Heat treatment reduces oxidative stress and protects muscle mass during immobilization

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Selsby, Joshua T., and Stephen L. Dodd. Heat treatment reduces oxidative stress and protects muscle mass during immobilization. Am J Physiol Regul Integr Comp Physiol 289: R134–R139, 2005. First published March 10, 2005; doi:10.1152/ajpregu.00497.2004.—This study examined the role of heating on oxidative stress and muscle mass in immobilized limbs. Rats were divided into three groups (n = 9/group): a control group (Con), an immobilized group (Im), and an immobilized and heated group (ImH). Rats were immobilized in the plantarflexed position for 8 days. The core temperature of the ImH group was elevated to 41–41.5°C on alternating days and maintained for 30 min before cooling. On day 8, both heat shock protein 25 (HSP25) and HSP72 were markedly elevated in the ImH compared with the Im group, whereas results in the Im group were not different from Con. Most notably, the ImH group had significantly larger solei compared with the Im group, which were less than those shown in the Con group. Furthermore, immobilization alone caused a significant increase in oxidative damage, and the addition of heating to immobilization significantly reduced oxidative damage. In an effort to further identify the cause of this protective effect, antioxidant enzyme activities were assessed. Cu/ZnSOD was sharply elevated in Im compared (P < 0.025) with that in the Con and reduced in the ImH group compared with that in the Im group (P < 0.025). Catalase was elevated 8% (P < 0.025) in the Im group compared with the Con group and was similar to the ImH group. Glutathione peroxidase, glutathione reductase, and MnSOD did not differ between groups. These data indicate that heating provides protection against oxidative stress and preserves muscle mass during disuse atrophy. These data also suggest that antioxidant protection is not conferred via antioxidant enzymes, and HSPs may play an important role.

free radicals; antioxidant; rat; soleus

SKELETAL MUSCLE IS A TISSUE that will readily adapt in response to changes in loading pattern. In the case of increased load, the response is hypertrophy. In contrast, atrophy occurs with a reduction in load. The most obvious indication of atrophy is a reduction in muscle mass and cross-sectional area (4, 11, 16, 28). Despite the well-known characteristics of an atrophying fiber, little is known about the basic mechanisms of atrophy.

Two models commonly used to induce atrophy are cast immobilization and hindlimb unweighting (HLU) (for review, see Refs. 1, 30). With HLU, there is an initial reduction in recruitment, which is nearly absent by 1 wk. However, with immobilization, there is a reduction in recruitment that persists for the duration of immobilization (3, 17). Despite the fact that the unweighted soleus is capable of contracting, atrophy still occurs because the muscle is only capable of very low-force contractions as the only load is provided by the mass/inertia of the foot. During immobilization, although the muscle is capable of producing force through an isometric contraction, EMG activity is drastically reduced; therefore, few fibers are recruited. Furthermore, when the soleus is immobilized in a shortened position, any fiber recruitment will result in very low-force contractions because the actomyosin complexes are in a near-maximally shortened position. Although differing in their nature, HLU and immobilization both result in dramatic losses of skeletal muscle mass. This loss in mass appears to be primarily of type I fibers, with a minor contribution from type II fibers during HLU and both types I and II during immobilization (8, 46). Nevertheless, both models result in a soleus that has undergone a type II shift and that has developed contractile properties reminiscent of this fiber type (15, 36, 47, 49).

Several reports have strongly implicated oxidative stress as partially causative of disuse atrophy as both damage to lipids and proteins have been detected (4, 21–26, 28). In addition to oxidative damage present during muscle disuse, antioxidant enzymes respond in a manner that suggests an elevated free radical content (22, 28). Indeed, Kondo et al. (26) were able to show elevated hydroxyl radicals in immobilized tissue. This group also showed that xanthine oxidase and free iron were contributing sources of free radical production during immobilization (23–25).

Several studies have furthered these observations and utilized antioxidant supplementation in an effort to reduce oxidative damage and preserve muscle mass and mass cross-sectional area with varying degrees of success during disuse atrophy (4, 19, 21, 24). Kondo et al. (21, 24) supplemented immobilized animals with vitamin E and reduced oxidative damage and increased muscle mass in immobilized animals. In addition, Appel et al. (4) found larger muscle cross-sectional area in vitamin E-supplemented animals following immobilization. In contrast, Koesterer et al. (19) found that an antioxidant cocktail containing vitamin E failed to preserve muscle mass during unweighting.

In addition to conventional antioxidants, heating has been used as a countermeasure to disuse atrophy (32). At the completion of one bout of whole body heating, Naito et al. (32) unweighted the hindlimbs of rats for 8 days. After the unweighting period, heat-treated animals had higher heat shock protein 72 (HSP72) levels as well as an attenuated soleus muscle mass loss compared with suspended rats not receiving heat treatment. Naito et al. (32) proposed that expression of HSP72 was necessary for maintenance of protein synthesis rates in the heated hindlimbs, whereas a nonheated suspended group would be characterized by a decline in protein synthesis. It has been hypothesized that increased expression of HSP72 enhances protein synthesis by maintaining the elongation phase of translation.
of protein synthesis. Furthermore, it has been hypothesized that HSP72 protects proteins from proteolysis by refolding damaged proteins.

It is now well accepted that heating animals increases not only HSP72 but many other HSPs as well; among them is HSP25 (HSP27 in humans). The most recent evidence suggests that these proteins can assist in the maintenance of protein synthesis rates, refold damaged proteins, scavenge free radicals, and inhibit apoptosis (6, 13, 27, 29, 40, 41). By chaperoning nascent polypeptides, the rate of protein synthesis may remain elevated, whereas the reduction in synthesis rates may be prevented. By refolding damaged proteins, they are not degraded and hence remain functional. If free radicals are removed, they cannot contribute to protein damage, leading to their subsequent degradation. Finally, preserving myonuclei may help to preserve the protein synthesis potential of a muscle. Therefore, the purpose of this investigation was to determine the effect of heating on oxidative stress and muscle mass during immobilization. We hypothesized that heating during immobilization would decrease oxidative damage and result in a larger muscle mass compared with a group exposed to immobilization alone.

**METHODS**

Experimental design. All procedures and experiments were conducted with the approval of the Institutional Animal Care and Use Committee at the University of Florida. Animals were housed in a 12:12-h light-dark photoperiod in an environmentally controlled room. Upon arrival in the facility, animals were handled daily for 1 wk before the initiation of experiments in an effort to minimize contact stress. Male Sprague-Dawley rats were randomly divided into three groups: a control group (Con; n = 9), an immobilized group (Im; n = 9), and an immobilized and heated group (ImH; n = 9). The hindlimbs of animals were casted in the plantarflexed position for a period of 8 days. Animals given the heat therapy were heated 24 h before immobilization and then on alternating days during the immobilization period. The Im and ImH animals were pair fed, whereas the Con animals were fed ad libitum. After completion of the 8-day immobilization period, a surgical plane of anesthesia was induced via intraperitoneal pentobarbital injection and the cast was removed.

Then, the soleus was removed, trimmed of excess fat, tendon, and nerve, blotted dry, weighed, and immediately frozen in liquid nitrogen for subsequent analysis. The final heat treatment was given 48 h before death.

*Immobilization.* Anesthesia was induced with a 5% isoflurane gas-oxygen mixture and maintained with a 1.5–2% isoflurane gas-oxygen mixture administered through a calibrated air flowmeter (Veterinary Equipment and Technical Service, Gainesville, FL). Animals were immobilized bilaterally in the plantarflexed position as to cause maximal atrophy in the triceps surae muscle group in accordance with the model described by Booth and Kelso (9) with modifications. Brieﬂy, animals were wrapped in a protective adhesive (Medipore Dress-it, 3M, St. Paul, MN) so that the animals would not come in contact with the plaster. The wrap began in the supra-abdominal area, below the level of the ribs, and continued down the abdomen of the animal and stopped in the infra-abdominal area such that urination would not be affected and continued down the hindlimbs. A quick-drying plaster was then applied and allowed to dry (Specialist, Johnson and Johnson, New Brunswick, NJ). Finally, a Plexiglas wrap was applied so that rats could not chew through the cast (Scotchcast Plus, 3M).

*Heat treatment.* Animals were anesthetized using isoflurane, as detailed above. A rectal probe was inserted and secured to the tail to ensure that it would not become displaced (YSI, Yellow Springs, OH). The animal was then wrapped in a prewarmed thermal blanket (Kaz, Hudson, NY), keeping the tail and head exposed. The tail was exposed because it was the anchor for the rectal probe. The head was visible to ensure that the nose cone remained in place to prevent accidental recovery from anesthesia.

Core temperature was continuously monitored and recorded every 2 min. Heating time started as soon as the core temperature of the animal breached 41°C, and temperature was maintained at 41–41.5°C for 30 min. At 30 min, the animal’s core temperature was lowered via convection cooling and continually monitored until the core temperature dropped below 39.9°C. Our preliminary work has shown that this produces an elevation in HSP expression that peaks between 24 and 48 h before the unheated control levels are reached by 48 h (data not shown). This is in contrast to local heating, which produces a peak in HSP levels that is resolved by 8 h postheating (34). Immobilized animals not receiving a heat treatment were given the same treatment except that core temperature was maintained at ~37°C for the 30-min period.

Western blot. Muscle was homogenized utilizing the technique of Solaro et al. (42). Briefly, frozen tissue was homogenized in sucrose buffer at a mass-to-buffer ratio of 20:1. The resulting homogenate was then centrifuged at 1,000 g for 10 min, and the supernatant was removed and the pellet discarded. Protein concentration was then determined by use of the biuret technique of Watters (48). Samples were diluted to 1 mg/ml in sample buffer containing 62.5 mM Tris (pH 6.8), 1.0% SDS, 0.01% bромphenol blue, 15.0% glycerol, and 5% β-mercaptoethanol. Samples were denatured via heating to 60°C for 15 min in a glass bead heater.

Precisely 10 μg of protein were loaded into 4–20% vertical precast gels (Cambrex, Rockland, ME). Samples were then electrophoresed at room temperature for 30 min at 50 V followed by 90 min at 120 V (Bio-Rad, Hercules, CA). Gels were removed from the electrophoresis apparatus and allowed to cool for 15 min in transfer buffer containing 25 mM Tris, 192 mM glycine, 0.02% SDS, and 20% methanol (pH 8.3). After the conditioning period, horizontal electrophoresis (100 V, 60 min, 4°C) was performed, and proteins were transferred to a nitrocellulose membrane with a pore diameter of 0.2 μm (Bio-Rad). Membranes were then stored in Tris-buffered saline containing 0.1% Tween 20 (TTBS). Membranes were blocked by exposure to a 5% dehydrated milk TTBS solution for 60 min. Membranes were washed for 10 min three times and exposed to the appropriate primary antibody as follows: HSP25 (SPA 801, Stressgen, Victoria, British Columbia; primary: 1:10,000; secondary: 1:2,000), HSP72 (SPA 810, Stressgen; primary: 1:1,000; secondary: 1:1,000), 4-hydroxy-2-nonenol (HNE; HNE11-S, Alpha Diagnostic International, San Antonio, TX; primary: 1:500; secondary: 1:1,000), and nitrotrosine (no. 9691, Cell Signaling Technology, Beverly, MA; primary: 1:1,000; secondary: 1:2,000) for 90 min. The membranes were washed three times at 10 min each and exposed to the appropriate secondary antibody for 60 min (Amersham, Little Chalfont, Buckinghamshire, UK). HNE is a highly cytotoxic compound formed by free radical attack of fatty acids. Nitrotrosine is a product of reactive nitrogen species acting on tyrosine residues. These are included as markers of oxidative stress.

The secondary antibody was diluted in TTBS containing 1.5–2% milk protein. Membranes were then washed three times at 10 min each and exposed to enhanced chemiluminescence (Amersham) for 2 min. Finally, the membranes were placed in a Kodak Image Station 440 CF developer, and the emitted signal was captured. The signal was analyzed using Kodak ID image analysis software (Eastman Kodak Scientific Imaging Systems, Rochester, NY).

**Enzymatic assays.** All enzymatic assays were performed in triplicate in microplates using a Spectramax 190 microplate reader ( Molecular Devices, Downingtown, PA) in whole homogenate diluted 1:100 in PBS buffer. Glutathione peroxidase (GPX) activity was determined by the method of Flohe and Gunzler (12); glutathione reductase (GR) activity was determined by the method of Carlberg and Yuan (44).
and Mannervik (10); catalase activity was determined by the method of Aebi (2); and superoxide dismutase (MnSOD and CuZnSOD) activities were determined simultaneously by the method of McCord and Fridovich (31).

**Statistical analysis.** Data were analyzed using a t-test comparing Con to Im and Im to ImH. At no time was Con compared with ImH. Inflation of /H9251 was corrected via the Bonferroni correction; /H9251 was set a priori at /H11021 0.05; however, the correction requires /H11021 0.025 for significance to be achieved. Data are reported as means ± SE.

**RESULTS**

Body weights before the study began were not different between the groups (Con = 321 ± 6 g, Im = 318 ± 3 g, and ImH = 319 ± 7 g). After the 8-day period of immobilization, body weight in the Im group was significantly less than that for the Con group (Im = 269 ± 3 g; Con = 347 ± 4 g). Application of the heating protocol did not correct this reduction (ImH = 265 ± 4 g).

There was no difference in HSP25 (Fig. 1, A and B) or HSP72 (Fig. 1, C and D) expression between the Im and Con groups. However, there was a 75% increase in HSP25 and a sevenfold increase in HSP72 in the ImH group compared with the Im group.

In regard to the soleus muscle mass, there was an ~40% reduction in soleus mass in the Im group compared with the Con group. The reduction in mass was attenuated by nearly 20% in the ImH group compared with the Im group, resulting in a significantly larger muscle (Fig. 2A). Furthermore, when expressed relative to body weight, muscle mass was nearly 20% smaller in the Im group compared with the Con group. Heat treatment was able to attenuate this reduction by 50% (Fig. 2B).

Oxidant damage was determined by detection of HNE compounds as well as nitrosylated tyrosine residues. HNE was increased 33% in the Im group compared with the Con group, and heat treatment significantly reduced this elevation (Fig. 3, A and B). In a similar pattern, nitrotyrosine was increased 35% in the Im group compared with the Con group, and heat treatment significantly reduced this elevation (Fig. 3, C and D). Thus heat treatment does reduce the oxidative stress encountered during immobilization.

Finally, we wanted to determine whether HSPs provided the antioxidant effect observed. By eliminating the antioxidant enzymes as contributors to the overall antioxidant effect observed in the ImH, we proposed that evidence for the protective effects of HSPs would be gained. To determine this, we measured the activities of MnSOD, CuZnSOD, catalase, and the glutathione-handling enzymes GPX and GR (Table 1). There was no change in the activities of MnSOD, GPX, or GR. Catalase was significantly elevated in the Im group by ~10% compared with that shown in the Con group, and results for the ImH group were similar to those found in the Im group. CuZnSOD was significantly elevated with immobilization by 50% compared with that shown in the Con group, but heating eliminated this increase. These changes are supportive of the

**Fig. 1.** HSP25 representative blot (A) and relative expression (B) and HSP72 representative blot (C) and relative expression (D) after heating on alternating days for 8 days during hindlimb immobilization. There is no difference between control (Con) and immobilized (Im) animals. Animals receiving the heat treatment (ImH) had significantly more HSP25 and HSP72 than Im animals. OD, optical density. #Significantly different from Im (P < 0.025).

**Fig. 2.** After the immobilization period, the soleus muscle was removed, cleaned, and weighed (A). Immobilization caused a significant reduction in soleus muscle weight, whereas heating reduced the atrophy experienced by the muscle. Soleus muscle weight was then expressed relative to the body weight in grams (B). Immobilization resulted in a significant reduction in muscle-to-body weight ratio, whereas heating attenuated this loss. *Significantly different from Con and #significantly different from Im (P < 0.025).
antioxidant effects of HSPs as none of the enzyme activities increased in response to heat treatment; in fact, CuZnSOD activity was lower, suggesting a reduced superoxide content.

DISCUSSION

This study investigated the possibility that heat treatment may attenuate oxidative stress and disuse atrophy induced via hindlimb immobilization. HSPs increased in the ImH group after heat treatment. Absolute muscle mass and muscle mass-to-body mass ratio were significantly higher in animals in the ImH group compared with that shown in the Im group. To our knowledge, this is the first study to show that heating significantly reduced oxidative stress in the soleus during immobilization. Finally, antioxidant enzyme activities in the ImH group changed in a manner consistent with reduced oxidant stress.

A loss of Ca$^{2+}$ homeostasis within the cell may be responsible for the generation of free radicals (24, 26), and the associated oxidative stress may have contributed to the atrophy seen during immobilization. To assess this possibility, we measured two indexes of oxidative damage. Immobilization resulted in an increase in both HNE and nitrotyrosine compared with that shown in the Con group, indicating that there was an oxidant stress in immobilized muscle. This oxidative stress could be the result of an increased free radical production, a decreased antioxidant status, or a combination of the two. Both indexes were decreased in the ImH group compared with that shown in the Im group, indicating that heating was effective in reducing oxidant damage (Fig. 3). Because our heat treatment was clearly effective as an intervention to increase HSPs (Fig. 1), it is plausible, therefore, that the most important role of HSPs in this model may be their ability to buffer free radicals.

Given that HSPs have been shown repeatedly to decrease oxidative stress in a variety of models, it seems reasonable to suggest that they are providing protection against oxidant damage in this study. Specifically, transfected cells receiving an HSP25 or HSP72 expression gene demonstrated significantly higher survivability when exposed to H$_2$O$_2$ (20, 39) as well as reduced lactate dehydrogenase release (20). Additionally, transfected hearts overexpressing HSP72 suffered less mitochondrial dysfunction after ischemia reperfusion injury and had higher ventricular function compared with control hearts (18). Furthermore, gene array studies have shown that the only discernable trend is an increase in HSPs. Comparatively, these studies demonstrate inconsistent changes in other antioxidant substances, including MnSOD, CuZnSOD, cata-
lase, and various glutathione handling enzymes (38, 43, 44, 50). For example, in one investigation, glutathione-S-transferase and MnSOD are reduced, whereas in another both are increased after heating (38, 50). Nevertheless, the possibility remains that proteins other than HSP72 or HSP25 are responsible for the observed protective effect.

To further implicate HSPs as providing the antioxidant effect observed in this study, several antioxidant enzyme activities were evaluated, including MnSOD, CuZnSOD, catalase, and the glutathione handling proteins GPX and GR (Table 1). MnSOD activity did not change as a result of immobilization, in agreement with previous work (22, 25), and may suggest that superoxide is not elevated in the mitochondria (5, 14). CuZnSOD was significantly increased with immobilization, supporting what has been found previously (22, 25, 28). This likely suggests an elevation of superoxide in the cytosol of immobilized skeletal muscle (14). Heating, however, caused a reduction in the CuZnSOD activity, suggesting a reduction in superoxide in the ImH group (5, 14). In accordance with the literature, GPX activity did not change after immobilization for 8 days (22, 25). The lack of change exhibited by GR activity in this investigation differs from what would be expected from the literature. In two investigations, Kondo et al. (22, 25) showed an increase in GR activity over the same time period. Although seemingly unlikely, it is possible that the difference in animal type could lead to these dissimilar results. Catalase activity is elevated with immobilization and is in good agreement with what has been observed before in immobilization studies of similar duration (22, 25). The changes in enzyme activity allow for the speculation that an increased activity of CuZnSOD resulted in the increased production of H$_2$O$_2$. Because there was no increase in GPX and only a slight increase in Cat, it seems likely that there was an increased production in OH$^-$ via fenton chemistry. Indeed, Kondo et al. detected both an increased level of iron, needed for fenton chemistry, as well as OH$^-$ after immobilization. Furthermore, we report an increase in oxidative stress after immobilization.

In addition to reduced oxidative damage, muscles in the ImH group were significantly larger than those in the Im group, indicating that heating protected muscle mass. Support for our findings comes from a study by Naito et al. (32), which used heating to protect muscle mass during hindlimb suspension. Naito et al. (32) demonstrated that a single bout of heat for 60 min was sufficient to detect an increase in HSP72 8 days later. Furthermore, muscle atrophy was reduced in the heated-suspended group by $\sim$32%. Heating appears to be protective in both HLU and immobilization models, indicating a robust response to HSPs in disparate unloading models. Moreover, another indicator that oxidant stress plays a role in causing the loss of muscle mass is the observation that vitamin E has had some success as a countermeasure to disuse atrophy. It reduced the loss of cross-sectional areas by $\sim$66% (4) in one investigation and attenuated losses in muscle mass by $\sim$20% in another (21). However, in a third study, vitamin E did not protect against disuse atrophy (19).

Finally, it is noteworthy that HSP levels did not decrease during disuse in the Im group compared with the Con group. In several investigations, including those measuring mRNA and those measuring protein, reductions in HSP expression/content were detected (7, 32, 45). Furthermore, Ku et al. (27) detected a reduction in HSP70 associated with the polysomes following unloading. However, in this investigation and another (33), no such change is detected. Resolution to this disparity can be found when the gender of the animal used in each study is compared. Generally speaking, female animals tend to reduce HSP levels, whereas males do not. One notable exception to this trend was shown by Oishi et al. (35) who found a reduced HSP72 content following unloading in male rats.

In summary, we successfully reduced atrophy during 8 days of immobilization by application of a heat stress, which caused the elevation of both HSP25 and HSP72. Furthermore, this study is the first to show a reduction in oxidative stress in heated animals after immobilization. These data suggest that there may be an interaction between HSPs and oxidative stress under these conditions. The lack of an increase in antioxidant enzyme activity as well as data from gene array studies further supports the notion that HSPs provided protection from oxidative stress.

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REFERENCES


