Metabolic profile of myosin heavy chain-based fiber types in the rat soleus after spinal cord transection

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Abstract

Fully differentiated muscle fibers can undergo considerable phenotypic changes in order to adjust to changing conditions of the physiological environment. It is generally accepted that the electrical impulses a muscle receives play a role in modulating the quantities of metabolic proteins (glycolytic and oxidative enzymes) and types of contractile proteins (myosin heavy chain, MHC) that are expressed. Research has shown that decreased neuromuscular activation following spinal cord transection (ST) results in adaptations in the physiological characteristics of paralyzed muscles, including atrophy and an accompanying loss of force production, and transformations of contractile and metabolic proteins toward a more fatigable state (Talmadge et al., 1993; Castro et al., 2000). However, it remains unclear whether or not a strong interdependence of energy metabolism and MHC isoform composition persists. Therefore, the goal of this study was to identify and quantify relative myosin heavy chain (MHC) isoform expression and metabolic enzyme profile adaptations at multiple time points (1, 3 and 6 months) in soleus fibers of rats following spinal cord transection (ST).

To accomplish this, female Sprague-Dawley rats (~150 g, n = 15) were subjected to complete transection of the spinal cord at a mid-thoracic level. Age and weight-matched, non-operated rats served as controls (n = 15). The soleus was processed for quantitative single fiber histochemical analyses for succinate dehydrogenase (SDH, oxidative marker) and α-glycerophosphate dehydrogenase (GPD, glycolytic marker) activities (~30 fibers/muscle) and immunohistochemical analysis for MHC isoform composition. The total number of soleus fibers analyzed was ~900.

Oxidative capacity was increased in muscle fibers at all time points after ST. Specifically, SDH activity was significantly higher than controls by 142, 127 and 206% at 1, 3 and 6 months post-ST, respectively. ISDH, a measure of total oxidative power, also increased in muscle fibers at all time points after ST. For example, 6 months after ST ISDH activity was 93% higher than controls (91.8–3.8 vs. 47.6–0.9 OD x 10^{-3}, respectively).

Glycolytic capacity peaked one month after ST. Thereafter, glycolytic capacity of all fibers steadily declined. For example, by 6 months, GPD activity had declined by 76% compared to 1 month GPD activities (3.3–0.2 vs. 13.7–1.4 OD x 10^{-3}, respectively). These data suggest that the increases in glycolytic capacity are transient as fibers transition toward a faster MHC phenotype and then return towards control levels as fibers of a given type become phenotypically stable .

The GPD/SDH ratio, an index of metabolic substrate utilization, peaked at one month after ST (394–41) and significantly decreased at 3 months (224–10) and at 6 months (95–7) after ST. Therefore, a shift occurred such that a greater dependence on oxidative metabolism was apparent.

These data suggest that the oxidative capacities of soleus muscle fibers are not compromised after ST. In fact, as the fibers transitioned toward faster MHC isoforms, the GPD/SDH ratio was maintained or decreased, suggesting a reliance on oxidative metabolism regardless of MHC isoform composition. This might imply a dissociation between the contractile and metabolic characteristics of paralyzed soleus muscle fibers. However, these data are consistent with previous data and suggest that the increased

fatigability observed after chronic reductions in neuromuscular activity are not due to compromised capacities for ATP synthesis.

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Chapter 1

Introduction

Introduction

Statement of Problem

It is estimated that over 200,000 people are living with spinal cord injuries (SCI) and that nearly 10,000 new cases are reported annually. Fortunately, the majority of these injuries are incomplete, meaning that some degree of motor or sensory function persists. Because of this, rehabilitation of paralyzed limbs below the level of injury is an option. Research has focused on several therapeutic modalities that stress neuromuscular activation, including functional electrical stimulation (Andersen et al., 1996; Wheeler et al., 1996), step training (Edgerton et al., 1992; Roy et al., 1999), passive cycling (Mohr et al., 1997) and weight support activity (Jiang et al., 1990). These modalities have been developed to counter the negative effects on musculature, mainly in an attempt to restore and maintain force production.

SCI results in adaptations in the physiological characteristics of the paralyzed muscles, including atrophy and an accompanying loss of force production, and transformations to contractile and metabolic proteins toward a more fatigable state (Talmadge et al., 1993; Gordon et al., 1994; Castro et al., 2000). However, it remains unclear how these adaptations interrelate with the increased fatigability reported in skeletal muscle.

Castro et al. (1999) have demonstrated that both succinate dehydrogenase (SDH, a marker enzyme for oxidative capacity) and α-glycerophosphate dehydrogenase (GPD, a marker enzyme for glycolytic capacity) activities increased in vastus lateralis (VL) muscle fibers (in all fiber types) of SCI individuals from 6 to 24 weeks after injury. These data suggested that the increased susceptibility to fatigue after SCI was not due to

a deficiency in enzymatic activity related to ATP synthesis. They also showed relatively small changes in the MHC isoform content of the VL in the same timeframe after SCI. Since fibers with the fast isoforms of MHC have higher ATP utilization rates than fibers with the slow MHC isoform, the MHC isoform content of the fiber could contribute to a higher ATP utilization rate and subsequent fatigue. However, MHC isoform content of the VL was not changed in short-term SCI individuals suggesting that an increased rate of ATP utilization was not involved.

In an effort to assess if the causes for increased fatigability were similar in an animal model of SCI, the rat model of complete spinal cord transection (ST) was used. In this study, we assessed the levels of SDH and GPD activity in rat soleus fibers in order to determine if they had an influence on the increased fatigability observed in this model for SCI (Talmadge et al., 1999). Therefore, the aim of this investigation was to identify the oxidative and glycolytic properties of the rat soleus after SCI and to identify possible relationships between these metabolic systems, myosin heavy chain composition and CSA at multiple time points after spinal cord transection (ST).

Significance of Study.

Few studies have analyzed phenotypic alterations in the metabolic and contractile properties of paralyzed musculature over multiple time points. Most studies cannot fully discriminate the time course of adaptations between these two protein systems. Furthermore, there is limited information about how these two systems co-adapt following spinal cord injury at the single fiber level. Studies on whole muscle adaptations yield useful information. However, most muscles are composed of a variety of fiber types that may show differential abilities to adapt. Therefore, analysis at the single fiber level over multiple time points is necessary. This study has attempted to elaborate and clarify the true complexity of some of the adaptations that occur in paralyzed skeletal muscle.

Chapter 2

Review of Literature

Review of Literature

The adult rat hindlimb muscle contains four different myosin heavy chain (MHC) isoforms: types I, IIa, IIx and IIb. These isoforms are expressed in fiber types I, IIA, IIX and IIB, respectively (Schiaffino et al., 1989). In addition, hybrid fibers containing types I and IIa MHC, types IIa and IIx MHC and types IIx and IIb occur under normal conditions. This functional categorization has been shown to correlate with three histochemically assessed fiber types: slow-twitch oxidative (SO; type I), fast-twitch oxidative-glycolytic (FOG; type IIA) and fast-twitch glycolytic (FG; types IIX and IIB) (Barnard et al., 1971). Histochemical analysis for metabolic enzymes of oxidative and glycolytic pathways allows for distinct categorization of four fast fiber phenotypes in rat muscle (Rivero et al., 1998, 1999).

Fibers in adult mammalian skeletal muscle have specific and limited combinations of contractile and metabolic profiles. For example, it has been shown that fibers with the highest myosin ATPase (enzyme regulating speed of contraction) activity had type IIb MHC isoforms and the highest glycolytic potential. Fibers that contained type I MHC isoforms had the lowest myosin ATPase activity and high oxidative potential (Hoffman et al., 1990, Rivero et al., 1998). Interfiber analysis has revealed that FOG (IIA) fibers in the rat generally have relatively higher oxidative enzyme activities than SO fibers (Hauschka et al., 1988; Rivero et al., 1999). Thus, muscle fibers can be categorized into distinct and stable types. However, upon perturbations to load, neuromuscular activation or hormonal status, muscle fibers have the capacity to change phenotypic expression of metabolic proteins and expression of MHC isoforms (Jiang et

al., 1991; Talmadge et al., 1996,1999; Bamman et al., 1998; Jakubiec-Puka et al., 1999; Putman et al., 1999; Roy et al., 1999).

It is generally accepted that the electrical impulses a muscle receives play a role in modulating the quantities of metabolic proteins (glycolytic and oxidative enzymes) and types of contractile proteins (MHC) that are expressed. In turn, these two protein systems regulate force generation, velocity of force development, muscle fatigue and metabolic substrate utilization. However, the interrelationships between the metabolic enzyme profile and the MHC characteristics of a fiber that is transitioning from one phenotype to another are unknown.

To determine how the interrelationships between metabolic enzyme profile and MHC are affected during myofiber transformation, the model of spinal cord transection (ST) were used. Specifically, ST results in reduction in neuromuscular electrical activity and mechanical loading (Roy et al., 1999). Electromyography recordings have reported marked reductions in muscle activation following ST (Alaimo et al., 1980). Chronic spinal transection has been shown to affect the metabolic and contractile properties of cat soleus muscle (Baldwin et al., 1984; Hoffman et al., 1990; Jiang et al., 1990). However, the interrelationships among these properties have not been assessed in single fibers during the stages of myofiber transformation.

Metabolic enzyme distribution in specific fiber types

In muscle fibers, the mitochondria are located throughout the fiber, alongside the myofibrils and in the subsarcolemmal region. Succinate dehydrogenase (SDH, EC1.3.99.1), a flavin-linked enzyme, is a metabolic enzyme associated with both the Krebs cycle and the electron transport chain. It is positioned at the inner mitochondrial membrane. For these reasons, quantitative analysis of SDH protein expression has been suggested as a marker for oxidative potential (Martin et al., 1985; Blanco et al., 1988). Skeletal muscle fiber types have different levels of SDH enzymatic activity. Rivero (1998) has suggested that the greatest average levels of succinate dehydrogenase activity (.501 OD min^{-1}) occur in MHC type IIa fibers followed in rank order by MHC types IIx, I and IIb. Previous research supports the interpretation that type IIa fibers have a higher oxidative potential than that of type I fibers (Baldwin et al., 1972; Blanco et al., 1982, Hauschka et al., 1988). Categorizing fibers via qualitative myofibrillar ATPase under alkaline conditions, Martin et al. (1988) has suggested a tendency for type I fibers to have a greater oxidative potential than fast type II fibers. However, the inherent limitations to this alkaline pre-incubation procedure did not allow for classification of fast MHC isoforms (IIa, IIx and IIb), nor did it allow for identification of hybrid fiber types. Following a more comprehensive alkaline incubation protocol, Sieck et al. (1995) has suggested that type I and IIa fibers had the highest SDH activity followed in rank order by type IIx and IIb. In general, Rivero et al. (1988) has shown that hybrid fibers will express intermediate values according to respective pure MHC isoform percentages.

Cytosolic (EC1.1.1.8) and mitochondrial (EC1.1.99.5) α-glycerophosphate dehydrogenase are components of the glycerol phosphate shuttle. This shuttle is

responsible for transporting NADH, produced by glycolysis, from the cytosol into the mitochondria. In this way, free cytosolic NAD^+ is replenished; allowing glycolysis to continue and ATP is generated from the NADH within the mitochondria. Studies have shown that the activity of this shuttle is critical in maintaining maximal glycolytic flux (MacDonald et al., 1981; Giroix et al., 1991). Thus, GPD activity can be used as a marker for glycolysis. Supporting this idea, Peter et al. (1972), demonstrated that GPD activity was highly correlated with other enzymes of glycolysis in mammalian skeletal muscle. However, at present it is not known if the histochemical assay for GPD measures the activities of the cytosolic, mitochondrial, or both enzymes. For this reason, quantitative analysis of GPD protein expression has been suggested as a marker of glycolytic potential (Lowry et al., 1972; Martin et al., 1985). Skeletal muscle fiber types have different levels of GPD protein expression. Rivero et al. (1999) have reported the highest GPD activities in type IIb fibers (.170 OD min^{-1}) followed by type IIx (.150 OD min^{-1}), IIa (.101 OD min^{-1}) and type I (.071 OD min^{-1}). Similarly, several other studies have reported that the highest GPD activities occurred in type IIB fibers followed in rank order by IIX, IIA and I isoforms (Hoffman et al., 1990; Jiang et al., 1990). Research has shown that hybrid fiber types have intermediate activities between the respective pure fiber types, except IIX/B fibers, which showed a higher GPD activity than pure IIB fibers (.183 OD min^{-1} vs. .170 OD min^{-1}, respectively) (Rivero et al., 1999).

In summary, studies have concluded that glycolytic enzyme activities are higher in fast muscles than in slow muscles, while the opposite is the case for oxidative enzymes (Peter et al., 1972). Microanalysis at the single fiber level has reflected this scheme (Wank et al., 1994; Rivero et al., 1998). Immunohistochemical analysis of fiber type

composition coupled with histochemical analysis of metabolic enzyme activity has suggested that the oxidative capacity is greatest in MHC type IIa and type I fibers followed by MHC type IIx and IIb fibers. Furthermore, research has shown that glycolytic capacity is greatest in MHC type IIb fibers followed in rank order by IIx, IIa and I fiber types. Metabolic profile analysis of hybrid fiber types has suggested that enzyme activities have intermediate values. These reported values are averages of the respective pure fiber type composition.

Effects of decreased activity on fiber types

Myosin heavy chain protein expression is, in part, regulated by the quantity of neuromuscular activation. Skeletal muscle responds by altering the phenotypic expression of MHC proteins to adjust to the new metabolic and functional demands required by the muscle. Accordingly, several studies have manipulated or removed neuromuscular activation to better analyze the resultant effects on MHC protein expression. To accomplish this, a variety of models including hindlimb suspension, space flight, spinal cord isolation and spinal cord transection have been employed to minimize neuromuscular activities. (Roy et al., 1991).

Hindlimb suspension results in the elimination of weight-support activity without surgical intervention compromising the nervous system. It is well accepted that hindlimb suspension is accompanied by a progressive decrease in the percentage of slow type I fibers in the rat soleus. In addition, there is a general shift toward a faster MHC composition (Roy et al., 1987; Takahashi et al., 1991; Oishi, 1993; Talmadge et al., 1996; Caiozzo et al., 1997). Bigard et al. (1997) has reported an increase in the percentage of fibers staining positively for MHC type IIa and *de novo* expression of MHC types IIx and IIb after 21 days of hindlimb suspension. Research has supported this interpretation that hindlimb suspension down-regulated the slow type I MHC isoform content of the soleus muscle while simultaneously increased the fast type IIa/IIx MHC isoform content (Ohira et al., 1992; Caiozzo et al., 1998).

Space flight has been suggested as a model of disuse because skeletal muscles are chronically unloaded due to the elimination of gravitational effects (see Edgerton et al., 1991 for review). Several researchers have reported increases in fast MHC isoforms in

rat soleus muscles following 7-12 days of spaceflight (Martin et al., 1988; Miu et al., 1990; Ohira et al., 1992; Caiozzo et al., 1994, 1996; Talmadge et al., 1996). For example, Miu (1990) reported that approximately 40% of the fibers in the soleus of flight rats expressed a fast myosin heavy chain compared with 22% in control rats. Likewise, 31% of the fibers in the soleus of flight rats expressed both fast and slow myosin heavy chains compared with 8% in control rats. Increased percentages of hybrid fibers types has also been supported by Baldwin et al. (1990), Martin et al. (1988) and Talmadge et al. (1996). However, Ohira et al. (1992) has suggested that the relative percentage of pure type I MHC does not decline after 14 days. Ohira has reported an increase in hybrid fiber types containing both slow and fast isoforms, however, this increase in co-expression was not at the expense of slow myosin heavy chain as previously suggested. Likewise, Riley et al. (1990) has reported no change in fiber type composition in soleus fibers of rats subjected to 12.5 days of microgravity during space flight. However, Riley did report a decrease in type I MHC in rat adductor longus single fibers.

Spinal cord isolation (SI) involves the isolation of the lumbar spinal cord via a rostral and a caudal cord transection and a bilateral dorsal rhizotomy between the two transection sites (Tower, 1937). This procedure eliminates all supraspinal, infraspinal and peripheral input to the motoneurons isolated in the lumbar portion of the spinal cord. Several researchers have suggested that SI results in a shift of the MHC isoform pattern in the soleus to resemble that of a fast muscle (Eldridge et al., 1984; Baldwin et al, 1989; Pierotti et al., 1989). Graham et al. (1992) reported a 45% increase in soleus fibers staining positive for fast MHC isoforms following SI. Talmadge et al. (1996) reported that SI resulted in decreased type I and increased type IIa MHC, as well as *de novo*

expression of IIb MHC compared to control levels. After SI, 48% of the fibers were type I, 11% were type IIa, 1% were type IIb and 25% contained hybrid fiber types.

Spinal cord transection (ST) reduces supraspinal input to the lumbar region of the spinal cord. ST causes adaptations to skeletal muscle properties that are similar in direction but less in magnitude compared to spinal cord isolation. Talmadge (1996) suggested that SI resulted in a greater reduction (~ 50% of total population) in the proportion of type I MHC fibers compared to ST (~ 35% of total population). Almost all studies show a decrease in the percentage of slow, type I fibers and an increase in the percentage of fast fibers following ST (Baldwin et al., 1984; Lieber et al., 1986; Hoffman et al., 1990; Jiang et al., 1990; Talmadge et al., 1995, 1999). Talmadge (1995, 1999) reported that soleus muscle single fibers express fast MHC isoforms as early as 15 days post-ST. In accordance, Jiang et al. (1990) reported a 45% increase of soleus fibers containing fast isoforms compared to controls. Furthermore, Talmadge has reported (1999) that nearly 50% of all fibers maintained co-expression of slow and fast MHC up to one year post-ST. Maintenance of hybrid fiber types has previously been reported up to 6 months (Hoffman et al., 1990; Jiang et al., 1990) following ST. Furthermore, Castro et al. (1999, 2000) reported an increase in type IIx MHC at the expense of type IIa in vastus lateralis fibers from spinal cord injured (SCI) individuals 24 weeks after injury.

These data support the idea that alterations in neuromuscular activity cause adaptations to MHC protein expression. By minimizing or removing neuromuscular activation via surgical procedures (ST and SI, respectively), the predominantly slow soleus transitions toward characteristics of a faster muscle. These adaptations are similar to those that occur during unloaded conditions (hindlimb suspension and spaceflight).

Overall, there is a down-regulation of slow myosin and an up-regulation of fast myosin to support the functional demands due to reduced neuromuscular activation and loading. Analysis at the single fiber level has suggested that phenotypic transitions to MHC protein expression can follow the step-wise continuum I _ I/IIa _ IIa _ IIa/IIx _ IIx _ IIx/IIb _ IIb (Pette et al., 1990; Talmadge et al., 1999) as they acquire a faster phenotype, however, this step-wise continuum is not required (Talmadge et al., 1999). Talmadge (1995, 1999) and Grossman (1998) have suggested that soleus fibers transitioning from a type I phenotype toward a faster phenotype may skip the expression of an intermediate isoform (e.g., MHC IIa) and follow the scheme: I _ I/IIx _ IIx. Thus, normal levels of neuromuscular activity appear to be necessary for maintenance of normal adult MHC profile.

Effects of decreased activity on metabolic enzymes

Metabolic protein expression is, in part, regulated by the quantity of neuromuscular activation. Skeletal muscle responds by altering the phenotypic expression of metabolic proteins to adjust to the new energy demands required by the muscle. Several studies have manipulated or removed neuromuscular activation to better analyze the resultant effects on the metabolic profile of skeletal muscle. To accomplish this, a variety of models including hindlimb suspension, space flight, spinal cord isolation and spinal cord transection have been employed to minimize neuromuscular activities. (for review, see Roy et al., 1991).

Several researchers have suggested that the oxidative capacity of soleus fibers is maintained or elevated following hindlimb suspension (Hauschka et al., 1985; Fitts et al., 1989; Graham et al., 1989; Ohira et al., 1992). For example, Ohira et al. (1992) has reported no significant difference in SDH activity of rat soleus muscle following 14 days of hindlimb suspension compared to controls (34.0 – 3.8 vs. 33.8 – 4.8 OD/min x 10^{-3}, respectively). This suggested that fibers were not experiencing an appreciable loss of oxidative potential. However, integrated SDH activity (SDH activity x CSA), a measure of total oxidative capacity, decreased significantly in slow, type I MHC fibers. This decline is due in large part to fiber atrophy. Likewise, independent research performed by Graham (1989) and Hauschka (1987) has corroborated the notion that integrated SDH activity declines following 7 or 28 days of hindlimb suspension, respectively. GPD activity, a marker of glycolytic metabolism, has been shown to nearly double in slow fibers in response to 28 days of hindlimb suspension (Hauschka et al., 1989). However, Ohira et al. (1992) showed no appreciable difference in GPD activity following 14 days

of hindlimb suspension. This discrepancy may be because more time was necessary for metabolic enzymes to adjust to changes in environment.

Very little research exists on long duration space flights (> 14 days) and the effect on metabolic enzymes. Data acquired after 12.5 days of flight has suggested that mean SDH activity is unchanged compared to control rats (Miu et al., 1990). However, integrated SDH activity was decreased in soleus single fibers. In contrast, independent research performed by Manchester (1990) and Desplanches (1990) reported no alteration to oxidative capacity following 7 days of space flight. Spaceflight also results in an increase in glycolytic potential. Martin et al. (1988) suggested that mean GPD activity was approximately twice that observed in ground-based controls in soleus muscle fibers following 12.5 days of spaceflight. Several other studies have corroborated the notion that GPD activity increases following 7-12.5 days of spaceflight (Miu et al., 1990; Manchester et al.; 1990; Edgerton et al., 1995), suggesting a general increase in the glycolytic potential of the soleus muscle (Roy et al., 1991).

Graham et al. (1992) showed that mean SDH concentrations were unaffected 6 months after spinal cord isolation, however, integrated SDH activity was decreased by 55% in slow soleus fibers and by 40% in fast soleus fibers. Research using the SI model has supported this interpretation (Roy et al., 1987; Jiang et al., 1991). Using a tetrodotoxin-induced muscle paralysis that produces similar effects as spinal isolation, Michel et al. (1994) reported a reduction in oxidative potential and an increase in glycolytic potential among fast fibers. Spinal isolation also results in an increase in glycolytic potential. Research has suggested that integrated GPD activity increases in response to spinal isolation (Graham et al., 1992; Roy 1987; Jiang 1991). In general, the

fibers of soleus muscles following SI are characterized by reduced cross sectional areas, reduced integrated SDH activities and elevated integrated GPD activities.

Based on quantitative histochemical assays, SDH activities of kitten soleus fibers have been shown to be elevated following ST (Roy et al., 1984; Baldwin et al., 1984). However, data from adult cats has suggested that SDH activity in soleus fibers is maintained at levels equivalent to controls (Roy et al., 1986; Hoffman et al., 1990). Interestingly, Jiang et al. (1990) has suggested that medial gastrocnemius muscle fibers have reduced SDH activity in both slow and fast fibers following ST. Castro et al. (1999, 2000) has demonstrated that SDH activities were increasing in vastus lateralis muscle fibers from SCI individuals from 6 to 24 weeks after injury. These data suggest that adaptations of oxidative potential may be muscle specific.

GPD activities have been shown to increase following spinal transection (Baldwin et al., 1984; Jiang et al., 1990; Hoffman et al., 1990). Castro et al. (1999, 2000) has reported increasing GPD activities in vastus lateralis muscle fibers from SCI patients from 6 to 24 weeks after injury. Hoffman et al. (1990) has suggested that the GPD/SDH ratio, an index of metabolic substrate utilization, was 48% higher in the soleus muscles of spinal transected cats. These results suggested a shift toward a greater dependence on glycolytic metabolism and a decreased reliance on oxidative metabolism. However, more extreme adaptations in GPD activity are seen in slow type fibers compared to fast type fibers. Jiang et al (1990) has reported a 100% increase in GPD activity in slow soleus fibers of ST cats, but no increase in GPD activity of fast fibers. These data suggest that adaptations in metabolic characteristics may occur without adaptations in contractile protein isoforms.

In order to support the new functional and metabolic demands required by soleus muscle fibers under conditions of decreased activity, metabolic enzyme protein content transitions toward a faster muscle phenotype (i.e., increased glycolytic capacity). Most studies have suggested a coordination in the activity of GPD and the type of myosin. However, mean SDH activity appears to be resistant to decreased levels of activity and unloading. This suggests that soleus fibers are not experiencing appreciable losses in oxidative potential. However, integrated SDH activity has consistently been shown to decrease mainly due to fiber atrophy. Thus, there seems to be a minimum level of oxidative potential in the soleus that is independent of activity level.

Most of the studies reviewed analyzed only a single time point following the model of unloading. Therefore, the actual phenomenon and true complexities of the transformations to the metabolic and contractile proteins might have been overlooked.

Literature Review Conclusion

Fully differentiated muscle fibers can undergo considerable phenotypic changes in order to adjust to changing conditions of the environment. Research has shown that decreased neuromuscular activation results in a transition from slow to fast characteristics. For example, the soleus expresses increasing percentages of fast type IIa and IIx MHC at the expense of slow type I. In addition, metabolic proteins transition to support the new energy demands required by the muscle, such that glycolytic activity ,as measured via GPD enzymatic activity, increases and SDH activity decreases. However, whether or not a strong interdependence of energy metabolism and MHC isoform content persists following ST is unclear.

Specific Aims and Hypothesis

1. **To identify the oxidative and glycolytic properties of rat soleus fibers after short and long term reductions in neuromuscular activity.**

2. **To identify the interrelationships among metabolic enzymes, contractile protein isoforms and fiber size of rat soleus fibers at multiple time points after ST.**

Specifically, it is hypothesized that (1) slow fibers will transition toward characteristics of a faster fiber type; (2) fibers initially with fast characteristics will maintain those characteristics or continue adapting toward an even faster phenotype; (3) intrafiber SDH to GPD ratio will decrease as fibers adapt toward faster characteristics; (4) an interdependence between MHC expression and GPD activity will be demonstrated, and (5) CSA will decrease independently of MHC, SDH or GPD adaptations following ST.

The goal of the proposed research will be to identify and quantify relative MHC isoform expression and metabolic enzyme profile adaptations in soleus fibers of rats following ST. It is hypothesized that metabolic and contractile properties of soleus fibers need to be coordinately regulated during myofiber transformations resulting from reduced neuromuscular activation, in order to support the functional demands placed on the muscle. Therefore, the proposed research will attempt to address the following question. How are the transformations in expression of metabolic and contractile proteins coordinated during adaptation of a single fiber from one phenotype to another? There is limited information about how these different protein systems co-adapt following spinal

cord injury at the single fiber level. Although studies on whole muscles yield insights into muscle-specific properties, the wide range of fiber type differences may be masked. Therefore, only single fiber analysis can provide an appreciation of the true complexity of skeletal muscle (Caiozzo et al., 1998; Pette et al., 1999).

Chapter 3

Research Design and Methods

Research Design and Methods

Experimental animals.

The rat soleus muscle is an ankle extensor located in the posterior compartment of the lower leg. Approximately 90% of fibers in soleus muscle are slow type I, and 10% are fast oxidative-glycolytic type IIa (Miu et al., 1990; Bigard et al., 1997). Slow twitch fibers have been shown to be most affected by spinal transection (Baldwin et al., 1984; Hoffmann et al., 1990; Jiang et al., 1990).

Soleus muscles from control and ST female Sprague-Dawley rats have been obtained. Details of surgery and sample preparation have been previously published (see Talmadge et al. 1999). Briefly, 150-g female rats were anesthetized with a mixture of zylazine (10 mg/kg body mass) and ketamine (75 mg/kg body mass). A dorsal midline incision was made and the muscles overlying the spinal column at T_6-T_{10} were separated from the spinal column. A laminectomy was performed at T_7-T_8. Two drops of Lidocaine (2%) were applied to the exposed spinal cord and the spinal cord was completely transected with micro-dissection scissors. A probe was inserted to verify the completeness of the transection. The overlying muscle and skin were sutured and animals received daily injections of Baytril (5 mg/kg body mass; IM) for the first two weeks post-surgery to prevent infection. Analgesics delivered orally by water bottle were supplied to all animals (acetaminophen, 300 mg/kg body mass). After the experimental period expired, rats were killed with a lethal dose of pentobarbital sodium (100 mg/kg body mass; IP). The soleus was excised, weighed, frozen in melting isopentane and stored at -80°C in preparation for quantitative analysis.

Experimental design.

Rats did not significantly differ in size (~150 g body weight) or age at the beginning of the study. Rats were assigned to six groups (n=5/group): (1) 30 day control; (2) 30 day post-ST; (3) 90 day control; (4) 90 day post-ST; (5) 180 day control; and (6) 180 day post-ST. Control animals were age-matched.

Quantitative histochemistry.

For each metabolic enzyme assay (SDH and GPD), five successive serial sections were cut on a cryostat (Microm HM505 N) at -20°C and adhered to coverslips. Three of the sections were incubated in a medium containing the substrate; two sections were incubated in medium without substrate to serve as reaction blanks (Figure 1). In accordance with Blanco et al. (1988), staining for the oxidative marker SDH was performed on 10μm thick sections and incubated in 100 mM potassium phosphate buffer medium (containing in mM: 1.0 mPMS, .75 NaN_3, 1.5 NBT, 5 EDTA, 110 Succinic Acid; pH 7.6) for 8 minutes. Staining for the glycolytic marker GPD was performed on 14μm thick sections and incubated in 100 mM potassium phosphate buffer medium (containing in mM: 0.02 mPMS, 1 NaN_3, 1.2 NBT, 6.3 GPD; pH 7.4) for 11 minutes according to Martin et al. (1985). All sections were digitized within 2-3 hours following initial reactions (Nikon Eclipse E400 microscope with COHU high performance CCD camera assembly). Randomly selected fibers were circumscribed and a CSA measurement was recorded. The intensity of staining of individual fibers was assessed using visual imaging software (Scion Image v1.62). For each fiber the average optical density (OD) determined from the sections incubated without substrate was subtracted

from the OD obtained from sections incubated with substrate. This density measurement reflects specific enzymatic activity. Integrated measurements (ISDH and IGPD) reflect total enzymatic capacity or power and are the products of specific enzymatic activity (SDH and GPD) and cross-sectional area.

Immunohistochemistry.

Transverse serial sections of 10μm were cut at -20°C and adhered to gelatin-coated slides. Sections were reacted with monoclonal antibodies (mAb) specific for rat MHC isoforms (Table 1). Avidin-Biotin peroxidase Complex (ABC) procedure was performed to link the peroxidase enzyme to the epitope recognized by the mAb. Briefly, samples were preincubated in a blocking solution for one hour. Excess blocking solution was removed and sections were incubated in monoclonal primary antibodies for two hours at room temperature or overnight at 4°C. Then, sections were washed with phosphate buffered saline (PBS) before a one-hour incubation in a biotinylated secondary antibody. Sections were then washed again prior to a one-hour incubation in ABC reagent. Finally, diaminobenzidine tetrahydrochloride (DAB) was used as a chromogen to visualize peroxidase enzymatic activity for all primary antibodies (Rivero et al. 1998). Comparing across the staining intensities from the seven primary antibodies and neighboring fibers allowed for final considerations of MHC fiber type (Figure 1). Between 30-40 single fibers per tissue section were analyzed for MHC isoform distribution.

Quantitative tissue analysis/Microphotometry.

A region free from artifact and containing a mixed population of MHC fiber types was selected from the sections stained for quantitative SDH, GPD and immunohistochemistry. For each fiber analyzed, a mean optical density (OD min^{-1}) was assessed and compared relative to background gray levels. Cross-sectional area (CSA μm^2) was tabulated simultaneously with enzyme activity analysis. Enzyme activity and CSA was calculated as the mean OD of the three tissue sections incubated in substrate minus the mean OD of the two NS sections. The same 30-40 single fibers per tissue section were analyzed for quantitative SDH, GPD and immunohistochemical analysis.

Fiber typing.

The fibers analyzed were classified according to their relative MHC content based on the immunohistochemical staining intensities. Fibers were classified according to MHC content by analyzing staining intensities from seven primary antibodies and neighboring fibers (see Table 1 in appendix).

Statistics.

All data are presented as means ± standard error. Data was stored as an Excel file and transferred to Sigma Stat 2.03 for data and graphical analysis. A two-way analysis of variance was employed for overall comparisons. Normal comparisons between groups were analyzed using Dunnett s (Dunn.) post-hoc tests. Non-normal comparisons were analyzed with Kruskal Wallis (KW) post-hoc tests. Significance was accepted at $p<0.05$.

Limitations.

Research was performed using only a rat model. Time points to analyze SDH and GPD activity, MHC isoform expression and CSA were fixed at 0 days (control), 30 days, 90 days and 180 days following spinal cord transection.

Basic Assumptions.

At the start of the investigation, it was assumed that rats were disease and pathogen free. It was also assumed that underlying factors within the muscle would not affect normal function. Furthermore, any alterations within the measured muscle parameters were a direct result of the reduced neuromuscular activation as accomplished via spinal cord transection.

Chapter 4

Results

Results

Distribution of slow and fast-twitch fibers.

The MHC content of individual fibers was assessed using a battery of primary antibodies specific to MHC isoforms (Table 1). Tissue sections serial to those used for quantitative histochemistry were used for the evaluation of MHC isoform composition. For all measures, the control group at all three time points (1, 3 and 6 months) have been collapsed into one group for graphical representation. No significant differences were observed among the three control groups.

In the control group (regardless of timepoint), ~95 and 2% of the soleus muscle fibers reacted with only slow type I MHC or fast type IIa MHC, respectively (Figure 2). Three percent of the fibers reacted with both the slow and fast antibodies.

One month after ST, however, fibers staining positively for MHC type I decreased significantly by 48% (Figure 2). Furthermore, fibers expressing both slow and fast MHC isoforms significantly increased compared to controls (13% vs. 1% of total population). These data suggest that MHC isoforms transition from slow to fast within 1 month following ST in the rat. Slow to fast transitions have been reported to occur as early as 15 days in the rat ST model (Talmadge et al., 1995b, 1999).

Three months after ST, fibers reacting with the slow type I MHC antibody significantly decreased to ~8% of the population (Figure 2). In addition, the percentages of fibers showing a positive reaction with both slow and fast antibodies and the antibodies designating MHC type IIx were significantly greater in ST rats compared to controls.

No difference was evident when comparing percentages of slow MHC expression between 3 and 6 months post-ST. No fibers stained for both slow and fast MHC 6 months after ST (Figure 2). However, fibers reacting positively for MHC type IIx and hybrid type IIa+IIx were significantly greater than controls, as well as, 1 and 3 months post-ST (Figure 2). These data indicate that adaptations were occurring in fibers containing slow and fast MHC (i.e., types I+IIa, I+IIa+IIx and I+IIx), because the percentage of fibers reacting with both the slow and fast myosin antibody was reduced after 6 months.

Fiber cross-sectional areas.

Compared to controls, the soleus CSAs for all fibers decreased significantly by 53, 49 and 41% following 1, 3 and 6 months ST, respectively (Figure 3). No significant difference in fiber atrophy was apparent between 1 and 3 months, however 6 months after ST; fibers had significantly greater CSAs than 1 month after ST (Figure 3). These data indicate that 6 months of normal body growth will curtail the atrophic response due to reduced neuromuscular activity. These data indicated that the atrophic response due to reduced neuromuscular activation is immediate (within 1 month in rats), however, normal body growth of the animal over time minimizes the degree of fiber atrophy.

Fiber type specific atrophy.

Type I fibers showed a similar progression of atrophy compared to total fiber atrophy. A significant decrease was apparent at 1 and 3 months post-ST compared to controls (Table 2). Even though type I fibers at 6 months were significantly smaller than controls (2707.4μm^2 vs. 3491.5μm^2, respectively); this subpopulation of fibers was significantly greater than those at 1 and 3 months post-ST.

Fibers staining positively for both slow and fast MHC at 1 and 3 months post-ST were significantly smaller than in control rats. However, no difference in CSA was seen in type IIa fibers at any timepoint post-ST or when compared to controls. This may be due to the small percentage of fibers staining positively for MHC type IIa (2.0, 3.3, 5.1 and 13.6% in controls, 1, 3 and 6 months post-ST, respectively).

No fibers in the control group stained positively for MHC type IIx, therefore, single fiber comparisons were made within the ST group at all three time points. The mean fiber sizes of type IIx and hybrid type IIa+IIx in the 3 and 6 months groups were 45-77% larger than 1 month post-ST, however, no difference was evident between 3 and 6 months.

The increase in size of type IIx and the IIa+IIx fiber types reflects the fact that (1) no IIx or IIa+IIx fibers were present in controls and, (2) the muscles were apparently growing in proportion to body weight (Talmadge, 2000) between 1 and 6 months after ST.

Metabolic measures and interrelationships among enzyme activities.

All Fiber Types

Mean SDH activity of the fibers regardless of fiber type (i.e., all fiber types combined) was significantly elevated at all time points post-ST compared to controls. Likewise, total integrated SDH activity (ISDH), a measure of the total oxidative capacity of the muscle, was significantly greater than control values at all time points post-ST (Figure 4). Six months after ST fibers displayed significantly greater SDH and ISDH values compared to 1 and 3 months post-ST.

GPD activity of the fibers regardless of fiber type was significantly elevated at all time points post-ST compared to controls. GPD activity in all fibers peaked at one month (89% increase compared to controls) and continued to decline thereafter (75% significant reduction in GPD activity at 6 months compared to 1 month). Likewise, total GPD activity (IGPD), a measure of the total glycolytic capacity of the muscle, was significantly greater at all time points post-ST compared to controls (Figure 5). At 6 months, fibers exhibited a decrease in glycolytic capacity compared to 3 months.

The GPD/SDH ratio significantly increased above control values up to 3 months post-ST, however, dramatically fell at 6 months post-ST (Figure 6). In fact, the GPD/SDH ratio did not differ when comparing controls and 6 months post-ST (133–10 vs. 95–7, respectively, $P<0.05$).

Type I Fibers

SDH activity of type I fibers was significantly elevated at all time points post-ST compared to controls (Figure 7). However, at 1 and 3 months post-ST, ISDH activity did not differ from controls, suggesting a maintenance of oxidative capacity. Yet, 6 months after ST, ISDH activity rose sharply above those reported during 3 months (92.1 vs. 47.7 OD/min x 10^{-3}, respectively, $p < 0.05$).

GPD activity and total glycolytic capacity were significantly elevated at 1 and 3 months post-ST compared to controls. Curiously, at 6 months post-ST, glycolytic activity in type I fibers was negligible (Figure 8).

GPD/SDH was not significantly different between controls, 1 and 3 months post-ST (Figure 9). Due to the lack of GPD activity at 6 months post-ST, GPD/SDH was negated.

Slow and Fast Hybrid Fibers

No fibers in the 6 months post-ST group were classified as slow and fast hybrid fibers. Therefore, single fiber comparisons were made between controls and within the ST groups at 1 and 3 months.

Fibers reacting positively for both slow and fast MHC in the 1 and 3 month groups showed significantly greater levels of SDH activity compared to controls (Figure 10). However, ISDH activity at these time points did not differ from control values suggesting a maintenance of total oxidative power in this subpopulation of fiber types.

GPD activity was significantly greater in the 1 and 3 months groups compared to controls. Furthermore, IGPD activity was also significantly elevated at these time points suggesting an increase in glycolytic capacity in the slow/fast hybrid subpopulation (Figure 11).

Compared to controls, GPD/SDH was significantly higher at 1 and 3 months post-ST (Figure 12). This suggests an increased reliance on glycolytic metabolism in this subpopulation of fiber types.

Type IIa Fibers

SDH and ISDH values were significantly greater at all timepoints post-ST compared to controls (Figure 13).

GPD activity was significantly greater at 1 and 3 months post-ST compared to controls (Figure 14). At 6 months post-ST, however, GPD and IGPD measures were significantly lower than 1 and 3 months post-ST, and did not significantly differ from controls. What s more, GPD/SDH reflected this scheme of increased oxidative metabolism and reduced glycolytic metabolism after ST. GPD/SDH dramatically fell at 6 months post-ST compared to controls, 1 and 3 months (Figure 15). This suggested that type IIa fibers relied more on oxidative metabolism to meet their energy requirements.

Type IIa & IIx Hybrid Fibers

No significant differences were evident in SDH or ISDH values between controls or any time point post-ST (Figure 16). This suggested maintenance of SDH activity and oxidative capacity following transection in this subpopulation of MHC-based fiber types.

GPD and IGPD measures did not differ between controls and 1 month post-ST, however a significant elevation in glycolytic capacity above control values was apparent at 3 months post-ST (Figure 17). However, at 6 months post-ST, GPD and IGPD measures were significantly lower than 1 and 3 months post-ST. What s more, both GPD and IGPD returned to control levels at 6 months post-ST.

GPD/SDH reflected this shift in metabolic demand. The ratio was significantly lower at 6 months post-ST compared to 1 and 3 months (Figure 18). In fact, there was no significant difference in GPD/SDH between controls and 6 months post-ST.

Type IIx Fibers

No fibers in the control group were classified as MHC type IIx. Therefore, single fiber analysis was only made within the ST group at the three time points (1, 3 and 6 months).

SDH activity showed a slight but significant decrease at 3 months compared to 1 month (51.6 – 2.4 vs. 28.6 – 1.9 OD/min x 10^{-3}, respectively, $p < 0.05$). However, no difference was evident between 1 and 6 months (Figure 19). What's more, total oxidative capacity as measured via ISDH did not change across all time points. This suggests a maintenance of oxidative power in this subpopulation of MHC-based fiber types.

GPD and IGPD activities were significantly lower at 6 months compared to both 1 and 3 months (Figure 20). GPD/SDH reflected this decrease in glycolytic capacity at 6 months (Figure 21). Type IIx fibers at 6 months reported a significantly reduced ratio compared to 1 and 3 months. This suggested type IIx fibers began to rely more on oxidative metabolism for energy resources.

Chapter 5

Discussion

Discussion

In this study, fiber size, oxidative capacity, glycolytic capacity and MHC composition were analyzed in the same fibers from rat soleus muscle following spinal cord transection. Because spinal cord transection results in reduced muscle use, it is expected that skeletal muscle properties would change following transection (Alaimo et al., 1984; Roy et al., 1984). Therefore, it was hypothesized that (1) slow fibers would transition toward characteristics of a faster fiber type; (2) fibers initially with fast characteristics would maintain those characteristics or continue adapting toward an even faster phenotype; (3) CSA would decrease independently of MHC, SDH or GPD adaptations following ST; (4) intrafiber GPD to SDH ratio would increase as fibers adapt toward faster characteristics, and (5) an interdependence between MHC expression and GPD activity would be demonstrated.

Furthermore, it was hypothesized that metabolic and contractile properties of soleus fibers would be coordinately regulated during myofiber transformations resulting from reduced neuromuscular activation, in order to support the functional demands placed on the muscle. Therefore, the proposed research attempted to address the following question. How are the transformations in expression of metabolic and contractile proteins coordinated during adaptation of a single fiber from one phenotype to another?

Slow-to-Fast MHC transformations

The MHC molecule is an excellent indicator of biochemical alterations, since it is a primary molecule regulating contractile velocity and myosin ATPase activities. Perhaps the most common observation in skeletal muscle of ST animals is an alteration in MHC isoform composition. Almost all studies show decreases in the percentage of slow MHC-based fibers and concomitant increases in the percentage of fast MHC-based fibers (Baldwin et al., 1984; Lieber et al., 1986, Hoffman et al., 1990; Jiang et al., 1990; Talmadge et al., 1995b, 1996, 1999).

Furthermore, it has been well documented in the rat model that ST results in increases in the proportion of fast fibers measured histochemically or biochemically (Dupont-Versteegden et al., 1998; Talmadge et al., 1995b, 1999). The current study is in concert with previous research. The percentage of fibers staining positively for slow type I MHC steadily decreased from 1 to 6 months with concomitant increases in fibers staining positively for fast type II MHC (Figure 2).

The fact that a small percentage (5.8%) of fibers stained positively for MHC type I after 6 months of recovery suggests that there may be a distinct subpopulation of type I fibers that are resistant to change. It could be argued that additional time is necessary for full suppression of MHC type I, however, Talmadge et al. (1999) have reported that approximately 12% of rat soleus fibers contained MHC type I twelve months after spinal cord transection. A popular hypothesis in the neuromuscular literature is that the patterns of impulses that reach a muscle play a role in the quantity of myosin proteins expressed (Lomo et al., 1974; Eisenberg et al., 1984; Pette et al., 1985). Thus, the residual amount of electrical activation in the rat soleus after ST may be sufficient to maintain a

subpopulation of MHC type I fibers, or a population of fibers is absolutely resistant to transformation.

Fibers staining positively for both slow and fast MHC steadily increased up to 3 months (Figure 2). However, no fibers stained positively for slow and fast MHC after 6 months of recovery suggesting that fibers were still in a transitional state after 3 months. This notion is supported by the fact that MHC hybrid type IIa + IIx and type IIx were significantly greater at 6 months compared to 3 months post-ST. Talmadge et al. (1999) have shown that 12 months post-ST rat soleus fibers contained high proportions of MHC hybrid type IIa + IIx (~ 45%) and pure MHC type IIx (40%). Very little expression of MHC type IIb (< 3%) was evident suggesting that transitioning toward a faster phenotype was nearing completion.

Cross-sectional Area

Since the pioneering work of Tower (1937), it has generally been accepted that paralyzed muscles because of spinal cord injuries undergo atrophy and an inability to generate and sustain maximal force. The atrophic response following SCI has been well documented in a variety of muscles in cats (Roy et al., 1984, 1999; Jiang et al., 1990, 1991), rats (Dupont-Versteegden et al., 1998) and humans (Lotta et al., 1991; Scelsi et al., 1992; Martin et al., 1992; Gordon et al., 1994; Castro et al., 1999). Spinal cord transection removes body weight bearing and has been shown to be especially detrimental to slow postural muscle in the cat (West et al., 1986; Roy et al., 1986, 1991; Hoffman et al., 1990). Roy et al. (1991) have suggested that slow plantar flexors (soleus) atrophy more than fast plantar flexors (gastrocnemius) and dorsi flexors (tibialis anterior). In general, the predominant fiber type in any given muscle is usually the most affected (Lieber et al., 1986; Roy et al., 1986; West et al., 1986).

The rats used in this study were transected at a young age and weighed ~150 grams. Therefore, the fact that the average CSA of all fibers was greater at 6 months relative to 3 months is not surprising (Figure 3). Using a spinal isolation (SI) model, Grossman et al. (1997) showed that adult, SI rats (~240 g) lost body weight until the eighth day of recovery. Thereafter, body weight steadily increased at a rate comparable to control rats until final termination at two months. This suggests that decreased neuromuscular activation will not hamper the normal growth patterns of maturing rats.

Analysis of all fibers revealed significant atrophy at 1, 3 and 6 months post-ST compared to controls. However, after 6 months of recovery, fiber CSA was significantly larger than 3 months. Likewise, type I fibers were significantly larger at 6 months

compared to 1 and 3 months post-ST. Although the percentage of fibers staining positively for MHC type I steadily decreased with time, West et al. (1986) have suggested that CSA is not associated with a reduction of type I fibers in ST cats. Fibers comprised entirely of fast MHC isoforms (IIa, IIa+IIx and IIx) showed greatest atrophy after one month; however, fiber size did not differ from controls at any time point thereafter. This might suggest that normal body growth of the animal will curtail the atrophic response in fast fibers following spinal cord transection. It is possible that the shift in MHC isoform composition from slow to fast acts as a secondary mechanism to spare muscle mass. Thus, the muscle will remain in a condition to respond favorably to a future acquisition of normal activity.

Interdependence among Oxidative and Glycolytic Capacities and MHC isoforms

Based on quantitative histochemical assays, research has shown SDH activity to be either maintained or increased following SCI in kittens (Roy et al., 1984; Baldwin et al., 1984), cats (Roy et al., 1986; Hoffman et al., 1990), rats (Lieber et al., 1986; Midrio et al., 1988) and humans (Castro et al., 1999, 2000). Likewise, GPD activities have been shown to increase following ST (Baldwin et al., 1984; Lieber et al., 1986; Midrio et al., 1988; Jiang et al., 1990; Hoffman et al., 1990). The current study reflects a general trend in all fiber types such that oxidative capacity is maintained as glycolytic capacity is elevated up to three months post-ST. However, at 6 months, oxidative capacity significantly rises as glycolytic capacity decreases. By factoring in the muscle growth (i.e., OD/min * CSA), one possible explanation for the increase in ISDH and decrease in IGPD seen at 6 months could be the increased size of the fibers relative to 3 months post-ST. Had the animals not been growing and CSA been maintained, a preservation of oxidative capacity might have been seen, as previously suggested (Baldwin et al., 1984; Lieber et al., 1986; Midrio et al., 1988; Jiang et al., 1990; Hoffman et al., 1990; Castro et al., 1999, 2000). Furthermore, CSAs of fibers with fast MHC isoforms at 6 months did not significantly differ compared to 3 months. In addition, the oxidative capacity was maintained over these periods. This supports the interpretation that normal muscle growth heightened the total oxidative capacity of type I fibers and of all fibers on average at 6 months. This elevated oxidative capacity is reflected in the GPD/SDH ratios at 6 months post-ST. In all fiber types, GPD/SDH, an index of metabolic substrate utilization, is consistently and significantly decreased. This suggests a shift towards a greater dependence on oxidative metabolism and a decreased reliance on glycolytic

metabolism. In contrast, Hoffman (1990) has suggested that 6 months after SI, the SDH/GPD ratio was 48% lower in kittens transected at two-weeks of age. This data suggested an increased reliance on glycolytic metabolism. However, the current data suggests that this reliance on glycolytic metabolism following chronic reductions in neuromuscular activation is transient. With age (3 months to 6 months), a shift occurred such that a greater dependence for oxidative metabolism was apparent (Figure 6). GPD/SDH ratios did not differ between controls and 6 months post-ST. Therefore, this might suggest a dissociation from energy substrate metabolism and MHC isoform composition over time. It could be argued that the decrease in the GPD/SDH ratio was due to the consistent decrease in glycolytic capacity of all MHC isoforms at 6 months, however, GPD measures analyzed at 12 months post-ST were comparable to 6 month values (data not shown). Even though MHC isoforms transitioned toward a faster composition, fibers did not rely on glycolytic metabolism for ATP production as previously suggested (Baldwin et al., 1984; Hoffman et al., 1990). These data suggest that metabolic and contractile properties of soleus fibers do not require coordinated regulation during myofiber transformations resulting from reduced neuromuscular activation.

It was previously suggested that the residual amount of electrical activation in the rat soleus after ST may be sufficient to maintain a subpopulation of MHC type I fibers, or a population of fibers is absolutely resistant to transformation. This notion is again supported by analyzing the glycolytic capacities of slow type I fibers at 3 and 6 months post-ST. The elevated GPD activities at 3 months post-ST might result from the tendency of these fibers to acquire fast fiber (glycolytic) characteristics. The negligible

GPD activities at 6 months post-ST might represent the fact that most fibers have transitioned to a fast phenotype and the residual slow fibers may rely totally on oxidative metabolism. Thus, these non-transformed slow fibers may represent a subtype of slow fibers with very low glycolytic capacity.

It is also interesting to note that each fiber type showed distinctly different trends in enzymatic activity during the ST time periods. The only consistency between all fiber type groups was a decrease in the GPD/SDH ratio at 6 months, suggesting a general dependence on oxidative metabolism regardless of MHC isoform composition.

The population distribution of single-fiber GPD activities is consistent with a generalized increase across all fibers up to 3 months (Table 2). However, it is improbable that all fibers had similar increases because MHC composition is associated with the glycolytic potential of that fiber (Jiang et al., 1990; Rivero et al., 1999). GPD activity after three months of recovery from ST increased by 340 and 618% in pure slow and hybrid fast and slow MHC isoforms compared to controls, respectively. To the contrary, GPD activity in pure type IIa and hybrid type IIa and IIx increased by 178 and 470%, respectively. MHC type IIx showed no increase in GPD activity. Accordingly, GPD enzyme activity in those fibers that formerly expressed pure slow or hybrid fast and slow MHC isoforms increased relatively more than that in the fibers that initially expressed fast MHC isoforms (average combined increases of 479% compared to 216%, respectively).

It appears as though glycolytic (GPD) and oxidative (SDH) activities are becoming dis-coordinately regulated following ST. Such that, GPD measures temporarily rise as fibers transition toward a faster phenotype and then return towards

control levels as fibers of a given type become phenotypically stable (i.e., no further MHC transitions are occurring). Thus, in the end, the GPD activities match the corresponding fiber type. On the other hand, SDH values appear to increase and remain elevated 6 months after ST. Thus, the SDH values remain high for the corresponding fiber type resulting in a lowered GPD/SDH ratio for a given fiber type.

In paralyzed musculature an increase in the proportion of fast MHC fibers at the expense of slow MHC fibers has been shown, however, this study has suggested that paralyzed musculature retains a high reliance on oxidative metabolism. Thus, the normal classification scheme becomes and remains disrupted for up to 6 months post-ST.

Functional Implications

Because spinal cord transection results in reduced muscle use, muscle atrophy and slow-to-fast transformations, it is expected that the functional characteristics would change following transection. Lieber et al. (1986a) have suggested that the velocity of shortening and rate of relaxation increased in the soleus as reflected by a decrease in time-to-peak tension and increase in fusion frequency. The decrease in time-to-peak tension suggested that alterations occurred in the Ca^{2+} handling properties of the sarcoplasmic reticulum. Studies have suggested that reductions to cytosolic Ca^{2+} could potentially alter force generation and maintenance in contracting skeletal muscle (Williams et al., 1995). The increase in fusion frequency suggested that the transected soleus had a faster rate of contraction and relaxation. This observation suggests a slow-to-fast fiber type conversion. Additionally, the CSA of the soleus decreased significantly while generating the same absolute tension. Thus, a large increase in soleus specific tension (force per unit area) was observed. The current data suggests that this increase in specific tension may be due to a slow-to-fast fiber type conversion secondary to ST. Recently, Geiger et al. (2000) have supported the notion that maximum specific force depends on MHC composition in rat diaphragm muscle fibers. Fast fibers expressing MHC type IIx produced greater specific force than fibers expressing MHC type IIa or type I isoforms. Therefore, the observed transition towards a faster phenotype may serve to off set the fiber atrophy occurring in the muscle. However, some single-fiber contractile studies do not support this notion that intrinsic force generating capabilities are dependent on MHC isoform composition (see Talmadge, 2000 for review).

The current study showed maintenance of oxidative and glycolytic enzyme activities suggesting that paralyzed musculature preserves the means to generate ATP. Therefore, the onset of fatigue associated with spinal cord transection is unlikely to be due to a negative energy balance (i.e., ATP production does not support ATP demand) (Castro et al., 2000). The onset of fatigue might be the result of alterations to Ca^{2+} handling (see Williams et al., 1995 for review). Therefore, sarcoplasmic/endoplasmic Ca^{2+} ATPase (SERCA) dysfunction or alterations to the Ca^{2+} release channels might be a possible cause for enhanced fatigability in paralyzed musculature. However, the mechanisms of elevated muscle fatigability following spinal cord injury remain unclear.

Conclusions

These data suggest that the oxidative capacities of soleus muscle fibers are not compromised after ST. In fact, as the fibers transitioned toward faster MHC isoforms, the GPD/SDH ratio was either maintained or decreased, suggesting an increased reliance on oxidative metabolism. The dis-coordination between MHC expression and SDH activity was maintained 6 month post-ST, however the dis-coordination of MHC expression and GPD activity was merely transient. Thus, these temporal dis-coordinations might imply dissociations between the contractile and metabolic characteristics of paralyzed soleus muscle fibers. However, these data are consistent with previous data and suggest that the increased fatigability observed after chronic reductions in neuromuscular activity are not due to compromised capacities for ATP synthesis.

Future Directions

Several proposed mechanisms could potentially regulate contractile protein expression including transcriptional regulation and activation of signaling pathways. Currently, research has suggested that the myogenic regulatory factor (MRF) pathway involving myogenin and MyoD might be regulating the slow-to-fast transitions in MHC isoform expression following reduced neuromuscular activation (Hughes et al., 1997; Dupont-Versteegden et al., 1998; Modziak et al., 1999; Wheeler et al., 1999)

MyoD and myogenin may function to turn-on the expression of muscle specific proteins and are preferentially located in fast and slow fibers, respectively (Hughes et al., 1999). Dupont-Versteegden et al. (1998) have shown that the myogenin:MyoD ratio in the soleus muscle of transected rats was decreased and the percentage of fast MHC isoforms increased, which supports the idea that a high myogenin:MyoD ratio favors MHC type I expression. Furthermore, Hughes et al. (1999) have suggested that myogenin induces a shift from glycolytic to oxidative substrate utilization in transgenic mice. In contrast, Dupont-Versteegden et al. (1999) have shown that the myogenin:MyoD ratio was significantly elevated in the predominantly fast extensor digitorum longus (EDL), but no significant elevations in MHC type I were reported. Furthermore, genetic models using mice deficient in MyoD expression demonstrate normal levels of fast fiber types in the EDL and peroneus brevis muscles (Hughes et al., 1997).

Recently, the calcium-activated, phosphatase calcineurin and the family of transcription factors known as NF-AT (nuclear factor of activated T-cells) have been implicated in the regulation of fiber type specificity (Chin et al., 1998; Delling et al.,

2000). Since Ca^{2+} is released into the cytosol from the sarcoplasmic reticulum during contraction, reduced levels of neuromuscular activation could potentially act as a signal for calcineurin:NF-AT pathways. Upon dephosphorylation, NF-AT isoforms are able to translocate into the myonucleus, bind to a conserved DNA binding site and modify transcription. It has been suggested that calcineurin activity is higher in slow muscle fibers due to inherently chronic elevations in Ca^{2+} concentration (100 to 300 nM), whereas fast fibers result in short bursts of elevated Ca^{2+} to ~ 1 μm (Westerblad et al., 1991; Chin et al., 1996, 1998). Furthermore, it has been shown that administration of cyclosporin A, an inhibitor of calcineurin, increases the proportion of fast fibers in the rat soleus (Chin et al., 1998) and blocks the fast-to-slow MHC transition in functionally overloaded mouse plantaris muscles (Dunn et al., 1999). Therefore, it is plausible that the calcineurin:NF-AT pathway plays a role in ST models in which chronic Ca^{2+} concentrations are minimized due to reduced activity (Talmadge, 2000).

Since only a few studies have directly quantified the influence of MRFs and calcineurin:NF-AT mechanisms on MHC isoform expression, very little is known about their actual involvement with the activity-based regulation of MHC isoform or metabolic enzyme expression. Therefore, further research regarding the influence of these specific pathways is warranted. It is likely that MHC isoform and metabolic protein expression involves the complex interaction of multiple control pathways (Talmadge 2000).

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Appendix

mAb	I	I/IIA	IIA	IIA/X	IIX	IIX/B	IIB
Slow	+	+	-	-	-	-	-
Fast	-	+	+	+	+	+	+
SC-71	-	+	+	+	-	-	-
BF-35	+	+	+	+	-	-	+
BF-G6	-	-	-	-	-	+	+
RT-D9	-	-	-	+	+	+	+
BF-F3	-	-	-	-	-	+	+

Table 1. Immunohistochemical staining of muscle fiber types according to MHC isoform content as determined by Schiaffino et al. (1989). +, Positive reaction for mAb and MHC. -, No reaction for mAb and MHC.

Table 2. Enzyme activities and size of soleus fibers in control and ST rats				
Fiber Type	**Control**	**One Month**	**Three Months**	**Six Months**
All Fibers				
n	458	155	154	155
CSA	3428.5 – 49.9	1578.7 – 43.1 (a)	1749.6 – 32.6 (a)	2018.6 – 44.5 (a,b,c)
SDH	14.9 – 0.4	36.2 – 1.2 (a)	33.8 – 0.9 (a)	45.6 – 1.6 (a,b,c)
ISDH	47.6 – 0.9	58.2 – 2.7 (a)	58.0 – 1.7 (a)	91.8 – 3.8 (a,b,c)
GPD	1.4 – 0.1	13.7 – 1.4 (a)	7.2 – 0.3 (a,b)	3.3 – 0.2 (a,b,c)
IGPD	4.1 – 0.3	7.7 – 0.5 (a)	12.9 – 0.6 (a,b)	6.6 – 0.3 (a,c)
GPD/SDH	133 – 10	394 – 41 (a)	224 – 10 (b)	95 – 7 (b,c)
% Slow Fibers	94.5	48.7 (a)	8.4 (a,b)	5.8 (a,b)
n	436	75	13	9
CSA	3491.5 – 49.9	1559.9 – 43.8 (a)	1516.5 – 128.9 (a)	2707.4 – 244.7 (a,b,c)
SDH	14.3 – 0.3	28.5 – 1.1 (a)	34.0 – 2.9 (a,b)	35.2 – 1.8 (a,b)
ISDH	47.3 – 0.9	44.4 – 2.4	47.7 – 2.2	92.1 – 4.1 (a,b,c)
GPD	1.4 – 0.1	4.0 – 0.2 (a)	4.8 – 0.8 (a)	0.4 – 0.4 (a,b,c)
IGPD	4.1 – 0.3	6.2 – 0.4	7.2 – 1.0 (a)	0.0 – 0.0 (a,b,c)
GPD/SDH	132 – 10	143 – 22	152 – 54	0.0 – 0.0 (a,b,c)
% S/F Fibers	3.0	13.1 (a)	23.4 (a,b)	0.0 (b,c)
n	9	62	109	0
CSA (Dunn s)	2421.9 – 204.4	1669.4 – 88.2 (a)	1696.2 – 34.6 (a)	-
SDH (Dunn s)	20.5 – 2.0	42.0 – 1.8 (a)	72.1 – 6.5 (a)	-
ISDH (Dunn s)	50.0 – 6.7	72.5 – 5.4	45.1 – 2.8 (b)	-
GPD (Dunn s)	1.1 – 0.4	6.2 – 0.3 (a)	6.8 – 0.3 (a)	-
IGPD (Dunn s)	2.3 – 0.8	9.9 – 0.7 (a)	11.4 – 0.7 (a)	-
GPD/SDH	67 – 27	158 – 9 (a)	166 – 13 (a)	-
% IIa Fibers	1.0	3.3	5.1	13.6
n	5	5	8	21
CSA	2471.9 – 635.9	1304.8 – 116.4	1809.9 – 166.7	1752.6 – 131.6
SDH	14.4 – 3.5	59.2 – 2.9 (a)	54.8 – 1.9 (a)	67.3 – 2.1 (a)
ISDH	27.0 – 4.9	76.9 – 7.2 (a)	98.5 – 9.3 (a)	114.3 – 6.6 (a)
GPD	3.8 – 0.4	10.8 – 0.8 (a)	6.8 – 0.8 (a,b)	3.0 – 0.3 (b,c)
IGPD	8.4 – 1.2	13.9 – 1.4	14.8 – 2.6	4.8 – 0.6 (b,c)
GPD/SDH	421 – 170	185 – 20	127 – 17	43 – 5 (a,b,c)
% IIa + IIx Fibers	0.5	3.3	3.2	29.7 (a,b,c)
n	8	5	5	46
CSA	1725.8 – 110.9	1080.1 – 175.1	1913.2 – 167.2 (b)	1908.1 – 72.2 (b)
SDH (KW)	42.3 – 6.3	34.8 – 9.1	46.8 – 2.4	51.1 – 2.9
ISDH (KW)	76.7 – 14.6	42.0 – 16.8	90.2 – 10.1	100.1 – 7.6
GPD (Dunn s)	3.0 – 0.4	9.4 – 0.7	14.1– 1.9 (a)	2.8 – 0.2 (b,c)
IGPD (Dunn s)	5.3 – 0.9	10.6 – 2.3	24.7 – 4.1 (a)	5.1 – 0.4 (b,c)
GPD/SDH	90 – 24	294 – 88	310 – 55 (a)	65 – 6 (b,c)
% IIx Fibers	0.0	5.3	12.3 (a)	51.0 (a,b,c)
n	0	8	19	79
CSA	-	1425.0 – 130.9	2150.7 – 82.9 (b)	2075.2 – 54.6 (b)
SDH (Dunn s)	-	51.6 – 2.4	28.6 – 1.9 (b)	37.7 – 2.0
ISDH (KW)	-	72.4 – 6.3	60.9 – 4.5	80.9 – 5.5
GPD (Dunn s)	-	11.9 – 1.1	9.3 – 0.7	4.0 – 0.1 (b,c)
IGPD (Dunn s)	-	15.2 – 1.4	21.1 – 1.8	8.4 – 0.3 (b,c)
GPD/SDH	-	234 – 25	340 – 27	135 – 10 (b,c)

Table 2 (continued). Values are means – SEM; n, number of fibers expressing specific MHC isoform. CSA, cross-sectional area, m^2; SDH, succinate dehydrogenase activity, optical density OD/min $x10^{-3}$; ISDH, integrated succinate dehydrogenase activity, SDH x CSA; GPD, α-glycerophosphate dehydrogenase activity, optical density OD/min $x10^{-3}$; IGPD, integrated α-glycerophosphate dehydrogenase activity, GPDxCSA; GPD/SDH, ratio reflecting metabolic substrate usage. a, Significantly different from control, $P < 0.05$. b, significantly different from 1 month ST, $P < 0.05$. c, significantly different from 3 months ST, $P < 0.05$. , Controls (1,3 and 6 months) have been collapsed into one group. , Fibers that express both slow and fast myosin heavy chain.