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Rapid diaphragm atrophy following cervical spinal cord hemisection

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Abstract

A cervical (C2) hemileision (C2Hx), which disrupts ipsilateral bulbospinal inputs to the phrenic nucleus, was used to study diaphragm plasticity after acute spinal cord injury. We hypothesized that C2Hx would result in rapid atrophy of the ipsilateral hemidiaphragm and increases in mRNA expression of proteolytic biomarkers. Diaphragm tissue was harvested from male Sprague-Dawley rats at 1 or 7 days following C2Hx. Histological analysis demonstrated a reduction in cross-sectional area (CSA) of type I and IIa fibers in the ipsilateral hemidiaphragm at 1 but not 7 days post-injury. Type IIb/x fibers, however, had reduced CSA at both 1 and 7 days. To examine the mechanisms of C2Hx-induced diaphragm atrophy, a targeted gene array was used to screen mRNA changes for genes associated with skeletal muscle myopathy and myogenesis; this was followed by qRT-PCR validation. Changes in diaphragm gene expression suggested that profound myoplasticity is initiated immediately following C2Hx including activation of both proteolytic and myogenic pathways. We conclude that an immediate myoplastic response occurs in the diaphragm after C2Hx with atrophy occurring in ipsilateral myofibers within 1 day.

Keywords

spinal cord injury; diaphragm; atrophy; proteolysis; genes

1. Introduction

The mammalian diaphragm is capable of extremely rapid plasticity. For example, during mechanical ventilation (MV) the diaphragm can atrophy in as little as 8-12 hours (Powers et al., 2009). MV is associated with decreases in diaphragm activity and loading, and MV-induced atrophy of all fiber types has been confirmed by multiple, independent laboratories (reviewed in (Jaber et al., 2011; Powers et al., 2013)). Accordingly, it could be expected that spinal cord injuries which lead to a reduction in diaphragm activation and loading would also trigger rapid diaphragm remodeling.

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A key consideration, however, is whether or not spinal cord injury (SCI) directly impacts the phrenic motoneuron pool (*e.g.*, mid-cervical contusion) or if the spinal lesion spares phrenic motoneurons while altering their synaptic inputs (*e.g.*, lesions rostral to C3). Cervical contusion lesions which cause a significant loss of phrenic motoneurons result in a decrease in both diaphragm thickness and myofiber size (Nicaise et al., 2013; Nicaise et al., 2012). This response likely occurs as a result of neuronal cell death and the associated loss of synaptic and/or trophic input to diaphragm myofibers. In contrast, rostral cervical lesions which interrupt bulbospinal synaptic pathways while sparing the phrenic motor pool appear to have a relatively minimal impact on the diaphragm. For example, at 14 days following lateral spinal cord hemisection at C2 (C2Hx) there is no change in ipsilateral hemidiaphragm contractile force, a slight hypertrophy of type I fibers, and no change in the size of other fiber types (Miyata et al., 1995). The mechanisms responsible for the remarkable lack of atrophy in the ipsilateral hemidiaphragm two weeks following C2Hx, particularly in slow myofibers, are not established. However, the hypertrophy suggests that, paradoxically, anabolic signaling and increased protein synthesis are likely to occur in the ipsilateral hemidiaphragm at 14 days. To our knowledge, however, the impact of C2Hx on the diaphragm during the immediate post-injury period (*e.g.*, 1-7 days) has not been evaluated. Accordingly, the purpose of the present study was to determine the impact of acute C2Hx on diaphragm muscle fiber size and the concordant myoplasticity-related gene expression immediately following the injury. Because multiple studies have shown that reductions in diaphragm loading is associated with rapid atrophy, we hypothesized that immediately post-injury (*e.g.*, 24 hrs.) the ipsilateral hemidiaphragm would show increased expression of proteolytic biomarkers and myofiber atrophy. However, we predicted this would be a transient event since the literature also indicates that C2Hx triggers anabolic processes in the ipsilateral diaphragm by 14 days post-injury (Miyata et al., 1995).

2. Methods

All experiments were approved by the Institutional Animal Care and Use Committee. A total of 27 adult, male Sprague-Dawley rats from Harlan Laboratories (Indianapolis, IN) were studied. Animals were maintained on a 12:12-h light-dark cycle and provided food and water *ad libitum*. Rats were randomly assigned to the following experimental groups ($n=9$ per group): uninjured (control), 24 hours post C2Hx, or 7 days post C2Hx. The individual performing tissue harvest, processing and subsequent histological evaluations (LCG) was blinded regarding the experimental groups.

2.1 Surgical procedures

The surgical methods used to produce the C2Hx injury are consistent with our prior reports (Dougherty et al., 2012a; Dougherty et al., 2012b). Rats were anesthetized with xylazine (10 mg/kg, *s.c.*) and ketamine (140 mg/kg, *i.p.*, Fort Dodge Animal Health, IA, USA), and a dorsal cervical incision was made from the base of the skull to the C3 segment. The spinal cord was exposed dorsally at the C2 level and a left C2HX lesion was induced using a microscalpel followed by aspiration. The dura and overlying muscles were sutured and the skin was closed with stainless steel wound clips (Stoelting, IL, USA). Following surgery, rats were given an injection of yohimbine (1.2 mg/kg, *s.c.*, Lloyd, IA, USA) to reverse the

effect of xylazine, an analgesic (buprenorphine, 0.03 mg/kg, *s.c.*, Hospira, IL, USA) and sterile lactated ringers solution (5 ml, *s.c.*). Post-surgical care included administration of buprenorphine (0.03 mg/kg, *s.c.*) during the initial 48 hours post-injury, delivery of lactated ringers solution (5 ml/day, *s.c.*) and oral Nutri-cal supplements (1–3 ml, Webster Veterinary, MA, USA) as needed until adequate volitional drinking and eating resumed.

2.2 Diaphragm harvest

Rats were initially anesthetized using isoflurane anesthesia (3–4% in O₂) in a closed chamber followed by intraperitoneal injection of urethane (1.6g/kg; Sigma, St. Louis, MO). To extract the diaphragm, a transverse incision was made across the abdomen followed by a rostral transverse incision through the lower sternum and above the lower ribs for the purposes of maintaining the attachment of the diaphragm to the lower ribs to preserve the tissue at close to *in-vivo* muscle length. The tissue was then placed in a shallow dissection bath containing a cooled buffer solution aerated with a 95% O₂, 5% CO₂ gas mixture. The diaphragm was then pinned to a layer of silicone plastic (Sylgard) at a non-stressed length. The costal diaphragm was then divided into ipsilateral and contralateral hemidiaphragm sections (*i.e.*, relative to the C2Hx lesion). Within each hemidiaphragm section the mid-costal portion was removed (Poole et al., 1997) and either rapidly frozen in liquid nitrogen and stored at –80°C for molecular analyses, or rapidly frozen in isopentane cooled by liquid nitrogen for histological analysis. In the latter case, tissues were cryoprotected in optimum cutting temperature compound prior to sectioning and immunohistochemical analysis.

2.3 RNA isolation and cDNA synthesis

Mechanical lysis of frozen hemidiaphragm samples was performed by grinding flashfrozen samples into a powder using a frozen, sterilized mortar and pestle. Following tissue disruption, the tissue powder was added to a 2ml microcentrifuge tube containing 1ml of a protein denaturing guanidinium thiocyanate phenol-chloroform solution (TRIzol Reagent, Invitrogen, Life technologies, Carlsbad, California) and allowed to incubate for 2–3 min at room temperature, subsequently 200 µl of chloroform was added and tubes inverted 15 times to mix. High speed centrifugation (~12,000g at 4°C for 15 min) separated the biphasic mixture into a lower red phenol chloroform phase containing DNA and proteins and the upper colorless aqueous phase containing total RNA. The aqueous, total-RNA containing phase was removed by pipetting and further purified with RNeasy mini columns (RNeasy Mini Kit, QIAGEN Sciences, Valencia, CA). In this procedure, a column based high-salt buffer system selectively binds RNA to a silica membrane while eliminating remaining DNA contaminants, utilizing 70% ethanol, proprietary wash buffers and high speed centrifugation (~12,000 g). High-quality total RNA was then eluted in 100 µl of molecular grade water. To ensure purity and integrity, all RNA was quantified spectrophotometrically, and assessed by agarose gel electrophoresis. All samples used for arrays exhibited 260nm:280nm absorbance ratios of between 1.8–2.0, and demonstrated intact ribosomal 28S and 18S ribosomal RNA bands in an approximate ratio of 2:1. Complementary DNA (cDNA) was prepared from 1 µg purified total RNA using the RT² First Strand Kit (QIAGEN Sciences, Valencia, CA) from each animal. An initial incubation at 42°C for 5 min in genomic DNA (gDNA) elimination buffer was performed to degrade any contaminating cellular gDNA. Next, samples were mixed with reverse-transcription

mix, RT buffer, P2 reverse transcription primer and RNase free water and allowed to incubate at 42°C for 15min. After the initial reverse transcription incubation period, the reaction was subsequently halted by incubating the reaction at 95°C for 5min.

2.4 Gene array

The full results of this array, including raw cycle data, normalized cycle data and postnormalized fold change compared to control animals has been uploaded to the National Center for Biotechnology Information (NCBI) gene expression omnibus (GEO) and can be accessed at <http://www.ncbi.nlm.nih.gov/geo/> (accession number: GSE45021). cDNA samples from each control or experimental group were pooled and used as template for the Rat Skeletal Muscle Development and Disease RT² Profiler PCR Array (QIAGEN Sciences, Valencia, CA). This array was employed to screen 84 pathway or disease-focused genes related to muscle atrophy and regeneration. Equal amounts of cDNA from each diaphragm were pooled onto one array plate (N=3 per treatment group). For each array plate, a master mix was made to combine 24µl RT² SYBR Green/ROX Mastermix (QIAGEN Sciences Valencia, CA) and 1 µl pooled cDNA sample for each well. The RT² SYBR Green Mastermix SYBR green dye, HotStart DNA Taq Polymerase, dNTP mix, and ROX reference dye (used to normalize based on PCR machine optics) dissolved in an optimized PCR buffer (1×). A real-time 2-step quantitative polymerase chain reaction (PCR) was performed using a 7300 real-time PCR system (Applied Biosystems City State). The cycling parameters were as follows: 95°C for 10min (for the Hotstart Taq Polymerase); 40 cycles of (95°C for 15 sec, and 60°C for 1 min). Direct detection of PCR products was monitored by measuring the increase in fluorescence caused by the binding of SYBR Green dye to double-stranded DNA. Data were obtained as cycle threshold (Ct) values, where Ct was defined as the number of PCR cycles required for the SYBR green dye to cross the threshold (*i.e.* exceed the background). Baseline was set at 3 to 15 cycles and the threshold value set at 0.3 for all array plates in the experiment. All data were analyzed using the C_T method and SABiosystems (QIAGEN Sciences Valencia, CA) online gene array analysis software (<http://www.sabiosciences.com/pcrarraydataanalysis.php>). Gene results were normalized to a panel of housekeeping genes present on array plates.

2.5 Real-time PCR

Based on the results of the gene array, several genes were chosen for validation using gene-specific quantitative PCR primer assays (QIAGEN Sciences, Valencia, CA). cDNA from each individual animal was used as a template for qPCR using primers for the following genes: atrogen-1, GenBank NM_133521; MuRF1, GenBank NM_080903; agrin, GenBank NM_175754; Actinin alpha 3, GenBank NM_133424; Calpain 2, GenBank NM_017116; Calpain 3, GenBank NM_017117; Caspase 3, GenBank NM_012922; Interleukin 1 beta, GenBank NM_031512; Interleukin 6, GenBank NM_012589; Myogenic factor 5, GenBank NM_001106783; Myogenic factor 6, GenBank NM NM_013172; Myogenic differentiation 1, GenBank NM_176079; Myogenin, GenBank NM_017115; Nfkb1, GenBank XM_342346; Nitric oxide synthase 2, GenBank NM_012611; Tumor necrosis factor, GenBank NM_012675; Forkhead box O3, GenBank NM_001106395; Muscle skeletal receptor tyrosine kinase, GenBank NM_031061; Ribosomal protein L13A, GenBank NM_173340. For each animal/gene condition, samples were set up in triplicate, and the

housekeeping gene, Ribosomal protein L13A, was monitored to ensure equal amounts of template across experimental plates. Minus reverse transcriptase (-RT) and no template (NTC) negative controls were included to ensure an absence of gDNA contamination or reagent contamination, respectively. Data were obtained as CT values, analyzed using the standard curve method, and fold change compared to the control group calculated for each individual experimental animal. Subsequently, a dissociation (melting) curve analysis was performed to verify PCR specificity. For transcripts that were not detectable in the control group, relative mRNA levels are reported in arbitrary units.

2.6 Diaphragm Histology

All morphometric analyses were performed on the mid-costal region of the hemidiaphragm. Tissue sections from both the ipsilateral and contralateral mid-costal region were cut at 10 μ m using a cryotome. Histological sections were quantitatively assessed for myofiber cross sectional area (CSA). To enable CSA quantification sections were permeabilized for 5 min with 0.5% Triton X-100 in PBS, and then reacted with primary antibodies A4.840 to detect type I fibers (1:15; Developmental Studies, Iowa City, Iowa,) and SC-71 to detect type IIa fibers (1:50; Developmental Studies, Iowa City, Iowa,) and dystrophin (1:100 pre-diluted in 0.05mol/L Tris-HCl, pH 7.6, Lab Vision, Kalamazoo, MI) for 1hr at room temperature. After washes in PBS, slides were incubated in Alexa Fluor 350 goat anti-mouse IgM (1:333 Life technologies, Carlsbad, California), Alexa Fluor 488 goat anti-mouse IgG (1:133 Life technologies, Carlsbad, California) and rhodamine-conjugated goat anti-rabbit IgG (1:40, Life technologies, Carlsbad, California) at room temperature for 1hr for detection of type I, IIa and IIb/x muscle cell borders respectively. Slides were washed and mounted in Vectashield w/o DAPI (Vector Laboratories, Burlingame, CA) and cover slipped.

For all tissue sections, digital image acquisition was performed using an integrated DM4000M compound microscope and DFC425 camera (Leica Microsystems, Bannockburn, IL). Tissue sections were photographed using a 10 $\times$ objective, capturing approximately 200-300 muscle fibers per image. At least 2 images were taken per tissue section using a grid to avoid overlap between images. All images were analyzed using LAS Image Analysis software (Leica Microsystems, Bannockburn, IL). In all cases, 250-350 fibers were counted. CSA was initially measured in μ m², and was also normalized to values measured in tissues harvested from control, uninjured rats.

2.7 Statistical analyses

Changes in each variable across the three experimental conditions (i.e., control, 1- and 7 days post-C2Hx) were assessed using a 1-way ANOVA followed by Tukey post-hoc comparisons. All statistical tests were done using SigmaStat v2.03 software. All data are expressed as mean $\pm$ SE.

3. Results

3.1 Body weight

Body weight was similar across all experimental groups prior to C2Hx (control: 346 $\pm$ 7, 1 day: 339 $\pm$ 6, 7 day: 350 $\pm$ 8; p=0.55). The C2Hx injury resulted in an expected decrease in

body weight. At one day post injury, body weight declined by $6\pm 1\%$ compared to the pre-injury measurement, but body weight was not statistically different than control ($P=0.09$). By 7 days post-injury body weight had dropped by $12\pm 2\%$ and was significantly less when compared to control animals ($P=0.04$).

3.2 Myofiber size

Representative histological images from the ipsilateral and contralateral hemidiaphragm are shown in Fig. 1; the mean results of the blinded assessment of histological sections are provided in Figs. 2 and 3. Analysis of the normalized ipsilateral fiber size data (%control, Fig. 2) revealed a significant interaction experimental group (*i.e.*, control, 1 and 7 days post-C2Hx) and fiber type ($F_{4,40}=4.78$, $P=0.003$). Inspection of the data (Fig. 2) suggests that the size of all fiber types in the ipsilateral hemidiaphragm was reduced at 1 day post-C2Hx. Post-hoc analyses of the 1 day post-C2Hx data (% control) showed statistically significant changes in type IIa ($P=0.02$) and IIb/x fibers ($P=0.01$) and a strong trend in the type I fibers ($P=0.06$). At 7 days post-C2Hx, however, only the type IIb/x fibers remained smaller than control. Analyses of the non-normalized myofiber CSA (*i.e.*, μm^2) resulted in similar conclusions (Fig. 3). Specifically, both type I and IIb fibers showed statistically significant reductions in size at 1 but not 7 days post-C2Hx; type IIb/x fibers showed reductions at both 1 and 7 days.

Assessment of the contralateral hemidiaphragm histology did not reveal any significant changes in myofiber size after C2Hx, although there was a trend for reduced myofiber size. Normalized contralateral fiber size was not different across treatment groups ($F_{2,19}=1.37$, $P=0.276$) or fiber type ($F_{2,4}=0.04$, $P=0.956$) (Fig. 2). Consistent with those results, the CSA data (μm^2) were not different across the three treatment groups for type I ($P=0.36$), type IIa ($P=0.36$) or type IIb/x fibers ($P=0.29$) (Fig. 3).

3.3. Gene analysis

Histological analyses were coupled with quantification of myoplasticity-related mRNA gene changes in diaphragm tissue. Initially, we used an 84 gene microarray to screen for changes in genes associated with muscle atrophy and myogenesis in both the ipsilateral and contralateral hemidiaphragm. The microarray experiment suggested rapid changes in myoplasticity-related gene expression in the diaphragm after C2Hx, and we briefly comment on these initial findings. The full results from the microarray experiment have been deposited on the NCBI GEO website <http://www.ncbi.nlm.nih.gov/geo/> (accession number: GSE45021). Within the ipsilateral diaphragm, the microarray showed robust increases in several genes associated with muscle atrophy including calpain, caspase3 and nitric oxide synthase. In addition, a high fidelity biomarker of the ubiquitin proteasome pathway (UPP), MuRF1 showed a substantial increase. The myogenic regulatory factor, myogenin was also increased as were genes associated with myogenesis and neuromuscular junction plasticity.

Quantitative PCR was used to validate changes in expression for select myoplasticity-related genes that showed 2-fold or greater increases in the microarray experiment. As shown in Table 1, the PCR results showed increased expression in several genes in both the ipsilateral and contralateral diaphragm after C2Hx. Of particular interest, the E3 ligase MuRF1 was

increased in the ipsilateral diaphragm at one but not seven days post-injury. Due to variance in the response however, statistical analysis of the mRNA data was precisely at the threshold of significance ($P = 0.05$). The proteolytic markers caspase3 (1 day post-C2Hx) and calpain (7 days post-C2Hx) were also significantly elevated in the ipsilateral diaphragm. Myogenin was increased at 1- but not 7 days post-C2Hx, and TNF α was robustly increased in the ipsilateral diaphragm at the 1 day time point. In a few cases, genes were undetectable in the control tissue, but expression was induced in the diaphragm following C2Hx. These data are shown in Table 2, and include two myogenic transcription factors and an inflammatory marker. Within the contralateral diaphragm, C2Hx was associated with increased expression of genes associated with atrophy (atrogin-1) and proteolysis (calpain-3), and inflammation (IL6).

4. Discussion

Our primary finding is that the C2Hx model of cervical SCI results in rapid but transient atrophy of the ipsilateral hemidiaphragm. This conclusion is based upon a blinded histological of assessment diaphragm myofibers and is further supported by the immediate upregulation of genes linked to both proteolysis and muscle atrophy.

4.1 The response of the diaphragm to inactivity and denervation

Unilateral hemidiaphragm paralysis in humans following resection of the phrenic nerve causes atrophy of diaphragm type I myofibers by two weeks, with atrophy of fast fibers occurring but with a slower time course (Welvaart et al., 2011). The impact of SCI-induced unilateral paralysis on the diaphragm has previously been studied using the C2Hx model in rats (Mantilla et al., 2013; Mantilla and Sieck, 2009; Miyata et al., 1995; Zhan et al., 1997). At 14 days following C2Hx, there is no change in ipsilateral hemidiaphragm contractile force, a slight hypertrophy of type I fibers, and no change in the size of other fiber types (Miyata et al., 1995). However, at 8 weeks post-C2Hx, ipsilateral hemidiaphragm contractile force is reduced by approximately 40% (Mantilla et al., 2013). The reductions in contractile force occur in parallel with decreases in type IIB/x myofiber size, and hypertrophy is no longer evident in type I or IIA myofibers. These previous findings indicate that there are distinct phases of diaphragm adaptation to C2Hx. The current results are consistent with this suggestion. We noted a reduction in CSA of type I and IIA myofibers immediately (i.e., 1 day) post-C2Hx, but not after 7 days. Type IIB/x fibers, however, had reduced CSA at both 1 and 7 days following C2Hx. The atrophy immediately post-C2Hx is consistent with one prior report in which unilateral hemidiaphragm denervation in rats caused a net loss of muscle protein after five days (Argadine et al., 2009). The rapid atrophy after C2Hx is also consistent with the diaphragm response to mechanical ventilation (MV) (Powers et al., 2002; Shanely et al., 2004; Shanely et al., 2002). MV results in rapid activation of proteases in diaphragm myofibers (Jaber et al., 2011; Powers et al., 2009) with significant atrophy in as little as 8-12 hours. Upregulation of catabolic pathways during MV is also indicated by gene analyses in human diaphragm samples obtained during cardiothoracic surgery (Huang et al., 2011). Thus, the MV literature (reviewed in (Powers et al., 2013)) has clearly demonstrated the speed with which diaphragm muscle fiber atrophy can occur.

The initial diaphragm atrophy that we observed following C2Hx would be predicted if abrupt reduction (or elimination) in diaphragm contractile work leads to atrophy. A reduction of ipsilateral hemidiaphragm contractile work is certain to occur after C2Hx due to the initial hemidiaphragm paralysis (Goshgarian, 2003, 2009). Following chronic C2Hx, however, a return of ipsilateral hemidiaphragm activity is likely to impact morphology. The detailed time course and extent of phrenic neuromuscular recovery after C2Hx is difficult to definitively ascertain since that would require continuous monitoring of ipsilateral hemidiaphragm activity (*e.g.*, 24 hour monitoring of indwelling EMG signals). While numerous studies have assessed ipsilateral phrenic recovery after C2Hx, all have the caveat that diaphragm activity was assessed for only a small time window, or phrenic output was assessed during a terminal neurophysiological preparation. In terminal neurophysiological preparations, it is possible to induce inspiratory bursting in the quiescent ipsilateral phrenic nerve after C2Hx using respiratory stimulation (Fuller et al., 2003), pharmacological manipulations (Zhou and Goshgarian, 2000), contralateral phrenicotomy or cervical dorsal rhizotomy (Fuller et al., 2002; Goshgarian, 1981). To our knowledge, the earliest that spontaneous inspiratory bursting in the previously paralyzed hemidiaphragm has been described is one week post-C2Hx (Mantilla and Sieck, 2009). However, during the “normal” cage behavior of rats following C2Hx (*e.g.*, sniffs, sighs, postural movements, locomotion), the paralyzed hemidiaphragm may be transiently activated even in the initial days following the injury.

One explanation for the current results is that C2Hx results in a complete electrical silencing of the ipsilateral hemidiaphragm and elimination of contractile work during the initial hours to days post-injury. In turn, upregulation of atrophy-related genes (*e.g.*, Table 1) would initiate processes which result in reductions in myofiber size. Subsequently, the restoration of even a small amount of ipsilateral hemidiaphragm activity may reverse the atrophy process. For example, the MV literature shows that even short periods of diaphragm activity can attenuate protease upregulation and contractile impairments (Yang et al., 2013), and atrophy (Gayan-Ramirez et al., 2005). Accordingly, the absence of type I myofiber atrophy at one week (current study) and 2-8 weeks post-C2Hx (Mantilla et al., 2013; Mantilla and Sieck, 2009; Miyata et al., 1995; Zhan et al., 1997) may indicate that return of ipsilateral hemidiaphragm contractile activity has contributed to a reversal of the atrophy that was prominent at 1 day post-injury (Fig. 2).

It is also possible that contractile activity *per se* has only a minor impact on the diaphragm after C2Hx (Mantilla et al., 2013). In other words, factors not directly related to contractile work may be primarily responsible for the observed diaphragm atrophy. This hypothesis is supported by studies of unilateral hemidiaphragm denervation (Miyata et al., 1995; Zhan et al., 1997). For example, 14 days of unilateral hemidiaphragm denervation resulting from either phrenicotomy or tetrodotoxin application to a phrenic nerve results in hypertrophy of diaphragm type I and IIa fibers and atrophy of type IIb/x fibers (Miyata et al., 1995). The hypertrophic response is intriguing, and suggests that anabolic processes activated by stretch or unknown mechanisms are initiated following unilateral hemidiaphragm denervation. In any case, since slow diaphragm myofibers are primarily responsible for normal “eupneic” breathing (Mantilla and Sieck, 2011), the lack of type I and IIa fiber atrophy after C2Hx

leads to the suggestion that the diaphragm may be resistant to activity-dependent mechanisms of muscle atrophy (Miyata et al., 1995). The contralateral hemidiaphragm response in the current data set may be consistent with this view since there was an apparent dissociation between activity levels and the hemidiaphragm response. Although not measured in the current study, it is well established that contralateral activity increases following unilateral hemidiaphragm paralysis (Katagiri et al., 1994; Miyata et al., 1995; Rowley et al., 2005; Teitelbaum et al., 1993). In turn, increased activity might be predicted to cause hypertrophy of the contralateral hemidiaphragm following C2Hx. In contrast, however, the contralateral hemidiaphragm tended to show reduced fiber size, and also had a significant upregulation of protease mRNA. Insight into this response may come from the work of Reid and colleagues investigating inflammatory related processes and the diaphragm (Reid et al., 2002; Stasko et al., 2013). Circulating cytokines (*e.g.*, TNF α) can trigger proteolysis and atrophy (Li et al., 2005) and has been shown to trigger nitric oxidedependent contractile dysfunction in the diaphragm (Stasko et al., 2013). The diaphragm may be particularly susceptible to cytokine-mediated inflammatory processes due to both its extremely high blood flow and juxtaposition between the abdominal and thoracic cavities (M.B. Reid, personal communication). Consistent with this suggestion, the proinflammatory cytokines TNF α and IL-6 both showed increased mRNA expression in the diaphragm following C2Hx (Tables 1 and 2). Thus, an inflammatory response may be contributing to the observed diaphragm atrophy.

The mRNA changes in genes encoding myogenic regulatory factors indicate that in addition to atrophy pathways, C2Hx may have also triggered regeneration-related processes within the diaphragm. Specifically, mRNA for myogenin, MyoD, and Myf6, all of which contribute to the regulation of myogenesis and muscle regeneration (Zanou and Gailly, 2013), was upregulated in the ipsilateral hemidiaphragm after the injury (Table 1). This finding is consistent with preliminary immunohistological studies from our laboratory in which diaphragm tissues show an apparent increase in positive staining for embryonic myosin heavy chain by 7 days post-C2Hx (L.C. Gill, D.D. Fuller, unpublished). In adult animals, the myogenic process including activation of satellite cells is typically associated with muscle injury or repair (Tidball and Villalta, 2010). Accordingly, a myogenic response after C2Hx could reflect a “repair process” initiated by myofiber damage.

4.2 Conclusion

The C2Hx model has been utilized extensively for studies of the neural control of the diaphragm following cervical SCI (reviewed in (Goshgarian, 2009; Sandhu et al., 2009)). The present results add to the smaller body of literature focused on the myoplastic diaphragm response following SCI. Interpretation of the current data in the context of past results (Mantilla et al., 2013; Mantilla and Sieck, 2009; Miyata et al., 1995; Zhan et al., 1997) leads us to suggest the following overall model of how ipsilateral hemidiaphragm myofibers change following C2Hx. A rapid atrophy occurs in all fiber types during the initial hours–days post-injury, and is associated with increased expression of protease and atrophy-related genes. However, over days–weeks post-injury, anabolic processes are initiated which result in hypertrophy of slow myofibers (Miyata et al., 1995). Over even

longer intervals (e.g., weeks–months) a mild atrophy develops in fast myofibers, while slow fibers return to “normal” (Mantilla et al., 2013).

The physiological mechanisms determining the changes in diaphragm morphology after C2Hx cannot be definitively stated at this time. We hypothesize that the changes in the diaphragm over 1-7 days post-injury occur in response to both the reductions in diaphragm contractile work (i.e., paralysis), as well as increases in inflammation and cytokines (Li et al., 2005; Reid et al., 2002; Stasko et al., 2013). Since we did not quantify changes in muscle proteins, we cannot draw definitive conclusions from the mRNA data. Nevertheless, the rapid increase in the expression of genes associated with atrophy, myogenesis and the neuromuscular junction indicates a rapid and dynamic myoplastic response to conditions triggered by the C2Hx. Clearly, the diaphragm response to spinal cord injury is a complex process and further study is needed to define the mechanisms underlying diaphragm plasticity.

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References

1. Argadine HM, Hellyer NJ, Mantilla CB, Zhan WZ, Sieck GC. The effect of denervation on protein synthesis and degradation in adult rat diaphragm muscle. *Journal of applied physiology*. 2009; 107:438–444. [PubMed: 19520837]
2. Bober E, Lyons GE, Braun T, Cossu G, Buckingham M, Arnold HH. The muscle regulatory gene, Myf-6, has a biphasic pattern of expression during early mouse development. *The Journal of cell biology*. 1991; 113:1255–1265. [PubMed: 2045411]
3. Bodine SC, Latres E, Baumhueter S, Lai VK, Nunez L, Clarke BA, Poueymirou WT, Panaro FJ, Na E, Dharmarajan K, Pan ZQ, Valenzuela DM, DeChiara TM, Stitt TN, Yancopoulos GD, Glass DJ. Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science*. 2001; 294:1704–1708. [PubMed: 11679633]
4. Campbell RL, Davies PL. Structure-function relationships in calpains. *The Biochemical journal*. 2012; 447:335–351. [PubMed: 23035980]
5. Daniels MP. The role of agrin in synaptic development, plasticity and signaling in the central nervous system. *Neurochemistry international*. 2012; 61:848–853. [PubMed: 22414531]
6. Dougherty BJ, Lee KZ, Gonzalez-Rothi EJ, Lane MA, Reier PJ, Fuller DD. Recovery of inspiratory intercostal muscle activity following high cervical hemisection. *Respiratory physiology & neurobiology*. 2012a; 183:186–192. [PubMed: 22705013]
7. Dougherty BJ, Lee KZ, Lane MA, Reier PJ, Fuller DD. Contribution of the spontaneous crossed-phrenic phenomenon to inspiratory tidal volume in spontaneously breathing rats. *Journal of applied physiology*. 2012b; 112:96–105. [PubMed: 22033536]
8. Fuller DD, Johnson SM, Johnson RA, Mitchell GS. Chronic cervical spinal sensory denervation reveals ineffective spinal pathways to phrenic motoneurons in the rat. *Neuroscience letters*. 2002; 323:25–28. [PubMed: 11911982]
9. Fuller DD, Johnson SM, Olson EB Jr, Mitchell GS. Synaptic pathways to phrenic motoneurons are enhanced by chronic intermittent hypoxia after cervical spinal cord injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2003; 23:2993–3000. [PubMed: 12684486]
10. Gayan-Ramirez G, Testelmans D, Maes K, Racz GZ, Cadot P, Zador E, Wuytack F, Decramer M. Intermittent spontaneous breathing protects the rat diaphragm from mechanical ventilation effects. *Critical care medicine*. 2005; 33:2804–2809. [PubMed: 16352963]

11. Gomes MD, Lecker SH, Jagoe RT, Navon A, Goldberg AL. Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. *Proceedings of the National Academy of Sciences of the United States of America*. 2001; 98:14440–14445. [PubMed: 11717410]
12. Goshgarian HG. The role of cervical afferent nerve fiber inhibition of the crossed phrenic phenomenon. *Experimental neurology*. 1981; 72:211–225. [PubMed: 7202624]
13. Goshgarian HG. The crossed phrenic phenomenon: a model for plasticity in the respiratory pathways following spinal cord injury. *Journal of applied physiology*. 2003; 94:795–810. [PubMed: 12531916]
14. Goshgarian HG. The crossed phrenic phenomenon and recovery of function following spinal cord injury. *Respiratory physiology & neurobiology*. 2009; 169:85–93. [PubMed: 19539790]
15. Huang TT, Deoghare HV, Smith BK, Beaver TM, Baker HV, Mehinto AC, Martin AD. Gene expression changes in the human diaphragm after cardiothoracic surgery. *The Journal of thoracic and cardiovascular surgery*. 2011; 142:1214–1222. 1222 e1211–1220. [PubMed: 21463877]
16. Jaber S, Jung B, Matecki S, Petrof BJ. Clinical review: ventilator-induced diaphragmatic dysfunction—human studies confirm animal model findings! *Critical care*. 2011; 15:206. [PubMed: 21457528]
17. Katagiri M, Young RN, Platt RS, Kieser TM, Easton PA. Respiratory muscle compensation for unilateral or bilateral hemidiaphragm paralysis in awake canines. *J Appl Physiol*. 1994; 77:1972–1982. [PubMed: 7836225]
18. Li YP, Chen Y, John J, Moylan J, Jin B, Mann DL, Reid MB. TNF-alpha acts via p38 MAPK to stimulate expression of the ubiquitin ligase atrogin1/MAFbx in skeletal muscle. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2005; 19:362–370. [PubMed: 15746179]
19. Mantilla CB, Greising SM, Zhan WZ, Seven YB, Sieck GC. Prolonged C2 spinal hemisection-induced inactivity reduces diaphragm muscle specific force with modest, selective atrophy of type IIx and/or IIb fibers. *Journal of applied physiology*. 2013; 114:380–386. [PubMed: 23195635]
20. Mantilla CB, Sieck GC. Neuromuscular adaptations to respiratory muscle inactivity. *Respiratory physiology & neurobiology*. 2009; 169:133–140. [PubMed: 19744580]
21. Mantilla CB, Sieck GC. Phrenic motor unit recruitment during ventilatory and non-ventilatory behaviors. *Respiratory physiology & neurobiology*. 2011; 179:57–63. [PubMed: 21763470]
22. Miyata H, Zhan WZ, Prakash YS, Sieck GC. Myoneural interactions affect diaphragm muscle adaptations to inactivity. *Journal of applied physiology*. 1995; 79:1640–1649. [PubMed: 8594024]
23. Nicaise C, Frank DM, Hala TJ, Authalet M, Pochet R, Adriaens D, Brion JP, Wright MC, Lepore AC. Early phrenic motor neuron loss and transient respiratory abnormalities after unilateral cervical spinal cord contusion. *Journal of neurotrauma*. 2013; 30:1092–1099. [PubMed: 23534670]
24. Nicaise C, Putatunda R, Hala TJ, Regan KA, Frank DM, Brion JP, Leroy K, Pochet R, Wright MC, Lepore AC. Degeneration of phrenic motor neurons induces long-term diaphragm deficits following mid-cervical spinal contusion in mice. *Journal of neurotrauma*. 2012; 29:2748–2760. [PubMed: 23176637]
25. Nowak KJ, Ravenscroft G, Laing NG. Skeletal muscle alpha-actin diseases (actinopathies): pathology and mechanisms. *Acta neuropathologica*. 2013; 125:19–32. [PubMed: 22825594]
26. Poole DC, Sexton WL, Farkas GA, Powers SK, Reid MB. Diaphragm structure and function in health and disease. *Medicine and science in sports and exercise*. 1997; 29:738–754. [PubMed: 9219201]
27. Powers SK, Kavazis AN, Levine S. Prolonged mechanical ventilation alters diaphragmatic structure and function. *Critical care medicine*. 2009; 37:S347–353. [PubMed: 20046120]
28. Powers SK, Shanely RA, Coombes JS, Koesterer TJ, McKenzie M, Van Gammeren D, Cicale M, Dodd SL. Mechanical ventilation results in progressive contractile dysfunction in the diaphragm. *Journal of applied physiology*. 2002; 92:1851–1858. [PubMed: 11960933]
29. Powers SK, Wiggs MP, Sollanek KJ, Smuder AJ. Invited Review: Ventilator-induced diaphragm dysfunction: cause and effect. *American journal of physiology Regulatory, integrative and comparative physiology*. 2013

30. Reid MB, Lannergren J, Westerblad H. Respiratory and limb muscle weakness induced by tumor necrosis factor- α : involvement of muscle myofilaments. *American journal of respiratory and critical care medicine*. 2002; 166:479–484. [PubMed: 12186824]
31. Rowley KL, Mantilla CB, Sieck GC. Respiratory muscle plasticity. *Respiratory physiology & neurobiology*. 2005; 147:235–251. [PubMed: 15871925]
32. Sander A, Hesser BA, Witzemann V. MuSK induces in vivo acetylcholine receptor clusters in a ligand-independent manner. *The Journal of cell biology*. 2001; 155:1287–1296. [PubMed: 11748247]
33. Sandhu MS, Dougherty BJ, Lane MA, Bolser DC, Kirkwood PA, Reier PJ, Fuller DD. Respiratory recovery following high cervical hemisection. *Respiratory physiology & neurobiology*. 2009; 169:94–101. [PubMed: 19560562]
34. Senf SM, Dodd SL, Judge AR. FOXO signaling is required for disuse muscle atrophy and is directly regulated by Hsp70. *American journal of physiology Cell physiology*. 2010; 298:C38–45. [PubMed: 19864323]
35. Shanely RA, Van Gammeren D, Deruisseau KC, Zergeroglu AM, McKenzie MJ, Yarasheski KE, Powers SK. Mechanical ventilation depresses protein synthesis in the rat diaphragm. *American journal of respiratory and critical care medicine*. 2004; 170:994–999. [PubMed: 15297271]
36. Shanely RA, Zergeroglu MA, Lennon SL, Sugiura T, Yimlamai T, Enns D, Belcastro A, Powers SK. Mechanical ventilation-induced diaphragmatic atrophy is associated with oxidative injury and increased proteolytic activity. *American journal of respiratory and critical care medicine*. 2002; 166:1369–1374. [PubMed: 12421745]
37. Stasko SA, Hardin BJ, Smith JD, Moylan JS, Reid MB. TNF signals via neuronal-type nitric oxide synthase and reactive oxygen species to depress specific force of skeletal muscle. *Journal of applied physiology*. 2013; 114:1629–1636. [PubMed: 23558387]
38. Talbert EE, Smuder AJ, Min K, Kwon OS, Szeto HH, Powers SK. Immobilization-Induced Activation of Key Proteolytic Systems in Skeletal Muscles Is Prevented by a Mitochondria-Targeted Antioxidant. *Journal of applied physiology*. 2013
39. Teitelbaum J, Borel CO, Magder S, Traystman RJ, Hussain SN. Effect of selective diaphragmatic paralysis on the inspiratory motor drive. *J Appl Physiol*. 1993; 74:2261–2268. [PubMed: 8101520]
40. Tidball JG, Villalta SA. Regulatory interactions between muscle and the immune system during muscle regeneration. *American journal of physiology Regulatory, integrative and comparative physiology*. 2010; 298:R1173–1187.
41. Welc SS, Clanton TL. The regulation of interleukin-6 implicates skeletal muscle as an integrative stress sensor and endocrine organ. *Experimental physiology*. 2013; 98:359–371. [PubMed: 22941979]
42. Welvaart WN, Paul MA, van Hees HW, Stienen GJ, Niessen JW, de Man FS, Sieck GC, Vonk-Noordegraaf A, Ottenheijm CA. Diaphragm muscle fiber function and structure in humans with hemidiaphragm paralysis. *Am J Physiol Lung Cell Mol Physiol*. 2011; 301:L228–235. [PubMed: 21622847]
43. Yang M, Wang H, Han G, Chen L, Huang L, Jiang J, Li S. Phrenic nerve stimulation protects against mechanical ventilation-induced diaphragm dysfunction in rats. *Muscle & nerve*. 2013
44. Zanou N, Gailly P. Skeletal muscle hypertrophy and regeneration: interplay between the myogenic regulatory factors (MRFs) and insulin-like growth factors (IGFs) pathways. *Cellular and molecular life sciences : CMLS*. 2013
45. Zhan WZ, Miyata H, Prakash YS, Sieck GC. Metabolic and phenotypic adaptations of diaphragm muscle fibers with inactivation. *Journal of applied physiology*. 1997; 82:1145–1153. [PubMed: 9104851]
46. Zhou SY, Goshgarian HG. 5-Hydroxytryptophan-induced respiratory recovery after cervical spinal cord hemisection in rats. *Journal of applied physiology*. 2000; 89:1528–1536. [PubMed: 11007592]

Highlights

- Diaphragm tissue was harvested from Sprague-Dawley rats 1 or 7 days following cervical (C2) hemilesion (C2Hx)
- Histological analysis demonstrated a reduction in cross-sectional area of all myofiber types in the ipsilateral diaphragm 1 day post-injury
- PCR analyses indicated increases of proteolytic and myogenic genes in the diaphragm
- An immediate myoplastic response occurs in the diaphragm after C2Hx with atrophy occurring within 1 day

Figure 1. Representative histological sections from the medial costal hemidiaphragm
Tissue sections from the mid-costal hemidiaphragm region were harvested and cut on a cryotome at 10 μm thickness. Sections were then processed to immunochemically detect type I (blue) and IIa (green) myofibers as well as cell borders (red). Panel A shows hemidiaphragm sections obtained from a control, uninjured rat and panel B depicts sections obtained 1 day following the C2Hx injury. Tissue sections were obtained from the hemidiaphragm ipsilateral (Ipsi) and contralateral (Contra) to the C2Hx. C2Hx injuries were always done on the left side of the spinal cord, and thus in control rats the labels Ipsi and Contra indicate the left and right hemidiaphragm, respectively. Please see the text for a more detailed description of staining procedures and quantification methods. Scale bar = 200 μm .

Figure 2. Relative changes in myofiber size in both the ipsilateral and contralateral hemidiaphragm at 1- and 7 days following C2Hx

All morphometric analyses were performed on the mid-costal region of the diaphragm. Tissue sections were obtained from the hemidiaphragms of control uninjured rats, and also from rats at 1 and 7 days following C2Hx. In this figure, the data are expressed relative to myofiber CSA measured in hemidiaphragm samples from control, uninjured rats. As shown in the top panel, the normalized ipsilateral hemidiaphragm CSA was significantly reduced in all myofiber types at 1 day post-C2Hx. However, only the type IIb/x myofibers had CSA values less than the control data at 7 days post-injury. The bottom panel shows that the normalized CSA did not significantly change in contralateral hemidiaphragm at either 1 or 7 days following C2Hx. Please see the text for a discussion of these data including the trends for a reduction in fiber size in the contralateral hemidiaphragm. *, $P < 0.05$ compared to control value

Figure 3. The impact of C2Hx on diaphragm myofiber size (μm^2)

Tissue sections were obtained from the mid-costal hemidiaphragms of control uninjured rats, and also from rats at 1 and 7 days following C2Hx. For this figure, the CSA is expressed in μm^2 (see Fig. 2 for the normalized values). The ipsilateral hemidiaphragm had reductions in myofiber CSA for all fiber types at 1 day post-C2Hx (top panel). At 7 days, however, only the type IIb/x myofibers had CSA values less than the control values. Normalized CSA did not significantly change in contralateral hemidiaphragm following C2Hx, although trends can be noted in the data (bottom panel). *, $P < 0.05$ compared to control value

Table 1
Increased mRNA expression of myoplasticity-related genes in the diaphragm at 1- and 7 days following C2Hx

Myoplasticity genes were first screened using a micro-array, and then PCR was used to quantify changes in genes of interest. The micro-array data are available online at <http://www.ncbi.nlm.nih.gov/geo/> (accession number: GSE45021). Data points are based on analyses of ipsilateral (IL) and contralateral (CL) hemidiaphragm tissues harvested at 1- and 7 days post-C2Hx. The data are presented as a percent change in mRNA expression relative to expression in diaphragm tissues harvested from rats that did not receive any prior surgery (i.e., 2.0 indicates a 2 fold increase). **Bolded** values indicate a significant difference across time points (i.e., 1- vs. 7 day; $P < 0.05$); **bolded and italicized** values indicate that the overall treatment effect, as determined via the ANOVA, was at the cutoff for statistical significance ($P = 0.05$). Thus, post-hoc tests were not conducted on those data.

Gene	Description	Citation	1 day, IL	7 day, IL	1 day, CL	7 day, CL
MuRF1	Ubiquitin E3 ligase; upregulated during muscle	(Bodine et al., 2001)	42.0±21.1	1.1±0.7	5.1 ±4.3	0.3±0.3
Atrogin-1	Ubiquitin E3 ligase; upregulated during muscle	(Gomes et al., 2001)	4.8±3.6	2.1±0.9	0.3±0.1	3.0±1.8
FoxO3	Regulates MuRF1 and Atrogin-1	(Senf et al., 2010)	1.7±0.2	1.6±0.8	1.0±0.6	1.9±1.3
Caspase- 3	Proteolytic enzyme; increased during diaphragm	(Talbert et al., 2013)	3.4±0.4 *	0.6±0.4	3.0±0.7 *	3.6±0.5 *
Calpain 3	Proteolytic enzyme;	(Talbert et al., 2013)	1.2±0.5	28.8±6.6 *	1.9±0.2	31.6±9.7 *
Calpain 2	Proteolytic enzyme;	(Campbell and Davies, 2012)	1.4±0.2	1.2±0.3	0.9±0.3	1.3±0.3
Myogenin	Transcription factor; can initiate myogenesis	(Zanou and Gailly, 2013)	22.4±8.6	1.5±0.2	1.6±0.7	1.7±0.8
Myf5	Transcription factor; can initiate myogenesis	(Zanou and Gailly, 2013)	1.3±0.3	1.1±0.5	3.2±0.7	1.3±0.6
αActin-3	Actin-binding protein	(Nowak et al., 2013)	0.4±0.1	2.7±1.2	0.3±0.2	1.0±0.2
MuSK	Neuromuscular junction development	(Sander et al., 2001)	2.9±0.6	2.4±0.6	0.3±0.1	2.6±0.5 *
Agrin	Neuromuscular junction development	(Daniels, 2012)	0.9±0.2	1.2±0.1	0.7±0.1	1.5±0.2
TNFα	Inflammatory marker	(Stasko et al., 2013)	18.0±0.5 *	0.2±0.1	3.7±3.1	0.9±0.1
NOS2	Inflammatory marker	(Stasko et al., 2013)	2.9±0.7	2.6±0.7	0.7±0.5	6.1 ±2.8

* $p < 0.05$ vs. control.

Induction of mRNA expression of myoplasticity-related genes in the diaphragm at 1- and 7 days following C2Hx

Table 2

For the genes reported in this table, mRNA expression not detectable in hemidiaphragm tissue samples obtained from rats that did not receive any prior surgery. Therefore, mRNA data could not be expressed in relation to control values. Data are presented as arbitrary units calculated *via* the standard curve method (i.e., the values are not related to fold increases). Data points presented include 1- and 7 days post-C2Hx for the ipsilateral (IL) and contralateral (CL) hemidiaphragm.

Gene	Description	Citation	Control	1 day, IL	7 day, IL	1 day, CL	7 day, CL
MyoD1	Transcription factor; can initiate myogenesis	(Zanou and Gailly,	n.d.	0.16±0.0	1.0±0.2 *	0.2±0.0	1.6±0.4 *
Myf6	Transcription factor; can initiate myogenesis	(Bober et al., 1991)	n.d.	0.6±0.1	0.5±0.1	0.3±0.1	0.5±0.0
IL 6	Inflammatory marker	(Welc and Clanton, 2013)	n.d.	2.8±2.3	1.0±0.8	0.6±0.4	9.1±9.1

* 7 day data point is greater than the 1 day data point, P<0.05; n.d., not detectable.