THE EXPRESSION OF MUSCLE SPECIFIC GENES IN MECHANICAL VENTILATION AND REGENERATION

Summary of PhD thesis

Gábor Z. Rácz, MD

Tutor:
Ernő Zádor PhD

Department of Biochemistry
Faculty of Medicine
Albert Szent-Györgyi Medical and Pharmaceutical Center
University of Szeged
Szeged, Hungary
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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.

1. INTRODUCTION

Skeletal muscle is composed of different types of myofibers, which are the result of coordinated expression of distinct sets of structural proteins and metabolic enzymes. Most of the proteins involved in contractile function, for example the myosin heavy chain (MyHC), and the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA), exist as multiple isoforms, which are usually distributed in a myofiber type specific and coordinated manner. Although patterning of skeletal muscle fiber composition begins to appear during embryonic development, any muscle can be partially or completely remodelled by stimuli applied to fully mature adult myofibers, most importantly by different patterns of motor nerve activity and contractile work. Several key pathways have been implicated in the transcriptional control of muscle-specific genes including some of the MyHCs and SERCAs. First, the MRF 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. 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. 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.

1.1. 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. 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. 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, corticosteroid treatment, but mechanical ventilation by itself may also lead to inspiratory muscle dysfunction. Animal models aiming to reveal the deleterious effects of

controlled mechanical ventilation (CMV) on respiratory muscle function found that even short-term (1–4 days) CMV leads to decreased force-generating capacity of the diaphragm and to reduced diaphragm muscle mass. The decreased diaphragm protein levels, increased proteasome activity, and reduced level of insulin-like growth factor-I supports that atrophy is in process during CMV. The atrophy manifests with preferential decrease in the cross-sectional area of type IIa and IIb fibers. Similar alterations were observed in the intercostal, but not in the hindlimb muscles of the same ventilated animals. These changes could explain the reduced force generation.

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. 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. 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. 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. 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.

1.2. Background: Regeneration study

Skeletal muscle has a remarkable potential to regenerate. 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. 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) and the SERCA mRNA and protein levels. Those parameters demonstrate that in this regeneration system the fibers are totally destroyed but later recreated in the soleus muscle. A number of 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 *e.g.* during cyclosporin A treatment in the soleus

or in passively stretched rat soleus muscles. Studies monitoring the effect of orally administrated calcineurin inhibitors have suggested that calcineurin stimulates in vivo muscle regeneration. Constitutively active calcineurin selectively upregulates the slow-fiber-specific promoters and the feeding of calcineurin inhibitors decreases the number of MyHC1-expressing slow fibers in rat soleus muscle through neural control. Innervation also seems to be a prerequisite for the expression of the slow SERCA2a isoform.

2. AIMS OF THE STUDIES

2.1. 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, it can be assumed that the intermittent spontaneous breathing has a protective effect. Therefore we examined whether intermittent spontaneous breathing may protect the diaphragm against the detrimental effects of controlled mechanical ventilation.

2.2. Regeneration study

The role of calcineurin in defining the slow muscle fiber identity is widely accepted. The aim of this study was to compare the time scale of expression of the active calcineurin subunit and the change of calcineurin activity with the switch from fast- to slow-type myosin and SERCA isoforms during regeneration. The nerve-dependence of calcineurin expression and activity was also studied.

3. MATERIALS AND METHODS

3.1. Mechanical ventilation study

Study design and experimental procedures. *Series 1 (mechanical ventilation).* 52 male Wistar rats were randomly divided into three groups: (1) A control group, (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 mRNA levels and/or the protein levels of several factors that possibly influence diaphragm function were measured: MRF and Id-proteins; MyHC and SERCA isoforms; acetylcholine receptor.

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. A ventilator 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. 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).

Series 3 (intermittent mechanical ventilation). Adult male Wistar rats 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. (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.

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. 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. 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.

mRNA extraction and RT-PCR. (In *Series 1 and 2*) Part of the diaphragm and the whole gastrocnemius were taken, total RNA was isolated using a modified guanidinium isothiocyanate-CsCl method. Samples of total RNA were subjected to oligo(dT)-primed first-strand cDNA synthesis and PCR.

Myosin heavy chain extraction and separation. (In *Series 1 and 2*) Myosin heavy chain isoforms from the diaphragm and gastrocnemius were extracted and SDS-PAGE with 8% separating gel containing 30% glycerol and 4% stacking gel was running during 24 hours. The gels were stained with silver nitrate, air dried, and scanned.

Western blot quantification of MyoD and myogenin. (In *all series*) Frozen muscle samples were homogenized and proteins were separated by SDS-PAGE, blotted. MyoD and myogenin were detected by indirect immunohybridization and developed by reaction of the conjugated peroxidase.

Measuring of diaphragm contractile properties. (In *Series 3*) Segments of the costal diaphragm were removed for measurement of *in vitro* contractile properties. 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 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.

3.2. Regeneration study

Animals and treatment. In narcotized three month old Wistar rats, the soleus muscle was injected with 20 μg venom of the mainland tiger snake (*Notechis scutatus scutatus*). On day 1, 3, 5, 7, 10, 21 or 28 the entire soleus muscle was removed. 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.

mRNA extraction and RT-PCR. The total RNA was isolated as described by Chomczynski and Sacchi. Samples of total RNA of each soleus was subjected to oligo(dT)-primed first-strand cDNA synthesis and multiplex PCR (MyHC or SERCA isoforms together with the internal control GAPDH). 5μl of the primary amplification product were radiolabelled with [α-^{32}P]dCTP in 2 additional cycles. After electrophoresis, the gels were air-dried and ^{32}P-spots were quantified.

Protein extraction, electrophoresis and Western-blots. Different methods were used for the extraction of MyHC, SERCA and calcineurin A. The suitable protein extracts were electrophoresed and blotted. SERCA, MyHC isoforms and calcineurin A were detected by indirect immunohybridization and developed by reaction of the conjugated peroxidase or Vistra EFC kit.

Calcineurin activity assay. Untreated, 5 days and 10 days old regenerating and denervated regenerating muscles were homogenised and centrifuged in the following solution: 50 mM Tris-HCl, (pH 8), 250 mM sucrose, 10 μg/ml leupeptin, 0.2 mM PMSF, 2 mM EGTA, protease inhibitor cocktail, 5 mM ascorbic acid, 0.15% β-mercaptoethanol, 0.5 mM DTT. Enzyme activity was measured by a colorimetric assay kit. The principle of the measurement is the following: the sample calcineurin A dephosphorilates the p-Ser phosphopeptide substrate in the presence of 0.5 μM calmodulin, the released inorganic phosphate is detected by Malachite Green reagent.

4. RESULTS & DISCUSSION

4.1. Mechanical ventilation study

General findings. Mortality was 25% in the mechanically ventilated (CMV) rats, it was higher in the spontaneously breathing group (SB) (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. Arterial blood pressure and blood gases for anesthetized rats were not significantly different between the groups and remained in the normal range for all studied variables.

Effect of controlled mechanical ventilation. Compared to controls (C), diaphragm myogenin mRNA levels were significantly increased in the CMV group (+67%, $p<0.01$ vs C) while they tended to increase in the spontaneously breathing group (+32%, not significant). Myf-5 mRNA increased along with myogenin after mechanical ventilation (+107%, $p<0.01$ vs C), however, it also increased significantly in the spontaneously breathing group (+130%, $p<0.01$ vs C). By contrast, the level of MyoD mRNA decreased both in the spontaneously breathing rats (–33%, $p<0.01$ vs C) and even more so in mechanically ventilated animals (–56%, $p<0.001$ vs C), 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 C) and more particularly after mechanical ventilation (–70%, $p<0.001$ vs C). In addition, this ratio was significantly decreased by 53% in the mechanically ventilated group compared to the spontaneously breathing group ($p<0.01$ vs SB). mRNA levels of MRF4, a factor involved in the later stages of myogenic transformation pathway, remained unchanged. On the protein level, diaphragm MyoD was significantly reduced in mechanically ventilated group (–49%, $p<0.05$ vs C and SB) and remained unchanged in spontaneously breathing animals while myogenin tended to increase (SB: +115% and mechanically ventilated: +59% vs C, $p=0.08$).

Inhibitor of DNA-binding protein-1 (Id1) mRNA levels were significantly and equally reduced both in the diaphragm of CMV and SB animals (–30%, $p<0.001$), while Id2 and Id3 mRNA showed no changes and Id4 mRNA was not detectable.

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 ($p<0.05$). 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). The slow-type SERCA2a mRNA did not change in the experiment. The mRNA level of acetylcholine receptor α-subunit showed a marked but not significant increase in the diaphragm of the SB (+140%) and CMV (+120%) groups.

The observed changes 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. The caloric intake (173 kcal/kg/day) was similar to that used in the studies of other authors. 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. 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.

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. 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. 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. 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.

Effect of passive movement on the gastrocnemius muscle. Passive shortening combined with immobilization (I+PS) caused a significant reduction in gastrocnemius MyoD mRNA (–23%, $p<0.05$ vs C). However, after immobilization alone (I), more pronounced decay was observed (–44%, $p<0.001$ vs C and –21%, $p<0.05$ vs I+PS). It is worth to note that myogenin (I: +52% and I+PS: +70%, $p<0.05$ vs C) and myf-5 (I: +34% and I+PS: +29%, not significant) mRNA increased after immobilization and also after passive shortening as was also the case in the diaphragm of mechanically ventilated and spontaneously breathing groups. As a result, MyoD/myogenin ratio decreased similarly after immobilization (–60%, $p<0.001$ vs C) and after immobilization combined with passive shortening (–51%, $p<0.001$ vs C). MRF4 mRNA did not change whatever was the condition. Compared to controls, gastrocnemius MyoD was significantly increased after immobilization (+40%, $p<0.01$ vs C) and after immobilization and passive shortening (+52%, $p<0.001$ vs C). For myogenin, a tendency to increase was also observed in both groups (I: +36% and I+PS: +44% vs C) compared to controls but these increases failed to reach statistical significance. 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. 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 C) while the other Id isoform mRNA did not change.

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. Similar effects were reported in electrically silent muscles: unweighting the hindlimb muscles lead to MyoD accumulation. 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 and mRNA content of MRFs, especially for MyoD and myogenin are similar to that of the diaphragm.

The beneficial effect of intermittent mechanical ventilation. 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. 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. 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.

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). MyoD protein was tended to decrease in the ISB5 group (–27%). 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). 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). The decrease in myogenin protein levels was significantly different between the CMV#2 and the ISB5 groups (p<0.0001). 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. 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.

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. However, not uncommonly, patients with acute respiratory distress syndrome receive passive ventilation through sedation with or without paralysis during mechanical ventilation. Thus the choice of controlled mechanical ventilation in this study is in accordance with clinical practice.

4.2. Regeneration study

Levels of MyHC and SERCA isoforms in the regenerating soleus. 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. First, the mRNAs of the neonatal MyHC, the fast-type MyHC2x, and of the neonatal SERCA1b appeared on day 1 of regeneration. However, after showing a transient increase, peaking at days 5 and 7, they normalized again toward day 28. The mRNAs of the fast-type MyHC2a and SERCA1a isoforms 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 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. The slow MyHC1 appeared on day 7, continuously increased until day 28, and then reached a value equal to the untreated controls. It is noteworthy that 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, so the appearance of slow SERCA mRNA and protein precede the slow MyHC by 2–4 days in the differentiation stage.

Level of calcineurin A in the regenerating soleus. The focus was set on days 5 and 10, time points representing the situation, respectively, before and after the muscle comes again under neuronal control. 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, presumably because of the high levels of reactive oxygen and nitrogen species, which are known to be deleterious to calcineurin. It re-emerged on days 5–10, after which it remained at about the normal level until day 28 of regeneration. The early expression 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, and macrophages apparently do not express high levels of CnA. 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. 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. Therefore, the restoration of the calcineurin activity was strictly dependent on the nerve and it correlated with the levels of CnA. Our group also measured the level of calcineurin Aα, Aβ and MCIP1.4 mRNA in this experiment. 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.

These data 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. 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, an approach extending our study found that inhibition of calcineurin does not prevent SERCA2a expression in regenerating muscles.

5. 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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