Heat stress protects against mechanical ventilation-induced diaphragmatic atrophy

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Heat stress protects against mechanical ventilation-induced diaphragmatic atrophy. J Appl Physiol 117: 518–524, 2014. First published July 24, 2014; doi:10.1152/japplphysiol.00170.2014.—Mechanical ventilation (MV) is a life-saving intervention in patients who are capable of maintaining adequate pulmonary gas exchange due to respiratory failure or other disorders. Unfortunately, prolonged MV is associated with the development of respiratory muscle weakness. We hypothesized that a single exposure to whole body heat stress would increase diaphragm expression of heat shock protein 72 (HSP72) and that this treatment would protect against MV-induced diaphragmatic atrophy. Adult male Wistar rats (n = 38) were randomly assigned to one of four groups: an acutely anesthetized control group (CON) with no MV; 12-h controlled MV group (CMV); 1-h whole body heat stress (HS); or 1-h whole body heat stress 24 h prior to 12-h controlled MV (HSMV). Compared with CON animals, diaphragmatic HSP72 expression increased significantly in the HS and HSMV groups (P < 0.05). Prolonged MV resulted in significant atrophy of type I, type IIa, and type IIx fibers in the costal diaphragm (P < 0.05). Whole body heat stress attenuated this effect. In contrast, heat stress did not protect against MV-induced diaphragm contractile dysfunction. The mechanisms responsible for this heat stress-induced protection remain unclear but may be linked to increased expression of HSP72 in the diaphragm.

heat shock protein; proteolysis; antioxidants; respiratory muscle weakness

MECHANICAL VENTILATION (MV) is a life-saving intervention in patients who are capable of maintaining adequate pulmonary gas exchange due to respiratory failure or other disorders (14). Unfortunately, prolonged MV is associated with the development of respiratory muscle weakness, specifically weakness of the diaphragm (9, 19). MV-induced respiratory muscle weakness is significant because it may contribute to difficulty in weaning from the ventilator (8), which in turn is associated with increased patient mortality and morbidity (7). Therefore, it is important to develop clinical strategies to protect patients against MV-induced diaphragmatic weakness.

It has been established that whole body heat stress performed 24 h prior to locomotor muscle inactivity prevents or attenuates disuse locomotor muscle atrophy by inducing 72-kDa heat shock protein (HSP72). HSP72 is a highly conserved stress protein that can be induced in skeletal muscle fibers by both environmental (e.g., heat stress) and intracellular stresses (e.g., disturbed pH) (4, 28), eccentric exercise (24, 25), and oxidative stress (47). Although the precise mechanisms underlying the protective effect of increased HSP72 expression against disuse muscle atrophy are unclear, it is thought that HSP72 prevents proteolysis by inhibiting key atrophy signaling pathways (36). Further, increased HSP72 expression may also protect against muscle atrophy by assisting in the refolding of oxidized proteins, thus protecting these proteins from proteolysis (20, 26). Regardless of the mechanisms responsible for HSP72-mediated protection against limb muscle atrophy, increased muscle levels of HSP72 clearly show a protective effect against inactivity-induced atrophy of locomotor skeletal muscles.

While exposure of animals to heat stress has been shown to be protective against disuse locomotor muscle atrophy (26, 35), it is unclear whether whole body heat stress can protect the diaphragm against MV-induced atrophy. Therefore, the present study was performed to determine whether whole body heat stress performed 24 h prior to prolonged MV could protect the diaphragm against MV-induced atrophy. We hypothesized that a single exposure to whole body heat stress would increase the expression of HSP72 in the diaphragm, and that this treatment would protect against MV-induced diaphragmatic atrophy.

MATERIALS AND METHODS

Animals. All experimental protocols were approved by the institutional ethics review committee of Juntendo University (H24-03), and the experiments were performed according to the American Physiological Society “Guiding Principles in the Care and Use of Animals.”

Adult male Wistar rats (3 mo old) were randomly assigned to one of four groups: 1) an acutely anesthetized control group (CON, n = 9) with no MV; 2) a 12-h controlled MV group (CMV, n = 10); 3) 1-h whole body heat stress group (HS, n = 10); or 4) 1-h whole body heat stress 24 h prior to 12-h controlled MV (HSMV, n = 9). All rats were maintained under a 12:12-h light-dark cycle with standard chow and water ad libitum for at least 1 wk before use.

Experimental design. Control animals were acutely anesthetized using pentobarbital sodium (ip injection of 60 mg/kg body wt) and the diaphragm was quickly removed. Animals in the HS and HSMV groups were exposed to 1-h whole body heat stress using a heat chamber. To ensure the same time intervals between heat stress and dissection with HSMV rats, rats from the HS group were anesthetized immediately following the 12-h control MV, CMV and HSMV animals were killed and diaphragm was removed. The time interval between completion of heat stress and initiation of controlled MV in the HSMV group was 24 h. After the entire diaphragm was removed, the costal diaphragm was divided into several segments for subsequent analysis.

Application of heat stress. Animals in HS and HSMV groups were exposed to heat stress (40–41°C, 60 min) in a heat chamber (TVG321AA; Advantec, Tokyo, Japan) without anesthesia (26, 41).
This heat stress increased the colonic temperature of the animals to ~40°C following 30 min of heat exposure (LTSA; Gunma, Saitama, Japan). Specifically, following 60 min of heat stress, colonic temperature increased to 41.1–41.4°C, suggesting that colonic temperature was maintained above 40°C for 30 min.

Mechanical ventilation. All surgical procedures were performed using aseptic technique. Animals from the CMV and HSMV groups were anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg body wt), tracheostomized, and mechanically ventilated (tidal volume = 0.6 ml/100 g body wt, 80 breaths/min, humidified ambient air) with a volume-cycled ventilator (SAR-1000; CWE, Ardmore, PA) for 12 h. ECG and heart rate were monitored via a lead-II ECG using needle electrodes placed subcutaneously. The carotid artery was cannulated to permit the continuous measurement of arterial blood pressure and periodic blood sampling (every 4 h) for analysis of arterial blood gas (cobas b 221; Roche Diagnostics, Tokyo, Japan). Arterial P\textsubscript{O\textsubscript{2}}, P\textsubscript{CO\textsubscript{2}}, and pH were maintained within their respective normal ranges by adjustment of tidal volume. A venous catheter was placed in the jugular vein for continuous infusion of pentobarbital sodium (10 mg·kg\textsuperscript{-1}·h\textsuperscript{-1}). Body (i.e., colonic) temperature was maintained at 36.5–37.5°C with a water-circulating warming pad. Continuous care during MV included expressing the bladder, lubricating the eyes, rotating the animal, and passive limb movement. Animals also received intramuscular injections of glycopyrrolate (0.04 mg/kg) prior to MV every 2 h during MV to reduce airway secretions.

Histological measures. Serial cross-sections from frozen costal diaphragm muscle samples were cut at a thickness of 10 \mu m using a microtome (CM3050S; Leica, Wetzlar, Germany) at ~20°C. Immunohistochemical staining was performed to identify muscle fiber types. Unfixed cryosections were rinsed in 0.1 M phosphate-buffered saline (PBS) and incubated in 0.3% Triton X-100/0.1 M PBS before blocking with 3% bovine serum albumin (BSA) in 0.1 M PBS at room temperature. Primary antibodies used for fiber typing were: M8421 (type I, 1:500; Sigma, St. Louis, MO), M4276 (type II, 1:500; Sigma), SC-71 (type IIA, 1:400), and BF-35 (all fibers except type Ix, 1:200). All antibodies were diluted in 3% BSA/0.1 M PBS and applied to sections overnight at 4°C. After rinsing with 0.1 M PBS, Alexa Fluor 488 goat anti-mouse IgG antibody (A11003; Invitrogen, Carlsbad, CA) was applied as secondary antibody (1:500). Hematoxylin and eosin staining was also performed for measurement of cross-sectional area (CSA). Sections were viewed under a fluorescence microscope (DM5000B; Leica) with a 10x objective and captured using a digital camera (IM 500; Leica). Diaphragm muscle fiber CSA was determined using Scion Image (NIH, Bethesda, MD).

Biochemical measures. Frozen costal diaphragm muscle samples were minced, and a portion of the muscle (~80 mg) was homogenized in 10 volumes of ice-cold homogenization buffer [20 mM HEPES, pH 7.4, 60 mM dithiothreitol, 1% (wt/vol) Triton X-100] containing protease inhibitor cocktail (Complete EDTA-free and Phe/STHD: Roche, Penzberg, Germany). The homogenates were centrifuged at 12,000 g for 20 min at 4°C to collect the cytosolic fraction. The supernatant protein concentration was determined using a Bio-Rad mini protein assay kit (Bio-Rad, Hercules, CA). The insoluble pellets corresponding to the particulate fraction were subsequently washed three times in 5 volumes of ice-cold homogenization buffer and centrifuged at 12,000 g for 5 min at 4°C. The pellets were then resuspended in 10 volumes of lysis buffer consisting of 20 mM HEPES (pH 7.4), 250 mM NaCl, and 1% (wt/vol) sodium dodecyl sulfate (SDS), and centrifuged at 17,000 g for 5 min at 4°C. The supernatant was saved and the protein concentration of the solubilized particulate fraction was determined using a BCA Protein Assay kit (Thermo Scientific, Rockford, IL).

Protein extracts were solubilized in sample buffer [30% glycerol, 5% 2-mercaptoethanol, 2.3% SDS, 62.5 mM Tris-HCl (pH 6.8), and 0.05% bromophenol blue] at 2 mg/ml and incubated at 95°C for 5 min. Proteins were then loaded onto 6.5–12.5% SDS-polyacryl-
post hoc tests were used for multiple comparisons. In all analyses, variances, and then a two-way analysis of variance (ANOVA) or Forsythe test was performed to determine the equality of group performed in two groups (i.e., CMV and HSMV); these data were significance.

Muscle (in g/cm3) (31, 34).

Table 1. Initial body weight, blood gas, and arterial blood pressure at completion of 12-h of mechanical ventilation

<table>
<thead>
<tr>
<th>Variable</th>
<th>CON</th>
<th>HS</th>
<th>CMV</th>
<th>HSMV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>303.6 ± 3.4</td>
<td>307.5 ± 2.0</td>
<td>304.1 ± 2.9</td>
<td>304.2 ± 2.9</td>
</tr>
<tr>
<td>PaO2, mmHg</td>
<td>NA</td>
<td>NA</td>
<td>82.2 ± 2.5</td>
<td>84.7 ± 2.5</td>
</tr>
<tr>
<td>PaCO2, mmHg</td>
<td>NA</td>
<td>NA</td>
<td>35.1 ± 1.0</td>
<td>36.1 ± 1.0</td>
</tr>
<tr>
<td>pH</td>
<td>NA</td>
<td>NA</td>
<td>7.49 ± 0.01</td>
<td>7.49 ± 0.01</td>
</tr>
<tr>
<td>Arterial blood pressure, mmHg</td>
<td>NA</td>
<td>NA</td>
<td>81.7 ± 14.2</td>
<td>85.1 ± 18.6</td>
</tr>
</tbody>
</table>

Values are means ± SE. PaO2, arterial PO2; PaCO2, arterial PCO2; NA, not applicable; CON, control (n = 9); HS, 1-h whole body heat stress (n = 10); CMV, 12-h controlled mechanical ventilation (n = 10); HSMV, 1-h whole-body heat stress 24 h prior to 12-h controlled mechanical ventilation (n = 9). No significant differences were observed among groups for any of the variables.

Results

Systemic and biological responses to MV. The systemic and biological responses to MV are shown in Table 1. Blood gases and arterial blood pressure were maintained within their respective normal ranges during 12-h MV and did not differ between the two MV groups. Heart rate (300–400 beats/min) and body temperature (36.5–37.5°C) were maintained within their respective normal ranges during the MV period. On completion of MV, no visual abnormality of the lungs or peritoneal cavity was observed.

Diaphragmatic fiber atrophy. Fiber CSA was determined for individual fiber types in all groups (Fig. 1). Similar to previous studies, prolonged MV resulted in significant atrophy of type I, type IIa, and type IIx fibers in the costal diaphragm. Note that prolonged MV resulted in a greater rate of atrophy in type II fibers compared with type I fibers. This greater rate of fiber atrophy resulted in an increase in the percentage of CSA occupied by type I fibers (Table 2). No significant difference in CSA area was observed for type I, type IIa, or type IIx fibers between the CON and HSMV animals, indicating that heat stress performed prior to MV attenuated MV-induced diaphragmatic atrophy. Further, no differences existed in muscle fiber type among the experimental groups.

HSP72 expression in the diaphragm. Compared with CON animals, diaphragmatic HSP72 expression increased significantly in the HS and HSMV groups (Fig. 2). Moreover, HSP72 expression levels in the diaphragm were greater in HSMV than in CMV animals. These results indicate that whole body heat stress as performed in this study significantly increased HSP72 expression in diaphragm muscle.

Autolyzed calpain-1, cleaved caspase-3, and ubiquitinated protein expression in the diaphragm. Prolonged MV significantly increased cleaved (activated) caspase-3 expression in diaphragm muscle in the CMV group (Fig. 3). Heat stress

![Fig. 1. Fiber cross-sectional area (CSA) in diaphragm muscle fibers from all four experimental groups. Values are means ± SE. *Significantly different from all groups (P < 0.05); **P < 0.01). CON, control (n = 9); HS, 1-h whole body heat stress (n = 10); CMV, 12-h controlled mechanical ventilation (n = 10); HSMV, 1-h whole body heat stress 24 h prior to 12-h controlled mechanical ventilation (n = 9).](http://jap.physiology.org/)

![Fig. 2. Heat shock protein 72 (HSP72) expression in diaphragm muscle. Values are means ± SE. #Significantly different from CON (P < 0.05). †Significantly different from CMV (P < 0.05). CON, n = 9; HS, n = 10; CMV, n = 10; HSMV, n = 9.](http://jap.physiology.org/)
decreased MV-induced expression of cleaved caspase-3 by 39%; however, the difference was not significant. In contrast, no significant difference among groups was observed for autolyzed calpain-1 or ubiquitinated protein levels. 

**SOD levels in the diaphragm.** No significant difference in SOD-1 or SOD-2 expression was observed among groups (Fig. 4). These results indicated that neither 12-h MV nor heat stress affected the expression levels of SOD-1 and SOD-2.

**Levels of oxidized proteins in the diaphragm.** Levels of oxidized proteins in the diaphragm increased significantly in response to prolonged MV (Fig. 5). Although heat stress tended to protect the diaphragm against MV-induced protein oxidation, the effect was not statistically significant.

**Actin and myosin protein content.** Actin content in the costal diaphragm decreased significantly as a result of prolonged MV, but this loss of actin protein was significantly attenuated by heat stress (Fig. 6). No change in myosin content was observed among groups.

**Diaphragm contractile properties.** To examine whether heat stress can protect against diaphragmatic contractile dysfunction, we measured both in vitro maximal isometric twitch force and the force-frequency responses in excised strips of costal diaphragm muscle. Compared with controls, 12 h of controlled MV resulted in a significant reduction in diaphragm muscle force production at all stimulation frequencies above 15 Hz (Fig. 7). Note that heat stress did not rescue the diaphragm from MV-induced contractile dysfunction.

**DISCUSSION**

**Overview of principal findings.** Application of heat stress did not protect against MV-induced diaphragmatic contractile dysfunction. A detailed discussion of these and other major findings follows.

**Heat stress protects against MV-induced atrophy.** In agreement with previous studies in limb skeletal muscle suggesting that heat stress can protect against muscle atrophy (11, 26, 35), our results indicated that whole body heat stress has a protective effect against MV-induced diaphragmatic atrophy. Importantly, this heat stress-induced protection against ventilator-induced atrophy was observed in all diaphragm muscle fiber types. Note that the magnitude of MV-induced diaphragmatic
atrophy observed in the present study was similar to those described in previous reports (10, 17, 22, 30, 38).

HSP72 level in type I fibers typically exceeds that in type II fibers (13, 18). Nonetheless, although heat stress increased HSP72 levels in the diaphragm, it did not alter the relative percentages of type I and II fibers in this key inspiratory muscle. Regarding HSP72 in skeletal muscle, previous studies have postulated that heat stress-induced expression of HSP72 in skeletal muscle is responsible for the protective effect of whole body heat stress against disuse locomotor muscle atrophy (26, 35). In this regard, exposing animals to brief periods of whole body heat stress has been shown to significantly elevate HSP levels in rat tissue (5), and the magnitude of this response depends on muscle fiber type. For example, Oishi et al. (28, 29) demonstrated that heat stress results in small increases in the expression of HSP72 in the soleus muscle, which has a high percentage of type I fibers, whereas heat stress promotes a much larger increase in HSP72 expression in the plantaris muscle, which is composed primarily of type II muscle fibers. Note that compared with a previous study (29) the magnitude of heat stress-induction of HSP72 in the diaphragm was lower than that of fast muscle fibers in locomotor skeletal muscles; however, it was similar to the magnitude of induced HSP72 expression in the diaphragm following a short period of endurance exercise training (38). Importantly, endurance exercise training prior to MV has been shown to have a preventive effect against MV-induced diaphragmatic atrophy (38). Therefore, it is possible that the protective effect of heat stress against MV-induced diaphragmatic atrophy is directly linked to the increase in HSP72 levels in the diaphragm.

With regard to the mechanisms underlying the protective effect of HSP72, it has been established that HSP72 can protect against calcium- and mitochondrial-related damage in muscle myotubes (21). In addition, HSP72 is known to inhibit apoptosis through caspase-dependent and -independent mechanisms (33). Previous work has shown that HSP72 attenuates the expression of the activated form of caspase-3, suggesting that HSP72 plays a role in protection against apoptosis (44). In the present study, the expression of cleaved caspase-3 increased significantly in the diaphragm following 12 h of MV (Fig. 3). In contrast, prior heat stress successfully protected the diaphragm against MV-induced cleaved caspase-3 expression in the diaphragm (Fig. 3). This finding is important because pharmacological inhibition of caspase-3 activation in the diaphragm can attenuate MV-induced diaphragmatic atrophy (22, 27). Therefore, prior heat stress may protect the diaphragm against MV-induced atrophy, at least in part, by preventing caspase-3 activation. With regard to the role of protease activation in MV-induced diaphragm atrophy, previous studies indicated that prolonged MV activates numerous proteolytic systems in the diaphragm, including calpain and caspase-3.

Fig. 5. Protein oxidation as determined by normalization of protein carbonyl levels against Ponceau S-stained protein expression. Values are means ± SE. #Significantly different from CON (P < 0.05). CON, n = 9; HS, n = 10; CMV, n = 10; HSMV, n = 9.

Fig. 6. Actin and myosin protein content. Values are means ± SE. #Significantly different from CON (P < 0.05). CON, n = 9; HS, n = 10; n = 10; CMV, n = 10; HSMV, n = 9.

Fig. 7. Effect of 12-h controlled mechanical ventilation on diaphragmatic muscle force-frequency responses in vitro. Values are means ± SE. #Significantly different from CON (P < 0.05). $Significantly different from HS (P < 0.05). CON, n = 9; HS, n = 10; CMV, n = 10; HSMV, n = 9.
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(22, 37). Emerging evidence suggests that MV-induced diaphragmatic atrophy is dependent on the activation of both calpain and caspase-3 via a cross-talk activation mechanism (27). In contrast to these reports, Supinski et al. (40) concluded that calpain and caspase-3 are activated in parallel and remain independently active in septic animals exposed to prolonged MV.

It was reported recently that nuclear factor of activated T cells (NFAT) proteins function in cellular adaptations to environmental changes (15, 16, 45). Indeed, NFAT-regulated gene expression is activated in response to cellular heat stress (12). NFAT activity is regulated by calcineurin and heat shock transcription factor 1 (HSF1) (12). Further, HSF1 also regulates the expression of HSPs. It follows that NFAT may also contribute to the protective effect of heat stress against MV-induced diaphragmatic atrophy. However, further studies are required to determine if NFAT activation contributes to heat stress-induced protection against diaphragmatic atrophy.

In the present study, calpain-1 expression increased in the diaphragm during prolonged MV, but the change did not reach statistical significance. Similarly, the levels of ubiquitinated proteins tended to increase in the diaphragm during MV, but these increases were not statistically significant. Nonetheless, these results do not preclude a potential role for the ubiquitin-proteasome pathway in diaphragmatic atrophy because the 20S protease can degrade nonubiquitinated proteins (43).

Heat stress does not protect against MV-induced decreases in diaphragmatic specific force production. Although several studies have investigated the effects of heat stress on locomotor muscle atrophy, there has been no investigation to date regarding the impact of heat stress on muscle contractile dysfunction induced by prolonged disuse. In this first report on this important issue, we found that 1 h of heat stress 24 h prior to MV does not confer protection against MV-induced diaphragm contractile dysfunction.

It has been reported that heat stress increases the antioxidant capacity of skeletal muscles (35). Oxidants decrease calcium release and calcium sensitivity of the contractile apparatus (1, 23) and impair sarcormic protein function (3). Indeed, administration of antioxidants prevents endotoxin-induced diaphragm dysfunction (39). In this study, oxyprotein levels increased significantly after prolonged MV, as expected. Contrary to our expectations, however, heat stress did not attenuate oxyprotein levels. Moreover, heat stress did not increase SOD-1 or -2 levels in this study. While it has been established that oxidative stress plays a necessary role in the development of ventilator-induced diaphragmatic dysfunction, whole body heat stress apparently does not elevate diaphragmatic oxidants to a level that provides protection. Yamashita et al. (46) reported that peak SOD values were observed after 48 h of whole body heat stress at 41ºC. Thus it is possible that the time interval between heat stress and MV in the present study was not sufficient to increase antioxidant capacity.

Finally, prolonged MV is known to result in a progressive impairment of diaphragm specific force production at all stimulation frequencies (2, 31). Further, prolonged MV also results in diaphragm fiber atrophy (32). Therefore, the combination of MV-induced diaphragm fiber atrophy and the MV-induced reduction in diaphragm specific force production act in concert to decrease total force generation in the diaphragm. Although heat stress performed before MV was not successful in protecting the diaphragm against MV-induced reductions in diaphragm specific force production, it was effective in protecting against MV-induced diaphragmatic atrophy. This is important because several lines of evidence suggest that depressed inspiratory muscle strength contributes to difficult weaning [6, 42; see Powers et al. (32) for more details on the influence of prolonged MV on diaphragmatic contractile dysfunction and the impact of impaired inspiratory muscle function on weaning difficulties].

Conclusions. In summary, 1 h exposure of animals to whole body heat stress 24 h prior to prolonged MV does not protect against MV-induced diaphragm contractile dysfunction but does provide protection against MV-induced diaphragmatic atrophy. The mechanisms underlying this heat stress-induced protection remain unclear but may be linked to increased expression of HSP72 and subsequent protection against caspase-3 activation in the diaphragm. Regardless of the mechanisms responsible for this protective effect, the discovery that heat stress provides protection against MV-induced diaphragm atrophy is a new and important finding that could have significant clinical implications in the prevention of MV-induced diaphragmatic weakness.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES

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