3=marked degree of changes, 4=very marked degree of changes. All evaluations were performed blinded of the origin of the samples.

**Estimation of vascular tachykinin-like immunoreactions**

The levels of tachykinin-like immunoreactions for the blood vessel walls (capillaries excluded) were evaluated based on the immunofluorescence intensity seen after staining with the polyclonal tachykinin antibody sc-14104. (0= no reaction, 1=weak reaction, 2=moderate reaction, 3=high level of reaction). The evaluation was performed blinded to the origin of the samples. The degree of fluorescence staining intensity of each of the vessels identified in the cross-section was estimated. The scores of all vessels were then pooled to give mean fluorescence intensity for all blood vessels in the analyzed muscle. Thereafter, the mean intensity for the entire animal group was defined.

**Capillary/blood vessel variables**

Capillary density (CD) was calculated as the total number of capillaries per mm² muscle cross-section (paper IV). The number of capillaries around fibers (CAF) included all capillaries in contact or nearly in contact with individual muscle fibers. Capillaries related to each fiber relative to its cross-sectional area (CAFA) were calculated according to the formula: CAFA / fiber cross-sectional area (CSA) x 10³. This variable relates CAF to fiber size. Vessel densities (VD), i.e densities of vessels larger than capillaries, were calculated as the total number of identified blood vessels/per mm² muscle cross-section area (paper III).
Statistical analysis

The statistical analyses were conducted for all the measured variables separately and combined (paper I, III, IV and paper V). A two-way ANOVA model was used to examine significant differences in mean values between the three different experimental groups and the controls in the exercised and non-exercised side. This test was also used for analysis of differences between the exercised and non-exercised side. Residuals were examined for each trait and it showed that the residual distribution were normal or approximately normal. A multivariate analysis of variance test (MANOVA) was used to examine significant differences between the independent variables in each of the four groups. A Pearson r test was used to measure correlations. The test included analysis both within and between the exercised and non-exercised sides. The statistical analysis was performed by using the statistical software SAS/STAT vers, 9.2 (SAS Institute Inc, USA). In addition, an independent t-test was used in paper II and a one way ANOVA and a paired t-test were used for the evaluation of differences in mean values in paper III and V by using SPSS (PASW Statistics 20). All data are expressed as mean and standard deviations. A p-value < 0.05 was considered to be significant.

Ethics statement

The animal studies have been conducted according to national and international guidelines. The study protocol was approved by the local ethical committee at Umeå University (A34/07 and A95/07). The approval was obtained before the start of the experiments. A licensed breeder had bred all animals for the sole purpose of being used in animal experiments. All efforts were made to minimize animal suffering.

RESULTS

Summary of the findings

The used experimental model caused bilateral muscle changes, inflammatory cell infiltration (myositis) and fibrosis in focal regions of both muscles, and especially after an experiment length of 6w. The histopathological alterations in the affected areas included changes in muscle fiber and nerve morphologies and alterations in the vascularization of the muscle tissue. Furthermore, a bilateral upregulation in the immunohistochemical expression of tachykinin and NK-1R was observed in nerve fascicles and blood vessel walls in affected areas. Expressions bilaterally of tachykinin and NK-1R were seen in infiltrated white blood cells at both mRNA and protein levels. EIA analyses showed that the concentration of tachykinin in the muscle tissue increased in relation to the length of the experiment regimen in both muscles and both sides. The injection experiments leading to increased tachykinin effects led to an aggravation of the morphological changes and increased tachykinin and NK-1R expressions in the muscles. The experiment also caused changes in muscle fiber type
composition, fiber size and capillary supply in both the exercised and non-exercised muscles after 3 and 6w of experiment length. The bilateral fiber and capillary changes mirrored each other to a large degree, but were generally of a lower magnitude in the contralateral muscles.

**Figure 5.** Muscle cross-sections from the soleus muscle of a control (A), the 1w (B) and 6w exercise groups (C, F-H), and the 6w non-exercised group (D-E). Sections A-F are stained with hematoxylin & eosin. Section G and H are double stained for laminin (G) and CD31 (H). Note the pronounced inflammatory infiltration and a marked presence of loose connective tissue (C, D) and the necrotic fiber (arrow, B). Note also the large variations in fiber size in (B-F), occurrence of fiber split (arrows, E, F), and frequent internal nuclei (C-F). Figures F-G (serial sections) shows a split fiber with internal nuclei (arrowhead) and an internalized capillary (arrow, G and H).

**Morphological muscle changes (paper I, IV)**

After 3 and 6w of E/EMS, marked structural tissue changes and various degrees of myositis were observed bilaterally in restricted regions of the muscle samples in both the exercised and the contralateral non-exercised soleus and gastrocnemius muscles (Fig. 5). In the affected areas, the morphological changes were mainly characterized by increased fiber size variability, changes in fiber form, increased numbers of fibers with internal nuclei, fiber split, fibers expressing developmental MyHCs, fibrosis, and signs of axon loss in the nerve fascicles. Necrotic fibers frequently occurred in these areas and infiltrations of fat were in some cases seen in the foci of the inflamed and fibrotic areas. Myositis areas containing loose connective tissue and a large number of white blood cells contained a high density of capillaries with enlarged lumen and/or an increased number of small arterioles or venules, while other adjacent areas, had a low number of capillaries or nearly lacked capillaries (Fig. 6). The soleus
muscle was generally more affected than the gastrocnemius muscle, although there was a large inter-individual variability in the severity of abnormalities for both muscles. The control muscles showed a normal morphology with no signs of inflammation and almost no structural abnormalities.

**Figure 6.** Muscle cross sections from the non-exercised gastrocnemius muscle after 3w of E/EMS (A-F). The sections are stained with H&E (A), CD 31 (B) and developmental MyHC and DAPI (nuclei) (C). Sections A-C are serial (asterisk). Sections D-F are double stained for laminin and CD 31. Note infiltration of inflammatory cells and a marked presence of loose connective tissue in (A), a low capillary supply of muscle fibers, but a high number of larger vessels in the connective tissue (B) and a high number of small fibers containing developmental MyHC (C). Note also the normal capillary network (D) (a capillary marked with an arrow), the low number of capillaries around fibers (E), and the high number of comparatively larger vessels (arrow) in an area with myositis (F).

**Fiber type composition and vascular supply (paper III, IV)**

**Soleus muscle**

**Fiber type frequency.** There were no statistical differences after 3w of EMS/E as compared to controls; there were also no significant changes in fiber type composition between exercised and non-exercised sides. However, after 6w of
E/EMS, the non-exercised side showed a significantly lower mean frequency of MyHCl fibers compared to controls (88% and 98%, respectively).

**Fiber cross-sectional area (CSA).** There was no change in CSA in either side after 1w of E/EMS. At 3w, the mean CSA value was smaller compared to the 1w group in both the exercised and non-exercised sides. Lower mean CSA values compared with controls were present for all fiber types, the decrease being significant for MyHCIIa fibers in the non-exercised side. After 6w, the exercised side showed a CSA in a similar range as that seen in controls, CSA was still lower in the non-exercised side than at 1w. For fiber types, CSA values at 6w were in the non-exercised side smaller for MyHCl fibers compared to the 1w group and MyHCIIa fibers were in the exercised side significantly smaller compared to the controls. See Fig. 7.

**The number of capillaries around fibers (CAF).** After 3w, there were significantly lower mean CAF values in the exercised side, but no difference in non-exercised side, as compared to controls. However, after 6w, the CAF value in the non-exercised side was at a similar low level as in the exercised side. The low CAF values were mainly present among the predominating MyHCl fibers in the exercised side, but lower CAF for MyHCIIa fibers were also present in the non-exercised side at 6w. Comparison of CAF between the two sides showed that the exercised side had significantly lower values compared to the non-exercised side after 1 and 3w of E/EMS. See Fig. 7.

**The number of capillaries around fibers relative to fiber area (CAFA).** When relating the CAF with CSA in the exercised side, there were no differences between controls and the 1w, 3w and 6w experimental groups. However, after 3w of EMS/E, CAFA in the non-exercised side was significantly higher than in the controls and 1w group. Comparison between both sides showed that there were significantly higher CAFA values in the non-exercised than in the exercised side after 3 and 6w of EMS/E. See Fig. 7.

**Capillary Density (CD).** In the exercised side, the mean CD did not differ between controls and the three experimental groups. In the non-exercised side, the CD was significantly higher in the 3 and 6w groups as compared to the control and 1w group. Comparison between both sides showed that the CD values were significantly higher in the non-exercised than in the exercised side after 3 and 6w of EMS/E. See Fig. 7.

**Blood vessel density (VD).** After 3w, especially after 6w of EMS/E, the density of arteries/arterioles and venules/veins in the exercised as well the non-exercised sides was significantly increased as compared to the control group. There was no significant difference between exercised and non-exercised sides.
Figure 7. Graphs showing fiber area (CSA) and capillary parameters in the exercised and non-exercised soleus muscle after 1, 3 and 6w of E/EMS. Exercised side is defined by continuous black line, non-exercised side by dotted line. Significant difference (p<0.05) in exercised side is marked with E and difference in the non-exercised side is marked with N. Significance to controls are marked (c) and to 1w group (1) or 3w group (3). Significant differences (p<0.05) between exercised and non-exercised sides are marked with an asterisk (*).

Gastrocnemius muscle

**Fiber type frequency.** After 3w of E/EMS, the mean frequency MyHCI fibers in the exercised side were lower than in controls. The mean frequency values for MyHCIIx/b fibers were increased, although not significant. At 6w, the frequency of MyHCI fibers in the exercised side was significantly lower than in controls (10% vs. 21%) and the frequency of MyHCIIa fibers in the non-exercised side was significantly higher (26% vs. 16%). Comparison between the exercised and non-exercised sides showed a lower proportion of MyHCI fibers in the exercised side at 3 and 6w, and higher proportion of MyHCIIa fibers in the non-exercised side at 6w.

**Fiber cross-sectional area (CSA).** After 1w, no changes in CSA were observed in either the exercised or non-exercised sides. After 3w, both sides the fiber area was significantly smaller compared to controls. Smaller fiber size than in controls was observed in the non-exercised side for MyHCI fibers and in both sides for MyHCIIx/b fibers. After 6w of E/EMS, both the exercised and non-exercised sides had larger CSA than at 3w, i.e. the CSA was in the same range as in the controls. Concerning fiber types, larger CSA was found for MyHCI, MyHCIIa and MyHCIIx/b fibers in the
exercised side and for MyHCl fibers in the non-exercised side. MyHClIIX/b fibers were in the non-exercised side significantly lower at 6w compared to controls. Comparison of CSA in both sides showed that CSA was at 6w significantly larger in the exercised than in the non-exercised side. See Fig. 8.

**Figure 8.** Graphs showing fiber area (CSA) and capillary parameters in the exercised and non-exercised gastrocnemius muscle after 1, 3 and 6w of E/EMS. Exercised side is defined by continuous black line, non-exercised side by dotted line. Significant difference (p<0.05) in the exercised side is marked with E and difference in the non-exercised side is marked with N. Significance to controls are marked (c) and to 1w group (1) or 3w group (3). Significant differences (p<0.05) between exercised and non-exercised sides are marked with an asterisk (*).

**The number of capillaries around fibers (CAF) (Paper IV).** Both the exercised and non-exercised side showed, with exception for the exercised 1w group, significantly lower CAF values in the 1, 3 and 6w groups compared to the controls. At 3w, lower CAF than in controls was present among MyHClI, MyHClIIXa and MyHClIIX/b fibers in the exercised side and for MyHClIIXa and MyHC IIX/b fibers in the non-exercised side. At 6w, CAF for MyHC IIX/b fibers was significantly lower than in the controls in both sides, whereas CAF for MyHClIIXa fibers were only lower in the non-exercised side. See Fig. 8.

**The number of capillaries around fibers relative to fiber area (CAFA).** In the exercised side, there were no differences in CAFA values between the controls and the 1, 3 and 6w experimental groups. In the non-exercised side, CAFA were larger at
3w than in controls and 1w group. The non-exercised side showed significantly higher CAFA than the exercised side at both 3 and 6w. For fiber types, no statistical differences in CAFA were observed. See Fig. 8.

**Capillary Density (CD).** In the 1 and 3w groups, the CD values in the exercised and non-exercised sides were in similar range as in the controls. After 6w of EMS/E, the CD in the exercised side showed lower CD values compared to controls, whereas there was no significant differences in the non-exercised side. Comparison of both sides showed significantly lower CD values at 6w in the exercised side than non-exercised side. See Fig. 8.

**Blood vessel density (VD).** After 3 and 6w of E/EMS, the density of blood vessel (arterioles/arteries and venules/veins) was increased in the exercised as well non-exercised sides. There were significant increases as compared to the control group, whereas there were no significant differences between exercised and non-exercised sides.

**Changes in nerve tissue (paper I-V)**

**Morphological changes**

After 1w of EMS/E, the histological appearance of the nerve fascicles appeared normal. However, after 3w, and especially after 6w of EMS/E, histological changes could be observed in some nerve fascicles. The affected nerve fascicles were located in the myositis areas of both the exercised and non-exercised sides of the soleus and gastrocnemius muscles. The changes within the nerve fascicles were increased numbers of cell nuclei, a higher amount of connective tissue and an occurrence of cellular structures with a ballooned, “swollen” and foamy cytoplasm (Fig. 9).

**β-III tubulin and S-100beta immunoreactions**

The immunohistochemical staining with the β-III tubulin antibody showed that a subpopulation of the axons in the nerve fascicles with an abnormal morphology was unstained. This was observed for both the soleus and gastrocnemius muscles of both legs after 3 and 6w of E/EMS. Immunostaining for white blood cell markers indicated that there were only occasionally white blood cells within the nerve fascicles and the cells present were immunohistochemically positive for the neutrophil/T-cell markers. The immunohistochemical staining with the S-100beta antibody with DAPI in mounting medium showed that a pink staining reaction occurred in nuclei of Schwann cells of affected nerve fascicles of myositis areas. In the nerve fascicles of normal muscle tissue, the nuclei exhibited the characteristic blue DAPI reaction.
Tachykinin-like immuno-reactions

Overall visualization of tachykinin reactions are shown in Fig. 10. Only a few nerve fibers showing tachykinin-like immunoreactions were seen in the nerve fascicles in muscles of the soleus and gastrocnemius muscles of control animals and in normally appearing muscle areas of the experimental animals. Tachykinin-like immunoreactions were, on the other hand, frequently observed in nerve fibers in parts of some nerve fascicles located in myositis or adjacent areas of both exercised and non-exercised soleus and gastrocnemius muscles in the 3 and 6w groups (Fig 10G, H). For the characterisation of the immunoreactions, double staining with β-III Tubulin was performed. This staining verified that the immunoreactions indeed were located in nerve fibers. Immunoreactions were also characterized after double stainings with S-100beta. It was noted that these tachykinin-like immunoreactions were found in association with S-100beta immunoreactive cells, i.e. Schwann cells. All the tachykinin-like immunoreactions seen in nerve fascicles and as fine nerve fibers were abrogated by preabsorption with SP peptides.

NK-1R immunoreactions

No NK-1R immunoreactions were found in the nerve fascicles of control samples and in normally appearing muscle areas of experimental animals. Occasional immunoreactive nerve fibers were seen in association with blood vessels. There were
marked immunoreactions for NK-1R in nerve fascicles located in myositis areas and in the close proximities of these areas. This was observed for the exercised and non-exercised sides of both muscles. These NK-1R immunoreactions were especially prominent in the 6w group but reactions were also seen in myositis areas of the 1 and 3w groups. Double-staining for S-100beta/NK-1R revealed that Schwann cells enclosed the NK-1R immunoreactions in these cases. By using double staining for β-III tubulin/NK-1R, it was verified that NK-1R reactions in these areas indeed corresponded to nerve structures.

**AChE immunoreactions (paper I)**

Normal AChE immunoreactions were seen in endplate regions of muscles of controls and in normal muscle areas of experimental animals. After 3w, and especially after
6w of E/EMS, some muscle fibers in the affected areas of both the exercised and non-exercised sides exhibited an abnormal staining pattern for AChE. The muscle fibers were in these cases encircled by AChE reactivity. Necrotic muscle fibers showed on the other hand, no AChE activity.

**Infiltration of white blood cells (paper I-III)**

White blood cells were frequently seen in the affected muscle tissue areas. Based on immunohistochemical analysis, cells were demarcated by antibodies against neutrophil/T-cell marker (MCA805G), macrophage marker (M0814) and eosinophil marker (MAB1087). Macrophages were often seen within and around necrotic muscle fibres. The neutrophil/T-cell-reactive cells were observed around damaged muscle fibres and in areas with fibrosis, and eosinophils were generally widely spread over the affected areas in the muscle tissue, but could be found within necrotic fibres as well. The eosinophils were also easily distinguished in the H&E stained sections.

**Tachykinin reactions**

Tachykinin-like immunoreactions were associated with the white blood cells of the inflammatory infiltrates of the myositis areas. These reactions were verified via preabsorption control staining using SP peptides. Double staining showed that reactions for both antibodies (polyclonal and monoclonal) were detected in the same cells. The reactions frequently occurred as granular reactions.

The results of co-localization studies showed that the cells exhibiting expression for T-cells/neutrophil marker did not exhibit tachykinin-like immunoreactions, whilst cells expressing reaction for CD68 often showed tachykinin-like immunoreactivity and those stained for eosinophil marker were always seen to exhibit co-localization with tachykinin-like immunoreactions.

Expression of tachykinin mRNA was detected as black intracellular reaction in cells of the inflammatory cell infiltrates of the experimental animals. The occurrence of specific mRNA was evidenced via parallel staining with antisense and sense probes (Figs 10 I, J).

**NK-1R reactions**

Immunoreactions for NK-1R occurred for some of the white blood cells that were dispersed in the inflammatory infiltrates (Fig. 11). These reactions were observed both for the exercised and the non-exercised sides and for both muscles. The reactions were mainly intracellularly located in the white blood cells and showed frequently a granular appearance. The specificity of the reactions was verified via preabsorption staining. The cells showing NK-1R reactions were macrophages or eosinophils (Fig 11C). There were no NK-1R immunoreactions in cells demarcated by the neutrophil/T-cell antibody. Double staining for tachykinin and NK-1R showed
that there was partly a co-localization between these molecules in the cells. In situ hybridization studies showed NK-1R mRNA reactions in the infiltrated white blood cells as verified by staining with antisense and sense probes.

**Figure 11.** Immunoreactions for NK-1R in the exercised gastrocnemius muscle specimen of the 1 week group. Specific reactions are shown for white blood cells of inflammatory infiltrates (A and B). Section (B) enlarged magnification. Section (C) shows double staining for NK-1R (green) and eosinophil marker (reddish). There is co-localization of staining in eosinophils (arrows).

**Muscle fiber reactions concerning NK-1R (paper II)**

NK-1R immunoreactions were observed in parts of necrotic muscle fibers that had a pronounced infiltration of white blood cells (Fig. 12). The NK-1R immunoreactions were mainly found in white blood cells infiltrating the fibers. NK-1R immunoreactions were also seen in muscle fibers that based on their desmin reaction pattern were considered to be in a regenerative stage (Fig 12 E, F). These fibers showed a marked desmin immunoreaction, whilst the necrotic muscle fibers were devoid of desmin immunoreaction.
Reactions in blood vessel walls (paper II and III)

Tachykinin reactions

Tachykinin-like immunoreactions were noted for the walls of arteries/arterioles as well as veins/venules. The reactions were particularly detected in the smooth muscle layer of the vessels but could sometimes be seen in the endothelial layer. The reactions were seen by using antibody sc-14104 but not by using antibody 8450-0505. The reactions were abrogated after preabsorption with SP peptide. The

Figure 12. Sections from the exercised side (A-D) and non-exercised (E-F) sides of soleus muscle (6w group) double stained for desmin (A, C, E) and NK-1R (B, D, F) with DAPI (nuclei). Figures show normal appearing muscle fibers with striated desmin immunoreaction pattern (A) and lack of NK-1R reaction (B). The necrotic muscle fiber, which is infiltrated by white blood cells, shows none or weak desmin staining reaction (C). There is a NK-1R reaction in the areas containing inflammatory cells (D). A muscle fiber (asterisk) with a marked desmin reaction (E) and point-like NK-1R reactions (arrow) is shown in higher magnification (F). Note the presence of internal nuclei in this fiber (E).
immunoreactions were to various degrees seen in all groups, but were clearly strongest in the 3 and 6w groups. Especially strong reactions were visible for the vessels in the myositis areas and in adjacent regions. The reactions in the blood vessel walls in control and 1w groups showed generally moderate or weak reactions while some vessel walls were unreactive. No reactions were found in capillaries in any of the groups.

The results of assessment of degrees of intensity of specific immunoreactions in blood vessel walls showed that a significant increase occurred after 3 and 6w as compared to 1w and controls. There was no significant difference between exercised and non-exercised sides in the soleus muscle or in the gastrocnemius muscle after 1, 3 and 6w of E/EMS (Fig. 13).

In situ hybridization revealed tachykinin mRNA reactions in the endothelial layer and the smooth muscle layer of blood vessel walls in vessels located in the areas with myositis in the exercised and the non-exercised side of both muscles.

![Figure 13. Graphs showing the scores of tachykinin-like immunoreaction intensities in blood vessel walls in exercised and non-exercised sides of soleus and gastrocnemius muscles in the 1, 3 and 6w groups and controls. Exercised side is presented as continuous line, non-exercised side as dotted line. Significant difference in the exercised side is marked with E and difference in the non-exercised side is marked with N. Significance to controls are marked (c) and to 1w group (1) or 3w group (3).](image)

**NK-1R reactions**

NK-1R immunoreactions were noted for the endothelial layer of some of the blood vessels in myositis areas and areas located in the close proximities. Immunoreactions were neither detectable in the walls of blood vessels of controls nor in those of normally appearing muscle tissue of experimental animals. Reactions were also in general not seen for the walls of large arteries of either of the groups.

In situ hybridization further strengthened the occurrence of NK-1R in the vessel walls. NK-1R mRNA reactions were thus seen in the blood vessel walls in the myositis areas and/or closely adjacent areas. These reactions were not only seen in the
endothelial layer but also in parts of the smooth muscle of the vessel walls. There were no reactions for NK-1R mRNA in the blood vessel walls in neither the control group nor the normally appearing muscle of experimental animals.

**Tachykinin concentration (paper III)**

Evaluations of the concentration of tachykinin in the muscle tissue by EIA showed that there was an increase after 1, 3 and 6w of E/EMS as compared to the controls. The increase was significant for exercised as well as non-exercised sides for the soleus muscle. For gastrocnemius muscle, there was a tendency of an increase at 3w as compared to controls. There was a significant increase after 6w E/EMS as compared to control, 1 and 3w groups for this muscle. This fact was observed for exercised as well as non-exercised sides. There was no significant difference in tachykinin concentration between exercised and non-exercised sides for both muscles (Fig. 14).

![Graphs showing changes in concentration of tachykinin in exercised and non-exercised soleus (A) and gastrocnemius (B) muscles in the 1, 3 and 6w groups and controls. Exercised side is presented as continuous line, non-exercised side as dotted line. E=exercised side, N=non-exercised side. E.c depicts statistical difference as compared to control group, E.1 as compared to 1w group, E.3 as compared to 3w group. N.c describes statistical difference as compared to control group, N.1 as compared to 1w group, N.3 as compared to 3w group.](image)

**Figure 14.**

Correlations between exercised and non-exercised sides (paper III)

A Pearson correlation test demonstrated that there was a positive and significant correlation between exercised and non-exercised sides with respect to tachykinin concentration ($r=0.906$, $p<0.001$). There was also a positive and significant correlation between the two sides with respect to intensity of tachykinin-like immunoreactivity in blood vessel walls ($r=0.601$, $p<0.001$). There was also a significant and positive correlation in VD between the two sides ($r=0.642$, $p<0.001$). These correlations were found after pooling of the soleus and gastrocnemius values of all experimental animals.
Pearson correlation tests for evaluation of correlations between exercised and non-exercised sides for the soleus and gastrocnemius muscles separately were also made. There was a positive correlation between the two sides concerning tachykinin-like immunoreaction intensity in blood vessel walls (r=0.687, p=0.002) and VD (r=0.809, p<0.001) for the soleus muscle. Concerning the gastrocnemius muscle, there was a positive correlation between the exercised and non-exercised sides with respect to tachykinin-like immunoreaction intensity in blood vessel walls (r=0.562, p=0.019) and tachykinin concentration (r=0.895, p<0.001).

**Local injections + 1w of E/EMS (paper V)**

**Morphological observations**

Marked morphological changes occurred in the 1w group of animals that had been given local peritendinous injections with C+Th and SP+C+Th. The changes conformed to a pronounced myosits, and occurrence of necrotic fibers, presence of very small and large fibers and fibers with internal nuclei. Wide areas of connective tissue were encountered in the muscle sections. There were clearly less morphological changes in the NaCl treated group than in the C+TH and SP+C+Th groups.

**Table 7.** Mean values of the scoring of morphological changes and tachykinin (SP) concentrations in response to 1w experiment with injections.

<table>
<thead>
<tr>
<th>Group</th>
<th>Scores for morphology</th>
<th>Tachykinin concentration (pg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SOL</td>
<td>GM</td>
</tr>
<tr>
<td>NaCl (n=5)</td>
<td>1.5±1.1</td>
<td>1.2±0.4</td>
</tr>
<tr>
<td>SP+C+Th (exp) (n=5)</td>
<td>2.3±0.8</td>
<td>2.0±1.3</td>
</tr>
<tr>
<td>SP+C+Th (non-exp) (n=5)</td>
<td>3.0±1.1</td>
<td>2.1±0.7</td>
</tr>
<tr>
<td>C+Th (n=6)</td>
<td>3.8±0.6</td>
<td>2.2±0.5</td>
</tr>
</tbody>
</table>

Statistical evaluations were made for the tachykinin concentration. Significance level was set at 0.05. # Significant difference as compared to NaCl group. ▲ Significant difference between the C+Th group and the SP+C+Th group (experimental side), □ Significant difference between the C+Th group and the SP+C+Th group (non-experimental side). Values within brackets (concerning tachykinin concentration, SOL) are the mean values for samples which CV (coefficient of variation) was above 15% were excluded (CV was above 15% for values for in total 3 samples out of the 42 samples analyzed).

**Results concerning tachykinin/NK-1R**

The tachykinin-like and NK-1R immunoeexpressions were more pronounced in the muscle tissue of the animals that had been given the local treatments with C+Th (and to some extent also those given SP+C+Th) than those injected with NaCl. The marked tachykinin-like/NK-1R immunoreactions were seen in white blood cells and in blood
vessels walls. Tachykinin-like/NK-1R immunoreactions occurred within large nerve fascicles and frequent tachykinin-like immunoreactive fine varicose nerve profiles were seen. The upregulation of the SP/NK-1 R immunoreactions was mainly found in myositis areas and areas adjacent to these. For illustrations, see Fig. 15.

The concentration of tachykinin in response to the injection treatment for the C+Th group was markedly increased as compared to the NaCl group for both soleus and gastrocnemius muscles (Table 7). There were also tendencies of higher concentrations for the SP+C+Th group than the NaCl group. Concerning the SP+C+Th group, there were no significant differences between the experimental and non-experimental sides for both muscles.

**Figure 15.** Muscle tissue from an exercised soleus muscle coupled with injections of C+Th (A-C) and series of sections of a non-exercised soleus muscle from the SP+C+Th group (D-F). Staining with H&E (A–D), and with polyclonal Abs against tachykinin (E) and NK-1R (F) and with a mAb against tachykinin (G). Note the very marked inflammatory infiltrate and the extensive occurrence of connective tissue (A–D), high number of necrotic fibers (A, D) and presence of internal nuclei (C). Necrotic fibers that were partly filled with white blood cells (triangle) (D) show immunoreaction for tachykinin (E) and NK-1R (F). Fibers with a more normal appearance (asterisk) lack tachykinin (E) and NK-1R (F) reactions. There are also NK-1R and tachykinin immunoreactions in white blood cells located in the connective tissue spaces that separate the muscle fibers (E-F). Frequent fine varicose nerve fibers exhibiting tachykinin like immunoreaction outside the wall of an arteriole (arrows) and in the connective tissue space (arrow, inset) are shown (F).
DISCUSSION

Opening remarks
The existence of cross transfer effects to the opposite side of the body after unilateral intervention and in inflammatory diseases is well known. However, knowledge on potential morphological and biochemical effects at the cellular level are limited and the understanding of the underlying processes is largely unknown or controversial. It is known that muscle overuse can cause muscle damage and inflammation under certain circumstances. Whether there is a cross transfer of unilateral deleterious muscle changes to the contralateral muscle is, however, so far unknown. The overall aim of the thesis was to experimentally study possible morphological, cellular and biochemical changes in both the ipsilateral and contralateral muscles after unilateral muscle overuse. The studies were focused on muscle fiber alterations, inflammation and the possible involvement of the tachykinin system.

Main findings
In this thesis, the major findings were that unilateral repetitive overuse exercise via EMS over time caused morphological tissue changes, myositis and modifications in the tachykinin system not only in the exercised muscle, but also in the non-exercised muscle in the contralateral leg. The most common bilateral histopathological changes included fiber necrosis, alterations in fiber size, infiltration of inflammatory cells and fibrosis. In addition, bilateral morphological changes within nerve fascicles were also observed. The most marked changes occurred in restricted areas of the muscle tissue, but alterations such as decreased fiber size, changes in fiber phenotype composition and decreased capillary supply were generally observed in the muscle samples. In parallel to these changes, there was a process of tissue repair including muscle fiber and nerve regeneration. In areas with marked myositis, the capillaries were often enlarged in size and the density of arterioles/venules was increased. On the other hand, in adjacent regions with faint or no inflammation, but an extensive amount of fibrous connective tissue, capillary density was usually low.

The concentration of tachykinin was over time bilaterally increased in both muscles. At the tissue level, the expressions of tachykinin and the SP-preferred receptor, the NK-1R, were bilaterally upregulated in nerve structures and blood vessel walls in the affected areas. Infiltrating white blood cells exhibited both tachykinin-like and NK-1R expressions. NK-1R was detected in white blood cells that had infiltrated into the necrotic muscle fibers as well as in regenerating muscle fibers. The expressions of these molecules in blood vessel walls and white blood cells was seen at both mRNA and protein levels. Local injections of C+Th and SP+C+Th for the 1w group markedly amplified the structural changes in the muscle tissue and led to increased tachykinin-like and NK-1R immunoreactivities in the myositis areas and increased tachykinin concentrations in the tissue.
The bilateral changes mirrored each other to a large extent. However, in some respects, the alterations were of a lower magnitude in the contralateral non-exercised muscles. There were also differences in the extent of the morphologic alterations in the soleus and gastrocnemius muscles. While histopathological tissue alterations and myositis were more severe in the soleus muscle, the changes in fiber phenotype composition, fiber size and muscle capillarization were more pronounced in the gastrocnemius muscle.

**Methodological aspects**

**The experimental model**

A rabbit exercise model was used that involved combined effects of unilateral passive flexion and extension of the ankle joint via EMS of the triceps surae muscle. The model was applied to induce muscle changes and myositis in the exercised muscles, as was verified by histological analysis of the muscle samples. It cannot be determined to what extent the histological abnormalities in the muscles are a consequence of the duration of the exercise or the use of EMS. It has to be considered that the histological abnormalities and inflammatory response in the muscles could, at least partly, relate to the use of EMS as the driving force to recruit motor units. It is known that EMS-evoked exercise can result in a significantly higher degree of muscle injury as compared with voluntary exercise (Crameri et al., 2007; Black and McCully, 2008; Nosaka et al., 2011). Due to an instantaneous recruitment of a very high number of motor-units, EMS causes a higher force and a considerably higher total work performed over time than in voluntary recruitment. EMS can also depolarize sensory neurons that generate an orthodromic sensory volley to the central nervous system, i.e. contractions are generated by a combination of recruitment by the stimulating electrodes and central recruitment (Bergquist et al., 2011). This results in a very high load during contractions that over time might have harmful effects on the muscle tissue.

**The muscles**

The advantage of using the triceps surae muscle is that it consists of two muscle parts, the soleus and gastrocnemius muscles. In other species, these muscles differ from each other in muscle fiber type composition (Gollnick et al., 1974; Armstrong et al., 1982). Our analysis of fiber phenotype composition confirmed that the rabbit, like other species, had a predominance of slow MyHCI fibers in the soleus muscle and a predominance of fast MyHClII fibers in the gastrocnemius muscle. This difference between the muscles may be one explanation as to why the two muscles to some part respond differently to the experiment. A drawback of the study was that no other non-exercised muscles than the homologous muscles in the contralateral side were studied. This aspect should be considered in future studies.
Muscle fiber phenotypes

It is well known that adult human muscles contain three major MyHC isoforms encoded by specific genes, MyHCI, MyHCIIa and MyHCIIx. However, other species, such as the rabbit, might contain an additional MyHC isoform, MyHCIIb (Schiaffino and Reggiani, 1994). Since we could not distinguish between MyHCIIx and MyHCIIb fibers with mAbs we used, in this study fibers containing these MyHC isoforms were grouped together and classified as one fiber type, namely MyHCIIx/b.

Morphological measurements

All the evaluations were made for the entire muscle samples, with exception for the measurements of muscle fiber CSA and capillary supply of muscle fibers and estimation of fiber phenotypes, where the areas with histopathological alterations, myositis and fibrosis were excluded as they could highly bias the calculations.

Cross transfer motor activity

A direct cross-transmission of motor nerve activity to the homologous contralateral muscle during the experiment could hypothetically explain some of the tissue alterations. Electromyography (EMG) recordings have demonstrated certain nerve activity in the contralateral muscles during unilateral exercise (Gregg, 1961; Panin et al., 1961; Devine et al., 1981). As we did not measure EMG activity in the contralateral muscles in this study, some motor activation of the contralateral muscle cannot be excluded. However, no contractions could be visually observed in the contralateral leg during the experimental sessions. If such cross transfer motor activity exists during the experiment, it should be very small and it is unlikely that such a limited nerve activity could cause the histopathological and other changes in the contralateral muscles observed in this study.

Unilateral muscle weakness

Some bilateral contralateral tissue alterations could hypothetically be related to a compensatory increased use of the contralateral muscles due to fatigue of the exercised leg after the experimental sessions. Weakness in the exercised leg could be compensated by an increased neuromuscular activity in the non-exercised leg in order to maintain posture during movements in-between the E/EMS sessions. Against this proposal is the fact that we did not observe significant amended movements or changed behaviors in the rabbits in-between the experimental periods.

Time pattern of muscle changes

After 1w of EMS/E, the exercised soleus muscle showed several structural alterations in the muscle tissue, including a mild focal accumulation of inflammatory cells. In this muscle, there was also bilaterally a significant increase in tachykinin
concentration as compared to the controls at this stage. Only a few morphological changes were found in the exercised gastrocnemius muscle and only limited changes were found in the homologous contralateral non-exercised muscles. At 3w, the severity of myositis and morphological changes significantly increased focally and the abnormalities were now present bilaterally for both the gastrocnemius and soleus muscles. The magnitude of these morphological abnormalities had significantly increased after 6w of E/EMS in both sides of the soleus muscle, while the changes in the gastrocnemius muscles were more or less in the same magnitude as in the 3w group. By this time, there were also bilateral morphological changes in the nerve fascicles, including a loss of axons and signs of Schwann cell activation. At 6w, the tachykinin system was bilaterally further up-regulated for both muscles. These observations show that there to some extent is a delay in the cross transfer effects to the contralateral side. It is also evident that the magnitude of morphological alterations and inflammatory cell infiltration increased with the duration of EMS/E in the soleus muscle, whereas it peaked after 3w in the gastrocnemius muscle. The tachykinin system was markedly upregulated for both muscles, especially the gastrocnemius muscle at 6w.

**Focal histopathological muscle changes**

It is evident that the overuse experiment over time focally affected the muscle tissue, leading to tissue damage, fiber necrosis and myositis and that this adverse process was cross-transfered to the contralateral side. While the deleterious processes in the exercised side is easy to understand, the background to the changes in the contralateral side is more difficult to interpret. Our results of a bilateral upregulation of the tachykinin system for the nerves in both the exercised and non-exercised muscles indicate that the nervous system is involved in this process (see below, Tachykinins). Further aspects on the finding of bilaterality are discussed below.

**Muscle repair after injury**

The bilateral tissue changes in focal areas including necrotic, atrophic and hypertrophic fibers, the high content of small fibers containing developmental MyHC and the extensive inflammation, suggests a parallel process of muscle derangement, degeneration and regeneration. Muscle regeneration is a complex process that involves the interaction of inflammatory cells, myogenic cells, blood vessels, nerves and extracellular matrix components (Ciciliot and Schiaffino, 2010). The initial event after fiber injury leading to fiber necrosis is activation and infiltration of mononucleated blood cells in the affected area. Inflammation is essential to remove necrotic tissue and to initiate a healing process where the damaged muscle undergoes biological repair. Depending on the type of inflammatory response, this phase may be followed by activation of myogenic satellite cells to proliferate, differentiate and fuse, leading to new fiber formation and reconstruction of damaged tissue (Charge and Rudnicki, 2004). Importantly, an intact microvascularization is required for an
adequate regeneration (Ciciliot and Schiaffino, 2010). In case of a defect vascularization, damaged muscle tissue can be replaced by fibrotic connective tissue. Our experimental model seems to involve all the different processes described above.

**The inflammatory process**

The inflammatory process is most likely a part of the complex biological response to the harmful stimuli of the exercised muscle. The simultaneous bilateral upregulation of tachykinin innervation in the tissue, which can be related to inflammatory effects, indicates that the nervous system is involved in the inflammatory processes in both sides. Whereas the frequent exercise via EMS causes injury and inflammation in the exercised side, a neuropeptide-induced inflammation might be involved in the establishment of the structural changes in the contralateral side. The involvement of the nervous system in the inflammation processes in the locomotor system is supported by the experimental findings that denervation of joints leads to regression of established rheumatoid arthritis (RA) and that protection from development of RA (Thompson and Bywaters, 1962; Glick, 1967) and psoriasis (Farber et al., 1986) can be obtained following skin denervation.

The inflammatory process is normally time-dependent, where neutrophils rapidly invade the muscle tissue after damage, later followed by phagocytic macrophages (Peake et al., 2005). Of the white blood cells detected in this study, macrophages and cells demarcated by the neutrophil/T-cells marker were commonly accumulated within the areas with severe inflammation and histopathological muscle changes, whereas eosinophils usually were more widely spread in and around these areas. Necrotic and damaged muscle fibers were invaded by phagocytic macrophages. Macrophages have been shown to promote muscle injury through the release of free radicals, but they also seem to participate in muscle repair and regeneration (Tidball, 2005). Previous studies have shown that phagocytic neutrophils invading into muscle tissue have the ability to release proteases for the removal of debris related to the injury and to produce high concentrations of cytolytic and cytotoxic molecules that can further damage the muscle tissue (Lowe et al., 1995; Tiidus, 1998). The degrading factors from the inflammatory cells may cause additional cell injury, which may contribute to the bilateral accumulation of histopathological muscle changes in the inflamed areas.

**Muscle fiber alterations**

The bilateral histological alterations in the muscle tissue, which were mainly observed in the areas with myositis and fibrosis and in adjacent regions to these areas, were characterized by a high variability in fiber size and a large number of fibers with internal myonuclei, split fibers, necrotic fibers and small fibers expressing developmental MyHCs. The high proportion of small-sized fibers probably relates to denervation atrophy or to newly formed regenerating fibers originating from activated satellite cells (Schiaffino and Partridge, 2008). Further support for a
regenerative process with newly formed fibers was the findings of expression of developmental MyHCs in some of the small-sized fibers and that many of these small fibers showed basophilic staining and contained central nuclei, reflecting a high protein synthesis. The concomitant presence of abnormally large hypertrophic fibers suggests a frequent activation of certain motor units or more likely, that activated satellite cells have fused to injured fibers and thereby increased the size of the fibers. The substantially increased number of fibers with internal myonuclei and the high number of split fibers support the latter mechanism. Fiber split and fibers with internal myonuclei are common findings in connection with muscle hypertrophy due to muscle overload (Bruusgaard et al., 2010) and in neuromuscular disorders (Swash and Schwartz, 1977). It is suggested that the mechanisms for fiber split relates to an incomplete fusion of activated and multiplying satellite cells to damaged fibers or to disturbed regeneration after segmental muscle fiber injury (Eriksson et al., 2006). An incomplete fusion of regenerating fibers within the same basal lamina is considered to be characteristic for muscle regeneration and the internalization of myonuclei in the fiber might be an outcome of this process (Eriksson et al., 2006). Once fusion of myogenic cells is completed, the fibers increase in size, and the internal myonuclei move to the periphery of the muscle fiber (Charge and Rudnicki, 2004).

Changes in muscle vascularization

Besides muscle fiber alterations, degradation of capillaries and an increased number of vessels with a larger size were present in the affected areas. Two interesting features should be pointed out. Firstly, in the focal areas with severe myositis there was an increased number of enlarged capillaries and vessels with a size more similar to arterioles or venoles. These observations imply that the inflammatory process dilates capillaries and triggers angiogenesis to increase blood flow to the area (Heil et al., 2006; Heil and Schaper, 2007). Secondly, adjacent areas with less inflammation but with an increased amount of connective tissue and fat infiltration had few or almost no capillaries. Muscle fibers in these areas were thus generally surrounded by few capillaries or lacked capillaries. Previous studies have shown that an inflammatory myopathy can damage the capillary endothelial cells and cause a destruction of capillaries (Dalakas, 2012). Since an intact microvascular supply is required for an adequate regeneration (Ciciliot and Schiaffino, 2010), an inflammatory process damaging capillaries might affect the possibility for muscle regeneration. Thus, when the inflammatory process is followed by defective muscle fiber regeneration, perhaps as a consequence of detrimental capillary degradation, parts of the affected area will in the long run be replaced by fibrous connective tissue. Since there is a decrease of oxygen-consuming contractile tissue in these areas, the need for oxygenation is reduced and the angiogenic process to form new vessels is probably low.

Changes in nerves

The bilateral deleterious processes also caused morphological changes in nerves
within or adjacent to the myositis areas. Signs of both axon degeneration and regeneration were thus observed in some nerves in these areas in the soleus and gastrocnemius muscles of both legs. A subpopulation of axons in the affected nerves was unstained for βIII-Tubulin, indicating axonal degeneration. The content of connective tissue and number of cell nuclei was substantially increased within the nerve fascicles.

Staining of the affected nerve fascicles with mAb S-100beta in combination with general DAPI staining for nuclei, indicated that a subpopulation of the Schwann cell nuclei revealed reactions for both these markers. It is reported that Schwann cells are activated in response to nerve damage and the S-100beta protein is known to play a crucial roles in axonal repair and regeneration and in CNS development (Hu et al., 2003; Duobles et al., 2008; Yardan et al., 2011). In the normal nerve fascicles, DAPI stains all nuclei, whereas the S-100beta protein generally is restricted to the cytoplasm and the membranes of the Schwann cells (Spreca et al., 1989). The findings made in our experimental model here used can imply that there is a bilateral process of regeneration and repair of injured nerve tissue in the muscles.

**AChE reaction pattern for affected muscle fibers**

AChE activity is normally restricted to a small area on the muscle fibers that represents motor-endplates in the neuromuscular junctions. In the myositis areas, both the exercised and non-exercised triceps surae muscles contained fibers which were more or less encircled by AChE reactivity. Denervated and regenerating fibers have been shown to produce AChE and the regenerating motor nerves can hereby play an indirect role by inducing the myofibers to produce synaptic AChE (Anglister, 1991). Our finding supports a suggestion that these disturbed motor-nerve innervations within the affected areas imply that a subpopulation of fibers is in a regenerative stage.

**Fibrosis in the muscle**

Within and adjacent to the areas with myositis there was an increased amount of fibrous connective tissue. Some of these areas contained loosely packed muscle fibers of various size and form whereas other areas lacked muscle fibers. After 6w of E/EMS, an extensive fat infiltration was observed in some of the fibrous areas. Fibrosis is essentially an excessive accumulation of extracellular matrix components, particularly collagen, which is the end result of events proceeding from tissue damage via inflammation (Mann et al., 2011). When regeneration of the muscle tissue does not occur, the fibrotic tissue can be infiltrated with adipocytes, causing fatty infiltration (Natarajan et al., 2010). Our findings of bilateral increased fibrosis and fatty infiltration with increased experimental length indicate that the experimental model not only causes injury and myositis but also an imbalance in the repair process with increased connective tissue synthesis and fat infiltration.
General bilateral muscle changes

Muscle fiber phenotype composition

There was a bilateral decrease in the proportion of MyHC-I fibers and a correlated increase in proportion of MyHC-II fibers in both the soleus and gastrocnemius muscles after the experimental intervention. The bilateral changes, which were relatively limited but significant, showed a shift towards a higher proportion of fibers with physiological characteristics of faster contraction and higher force production properties, but lower fatigue resistance, than in the normal situation. These changes suggest that the muscles to some extent have changed their physiological properties. Interestingly, voluntary exercise usually leads to a fiber type transition from fast to less fast fiber types (Pette and Staron, 2000, 2001). Slow to fast transition, as found in this study, has been observed after long-term complete inactivity, spinal cord injury, detraining (Pette and Staron, 2001; Schiaffino and Reggiani, 2011) or experimentally by using tonic low frequency EMS (Hamalainen and Pette, 1996). Since the impulse pattern to a muscle is the major cause for changes in muscle fiber phenotype composition (Hoh, 1975; Pette and Vrbova, 1985), the changes in fiber type composition might thus relate to the use of EMS. Alternatively, although less likely, the bilateral degenerative processes in the muscles may selectively affect the slow motor units more than the fast, or that there is enlargement of preferentially fast motor-units in case of reinnervation.

Significance of muscle phenotype

The cause of the more severe morphological abnormalities in the soleus than in the gastrocnemius muscle is unclear, but one explanation might be the characteristic known differences between the muscles in fiber phenotype composition. The soleus muscle is mainly being composed of slow twitch MyHC-I fibers whereas fast twitch MyHC-II fibers predominate in the gastrocnemius muscle. Previous studies have suggested that fast MyHC-II fibers are more susceptible to injury than slow MyHC-I fibers (Friden and Lieber, 1992; Vijayan et al., 2001). Nevertheless, exercise muscle overuse via EMS seems to affect muscles of different phenotype differently.

Muscle fiber size alterations

The bilateral decrease in fiber CSA after 1 and 3w of E/EMS in both the soleus and gastrocnemius muscles suggests a higher degradation than synthesis of skeletal muscle proteins during the experimental sessions. It seems that the muscle fiber metabolism is inadequate to meet the requirements of the overuse exercise via E/EMS, resulting in muscle fiber degeneration and fiber injury in some areas. Since this trend also was observed in the contralateral side for both muscles, the experiment caused a bilateral impact on the muscle metabolism. However, activation of myogenic cells adjacent to the affected areas might also, to some part, contribute to the decreased mean CSA values. An increased number of newly formed small sized
fibers in the “normal” areas might influence the measurements. Interestingly, the fiber size in the gastrocnemius muscle returned to normal values after 6w of E/MS in both the exercised and non-exercised sides, indicating that the muscle adapted to the intense exercise regime over time. This adaptation could relate to both an increased metabolism with balanced protein synthesis and an increased size of newly formed fibers.

**Capillary supply of muscle fibers**

A significant regression of capillaries around fibers was observed over time in both muscles during the experiment. The degradation of capillaries was bilaterally more extensive in the gastrocnemius muscle than in the soleus muscle. In the soleus muscle, especially in the non-exercised side, the degradation was observed in a later stage and at a lower magnitude than in the gastrocnemius muscle. Interestingly, the modification in number of capillaries per fiber was closely associated with changes in fiber area in all experimental groups, suggesting an adaption of the capillary network to muscle fiber degradation, or vice versa. Since studies have shown that strenuous exercise (Warhol et al., 1985; Brzank and Pieper, 1986), EMS (Koltzenburg et al., 1999; Hudlicka et al., 2003) and inflammation can damage the capillary endothelial cells and cause capillary degradation (Dalakas, 2012), any change in the extent of the microcirculation risks to upset the balance between muscle capillarisation and metabolic requirement. Thus, a limitation of blood supply in combination with an exaggerated physical activity can over time increase the risk for muscle fiber damage (Corsi et al., 1990). The lowered capillary supply, together with the increased proportion of MyHCII fibers, further support a general shift against faster and stronger contracting fibers, but with lower fatigue resistance.

**The tachykinin system**

**Comments on tachykinin antibodies and reaction patterns**

The continuous discoveries of new tachykinins have raised new questions not only on the roles that the various tachykinins have, but also on the nomenclature that should be used (Patachini et al., 2004). Concerning immunohistochemical examinations it is always crucial to know whether the antibody is specific for the molecule/structures specified by the manufacturer.

One of the tachykinin antibodies used for immunohistochemistry (sc-14104) is against a peptide mapping within an internal region of preprotachykinin-1 and is recommended for detection of mature SP and all isoforms of the protachykinin-1 (protachykinin A) precursor. It is well-known that protachykinin-1 is encoded by the TAC1 gene and that it is cleaved not only to yield SP but also neurokinin A (NKA), sometimes called "substance K", and several other tachykinin forms (Harmar et al., 1986; Krause et al., 1987). Both SP and NKA have been well studied and both are
shown to play important roles in neurogenic inflammation. SP acts primarily but not exclusively at the NK1 receptor and NKA primarily at the NK2 receptor (e.g. Bhatia et al 2003). Other tachykinins (endokinins and hemokinins) also show a significant preference for the NK-1R (Helyes et al., 2010).

It was noteworthy that the polyclonal tachykinin antibody (sc14104) gave reactions for structures that also showed reactions by in situ hybridization for demonstration of rabbit tachykinin (SP) mRNA. Tachykinin mRNA expression and immunoreactivity were thus noted for white blood cells as well as blood vessel walls. On the other hand, more restricted immunoreactivity was observed with the monoclonal SP antibody (8450-0505); no immunoreactions were seen for blood vessel walls. This may be related to the fact that this antibody recognizes only the C-terminal end of SP, and that the polyclonal tachykinin antibody detects a tachykinin variant that is present in the blood vessel walls. Both tachykinin antibodies showed similar reaction patterns for nerves structures and white blood cells.

Polyclonal antibodies can amplify signal from target protein with low expression level due to recognition of multiple epitopes, whilst monoclonal antibodies detect only a certain epitope on the antigen. It should nevertheless be stressed that the immunoreactions seen with both tachykinin antibodies were abolished by use of SP blocking peptide. The tachykinin-like immunoreactions in white blood cells were nevertheless comparatively weak compared with the reaction in nerves with both antibodies. This can be related to the known fact that there is a low sensitivity of commonly used immunohistochemical techniques in detecting tachykinin in non-neuronal cells (Erin and Ulusoy, 2009).

**Comments on tachykinin terminology**

It was decided to describe the overall immunohistochemical reactions with the tachykinin antisera as "tachykinin-like" immunoreactions/immunoreactivity. An alternative nomenclature would have been “SP-like”. Also in order to be consistent, the term tachykinin and not SP was furthermore used for the measurements that were made by EIA, despite the fact that less than 0.01% cross-reactivity was reported for the closely SP-related peptides neuropeptide K and neurokinin A.

**Comments on the tachykinin RNA probe**

It was found to be of extra importance to verify that the mRNA for tachykinin (in the situ hybridization studies) was not related to detectability of hemokinins as we noted tachykinin mRNA reactions in non-neuronal cells. It should here be recalled that hemokinin-1, which is encoded by the TAC4 gene, is a tachykinin peptide reported to predominantly be expressed in non-neuronal cells (Wang et al., 2010). It is thus known to be involved in immune functions and inflammation (Wang et al., 2010; Liu et al., 2011). "BLAST-Basic Local Alignment Search Tool” evaluation confirmed that the probe used not detect rabbit hemokinin-1.
Comments on NK-1R immunoreactions

It was observed that the NK-1R immunoreactions for white blood cells were principally detected within the cytoplasm of the cells. This shows that the receptor is captured in an internalized state, i.e. the NK-1R is being internalized after binding to SP. Alternately the feature represents newly synthesized receptors. Concerning neurons, it is well-known that the NK-1R undergoes rapid internalization after binding to SP has occurred and that the receptor thereafter recycles to the plasma membrane (Mantyh, 2002). The locations of the NK-1R intracellularly are transport vesicles and endosomes (Goto et al., 1998). Also in non-neuronal cells, there is a process of endocytosis and recycling of the NK-1R (Roosterman et al., 2004). It might be that the white blood cells are under continuous influence by tachykinin in an autocrine/paracrine fashion and that there is a continuous receptor internalization.

Reactions in nerve structures

The great majority of the nerve fascicles in the soleus and gastrocnemius muscles were entirely composed of myelinated nerve fibers. These nerve fascicles did not contain nerve fibers showing tachykinin-like or NK-1R immunoreactions. Only a few fine nerve fibers exhibiting tachykinin-like and NK-1R immunoreactions were seen in the normal muscle. This finding of very few such nerve fibers in normal muscle is in accordance with observations in previous studies showing that the number of SP-containing C-fibers is much lower in the innervations of muscle than the skin in the rat hindlimb (McMahon et al., 1984).

On the other hand, in the experimental animals, marked tachykinin-like and NK-1R immunoreactions were noted in nerve fascicles that were located in myositis areas and in the close proximities of these areas. That was observed for the exercised and non-exercised sides of both the soleus and gastrocnemius muscles. Frequent fine freely dispersed nerve fibers exhibiting tachykinin-like and NK-1R immunoreactions were also observed in inflammatory areas of both sides and both muscles.

The tachykinin-like and NK-1R immunoreactive nerve profiles were related to Schwann cells that not only showed cytoplasmatic S-100beta reactions, but also to certain extents appeared to show nuclear S-100beta reactions, which could represent activated Schwann cells. It may be that Schwann cells are activated in order to repair axons after nerve damage, where axons of regenerating nerve fibers are related to these Schwann cells (Hu et al., 2003; Duobles et al., 2008). It should, however, be recalled that the immunohistochemical reactions seen in the microscope are related to the section thickness, and “overlapping” reactions can occur. Thus interpretations of locations of immunoreactions should be made with caution.

The results in the present study are consistent with the previous finding of increases in the density of SP-immunoreactive nerve fibers in rat skeletal muscle in response to persistent inflammation (Reinert et al., 1998). In the present model, the increase was
noted for myositis areas and areas close to these. It was also observed that there sometimes was a co-localization between tachykinin-like and NK-1R reactions. This can be related to autoreceptor functions of tachykinin. Thus, NK-1R on sensory neurons is suggested to be related to a modulation of peripheral pathophysiological effector functions (Lever et al., 2003).

It is likely that nerve-related tachykinin can contribute to the focal inflammation, tachykinins hereby attracting and activating inflammatory cells (Chiu et al., 2012). Therefore, the increased tachykinin-like and NK-1R innervations can be involved in the inflammation processes in the myositis areas, and lead to vasoactive processes. It is also likely that the sensory innervation is rapidly affected by the overuse in the exercised side, leading to central effects. As will be discussed below, this can secondarily lead to the marked upregulation of the tachykinin system followed by myositis in the contralateral side. Nevertheless, not only tachykinins, but also other signal substances, are likely to be involved in the myositis processes. Studies in our research group have e.g. shown that there is an expression of the glutamate transporter VGluT2 and the glutamate receptor NMDR1 in white blood cells in the myositis process (Spang et al., 2012).

**Blood vessel wall-related reactions**

Tachykinin mRNA reactions and tachykinin-like immunoreactions were seen in the smooth muscle layer and to some extent in the endothelial layer of some blood vessel walls. It is well-known from the literature that tachykinins can be produced in the endothelium of blood vessels (Ralevic et al., 1990), but there are also reports that tachykinins can be produced in the smooth muscle cells of blood vessel walls (Munoz et al., 2010b). The intensity of tachykinin immunoreactivity in the vessel walls varied between different regions and the overall magnitude of the vessel reactions differed between the different groups. Strong reactions in the vessel walls were seen in the areas with myositis and/or adjacent regions to these. Evaluations of tachykinin-like immunoreactions revealed that there was an increase in the degree of immunoreactivity with increasing experimental length. NK-1R immunoreactions and NK-1R mRNA reactions were also found in the endothelium and the smooth layer of vessels in myositis areas. No such reactions were seen for normal muscle tissue.

The upregulation of the tachykinin system for the blood vessels in response to the experimental procedure suggests that tachykinins can be involved in vascular functions and angiogenic events in the myositis processes. In accordance with this suggestion, it is well-known that SP is a potent factor leading to vasodilation and neurogenic plasma extravasation (Lembeck and Holzer, 1979) and that SP is a potent and highly effective endothelium-dependent vasodilator of various vascular beds (Lam, 2000). Nitric oxide has been shown to have a role in the SP-induced vasodilation (Karabucak et al., 2005) and SP has been found to regulate vasodilator activity of CGRP (Brain and Williams, 1988). SP has also been shown to elicit relaxation of vascular smooth muscle by acting on receptors on the luminal surface of
endothelial cells, e.g. in the rabbit and dog renal and celiac arteries (Furchgott and Zawadzki, 1980; Ralevic et al., 1990). Furthermore, it has in several studies been shown that SP has angiogenic properties (Ziche et al., 1990; Fan et al., 1993; Kohara et al., 2010). The binding of tachykinin to NK-1R of vessel walls can play important roles in proinflammatory processes, i.e. in the activation and recruitment of leukocytes.

**White blood cell reactions**

Expression of tachykinin, as well as NK-1R, was detected in white blood cells at both protein and mRNA levels. As seen in the immunohistochemical double-stainings, the cells corresponded to macrophages and eosinophils. These findings are in accordance with previous observations that SP has been detected in macrophages (Bost et al., 1992) and eosinophils (Aliakbari et al., 1987), and that NK-1R is detected in several types of white blood cells (Bost et al., 1992; Sipka et al., 2010). Based on these findings it is of interest to note that tachykinin synthesised in non-neuronal cells, such as white blood cells, can be especially important in pathological conditions (Erin and Ulusoy, 2009). Non-neuronal SP is thus thought to be an important factor in psoriasis via binding to NK-1R (Amatya et al., 2011).

As mentioned before, it is frequently emphasized that tachykinins can have proinflammatory effects in inflammatory conditions (Zimmerman et al., 1992; De Swert et al., 2009). It is therefore tempting to suggest that tachykinins induce marked proinflammatory effects in the myositis process, recruiting and activating white blood cells. It is obvious that parts of the inflammatory-mediating effects can be produced via autocrine/paracrine actions as tachykinin-like and NK-1R immunoreactions generally were co-occurring in the white blood cells.

**Possible healing effects of tachykinins**

The effects of tachykinins in the myositis areas may not only be related to inflammatory destructive effects. The effects can actually be related to healing events. It is thus known that SP can be involved in corneal wound healing (Nishida, 2005). SP is also known to enhance wound closure in nitric oxide synthase knockout mice (Muangman et al., 2009) and to promote skin wound healing via stimulation of fibroblast proliferation and inhibition of apoptosis, possibly in this case in the long run leading to scar formation in the skin (Jing et al., 2010). An inflammatory component can actually on the whole be involved in the healing effects. Topical treatment of SP to skin wounds leads to an increase in inflammatory density in the healing process of skin wounds in genetically diabetic mice (Scott et al., 2008b). SP can also be involved in tendon healing (Burssens et al., 2005a; Carlsson et al., 2011).
Possible involvement in muscle fiber necrosis and regeneration

NK-1R was not only detected in white blood cells dispersed in the tissue but also in such cells that were infiltrated into the muscle fibers. Desmin-negative fibers infiltrated by leukocytes were considered to be degenerating/necrotic muscle fibers. On the other hand, another type of muscle fiber also showed NK-1R reactions (point like-reactions). These fibers showed marked desmin immunoreactions and were interpreted to be in a regenerative stage. This is in accordance with the known fact that there is an over expression of desmin during muscle regeneration processes (Gallanti et al., 1992).

Results of use of enzyme inhibitors

It was evident that the combination of E/EMS and the injections with the ACE- and NEP-inhibitors markedly increased the morphological changes of the tissue. There were clearly less effects in the NaCl group. The tissue changes in the C+Th and SP+C+Th groups were mainly found in areas with myositis and nearby regions. Pronounced tachykinin-like and NK-1R immunoreactions and elevated tachykinin concentrations were also seen for these groups. A central part to this effect may be the fact that tachykinin effects can be terminated by enzymes, out of which NEP and ACE are the most important (Matsas et al., 1984; Scholzen and Luger, 2004). However, one should not forget that enkephalins are also hydrolyzed by NEP (Vijayaraghavan et al., 1990) and that ACE, in addition to tachykinins, also degrades endothelin-1 and presumably CGRP as well (Yang et al., 1970). In any case, the immunohistochemical observation showed that there was an upregulation of NK-1R. It is also evident that there was a booster effect on endogenous SP production.

Summarizing points concerning the tachykinin system

It is evident that in parallel with the development of marked muscle derangement and myositis, an upregulation of the tachykinin system occurs in response to the E/EMS. The upregulation of tachykinin/NK-1R was not only found in the nerve innervation, but also in non-neuronal sources as blood vessel walls and white blood cells in the derangement/myositis process. Thus, tachykinins are likely to be of importance for the inflammation process in myositis areas. In accordance with this suggestion, SP and NK-1 R has in a large number of studies been shown to be of great importance in inflammation processes at different locations (Bhatia et al., 1998; Cao et al., 2000; Caviedes-Bucheli et al., 2007; Wu et al., 2007; Bhatia, 2010).

Interestingly, the findings that NK-1R was detected in the white blood cells infiltrating into necrotic muscle fibers as well as in muscle fibers showing regenerating characteristics, indicate that tachykinin are involved in both degeneration (necrosis) and regenerating processes for muscle fibers. This is completely new information and indicates that tachykinins can have a double-edged
function in relation to inflammation and damage in muscle tissue. A similar conclusion has been made for other tissues (Jing et al., 2010).

**What is the cause of the cross transfer effects?**

The outcome from these studies clearly indicates that there is a signaling system across the midline of the body. The findings provide support to previous suggestions that the nervous system can cause deleterious cross-transfer effects related to the locomotor system. For example there is a remarkably symmetrical distribution of inflammation in RA (Shenker et al., 2003). The symmetry of inflammation in RA is probably mediated by stimuli-specific responses of the sensory nerves, a unilateral proinflammatory stimulus causing a contralateral reaction (Shenker et al., 2003; Kelly et al., 2007). Moreover, a recent study on the effects of treatment with minimally invasive scraping technique for patients with bilateral Achilles tendinopathy showed that unilateral treatment mostly lead to curing of the chronic pain also on the non-treated side (Alfredson et al., 2012). A sensory cross over neuronal mechanism was suggested to be responsible for the contralateral effects on the non-operated side.

Other important findings supporting crosstalk of the nervous system are the previous reports of increased strength in the homologous contralateral muscle after unilateral exercise (Zhou, 2000; Munn et al., 2004; Carroll et al., 2006). Hypothetically the increased strength in the contralateral limb after the unilateral exercise described in previous studies is akin to a process of motor learning, i.e motor areas in the brain that are responsible for motor control have adapted to unilateral voluntary training and the opposite hemisphere in the brain may have access to these modifications (Carroll et al., 2006; Lee and Carroll, 2007). Alternatively, unilateral training might enhance the organization of the spinal and cortical motor pathways to the contralateral limb which results in an increased drive to the untrained limb (Lee and Carroll, 2007).

The mechanism behind the severe bilateral muscle damage and inflammation after unilateral E/EMS, as seen in the present study, and the reports of increased muscle strength commended on above may not be the same. The contralateral muscle alterations and inflammation seen in this study may thus be caused by another neuronal mechanism. Since there is some evidence for a commissural system in the spinal cord that mediates transmedian signaling with a fairly precise bilateral representation (Koltzenburg et al., 1999), nerve signals from the trained side may pass over to the contralateral muscles through commissural inter-neurons. Our findings of a cross-transfer upregulation of nerve-related tachykinin and NK-1R, indicate that tachykinin trelated to the nerves might be involved in the inflammatory process not only in the exercised muscle but also in the contralateral homologous muscle. It seems not far-fetched to suggest that the exercise-induced overuse at an early stage leads to an affection of the sensory innervation in the exercised side.
Secondary effect can then lead to sensory affection contralaterally. The precise background for the cross-over effect should nevertheless await further studies.

Even if our findings strongly indicate an involvement of the nervous system in the cross transfer effects, it cannot completely be excluded the possibility that the bilateral adaptive and degenerative changes in this study relate to changed levels of humoral substances that have exerted a modulating effect on the muscle motor system (Caiozzo et al., 1992; Larsson et al., 1995; Canepari et al., 1998). However, myositis and histopathological changes were only present in focal areas of the muscle tissue, a finding that contradicts a circulatory effect as a major cause. Furthermore, it is known that ligating the draining venous system of the inflamed area prior to an insult does not abolish the contralateral response (Levine et al., 1985b), while lesion of nociceptive nerves supplying either the contralateral or the ipsilateral limb prior to inflammatory insults, abolishes the contralateral responses (Shenker et al., 2003). Regardless of the cause, our study shows that the experimental model with frequent unilateral overuse via E/EMS causes cross-transfer effects in the fiber composition, vascular supply and nerve fascicle morphology in the muscles. Since some cross-transfer effects may be harmful and others not, the occurrences of contralateral cross-transfer processes should be considered in clinical situations with muscle damage and inflammation.
**SUMMARY**

In summary, the repetitive unilateral E/EMS led to the following changes in both the exercised and the non-exercised sides of the muscles:

- Both muscles contained focal inflammation and presence of necrotic fibers

- The areas with myositis contained muscle fibers in degenerative and regenerative stages

- The fiber size was generally decreased in both muscles after 3w of E/EMS

- There was a decreased capillarization of muscle fibers, especially in the gastrocnemius muscles. The degradation of capillaries correlated with the decrease in fiber size

- There was a shift, although restricted, in fiber phenotype composition against lower proportion of fibers containing MyHCI and higher proportion of fibers containing MyHCII in both muscles

- There were structural changes in the nerve fascicles and in the reactivity pattern of AChE on fibers

- An increased number of enlarged capillaries and/or arterioles/venoles were present in the focal areas with severe myositis. Furthermore, in the adjacent areas with less inflammation and fibrosis, the muscle fibers were generally surrounded by few capillaries

- The tachykinin system was upregulated in myositis areas. This was related to an increase in tachykinin expression in the nerves and the blood vessel walls. Tachykinin-like reaction was also present in the infiltrating white blood cells.

- The concentration of tachykinin in the muscle tissue increased in a time-dependent manner

- The expression of NK-1R was upregulated. NK-1R immunoreactions and NK-1R mRNA reactions were found in cells of the inflammatory infiltrates and in blood vessel walls. Axons within the affected areas showed NK-1R immunoreactivity.

- NK-1R immunoreactions were noted within necrotic and regenerating muscle fibers

- The combination of E/EMS and the injections with the ACE- and NEP-inhibitors led to marked morphological changes and a pronounced inflammation of the tissue. The combined treatment also led to marked tachykinin-like and NK-1R immunoreactions and to elevations in tachykinin concentrations.
CONCLUSIONS

It is evident that unilateral exercise via EMS causes muscle injury and myositis and other structural changes that are cross-transferred to the homologous contralateral muscle in the opposite side of the body. Our findings of an upregulation of the tachykinin system in the muscles of both sides indicate that the tachykinin system is involved in the cross transfer effects.
SVENSK SAMMANFATTNING

Ensidig träning kan ge viss styrkeeffekt även i kontralaterala muskler. Skadliga förändringar även kan överföras till motsatta sidan, vilket illustreras av den strikta symmetriska utbredningen av vissa kroniska inflammatoriska sjukdomar. Kunskapen om effekterna av överbelastning av muskler är begränsad, och det finns i stort sett ingen information om hur ensidig överbelastning påverkar kontralaterala muskler. Med den informationen som bakgrund har en studie utförts för att testa hypotesen att ensidig muskelöverbelastning orsakar förändringar i vävnadsstrukturer och i tachykinin systemet, med fokus på substans P (SP), inte bara i de tränaade musklerna, utan även i de kontralaterala musklerna. SP är en neuromodulator som är känd för att vara pro-inflammatorisk.

En experimentell kaninmodell användes där ensidig överbelastning av soleus och gastrocnemius musklerna utfördes genom träning med elektrisk muskelstimulering (E/EMS). Totalt 40 kaniner indelades slumpmässigt i sju grupper, varav två grupper tjänade som kontroller. Kaninerna sövdes och sattes sedan i en "spark maskin" för att utföra träning via EMS 2 timmar varannan dag. Den experimentella perioden för grupperna 1-3 var 1, 3 och 6 veckor, medan grupp 4-6 trädes i 1 vecka efter att ha injicerats utanför servänvaden med SP, NaCl, Captopril (C), en ACE-hämare, och med DL- Thiorphan (Th), som inhiberar aktiviteten av endopeptidas. En grupp medverkade inte i experimentet. Dagen efter sista E/EMS experimentet avlägnades soleus och gastrocnemius musklerna från både den experimentella och icke-experimentella sidan. Förändringar i muskel-struktur och i tachykinin systemet analyserades med enzym och immunhistokemiska metoder, in situ hybridisering och ELA teknik.

Efter 1 vecka av ensidig E/EMS, innehöll de tränaade musklerna i vissa områden en mild infiltration av inflammatoriska celler (myosit) och en mindre mängd morfologiskt förändrade muskelfibrer. Efter 3 och 6 veckor av E/EMS fanns det bilateralt lokal en distinkt myosit och muskelförändringar i både soleus och gastrocnemius musklerna. Förändringarna, som främst observerades i områden med myosit, bestod av en hög variabilitet i fiber storlek, fiber split, inre kärnor, nekrotiska fibrer, fibros, fettinfiltration och små fibrer som innehöll MyHCs relaterat till utveckling. Bilaterala morfologiska förändringar som förlost av axoner observerades även i nerver. Dessutom var uttrycket för tachykinin och den receptor som SP främst binder till, neurokinin-1 (NK-1R), bilateralt upp-reglerade i nervstrukturer och blodkärlsväggar. De infiltrerade vita blodkroppar i områdena med myosit, uttryckte tachykinin och NK-1R immunreaktioner. NK-1R reaktioner sågs också i nekrotiska och regenererande muskelfibrer.

Konzentrationen av tachykinin ökade signifikant både i soleus och gastrocnemius efter 3 och 6 veckor av E/EMS. Det fanns en signifikant korrelation mellan de två sidorna i koncentrationen av tachykinin och i intensiteten för tachykinin immunreaktion i blodkärlens väggar. Fiberstorlek och kapillärförsörjning av muskelfibrer var bilateralt minskade efter 3 veckor av EMS. Regionerna med myosit innehöll ett ökat antal kärl som var större än kapillärer, medan områden med ökad bindvävsinlagring innehöll få kapillärer. I båda musklerna var det en bilateral fibertyp förskjutning mot en lägre andel långsamma MyHCl fibrer och en högre andel snabba MyHCII fibrer. Lokala injektioner av C + Th och SP + C + Th gav en markant ökade uttryck för muskelvävnadens strukturer och ledde till ökade NK-1R och tachykinin uttryck i områdena med myosit och ökad tachykinin koncentration i vävnaden. Sammanfattningsvis ledde den repetitiva ensidiga muskel-överbelastningen via E/EMS med tiden till muskelkallas och myosit. De drabbade områdena innehöll både degenerativa och regenerativa förändringar i muskel och nervväv samt en upp-reglering av tachykinin systemet. Mest intressant är att förändringarna inte bara skedde i den tränaade sidan, utan även i den homologa kontralaterala muskeln. Tachykinin systemet verkar vara inblandat i erhållandet av vävnadförfaradningar och överföringseffekten.
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REFERENCES


Denko CW, Petricevic M (1978) Sympathetic or reflex footpad swelling due to crystal-induced inflammation in the opposite foot. Inflammation 3; 81-86.


Kelly S, Dunham JP, Donaldson LF (2007) Sensory nerves have altered function contralateral to a monoarthritis and may contribute to the symmetrical spread of inflammation. Eur J Neurosci 26; 935-942.


Koltzenburg M, Wall PD, McMahon SB (1999) Does the right side know what the left is doing? Trends Neurosci 22; 122-127.


Lembeck F, Holzer P (1979) Substance P as neurogenic mediator of antidromic vasodilation and neurogenic plasma extravasation. Naunyn Schmiedebergs Arch Pharmacol 310; 175-183.


Österlund C, Liu JX, Thornell LE, Eriksson PO (2013) Intrafusal myosin heavy chain expression of human masseter and biceps muscles at young age shows fundamental similarities but also marked differences. Histochem Cell Biol


