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Rapid diaphragm atrophy following cervical spinal cord hemisection

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ABSTRACT

A cervical (C2) hemilesion (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 reduction in cross-sectional area (CSA) of type I and IIa fibers in the ipsilateral hemidiaphragm at 1 but not 7 days. Type IIb/x fibers, however, had reduced CSA at 1 and 7 days. 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.

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 h (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.

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., 2012, 2013). 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 the best of 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 are associated with rapid atrophy, we hypothesized that immediately post-injury (*e.g.*, 24 h) 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

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experimental groups ($n = 9$ per group): uninjured (control), 24 h 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,b). 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 h 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 O_2) in a closed chamber followed by intraperitoneal injection of urethane (1.6 g/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% O_2, 5% CO_2 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 flash-frozen samples into a powder using a frozen, sterilized mortar and pestle. Following tissue disruption, the tissue powder was added to a 2 ml microcentrifuge tube containing 1 ml of a protein denaturing guanidinium thiocyanate phenol–chloroform solution (TRIzol Reagent, Invitrogen, Life technologies, Carlsbad, CA) 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 (~12,000 × *g* 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 260 nm:280 nm absorbance ratios of between 1.8 and 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 RT^2 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 15 min. After the initial reverse transcription incubation period, the reaction was subsequently halted by incubating the reaction at 95 °C for 5 min.

2.4. Gene array

The full results of this array, including raw cycle data, normalized cycle data and post-normalized fold change compared to control animals have 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 RT^2 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 24 μl RT^2 SYBR Green/ROX Mastermix (QIAGEN Sciences Valencia, CA) and 1 μl pooled cDNA sample for each well. The RT^2 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 10 min (for the Hotstart Taq Polymerase); 40 cycles of (95 °C for 15 s, 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 (C_t) values, where C_t 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 3–15 cycles and the threshold value set at 0.3 for all array plates in the experiment. All data were analyzed using the $\Delta\Delta C_t$ 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: atrogin-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