THE EXPRESSION OF MUSCLE SPECIFIC GENES IN MECHANICAL VENTILATION AND REGENERATION

PhD thesis

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LIST OF PUBLICATIONS RELATED TO THE THESIS

I. Gayan-Ramirez GN, Testelmans D, Maes K, *Rácz GZ*, Cadot PG, Zádor E, Wuytack FC, Decramer ML (2005) Intermittent spontaneous breathing protects the rat diaphragm from mechanical ventilation effects. *Crit Care Med* **33**(12):2804–2809.

II. Fenyvesi R, *Rácz GZ*, Wuytack F, Zádor E (2004) The calcineurin activity and MCIP1.4 mRNA levels are increased by innervation in regenerating soleus muscle. *Biochem Biophys Res Comm* **320**:599–605.

III. *Rácz GZ*, Gayan-Ramirez G, DePaepe K, Zádor E, Wuytack F, Decramer M (2003) Early changes in rat diaphragm biology with mechanical ventilation. *Am J Respir Crit Care Med* **168**(3):297–304.

IV. Zádor E, Szakonyi G, *Rácz G*, Mendler L, Ver Heyen M, Lebacq J, Dux L and Wuytack F (1998) Expression of the sarco/endoplasmic reticulum Ca^{2+}-transport ATPase protein isoforms during regeneration from notexin-induced necrosis of rat soleus muscle. *Acta Histochem* **100**:355–369.

LIST OF OTHER PUBLICATIONS, NOT RELATED TO THE THESIS

V. De Paepe B, *Rácz GZ*, Schroder JM, De Bleecker JL (2004) Expression and distribution of the nitric oxide synthases in idiopathic inflammatory myopathies. *Acta Neuropathologica Berl* **108**:37–42.

VI. De Paepe B, Schroder JM, Martin J-J, *Rácz GZ*, De Bleecker JL (2004) Localization of the α-chemokine SDF-1 and its receptor CXCR4 in idiopathic inflammatory myopathies. *Neuromusc Disord* **14**:265–273.

CONTENTS

1. ABBREVIATIONS

ATP	adenosine-triphosphate
CnA	calcineurin A
CMV	controlled mechanical ventilation
DAB	diamino-benzidin
DTT	dithiothreitol
EDTA	ethylene-diamine-tetraacetic acid
EGTA	ethylene-glycol-bis(β-aminoethyl ether)-*N*,*N'*-tetraacetic acid
GAPDH	glycerinaldehide-3-phosphate-dehidrogenase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Id	inhibitor of DNA-binding
ISB5	5 min of intermittent spontaneous breathing after 5 hr 55 min CMV
ISB60	60 min of intermittent spontaneous breathing after 5 hr CMV
MCIP	modulatory calcineurin interacting protein
mRNA	messenger ribonucleic acid
MyHC	myosin heavy chain
MRF	myogenic regulatory factor
NFAT	nuclear factor of activated T-cells
PMSF	phenylmethanesulfonyl-fluoride
PS	passive shortening
RT-PCR	reverse transcription, followed by polymerase chain reaction
SB	spontaneous breathing
SDS-PAGE	sodium-dodecyl-sulphate/polyacrylamide gel electrophoresis
SERCA	sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase

2. INTRODUCTION

Almost half a century ago, the cross innervation experiments of Buller and Eccles provided compelling evidence for the essential role of innervation in determining the properties of mammalian skeletal muscle fibers. They revealed that terminally differentiated muscle fibers are not inalterable but highly versatile entities capable to adapt their phenotype to the altered functional demands. This malleability of muscle fibers inspired the introduction of the term 'plasticity', now a commonly used term in neurophysiology (Buller *et al.,* 1960). Muscle adaptation plays crucial role in a variety of physiological and pathological conditions and processes, from marathon runner's training to muscle hypotrophy in case of muscle disuse during space flights.

Each muscle of the mature limbs and the trunk is a heterogeneous tissue composed of many muscle fibers. The fibers can be classified into different groups according to morphological (*e.g.* size, capillarization), physiological (*e.g.* contraction rate, fatigue resistance, maximal force generation) and biochemical features (*e.g.* succinate dehydrogenase level, myosin ATPase activity; *reviewed by* Pette and Staron 1993). The proportion of the different muscle fibers determines the gross properties of a muscle. The myofibers of normal muscles (*i.e.* not actually adapting to changing circumstances) exhibit a high degree of co-ordination in these attributions (Talmadge *et al.,* 1996; Quiroz-Rothe and Rivero 2001, 2004).

In the rodent musculature, myofibers that experience phasic patterns of contractile work—brief bursts of activity interspersed within long periods of inactivity—will assume the fast contracting, glycolytic phenotype (type IIB). Myofibers subjected to tonic patterns of work activity—sustained periods of repetitive contraction on a habitual basis—will take on fast oxidative (type IIA) or slow oxidative (type I) properties. The latest discovered type IIX fiber has intermediate properties between type IIA and IIB (Schiaffino *et al.,* 1989; Pette and Staron 1993; DeNardi *et al.,* 1993).

At the molecular level, the myosin molecules play predominant role in specifying the muscle fiber properties such as the contraction velocity and force. The myosin heavy chain (MyHC) component of the myosin molecule constitutes the primary basis for the aforementioned traditional classification of muscle fibers. In the different types of muscle fibers, different MyHC isoforms are present, called MyHC1, 2a, 2b and 2x. In addition, specific MyHC isoforms are expressed during the embryonal and neonatal developmental stages (Pette and Staron 1993; Schiaffino and Reggiani 1996). Hybrid fibers containing more than one MyHC isoform can exist even under normal circumstances. In addition to MyHCs,

many other proteins exist as multiple isoforms, all of which contribute to the physiological and biochemical differences between fiber types.

The SERCA pumps transport cytosolic free Ca^{2+} (which induce the contraction) to the sarcoplasmic reticulum after cell activation, bringing the Ca^{2+} concentration back to resting level, thus allowing relaxation (Figure 1). The relaxation kinetics of different muscles correlates with the different SR Ca^{2+}-ATPase activity (*reviewed in* Dux 1993) and the fiber type-specific expression of fast and slow myosins correlates with the expression of fast and slow SERCAs (Talmadge *et al.,* 1996). Among the three SERCA genes SERCA1 and SERCA2 are expressed in muscle and are alternatively spliced into different transcripts. In adult rat fast muscle fibers (type II) SERCA1a, in slow skeletal (type I) and heart muscle fibers SERCA2a is expressed. In developing muscle, the splice variant of SERCA1 gene, SERCA1b is transiently expressed (Brandl *et al.,* 1987). The SERCA2b protein is translated from three different transcripts and it is commonly expressed in most tissues (Misquitta *et al.,* 2002). SERCA3 isoforms are expressed in various cell types, such as white blood cells, platelets, Purkinje cells or secretory cells, including pancreatic β-cells (*reviewed in* Wuytack *et al.,* 2003).

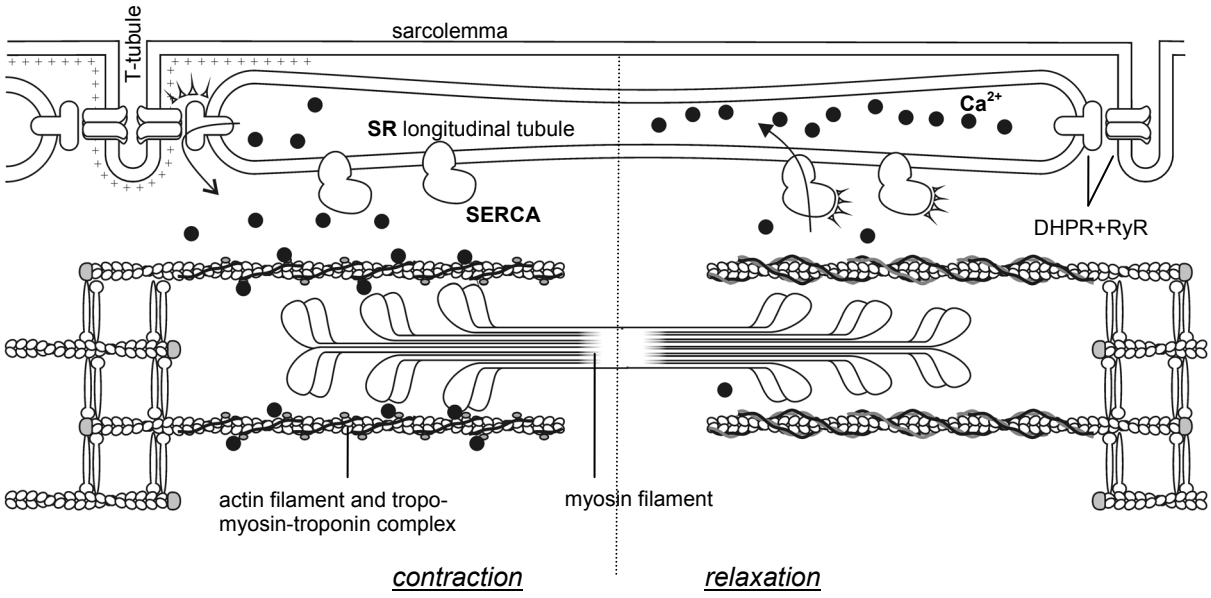

Figure 1. Schematic process of contraction and relaxation in one sarcomere and adjacent sarcoplasmic reticulum (SR). Depolarization of the sarcolemma is propagated along the T-tubule (left side). The activated dihydropyridine receptor (DHPR) opens the ryanodine receptor (RyR), so that Ca^{2+} (black dots) is released from the SR to the cytosol, initiating the contraction through the conformational change of the tropomyosin-troponin complex. When stimulation ceases, SERCA lowers the cytosolic Ca^{2+} concentration, thus inhibiting contraction.

Patterning of skeletal muscle fiber composition begins to appear during embryonic development, through interaction between myoblasts and the limb environment (Chevallier and Kieny 1982), independently of innervation (Butler *et al.,* 1982). However, any muscle can be partially or completely remodelled by stimuli applied to fully mature adult myofibers: by hormonal influences (e.g., thyroid hormone), but most importantly by different patterns of motor nerve activity and contractile work (Buller *et al.,* 1960; Pette and Vrbová 1985). These determinants may influence the fiber type-specific gene expression via diverse, but presumably not mutually exclusive pathways.

First, the four myogenic basic helix-loop-helix (bHLH) proteins, myogenin, MyoD, myf-5 and MRF4 were found to be regulated by electrical activity and to mediate the expression of a variety of downstream proteins in pre-existing muscle fibers (*reviewed in* Perry and Rudnicki 2000; Carlsen and Gundersen 2000). Also the differentiation of myogenic cells, during embryogenesis and in muscle culture experiments, has been shown to be regulated by these myogenic regulatory factors (MRF) (Molkentin and Olson 1996; *reviewed in* Sabourin and Rudnicki 2000). MRFs operate as heterodimers bound to ubiquitous bHLH factors called E-proteins, recognizing a 6-base pair DNA sequence, the CANNTG motif (also called E box), to activate a large number of muscle-specific genes. The inhibitor of DNA-binding proteins (Id; also called inhibitor of differentiation) are known inhibitors of myogenesis (Benezra *et al.,* 1990). Four Id proteins have been isolated from mammalian organisms (Melnikova and Christy 1996).The Id1 has been proposed as a mediator of muscle disuse atrophy (Gundersen and Merlie 1994), and it is upregulated in electrically silent muscles (Carlsen *et al.,* 2000). Id proteins contain the HLH dimerization motif but lack the domain rich in basic amino acids, which mediates DNA binding. As a consequence, they sequester bHLH factors, preventing the formation of active E-protein–MRF heterodimers, so that they block the expression of muscle specific genes and are therefore considered to act as dominant negative regulators of differentiation pathways.

Second, the temporal and chronic elevation of the intracellular concentration of calcium ions regulates many signalling pathways associated with muscle plasticity, such as fiber hypertrophy and shifts in fiber phenotype (*reviewed in* Olson and Williams 2000; Bassel-Duby and Olson 2003; Williams and Kraus 2005). Calcineurin, a Ca^{2+}-calmodulin-dependent protein serine/threonine phosphatase (also known as protein phosphatase 2B) has been implicated as a regulatory molecule involved in the transduction of contractile activity–based signals to molecular signals, so that it may have pivotal role in the regulation of muscle

growth and phenotypic gene expression in skeletal muscle (Chin *et al.,* 1998). Calcineurin A exists in three isoforms. Among these the α and β isoforms are present in skeletal muscle (Parsons 2003). Calcineurin is activated by calmodulin upon binding Ca^{2+} and dephosphorylates transcription factors, including the phosphorylated isoforms of the nuclear factor of activated T-cells (NFAT) (Figure 2). Upon dephosphorylation, NFAT isoforms translocate into the nucleus where they can bind to a conserved DNA binding site known as the NFAT response element and alter transcription, leading to enhanced expression of specific genes (Olson and Williams 2000, Schiaffino and Serrano 2002).

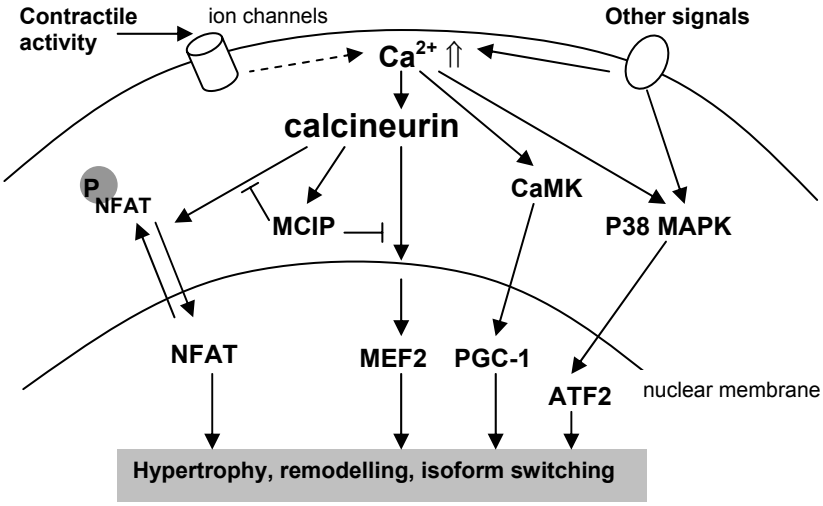

Figure 2. Molecular signalling pathways link changes in contractile activity to changes in gene expression that establish myofiber diversity. A tonic pattern of motor nerve activity promotes changes in intracellular calcium that trigger a variety of intracellular events that modify the function of nuclear transcription factors. Other signals are received by cell surface receptors to activate similar or parallel signaling events. Signalling proteins that participate in transducing effects of contractile activity to specific genes include ion channels, protein phosphatases and protein kinases (calcineurin, CaMK, p38MAPK), DNA-binding transcription factors (NFAT, MEF2, PGC-1, ATF2), and endogenous inhibitors (MCIP). (Williams and Kraus 2005).

2.1. Background: Mechanical ventilation study

Various modes of mechanical ventilation are essential intervention in the intensive care unit used for the management of respiratory failure. Difficulties in weaning patients from mechanical ventilation are frequent and concern about 20–30% of the patients after prolonged mechanical ventilation (Esteban *et al.,* 1995). Mechanically ventilated patients suffering from chronic obstructive pulmonary disease encounter weaning delay or difficulty even in 35%–67% (Sporn and Morganroth 1988; Tobin and Alex 1994). Weaning failure may be due to a variety of factors including unresolved primary illness, nosocomial infection, inadequate

ventilatory drive, upper airway disease, increased work of breathing, cardiac failure or respiratory muscle weakness (Sporn and Morganroth 1988; Davidson and Treacher 2002). Importantly, these factors are interrelated and several of them may be present in the same patient. Disuse atrophy is likely to develop in the diaphragm of patients under long-term mechanical ventilation, however, diaphragm inactivity is probably not the unique cause of weaning failure. Respiratory muscle weakness may develop during mechanical ventilation for causes other than disuse, including muscle disease, sepsis, sedation, oxidative stress (Shanely *et al.,* 2002; Zergeroglu *et al.,* 2003; Vassilakopoulos and Petrof 2004), corticosteroid treatment (Decramer and Stas 1992; Gayan-Ramirez *et al.,* 1999), but mechanical ventilation by itself may also lead to inspiratory muscle dysfunction (Goldstone 1994).

Assessment of respiratory muscle function is difficult in critically ill patients particularly because it requires voluntary manoeuvres highly dependent on patient motivation and cooperation. Therefore, animal models of controlled mechanical ventilation were developed in which the deleterious effects of controlled mechanical ventilation on respiratory muscle function were highlighted. The rationale of these studies is the deeper understanding of the molecular mechanisms of the muscle dysfunction that may develop in mechanically ventilated patients in order to facilitate the weaning strategies aiming to avoid this deleterious effect. Among the respiratory muscles, the diaphragm deserves special consideration, accounting for 70% of the resting ventilation. Histologically it is similar to skeletal muscles, with approximately 50% type I fibers in most species, and is therefore susceptible to the same physiological processes, including the development of fatigue or atrophy.

Numerous studies have consistently found that controlled mechanical ventilation (CMV) leads to decreased force-generating capacity of the diaphragm. The maximal transdiaphragmatic pressure generated during phrenic nerve stimulation *in vivo* was evidently reduced after even 1 day CMV in rabbits (Sassoon *et al.,* 2002), 3 days in piglets (Radell *et al.,* 2002) or 11 days in baboons (Anzueto *et al.,* 1997). *In vitro* contractility measurements on isolated diaphragm strips showed rapid, progressive and severe decline in contractile function after even 12 hours–3 days of CMV in rats (LeBourdelles *et al.,* 1994; Yang *et al.,* 2002; Powers *et al.,* 2002; Gayan-Ramirez *et al.,* 2003), and in rabbits (Sassoon *et al.,* 2002).

The morphological changes, *i.e.* reduced diaphragm muscle mass and/or muscle fiber atrophy observed in both short (less than 2 days) and longer term CMV could explain the reduced force generation. Two (Le Bourdelles *et al.,* 1994) or 4 days CMV (Yang *et al.,* 2002) leads to reduced diaphragm muscle mass without affecting hindlimb muscle weight.

This suggests also that diaphragm is particularly vulnerable to disuse effects. The decreased diaphragm protein levels, increased proteasome activity (Shanely *et al.,* 2002, 2004), and reduced mRNA level of the anabolic insulin-like growth factor-I (Gayan-Ramirez *et al.,* 2003) supports that atrophy is in process during CMV.

The majority of the muscle fiber protein content consists of the contractile proteins, including myosin heavy chains (MyHC). CMV results in modifications of MyHC expression, which gradually leads to fiber type transformation within the diaphragm. After 18 hours of CMV in rats both type I (slow) and type II (fast) fibers are reduced in size, but with the type II fiber population exhibiting a greater degree of atrophy (Shanely *et al.,* 2002). In rabbits, 2 days of CMV also resulted in atrophy of the respiratory muscles and a preferential decrease in the cross-sectional area of type IIa and IIb fibers (Bernard *et al.,* 2003). These changes marks an initial fast-to-slow fiber phenotype shift and must be at least partially responsible for the decline of force production. More prolonged CMV appears to result in different pattern. Four days of CMV leads to an increase of the number of hybrid fibers at the expense of fatigue-resistant pure slow fibers, without influencing the fast fiber number (Yang *et al.,* 2002).

In addition, subcellular structural abnormalities, such as disrupted myofibrils, or increased number of lipid vacuoles in the sarcoplasm of diaphragm muscle fibers have been found after 1–3 days of CMV in rabbits (Sassoon *et al.,* 2002; Zhu *et al.,* 2005). Similar alterations were observed in the intercostal (Bernard *et al.,* 2003), but not in the hindlimb muscles (Sassoon *et al.,* 2002) of the same ventilated animals.

It appears likely that one reason for these alterations to occur is inactivity and disuse atrophy, as it was shown in limb muscles in various studies (*reviewed in* Talmadge *et al.,* 2000). After 2–5 days of immobilization in shortened position, the gastrocnemius muscle, of which fiber type composition is similar to that of the diaphragm, show reduced levels of expression of MyHC1 and MHC2a mRNA transcripts, while the slow soleus did not show reductions in MyHC1 expression, but did show increased levels of expression of fast MyHC transcripts (Loughna *et al.,* 1990). However, the diaphragm differs from other striated muscles, because its total immobilization can not be achieved under *in vivo* experimental conditions. One key difference is the passive movement in CMV as the lungs are cyclically inflated and deflated (Decramer *et al.,* 1984). Unilateral and bilateral diaphragm denervation (without subsequent CMV) lead to significant muscle hypertrophy after 7 days, which is accompanied by increased number of hybrid fibers at the expense of pure fast fibers (Yang *et al.,* 1998). In this experiment, the passive stretch of the inactive, flaccid diaphragm by the

active hemidiaphragm or accessory muscles was proposed to cause the observed response. Obviously, these data show that controlled mechanical ventilation exerts early direct and deleterious effects on diaphragm function.

2.2. Background: Regeneration study

Skeletal muscle has a remarkable potential to regenerate (*reviewed in* Charge and Rudnicki 2004). The injury of muscle fibers activates dormant stem cells positioned between the basal membrane and the sarcolemma. These cells named as satellite cells (Mauro *et al.,* 1965), divide and give rise to myogenic precursor cells that either undergo one more mitosis or differentiate into myoblast and fuse to the injured fiber. In case of no fibers near to them the myoblasts fuse with each other. Skeletal muscle regeneration is a powerful system to dissect regulatory pathways involved in formation of identity of the slow muscle type (Serrano *et al.,* 2001; Murgia *et al.,* 2000). Previous studies demonstrated that a single injection of the snake toxin notexin induces a rapid and extensive necrosis of the muscle fibers, followed by a relatively rapid and complete regeneration process (Harris *et al.,* 1975; Whalen *et al.,* 1990; Grubb *et al.,* 1991). In our laboratory, the *in vivo* model of muscle regeneration has been thoroughly characterized by assessing a number of parameters, including the mRNA levels of myogenic regulatory factors (myoD, myf-5, myogenin, and MRF4) (Mendler *et al.,* 1998), skeletal α-actin and the SERCA mRNA and protein levels (Zádor *et al.,* 1996, 1998). Those parameters demonstrate that in this regeneration system the fibers are totally destroyed but later recreated in the soleus muscle.

The rate of contraction and of relaxation of a skeletal muscle fiber is largely determined by the myosin heavy chain (MyHC) and the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) isoforms, respectively (Schiaffino and Reggiani 1996). A number of experimental studies showed that the corresponding fast and slow isoforms of MyHC and SERCA, together with other non-contractile proteins were found to change in parallel in response to various stimuli. Such a coordinated change was observed during chronic low-frequency stimulation in overloaded plantaris muscles of the cat (Talmadge *et al.,* 1996); in muscles of euthyroid and hyperthyroid rats (Hämäläinen and Pette 1997); during cyclosporin A treatment (immunosuppressive drug, calcineurin A inhibitor) in the soleus (Bigard *et al.,* 2000, 2001); and in passively stretched rat soleus muscles (Zádor *et al.,* 1999). MyHCs and SERCAs are functionally very different molecules, but their coordinated expression may have functional

rationale. In the fast muscle fibers more calcium is released via the ryanodine receptors during the quick activity bursts than in the slow fibers (Baylor and Hollingworth 2003). The calcium uptake capacity and ATP utilization of the sarcoplasmic reticulum in fast myofibers is higher than in the slow fibers (Szentesi *et al.,* 2001), although the fast and slow SERCA isoforms possess almost identical (Lytton *et al.,* 1992; *reviewed in* Dux 1993) but slightly different Ca^{2+}-affinity and rate of Ca^{2+} transport (Dode *et al.,* 2005).

However, there are accumulating evidences suggesting that the coexpression and the coordination is not ultimate or less clear and in some fibers are even absent. In overloaded mouse soleus muscle a temporal dissociation between the relative expression of MyHC and SERCA can occur (Awede *et al.,* 1999). In human muscles adapting after spinal cord injury mismatch between SERCA2a and MyHC1 expressing fibers was observed (Talmadge *et al.,* 2002). Our group showed that the expression of SERCA2a mRNA occurs earlier than day 5 in the regenerating soleus muscle of the rat, *i.e.* before the completion of the reinnervation (Zádor *et al.,* 1996). Moreover, the transfection of the denervated regenerating soleus muscle with the H-*ras* oncogene, which is known to mimic the effect of innervation on the expression of MyHC1, did not affect the SERCA2a expression (Zádor and Wuytack 2003). The expression of the slow myosin isoform (MyHC1) is strictly dependent on slow type innervation (Whalen *et al.,* 1990; Murgia *et al.,* 2000), while that of the SERCA2a is not (Zádor and Wuytack 2003).

Studies monitoring the effect—and not the calcineurin activity—of orally administrated calcineurin inhibitors have suggested that calcineurin stimulates in vivo muscle regeneration (Friday and Pavlath 2001; Bigard *et al.,* 2000; Irintchev *et al.,* 2002; Sakuma *et al.,* 2003). Constitutively active calcineurin selectively upregulates the slow-fiber-specific promoters (Chin *et al.,* 1998) and oral administration of calcineurin inhibitors decreases the number of MyHC1-expressing slow fibers in rat soleus muscle (Chin *et al.,* 1998; Bigard *et al.,* 2000; Serrano *et al.,* 2001). It has been established that calcineurin mediates neuronal control of MyHC1 expression in regenerating muscle (Serrrano *et al.,* 2001), although also an alternative regulating pathway acting through H-*ras* has been reported (Murgia *et al.,* 2000). In accordance with this, formerly even the role of calcineurin in muscle differentiation has been debated (Schiaffino and Serrano 2002), partly because the direct link of NFAT to slow-muscle-specific promoters is still missing (Calvo *et al.,* 1999, Swoap *et al.,* 2000). Specifying slow fiber fate is probably a multifactorial process, the relative contribution of various signalling pathways remains to be established, because both the calcineurin-dependent

pathway (Serrano *et al.,* 2001) and a *ras*/MAPK pathway (Murgia *et al.,* 2000) can control the excitation-transcription coupling. Innervation also seems to be a prerequisite for the expression of the slow SERCA2a isoform (Briggs *et al.,* 1990; Germinario *et al.,* 2002), however, the direct connection between the innervation and expression of SERCA2a has not been found in the regenerating soleus (Zádor and Wuytack 2003; Zádor *et al.,* 2005).

3. AIMS OF THE STUDIES

3.1. Mechanical ventilation study

The question how mechanical ventilation affects the inspiratory muscles in patients with normal or already impaired inspiratory muscle function appears to be of great clinical relevance. Therefore, three aims were raised:

(1) To characterize the effects of mechanical ventilation on the diaphragm in terms of expression of transcription factors and key muscle proteins, which are known to associate with disuse/atrophy processes.

(2) Further aim is to unravel which of the consequences of mechanical ventilation, disuse-induced deconditioning or rhythmic passive shortening might be responsible for these effects. It was hypothesized that mechanical ventilation would alter the expression levels of contractile proteins and transcription factors and disuse-induced deconditioning and/or passive shortening would be implicated in the diaphragm alterations seen after mechanical ventilation. This effect was studied on the gastrocnemius muscle.

(3) If it appears that one reason for any deleterious alterations to occur is inactivity or disuse-induced deconditioning, the protective effect of the intermittent spontaneous breathing is assessed. It was examined whether intermittent spontaneous breathing during controlled mechanical ventilation may protect the diaphragm against the detrimental effects of mechanical ventilation.

3.2. Regeneration study

To further characterize the MyHC and SERCA coexpression, the time course of the MyHC and SERCA levels were studied in our regenerating soleus model. The nerve dependent fast-to-slow switch was compared to the nerve dependence of calcineurin expression and activity.

4. MATERIALS AND METHODS

4.1. Mechanical ventilation study

4.1.1. Study design and experimental procedures

According to the aims, three series of experiments were conducted.

Series 1 (mechanical ventilation). 52 male Wistar rats were randomly divided into three groups: (1) A control group, where no intervention was performed (group C, n=10). (2) A group of rats breathing spontaneously for 24 hrs, in which the same surgery was performed as in the mechanically ventilated rats (group SB, n=5). (3) A group of rats submitted to 24 hrs of anaesthesia and continuous controlled mechanical ventilation (group CMV, n=7).

The following factors were measured: the mRNA levels and/or the protein levels of several factors that possibly influence diaphragm function directly or indirectly: (1) Myogenic regulatory factors (MRF) and Id-proteins, as these transcription factors are known to regulate muscle adaptation. (2) MyHC and SERCA isoforms, as they are two of the main proteins having an impact on the rate of muscle force development and relaxation. (3) Acetylcholine receptor, as it is a good marker of decreased nerve-evoked electrical activity. In particular, the expression of the acetylcholine-receptor α-subunit was found to be regulated by nerve activity and myogenic regulatory factors (Prody and Merlie 1991; Merlie *et al.,* 1994).

Series 2 (effects of immobilization and rhythmic passive shortening on the gastrocnemius). 12 animals were instrumented as in the mechanical ventilation study, but in addition, the right hindlimb was immobilized, and the left hindlimb was also passively moved rhythmically (Figure 3). A ventilator (Harvard pump) was adapted such that it allowed attachment to the left hindlimb of the rat. Movements of the piston were translated into movements of the foot, producing thereby passive shortening of the gastrocnemius at 55 movements per minute. The device was calibrated such that the shortening was approximately 10% of resting muscle length, a change in length similar to the one undergone by the diaphragm during mechanical ventilation (Decramer 1984). The moved hindlimb (group I+PS, n=12) was compared with the contralateral side, which was immobilized at resting position and underwent the effects of anaesthesia and disuse-induced deconditioning (group I, n=12). The gastrocnemius muscle from the *Series 1* study control animals served as the true control group (group C, n=10).

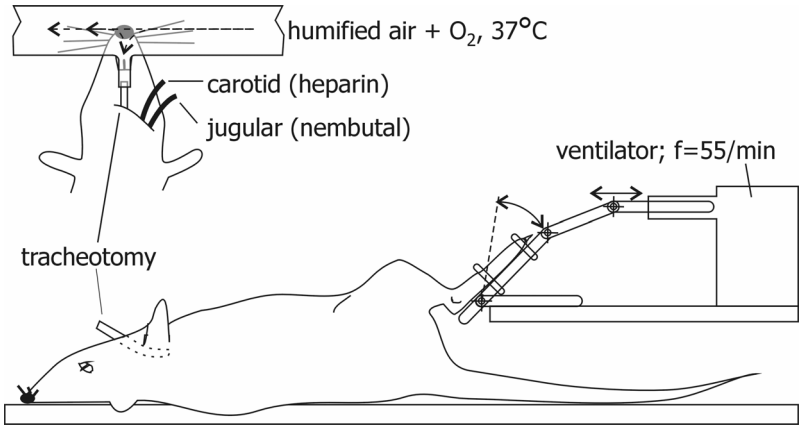

Figure 3. Experimental setup of the rhythmic passive shortening study. The rat is tracheotomized and breathed humidified air enriched with O_2 and maintained at 37C. During the 24 hours, continuous infusion of anesthetic) and heparin was given via the right jugular vein and carotid artery, respectively. In addition, both hindlimb were immobilized, but while the right hindlimb was solely immobilized, the left hindlimb was also passively moved rhythmically. Passive shortening of the left hindlimb occurred at 55 movements per minute (*f*=frequency). The device was calibrated such that the degree of gastrocnemius shortening was approximately 10% of resting muscle length, a change in length similar to the one experienced by the diaphragm during mechanical ventilation.

Series 3 (intermittent mechanical ventilation). Adult male Wistar rats (350–500 g) were randomly divided into five groups: (1) A control group consisting of awake animals free from intervention (group C#2, n=5). (2) A group of rats breathing spontaneously for about 24 hrs submitted to the same sham surgical procedure than the mechanically ventilated rats (group SB#2, n=5). (3) A group of rats submitted to 24 hrs of continuous controlled mechanical ventilation (group CMV#2, n=7). (4) A group of rats submitted to 24 hrs of controlled mechanical ventilation with intermittent spontaneous breathing (group ISB5, n=9). The animals were allowed to breathe 5 minutes spontaneously every 5 hrs 55 minutes of controlled mechanical ventilation. Hence, they were breathing 20 minutes spontaneously for 24 hrs of controlled mechanical ventilation. (5) A group of rats submitted to 24 hrs of controlled mechanical ventilation with intermittent spontaneous breathing (group ISB60, n=8), breathing 60 minutes spontaneously every 5 hrs of controlled mechanical ventilation. They were breathing 4 hrs spontaneously in total for about 24 hrs of controlled mechanical ventilation.

4.1.2. Common procedures

Except for the control rats, all animals were initially anesthetized with sodium pentobarbital (60 mg/kg intraperitoneally) and were tracheotomized, and their body

temperature was continuously monitored with an internal probe and maintained at 37°C. During the 24 hours, continuous infusion of anaesthetic (2 mg/100 g body weight/hr) and heparin (2.8 U/ml/hr) was given via the right jugular vein and carotid artery, respectively, using pressure pumps (Pilot A2; Fresenius, Schelle, Belgium). Constant levels of anaesthesia were controlled throughout the experiment by evaluating foot reflex, corneal reflex, arterial blood pressure, and breathing pattern (for the spontaneously breathing group). Animals also received enteral nutrition, including vitamins and minerals that were administered via a gastric tube. Animals breathed humidified air enriched with O_2 and maintained at 37°C. During the period of mechanical ventilation, animals were ventilated with a volume-driven small animal ventilator (Harvard Apparatus 665A, Holliston, MA). The tidal volume was set at ±0.5 ml/100 g and the respiratory rate was 55–60 breaths/min. After completion of the 24 hours, blood gas analysis was performed while part of the diaphragm and the whole gastrocnemius samples were removed and frozen in liquid N_2.

4.1.3. mRNA extraction and RT-PCR

(In *Series 1 and 2*) Part of the diaphragm and the whole gastrocnemius were taken, blotted, weighed, frozen in liquid nitrogen and stored at –80°C. Total RNA was isolated using a modified guanidinium isothiocyanate-CsCl method (Chirgwin *et al.,* 1979). Approximately 0.2 g of tissue was homogenised using an UltraTurrax homogeniser (Janke & Kunkel, Germany) in a solution containing 50% (w/v) guanidinium isothiocyanate, 25 mM EDTA, 0.5% lauryl-sarcosine and 0.1 M β-mercaptoethanol. The homogenate was layered on top of a solution containing 5.7 M CsCl, 25 mM sodium acetate (pH 5.0) and 10 mM EDTA. After ultracentrifugation at 20°C in an SW41 rotor (Beckman, Germany) at 100 000 g for approximately 16 hours, the supernatant was removed and the RNA pellet was dissolved in water. Sodium acetate (pH 5.2) was added to the solution to a final concentration of 0.3 M and the RNA was precipitated with 2.5 volumes of absolute ethanol. The RNA was then rinsed in 70% ethanol, vacuum dried and redissolved in water. The purity and quantity of the RNA preparations were determined by measurement of absorbance at 260 and 280 nm.

Samples of 4.5 μg of total RNA were subjected to oligo(dT)-primed first-strand cDNA synthesis in a volume of 20 μl (Gibco BRL ThermoScript kit; Life Technologies, Merelbeke, Belgium). After reverse transcriptase reaction, the first-strand cDNA mixture was subjected to polymerase chain reaction (Perkin Elmer kit, Lennik, Belgium). The number of PCR cycles was adjusted to avoid saturation of the amplification system. Amplification products were

Table 1. PCR specifications. Applicable to the mechanical ventilation study, as well as the regeneration study. Primers marked with * were used also in the regeneration study.
[#] PCR cycles in diaphragm / gastrocnemius, where applicable. Each cycle lasted for 1-1 minute.

Primer name	Ref.	Sequence (5' → 3') antisense - sense	Fragment size	PCR cycles	Temp. (°C)
myogenin	Mendler 1998	gacctgatggagctgtat agacaatctcagttgggc	688	30 / 24 [#]	94-60-72
Myf-5		gagccaagagtagcagccttcg gttctttcgggaccagacaggg	441	29 / 27 [#]	94-60-72
MyoD		tggcgcgctgccttctacg acacggccgcactcttccctg	221	28 / 35 [#]	94-60-72
MRF4		agagactgcccaaggtggagattc aagactgctggaggctgaggcatc	272	28 / 26 [#]	94-60-72
Id1		catgaaggtcgccagtcgcag gtccatctggttcctcagtgc	475	23 / 23 [#]	94-60-72 (coampli-fication)
Id2		gagcaaaaccccggtggac gctgtcattcgacataagctcaga	316		
Id3		aaggcgctgagcccggtg ctgcgttcgggaggtgcc	387		
Id4		gctgtgctgcagtgcgata gtcaccctgcttgttcacgg	286		
MyHC1*	Jaschinski 1998	gggcttcacaggcatccttag acagaggaagacaggaagaacctac	288	15	95-60-72
MyHC2a *		taaatagaatcacatggggaca tatcctcaggcttcaagatttg	310	20	95-55-72
M *		tcccaaagtcgtaagtacaaaatgg cgcgaggttcacaccaaa	120	20	95-58-72
MyHC2b *		ttgtgtgatttcttctgtcacct ctgaggaacaatccaacgtc	197	22	94-55-72
neoMyHC *		gcggcctcctcaagatgcgt gaaggccaaraargccatya	567	35	95-52-72
SERCA1a/b *	Zádor 1996	ttccatctgcctgtccatgtc ctggttacttccttctttcgtctt	248 / 206	21	94-60-72
SERCA1a *			248	21	94-60-72
SERCA2a *		ctccatctgcttgtccat gcggttactccagtattg	231	22	94-55-72
GAPDH *		tcctgcaccaccaactgcttagcc tagcccaggatgccctttagtggg	376	identical w/ that of the coamplified MyHC	
AChR-α subunit	Merlie 1994	acgttcgtctgaaacagcaatggg gccgtcataggtccaagtgccc	289	36	94-60-72
cyclophilin A	Gayan-R. 2003	aaccccaccgtgttcttc tgccttctttcaccttccc	400	25 / - [#]	94-60-72
L32		gcctctggtgaagcccaaga tctgatggccagctgtgctg	349	- / 24 [#]	94-60-72

identified by their sizes after electrophoresis on 6% (w/v) acrylamide gels (*Table 1*). After staining with Vistra Green, the fluorescence levels of the bands were quantified by means of a PhosphorImager model 425 (Molecular Dynamics, Sunnyvale, CA). Band intensities were normalized to the corresponding cyclophilin A (diaphragm) or L32 (gastrocnemius) amplification signals. As RT-PCR reaction is only a semiquantitative technique, only relative amounts were reported.

4.1.4. Myosin heavy chain extraction and separation

(In *Series 1 and 2*) Myosin heavy chain isoforms from the diaphragm and gastrocnemius were extracted as previously described in Maréchal *et al.,* (1995). SDS-PAGE with 8% separating gel containing 30% glycerol and 4% stacking gel was running during 24 hours at 270 V and 4°C. The gels were stained with silver nitrate (BioRad Silver stain plus kit; BioRad, Richmond, CA), air dried, and scanned. Bands were quantified using a gel documentation software system (Quanti One software; BioRad).

4.1.5. Western blot quantification of MyoD and myogenin

(In *all series*) Frozen muscle samples were homogenized according to the protocol of Sakuma *et al.,* (1999). Proteins (20 or 50 μg) were separated by SDS-PAGE (4% stacking gel and 12.5% separating gel) and then transferred onto polyvinyldifluoride membranes by semidry blotting. The blots were blocked and incubated with rabbit anti-MyoD, anti-myogenin antibodies and the corresponding secondary antibodies (*Table 2*). After revelation of the bands with tetramethylbenzidine, the blots were scanned and quantified using the QuantiOne software (BioRad).

For the two sets of electrophoresis (section 4.1.4. and 4.1.5.) protein concentration was measured with the Bradford methodology.

Table 2. Antibody specifications.

Antigen	Reference	Antibody name, source	Dilution
MyoD	Santa Cruz Biotechnology, Santa Cruz, CA	sc-760, rabbit polyclonal	1:400
myogenin		sc-576, rabbit polyclonal	1:400
secondary antibodies	Biosource, Nivelles, Belgium	peroxidase-conjugated rat anti-rabbit IgG	1:1000

4.1.6. Measuring of diaphragm contractile properties

(In *Series 3*) Segments of the costal diaphragm were removed for measurement of *in vitro* contractile properties. Briefly, two diaphragm bundles per animal were suspended in a tissue bath containing Krebs solution continuously aerated with 95% oxygen and 5% CO_2 maintained at 37°C. Optimal length for peak twitch force was established for each bundle, and after a 15 min thermoequilibration period the force-frequency relationship was established by stimulating the bundles at the following frequencies: 1, 25, 50, 80, 120, and 160 Hz (250-msec train duration, 0.2-msec pulse duration). After completion of this protocol, bundle length was measured at optimal length and weighted. Cross-sectional area was obtained by dividing bundle weight by muscle specific density and optimal length. Forces were expressed per unit cross-sectional area.

4.1.7. Statistical analysis

The individual reverse transcriptase-polymerase chain reaction results were normalized to the housekeeping gene mRNA data. Differences between groups were assessed with one-way analysis of variance followed by Newman–Keuls *post hoc* test using the GraphPad Prism 3 software or the SAS statistical package (SAS Institute, Cary, NC). Data are presented as mean + SE (*Series 1, 2*) or as mean + SD (*Series 3*).

(n *Series 3*) The evolution of the diaphragm force as a function of frequency has been analyzed using a nonlinear mixed-effects model. The control and SB groups were merged as well as the two ISB groups for subsequent analysis. The analysis was performed on three treatment groups, namely: control, ISB, and CMV. The three-variable logistic model fitting with the force-frequency profile was given by the following formula:

$$y = \frac{A}{1 + \exp[(freq - B)/C]}$$

where y is the force (g/cm^2), $freq$ is the frequency (Hz), A is the asymptotic value of the force y, B is the value of the frequency (Hz) at which the tissue reaches half of the asymptotic force, and C is the value of the additional frequency (Hz) required for the tissue to move from the half to approximately 3/4 of the asymptotic force. The overall difference between the groups was tested with the likelihood ratio test; p values were imposed to Bonferroni adjustments for multiple comparisons.

4.2. Regeneration study

4.2.1. Animals and treatment

Three-month old (300-360 g) adult male Wistar rats were used for the experiments, n=3 for each time points. The rats were narcotized by intraperitoneal injection of 1 ml 0.5% Na-pentobarbital per 100 g body weight. A 2 cm long incision was made laterally on the calf, the gastrocnemius muscle was shifted laterally. The soleus muscle was slightly lifted up from its bed and slowly injected at a point located approximately 1/3 from the distal end with 20 μg venom of the mainland tiger snake (*Notechis scutatus scutatus*) in 200 μl 0.9% NaCl using a 27G 20 mm injection needle. After injection, the wound of the gastrocnemius and the skin were suture closed. On day 1, 3, 5, 7, 10, 21 or 28 the entire soleus muscle was removed and the animals were killed with an overdose of Na-pentobarbital. Soleus muscles from freely moving rats served as normal controls (N). The removed muscle were blotted, weighed, frozen in liquid nitrogen and stored at –80°C. For the calcineurin expression experiment, regeneration was induced immediately after the denervation of the soleus, where the sciatic nerve was transected in the level of the thigh.

4.2.2. mRNA extraction and RT-PCR

The total RNA was isolated as described by Chomczynski and Sacchi (1987) and kept at –70°C. 2 μg total RNA of each soleus was subjected to oligo(dT)-primed first-strand cDNA synthesis in a volume of 20 μl. A 1 μl portion of the first-strand cDNA mixture was subjected to multiplex PCR according to the specifications shown in *Table 1*.

5μl of the primary amplification product were radiolabelled with [α-^{32}P]dCTP in 2 additional cycles with the same amplification buffer and conditions. The amplification products were analyzed by electrophoresis on 6% (w/v) acrylamide gel. The gels were air-dried and ^{32}P-spots were quantified by means of a PhosphorImager model 425 (Molecular Dynamics, Sunnyvale, CA, USA) and ImageQuaNT 2.0 software. Amplification products were identified by their sizes and sequences. MyHC and SERCA RT-PCR were done from the same RNA samples. SERCA1a and 1b were amplified by the same primer pair encompassing the optional 42 bp exon present in the adult SERCA1a mRNA but spliced out in the neonatal SERCA1b. So the ratio of both variants can be assessed. Band intensities were normalized to the corresponding GAPDH amplification signals.

4.2.3. Protein extraction

Extraction for MyHC electrophoresis. The isolated muscles were weighed and powdered in liquid nitrogen with mortar and pestle. The muscle powder was suspended in 8 volumes of myosin extraction solution (100 mM Na-pyrophosphate, 5 mM EGTA, 5 mM $MgCl_2$ and 300 mM KCl, pH 8.50). The suspension was stirred for 20 min at 4°C and centrifuged for 10 min at 10000 *g* in an Eppendorf centrifuge. The supernatant fraction was diluted with 1 vol glycerol and stored at –25°C (Hämäläinen and Pette 1997).

Extraction for SERCA electrophoresis. The isolated muscles were weighed and homogenized in 2.5 ml 0.25 M ice-cold sucrose and 5 mM HEPES, pH 7.5, with a glass/teflon Potter homogenizer. The homogenates were centrifuged at 1000 *g* for 10 min at 4°C. The supernatants were centrifuged at 200 000 *g* for 30 min at 4°C. The pellets, which represented the combined microsomal/mitochondrial fraction, were resuspended in 600 µl 0.25 M sucrose and kept at –20°C.

Extraction for calcineurin electrophoresis. The muscles were finely minced with scissors and homogenized in 30 vol of lysis buffer (320 mM sucrose, 10 µM $MgCl_2$, 5 mM KCl, 0.62 mM β-mercaptoethanol, 10 mM HEPES [pH 7.5]) and centrifuged for 10 min at 1000 *g*. The pellet was resuspended in 600 µl of extraction buffer (0.42 M NaCl, 20 mM HEPES [pH 7.9], 1 mM EDTA, 2 mM DTT, 25% glycerol).

Extraction for calcineurin activity assay. (Modified method of Mitsuhashi *et al.,* 2000) 5 and 10 day-old regenerating and denervated regenerating soleus muscles were rinsed in ice-cold washing solution containing 150 mM NaCl and 0.1 mM EGTA then frozen in liquid N_2 and kept –80°C until use. The frozen samples were homogenised with 4 vol of the buffer containing 60 mM Tris-HCl (pH 8), 250 mM sucrose, 10 µg/ml leupeptin, 0.2 mM PMSF, 2 mM EGTA, 10 µl/0.2 g tissue Sigma protease inhibitor, 5 mM ascorbic acid, 0.15% β-mercaptoethanol, 0.5 mM DTT. The homogenates were centrifuged at 20 000 *g* for 10 min, and the supernatants were diluted with equal volume of glycerol and stored at –20°C.

After all extraction procedure, protein concentrations were measured with the bicinchonin-acid methodology, according to the vendor's protocol.

4.2.4. Electrophoresis and Western-blots

The protein extracts (1:10 diluted for MyHC1 and SERCA2a and undiluted for MyHC2a and SERCA1) were supplemented with 0.5 volume loading buffer. 15 µl of this

solution were run on 6% (7.5% for SERCA) SDS-PAGE and then blotted onto Hybond C-extra (Amersham Pharmacia Biotech) membranes. The blots were incubated in 5% milk for 1 hour to block the non-specific binding sites. The specifications of the antibodies are described in *Table 3*. Incubations with primary and secondary antibodies lasted for 1 h. For visualization of the immunocomplexes nickel-enhanced diamino-benzidine (DAB) staining was used in the presence of 0.006% H_2O_2. Quantification was performed by densitometry, using the ScanPack 10.1 A20 software (Biometra, Göttingen, Germany).

10 μl of the calcineurin extract was submitted to SDS-PAGE and immunoblotting. The immunoblots were stained by Vistra EFC kit (Amersham Life Sciences) and quantified by fluorescence imaging on a Storm 840 device (Molecular Dynamics, Sunnyvale, CA).

Table 3. Antibody specifications. Abbreviations: wb, ihc – dilution used for Western-blots or immunohistochemistry, respectively.

Antigen	Reference	Antibody name, source	Dilution
MyHC1	Schiaffino 1989	BA-D5, mouse monoclonal	1:10
MyHC2a	Schiaffino 1989	SC-71, mouse monoclonal	1:15
SERCA2a	Eggermont 1990	R15, rabbit antiserum	1:500
SERCA1	Zubrzycka-Gaarn 1984	A3, mouse monoclonal	1:10
calcineurin A, all isoforms	Sigma	mouse monoclonal	1:2500
secondary antibodies	Dako A/S, Glostrup, Denmark	peroxidase-conjugated goat anti-mouse or swine anti-rabbit immunoglobulin	1:1000 wb 1:200 ihc

4.2.5. Calcineurin activity assay

Enzyme activity was measured by a colorimetric assay kit (Calcineurin Assay Kit, Calbiochem) according to the vendor's protocol. The principle of the measurement is the following: the sample calcineurin A dephosphatases the p-Ser phosphopeptide substrate in the presence of 0.5 μM calmodulin. The released inorganic phosphate is detected with Malachit Green reagent.

4.2.6. Number of experiments, statistics

For the PCR, calcineurin activity and the Western-blot studies 3 or 4 preparations, each from separate animals, were used for every time point. ANOVA was used to test for

statistically significant differences (p<0.05%) between the groups. Caclulation were performed with the GraphPad Prism 3 software. All data are presented as mean + SE.

Relationships between the time course of MyHC and SERCA mRNA or protein isoforms were assessed with the Pearson correlation coefficient.

5. RESULTS

5.1. Controlled mechanical ventilation study

5.1.1. General findings

Mortality. In the mechanical ventilation study, 12 animals out of the 16 studied survived the 24 hours in the mechanically ventilated group while only 9 out of 26 survived in the spontaneously breathing group. Thus, while mortality was 25% in the mechanically ventilated rats, it was higher in the spontaneously breathing group (65%) because in the latter group overdosing of anaesthesia led to apnoea and death. For the rhythmic passive shortening study, 10 out of 12 animals survived, such the mortality rate was 16%.

In the intermittent mechanical ventilation study mortality was 25% for the CMV#2, ISB60 and ISB5 groups, while it was 37% for the SB#2 group.

Blood gases and arterial blood pressure. As shown in Table 4, at the time of dissection, values for arterial blood pressure and blood gases for anesthetized rats were not significantly different between the groups. Moreover, these values remained in the normal range for all studied variables.

Table 4. Blood gas data and arterial blood pressure at dissection time in anesthetized rats. Values are mean ± SD in torr. *Series 1*: mechanical ventilation; *Series 2*: effects of immobilization and rhythmic passive shortening on the gastrocnemius; *Series 3*: intermittent mechanical ventilation.
SB: spontaneous breathing; CMV: controlled mechanical ventilation; PS: passive shortening;
ISB5: 5 minutes of intermittent spontaneous breathing after 5 hr 55 min CMV; ISB60; 60 minutes of intermittent spontaneous breathing after 5 hr CMV

Series 1 and 2	SB	CMV	PS
PaO_2	123±13	142±13	113±6
$PaCO_2$	31±5	33±5	42±2
pH	7.38±0.02	7.45±0.04	7.37±0.02
arterial blood pressure	130±7	104±11	128±8

Series 3	SB#2	ISB60	ISB5	CMV#2
PaO_2	161±28	94±21	116±46	130±54
$PaCO_2$	48±12	37±14	35±14	39±22
pH	7.33±0.03	7.42±0.06	4.49±0.12	7.44±0.05
arterial blood pressure	93±15	113±10	111±17	115±23

5.1.2. Myogenic regulatory factors

Series 1, mRNA levels. Compared to controls, diaphragm myogenin mRNA levels were significantly increased in the CMV group (+67%, $p<0.01$ vs controls) while they tended to increase in the SB group (+32%, not significant) (Figure 4A). Myf-5 mRNA increased along with myogenin after mechanical ventilation (+107%, $p<0.01$ vs controls), however, it also increased significantly in the spontaneously breathing group (+130%, $p<0.01$ vs controls). By contrast, the level of MyoD mRNA decreased both in the spontaneously breathing rats (–33%, $p<0.01$ vs controls) and even more so in mechanically ventilated animals (–56%, $p<0.001$ vs controls), the latter being also statistically significantly different from the spontaneously breathing group ($p<0.05$). As a consequence, compared to controls the MyoD/myogenin ratio, which may reflect the ongoing changes in isoform switch, decreased significantly in the spontaneously breathing group (–34%, $p<0.05$ vs controls) and more particularly after mechanical ventilation (–70%, $p<0.001$ vs controls) (Figure 4B). In addition, this ratio was significantly decreased by 53% in the mechanically ventilated group compared to the spontaneously breathing group ($p<0.01$ vs spontaneously breathing). mRNA levels of MRF4, a factor involved in the later stages of myogenic transformation pathway, remained unchanged.

Series 2, mRNA levels. Passive shortening combined with immobilization caused a significant reduction in gastrocnemius MyoD mRNA (–23%, $p<0.05$ vs controls). However, after immobilization alone, more pronounced decay was observed (–44%, $p<0.001$ vs controls and –21%, $p<0.05$ vs immobilization and passive shortening) (Figure 4C). It is worth to note that myogenin (immobilization: +52% and immobilization and passive shortening: +70%, $p<0.05$ vs controls) and myf-5 (immobilization: +34% and immobilization and passive shortening: +29%, not significant) mRNA increased after immobilization and also after passive shortening (Figure 4C) as was also the case in the diaphragm of mechanically ventilated and spontaneously breathing groups (Figure 3A). As a result, MyoD/myogenin ratio decreased similarly after immobilization (–60%, $p<0.001$ vs controls) and after immobilization combined with passive shortening (–51%, $p<0.001$ vs controls) (Figure 3D). MRF4 mRNA did not change whatever the condition.

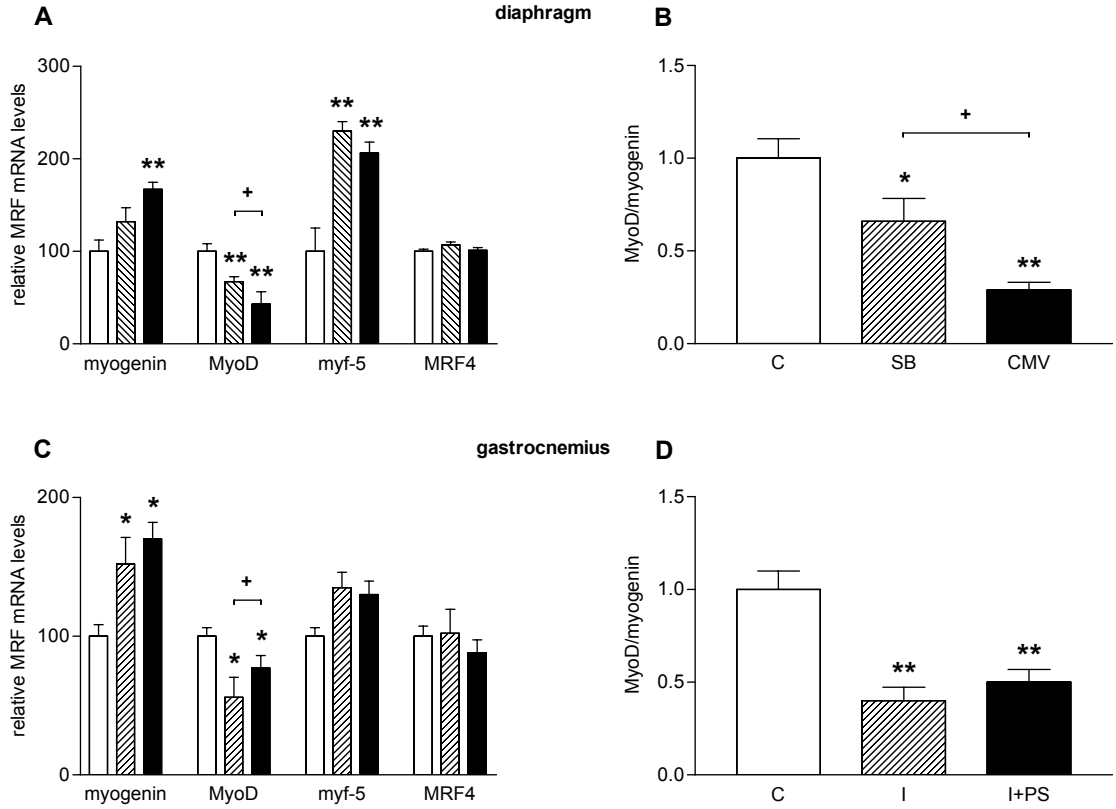

Figure 4. mRNA levels of myogenic regulatory factors (MRF) in the diaphragm (panels A and B) of control rats (C, open bars), spontaneously breathing rats (SB, hatched bars), and rats under mechanical ventilation (CMV, solid bars) and in the gastrocnemius (panels C and D) of control rats (C, open bars), after immobilization (I, hatched bars), and after both immobilization and passive shortening (I+PS, solid bars). Values were normalized to the corresponding house-keeping gene amplification signals. Values are means + SE. * $p<0.05$; ** $p<0.01$; $^{+}$ $p<0.05$ between SB and CMV.

Series 1, protein levels. Compared to controls, diaphragm MyoD was significantly reduced in mechanically ventilated group (–49%, $p<0.05$ vs controls and spontaneously breathing) and remained unchanged in spontaneously breathing animals (Figure 5A) while myogenin tended to increase (spontaneously breathing: +115% and mechanically ventilated: +59%, $p=0.08$; graph not shown).

Series 2, protein levels. Compared to controls, gastrocnemius MyoD was significantly increased after immobilization (+40%, $p<0.01$ vs controls) and after immobilization and passive shortening (+52%, $p<0.001$ vs controls) (Figure 5B). For myogenin, a tendency to increase was also observed in both groups (immobilization: +36% and immobilization and passive shortening: +44%) compared to controls but these increases failed to reach statistical significance (graph not shown). These changes in protein expression thus did not follow the changes in mRNA levels for MyoD whereas for myogenin a close accordance with the mRNA changes was present.

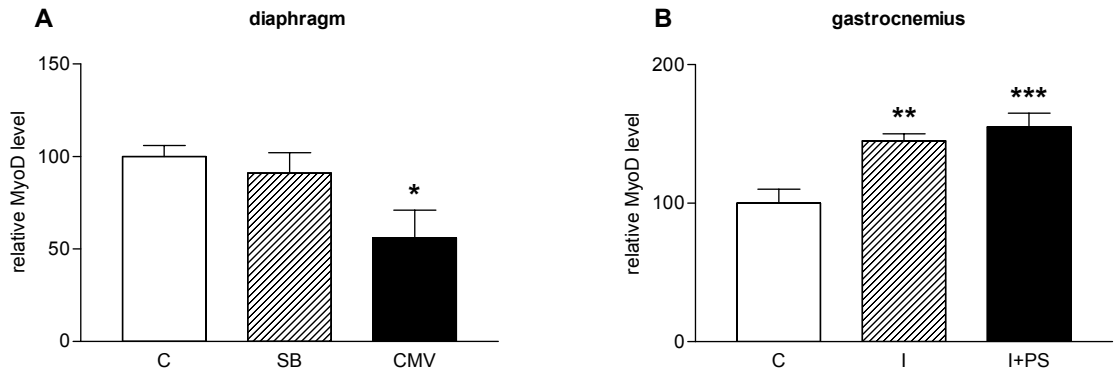

Figure 5. Western-blotting data of MyoD in diaphragm (A) of control rats (C), spontaneously breathing rats (SB), rats under controlled mechanical ventilation (CMV) and in the gastrocnemius (B) of control rats (C) after immobilization (I) and after both immobilization and passive shortening (I+PS). Values are means + SE. ** p0.05 vs C; *** p<0.001 vs C.

Series 3, protein levels. Compared to controls and spontaneously breathing animals, diaphragm MyoD protein expression was significantly decreased after ISB60 (–35%, p<0.0001) and even more after mechanical ventilation (–73%, p<0.0001) (Figure 6A). MyoD protein was also decreased in the ISB5 group (–27%) but it failed to reach statistical significance. Most importantly, the diaphragm MyoD protein levels in the CMV#2 group were significantly different from that of both the ISB5 and the ISB60 (p<0.0001) (Figure 6A). For myogenin, the same pattern was observed with myogenin protein levels being particularly decreased in the CMV#2 group (–90%, p<0.0001 vs C#2 and SB#2) and to a lesser extent in the ISB5 (–56%, p<0.0001 vs C and SB) and ISB60 (–67%, p<0.0001 vs C#2 and SB#2) (Figure 6B). The decrease in myogenin protein levels was significantly different between the CMV#2 and the ISB5 groups (p<0.0001).

5.1.3. Inhibitor of the DNA-binding protein isoforms

Series 1. Reverse-transcriptase polymerase chain reaction data showed that inhibitor of DNA-binding protein-1 (Id1) mRNA levels were significantly and equally reduced both in the diaphragm of mechanically ventilated and spontaneously breathing animals (–30%, p<0.001), while Id2 and Id3 mRNA showed no changes and Id4 mRNA was not detectable. (Graph is not shown.)

Series 2. In the immobilized and passively moved but also in the solely immobilized gastrocnemius, Id3 mRNA decreased equally (–27% and –25%, respectively, p<0.01 vs controls) while the other Id isoform mRNA did not change. (Graph is not shown.)

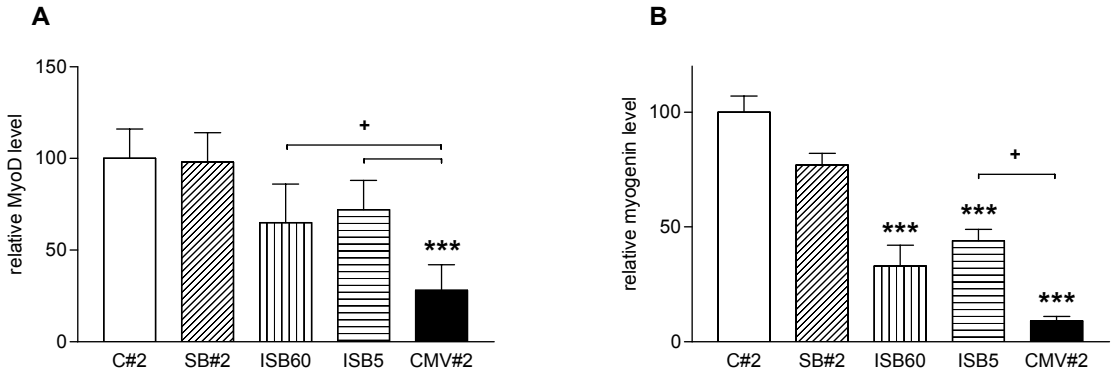

Figure 6. Western-blotting data of MyoD (A) and myogenin (B) in the diaphragm of the five groups: control (C#2), spontaneous breathing (SB#2), intermittent spontaneous breathing 60 mins/5 hrs of controlled mechanical ventilation (ISB60) or 5 mins/5 hrs and 55 mins of controlled mechanical ventilation (ISB5), and controlled mechanical ventilation (CMV#2). Values are means + SD. *** $p<0.0001$ vs controls and SB; + $p< 0.0001$ vs ISB5, $^{+}$ $p<0.05$ between the marked groups.

5.1.4. MyHC and SERCA isoforms and acetylcholine receptor α-subunit

Series 1. The fast MyHC2a mRNA decreased in the diaphragm of the spontaneously breathing group (–10%, $p<0.05$ vs controls) and even more so in the mechanically ventilated group (–20%, $p<0.001$ vs controls). The decrease in the latter was moreover significantly different from the spontaneously breathing group ($p<0.05$) (Figure 7A). Also MyHC2b mRNA decreased, but this reached statistical significance only in the mechanically ventilated animals (–19%, $p<0.05$ vs controls). MyHC1 and 2x mRNA did not change during the experiment.

The fast muscle fiber-specific SERCA1a mRNA decreased significantly in the diaphragm of the mechanically ventilated group (–21%, $p<0.05$ vs controls) (Figure 7B). The slow-type SERCA2a mRNA did not change in the experiment.

The mRNA level of acetylcholine receptor α-subunit showed a marked increase in the diaphragm of the spontaneously breathing (+140%) and mechanically ventilated (+120%) groups (Figure 7B). These increases reached statistical significance with one way analysis of variance ($p<0.05$) but not with the Newman–Keuls post hoc test.

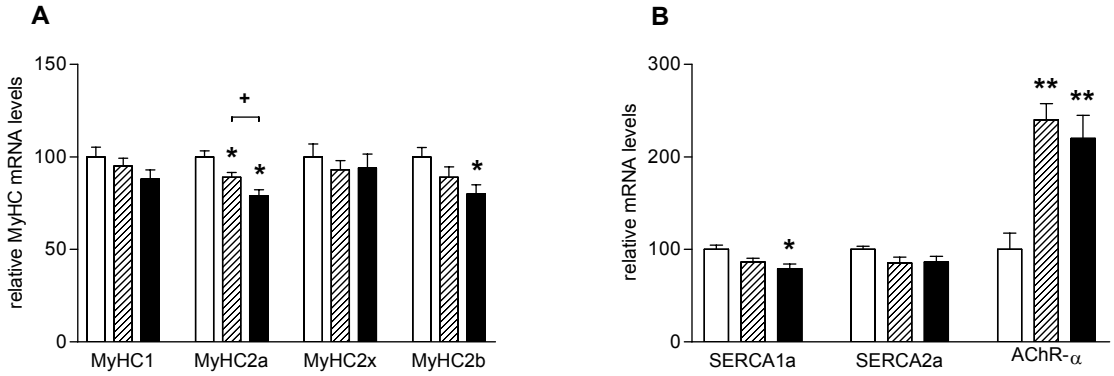

Figure 7. mRNA levels of myosin heavy chain isoforms (MyHC) (A), sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) isoforms, and acetylcholine receptor α-subunit (AChR-α) (B) determined by RT-PCR in the diaphragm of control rats (open bars), spontaneously breathing rats (SB, hatched bars), and rats under controlled mechanical ventilation (CMV, solid bars). Values were normalized to the corresponding housekeeping gene amplification signals. Values are means + SE. * p<0.05 and ** p<0.001 vs control; + p<0.05 CMV vs SB.

5.1.5. Diaphragm contractile properties

Series 3. The profile of the diaphragm force-frequency curve of the controls and SB#2 group was significantly different from that of the ISB and CMV groups. More specifically, the mean asymptotic force was less in the ISB (p=0.008) and CMV#2 (p<0.005) groups compared with controls and SB group (Figure 8). Also, the mean frequency at which the diaphragm reached half of the asymptotic force was significantly different in the ISB (p=0.0006) and CMV ($p<0$.0001) groups compared with control and SB groups. Twitch characteristics (time-to-peak tension and half-relaxation time) were otherwise similar.

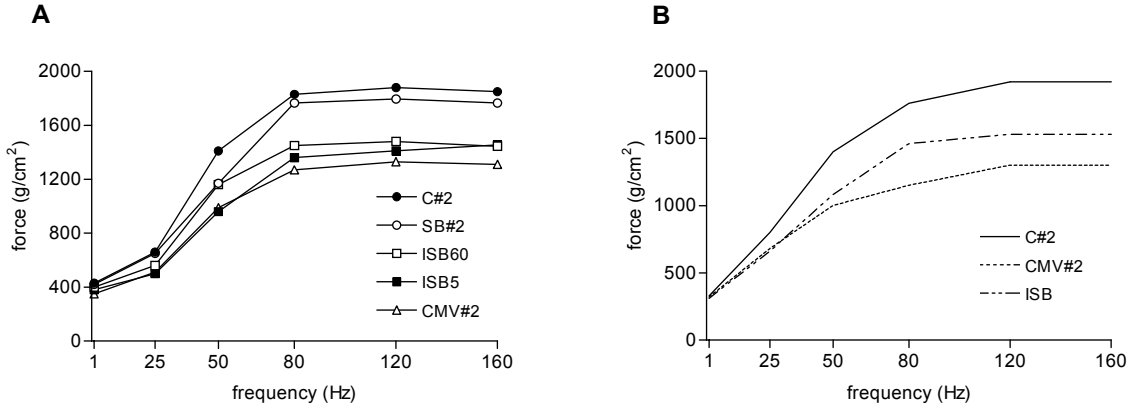

Figure 8. (A) diaphragm force generated at 1, 25, 50, 80, 120, and 160 Hz during the force-frequency relationship in the five groups: control (●), spontaneously breathing (○), intermittent spontaneous breathing 60 mins/5 hrs of controlled mechanical ventilation (□) or 5 mins/5 hrs and 55 minutes of controlled mechanical ventilation (■), and controlled mechanical ventilation (△). (B) Force-frequency relationship depicted in A after fitting with the nonlinear mixed effects model using three treatment groups: control group (control and spontaneous breathing), intermittent spontaneous breathing group (ISB), and controlled mechanical ventilation group (CMV).

5.2. Regeneration study

The local injection of notexin into a soleus muscle completely degraded the myofibers. The necrosis was followed by a relatively fast regeneration process which, judged in terms of light-microscopical morphology, was largely completed in 28 days.

5.2.1. Levels of MyHC and SERCA isoforms in the regenerating soleus

First, the mRNA of the neonatal MyHC, the fast-type MyHC2x, and of the neonatal SERCA1b appeared on day 1 of regeneration (Figure 9A). However, after showing a transient increase, peaking at days 5 and 7, they normalized again toward day 28.

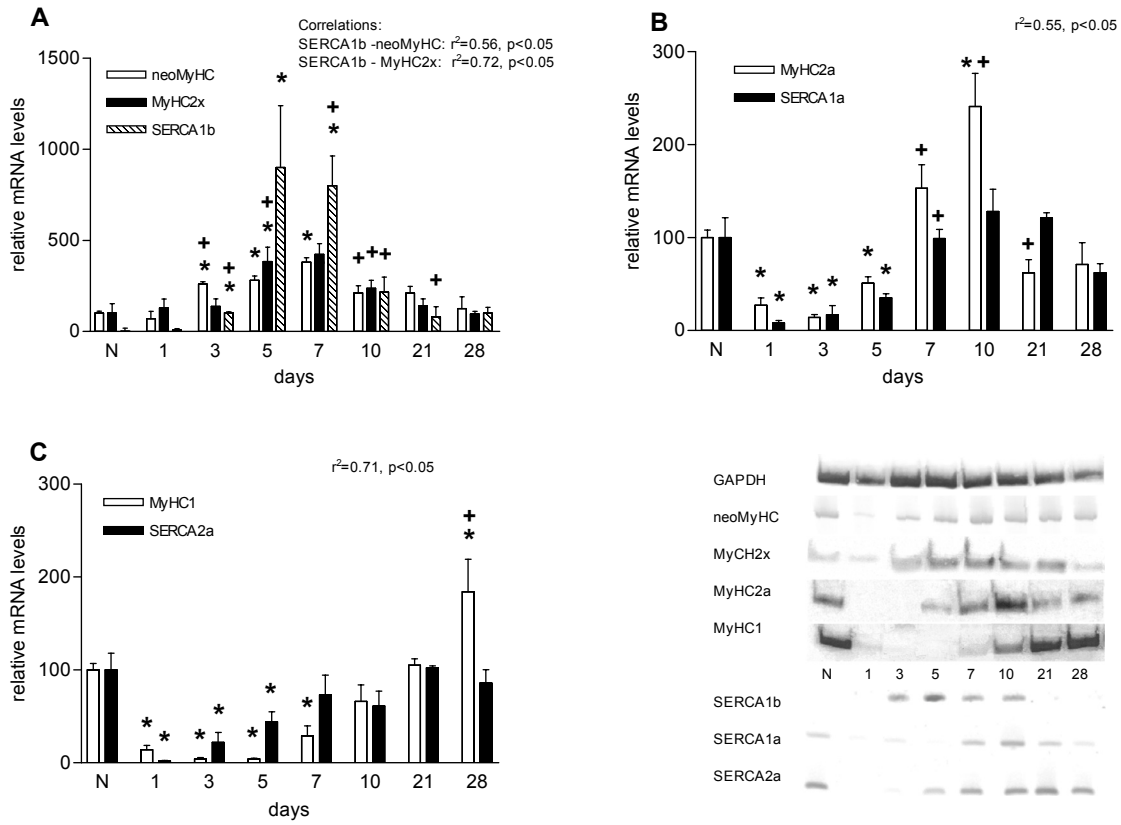

Figure 9. Time course of mRNA levels during the regeneration of soleus after notexin-induced necrosis. (A) neoMyHC, MyHC2x and SERCA1b, (B) MyHC2a and SERCA1a, (C) MyHC1 and SERCA2a.
The values cannot be compared between panels and are normalized to the control values of normal soleus, which are taken as 100%. Values are means + SE. * $p<0.05$ vs controls; $^{+}$ $p<0.05$ significant difference between two successive time points. Representative gels are shown in the lower part of the figure.

The mRNAs of the fast-type MyHC2a and SERCA1a isoforms (Figure 9B) nearly disappeared from the muscle on days 1–3 after injection of the toxin, but they were increased

again on day 5, and after passing through a maximum on day 10, gradually declined to the normal level. The mRNA of the slow MyHC1 (Figure 9C) reappeared on day 7 and showed a monotonous gradual increase until day 28, where it reached a significantly higher level than the initial control. The transcript of the slow SERCA2a was already increasing on days 3–5 and reached practically the normal level on day 7 then it did not change until day 28.

The expression of the main fast and slow myosin and SERCA protein isoforms corresponded well to their transcript levels. The fast MyHC2a and SERCA1a protein started to recover on day 5, reached their maximum on day 10, and declined to the level of control muscles (Figure 10A). The slow MyHC1 appeared on day 7, continuously increased until day 28, and then reached a value equal to the untreated controls (Figure 10B). The slow type SERCA2a was already detectable on days 5–10, and then it increased on day 21 to the normal level and remained there until day 28.

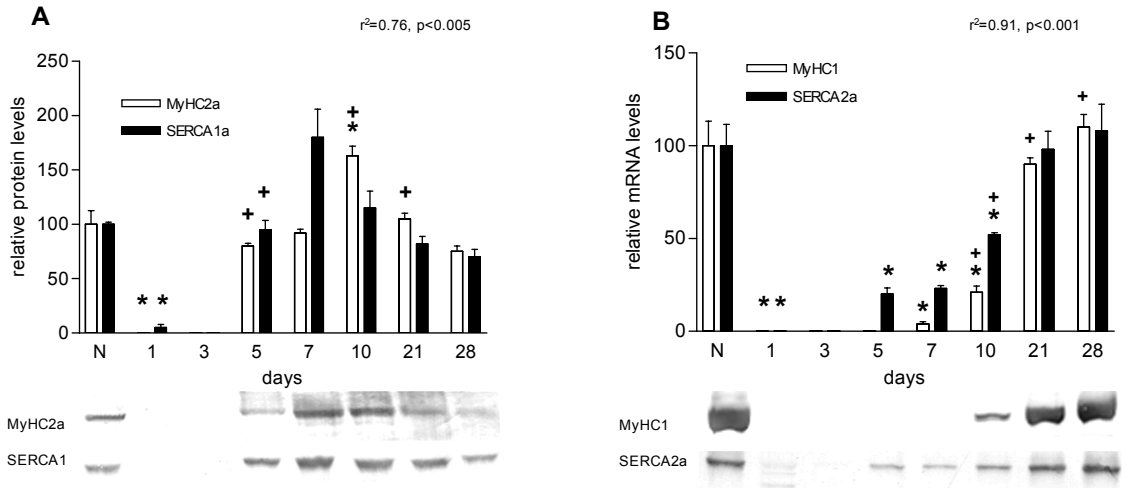

Figure 10. Time course of the levels of MyHC2a and SERCA1 (A) and of MyHC1 and SERCA2a (B) proteins in regenerating soleus. The values cannot be compared between panels. Values are means + SE. * $p<0.05$ vs controls; + $p<0.05$ significant difference between two successive time points. Representative gels are shown in the lower part of the figure.

5.2.2. Level of calcineurin A in the regenerating soleus

In the normal muscles, the active subunit of calcineurin, the calcineurin A (CnA) protein was readily detectable by Western blotting but after notexin injection it transiently disappeared on days 1–3, re-emerged on days 5–10, after which it remained at about the normal level until day 28 of regeneration. In denervated regenerating muscles, like in innervated controls, the CnA protein levels were also low on day 5, but they failed to

normalize after 10 days (Figure 11A). The calcineurin enzyme activity also dropped to about 50% of the control level on day 5 of regeneration and completely recovered on day 10 in innervated muscles. In contrast, in denervated muscles no recovery was observed (Figure 11B). Therefore, the restoration of the calcineurin activity was strictly dependent on the nerve and it correlated with the levels of CnA.

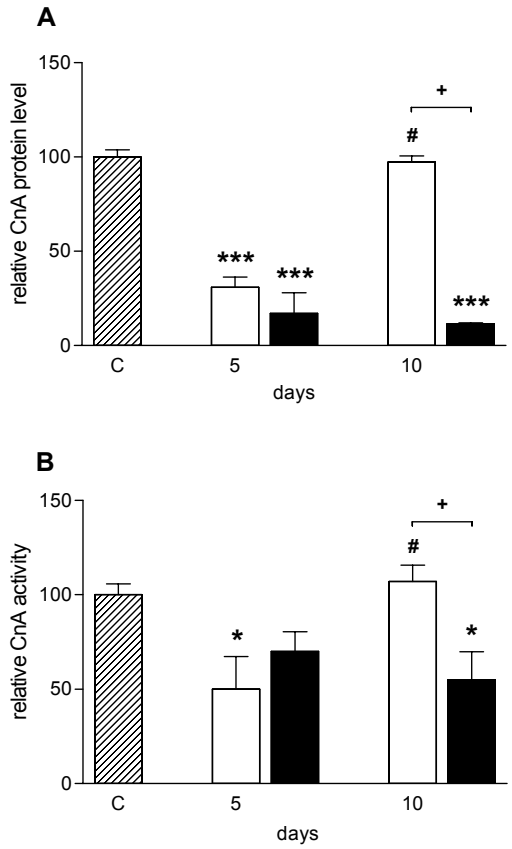

Figure 11. Calcineurin A protein levels (A) and enzyme activity (B) in regenerating rat soleus in the presence (open bars) and absence (solid bars) of innervation on days 5 and 10. Values are means + SE, n=3. Significant ($p<0.05$) and highly significant ($p<0.001$) differences compared to control muscles (hatched bars) are marked with * and ***; between innervated and denervated muscles at the same time point with + and +++, respectively. Significant difference ($p<0.05$) between day 5 and 10 is marked with #.

6. DISCUSSION

6.1. Mechanical ventilation study

The present results showed that anaesthesia, and more particularly controlled mechanical ventilation, alters the mRNA levels of the muscle regulatory factors (MyoD, myogenin), the myosin heavy chain isoforms (MyHC), the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) isoforms and diaphragm force as early as after 24 hours. The rhythmic passive movement of the gastrocnemius muscle does not exert any additional effect compared to immobilization, and these changes were similar to those seen in the diaphragm after controlled mechanical ventilation. Spontaneous breathing during the course of controlled mechanical ventilation may help to maintain diaphragm function and, more particularly, to prevent diaphragm atrophy and downregulation of key muscle regulatory factors. This suggests that intermittent spontaneous breathing may protect the diaphragm against the deleterious effects of controlled mechanical ventilation.

The changes observed in this study are likely to be related to the effects of controlled mechanical ventilation, as blood gas levels and arterial blood pressure, which can influence muscle force and/or transcription rate, remained within the normal range. Infection is unlikely to develop over the 24-hour time period (LeBourdelles *et al.,* 1994). The caloric intake (173 kcal/kg/day) was similar to that used in the studies of other authors (Powers *et al.,* 2002; Radell *et al.,* 2002; Sassoon *et al.,* 2002). Although nutritional deprivation may have occurred in the model, the contribution of undernutrition is probably not essential, as it is known that undernutrition does not reduce diaphragmatic maximal tetanic tension (Kelsen *et al.,* 1985) nor does it affect diaphragmatic insulin-like growth factor-I mRNA (Gayan-Ramirez *et al.,* 1999), whereas both changes were observed in the model. Moreover, undernutrition even if present would not explain the differences between the spontaneously breathing rats and the rats under controlled mechanical ventilation. Finally, the significant differences between spontaneous breathing and mechanically ventilated groups in the MyoD/myogenin ratio, or the mRNA level of MyHC2a show that although anaesthesia itself had an effect on diaphragm properties, it was markedly enhanced by controlled mechanical ventilation. The overall pattern of the changes induced by controlled mechanical ventilation points in the direction of a reduction in the mRNA levels of the fast isoforms of the respective muscle proteins (MyHC and SERCA) and fast (type IIX/B) muscle fiber cross-sectional area, paralleled by a reduction in the levels of MyoD and myogenin. These alterations are expected to favour a slow

oxidative profile. This is in line with a previous report showing that although all diaphragm fibers atrophied after 18 hours of controlled mechanical ventilation, the effect on type II fibers was greater than on type I fibers (Shanely *et al.,* 2002). Increases in citrate-synthase activity in the diaphragm of mechanically ventilated rats further confirmed that oxidative capacity of the diaphragm increased after short-term controlled mechanical ventilation (Shanely *et al.,* 2003).

In mature adult skeletal muscles, the role of the muscle specific transcription factors (MRFs) is still not fully established, but their implication in situations such as denervation, immobilization, electrostimulation, mechanical loading, and hormone treatment has been demonstrated (Hughes *et al.,* 1993; Mozdziak *et al.,* 1999; Zádor *et al.,* 1999; Carlsen and Gundersen 2000). MyoD in particular may be the master regulatory factor for skeletal myogenesis, as its expression in fibroblasts is sufficient to convert these cells into myoblasts (Weintraub *et al.,* 1989) and MyoD(-/-) cells are differentiation defective (Cornelison *et al.,* 2000). Also, MyoD is required for expression of MyHC2b *in vivo* in fast muscles (Wheeler *et al.,* 1999), in the mouse diaphragm and soleus, but not in all muscle (Seward *et al.,* 2001). The importance of MyoD in regulating diaphragm force has been highlighted as MyoD inactivation was associated with a downward shift of the diaphragm force-frequency relationship and a decrease in its maximal tetanic tension (Staib *et al.,* 2002).

In line with these data, the present study showed a downregulation of diaphragm MyoD protein expression together with a reduction in MyHC2b mRNA, type IIX/B fiber cross section area and diaphragm force after controlled mechanical ventilation. This close association between MyoD levels and diaphragm force-generating capacity is further underlined by the positive relationships found between diaphragm MyoD protein expression and diaphragm force (Staib *et al.,* 2002; Gayan-Ramirez *et al.,* 2005). Intriguingly, diaphragm myogenin protein levels followed the same pattern as MyoD, and the same relationships were also found between myogenin and diaphragm force. However, because of the low levels of MyHC2b in the rat diaphragm, the functional implication of this decrease should be interpreted with care.

The repercussion of increased myogenin protein levels on diaphragm function is not known because the role of myogenin in mature muscles has mainly been investigated in relation to denervation and regeneration. However, it is well known that MyoD and myogenin both contribute to satellite cell activation, but whereas MyoD is primarily involved in satellite cell proliferation, myogenin is rather implicated in the differentiation process. Other authors

emphasize the selective accumulation of MyoD in fast, and myogenin and myf-5 in slow muscle fibers, respectively (Sakuma *et al.,* 1999; Muroya *et al.,* 2002) as well as their role in maintaining the slow fiber phenotype (Hughes *et al.,* 1993, 1999). Whether the increases in MyoD and myogenin protein levels seen in the present study are related to satellite cell activation or to changes in myofiber nuclei remains to be established.

Increase of myf-5 mRNA was similar in the spontaneous breathing animals and in the rats submitted to controlled mechanical ventilation; it was likely to reflect the effect of anaesthesia and/or surgical procedure. Intriguingly, similar increase in myf-5 mRNA level was observed in the diaphragm after surgical procedure and anaesthesia in a model of lung volume reduction surgery in hamsters (Reynders *et al.,* 2002). The functional implication of elevated myf-5 mRNA in these two models is, however, not known. As MyoD mRNA decreased in our study in both anesthetized groups (but to a greater extent after controlled mechanical ventilation), it might be suggested that the similar increase in myf-5 mRNA in the anesthetized animals represents a compensatory response for this MyoD downregulation, based on data in knockout MyoD mice, high levels of myf-5 mRNA have been reported to maintain normal muscle phenotype (Rudnicki *et al.,* 1992).

The decrease in the inhibitor of DNA-binding protein-1 (Id1) mRNA observed in the present study was similar in spontaneously breathing and mechanically ventilated animals, suggesting thereby that this decrease was not caused by mechanical ventilation but was rather related to anaesthesia or surgical procedure. Similar decreases in the Id1 mRNA were previously observed in the hamster diaphragm after anaesthesia and surgical procedure (Reynders *et al.,* 2002). Whether the decrease in the Id1 mRNA would be a compensatory mechanism in response to anaesthesia and/or surgical procedure cannot be concluded from these data and is beyond the aim of this study.

The additional data on gastrocnemius showed that passive shortening did not further affect the expression levels of the studied factors compared with immobilization-induced deconditioning. The mRNA and protein changes in gastrocnemius were similar in nature to those observed in the diaphragm after mechanical ventilation except for MyoD protein expression, which increased in the gastrocnemius but decreased in the diaphragm. Whether this increase in gastrocnemius MyoD protein is transient and will be followed by a decrease as expected from its mRNA expression is impossible to predict from these data. Although still controversial, several studies have shown that the MyoD/myogenin is highly correlated with

muscle fiber phenotype, and the transformation from slow to fast phenotype is associated with decreased myogenin and elevated MyoD mRNA expression in hindlimb muscles (Talmadge *et al.,* 2000; Seward *et al.,* 2001). Similar effects were reported in electrically silent muscles: unweighting the hindlimb muscles lead to MyoD accumulation (Mozdziak *et al.,* 1999). In contrast to the diaphragm, in the immobilized gastrocnemius the Id3 mRNA level was decreased, Id1 was unaffected. Besides the relatively well-studied role of Id-proteins in the muscle differentiation (Melnikova and Christy 1996), little is known about the relationship between the Id-s and fiber type shift in mature muscle. Four days after denervation the Id1 mRNA level increases in rat hindlimb muscle (Gundersen and Merlie 1994), but Adams *et al.,* (1995) reported peak levels of Id1 mRNA 2 months after denervation of rat hindlimb muscle. To date, the role of Id3 has been studied only in embryogenesis studies (Melnikova *et al.,* 1996, 1999).

However, the data of gastrocnemius experiments should be interpreted in the perspective of the limitations of the study. Caution should be taken when extrapolating conclusions from experiments on the gastrocnemius to the diaphragm, as many anatomical and functional differences exist between these two muscles, *i.e.* the activity patterns of the gastrocnemius and the diaphragm are dissimilar, as the diaphragm has a longer duty cycle and is activated throughout the whole life and is likely to be particularly susceptible to the effects of inactivity. Still, among all the skeletal muscles, the gastrocnemius was probably the most suitable muscle to examine immobilization-induced deconditioning and passive shortening, because the fiber composition (Maltin *et al.,* 1989) and mRNA content of MRFs, especially for MyoD and myogenin are similar to that of the diaphragm (Kraus and Pette 1997). Fiber composition was a key factor, as immobilization-induced deconditioning is known to preferentially affect slow-type muscle and to a lesser extent fast-type muscle. However, according to a more recent study, after 24 hours of CMV disrupted myofibrils can be detected in the rabbit diaphragm, but not in the (resting) soleus, which underlies the inherent difference of the diaphragm compared to other skeletal muscles (Zhu *et al.,* 2005).

In the next set of experiments, a strategy was used to maintain the diaphragm intermittently active during the course of controlled mechanical ventilation, which is basically similar to that is applied in the human medicine when the prevention of the diaphragm disuse is necessary. It was hypothesized that this strategy would help to minimize diaphragm disuse atrophy occurring with controlled mechanical ventilation. Although this strategy did not result

yet in a significant improvement in diaphragm force, it was obviously sufficient to minimize the effects of controlled mechanical ventilation on diaphragm intrinsic properties. Whereas continuous mechanical ventilation resulted in a type I and more severe type IIX/B fiber atrophy, this was not the case in the groups allowed to intermittently breathe spontaneously during the course of controlled mechanical ventilation (Gayan-Ramirez *et al.,* 2005). As it was expected, both MyoD and myogenin protein levels were less decreased in the diaphragm after intermittent spontaneous breathing compared with continuous controlled mechanical ventilation. These findings are of particular interest as they show for the first time that diaphragm atrophy, due to mechanical ventilation, can be prevented by maintaining the diaphragm active for a short while during mechanical ventilation. Even a very short diaphragm activity as low as 20 minutes for 24 hours of controlled mechanical ventilation was sufficient.

Although methods for weaning patients from mechanical ventilation vary, they all generally consist of gradually making the patient assume a greater proportion of the work of breathing over time. In other words, the diaphragm of the patient is "trained" to regain the strength it lost during the period it was inactive. Actually, promising experiments have been conducted in which electrical stimulation of the diaphragm (Radeczki and Tomatis 1976; Fodstad *et al.,* 1983; Glenn *et al.,* 1984; Elefteriades *et al.,* 2002), resistance training (Belman *et al.,* 1981; Aldrich *et al.,* 1989), and strength training (Tobin and Alex 1994; Chao and Scheinhorn 1998) were separately used in an attempt to train the diaphragm of patients under mechanical ventilation, but data need to be extended to a larger population. In particular, diaphragm pacing may probably be used as training method to prevent the diaphragm from disuse atrophy during long-term mechanical ventilation but also during temporary ventilation in patients with high risk of developing respiratory muscle fatigue (Pavlovic and Wendt 2003). These data may open the door to preventing muscle function alterations instead of curing them once they are established.

The potential mechanisms by which intermittent spontaneous breathing may preserve diaphragm function are probably complex. However, we might hypothesize that the decrease in diaphragm insulin-like growth factor-I expression seen after controlled mechanical ventilation (Gayan-Ramirez *et al.,* 2003) could result in the decrease in MyoD expression, which may in turn lead to decreased expression of contractile proteins and thus decreased diaphragm force. Intermittent spontaneous breathing by preserving MyoD protein expression probably contributes to maintaining the levels of contractile proteins, thereby protecting

diaphragm properties from the effects of controlled mechanical ventilation. Further studies are required to highlight these mechanisms.

Finally, it is necessary to mention that the data of the present study pertain to an experimental model of mechanical ventilation in healthy animals. The relevance of these data to the clinic is hypothetical, and it seems premature to attempt to extrapolate these data to patients. It remains first to be determined the extent to which the data of the present study may be pertinent to patients. Moreover, the present study evaluated the effect of controlled mechanical ventilation, rather than assist-control, a mode more frequently used in clinical practice. Assist-control preserves diaphragmatic contractions and attenuates the force loss compared to complete inactivity (Sassoon *et al.,* 2004). However, not uncommonly, patients with acute respiratory distress syndrome receive passive ventilation through sedation with or without paralysis during mechanical ventilation (Esteban *et al.,* 2000). Thus the choice of controlled mechanical ventilation in this study is in accordance with clinical practice.

6.2. Regeneration study

The role of calcineurin in defining the slow muscle fiber identity is widely accepted (Bigard *et al.,* 2000; Talmadge *et al.,* 2004). The aim of this study was to compare the nerve-dependent increase of calcineurin expression and activity with the switch from fast- to slow-type myosin and SERCA isoforms in the time scale of regeneration.

During the regeneration, each MyHC type appears in a particular sequence: the fast isoforms, otherwise missing or present in low quantity in the normal slow soleus are expressed earlier. In general, the SERCA isoform levels change parallel to the corresponding MyHC. The time pattern of fast and neonatal isoforms, whose expression do not require innervation, is very similar. It is worth noting that the neonatal MyHC protein was also detected in the normal soleus by others (Whalen *et al.,* 1990; Jerkovic *et al.,* 1997; Noirez *et al.,* 2000). In contrast, in the differentiation stage (day 5–7, *i.e.* when innervation occurs) the appearance of slow SERCA mRNA and protein precede the slow MyHC by 2–4 days.

It is well established that in the denervated regenerating rodent soleus muscle, no slow MyHC1 is expressed (DeNardi *et al.,* 1993; Schiaffino *et al.,* 1998). The nerve dependence of SERCA2a expression was debated (Germinario *et al.,* 2002), however, the latest observations suggest that the initiation of the expression is not directly dependent, but the long term maintenance is modulated by the innervation (Zádor *et al.,* 2003).

CnA activity and protein could be detected consistently only after the fourth day of regeneration, when it is most likely of myofiber origin, because even in this early stage of regeneration, primitive desmin-expressing myofibers/myotubes already dominate over the other type of cells that populate a regenerating muscle (Mendler *et al.,* 1998). We focused on days 5 and 10, time points representing the situation, respectively, before and after the muscle comes again under neuronal control (Grubb *et al.,* 1991). The calcineurin activity was clearly increased by innervation in this experiment. This implies the role of upstream regulators of calcineurin, such as Ca^{2+} and calmodulin, in mediating the nerve effect.

Calcineurin activity might be absent from 1 to 5 day regenerating muscle because of the high levels of reactive oxygen and nitrogen species, which are known to be deleterious to calcineurin (Sommer *et al.,* 2000). Indeed, inflammatory cells, macrophages are prevailing in regenerating muscle on the first three days after toxin injection (Zádor *et al.,* 2001) and the reactive superoxide and $NO^{\bullet}$ produced by these cells might cause calcineurin to be degraded (Mitsuhashi *et al.,* 2000). It has been reported that macrophages are not expressing detectable amounts of CnA in early regenerating muscle (Sakuma *et al.,* 2003).

Our group also measured the level of calcineurin Aα, Aβ and MCIP1.4 mRNA in this experiment (Fenyvesi and Rácz 2005). We found that mRNA levels of CnAα and Aβ did not changed significantly; however, the trends were similar to the time course of the protein. The modulatory calcineurin interacting protein (MCIP1.4) is strongly induced by the calcineurin activity because its promoter includes a repeat of NFAT-binding sites (Rothermel *et al.,* 2003). At day 10, MCIP 1.4 mRNA level was increased in innervated, but not in denervated-regenerating muscles when compared to day 5. This shows that the expression of the MCIP1.4 mRNA mirrors the changes in protein and calcineurin activity both under conditions of innervated and denervated regeneration. This was also supported by the nerve dependence of MCIP1.4 mRNA level. In accordance, in the more advanced stages of regeneration of myofibers (day 10) compared to the less developed primitive myotubes (day 5), the increase was more pronounced, which also suggest dependence on innervation. The relative in vivo effects of the calcineurin-NFAT and MEF2 signalling pathways have long been debated (Schiaffino and Serrano 2002; Bassel-Duby *et al.,* 2003), although it is well established that the control of slow muscle fiber specification is mediated by calcineurin (Serrano *et al.,* 2001; Schiaffino and Serrano 2002), and that NFAT dephosphorylated by calcineurin is translocated to the nucleus (Meißner *et al.,* 2001). When slow innervation is established the resting free calcium level is increased in the sarcoplasm and this activates Ca^{2+}-calmodulin and

downstream calcineurin (Schiaffino and Serrano 2002; Bassel-Dubby *et al.,* 2003; Williams and Kraus 2005).

This work supports that the nerve-dependent increase of calcineurin activity is a critical part of slow muscle differentiation and is an essential step to translocate transcription factors to the nucleus in order to generate larger amounts of slow myosin mRNAs. Which regulators in this process mediate the nerve influence on calcineurin remains unknown. For example, the role of calcineurin in muscle adaptation to overload has been recognized (Bigard *et al.,* 2001). However, when calcineurin is overexpressed in muscle, it did not promote adaptation to overload (Dunn *et al.,* 2000). This highlights the importance of regulators (like Ca^{2+} and calmodulin) acting upstream of calcineurin in the calcineurin signalling pathway, probably acting on other branching regulatory pathways. Since in regenerating soleus muscle, calcineurin protein and activity but not its mRNA level showed an innervation-dependent increase, either the rate of CnA translation or the stability of the protein must be affected in these conditions. Taken together these results show that the nerve-dependent increases of calcineurin (and MCIP1.4 mRNA) levels preceded the replacement of fast myosin transcripts by the slow type MyHC1 mRNA, on days 5–10, but not the increase of slow SERCA2a mRNA. Finally, in accordance with this observation calcineurin was not found to regulate SERCA2a expression in regenerating muscles (Zádor *et al.,* 2005).

7. CONCLUSION

The first part of the study showed that controlled mechanical ventilation exerted early and severe alterations in gene expression that may play a role in diaphragm dysfunction and atrophy observed during mechanical ventilation. Passive shortening did not exert additional effect compared to disuse. Data of deconditioning were very similar to those observed in the diaphragm during controlled mechanical ventilation suggesting a predominant role of deconditioning in MV. Maintaining the diaphragm active even for a relatively short period of time during the course of controlled mechanical ventilation was associated with preservation of the diaphragm fiber dimensions and expression of transcription factors. This study highlights the fact that intermittent spontaneous breathing was an efficient tool to protect the diaphragm against the detrimental effects of controlled mechanical ventilation.

Two of the markers (MyHC, SERCA) that were examined in the mechanical ventilation study changed more dynamically in the regeneration model. This allowed a deeper insight into the regulation of these proteins. In non-adapting muscles the fast and slow MyHC and SERCA isoforms are expressed in a coordinated fashion. One candidate regulator of MyHC1 is calcineurin, of which activity was measured in innervated and denervated regenerating soleus muscle. The detailed time course of the regeneration revealed that the re-expression of SERCA2a precedes the MyHC1 and the CnA activity increase. This suggests the different regulation of these proteins, which is confirmed by other studies in our laboratory.

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PUBLICATIONS RELATED TO THE THESIS

I.

Gayan-Ramirez GN, Testelmans D, Maes K, *Rácz GZ*, Cadot PG, Zádor E, Wuytack FC, Decramer ML (2005) Intermittent spontaneous breathing protects the rat diaphragm from mechanical ventilation effects. *Crit Care Med* **33**(12):2804–2809.

Intermittent spontaneous breathing protects the rat diaphragm from mechanical ventilation effects*

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Objective: **Short-term mechanical ventilation has been proven to reduce diaphragm force and fiber dimensions. We hypothesized that intermittent spontaneous breathing during the course of mechanical ventilation would minimize the effects of mechanical ventilation on diaphragm force and expression levels of transcription factors (MyoD and myogenin).**

Design: **Randomized, controlled experiment.**

Setting: **Animal basic science laboratory.**

Subjects: **Male Wistar rats, weighing 350–500 g.**

Interventions: **Anesthetized and tracheotomized rats were submitted to either 24 hrs of spontaneous breathing (SB, n = 5), 24 hrs of continuous controlled mechanical ventilation (CMV, n = 7), or controlled mechanical ventilation with intermittent spontaneous breathing: 60 mins every 5 hrs of mechanical ventilation repeated four times (ISB60, n = 8) or 5 mins every 5 hrs 55 mins of mechanical ventilation repeated four times (SB5, n = 9). They were compared with control animals free from intervention (C, n = 5).**

Measurements and Main Results: **The profile of the diaphragm force-frequency curve of the controls and SB group was significantly different from that of the ISB and CMV groups; especially, the mean asymptotic force was less in the ISB and CMV compared with controls and SB. CMV resulted in a significant decrease in the diaphragm type I (−26%, $p < .05$ vs. C) and type IIx/b (−39%, $p < .005$ vs. C and SB) cross-sectional area, whereas this was not observed in the ISB groups. Diaphragm MyoD protein expression was significantly decreased after ISB60 (−35%, $p < .0001$ vs. C and SB) and even more after CMV (−73%, $p < .0001$ vs. others). The same pattern was observed with myogenin protein levels. Positive relationships between diaphragm MyoD and myogenin protein levels and diaphragm force were observed.**

Conclusions: **The data demonstrated that intermittent spontaneous breathing during the course of mechanical ventilation may minimize the deleterious effect of controlled mechanical ventilation on diaphragm force, fiber dimensions, and expression of transcription factors. (Crit Care Med 2005; 33:2804–2809)**

KEY WORDS: mechanical ventilation; myogenic regulatory factors; diaphragm; intermittent spontaneous breathing

Mechanical ventilation is an essential intervention in the intensive care unit used for the management of respiratory failure. Difficulties in weaning patients from mechanical ventilation are frequent and concern about 20–30% of patients after prolonged mechanical ventilation (1). In patients with chronic obstructive pulmonary disease, the number of patients needing a weaning procedure varies even from 35% to 67% (2). Weaning failure may be due to a variety of factors including inadequate ventilatory drive, respiratory muscle weakness, respiratory muscle fatigue, increased work of breathing, or cardiac failure (2). There is, however, accumulating evidence that weaning problems are associated with failure of the respiratory muscles to resume ventilation (2–4). Although many factors other than mechanical ventilation (e.g., sepsis, sedation, corticosteroid treatment) may contribute to respiratory muscle weakness in ventilated patients, mechanical ventilation by itself may also cause inspiratory muscle dysfunction (3, 4).

Assessment of respiratory muscle function is difficult in critically ill patients particularly because it requires voluntary maneuvers highly dependent on patient motivation and cooperation. Therefore, animal models of controlled mechanical ventilation were recently developed in which the deleterious effects of controlled mechanical ventilation on diaphragm function were highlighted. Reduced forces generated by the diaphragm *in vitro* (5, 6) related to the time spent on the ventilator (6, 7), and decreases in diaphragm fiber cross-section were reported as early as after 18–24 hrs of controlled mechanical ventilation. Moreover, several markers of atrophy were stimulated by controlled mechanical ventilation as shown by the concomitantly decreased diaphragm protein levels, increased proteasome activity (8), reduced insulin-like growth factor-I messenger RNA levels (5), and decreased MyoD expression levels in the diaphragm (9). Controlled mechanical ventilation also resulted in a rapid onset of oxidative injury in the diaphragm (10). Obviously, the data showed that controlled mechanical ventilation exerts early direct and deleterious effects on diaphragm function.

***See also p. 2852.**

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It appears likely that one reason for these alterations is inactivity and disuse atrophy. In theory, this could be prevented by intermittent spontaneous breathing. Therefore, we developed an animal model to examine whether intermittent spontaneous breathing during controlled mechanical ventilation may protect the diaphragm against the detrimental effects of mechanical ventilation.

To address this question, diaphragm contractile properties were examined *in vitro*, and the protein levels of myogenic regulatory factors (MyoD and myogenin) were measured in the diaphragm. The transcription factors, MyoD and myogenin, are known to stimulate myogenesis (11), and their expression was altered in the diaphragm after 24 hrs of controlled mechanical ventilation (9). Moreover, the importance of MyoD in regulating diaphragm force has been recently highlighted, as MyoD inactivation was associated with a downward shift of the diaphragm force-frequency relationship and a decrease in its maximal tetanic tension (12).

We hypothesized that intermittent spontaneous breathing would preserve diaphragm force, fiber dimensions, and the expression levels of diaphragm transcription factors, thereby protecting diaphragm function against the deleterious effects of controlled mechanical ventilation.

MATERIALS AND METHODS

Experimental Procedures, Study Design. The study was approved by the Animal Experiments Committee of the Medical Faculty of the Katholieke Universiteit, Leuven, and corresponded to the stipulations of the Helsinki declarations.

The study was performed on adult male Wistar rats (350–500 g) randomly divided into five groups:

1. A control group consisting of awake animals free from intervention (C, n = 5).
2. A group of rats breathing spontaneously for about 24 hrs submitted to the same surgical procedure than the mechanically ventilated rats (SB, n = 5).
3. A group of rats submitted to about 24 hrs of continuous controlled mechanical ventilation (CMV, n = 7).
4. A group of rats submitted to about 24 hrs of controlled mechanical ventilation with intermittent spontaneous breathing (ISB5, n = 9). The animals were allowed to breathe 5 mins spontaneously every 5 hrs 55 mins of controlled mechanical ventilation. Hence, they were breathing 20 mins spontaneously for about 24 hrs of controlled mechanical ventilation.
5. A group of rats submitted to about 24 hrs of controlled mechanical ventilation with intermittent spontaneous breathing (ISB60, n = 8), breathing 60 mins spontaneously every 5 hrs of controlled mechanical ventilation. They were breathing 4 hrs spontaneously in total for about 24 hrs of controlled mechanical ventilation.

Rats were initially anesthetized with sodium pentobarbital (60 mg/kg intraperitoneally) and then tracheotomized while their body temperature was continuously maintained at 37°C. The right carotid artery was cannulated for measurement of arterial blood pressure, pH, and blood gases. During the experiment, continuous infusion of both anesthesia (sodium pentobarbital 2 mg/100 g/mL/hr) and heparin (2.8 units/mL/hr) was given via the right jugular vein and carotid artery, respectively, using osmotic pumps (Pilot A2, Fresenius, Schelle, Belgium). Anesthesia depth was controlled throughout the experiment by evaluating foot reflex, corneal reflex, and arterial blood pressure. Anesthetized animals received 69 kcal/day enteral nutrition (liquid diet 821338, SDS, Essex, UK) administered every 2 hrs via a gastric tube. The diet was composed of 24% protein, 9% energy, 57% carbohydrate, 5% fiber, and 5% supplement including a vitamin and mineral mix. Anesthetized animals breathed humidified air enriched with oxygen (F_{IO_2} around 40%) and maintained at 37°C. During the period of mechanical ventilation, animals were ventilated with a volume-driven small-animal ventilator (Harvard Apparatus 665A, Holliston, MA), the tidal volume was set at ±0.5 mL/100 g body weight, and the respiratory rate was 55–60 breaths/min. For the animals breathing intermittently spontaneously, periods of spontaneous breathing were achieved by disconnecting the animals from the ventilator while breathing the same gas mixture as during controlled mechanical ventilation. They returned under controlled mechanical ventilation when the duration of spontaneous breathing was reached. Level of anesthesia was kept similar before and during the periods of spontaneous breathing as well as throughout the study duration. No positive end-expiratory pressure was applied during the experiment. Continuing care to the animals was performed including clearing the bladder, lubricating the eyes, rotating the animal, and passive movement of the limbs. Mechanical ventilation duration was similar between the groups and averaged 22.3 ± 1.9 hrs for the CMV group, 21.6 ± 1.3 hrs for the ISB60 group, and 24.1 ± 2.4 hrs for the ISB5 group, whereas spontaneous breathing duration averaged 24.3 ± 2.4 hr for the SB group.

In preliminary experiments, electromyographic activity of the diaphragm was measured in a few animals of each anesthetized group to ensure that no electrical activity was present in the diaphragm during the period of controlled mechanical ventilation. Wire electrodes (coated diameter, 112 μm; bare diameter, 50 μm; A-M systems, Carlsborg, VA) were placed in the costal diaphragm and were connected to a DISA 05A02 preamplifier where the signal was visualized.

On completion of the experiment, an arterial blood sample (90 μL) was taken and analyzed for Pa_{O_2}, Pa_{CO_2}, and pH by using a blood gas analyzer (1610, Instrumentation Laboratories, Zaventem, Belgium). Segments of the costal diaphragm were removed for measurement of *in vitro* contractile properties as previously described (13). Briefly, two diaphragm bundles per animal were suspended in a tissue bath containing Krebs solution continuously aerated with 95% oxygen and 5% CO_2 maintained at 37°C. Optimal length for peak twitch force was established for each bundle, and after a 15-min thermoequilibration period the force-frequency relationship was established by stimulating the bundles at the following frequencies: 1, 25, 50, 80, 120, and 160 Hz (250-msec train duration, 0.2-msec pulse duration). After completion of this protocol, bundle length was measured at optimal length and weighted. Cross-sectional area was obtained by dividing bundle weight by muscle specific density and optimal length. Forces were expressed per unit cross-sectional area.

Part of the remaining costal diaphragm was frozen in liquid nitrogen to examine protein expression of MyoD and myogenin using Western blotting as described subsequently. The other part of the costal diaphragm was fold, cut transversely, and frozen in isopentane cooled with liquid nitrogen to subsequently perform histologic and morphologic analysis as described subsequently. In addition, weights of the diaphragm and the gastrocnemius were measured. Animals were killed by injecting a bolus of sodium pentobarbital into the heart.

Histologic and Morphologic Analysis. Serial sections of the costal diaphragm were cut at 10-μm thickness with a cryostat kept at −20°C. Sections were stained with hematoxylin and eosin and analyzed qualitatively for structural abnormalities by an expert not aware of the experimental design of the study. Other sections were stained for adenosine triphosphatase after acid preincubation at pH 4.5 and 4.3, and fibers were identified according to their histochemical reactions as slow-twitch type I, fast-twitch type IIa, or fast-twitch type IIx/b fibers. Cross-sectional areas of each fiber type were determined from the number of pixels within the outlined borders using a Leitz Laborlux S. microscope (Wetzlar, Germany) at ×20 magnification, connected to

Table 1. Blood gas data and arterial blood pressure at dissection time in anesthetized rats either breathing spontaneously or under continuous controlled mechanical ventilation or submitted to intermittent spontaneous breathing (60 mins or 5 mins)

	Spontaneous Breathing	Intermittent Spontaneous Breathing—60 Mins	Intermittent Spontaneous Breathing—5 Mins	Continuous Controlled Mechanical Ventilation	*p* Values
Pa_{O_2}	161 ± 28	94 ± 21	119 ± 46	130 ± 54	.08
Pa_{CO_2}	48 ± 12	37 ± 14	35 ± 14	39 ± 22	.57
pH	7.33 ± 0.03	7.42 ± 0.06	7.49 ± 0.12	7.44 ± 0.05	.16
Arterial blood pressure	93 ± 15	113 ± 10	111 ± 17	115 ± 23	.14

Values are mean ± SD expressed in torr.

a computerized image system (Quantimet 500, Leica, Cambridge Ltd., UK) as previously described (5). Fiber proportion was also calculated.

Western Blot Quantification of MyoD and Myogenin. Frozen diaphragm samples were homogenized according to the protocol of Sakuma et al. (14). After polyacrylamide gel electrophoresis, the proteins were transferred onto a polyvinyldifluoride membrane by semidry blotting. After incubation with polyclonal rabbit anti-MyoD (1/400, sc-760, Santa Cruz Biotechnology, Santa Cruz, CA) or antimyogenin (1/400, sc-576, Santa Cruz Biotechnology) and horseradish peroxidase-conjugated rat antirabbit immunoglobulin G (1/1000, Biosource, Nivelles, Belgium) or swine antirabbit immunoglobulin G (1/1000, Dako, Heverlee, Belgium), the bands were revealed with tetramethylbenzidine and quantified using the Quanti One software (BioRad, Richmond, CA).

Statistical Analysis. The evolution of the diaphragm force as a function of frequency has been analyzed using a nonlinear mixed-effects model (15). The control and SB groups were merged as well as the two ISB groups for subsequent analysis. The analysis was performed on three treatment groups: namely control, ISB, and CMV. The three-variable logistic model fitting with the force-frequency profile was given by the following formula:

$$y = \frac{A}{1 + \exp[(freq - B)/C]}$$

where

y is the force (g/cm^2),

Freq is the frequency (Hz),

A is the asymptotic value of the force y,

B is the value of the frequency (Hz) at which the tissue reaches half of the asymptotic force, and

C is the value of the additional frequency (Hz) required for the tissue to move from the half to approximately 3/4 of the asymptotic force.

The overall difference between the groups was tested with the likelihood ratio test; *p* values were imposed to Bonferroni adjustments for multiple comparisons. The analysis to fit the nonlinear models and the Bonferroni corrections were implemented in S-PLUS.

For the data other than forces, comparisons between the five groups were performed using a one-way analysis of variance followed by a Newman-Keuls' test as *post hoc* test. Relationships between MyoD and myogenin protein levels with forces were assessed with the Pearson correlation coefficient. Statistical analysis was performed with the SAS statistical package (SAS Institute, Cary, NC). Data are presented as mean ± SD.

RESULTS

Blood Gases and Arterial Blood Pressure. As shown in Table 1, at the time of dissection, values for arterial blood pressure and blood gases for anesthetized rats were not significantly different between the groups. Moreover, these values remained in the normal range for all studied variables.

Body Weight and Muscle Mass. Initial body weight was significantly lower in the CMV and ISB5 groups compared with C and SB groups, as was the body weight in the ISB60 group compared with the SB group (Table 2). Diaphragm mass was significantly decreased in the CMV group compared with C (−22%, $p < .05$) and SB (−23%, $p < .05$) groups, whereas it remained unchanged in the intermittent spontaneous breathing groups. Similarly, gastrocnemius mass was lower in the ISB5 and CMV groups compared with C and SB groups. These effects disappeared when muscle mass was expressed as a percentage of respective body weight (Table 2).

Diaphragm Contractile Properties. The profile of the diaphragm force-frequency curve of the controls and SB group was significantly different from that of the ISB and CMV groups. More specifically, the mean asymptotic force was less in the ISB ($p = .008$) and CMV ($p = .005$) groups compared with controls and SB group (Fig. 1). Also, the mean frequency at which the diaphragm reached half of the asymptotic force was significantly different in the ISB ($p = .0006$) and CMV ($p < .0001$) groups compared with control and SB groups. Twitch characteristics (time-to-peak tension and half-relaxation time) were otherwise similar between the groups.

Histologic and Histochemical Analysis. Diaphragm fiber proportion was similar between the five groups (pooled values for type I, 42 ± 6; type IIa, 30 ± 4; and type IIx/b, 29 ± 6%). There were no changes in diaphragm fiber dimensions in the SB and ISB groups compared with controls (Fig. 2). By contrast, CMV resulted in a significant decrease in the diaphragm type I (−26%, $p < .05$ vs. C) and type IIx/b (−39%, $p < .005$ vs. C and SB) cross-sectional area (Fig. 2).

Western Blot: Analysis of MyoD and Myogenin. Compared with controls and SB group, diaphragm MyoD protein expression was significantly decreased after ISB60 (−35%, $p < .0001$) and more severely after CMV (−73%, $p < .0001$, Fig. 3, *top panel*). Diaphragm MyoD protein was also slightly decreased in the ISB5 group (−27%), but this decrease failed to reach statistical significance. Most important, the diaphragm MyoD protein levels in the CMV group were significantly different from those of the ISB5 and the ISB60 groups ($p < .0001$, Fig. 3, *top panel*).

For myogenin, the same pattern was observed, with myogenin protein levels being particularly decreased in the CMV group (−90%, $p < .0001$ vs. C and SB) and to a lesser extent in the ISB5 (−56%, $p < .0001$ vs. C and SB) and ISB60 groups (−67%, $p < .0001$ vs. C and SB, Fig. 3, *bottom*). Interestingly, the decrease in myogenin protein levels was significantly different between the CMV and the ISB5 groups ($p < .0001$, Fig. 3, *bottom*).

There were also significant relation-

Table 2. Body weight (in grams) and muscle mass (in mg) in the five groups

	Control	Spontaneous Breathing	Intermittent Spontaneous Breathing—60 Mins	Intermittent Spontaneous Breathing—5 Mins	Continuous Controlled Mechanical Ventilation
Body weight	480 ± 44	507 ± 14	396 ± 35[a]	408 ± 39[a,b]	381 ± 72[a,b]
Diaphragm	608 ± 77	615 ± 50	517 ± 86	535 ± 38	474 ± 50[a,b]
Diaphragm/body weight	0.126 ± 0.006	0.121 ± 0.009	0.131 ± 0.024	0.132 ± 0.014	0.134 ± 0.018
Gastrocnemius	2414 ± 257	2402 ± 84	1994 ± 229	1945 ± 143[a,b]	1882 ± 332[a,b]
Gastrocnemius/body weight	0.503 ± 0.029	0.474 ± 0.028	0.493 ± 0.052	0.478 ± 0.022	0.486 ± 0.027

Muscle masses are also expressed as percent body weight to normalize for differences in body weight between the groups.
[a]$p < .05$ vs. spontaneous breathing; [b]$p < .05$ vs. control. Values are mean ± SD.

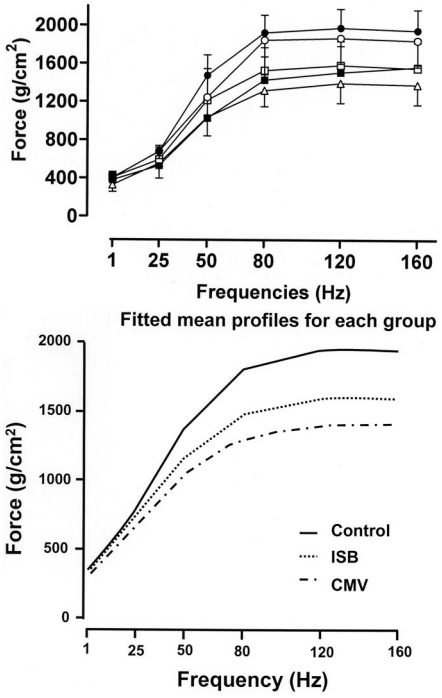

Figure 1. *Top panel*, diaphragm force generated at 1, 25, 50, 80, 120, and 160 Hz during the force-frequency relationship in the five groups: control (*filled circles*), spontaneously breathing (*open circles*), intermittent spontaneous breathing 60 mins/5 hrs of controlled mechanical ventilation (*open squares*) or 5 mins/5 hrs and 55 mins of controlled mechanical ventilation (*filled squares*), and controlled mechanical ventilation (*open triangles*). Values are mean and SD, expressed in absolute values. *Bottom panel*, force-frequency relationship depicted in *A* after fitting with the nonlinear mixed effects model using three treatment groups: control group (control and spontaneous breathing), intermittent spontaneous breathing group (*ISB*), and controlled mechanical ventilation group (*MV*).

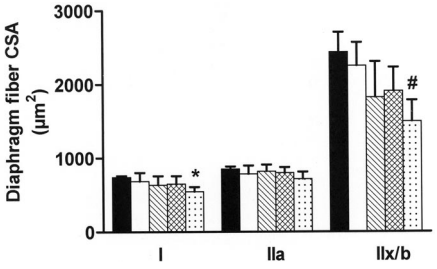

Figure 2. Diaphragm cross-sectional area (*CSA*) in the diaphragm of type I, type IIa, and type IIx/b fibers in the controls (*filled bars*), rats submitted to either spontaneous breathing (*open bars*), intermittent spontaneous breathing 60 mins every 5 hrs of controlled mechanical ventilation (*hatched bars*) or 5 mins every 5 hrs and 55 mins of controlled mechanical ventilation (*cross-hatched bars*), or continuous controlled mechanical ventilation (*dotted bars*). Values are mean and SD. *$p < .05$ vs. controls, #$p < .005$ vs. controls and spontaneously breathing group.

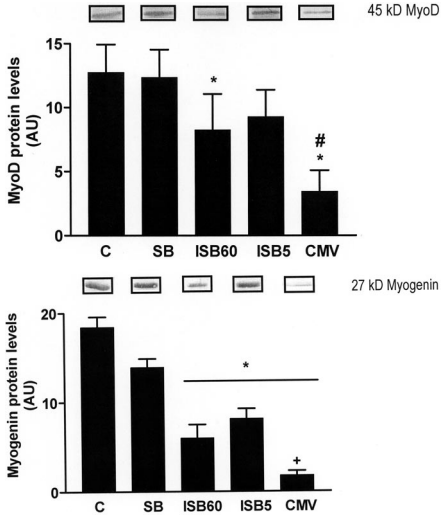

Figure 3. Western-blotting data of MyoD (*upper panel*) and myogenin (*lower panel*) in the diaphragm of the five groups: control (*C*), spontaneous breathing (*SB*), intermittent spontaneous breathing 60 mins/5 hrs of controlled mechanical ventilation (*ISB60*) or 5 mins/5 hrs and 55 mins of controlled mechanical ventilation (*ISB5*), and controlled mechanical ventilation (*CMV*). Each band represents representative sample for each group. Values are means and SD. *$p < .0001$ vs. controls and SB; #$p < .0001$ vs. ISB5 and ISB60; +$p < .0001$ vs. ISB5.

ships between diaphragm MyoD and myogenin protein levels and diaphragm force (Fig. 4 for tetanic tension). Thus, MyoD was positively correlated with diaphragm force especially with force generated at high frequencies (Table 3). These positive correlations were also observed with diaphragm myogenin protein levels (Table 3). In addition, diaphragm MyoD and myogenin protein levels were positively and strongly correlated ($r = .86$, $p < .0001$).

DISCUSSION

The present study is the first showing that spontaneous breathing during the course of controlled mechanical ventilation may help to maintain diaphragm function and, more particularly, to prevent diaphragm atrophy and down-regulation of key muscle regulatory factors. This suggests that intermittent spontaneous breathing may protect the diaphragm against the deleterious effects of controlled mechanical ventilation.

Recently, several groups have developed animal models to examine the extent to which continuous controlled mechanical ventilation could affect diaphragm function. Twelve to 24 hrs of controlled mechanical ventilation resulted in reduced *in vitro* diaphragmatic forces (5, 6), the diaphragmatic dysfunction increasing with the time spent on the ventilator (6, 7). Also *in vivo* diaphragmatic force was reduced after 24 hrs of controlled mechanical ventilation as shown by the decrease in maximal transdiaphragmatic pressure generated during phrenic nerve stimulation (7). Clearly, these data showed that continuous controlled mechanical ventilation exerted very early detrimental effects on diaphragm function. In the present study, a strategy was used to maintain the diaphragm intermittently active during the course of controlled mechanical ventilation. We hypothesized that this strategy would help to minimize diaphragm disuse atrophy occurring with controlled mechanical ventilation. Although this strategy did not result yet in a significant

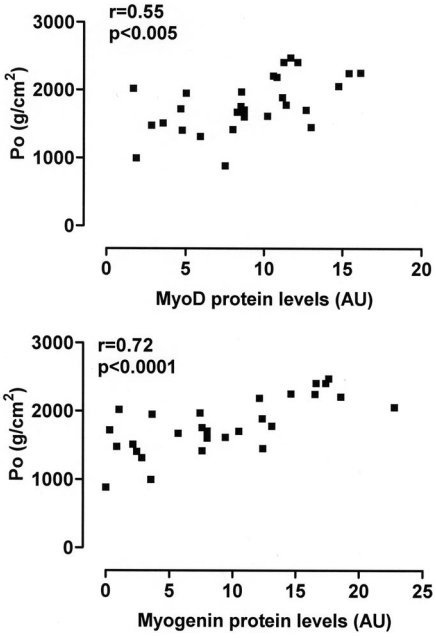

Figure 4. Correlation between diaphragm MyoD (*upper panel*) and myogenin (*lower panel*) protein levels (expressed in arbitrary unit) and maximal tetanic tension (*Po*).

Table 3. Relationships between diaphragm MyoD and myogenin protein levels and diaphragm force generated at different frequencies

	MyoD Protein	Myogenin Protein
80 Hz	r = .58	r = .66
	$p < .005$	$p < .001$
120 Hz	r = .50	r = .64
	$p < .01$	$p < .001$
160 Hz	r = .55	r = .72
	$p < .005$	$p < .0001$

improvement in diaphragm force, it was obviously sufficient to minimize the effects of controlled mechanical ventilation on diaphragm intrinsic properties. Indeed, whereas continuous mechanical ventilation resulted in a type I and IIx/b fiber atrophy, this was not the case in the groups allowed to intermittently breathe spontaneously during the course of mechanical ventilation. These findings are of particular interest as they show for the first time that diaphragm atrophy, due to mechanical ventilation, can be prevented by maintaining the diaphragm active for a short while during mechanical ventilation. Even a very short diaphragm activity as low as 20 mins for 24 hrs of controlled mechanical ventilation was sufficient.

In mechanically ventilated patients, causes of failure to wean from mechanical ventilation are various and include inadequate ventilatory drive, increased work of breathing, excessive CO_2 production, cardiac failure, respiratory muscle weakness, and respiratory muscle fatigue. Importantly, these factors are interrelated and several of them may be present in the same patient. Additionally, diaphragm inactivity may also contribute to weaning failure although it is probably not the unique cause of weaning failure. Obviously, disuse atrophy is likely to develop in the diaphragm of patients under mechanical ventilation. Although methods for weaning patients from mechanical ventilation vary, they all generally consist of gradually making the patient assume a greater proportion of the work of breathing over time. In other words, the diaphragm of the patient is "trained" to regain the strength it lost during the period it was inactive. Actually, promising experiments have been conducted in which either electrical stimulation of the diaphragm (16–19), resistance training (20, 21), or strength training (22, 23) was used in an attempt to train the diaphragm of patients under mechanical ventilation, but data need to be extended to a larger population. In particular, diaphragm pacing may probably be used as training method to prevent the diaphragm from disuse atrophy during long-term mechanical ventilation but also during temporary ventilation in patients with high risk of developing respiratory muscle fatigue (24). Our data suggest that even very short bouts of activity would be efficient in minimizing disuse atrophy induced by controlled mechanical ventilation. These data may open the door to preventing muscle function alterations instead of curing them once they are established. However, in ventilated patients, respiratory muscle weakness may develop during mechanical ventilation for causes other than disuse (25); these include protein-calorie malnutrition, electrolyte disturbances, corticosteroid treatment, sepsis, and sedation (2).

Interestingly, even at the molecular levels, both MyoD and myogenin protein levels were less decreased in the diaphragm after intermittent spontaneous breathing compared with continuous controlled mechanical ventilation. In mature adult skeletal muscles, the role of these muscle specific transcription factors is not fully established, but their implication in situations such as denervation, immobilization, electrostimulation, mechanical loading, and hormone treatment has been demonstrated (26–29). MyoD in particular is the master regulatory gene for skeletal myogenesis, as its expression in fibroblasts is sufficient to convert these cells into myoblasts (30) and MyoD −/− cells are differentiation defective (31). Also, MyoD deletion resulted in a diaphragm fast-to-slow shift in the myosin heavy chain phenotype (12) and a downward shift of the force-frequency relationship. These data suggest that MyoD probably plays an important role in maintaining diaphragm force. This is in line with our data showing a down-regulation of diaphragm MyoD protein expression together with a reduction in diaphragm force after continuous controlled mechanical ventilation being reversed by intermittent spontaneous breathing. This close association between MyoD levels and diaphragm force-generating capacity is further underlined by the positive relationships found between diaphragm MyoD protein expression and diaphragm force. Intriguingly, diaphragm myogenin protein levels followed the same pattern as MyoD, and the same relationships were also found between myogenin and diaphragm force. The repercussion of increased myogenin protein levels on diaphragm function is not known because the role of myogenin in mature muscles has mainly been investigated in relation to denervation and regeneration. However, it is well known that MyoD and myogenin both contribute to satellite cell activation, but whereas MyoD is primarily involved in satellite cell proliferation, myogenin is rather implicated in the differentiation process. Whether the increases in MyoD and myogenin protein levels seen in the present study are related to satellite cell activation or to changes in myofiber nuclei remains to be established.

The potential mechanisms by which intermittent spontaneous breathing may preserve diaphragm function are probably complex and were beyond the aim of the present study. However, we might hypothesize that the decrease in diaphragm insulin-like growth factor-I expression seen after continuous mechanical ventilation could result in the decrease in MyoD expression, which may in turn lead to decreased expression of contractile proteins and thus decreased diaphragm force. Intermittent spontaneous breathing by preserving MyoD protein expression probably contributes to maintaining the levels of contractile proteins, thereby protecting diaphragm

The data demonstrated that intermittent spontaneous breathing during the course of mechanical ventilation may minimize the deleterious effect of controlled mechanical ventilation on diaphragm force, fiber dimensions, and expression of transcription factors.

properties from the effects of continuous mechanical ventilation. Further studies are required to highlight these mechanisms.

Finally, it is necessary to mention that the data of the present study pertain to an experimental model of mechanical ventilation in healthy animals. The relevance of these data to the clinic is hypothetical, and it seems premature to attempt to extrapolate these data to patients. Furthermore, the use of intermittent spontaneous breathing in patients under mechanical ventilation is obviously not feasible. However, other strategies may be considered. It remains first to be determined the extent to which the data of the present study may be pertinent to patients.

CONCLUSIONS

The present study showed that maintaining the diaphragm active even for a relatively short period of time during the course of controlled mechanical ventilation was associated with preservation of the diaphragm fiber dimensions and expression of transcription factors. This study highlights the fact that intermittent spontaneous breathing was an efficient tool to protect the diaphragm against the detrimental effects of controlled mechanical ventilation.

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The calcineurin activity and MCIP1.4 mRNA levels are increased by innervation in regenerating soleus muscle☆

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Abstract

The level of active subunit of calcineurin and the calcineurin (Cn) enzyme activity are increased in innervated but not in denervated slow type regenerating skeletal soleus muscle. These nerve-dependent increases were not accompanied by similar increases in the mRNA levels. The changes in the mRNA level of the modulatory calcineurin interacting protein, MCIP1.4, reflected the calcineurin activity and did not increase in denervated regenerating muscles compared to the innervated regenerating controls. The increases in Cn activity and in MCIP1.4 mRNA levels occurred before the switch from fast to slow-type myosin heavy chain isoforms, a phenomenon similarly known to be dependent on innervation. This highlights the role of mediators, acting between the nerve and calcineurin, in the formation of slow fiber identity.

Keywords: Calcineurin; MCIP1.4; Myosin heavy chain; SERCA; Regeneration

The expression of the slow myosin isoform (MyHC1) is strictly dependent on a slow type innervation and is characteristic for the slow fibers [1,2]. Calcineurin mediates the nerve effect on slow-fiber-type gene transcription by dephosphorylating transcription factors like NFAT, MEF2 and rendering their translocation to the nucleus [3,4]. Constitutively active calcineurin selectively upregulates the slow-fiber-specific promoters [5] and oral administration of calcineurin inhibitors decreases the number of MyHC1-expressing slow fibers in rat soleus muscle [5,6]. It has been established that calcineurin mediates neuronal control of MyHC1 expression in regenerating muscle [7], although also an alternative regulating pathway acting through H-ras has been reported [2]. We have recently shown that the expression of slow-muscle-specific sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA2a) is not innervation- and ras-dependent in regenerating soleus, in contrast to MyHC1 [8]. Apart from its independence from nerve activity, SERCA2a expression is regulated translationally and posttranslationally in regenerating muscle, while in the case of MyHCI the regulation is primarily at the transcript level [2]. This clearly shows that innervation through calcineurin cannot coregulate SERCA2a and MyHCI as was proposed earlier [6,9,10]. In accordance with this, the role of calcineurin in muscle differentiation has been debated [4], partly because the direct link of NFAT to slow-muscle-specific promoters is still missing [11,12]. Specifying slow fiber fate is probably a multifactorial process, as it has been suggested by studies on the phenotypic adaptation of cat soleus [13]. Therefore, we found it important to further study the effect of innervation on individual genes involved in differentiation of slow fibers. In this study we focused on calcineurin, which has been reported to control muscle regeneration [14–16] but whose activity has not been measured yet in regenerating muscle (reviewed by [17]). We present here new data indicating that the level of

☆ *Abbreviations:* CnA, calcineurin A subunit; DAB, diamino-benzidine; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; MCIP, modulatory interacting protein; MyHC, myosin heavy chain; RT-PCR, reverse-transcriptase polymerase chain reaction; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; SR, sarcoplasmic reticulum.

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calcineurin protein and its enzyme activity is elevated in a nerve-dependent manner in regenerating soleus muscle whereas the levels of the corresponding mRNA of CnAα and CnAβ subunits are not changed. The modulatory calcineurin interacting protein MCIP1.4, which bears multiple NFAT-binding sites in its promoter, is shown to reflect the changes in calcineurin activity and hence appears a good reporter for general calcineurin activity in differentiating muscle cells [18]. The increase of calcineurin activity and MCIP1.4 precedes the switch from fast myosin to slow-type MyHC1 isoform but not the expression of SERCA2a.

Materials and methods

Animals and treatment. Three-month-old (300–360 g) adult male Wistar rats were treated with notexin and occasionally denervated before the toxin administration as in [8]. On days 1, 3, 5, 7, 10, 21 or 28 the entire soleus muscle was removed and the animals were killed with an overdose of Na-pentobarbital.

RT-PCR. The total RNA was isolated as previously described in [19] and kept at −70 °C. Two microgram of total RNA of each soleus was subjected to oligo(dT)-primed first-strand cDNA synthesis in a volume of 20 μl as in [20]. A 1-μl portion of the first-strand cDNA mixture was subjected to multiplex PCR according to the specifications shown in Table 1.

The PCR products were identified by their sizes and sequences and quantified by means of a PhosphorImager model 425 (Molecular Dynamics, Sunnyvale, CA, USA) and ImageQuaNT 2.0 software. MyHC and SERCA RT-PCR was done from the same RNA samples. SERCA1a and 1b were amplified by the same primer pair encompassing the optional 42-bp exon present in the adult SERCA1a mRNA but spliced out in the neonatal SERCA1b. In this way the ratio of both variants can be assessed.

Preparation of muscle extracts for electrophoresis. The myosin was extracted according to [21]. The isolated muscles were weighed and homogenized in liquid nitrogen with mortar and pestle. The muscle powder was suspended in 8 volumes of myosin extraction solution (100 mM Na–pyrophosphate, 5 mM EGTA, 5 mM $MgCl_2$, and 300 mM KCl, pH 8.50). The suspension was stirred for 20 min at 4 °C and centrifuged for 10 min at 10,000*g* in an Eppendorf centrifuge. The supernatant fraction was diluted with 1 vol glycerol and stored at −25 °C. For extraction of SERCA proteins, the muscles were homogenized in 2.5 ml ice-cold 0.25 M sucrose in 5 mM Hepes, pH 7.5, using a glass/teflon Potter homogenizer. The homogenates were centrifuged at 1000*g* for 10 min at 4 °C. The supernatants were centrifuged at 200,000*g* for 30 min at 4 °C. The pellets, which represented the combined microsomal/mitochondrial fraction, were resuspended in 600 μl of 0.25 M sucrose and kept at −20 °C. Calcineurin was measured in whole muscle extracts made as in [22,23].

Immunoblots. For electrophoresis of myosin heavy chain, the crude myosin extracts (1:10 diluted for MyHC1 and SERCA2a and undiluted for MyHC2a and SERCA1) were supplemented with 0.5 volume loading buffer, 15 μl of this solution was run on 6% SDS–PAGE (7.5% for SERCA immunoblots) and then blotted as described in [24]. The primary antibodies used were: monoclonal antibody for MyHC1 (mouse, clone BA-D5, 1:10 [25]), for MyHC2a (SC-71, mouse, 1:15 [25]), SERCA2a-specific antiserum (R15, rabbit, 1:500 [26]), monoclonal antibody for SERCA1—(A3, mouse, 1:30 [27]), and a monoclonal against all isoforms of calcineurin A (mouse, 1:2500; Sigma). Secondary antibodies were peroxidase-conjugated goat anti-mouse immunoglobulin (1:1000) or peroxidase-conjugated swine anti rabbit (1:1000) (Dako A/S, Glostrup, Denmark). The immunoblots for calcineurin A were stained by the Vistra EFC kit (Amersham Life Sciences) and quantified by fluorescence imaging on the Storm 840 (Molecular Dynamics, Sunnyvale, CA).

Calcineurin activity assay. Homogenates of fresh muscles were made as described in [23]. Enzyme activity was measured from the homogenate by a colorimetric assay kit (Calbiochem) according to the vendor's protocol.

Number of experiments, statistics. For the PCR and the Western blot studies 3 or 4 preparations, each from separate animals, were used for every time point. For the immunohistochemical studies the notexin-treated and their contralateral muscles from three animals were used for every time point. ANOVA or t-probe was used to test for statistically significant differences ($p < 0.05$) between the groups.

Results and discussion

The local injection of notexin into a soleus muscle completely degraded the myofibers, this fiber necrosis was followed by a relatively fast regeneration process

Table 1
Primers and cycle numbers, denaturing, annealing, and extension temperatures (each applied for 1–1–1 min) used in PCR

Primer	Sequence	Fragment size	PCR cycles	Temperatures (°C)
MyHC I	5′-acagaggaagacaggaagaacctac-3′ 5′-gggcttcacaggcatccttag-3′	288	15	95–60–72
MyHC IIa	5′-tatcctcaggcttcaagatttg-3′ 5′-taaatagaatcacatggggaca-3′	309 bp	20	95–55–72
MyHC IIx	5′-cgcgaggttcacaccaaa-3′ 5′-tcccaaagtcgtaagtacaaaatgg-3′	121 bp	20	95–58–72
neoMyHC	5′-gaaggccaagaaggccatca-3′ 5′-gcggcctcctcaagatgcgt-3′	567	35	95–52–72
SERCA1a/SERCA1b	Zador et al. [19]	248/206	21	94–60–72
SERCA2a		231	21	94–55–72
GAPDH		376	Depending on the coamplified MyHC	
MCIP1.4	5′-aaggaacctccagcttgggct-3′ 5′-ccctggtctcactttcgctg-3′	159	23	95–54–70
CnA α	5′-agttgtcgactacggacag-3′ 5′-aagtcaaagaattgtccatgg-3′	265	28	95–50–72
CnA β	5′-tgaagtaggaggatcacctg-3′ 5′-tcagaaggatcagaccacag-3′	405	26	95–50–72

which, at least judged in terms of light-microscopical morphology, was largely completed in 28 days. In the normal muscles, the calcineurin A (CnA) protein was readily detectable by Western blotting but after necrosis it transiently disappeared on days 1–3 after notexin injection (Fig. 1A), it re-emerged on days 5–10, after which it remained at about the normal level until day 28 of regeneration. From day 4 of regeneration onwards, the CnA protein found in the assay is most likely of myofiber origin, because even in this early stage of regeneration, primitive myofibers/myotubes already dominate over the other type of cells that populate a regenerating muscle [28]. We focused our study on days 5 and 10, i.e., time points representing the situation, respectively, before and after the muscle comes again under neuronal control [29]. In denervated regenerating muscles, like in innervated controls, the CnA protein levels were also low on day 5, but they failed to normalize after 10 days (Fig. 1B). The calcineurin enzyme activity also dropped to about 50% of the control level on day 5 of regeneration and completely recovered on day 10 in innervated muscles. In contrast, in denervated muscles no recovery was observed (Fig. 1C). Therefore, the restoration of the calcineurin activity was strictly dependent on the nerve and it correlated with the levels of CnA.

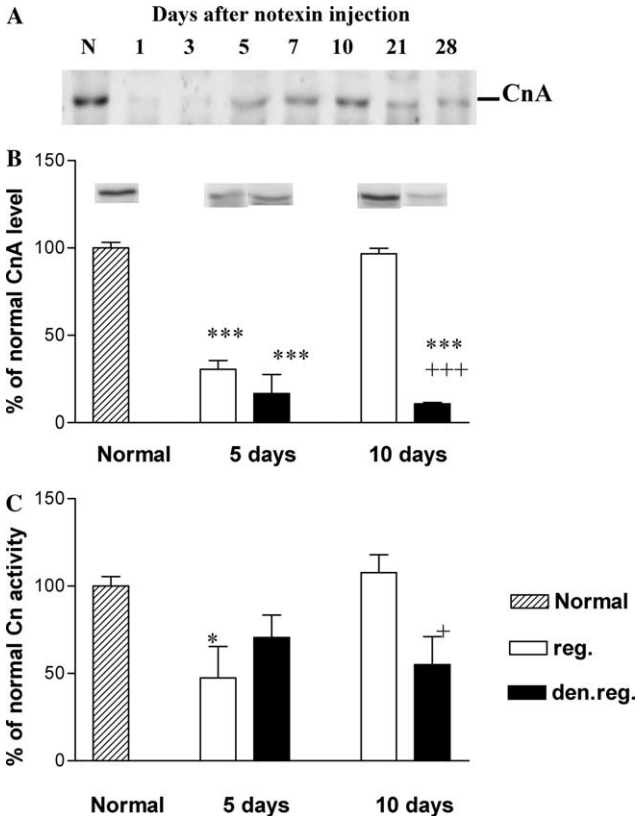

Fig. 1. Calcineurin protein and enzyme activity in regenerating rat soleus. (A) The calcineurin A protein levels in regenerating soleus. (B) Calcineurin A protein levels and (C) enzyme activity in regenerating soleus in the presence (white columns) and absence (black columns) of innervation on days 5 and 10. Values are means ± SE, $n = 3$. Significant ($p < 0.05$) and highly significant ($p < 0.001$) differences compared to control muscles are marked with * and ***; and between innervated and denervated muscles with + and +++, respectively.

Calcineurin A exists in three isoforms. Among these the α and β isoforms are present in skeletal muscle [30]. We checked the mRNA levels of the CnAα and CnAβ and, in contrast to what we observed for the protein levels, found no change in the CnAα or CnAβ mRNAs in innervated nor in denervated regenerating muscle on days 5 and 10 (Figs. 2A and B). This shows that the level of CnA expression is largely regulated at the protein level.

It is expected that the expression of the modulatory calcineurin interacting protein (MCIP1.4) is strongly induced by the calcineurin activity because its promoter includes a cluster of NFAT-binding sites [18,31,32]. We therefore measured the level of MCIP1.4 mRNA after 5 and 10 days in innervated-regenerating and in denervated-regenerating muscles and found that at day 10 its level was increased in innervated—but not in denervated-regenerating muscles when compared to day 5 (Fig. 3). This shows that the expression of the MCIP1.4 mRNA mirrors the changes in CnA protein and calcineurin activity both under conditions of innervated and denervated regeneration.

Because the role of calcineurin in defining the slow fiber identity is widely accepted, we tried to correlate the nerve-dependent increase in calcineurin expression and activity to the switch from fast- to slow-type myosin and from SERCA1 to SERCA2 mRNAs in regenerating soleus [1,19,24].

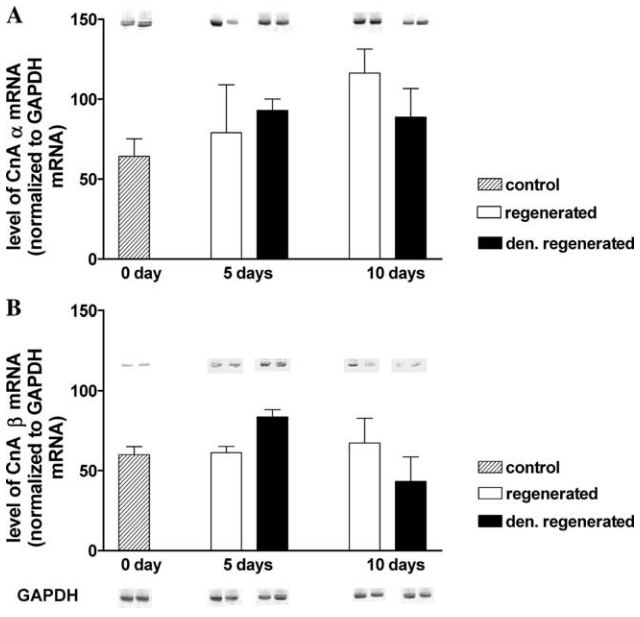

Fig. 2. The mRNA levels of CnAα (A) and CnAβ (B) in innervated and denervated regenerating soleus. Symbols as in Fig. 1, $n = 4$.

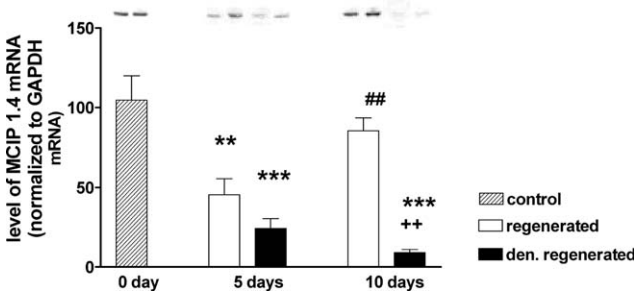

Fig. 3. The mRNA level of modulatory calcineurin interacting protein, MCIP1.4, in innervated and denervated regenerating soleus muscle. Symbols as in Fig. 1. Very significant differences ($p < 0.01$) to the control are indicated with **, between the innervated regenerated and denervated-regenerated muscles with ++, and between two sequential time points are marked with ##. *** as in Fig. 1

First in normal adult soleus muscle, the mRNA of the neonatal MyHC, the fast-type MyHC2x, and of the neonatal form of the fast-type SERCA1b appeared on day 1 of regeneration (Fig. 4A). However, after showing a transient increase, peaking at days 5 and 7, they normalized again toward day 28. It is worth noting that the neonatal MyHC protein was also detected in the normal soleus by others [1,33–35].

The mRNAs of the fast-type MyHC2a and SERCA1a isoforms (Fig. 4B) nearly disappeared from the muscle on days 1–3 after injection of the toxin, but they were increased again on day 5, and after passing through a maximum on day 10, gradually declined to the normal level.

The message of the slow MyHC1 (Fig. 4C) reappeared on day 7 and showed a monotonous gradual increase until day 28, where it reached a higher level than the initial control. The transcript of the slow SERCA2a was already increased on days 3–5, otherwise it changed in parallel with the slow MyHC1 mRNA.

The expression of the main fast and slow protein myosin and SERCA isoforms corresponded well to their respective transcript levels. The fast MyHC2a and SERCA1a protein started to recover on day 5, reached

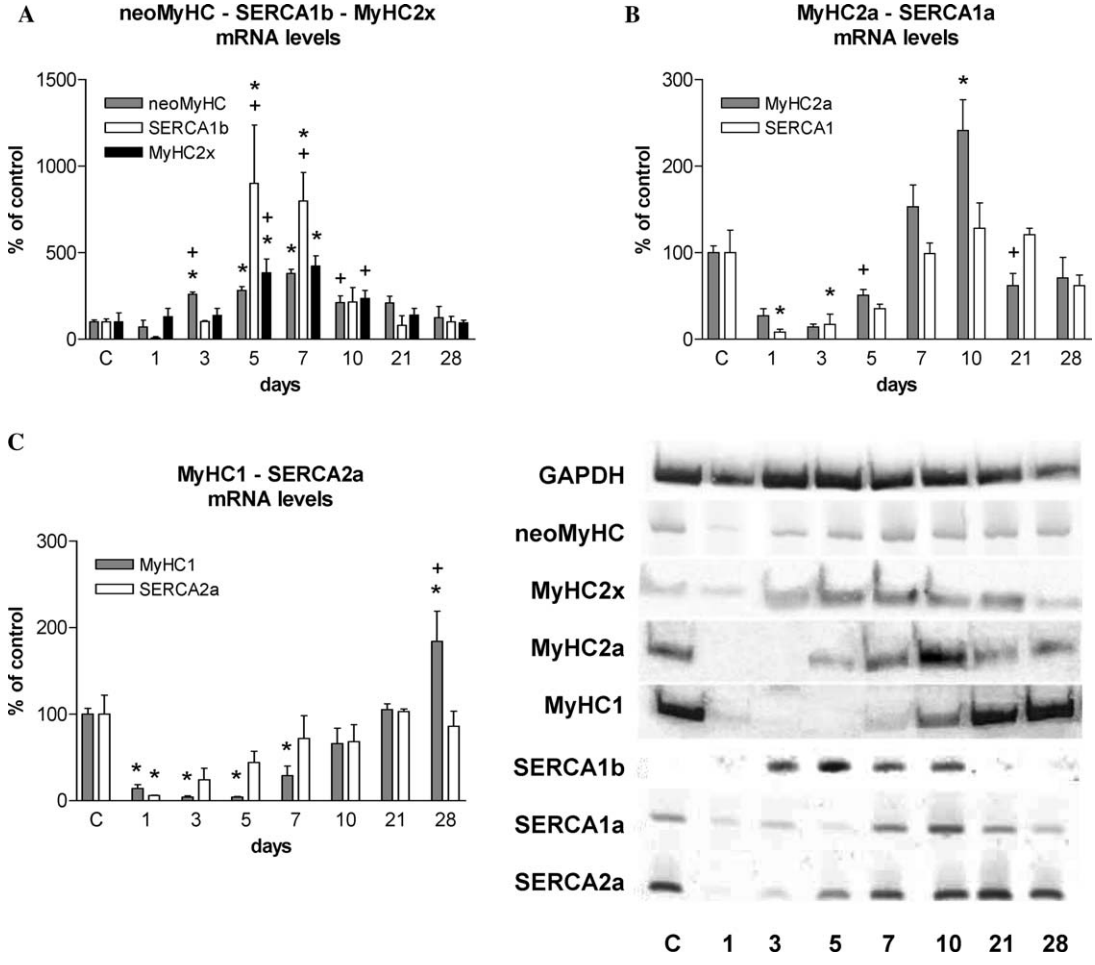

Fig. 4. The mRNA levels (A) of neoMyHC, SERCA1b, and MyHC2x, (B) MyHC2a and SERCA1a, and (C) MyHC1 and SERCA2a isoforms during the regeneration of soleus after notexin-induced necrosis. The values on the ordinate cannot be compared between panels and are normalized to the control values of (normal soleus) which are taken as 100%. Significant ($p < 0.05$) differences compared to C are marked with *; significant differences between two adjacent columns are marked with +. Obviously significant differences between control and zero levels are not marked. Representative gels are shown in the lower part of the figure.

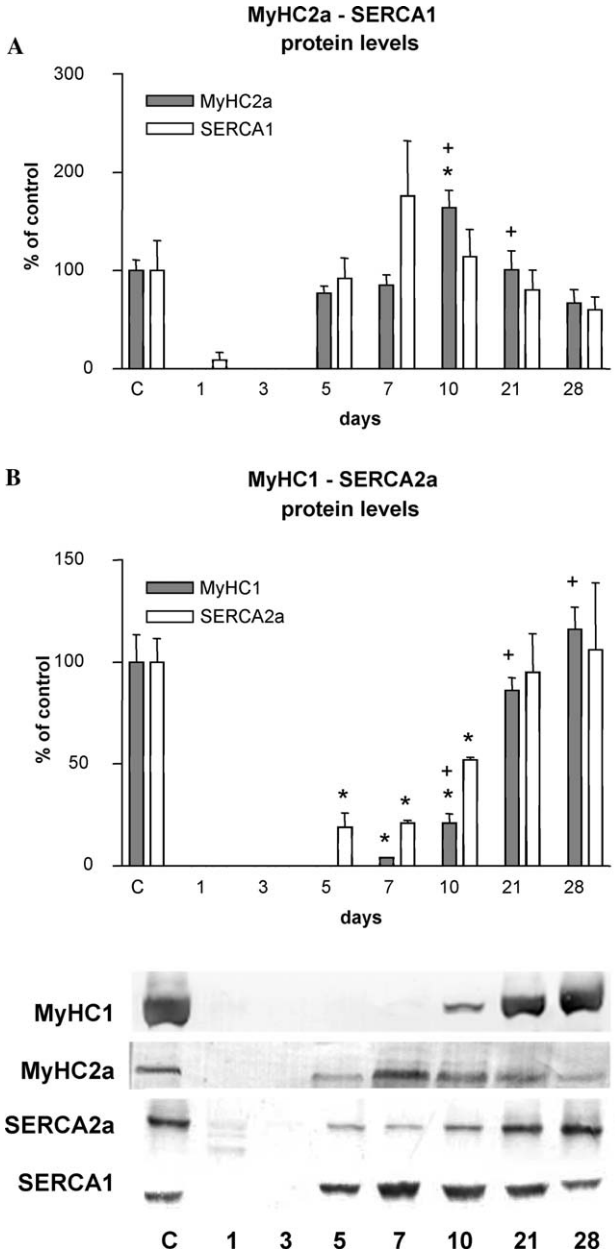

Fig. 5. The levels (A) of MyHC2a and SERCA1 and (B) of MyHC1 and SERCA2a proteins in regenerating soleus. The values cannot be compared between panels. Symbols as in Fig. 4. Representative immunoblots are shown in the lower part of the figure.

their maximum on day 10, and declined to the level of control muscles (Fig. 5A).

The slow MyHC1 appeared on day 7, continuously increased until day 28, and then reached a value approximately equal to the untreated controls (Fig. 5B). The slow type of SR Ca^{2+} pump (SERCA2a) was already detectable on days 5–10 [24], then it increased on day 21 to the normal level and remained there until day 28.

Taken together these results show that the nerve-dependent increases of calcineurin and MCIP1.4 mRNA levels preceded the replacement of fast myosin transcripts by the slow type MyHC1 mRNA, on days 5–10, but not the increase of slow SERCA2a mRNA.

Studies monitoring the effect of orally administrated calcineurin inhibitors (and not the calcineurin activity) have suggested that calcineurin stimulates in vivo muscle regeneration [15,16]. We could detect the CnA activity consistently only after the fourth day of regeneration, i.e., when desmin-expressing myofibers have occupied practically the entire volume of the muscle [28]. Calcineurin activity might be absent from 1 to 5 day regenerating muscle because of the high levels of reactive oxygen and nitrogen species, which are known to be deleterious to calcineurin [36]. Indeed, inflammatory cells, macrophages are prevailing in regenerating muscle on the first three days after toxin injection [37] and the reactive superoxide and NO produced by these cells might cause calcineurin to be degraded [23]. It has been reported that macrophages are not expressing detectable amounts of calcineurin in early regenerating muscle [16]. The level of the CnA protein subunit correlated well with the calcineurin activity [23]. The calcineurin activity was clearly increased by innervation in our experiments. This was also supported by the nerve dependence of MCIP1.4 mRNA level. The level of MCIP1.4 is also elevated during myogenic differentiation of C2C12 cells [31]. In accordance, we also found significant increase in the more advanced stages of regeneration of myofibers (day 10) compared to the less developed primitive myotubes (day 5), this increase was in our experiments also dependent on innervation. The relative in vivo effects of the calcineurin-NFAT and MEF2 signalling pathways have long been debated [4,17], although it is well established that the control of slow muscle fiber specification is made by calcineurin [4,7], and that NFAT dephosphorylated by calcineurin is translocated to the nucleus [38]. When slow innervation is established the resting free calcium level is increased in the sarcoplasm and this activates Ca^{2+}–calmodulin and downstream calcineurin [3,4]. We suggest that the nerve-dependent increase of calcineurin activity is a critical part of slow muscle differentiation and is an essential step to translocate transcription factors to the nucleus in order to generate larger amounts of slow myosin mRNAs. Which regulators in this process mediate the nerve influence on calcineurin remains unknown. The role of calcineurin in muscle adaptation to overload has been recognized. However, when calcineurin is overexpressed in muscle, it did not promote adaptation to overload. This highlights the importance of regulators (like Ca^{2+} and calmodulin) acting upstream of calcineurin in the calcineurin signalling pathway [39]. Since in regenerating soleus muscle, calcineurin protein and activity but not its mRNA levels show an increase which is innervation-dependent, either the rate of CnA translation or the stability of the protein must be affected in these conditions. Whether

the mediators are the same in innervated regeneration as in overload needs to be investigated.

Acknowledgments

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III.

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Early Changes in Rat Diaphragm Biology with Mechanical Ventilation

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To better characterize the effects of 24-hour mechanical ventilation on diaphragm, the expression of myogenic transcription factors, myosin heavy chains, and sarcoplasmic/endoplasmic reticulum calcium-ATPase pumps was examined in rats. In the diaphragm of mechanically ventilated animals, the mRNA of MyoD, myosin heavy chain-2a and -2b, and sarcoplasmic/endoplasmic reticulum calcium-ATPase-1a decreased, whereas myogenin mRNA increased. In the diaphragm of anesthetized and spontaneously breathing rats, only the mRNA of MyoD and myosin heavy chain-2a decreased. MyoD and myogenin protein expression followed the changes at the mRNA, whereas the myosin heavy chain isoforms did not change. Parallel experiments involving the gastrocnemius were performed to assess the relative contribution of muscle shortening versus immobilization-induced deconditioning on muscle regulatory factor expression. Passive shortening produced no additional effects compared with immobilization-induced deconditioning. The overall changes followed a remarkably similar pattern except for MyoD protein expression, which increased in the gastrocnemius and decreased in the diaphragm while its mRNA diminished in both muscles. The early alterations in the expression of muscle protein and regulatory factors may serve as underlying molecular basis for the impaired diaphragm function seen after 24 hours of mechanical ventilation. Whether immobilization-induced deconditioning and/or passive shortening play a role in these alterations could not be fully unraveled.

Keywords: mechanical ventilation; myogenic regulatory factors; myosin heavy chain; diaphragm; passive shortening

Mechanical ventilation is commonly used in the management of respiratory failure. In 20–50% of patients after successful treatment of the original illness, weaning from mechanical ventilation may be problematic (1). Respiratory muscle dysfunction is the major factor in producing weaning problems.

Recently, animal models of mechanical ventilation were developed in which the effects of anesthesia and the effects produced by mechanical ventilation were distinguished. At first, it was shown that already 12–24 hours of mechanical ventilation resulted in reduced forces generated by the diaphragm *in vitro* (2, 3). The magnitude of diaphragmatic dysfunction increased with the time spent on the ventilator (3, 4). Conversely, maximal transdiaphragmatic pressure generated during phrenic nerve stimulation was decreased after 24 hours of controlled mechanical ventilation and myofibril damage occurred after 3 days (4). Furthermore, a decrease in diaphragm fiber cross-section was noticed as early as after 18–24 hours of controlled mechanical ventilation, although the diaphragm mass remained unchanged (2, 5). The concomitantly decreased diaphragm protein levels, increased proteasome activity (5), and reduced insulin-like growth factor-I mRNA levels (2) suggest that an atrophy process would likely follow.

The question how mechanical ventilation affects the inspiratory muscles in patients with normal or already curtailed inspiratory muscle function appears to be of great clinical relevance but is difficult to assess in patients. Therefore, this study was performed (*1*) to further characterize the effects of mechanical ventilation on the diaphragm in terms of expression of transcription factors and key muscle proteins and (*2*) to unravel which of the consequences of mechanical ventilation, immobilization-induced deconditioning, or rhythmic passive shortening might be responsible for these effects. To address the first question, we measured the mRNA levels and/or the protein levels of several factors that possibly influence diaphragm function directly or indirectly. These factors included (*1*) myogenic regulatory factors, as these transcription factors are known to stimulate myogenesis (6) and the change in MyoD/myogenin ratio may drive muscle fiber phenotype adaptation (7–9); (*2*) the Id-type inhibitors of DNA-binding protein that are known inhibitors of myogenesis (10) (indeed, the inhibitor of DNA-binding protein-1 has been proposed as a mediator of muscle disease atrophy [11]); (*3*) myosin heavy chain and sarcoplasmic/endoplasmic reticulum calcium ATPase isoforms, as they are two of the main proteins having an impact on, respectively, the rate of muscle force development and relaxation; and (*4*) acetylcholine receptor, as it is a good marker of decreased nerve-evoked electrical activity (12). In particular, the expression of the acetylcholine-receptor α-subunit was found to be regulated by myogenic factors (13–15).

To address the second question, the gastrocnemius muscle was either immobilized or subjected to passive shortening in the same range as experienced by the diaphragm during mechanical ventilation.

We hypothesized that (*1*) mechanical ventilation would alter the expression levels of contractile proteins and transcription factors and (*2*) immobilization-induced deconditioning and/or passive shortening would be implicated in the diaphragm alterations seen after mechanical ventilation.

METHODS

Experimental Procedures and Study Design

Two series of experiments were conducted. First, we examined the consequences of 24 hours of mechanical ventilation on the expression of several factors in the diaphragm. Second, we determined the effects of 24 hours of

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rhythmic passive shortening and immobilization-induced deconditioning on the expression levels of these factors in the gastrocnemius. The mRNA and/or the protein expression levels of a number of factors were examined in the diaphragm and the gastrocnemius. These factors include the muscle regulatory factors (myogenic determination or MyoD, myogenin, myf-5; herculin or MRF4), the inhibitors of DNA-binding proteins, the myosin heavy chain isoforms, the sarcoplasmic/endoplasmic reticulum calcium ATPase pumps, and acetylcholine receptor α-subunit.

Effects of mechanical ventilation on the diaphragm. In the mechanical ventilation study, 52 male Wistar rats were randomly divided into three groups: (*1*) a control group, where no intervention was performed (n = 10); (*2*) anesthetized and spontaneously breathing rats in which the same surgery was performed as in the animals under mechanical ventilation (n = 26); and (*3*) anesthetized and mechanically ventilated animals (n = 16). These were the same animals as in a previous publication (2).

Effects of immobilization and rhythmic passive shortening on the gastrocnemius. In this study, 12 animals were instrumented as in the mechanical ventilation study, but in addition, both hindlimbs were immobilized. Whereas the right hindlimb was only immobilized, the left hindlimb was also passively moved rhythmically (Figure 1). A ventilator (Harvard pump) was adapted such that it allowed attachment to the left hindlimb of the rat. Movements of the piston were translated into movements of the foot, producing thereby passive shortening of the gastrocnemius at 55 movements per minute. The device was calibrated such that the degree of shortening was approximately 10% of resting muscle length, a change in length similar to the one undergone by the diaphragm during mechanical ventilation (16). The moved hindlimb was compared with the contralateral side, which was immobilized at resting position and underwent the effects of anesthesia and immobilization-induced deconditioning. The gastrocnemius muscle from the freely moving and ad libitum fed animals from the mechanical ventilation study served as the true control group.

Common procedures. Except for the control rats, all animals were initially anesthetized with sodium pentobarbital and were tracheotomized, and their body temperature was continuously monitored with an internal probe and maintained at 37°C. During the 24 hours, continuous infusion of anestheticum and heparin was given via the right jugular vein and carotid artery, respectively, using pressure pumps (Pilot A2; Fresenius, Schelle, Belgium). Constant levels of anesthesia were controlled throughout the experiment by evaluating foot reflex, corneal reflex, arterial blood pressure, and breathing pattern (for the spontaneously breathing group). Animals also received enteral nutrition, including vitamins and minerals that were administered via a gastric tube. Animals breathed humidified air enriched with O_2 and maintained at 37°C. Mechanically ventilated rats were ventilated with a respiratory rate of 55 breaths per minute.

After completion of the 24 hours, blood gas analysis was performed while diaphragm and gastrocnemius samples were removed and frozen in liquid nitrogen.

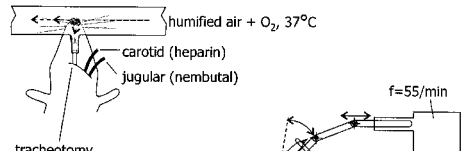

Figure 1. Experimental setup of the rhythmic passive shortening study. The rat is tracheotomized and breathed humidified air enriched with O_2 and maintained at 37°C. During the 24 hours, continuous infusion of anestheticum (nembutal) and heparin was given via the right jugular vein and carotid artery, respectively. In addition, both hindlimbs were immobilized, but while the right hindlimb was solely immobilized (data not shown), the left hindlimb was also passively moved rhythmically. Passive shortening of the left hindlimb occurred at 55 movements per minute (f = frequency). The device was calibrated such that the degree of gastrocnemius shortening was approximately 10% of resting muscle length, a change in length similar to the one experienced by the diaphragm during mechanical ventilation.

Histologic Procedure: Mechanical Ventilation Study Only

For the mechanical ventilation study, a diaphragm sample was fixed to a cork holder with its fibers oriented perpendicularly to the surface and was frozen in isopentane cooled in liquid nitrogen. Serial cross-sections parallel to the cork were stained with hematoxylin and eosin. Qualitative examination of these slides was performed by an expert who was not aware of the experimental design of the study.

mRNA Extraction and Reverse Transcriptase-Polymerase Chain Reaction

Total RNA of diaphragm and gastrocnemius was isolated using the guanidinium isothiocyanate procedure with cesium chloride method (17), as previously described (18). After reverse transcriptase reaction (Gibco BRL ThermoScript kit; Life Technologies, Merelbeke, Belgium), the first-strand cDNA mixture was subjected to polymerase chain reaction (Perkin Elmer kit, Lennik, Belgium). The number of polymerase chain reaction cycles was adjusted to avoid saturation of the amplification system. Amplification products were identified by their sizes after electrophoresis on 6% (wt/vol) acrylamide gels. After staining with Vistra Green, the fluorescence levels of the bands were quantified by means of a PhosphorImager model 425 (Molecular Dynamics, Sunnyvale, CA). Band intensities of the amplified fragments were normalized to the corresponding cyclophilin A (diaphragm) or L32 (gastrocnemius) amplification signals. No attempts were made to quantify the amount of the different target mRNAs, as reverse transcriptase-polymerase chain reaction is only a semiquantitative technique; only relative measurements are reported in the text.

Myosin Heavy Chain Extraction and Electrophoresis

Myosin heavy chain isoforms from the diaphragm and gastrocnemius from both studies were extracted as previously described (19). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis with an 8% separating gel containing 30% glycerol and a 4% stacking gel was performed during 24 hours at 270 V and 4°C. The gels were stained with silver nitrate (BioRad Silver stain plus kit; BioRad, Richmond, CA), air dried, and scanned. Bands were quantified using a gel documentation software system (Quanti One software; BioRad).

Western Blot Quantification of MyoD and Myogenin

Frozen muscle samples were homogenized according to the protocol of Sakuma and colleagues (20). Proteins (20 or 50 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4% stacking gel and 12.5% separating gel) and then transferred onto polyvinyldifluoride membranes by semidry blotting. The blots were blocked and incubated with polyclonal rabbit anti-MyoD (sc-760; Santa Cruz Biotechnology, Santa Cruz, CA) and antimyogenin (sc-576; Santa Cruz Biotechnology). Horseradish peroxidase-conjugated rat anti-rabbit IgG (Biosource, Nivelles, Belgium) was used as secondary antibody. After revelation of the bands with tetramethylbenzidine, the blots were scanned and quantified using the Quanti One software (BioRad).

Statistical Analysis

The individual reverse transcriptase-polymerase chain reaction results were normalized to the housekeeping gene mRNA data. All data are presented as mean ± SE. Differences between groups were assessed with one-way analysis of variance followed by Newman-Keuls post hoc test using the GraphPad Prism 3 software.

RESULTS

General Findings

Mortality. In the mechanical ventilation study, 12 animals out of the 16 studied survived the 24 hours in the mechanically ventilated

group, whereas only 9 out the 26 studied survived in the spontaneously breathing group. Thus, although mortality was 25% in the mechanically ventilated rats, it was higher in the spontaneously breathing group (65%) because in the latter group overdosing of anesthesia led to apnea and death. For the rhythmic passive shortening study, 10 out of 12 animals survived such that the mortality rate was 16%.

Blood gases and arterial blood pressure. Blood gas analysis showed that $P_{A_{O_2}}$ remained in the normal range for the anesthetized animals in the mechanical ventilation study (spontaneously breathing rats, 123 ± 13; and mechanically ventilated rats, 142 ± 13 mm Hg) and in the rhythmic passive shortening study (113 ± 6 mm Hg). This was also the case for $P_{A_{CO_2}}$ (spontaneously breathing rats, 31 ± 5; mechanically ventilated rats, 33 ± 5; and rhythmic passive shortening study, 42 ± 2 mm Hg) and pH (spontaneously breathing rats, 7.38 ± 0.02; mechanically ventilated rats, 7.45 ± 0.04; and rhythmic passive shortening study, 7.37 ± 0.02).

Arterial blood pressure was similar in the different groups and averaged 130 ± 7, 104 ± 11, and 128 ± 8 mm Hg for the spontaneously breathing, mechanically ventilated groups, and the rhythmic passive shortening study, respectively.

Histological Analysis: Mechanical Ventilation Study Only

Qualitative examination of the diaphragm sections stained with hematoxylin and eosin did not reveal any abnormalities in diaphragm histology whatever the condition except for the fact that diaphragm fiber dimensions were smaller in the spontaneously breathing and mechanically ventilated groups.

Reverse Transcriptase-Polymerase Chain Reaction Data

Myogenic regulatory factors.

Mechanical ventilation study. Compared with control group, diaphragm myogenin mRNA levels were significantly increased in the mechanically ventilated group (67%, $p < 0.01$ vs. control group), whereas they tended to increase in the spontaneously breathing group (32%, p = NS) (Figure 2A). Myf-5 mRNA increased together with myogenin after mechanical ventilation (107%, $p < 0.01$ vs. control group); however, it also increased significantly in the spontaneously breathing group (130%, $p < 0.01$ vs. control group) (Figure 2A). In contrast, the level of MyoD mRNA decreased both in the spontaneously breathing rats (−33%, $p < 0.01$ vs. control group) and even more so in mechanically ventilated animals (−56%, $p < 0.001$ vs. control group), the latter being also statistically significantly different from the spontaneously breathing group ($p < 0.05$) (Figure 2A). As a consequence, compared with control group, the MyoD/myogenin ratio, which may reflect the ongoing changes in isoform switch, decreased significantly in the spontaneously breathing group (−34%, $p < 0.05$ vs. control group) and more particularly after mechanical ventilation (−70%, $p < 0.001$ vs. control group) (Figure 3A). In addition, this ratio was significantly decreased by 53% in the mechanically ventilated group compared with the spontaneously breathing group ($p < 0.01$ vs. spontaneously breathing). mRNA levels of MRF4, a factor involved in the later stages of myogenic transformation pathway, remained unchanged (Figure 2A).

Rhythmic passive shortening study. Passive shortening combined with immobilization caused a significant reduction in gastrocnemius MyoD mRNA only (−23%, $p < 0.05$ vs. control group). However, after immobilization alone, a slightly more pronounced decay was observed (−44%, $p < 0.001$ vs. control group, and −21%, $p < 0.05$ vs. immobilization and passive shortening) (Figure 2B). It is worth to note that myogenin (immobilization, 52%; immobilization and passive shortening, 70%, $p < 0.05$ vs. control group) and myf-5 (immobilization, 34%; immobilization and passive shortening, 29%; p = NS) mRNA increased after immobilization and also after passive shortening (Figure 2B), as was also the case in the diaphragm of mechanically ventilated and spontaneously breathing groups (Figure 2A). As a result, the MyoD/myogenin ratio decreased similarly after immobilization (−60%, $p < 0.001$ vs. control group) and after immobilization combined with passive shortening (−51%, $p < 0.001$ vs. control group) (Figure 3B). MRF4 mRNA did not change whatever the condition.

Inhibitor of the DNA-binding protein isoforms. **Mechanical ventilation study.** Reverse transcriptase-polymerase chain reaction data showed that inhibitor of DNA-binding protein-1 mRNA levels were significantly and equally reduced both in the diaphragm of mechanically ventilated and spontaneously breathing animals (−30%, $p < 0.001$), whereas inhibitor of DNA-binding protein-2 and inhibitor of DNA-binding protein-3 mRNA showed no changes, and inhibitor of DNA-binding protein-4 mRNA was not detectable.

Rhythmic passive shortening study. In the immobilized and passively moved but also in the solely immobilized gastrocnemius, inhibitor of DNA-binding protein-3 mRNA decreased equally (−27% and −25%, respectively, $p < 0.01$ vs. control group), whereas the other inhibitor of DNA-binding protein isoform mRNA did not change.

Myosin heavy chain isoforms: mechanical ventilation study only. The fast myosin heavy chain-2a mRNA decreased in the diaphragm of the spontaneously breathing group (−10%, $p < 0.05$ vs. control group) and even more so in the mechanically ventilated group (−20%, $p < 0.001$ vs. control group). The decrease in the latter was moreover significantly different from the spontaneously breathing group ($p < 0.05$) (Figure 4A). Also myosin heavy chain-2b mRNA decreased, but this reached statistical significance only in the mechanically ventilated animals (−19%, $p < 0.05$ vs. control group) (Figure 4A). Myosin heavy chain-1 and myosin heavy chain-2x mRNA did not change during the experiment (Figure 4A).

Sarcoplasmic/endoplasmic reticulum calcium ATPase isoforms: mechanical ventilation study only. Sarcoplasmic/endoplasmic re-

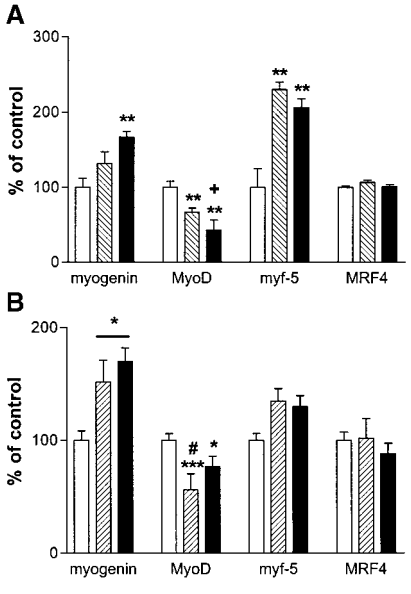

Figure 2. mRNA levels of myogenic regulatory factors determined by reverse transcriptase-polymerase chain reaction in the diaphragm (*A*) of control rats (*open bars*, n = 9), spontaneously breathing rats (*hatched bars*, n = 9), and rats under mechanical ventilation (*solid bars*, n = 12) and in the gastrocnemius (*B*) of control rats (*open bars*, n = 9), after immobilization (*hatched bars*, n = 10), and after both immobilization and passive shortening (*solid bars*, n = 10). Values were normalized to the corresponding house-keeping gene amplification signals. Values are means and SE. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus control; $^{+}p < 0.05$ versus spontaneously breathing; $^{\#}p < 0.05$ versus immobilization and passive shortening.

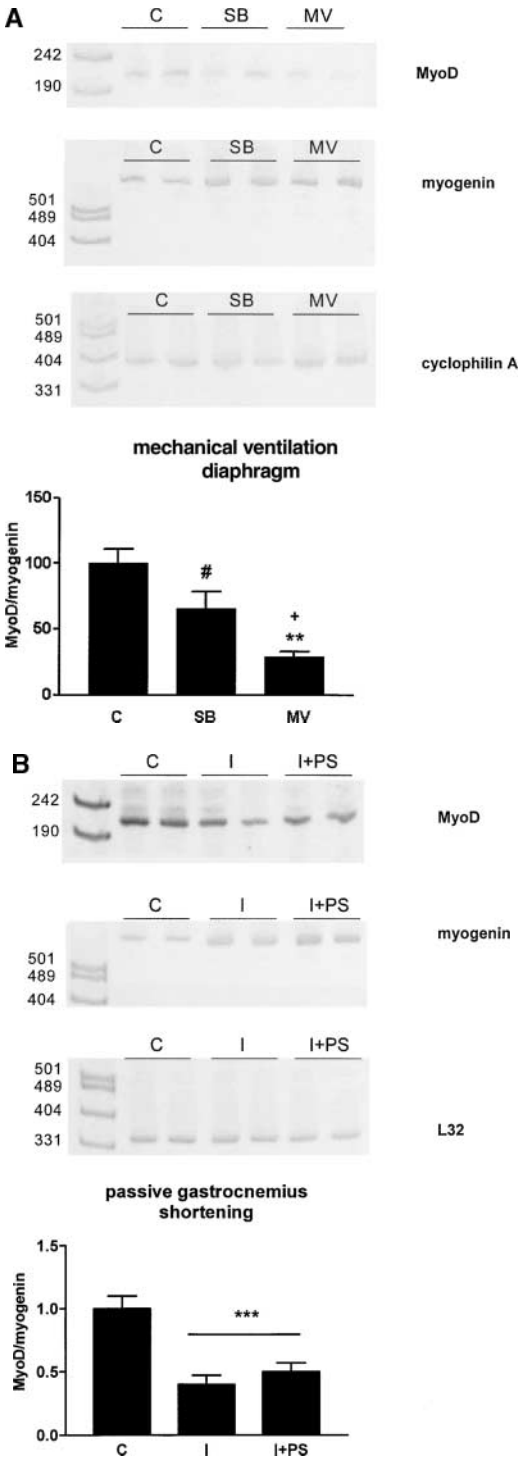

Figure 3. MyoD/myogenin ratio in the diaphragm (*A*) of control rats (C, n = 9), spontaneously breathing rats (SB, n = 9), and rats under mechanical ventilation (MV, n = 12) and in the gastrocnemius (*B*) of control rats (C, n = 9), after immobilization (I, n = 12), and after both immobilization and passive shortening (I + PS, n = 12). Representative reverse transcriptase-polymerase chain reaction results are provided for MyoD, myogenin, and housekeeping gene (cyclophilin A for the diaphragm and L32 for the gastrocnemius). Each *lane* represents a sample with the first lane being the molecular marker. The values of the bar graph were normalized to the corresponding housekeeping gene amplification signals. Values are means and SE. *$p < 0.05$, ***$p < 0.001$ versus C, $^{+}p < 0.05$ versus SB.

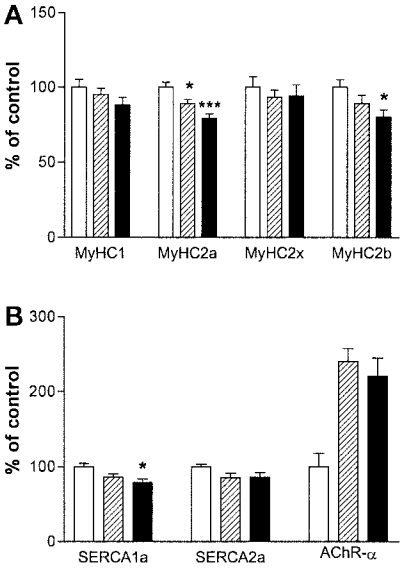

Figure 4. mRNA levels of myosin heavy chain isoforms (MyHC) (*A*), sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) isoforms, and acetylcholine receptor α-subunit (AChR-α) (*B*) determined by reverse transcriptase-polymerase chain reaction in the diaphragm of control rats (*open bars*, n = 9), spontaneously breathing rats (*hatched bars*, n = 9), and rats under mechanical ventilation (*solid bars*, n = 12). Values were normalized to the corresponding housekeeping gene amplification signals. Values are means and SE. *$p < 0.05$ and ***$p < 0.001$ versus controls.

ticulum calcium ATPase-1a mRNA, which is characteristic of the fast muscle fibers, decreased significantly only in the diaphragm of the mechanically ventilated group (−21%, $p < 0.05$ vs. control group) (Figure 4B). The slow-type sarcoplasmic/endoplasmic reticulum calcium ATPase-2a mRNA did not change in the experiment (Figure 4B).

Acetylcholine receptor α-subunit: mechanical ventilation study only. The mRNA level of acetylcholine receptor α-subunit showed a marked increase in the diaphragm of the spontaneously breathing (140%) and mechanically ventilated (120%) groups (Figure 4B). These increases reached statistical significance with one-way analysis of variance ($p < 0.05$) but not with the Newman-Keuls post hoc test.

Myosin Heavy Chain Electrophoresis

For both studies, there were no changes in the relative proportion of the different myosin heavy chain isoforms between the different groups. For each study, the proportions of the different myosin heavy chain isoforms are reported in Table 1.

Western Blot: Analysis of MyoD and Myogenin

Mechanical ventilation study. Compared with the control group, diaphragm MyoD was significantly reduced in mechanically ventilated group (−49%, $p < 0.05$ vs. control group and spontaneously breathing) and remained unchanged in spontaneously breathing animals (Figure 5), whereas myogenin tended to increase (spontaneously breathing, 115%; mechanically ventilated, 59%; $p = 0.08$).

Rhythmic passive shortening. Compared with the control group, gastrocnemius MyoD was significantly increased after immobilization (40%, $p < 0.01$ vs. control group) and also after immobilization and passive shortening (52%, $p < 0.001$ vs. control group). For myogenin, a tendency to increase was also observed in both groups (immobilization, 36%; immobilization and passive shortening, 44%) compared with the control group, but these increases failed to reach statistical significance. These changes in protein expression thus did not follow the changes in mRNA expression for MyoD, whereas for myogenin, a close accordance with the mRNA changes was present.

TABLE 1. PERCENTAGE OF THE DIFFERENT MYOSIN HEAVY CHAIN ISOFORMS IN THE DIAPHRAGM OF THE MECHANICAL VENTILATION STUDY AND IN THE GASTROCNEMIUS OF THE PASSIVE SHORTENING STUDY

	Mechanical Ventilation: Diaphragm		
	Myosin Heavy Chain-1	Myosin Heavy Chain-2a	Myosin Heavy Chain-2x
Control group	25 ± 1	41 ± 2	34 ± 2
Spontaneous breathing	23 ± 1	39 ± 1	37 ± 1
Mechanical ventilation	22 ± 1	41 ± 2	37 ± 1
	Passive Shortening: Gastrocnemius		
	Myosin Heavy Chain-1	Myosin Heavy Chain-2a	Myosin Heavy Chain-2x
Control group	33 ± 3	32 ± 2	35 ± 7
Immobilization	38 ± 2	27 ± 1	35 ± 1
Immobilization and passive shortening	34 ± 1	31 ± 1	35 ± 1

Values are mean ± SE. Data are expressed as percentages.

DISCUSSION

This study showed that anesthesia, but more particularly controlled mechanical ventilation, alters the mRNA levels of the muscle regulatory factors (MyoD, myogenin), the myosin heavy chain isoforms, and the sarcoplasmic/endoplasmic reticulum calcium ATPase isoforms as early as after 24 hours. The overall pattern of these changes points in the direction of a reduction in the mRNA levels of the fast isoforms of the respective muscle proteins (myosin heavy chain and sarcoplasmic/endoplasmic reticulum calcium ATPase) paralleled by a reduction in the levels of MyoD, whereas the mRNA of myogenin, a factor that is often found together with the slow isoforms of the previously mentioned proteins, is increased. MyoD and myogenin protein expression followed the corresponding changes seen at the mRNA level, whereas no significant changes were noticed in the myosin heavy chain protein isoforms. In addition, our data on gastrocnemius showed that passive shortening did not further affect the expression levels of the studied factors compared with immobilization-induced deconditioning. These mRNA and protein changes in gastrocnemius were similar in nature to those observed in the diaphragm after mechanical ventilation except for MyoD protein expression, which increased in the gastrocnemius but decreased in the diaphragm. Whether this increase in gastrocnemius MyoD protein is transient and will be followed by a decrease as expected from its mRNA expression is impossible to predict from these data.

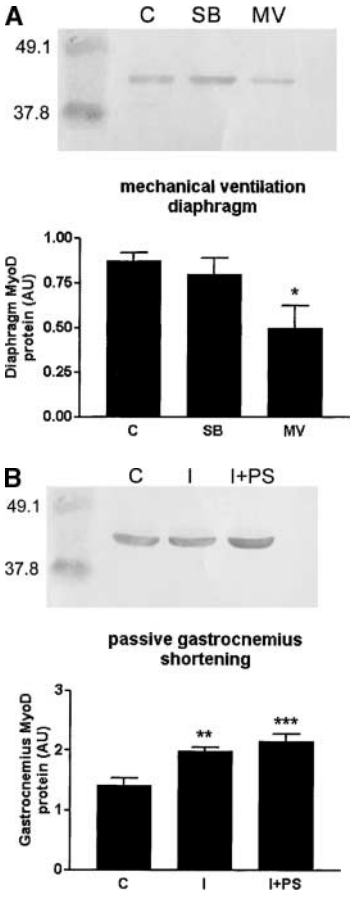

Figure 5. Western-blotting data of MyoD in the diaphragm (*A*) of control rats (C, n = 9), spontaneously breathing rats (SB, n = 9), and rats under mechanical ventilation (MV, n = 12) and in the gastrocnemius (*B*) of control rats (C, n = 9) after immobilization (I, n = 12) and after both immobilization and passive shortening (I + PS, n = 12). Representative samples are provided for each group and each muscle. Each *lane* represents a sample with the first lane being the molecular marker. Values are means and SE. *$p < 0.05$ versus C and SB; *$p < 0.01$ and ***$p < 0.001$ versus C.

The changes observed in this study are likely to be related to the effects of the mechanical ventilation, as blood gas levels and arterial blood pressure, which can influence muscle force and/or transcription rate, remained within the normal range. Infection was unlikely to develop over the 24-hour time period (3, 21). Although nutritional support was limited to 69 kcal/day (corresponding to 173 kcal/kg/day) in the anesthetized animals, this caloric intake was similar to that used in the study of Powers and colleagues (3) and was much higher than in other animal models of mechanical ventilation where longer duration of mechanical ventilation was performed (50 kcal/kg/day up to 4 days of mechanical ventilation [22], 30–35 kcal/kg/day up to 5 days of mechanical ventilation [23], and 100 cal/kg/day up to 1 or 3 days of mechanical ventilation [4]). That nutritional deprivation may have occurred in our model is possible, but the contribution of undernutrition is probably not essential, as it is well know that undernutrition does not reduce diaphragmatic maximal tetanic tension (24–27) nor does it affect diaphragmatic insulin-like growth factor-I mRNA (28), whereas both were observed in our model. Moreover, undernutrition even if present would not explain the differences between the spontaneously breathing rats and the rats under mechanical ventilation. Finally, the significant differences between spontaneous breathing and mechanically ventilated groups in the mRNA expression of MyoD/myogenin ratio, myosin heavy chain-2a, myosin heavy chain-2b, sarcoplasmic/endoplasmic reticulum calcium ATPase-1a show that although anesthesia itself had an effect on diaphragm properties, it was markedly enhanced by mechanical ventilation.

The myogenic regulatory factors belong to the basic helix–loop–helix family and are muscle specific. They form heterodimers with the E proteins, and because they contain a basic DNA-

binding domain, they bind DNA in a sequence-specific manner at sites known as E boxes. Because this DNA motif is present in the promoters of many skeletal muscle-specific genes, gene activation is mediated in a myogenic regulatory factor-dependent manner. During embryogenesis, the myogenic regulatory factors stimulate myoblast determination and differentiation (29). The myogenic regulatory factors have been shown to be negatively regulated by the helix–loop–helix inhibitor of the DNA-binding protein, which lacks the basic DNA-binding domain (10). Although the role of the myogenic regulatory factors and the inhibitor of the DNA-binding proteins in adult mature skeletal muscles is not fully established, the expression levels of these transcription factors have been shown to be modulated by various types of intervention such as denervation, immobilization, electrostimulation, mechanical loading, and hormone treatment (8, 9, 12, 15).

Mechanical Ventilation Study

This study is the first showing that 24 hours of controlled mechanical ventilation leads to marked changes in the mRNA levels of key muscle proteins and regulatory factors. For the myosin heavy chain isoforms and the sarcoplasmic/endoplasmic reticulum calcium ATPase pumps, mechanical ventilation led to a reduction in the mRNA levels of the fast isoforms, these effects being independent of anesthesia. These alterations are expected to favor a slow oxidative profile. This is in line with a previous report showing that although all diaphragm fibers atrophied after 18 hours of controlled mechanical ventilation, the effect on type II fibers was greater than on type I fibers (5). Increases in citrate synthase activity in the diaphragm of mechanically ventilated rats further confirmed that oxidative capacity of the diaphragm increased after short-term controlled mechanical ventilation (30). In agreement with Shanely and colleagues (5), no significant changes in myosin heavy chain isoform distribution were observed in this study after 24 hours of controlled mechanical ventilation, whereas with more prolonged (3–7 days) mechanical ventilation combined with antibiotic treatment and neuromuscular blockade, an increased proportion of diaphragm fibers coexpressing myosin heavy chain-1 and -2 at the expense of the pure myosin heavy chain-1 population was reported in rats (22). Caution should, however, be taken, as antibiotics and neuromuscular blocking agents in addition to mechanical ventilation may have contributed to this phenotype adaptation, as they are known to affect inspiratory muscle contractility (31).

For the muscle regulatory factors, the alterations of MyoD and myogenin mRNA were clearly more pronounced after mechanical ventilation than during anesthesia. Although the role of the myogenic regulatory factors in adult muscle is not well defined, MyoD and myogenin have been reported to be implicated as regulators of fiber phenotype (32). In fact, MyoD mRNA is predominantly located in fast twitch muscle, whereas myogenin mRNA is mainly found in slow-twitch muscle (8). Although still controversial, several studies have shown that the MyoD/myogenin is highly correlated with muscle fiber phenotype such that a transformation from slow to fast phenotype was associated with a decreased myogenin and elevated MyoD mRNA expression (32). Thus, the reduction in the MyoD/myogenin ratio after mechanical ventilation both at the mRNA and the protein levels suggests phenotypic adaptation toward a slower profile. This is in line with the effects observed in this study for the myosin heavy chain isoforms and sarcoplasmic/endoplasmic reticulum calcium ATPase pumps at the mRNA levels. Especially the decrease in myosin heavy chain-2b mRNA observed after mechanical ventilation further underlines the already known strong correlation between MyoD and myosin heavy chain-2b gene expression (33). However, because of the low levels of myosin heavy chain-2b in the rat diaphragm, the functional implication of this decrease should be interpreted with care. No changes in myosin heavy chain isoforms were observed, however, at the protein level in the diaphragm of the mechanically ventilated rats despite alterations toward a slower profile at the mRNA levels. Alterations in MyoD and/or myogenin expression may induce adaptations in the intrinsic properties of the fiber without altering fiber phenotype (34, 35). That the diaphragm in mechanically ventilated rats moves toward a slower profile is further confirmed by the data of Shanely and colleagues showing increases in citrate synthase activity after 18 hours of controlled mechanical ventilation without changes in fiber proportion (5).

Furthermore, caution should also be taken because the role of MyoD/myogenin in regulating myosin heavy chain isoform expression is model dependent, and whereas it seems to be valid for hindlimb unloading, it is not supported in tetrodotoxin-induced paralysis or after short-term spinal cord transection (32). Moreover, although it would be expected that MyoD knockout mice would have reduced proportions of fast fibers, these mice showed a remarkably normal fiber-type distribution (36). Hughes and colleagues, however, showed that a loss of the MyoD function caused by gene disruption resulted in a subtle shift toward a slower profile in fast muscles (35). On the other hand, although mice overexpressing myogenin showed no alterations in myosin heavy chain proportion, a shift of enzyme activity from glycolytic to oxidative metabolism was reported in the muscles of these transgenic mice (34). Nonetheless, the diaphragm seems to behave differently than peripheral skeletal muscle, as recently MyoD deletion has been reported to result in a fast-to-slow shift in the myosin heavy chain phenotype of the diaphragm (37). This MyoD elimination was associated with a downward shift of the diaphragm force–frequency relationship and a decrease in the maximal tetanic tension. This further supports the view that the downregulation of MyoD seen in our study after mechanical ventilation may have contributed to the previously reported reduction in diaphragm function (2).

In this study, an increased myf-5 mRNA was also observed, but because this increase was similar in the spontaneous breathing animals and in the rats submitted to mechanical ventilation, it was likely to reflect the effect of anesthesia and/or surgical procedure. Intriguingly, we previously observed similar increases in myf-5 mRNA levels in the diaphragm after surgical procedure and anesthesia in a model of lung volume reduction surgery in hamsters (38). The functional implication of elevated myf-5 mRNA in these two models is, however, not known, but our present data show that this increase is not specific for mechanical ventilation effects. As MyoD mRNA decreased in our study in both anesthetized groups (but to a greater extent after mechanical ventilation), it might be suggested that the similar increase in myf-5 mRNA in the anesthetized animals represents a compensatory response of myf-5 for this MyoD downregulation. Indeed, in knockout MyoD mice, high levels of myf-5 mRNA have been reported to maintain normal muscle phenotype (36). This is further supported by the fact that the proportion of the myosin heavy chain isoforms was unaltered in this study. Finally, recent data suggest that like MyoD, myf-5 may also influence myosin heavy chain isoform expression (39). This further supports the idea that myf-5 may compensate for MyoD downregulation to maintain the normal diaphragm phenotype.

The decrease in the inhibitor of DNA-binding protein-1 mRNA observed in the present study was similar in spontaneously breathing and mechanically ventilated animals, suggesting thereby that this decrease was not caused by mechanical ventilation but was rather related to anesthesia and/or surgical procedure. Similar decreases in the inhibitor of DNA-binding protein-1 mRNA were previously observed in the hamster diaphragm after anesthesia and surgical procedure (38). As the inhibitors of DNA-binding protein are known to negatively regulate muscle growth, such a

decrease in the inhibitor of DNA-binding protein-1 mRNA levels is expected to result in muscle hypertrophy. Whether this decrease in the inhibitor of DNA-binding protein-1 mRNA would be a compensatory mechanism in response to anesthesia and/or surgical procedure cannot be concluded from these data and is beyond the aim of this study.

Rhythmic Passive Shortening Study

This study is the first attempting to develop an animal model examine which of the consequences of mechanical ventilation (immobilization-induced deconditioning or rhythmic passive shortening) may be involved in the diaphragm dysfunction caused by mechanical ventilation. Our data showed that similar results were observed after gastrocnemius immobilization alone or combined with rhythmic passive shortening. More specifically, while MyoD mRNA was decreased, myogenin mRNA was increased as was myf-5 mRNA, although the latter did not reach statistical significance. In contrast, MyoD protein expression did not parallel the changes seen at the mRNA levels as MyoD significantly increased after immobilization and also after immobilization combined with rhythmic passive shortening. That MyoD protein increased while its mRNA decreased may reflect a transient response of MyoD protein that will be followed by a decrease in a later stage as expected from its RNA expression. The potential mechanisms responsible for the discrepancy between MyoD protein and mRNA are not known and are difficult to unravel especially because the regulation of MyoD is still not fully understood. Indeed, it is well known that MyoD is able to autoregulate its own transcription positively and that the turnover of both MyoD protein and mRNA is high (40). However, the function of this positive feedback loop *in vivo* is not clear. In fact, discrepancy between MyoD mRNA and protein has previously been reported in limb muscle after denervation (41, 42) and regeneration (43). Unfortunately, in none of these studies was a rationale for such discrepancy provided. We thus hypothesized that the increased MyoD protein observed in the gastrocnemius in this study may reflect a compensatory mechanism to the decrease in its mRNA to stabilize MyoD protein. On the other hand, knowing that MyoD turnover is high, MyoD effects are probably better related to its protein level rather than to its mRNA level.

The data of this study show that the gastrocnemius is affected by 24 hours inactivity but that the inactivity-induced effects are less pronounced than those observed in the diaphragm probably because more time is needed for the gastrocnemius to be similarly affected by inactivity as compared with the diaphragm. The nature of these effects is, however, similar in both muscles, except for MyoD protein expression, which increases in the gastrocnemius while it decreases in the diaphragm after mechanical ventilation. Whether this would lead to fiber phenotype adaptation at a later stage remains to be determined. Our data also show that 24 hours of gastrocnemius passive shortening did not produce any effects in addition to those induced by immobilization-induced deconditioning whose effects developed rapidly and predominated during the first 24 hours at least. However, an effect of passive shortening should not be completely excluded as more time might be necessary for the stretch effect to develop.

Finally, caution should be taken when extrapolating conclusions from experiments on the gastrocnemius to the diaphragm, as many anatomical and functional differences exist between these two muscles. However, among all the skeletal muscles, the gastrocnemius was probably the most suitable muscle to examine immobilization-induced deconditioning and passive shortening. Indeed, first it best fits to the diaphragm for what concerns its fiber composition (25) and mRNA content of myogenic regulatory factors, especially for MyoD and myogenin (44). Fiber composition was a key factor to assess, as immobilization-induced deconditioning is known to preferentially affect slow-type muscle and to a lesser extent fast-type muscle. Thus, in order not to bias a potential effect, a mixed muscle such as the gastrocnemius was preferentially chosen to mimic better what happens to the diaphragm. Second, gastrocnemius access makes it easy to control for resting position and to adjust shortening during passive shortening. Third, the muscle is large enough to obtain sufficient material for several measurements. Of course, the activity patterns of the gastrocnemius and the diaphragm are very dissimilar, as the diaphragm has a longer duty cycle and is activated throughout the whole life and is likely to be particularly susceptible to the effects of, for example, inactivity (45).

On the other hand, because fiber length is a major issue, care was taken to induce immobilization and/or passive shortening of the gastrocnemius at a resting length that is known to be close to its optimal length. Of course, spatial arrangement and recruitment profiles are different between the two muscles; however, obviously, these parameters could not be controlled, and as such, they contribute to the limitations of the model. It is also true that 10% shortening may be more (or less) harmful for the diaphragm than for the gastrocnemius. However, it was reasonable to apply the same relative level of shortening to the gastrocnemius as would be experienced by the diaphragm during mechanical ventilation, as actually no literature is available to describe the effect of various extents of shortening on the gastrocnemius function. In fact, there is no skeletal muscle that would perfectly match the properties of the diaphragm, and for this reason, the data of gastrocnemius experiments should be interpreted in the perspective of the limitations of the study.

Conclusions

The data of this study emphasize that short-term mechanical ventilation results in important and multiple alterations of muscle protein and transcription factors at both the mRNA and also the protein levels. Part of these changes is likely to contribute to the diaphragm dysfunction caused by mechanical ventilation. Whether the effects of immobilization-induced deconditioning/unloading and rhythmic passive shortening would play an important role in the diaphragm alterations seen after mechanical ventilation could not be fully unraveled by this study. Further studies are needed to map the complexity of this phenomenon to improve prevention of the weaning problems and to develop strategies to minimize the effects of mechanical ventilation on diaphragm function.

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IV.

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Expression of the sarco/endoplasmic reticulum Ca^{2+}-transport ATPase protein isoforms during regeneration from notexin-induced necrosis of rat soleus muscle

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Summary

Expression levels of fast-twitch (SERCA1), slow-twitch (SERCA2a) and "housekeeping" (SERCA2b) isoforms of the sarcoplasmic reticulum Ca^{2+}-transport ATPase were monitored during regeneration of rat soleus muscles following necrosis induced by the toxin notexin at the tissue level by Western blot analysis and at the cellular level by immunocytochemical analysis. Due to necrosis, levels of muscle-specific SERCA1 and SERCA2 a isoforms dropped to low levels on the third day after injection of the toxin. Subsequently, during regeneration both isoforms recovered but with a different time course. Expression of the fast type SERCA1 increased first. This type showed its most pronounced increase between day 3 and 10. Expression of the slow type SERCA2 a was biphasic. After an increase to approximately one third of the control value on days 5–10, it showed its main increase up to the control level between day 10 and 21. Expression levels of the housekeeping SERCA2b isoform remained relatively constant throughout the 4 weeks of regeneration. Between day 10 and 28, when new innervation is established, SERCA2 a expression spread gradually over almost all fibers whereas the number of SERCA1-expressing fibers decreased and only a limited number of fibers co-expressed SERCA1 and SERCA2a. At 4 weeks of regeneration, expression of the fast isoform was found only in 12% of the fibers, whereas the slow form was found in 98% of the fibers. In the contralateral untreated soleus muscles, 26% SERCA1-positive and 81% SERCA2a positive fibers were observed. Immunocytochemical analysis showed that SERCA1 and SERCA2 a were co-expressed with fast and slow myosin isoforms in fibers of normal muscles but in regenerated muscle only slow myosin and show SERCA isoforms correlated. The results show that during regeneration levels of fast and slow SERCA proteins change in a similar way as their rnRNAs do. However, in regenerated soleus, unlike in normal muscle, expression of slow SERCA is coregulated only with the slow myosin isoform. This finding is in agreement with the fact that the number of slow type fibers is increased in regenerated soleus.

Key words: SERCA protein level – soleus-regeneration – myosin isoforms – skeletal muscle – necrosis – Ca^{2+}-ATPase

List of abbreviations: DAB, 3,3'-diaminobenzidine; MHC 1, type 1 myosin heavy chain; MHC 2a, type 2a myosin heavy chain; PAGE, polyacrylamide-gel electrophoresis; SERCA, sarcolendoplasmic reticulum Ca^{2+}-ATPase; S.E.M., standard error; SR, sarcoplasmic reticulum

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Introduction

Relaxation of skeletal muscle largely depends on sarco/endoplasmic reticulum Ca^{2+}-transport ATPase (SERCA), which removes Ca^{2+} from the sarcoplasm into the sarcoplasmic reticulum (SR). Four different isoforms of SR Ca^{2+}-ATPases have been described in skeletal muscle. They are encoded by two genes (McLennan et al., 1992). SERCA1 a/b splice variants are mainly expressed in fast-twitch muscle, but they are detectable in show-twitch muscles too (Brandl et al., 1987; Wu and Lytton, 1993; Wu et al., 1995). SERCA2 a protein is expressed largely in slow-twitch fibers and cardiac muscle, but is also present in fast-twitch muscles (Brandl et al., 1987; Wu and Lytton, 1993; Wu et al., 1995). In a previous study, we documented time-dependent changes in the levels of mRNAs encoding SERCA1 a/b and SERCA2 a/b in rat soleus muscle during regeneration from necrosis (Zador et al., 1996). Necrosis was induced by intramuscular injection of notexin venom from the mainland tiger snake (Harris and Johnson, 1978), and concomitant regeneration can be used as a model of muscle recovery from degenerative disorders or after heavy exercise. It was concluded that during regeneration, appearance of mRNAs coding for different SERCA isoforms showed a similar time course as in normal development.

The present study first investigates expression of SERCA1, SERCA2a and SERCA2b at the protein level in whole muscle. Any discrepancy in time-dependent expression patterns of the mRNAs (Zador et al., 1996) and corresponding protein patterns would indicate that posttranscriptional mechanisms control SERCA expression. Indeed, cellular levels of different SERCA proteins depend on several factors. These include efficiency of gene transcription, mRNA stability, efficiency of translation and protein stability. According to Khan et al. (1990), cardiac muscle which expresses SERCA2a,

contains 70 times more SERCA2 protein, but only 7 times more SERCA2 mRNA compared to stomach which expresses predominantly SERCA2b. Additional control of SERCA2 expression on the translational level has also been reported for canine latissimus dorsi (Hu et al.. 1995). However, Wu and Lytton (1993) report both for cardiac muscle (SERCA2a) and for stomach or aorta smooth muscle (mainly SERCA2 b) similar ratios of 2 x 10^5 pro-tein molecules/mRNA. A second part of the present study deals with the expression of SERCA protein isoforms in individual muscle fibers. Our previous study on mRNA levels (Zador et al., 1996) did not provide information on changes in morphology of muscle cells, nor on distribution patterns of the different SERCA isoforms amongst individual fibers during regeneration. It is now concluded that an initially relatively higher expression of SERCA1 compared to SERCA2 in myoblasts and fibers changes between 10 and 28 days of regeneration, i.e. at the time that fibers become reinnervated, in favour of SERCA2 a. At that time nearly all fibers express SERCA2 a, but a number of fibers go on co-expressing SERCA1 and SERCA2. However, the number of SERCA1-positive fibers is then much lower than in control soleus.

We also confirmed by immunostaining that SERCA1 and SERCA2 a are expressed in type II A (fast-twitch, oxidative glycolytic) and type I (slow twitch) fibers, respectively, in the normal soleus muscle. After regeneration however, when the number of type II A fibers declined, only SERCA2 a corresponded to the expression of slow myosin.

Material and Methods

Animals and treatment. 300–360 g male Wistar rats were used for the experiments. The rats were anaesthetised by injection of 1 ml 0.5% pentobarbital sodium per 100 g body weight. A small incision was made in the m. gastrocnemius, the m. soleus slightly lifted from its bed and slowly injected at a point located approximately 1/3 from the distal end with 20 μg venom of the mainland tiger snake (*Notechis scutatus scutatus*) in 200 μl 0.9% NaCl (Sigma, St. Louis, MO, USA) using a 27G 3/4 injection needle. After injection, the wound of the gastrocnemius and the skin were suture closed. At times ranging from day 3 to 28 after injection, the entire soleus muscle was removed and the animals were sacrificed with an overdose of pentobarbital sodium.

Sampling and hematoxylin-eosin staining of the soleus muscles. The dissected soleus muscles were frozen in isopentane cooled by liquid nitrogen and kept at –70°C. A biopsy was taken from the central part of each frozen soleus before homogenisation, frozen sectioned and stained by hematoxylin-eosin to monitor necrosis and the subsequent regeneration process. The time course of regeneration and the optimal dose of snake venom was established in a separate set of experiments and found to be remarkably reproducible (Zador et al., 1996).

Preparation of fragmented muscle membranes and immunoblots. The muscles, for which the regeneration state was documented by hematoxylin-eosin staining of sections taken from the central part of the muscles, were finely minced with scissors and homogenised in 2.5 ml ice-cold 0.25 M sucrose in 5 mM HEPES, pH 7.5 using a glass/Teflon Potter homogeniser. The homogenates were centrifuged at 1,000 g for

10 min at 4°C and the pellets discarded. The supernatants were centrifuged at 200,000 g for 30 min at 4°C. The pellets, which represented the combined mitochondrial and microsomal fractions, were washed in homogenisation buffer and resuspended in 300 μl of 0.25 M sucrose. The protein content of these fractions was measured and found to be similar to that of normal muscle on days 1, 3, 5, 21 and 28 but it was lower on days 7 and 10 (see legend of Fig. 1). 5 μl of these suspensions were electrophoresed on a Laemmli-type of 7.5% SDS PAGE and then electroblotted onto immobilon-P (Millipore). The SERCA1 proteins were detected by a 1:5 dilution of culture supernatant of the A 3 mouse anti-SERCA1 monoclonal antibody (Zubrzycka-Gaarn et al., 1984). The blots were quenched in l0mM Tris.HC1, pH 7.5, 0.9% NaC1, 0.05% TWEEN-20 (TBST) for 1 h. The antibodies were also dissolved in TBST solution. The SERCA2 a and SERCA2b proteins were detected by a 1: 500 dilution of SERCA2 a- and SERCA2 h-specific rabbit antisera (Wuytack et al., 1989 Eggermont et al., 1990). Incubations with primary antibodies and secondary antibodies (peroxidase-conjugated rabbit anti-mouse immunoglobulins or swine anti-rabbit immunoglobulins, 1:1000; Dako A/S, Glostrup, Denmark) lasted for 1 h. For visualisation of the immunocomplexes nickel-enhanced diaminobenzidine (DAB), precipitations were produced by staining for peroxidase activity in the presence of 0.006% H_2O_2 (Wuytack et al., 1994). Quantitification was performed by densitometer scanning, using a ScanPack 10.1 A20 program (Biometra, Göttingen, Germany). Amounts of sample were always applied within the linear range of the assay as determined by a dilution series of the sarcoplasmic reticulum fraction.

Controls to ascertain specificity of the SERCA antibodies were performed. The A3 SERCA1 specific antibody did not cross react on immunoblots with serial dilutions of combined mitochondrial and microsomal fraction of rat heart known to express SERCA2a but not SERCA1. On the other hand, SERCA2 a was detected in the same microsomes by the R-15 SERCA2 a specific antibody (data not shown).

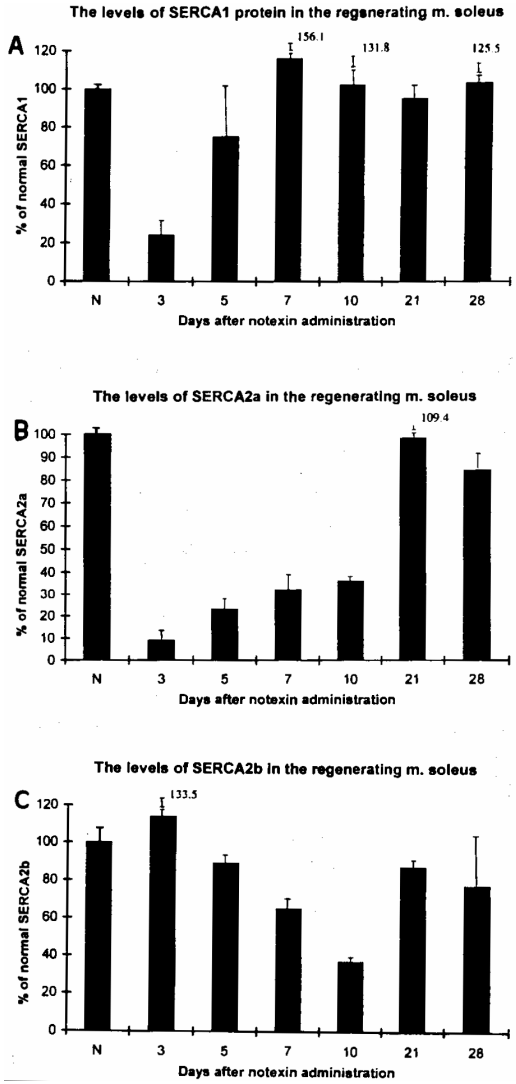
Immunocytochemistry. Soleus muscles were cryoprotected with 20% sucrose and frozen in isopentane cooled by liquid nitrogen. Each of the regenerated muscles and their contralateral counterparts were cut into 5 transverse blocks by a device incorporating equally spaced razor blades. Serial cryosections of 20 μm thickness were cut from each block and processed for immunostaining. Sections were incubated in 1% BSA and 10% normal rabbit or goat serum in PBS for 20 min to block nonspecific binding sites. Sections were incubated overnight with the SERCA1-specific antibody (A3, mouse monoclonal, 1:10), the SERCA2a-specific rabbit serum (R-15, 1:500), the SERCA2 b-specific rabbit serum (R2-88, 1: 500) the type 1 myosin heavy chain (MHC) specific antibody (BA-G5, mouse monoclonal, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, 1:10) or the type 2a MHC-specific antibody (SC-71, mouse monocional, Schiaffino et al., 1989, Deutsche Sammlung von Mikroorganismen und Zellkulturen, 1:20) and then with the secondary antibody (peroxidase-conjugated rabbit anti-mouse immunoglobulins, Dako, 1:200 or goat anti-rabbit, Sigma, 1:200) for 30 min. The immunocomplexes were visualized by DAB staining of peroxidase activity in the presence of 0.006% H_2O_2. Control sections were immunostained in the absence of primary antibody.

Sampling procedure. All quantitations were performed on immunostained sections from each block. The Visual field of the l0x objective/12.5x eyepiece (approximately 0.92 mm^2) was chosen as sample area. Starting from the medial and superficial part of the muscle, 2–7 areas were taken from each section, depending on the size of the whole muscle cross section (Lexell et al., 1994).

Images of serial sections stained with the SERCA and myosin antibodies were

compared using Global Vision System software (Analis Belgium, Version 1.2, Namur, Belgium).

Number of experiments, statistics. For the immunoblots, 3 preparations, each from separate animals, were used for every time point studied. For the immunohistochemical experiments four animals were used for every time point. A Student's t-test indicated that after 28 days of regeneration ratios of SERCA1/SERCA2 expressing fibers differed significantly ($p < 0.05$) from that in the normal untreated soleus.



Figs. 1 A–C

Results

Levels of SERCA proteins in fragmented muscle membranes. Levels of SERCA1, SERCA2a and SERCA2b proteins during regeneration from notexin induced necrosis were assessed on immunoblots of the combined mitochondrial/microsomal muscle fractions by means of specific antibodies. Prelimin-

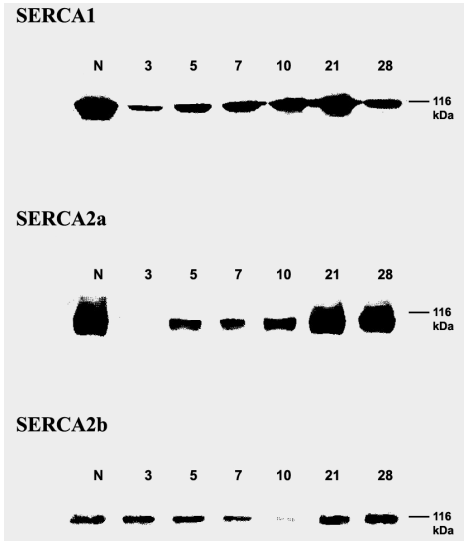


Fig. 1 D. Examples of the immunoblots are shown.

◀

Figs. 1 A–C. Levels of sarcoplasmic reticulum Ca^{2+}-ATPases during regeneration. Immunoblots were stained with A3 monoclonal antibody (SERCA1-specific). R-15 antiserum (SERCA2a-specific) and R2-88 antiserum (SERCA2b- specific). N, normal untreated soleus; 3, 5, 7, 10. 21, 28, days after notexin administration. The level of SERCA1 (A) is significantly decreased on day 3. The level of SERCA2 a (B) is significantly lower on days 3, 5, 7 and 10. The level of SERCA2b (C) is significantly below that of the untreated controls on days 7 and 10. The values are normalized the control soleus (N). Note that equivalent parts of the membrane fractions were analysed. The average protein contents (mean ± S.E.M., n = 3) of the samples applied to a gel slot were the following: N: 9.5 ± 0.8 μg, 3d: 9.5 ± 0.5 μg, 5d: 7.4 ± 0.3 μg, 7d: 7.8 ± 0.9 μg, 10d: 4.9 ± 1 μg, 21d: 10.6 ± 1.4 μg, 28d: 9.8 ± 1.2 μg. The protein content of fractions on day 10, when the SERCA2b level was decreased, was significantly lower than in the fractions obtained on other days ($p < 0.05$).

ary experiments showed that in the normal soleus approximately 40% of the Ca^{2+}-pumps (SERCA1, SERCA2a and SERCA2b immunostaining) were recovered in the mitochondrial fraction (pellet of a 15 min x 10,000 g spin of the postnuclear supernatant) and approximately 60% in the microsomal fraction (pellet of a 30 min x 200,000 g spin of the postmitochondrial supernatant). Since morphological changes which occur during muscle regeneration could cause a redistribution of pumps between mitochondrial and microsomal fractions, we preferred to use combined mitochondrial/microsomal fractions for our assays. For each time point, the same volume of membrane fraction (i.e. 5 μl out of a total of 300 μl) was applied on the gel. Therefore, signals on the immunoblots directly relate to the total amount of expressed protein.

SERCA1, SERCA2a and SERCA2b followed different time courses of expression during muscle regeneration. Three days after administration of

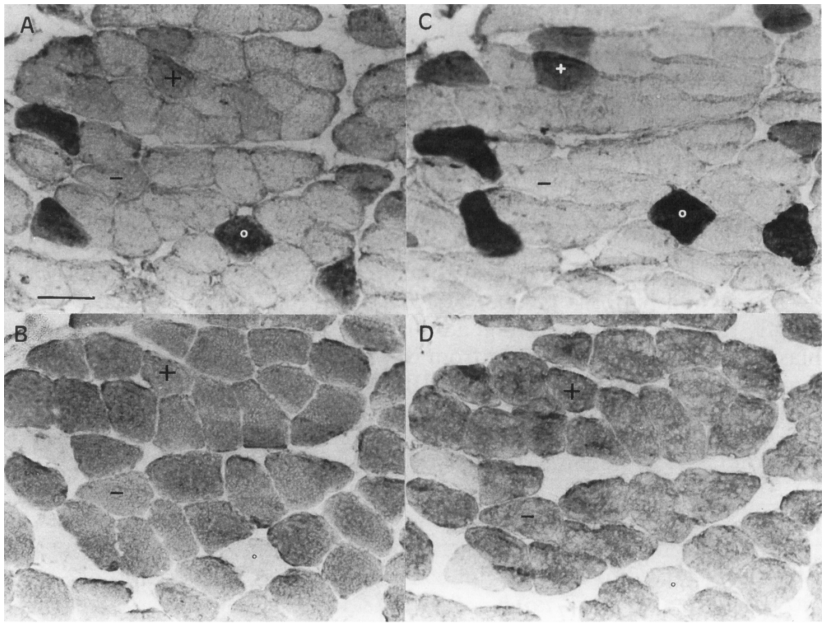


Fig. 2. Expression of sarco/endoplasmic reticulum Ca^{2+}-ATPases in fibers of normal soleus muscle. Serial transverse sections showing (A) fibers expressing the fast-type SERCM isoform stained by the A3 monoclonal antibody, (B) fibers expressing the slow-type SERCA2a isoform stained by the R-15 antiserum, (C) fibers stained by the MHC2a (fast-type) SC-71 antibody, (D) fibers stained by the MHC1 (slow-type) BA-G5 antibody. Note fibers stained for SERCA1 (o) or SERCA2 a (–) only, fibers expressing both SERCA1 and SERCA2 a Ca^{2+}-ATPases (+) and fast fibers coexpressing MHC2 a with SERCA1 (o) or slow fibers coexpressing MHC1 with SERCA2a (–). The scale bar is 50 μm.

the toxin, the SERCA1 level in the fragmented membranes was strongly reduced. but it largely recovered between day 5 and 21. In some animals, SERCA1 declined again on day 28 to a value below the level in normal controls (Fig. 1 A), but the mean levels were not significantly different from controls. The level of slow-type Ca^{2+}-pump protein (SERCA2 a) was also strongly reduced on the third day after administration of the toxin. On days 5, 7 and 10, SERCA2 a recovered to a level at approximately 1/3 of the control level. On day 21, a marked further increase in the SERCA2 a level was observed to a value that remained constant to day 28 (Fig. 1 B). Whereas the amount of muscle-specific SERCA1 and SERCA2 a Ca^{2+} pumps changed dramatically, the level of the "housekeeping" SERCA2b isoform remained relatively constant throughout the whole regeneration period (Fig. 1 C), except on days 7 and 10, when this isoform declined because of reduced protein content of the membrane fractions. Examples of the immunoblots are shown in Fig. 1 D.

Immunocytochemical detection of SERCA proteins during regeneration. On transverse sections of normal soleus muscles, SERCA1 protein was coex-pressed with the fast type 2 a MHC. Similarly, a strong correlation of immuno-staining was observed for SERCA2a and type 1 MHC (Fig. 2 A–D; Table 1).

In normal soleus muscles, SERCA1 protein was found to be expressed in 26.3±2.6 (mean±S.E.M.)% of the fibers, whereas SERCA2 a could be demonstrated in 80.7±4.3% of the fibers. This suggests that at least a few percent of fibers coexpress both isoforms. Indeed on consecutive sections, the occasional simultaneous expression of SERCA1 and SERCA2 a in single fibers was confirmed (Fig. 2A,B). The SERCA2b isoform was faintly but uniformly expressed and found in more than 98% of the fibers (data not shown).

Expression patterns of both SERCA1 and SERCA2 a showed a characteristic time course during regeneration of soleus muscle (Figs. 3, 4). Myoblasts expressed both isoforms from day 3 onwards. On day 10, a large number of the fibers coexpressed SERCA1 and SERCA2a (Figs. 3 A, B).

Table 1. Coexpression of SERCA1 with MHC2 a and SERCA2 a with MHC1 in normal soleus muscles

Blocks	MHC2a + SERCA1	MHC1 + SERCA2a
1	94.2 ± 1.5	99.8 ± 0.2
2	93.6 ± 2.7	99.6 ± 0.2
3	96.8 ± 1.7	99.1 ± 0.3
4	95.2 ± 2.7	98.9 ± 0.6
5	93.5 ± 1.5	99.1 ± 0.3

Percentage of MHC2 a-expressing fibers which also expressed SERCA1 (column 2) and the percentage of MHC1-expressing fibers which coexpressed SERCA2 a (column 3) are given for a series of 5 blocks from proximal to distal end of rat soleus muscles (mean ± SEM, n = 4). Note that in whole soleus muscles an average 94.6 ± 0.76% of the MHC2a-expressing fibers was also expressing SERCA1 and 99.1 ± 0.2% of the MHC1-expressing fibers expressed SERCA2 a.

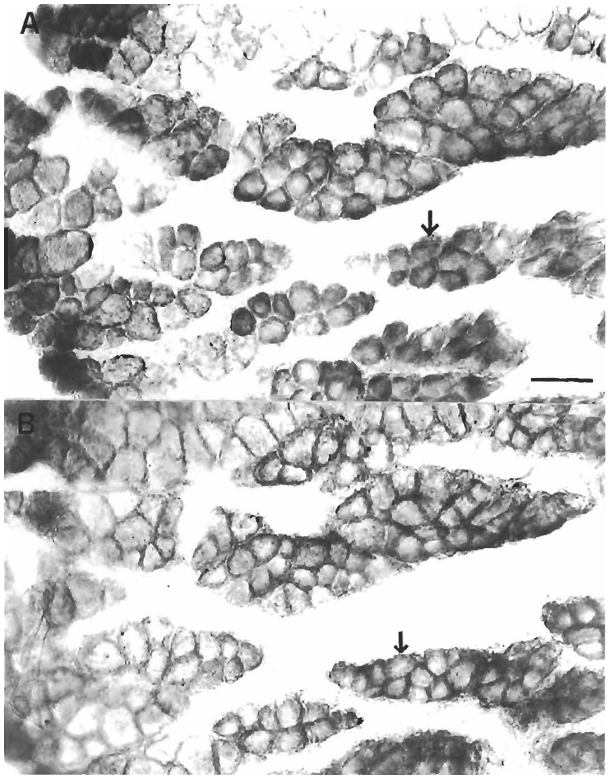


Fig. 3. Expression of sarcoplasmic reticulum Ca^{2+}-ATPases in muscle fibers of regenerating soleus on day 10. Transverse sections were stained with fast type SERCA1-specific A3 monoclonal antibody (A) or slow-twitch type SERCA2a-specific R-15 antiserum (B). Note fibers stained for both SERCA1 and SERCA2a (arrows). The scale bar is 50 μm.

Characteristically, a few fibers were found to express more SERCA1 than most of the other fibers. Such a difference in relative expression levels between fibers was not observed for SERCA2a. After 3 weeks, expression of both isoforms resembled that on day 10, i.e. SERCA1 was expressed in higher amounts in a few fibers as compared with the others, whereas SERCA2a was detected in nearly all fibers. Whalen et al. (1990) reported that after 4 weeks regenerated soleus showed less type I A fibers and more type I fibers in terms of expression of myosin heavy chain isoforms. We now found that fast MHC2a was coexpressed with SERCA1 and slow type MHC1 with SERCA2 a isoform in regenerated soleus. After 4 weeks of regeneration, the number of SERCA1 expressing fibers had also dramatically declined. SERCA1 was expressed only in 114+1.1 % of the fibers (Fig. 4 A;

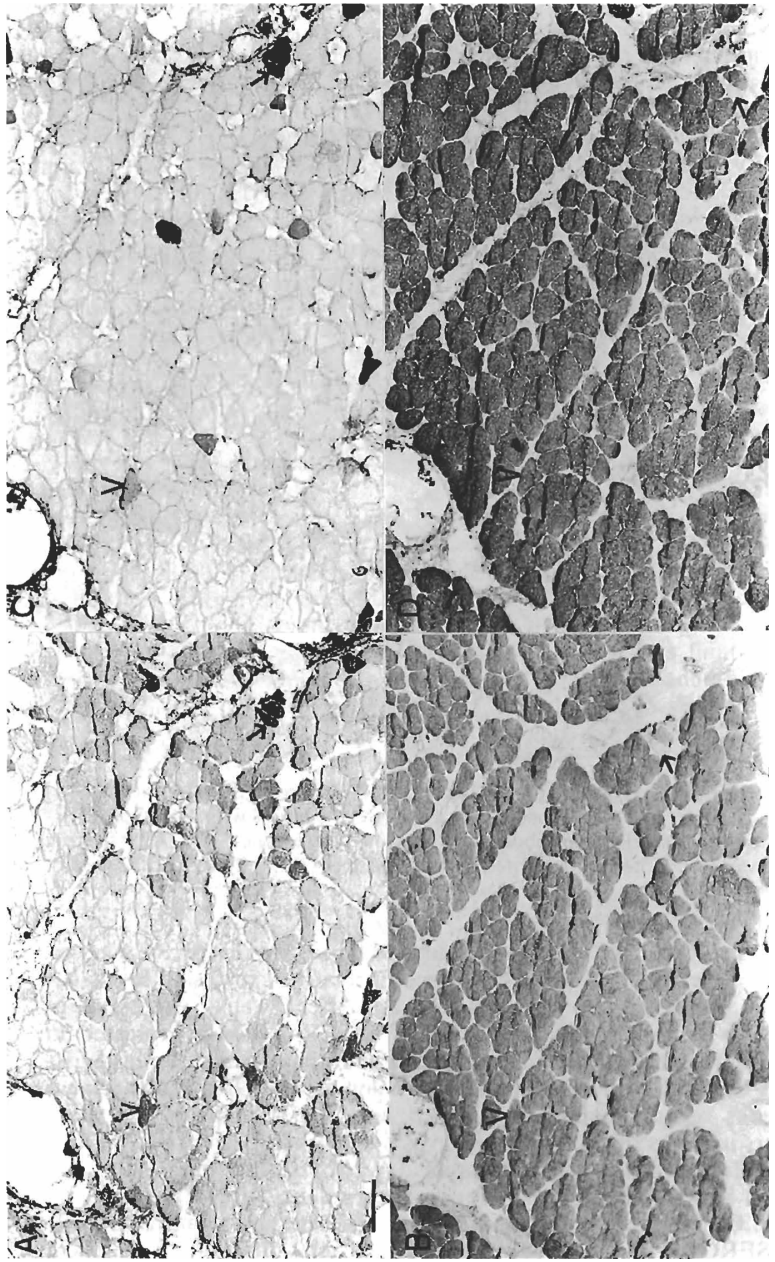


Fig 4. Expression of sarco/endoplasmic reticulum Ca^{2+}-ATPase and myosin isoforms in muscle fibers on serial sections on day 28 of regeneration. Transverse sections were stained with the fast type SERCA1-specific A3 monoclonal antibody (A), the slow type SERCA2a-sepcific R-15 antiserum (B), the MHC2a antibody (C) or the MHC1 antibody (D). Although the majority of the fibers coexpressed SERCA2a and MHC1, note that some fibers also expressed SERCA 1 and MHC2a (v) A few fibers coexpressed SERCA1 and MHC2a, but not SERCA2a and MHC1 (arrow) The scale bar is 100 μm.

Table 2). Furthermore, the characteristic variability in expression level among SERCA fibers observed at 3 weeks persisted after 4 weeks, although only a minority of the fibers showed high SERCA1 expression. At that time nearly all fibers (98.2 ± 0.6%) expressed SERCA2 a (Fig. 4 B; Table 3) together with slow myosin (Fig. 4D). MHC1 showed typically less variation in expression levels between the fibers than SERCA1. Hence on day 28 of regeneration, SERCA2 a was clearly the dominating isoform, whereas in untreated contralateral muscle, which acted as a control, expression of SERCA1 and SERCA2 a was substantially mixed (Fig. 2A, B). After 5 months of regeneration. the ratio of SERCA1- and SERCA2a-expressing fibers remained similar to that observed on day 28 (Figs. 5 A, B).

Table 2. Expression of SERCA1 in fibers of 4 regenerated soleus muscles and their contralaterals

Blocks	Regenerated m. soleus	Contralateral m. soleus
1	14.7 ± 2.1	26.6 ± 4.9
2	11.9 ± 1.6	27.1 ± 3.6
3	13.9 ± 1.9	26.4 ± 2.4
4	13.8 ± 1.7	27.3 ± 2.2
5	11.6 ± 1.5	22.8 ± 2.2

Percentage of fibers (mean ± S.E.M., n = 4) showing positive immunostaining for SERCA1 is given for 5 blocks from proximal to distal end of soleus muscles regenerated after notexin induced necrosis. Note that the SERCA1 was expressed on average in 12.4 ± 1.1% of the fibers of regenerated and in 26.3 ± 2.6% of fibers of untreated contralateral soleus muscles. The difference between numbers of SERCA1-expressing fibers in regenerated and normal soleus muscles was significant ($P < 0.05$) in each block. but significant differences were not observed between the five different blocks of regenerated soleus muscle or between five blocks of contralateral muscles.

Table 3. Expression of SERCA2 a in fibers after 4 weeks of regeneration and in contralateral controls

Blocks	Regenerated m. soleus	Contralateral m. soleus
1	97.9 ± 0.7	79.7 ± 7.7
2	98.3 ± 0.5	81.6 ± 4.0
3	97.9 ± 0.9	79.5 ± 5.1
4	98.5 ± 0.7	79.4 ± 4.7
5	98.6 ± 0.7	79.0 ± 6.3

Percentage (% of total fibers ± S.E.M., n = 4) of SERCA2a-positive fibers is given for 5 blocks from proximal to distal ends of soleus muscles regenerated after notexin induced necrosis. Note that SERCA2 a was expressed on average in 98.2 ± 0.6% of the fibers of regenerated and in 80.7 ± 4.9% of fibers of contralateral soleus muscles. The difference between numbers of SERCA2 a-expressing fibers in regenerated and nor mal soleus muscles was significant at the $P < 0.05$ level in each block, but significant differences were not observed between the five different blocks of regenerated soleus muscles or between the five blocks of contralateral muscles.

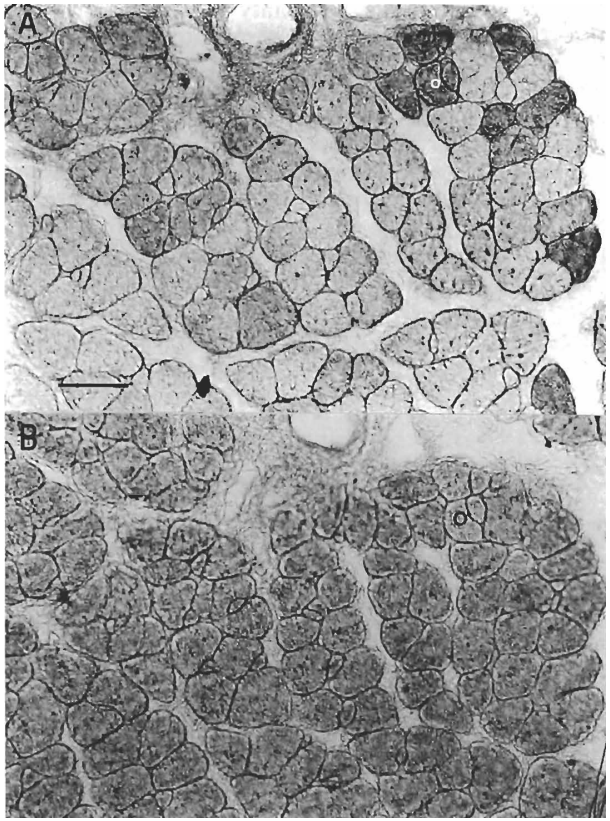


Fig. 5. Expression of fast and slow sarcoplasmic reticulum Ca^{2+}-ATPases in muscle fibers after 5 months of regeneration. Serial sections were stained with (A) fast type SERCA1-specific and (B) slow type SERCA2a-specific antibody. Fibers stained for both SERCA1 and SERCA2 a (o).The scale bar is 50 μm.

Discussion

Our results show that protein levels of SERCA1 and SERCA2a isoforms mainly depend on the differentiation state of muscle fibers, whereas SERCA2b is present in myoblasts, myotubes and in non-muscle cells. However, the SERCA isoforms characteristic for fast- and slow-twitch muscle increased with different time courses during regeneration; the fast-twitch SERCA1 increased predominantly between day 3 and 10, whereas the expression of the slow-twitch SERCA2 a isoform increased mostly between day 10 and 21. It is of interest that reinnervation of regenerating soleus is nearly complete after 10 days (Grubb et al., 1991). Therefore, the switch be-

tween the two isoforms appears to follow the establishment of new reinnervation.

It was not possible in our experiments to deduce the molar ratio of SERCA1 and SERCA2a proteins from the immunoreactions because different antibodies were used. However, changes in SERCA1 and SERCA2 transcript levels showed during regeneration time courses similar to their respective proteins (Zador et al., 1996). Therefore, SERCA1 and SERCA2a expression is probably largely pretransiationaily controlled. The monoclonal antibody A3 does not discriminate between neonatal and adult variants of SERCA1. Indeed, the only difference between neonatal and adult forms of SERCA1 is confined to their extreme C termini where terminal G994 of the adult form is replaced by an extended tail 994-DPEDERRK-1001 in the neonatal form (Wu and Lytton. 1993). This might explain why a specific antibody for SERCA1a is not available. Both during normal ontogeny and during regeneration, the neonatal transcript appeared before the adult one (Zador et al., 1996). Therefore, it seems likely that the SERCA1 isoform detected on days 3 to 5 of regeneration mainly represents the neonatal isoform of the protein (Brandl et al., 1987).

In early stages of regeneration of soleus, SERCA1 was found to be expressed in many myoblasts and it remained the major isoform in the newly formed muscle fibers during the first 10 days. After 4 weeks, SERCA1 was expressed in 12% of the fibers, i.e., a 2-fold lower value ($p < 0.05$) than observed in normal rat soleus muscle (26.3%). We did not observe any differences between the ratio of fiber types in the proximal, middle and distal parts of soleus muscles as were reported by Punkt et al. (1998). However, these authors used fiber type related enzyme activity and not immunostaining. Therefore, this discrepancy must be due to the methodology applied. Moreover. it is interesting to note that individual fibers showed variable levels of staining with the A 3 antibody. This most likely points at different levels of expression of the fast type Ca^{2+}-pump. Since we used in these assays a monoclonal antibody, recognising only a single epitope on SERCA1, a change in reactivity due to a change in epitope presentation as a result of altered conformation may not be excluded (reviewed by Dux, 1993).

Statistical analysis of our data confirmed that in fibers of normal soleus muscle, the type 2A fast MHC isoform is coexpressed with SERCA1. Similarly, the slow myosin and the SERCA2 a also coincided. However, in the re generated muscle MHC1 and SERCA2 a were present in almost all fibers. This indicates that the regenerated soleus, even after a prolonged period of time, does not return to its condition prior to necrosis but instead expresses clearly the slow isoforms of the pumps and myosin proteins in a larger number of fibers. It should be remarked that the activity of the affected leg in the animals appeared to be normal after 4 weeks of regeneration. Therefore, changes in contractile activity cannot be held responsible for the lack of apparent complete recovery. Moreover, adult soleus muscle, if deprived of stretch and contractile activity, is known to switch back to transcribing the fast myosin heavy chain gene (Goldspink et al., 1992). This transition towards a more slow-type of muscle appears to be permanent since even

5 months after injection of the toxin, increased levels of SERCA2 a were still observed. After a similar period of recovery from notexin-induced necrosis, an increased ratio of slow/fast myosin containing fibers has also been described (Whalen et al., 1990).

Single fibers were found to coexpress the slow and fast SERCA isoforms. This is similar to what was observed for myosin isoforms, where coexpression of fast and slow isoforms was also shown (Whalen et al., 1990). In rats, each fiber receives its own innervation from individual motor units (Torella et al., 1993) and chronic low-frequency stimulation studies led to the conclusion that a different stimulation pattern is required for the induction of SERCA2 a or SERCA1 (Leberer et al., 1989), similar to that of slow and fast myosin isoforms (Thomas and Ranatunga, 1993). Coexpression of slow and fast type of SERCA pumps both in normal and regenerated soleus suggests co-induction of SERCA1 and SERCA2a. Coexpression of low levels of SERCA2a with the dominant SERCA1 pump has also been reported in the fast-twitch muscle (Wu and Lytton, 1993).

In conclusion, during regeneration levels of muscle-specific SERCA isoforms undergo 1arge time-dependent changes. Changes in both myosin isoforms and the Ca^{2+}-pump isoforms reflect alterations of the fiber types in regenerating soleus probably controlled by a common factor.

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