ONLINE DATA SUPPLEMENT

CASPASE-3 REGULATION OF MYONUCLEAR DOMAIN DURING MECHANICAL VENTILATION INDUCED DIAPHRAGMATIC ATROPHY

Joseph M. McClung, Andreas N. Kavazis, Keith C. DeRuisseau, Darin J. Falk, Melissa A. Deering, Youngil Lee, Takao Sugiura, and Scott K. Powers

METHODS

Experimental design. This experiment was approved by the University of Florida Animal Care and Use Committee (IACUC) and followed the guidelines for animal experiments established by the American Physiological Society. Healthy, young, adult female (four months old) Sprague-Dawley rats were individually housed and fed rat chow and water *ad libitum* and were maintained on a 12 hour light/dark cycle for three weeks prior to initiation of these experiments. To examine the effect of caspase-3 and MV on myonuclear domain and myofiber size in the diaphragm, rats were randomly assigned to one of three experimental groups: 1) an acutely anesthetized control group (n = 6); 2) a 12-hour MV group (n = 6); and 3) a 12-hour MV group administered a specific, water soluble, caspase-3 inhibitor (n = 6; DEVD-CHO; *N*-acetyl-Asp-Glu-Val-Asp-al; BIOMOL International, Plymouth Meeting, PA) 3mg/kg administered intravenously 1-hour prior to the initiation of ventilation). DEVD-CHO was chosen due to its specificity as a caspase-3 inhibitor and previous work establishing the effectiveness of this inhibitor at this dosage in skeletal muscle (E7). Due to the results of this initial study, another group of animals was randomly assigned to additional experimental groups: 4) acute anesthesia control (n=6); 5) 6-hours of MV (n=6); and 6) 6-hours of MV with DEVD-CHO administration (n=6). These later experiments were performed to investigate caspase-3 regulation of myonuclear domain alterations during the initial hours of MV.

Acutely Anaesthetized Controls. Control animals were subjected to an acute plane of surgical anesthesia with an IP injection of sodium pentobarbital (60 mg/kg body weight). Segments of the costal diaphragm and the plantaris muscle were then removed, rapidly frozen in liquid nitrogen and stored at -80°C for subsequent biochemical and molecular

analysis. In addition, diaphragm and plantaris strips were mounted in Tissue-Tek O.C.T. compound (Sakura Finetek U.S.A., Torrance, CA) at an unstressed length for myofiber area, myonuclear domain, and myonuclear apoptosis measurements.

Mechanical Ventilation. All surgical procedures were performed using aseptic techniques. Animals randomly selected for MV were anesthetized with an IP injection of sodium pentobarbital (60 mg/kg body weight). After reaching a surgical plane of anesthesia, the animals were tracheostomized and mechanically ventilated using a volume-driven small animal ventilator (Harvard Apparatus, Cambridge, MA). The ventilator delivered all breaths; hence, this mode of ventilation (i.e., controlled MV) results in complete diaphragmatic inactivity (E5). The tidal volume was established at approximately 0.55 mL/100 grams of body weight with a respiratory rate of 80 breaths/minute. This respiratory rate was selected to mimic the breathing frequency of adult rats at rest. Additionally, positive end-expiratory pressure of 1 cm H_2O was used throughout the protocol. An arterial catheter was inserted into the carotid artery for constant measurement of blood pressure and periodic blood sampling for analysis of arterial pH and blood gases. Arterial blood samples (100 μL per sample) were removed during the first and last hour of MV and analyzed for arterial pCO_2, pO_2, and pH using a blood gas analyzer (model 1610, Instrumentation Laboratories Company, Lexington, MA). Anesthesia was maintained over the entire period of MV by continuous infusion of sodium pentobarbital (10 mg/kg body weight/hr) via a venous catheter was inserted into the jugular vein. Body temperature was maintained at 37 ± 0.5 ºC by use of a re-circulating heating blanket. Additionally, heart rate and electrical activity of the heart were monitored via a lead II ECG using needle electrodes placed subcutaneously.

Body fluid homeostasis was maintained via the administration of 2.0mL/kg/hour intravenous electrolyte solution. Continuing care during MV included expressing the bladder, removing airway mucus, lubricating the eyes, rotating the animal and passive movement of the limbs. This care was maintained throughout the experimental period at hourly intervals. Finally, intramuscular injections of glycopyrrolate (0.04 mg/kg/2 hours) were employed to reduce airway secretions during MV. Upon completion of MV, segments of the costal diaphragm and the plantaris muscle were removed, rapidly frozen in liquid nitrogen and stored at -80°C for subsequent biochemical and molecular analysis. In addition, diaphragm and plantaris strips were mounted in Tissue-Tek O.C.T. compound (Sakura Finetek U.S.A., Torrance, CA) at an unstressed length for myofiber area, myonuclear domain, and myonuclear apoptosis measurements.

Myofiber Cross Sectional Area and Morphological Analyses. Serial sections from frozen diaphragm and plantaris samples were cut at 10 μm using a cryotome (Shandon Inc., Pittsburgh, PA). Sections for cross-sectional area analysis were then dried at room temperature for 30 minutes and incubated in a PBS solution containing 0.5% Triton X-100. Sections were then rinsed in PBS and simultaneously exposed to primary antibodies specific to dystrophin protein (rabbit host, #RB-9024-R7, Lab Vision Corporation), myosin heavy chain Type I (mouse host, immunoglobulin M (IgM) isotype, A4.840, Developmental Studies Hybridoma Bank, Iowa City, IA (E3) and MHC Type IIa (SC-71, mouse host, immunoglobulin G (IgG) isotype, a kind gift from Takao Sugiura, Laboratory of Biomechanics and Physiology, Faculty of Liberal Arts, Yamaguchi University, Yamaguchi, Japan, (E1) primary antibodies in a dark humid chamber at room temperature for 1 hour. Sections were subsequently rinsed 3X in PBS and exposed to rhodamine red

anti-rabbit secondary antibody (R6394, Molecular Probes, Eugene, OR), Alexa Fluro 350 goat anti-mouse IgM isotype specific secondary antibody (AB1552, Molecular Probes, Eugene, OR), and Alexa Fluro 488 goat anti-mouse IgG isotype specific secondary antibody (A11011, Molecular Probes, Eugene, OR) diluted in PBS containing 0.5% Pierce Super Blocker (57535, Pierce) in a dark humid chamber at room temperature for 1hour. Sections were then washed in PBS and mounted with Vectashield (Vector Laboratories, Burlingame, CA). After mounting, slides were coverslipped and sealed for viewing via an inverted fluorescence microscope (Carl Zeiss Axiovert 200). Fiber typing utilizing this method allows for the individual visualization of the myofiber membrane protein dystrophin using the rhodamine filter set (red), Type I myosin using the DAPI filter set (blue), Type IIa myosin using the FITC filter set (green), and Type IIb/IIx fibers (non-stained/black) myofibers. Images were obtained at a 10X magnification and approximately 250 myofibers, which was chosen by determination of no additional change in standard deviation, were analyzed for myofiber cross-sectional area (μm^2) using Scion Image software (Scion Technologies, Frederick, MD) by a blinded investigator.

Myonuclear domain (μm^3 cytoplasmic myofiber volume/myonucleus) was determined in the corresponding serial sections taken from the diaphragm and plantaris. Sections were stained with hematoxylin and eosin (H&E) and images were obtained at a 10X magnification. Myonuclei for approximately 250 myofibers, which was chosen by determination of no additional change in myofiber standard deviation, were counted and matched to corresponding images of myofiber area by a blinded investigator.

For the determination of nuclear localization, digital images were taken from each hematoxylin and eosin-stained muscle section at a 10X magnification, and a minimum of

2,100 individual nuclei per sample were counted and analyzed for myofiber and non-myofiber localization per mm^3 of muscle area by a blinded investigator as previously described (E4). To reduce experimental bias, all nuclei present on digital images were quantified.

In situ TdT-mediated dUTP nick end labeling. The nuclei with DNA strand breaks were assessed using a fluorometric TdTmediated dUTP nick end labeling (TUNEL) detection kit according to the manufacturer's instructions (1684795; Roche Applied Science, Indianapolis, IN) with the following modifications. Eight-μm-thick frozen muscle cross sections from both diaphragm and plantaris muscles were cut in a cryostat at -20°C. Sections were air dried at room temperature, fixed in 4% paraformaldehyde in PBS, pH 7.4, at room temperature for 20 min, permeabilized with 0.2% Triton X-100 in 0.1% sodium citrate at 4°C for 2 min, and incubated with fluorescein-conjugated TUNEL reaction mixture in a humidified chamber at 37°C for 1 h in the dark. Positive control staining was performed by exposing tissue to DNAse treatment for 10-minutes prior to antibody exposure. Negative control staining was performed by omitting the TdT enzyme in the TUNEL reaction mixture. Sections were then labeled with dystrophin (#RB-9024-R7, Lab Vision Corporation, Fremont, CA) and exposed to rhodamine red secondary antibody (R6394, Molecular Probes, Eugene, OR) to visualize the sarcolemma. Sections were then mounted with DAPI Vectashield mounting medium. TUNEL- and DAPI-positive nuclei and dystrophin staining were examined under a fluorescence microscope. Images were obtained at a 40X magnification and approximately 800 labeled TUNEL- and DAPI positive nuclei localized beneath dystrophin staining were counted per sample to exclude non-muscle myofiber nuclei by a blinded investigator. To reduce experimental bias, all

myonuclei present on digital images were quantified. Data were expressed as TUNEL index by calculating the number of TUNEL-positive nuclei divided by the total number of nuclei (i.e., DAPI-positive nuclei) X 100 as previously described (E6).

Genomic DNA isolation. A section of the costal diaphragm was homogenized using a hand held glass/Teflon homogenizer in 1 mL DNAzol reagent (Molecular Research Inc., Cincinnati, OH) by applying as few strokes as possible. Following homogenization, 20 μL of Proteinase K (20 mg/mL) was added to the homogenate and samples were stored at room temperature for 3 hours. Samples were then centrifuged at 10,000 *x g* for 10 minutes (4°C) to sediment solid material. Following transfer to a new tube, DNA was precipitated by the addition of 0.5 mL of 100% ethanol. Samples were gently mixed by inversion and stored at room temperature for 3 minutes, and then centrifuged at 5,000 *x g* for 5 minutes (4°C) to pellet DNA. DNA was washed twice with 0.8 mL of 75% ethanol. At each wash DNA was suspended in ethanol by inverting the tube 3-6 times. DNA was subsequently sedimented by centrifugation at 1,000 *x g* for 1 minute (4°C), and resuspended in water (4 μL/mg tissue). Following spectrophotometric quantification of DNA (260 nm), samples were stored at -80°C.

Ligation mediated PCR ladder assay. The detection of nucleosomal DNA ladders of apoptotic nuclei was performed using a ligation mediated PCR (LM-PCR) ladder assay kit (Maxim Biotech, Inc. San Francisco, CA) according to the manufacturer's instructions. Briefly, 0.5 μg of genomic DNA was added to 1X ligation mix containing oligonucleotide adaptors. The oligonucleotides were annealed by heating to 55°C for 10 minutes and then gradually cooled to 10°C over 1 hour followed by incubation at 10°C for 10 minutes. Following the addition of T4 DNA ligase, samples were incubated at

16°C for 16 hours. Hot-start PCR containing the adaptor-ligated DNA was performed using forward and reverse primers supplied in the kit according to the following parameters: 72°C for 10 minutes (1 cycle), 94°C for 1 minute (1 cycle), 94°C for 1 minute and 72°C for 3 minutes (26 cycles), and 72°C for 10 minutes (1 cycle). Following PCR, samples were electrophoresed on 1.8% agarose gel containing 0.5 μg/mL ethidium bromide for 1 hour at 100 mA. DNA bands were visualized under UV transillumination using a gel documentation system (BioRad, Hercules, CA).

Cytosolic protein isolation. A section of the ventral costal diaphragm was homogenized 1:10 (v:v) (5 mM Tris-HCL, pH = 7.5; 5 mM EDTA, pH = 8.0) and centrifuged at 1,500 *x g* for 10 minutes (4°C). Cytosolic fraction was centrifuged at 10,000 *x g* for 10 minutes (4ºC) followed by an additional spin of the supernatant at 100,000 *x g* for one hour (4ºC).

Western Blot Analysis. Proteins (100 μg) from the cytosolic fraction were separated via polyacrylamide gel electrophoresis via 15% gradient polyacrylamide gels containing 0.1% SDS. After electrophoresis proteins were transferred to nitrocellulose membranes. Membranes were stained with Ponceau-S, visually inspected for equal protein loading/transfer and soaked in tris buffered saline (TBS) buffer prior to exposure to a 5% non-fat dry milk in TBS + 0.1% tween (TBST) blocking solution for 1hr. Membranes were then subsequently incubated with a primary anti-cleaved active caspase-3 antibody (#9664; Cell Signaling Technology, Danvers, Maryland) diluted 1:550 in 5% bovine serum albumin (BSA) in TBST solution overnight at 4ºC as previously described (E2). This step was followed by a 1hr incubation with a horseradish peroxidase-secondary antibody conjugate diluted 1:1000 in 5% milk in TBST at room temperature. Membranes were then treated with chemiluminescent reagents (ECL Plus; Amersham Life

Biosciences, Piscataway, NJ) and exposed to film. Membranes were subsequently stripped of bound antibodies using a commercially available stripping buffer (Restore Western Blot Stripping Buffer, Pierce, Rockford, IL), rinsed for 25 minutes in TBS and subsequently blocked in 5% milk in TBST solution for 1hr. Membranes were then incubated with total caspase-3 primary antibody (#9665; Cell Signaling Technology, Danvers, Maryland) diluted 1:550 in 5% BSA in TBST solution overnight at 4ºC. This step was again followed by a 1hr incubation with a horseradish peroxidase-secondary antibody conjugate diluted 1:1000 in 5% milk in TBST at room temperature. Membranes were then treated with chemiluminescent reagents (ECL Plus; Amersham Life Biosciences) and exposed to film. Digital images of these films were captured and analyzed using computerized image analysis software (Scion Technologies, Frederick, MD) producing an integrated optical density (IOD) for each sample.

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