

HIGHLIGHTED TOPIC | *Free Radical Biology in Skeletal Muscle*

Intermittent hyperthermia enhances skeletal muscle regrowth and attenuates oxidative damage following reloading

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Selsby JT, Rother S, Tsuda S, Pracash O, Quindry J, Dodd SL. Intermittent hyperthermia enhances skeletal muscle regrowth and attenuates oxidative damage following reloading. *J Appl Physiol* 102: 1702–1707, 2007. First published November 16, 2006; doi:10.1152/jappphysiol.00722.2006.—Skeletal muscle reloading following disuse is characterized by profound oxidative damage. This study tested the hypothesis that intermittent hyperthermia during reloading attenuates oxidative damage and augments skeletal muscle regrowth following immobilization. Forty animals were randomly divided into four groups: control (Con), immobilized (Im), reloaded (RC), and reloaded and heated (RH). All groups but Con were immobilized for 7 days. Animals in the RC and RH groups were then reloaded for 7 days with (RH) or without (RC) hyperthermia (41–41.5°C for 30 min on alternating days) during reloading. Heating resulted in ~25% elevation in heat shock protein expression ($P < 0.05$) and an ~30% greater soleus regrowth ($P < 0.05$) in RH compared with RC. Furthermore, oxidant damage was lower in the RH group compared with RC because nitrotyrosine and 4-hydroxy-2-nonenol were returned to near baseline when heating was combined with reloading. Reduced oxidant damage was independent of antioxidant enzymes (manganese superoxide dismutase, copper-zinc superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase). In summary, these data suggest that intermittent hyperthermia during reloading attenuates oxidative stress and improves the rate of skeletal muscle regrowth during reloading after immobilization.

oxidant stress; heat shock proteins; antioxidant

MUSCLE ATROPHY IS A CRITICAL health concern in several disease states and impacts the quality of life and ability to live independently in the elderly (28, 43). Often, atrophy is a necessary consequence of clinical interventions, such as bed confinement or limb immobilization. Independent of events leading to atrophy, reloading of the muscle is necessary to return to a preatrophy functional level. Much research has been dedicated to the determination of atrophic mechanisms. Comparatively little attention has been dedicated to the search for mechanisms and interventions that enhance regrowth during subsequent muscle reloading.

Reloading is marked by oxidative damage because cellular macromolecules are modified by free radicals (17). Furthermore, there is a cytosolic Ca^{2+} overload (2, 13, 37) contributing to a loss of Ca^{2+} homeostatic balance that likely exacerbates the oxidative stress response via xanthine oxidase-mediated superoxide anion radical ($\text{O}_2^{\bullet-}$) production (25, 29).

Specifically, elevated intracellular Ca^{2+} activates the protease calpain, which in turn converts xanthine dehydrogenase into the $\text{O}_2^{\bullet-}$ -producing xanthine oxidase. This oxidative stress response is also associated with contraction induced injury. Superoxide dismutase (SOD) supplementation attenuates the reduction in force associated with contraction-induced injury, indicating that loss in force generation is not solely mechanical in nature but that it is also related to production of reactive oxygen species (5, 47). Regardless of the source of free radicals, increased oxidant production during reloading results in increased lipid oxidation as well as oxidized glutathione (17). Furthermore, vitamin E administered concurrently with reloading results in a reduction in lipid oxidation and muscle mass recovers at a significantly faster rate (17).

In addition to chaperone qualities, heat shock proteins (HSPs) have repeatedly been shown to possess antioxidant properties. Recently, we used heating to induce HSPs and reduce oxidant damage and attenuate the muscle atrophy seen during immobilization (38). Studies in other tissues, designed to clarify the antioxidant roles of HSP 25 (HSP25) and HSP 72 (HSP72), have used molecular biology techniques to induce expression of these HSPs. HSP overexpression improved cell survivability and reduced cell damage in renal tubular cells and human neuroblastoma cells following H_2O_2 exposure (16, 46). Moreover, HSP72-overexpressing mutants recovered force significantly faster and had less damage following a lengthening contraction protocol than wild-type controls (24).

Thus it seems clear that HSPs can augment muscle regrowth by preventing oxidant stress. Based on this rationale, we hypothesized that intermittent hyperthermia during 1 wk of reloading after immobilization, will increase the rate of muscle regrowth and decrease oxidative damage. Findings from this study support these hypotheses and demonstrate, for the first time, that intermittent heating augments muscle regrowth and attenuates oxidative damage during reloading. Our data suggest a link between the enhanced regrowth rate, decreased oxidative damage and overexpression of HSPs.

METHODS

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. On arrival in the facility,

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Table 1. Body weight changes at various time periods during the investigation

	Con	Im	RC	RH
Initial body mass	357±3	357±3	351±5	358±4
Week 1 body mass	381±6	318±3*†	312±6*†	317±5*†
Week 2 body mass	405±9*	NA	314±6*†	301±4*†

Values are means ± SE given in g; $n = 10$ Con, 10 Im, 9 RC, 10 RH. Control (Con) animals spent weeks 1 and 2 with cage sedentary activity. Immobilized (Im), reloaded control (RC), and reloaded heated (RH) animals spent the first week immobilized. RC and RH were returned to cage sedentary activity during the second week while Im animals were killed following the first week. NA, not applicable. *Significantly different from the initial body mass within a group, $P < 0.05$. †Significantly different from Con between groups for the corresponding time point, $P < 0.05$.

animals were handled daily for 1 wk before the initiation of experiments in an effort to minimize contact stress. Male Sprague-Dawley rats (~350 g) were randomly divided into four groups including a control group (Con; $n = 10$), a group that was immobilized for 7 days (Im; $n = 10$), a group immobilized for 7 days and reloaded for 7 days (RC; $n = 10$), and a group immobilized for 7 days and reloaded for 7 days that received heat treatments on alternating days during reloading (RH; $n = 10$).

Animals were fed (ad libitum) and then matched to the RH group for feeding patterns 24 h before reloading (day of first heating). During the immobilization phase, no treatment differences in food consumption were observed.

Animal treatments. Animals were brought to a surgical plane of anesthesia via a 5% isoflurane gas oxygen mixture and maintained with a 1.5–2% isoflurane gas-oxygen mixture administered through a calibrated air flowmeter. Animals were cast immobilized bilaterally in the plantar-flexed position to cause atrophy in the triceps surae muscle group as previously described (38). After the immobilization period, animals were again anesthetized with isoflurane, and the casts were removed. Animals were then returned to their cages (RC and RH) for a period of 7 days or killed via lethal overdose (Im).

Heating was performed as described previously such that animal core temperature was increased to 41–41.5°C for 30 min under isoflurane induced anesthesia (38). The initial hyperthermic bout occurred 24 h before cast removal to ensure HSP overexpression before muscle reloading (RH). Additional animals were killed immediately following cast removal to ensure that a single heating bout did not attenuate atrophy. Animals not receiving a heat treatment (Con, Im, RH) were treated identically, except that core temperature was maintained for the 30-min period during a sham heating. We have found that HSP expression peaks between 24 and 48 h following

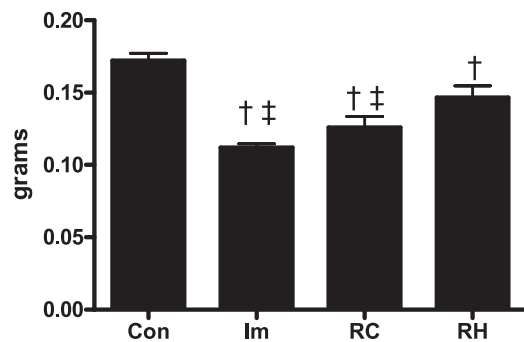


Fig. 1. Wet muscle mass in control animals (Con), following 1 week of immobilization (Im), immobilization followed by 1 wk of reloading (RC), or immobilization followed by 1 wk of reloading in combination with a heat treatment (RH). Values are means ± SE; $n = 10$ Con, 10 Im, 9 RC, 10 RH. †Different from Con, $P < 0.05$. ‡Different from RH, $P < 0.05$.

Table 2. Relative muscle mass, water content, and wet weight-to-dry weight ratio in the Con, Im, RC, and RH groups

	Con	Im	RC	RH
Relative muscle mass, mg/g	0.44±0.01	0.35±0.01*†	0.41±0.02†	0.48±0.03
%Water	78.2±0.5	77.5±0.4	78.1±0.5	78.5±0.7
Wet-to-dry weight Ratio	4.61±0.12	4.45±0.08	4.59±0.10	4.70±0.15

Values are means ± SE; $n = 10$ Con, 10 Im, 9 RC, 10 RH. *Different from Con, $P < 0.05$. †Different from RH, $P < 0.05$.

heating. To maintain HSP overexpression, heating and sham heating were performed every 48 h during the reloading phase. Animals were killed 48 h following the final heating bout; thus our results in heated animals represent sustained effects as opposed to transient hyperthermic responses that could be found if animals were killed immediately following a heating bout. Based on pilot experiments, we determined that increasing either peak core temperature or heating duration increased mortality.

Muscle removal and sample preparation. On the day they were killed, animals were anesthetized, and the soleus was removed, weighed, and immediately frozen in liquid nitrogen. Muscle was homogenized in a cell lysis buffer (no. 9803, Cell Signaling Technology) with a supplemental protease inhibitor cocktail [aprotinin, leupeptin, bestatin, pepstatin A, E-64 (AEBSF); no. 8340, Sigma] at a 20:1 mass-to-buffer ratio. The resulting homogenate was centrifuged at 300 g for 10 min to remove cellular debris. Protein concentrations were determined using the biuret technique of Watters (44).

To determine whether muscle water content was altered, total water content of muscles was determined by using a freeze-drying technique incorporating a vacuum pump with a negative pressure of ~1 mmHg. The measurement was terminated when the same weight was recorded three times in succession during a 48 h period.

Immunoblotting. Western blotting was done following standard techniques previously reported with precisely 15 µg/lane (38). Primary antibodies were used as follows: HSP 25 (SPA 801, Stressgen, Victoria, British Columbia, Canada), HSP 72 (SPA 810, Stressgen), nitrotyrosine (NT; no. 9691, Cell Signaling Technology, Beverly, MA) with an overnight primary incubation. Alternatively, a dot blot

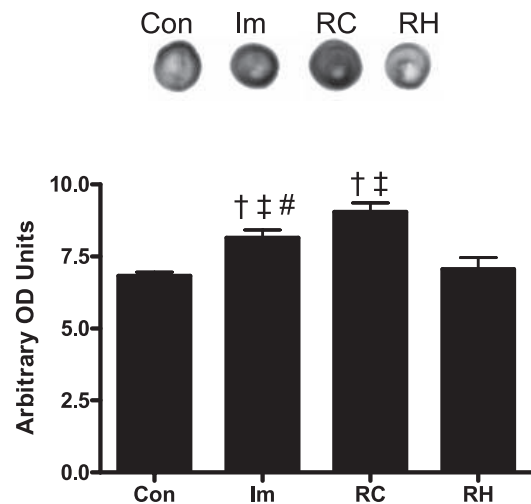
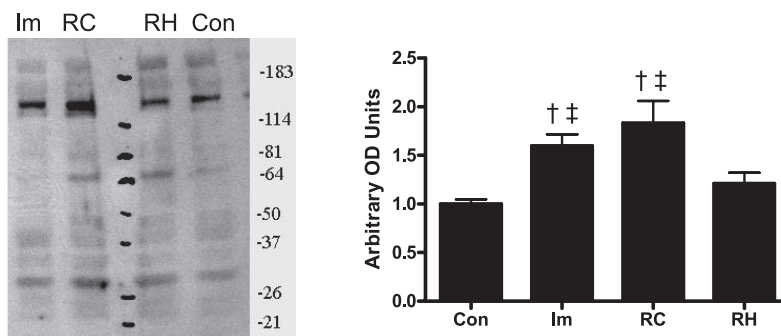


Fig. 2. 4-Hydroxy-2-nonenol as determined by immunoblotting in the Con, Im, RC, and RH groups. Values are means ± SE; $n = 8$ Con, 8 Im, 8 RC, 9 RH. †Different from Con, $P < 0.05$. ‡Different from RH, $P < 0.05$. #Different from RC, $P < 0.05$.

Fig. 3. Nitrotyrosine as determined by Western blotting in the Con, Im, RC, and RH groups. Optical density (OD) is determined by taking the sum total of each band's OD in the lane after correcting for local background. In the *middle* lane, pen marks denote molecular weight (nos. are at *right*). Values are means \pm SE; $n = 10$ Con, 7 Im, 7 RC, 9 RH. †Different from Con, $P < 0.05$. ‡Different from RH, $P < 0.05$.



was performed for 4-hydroxy-2-nonenol (HNE; HNE11-S, Alpha Diagnostic International, San Antonio, TX) such that 15 μ g of sample were added directly to the membrane, and then the procedure was continued as a normal Western blot following transfer per Hamilton et al. (11) with an overnight primary exposure (11). Nitric oxide (NO) or ONOO \cdot (resulting from the interaction of NO and O $_2^{\cdot-}$) modify proteins resulting in a characteristic epitope that is easily identified using antibody technology. Accordingly, Western blots were incubated with secondary antibody (Amersham, Little Chalfont, Buckinghamshire, UK), exposed using enhanced chemiluminescence (Amersham) and imaged in a Kodak Image Station 440 CF developer (Eastman Kodak Scientific Imaging Systems, Rochester, NY). The signal analysis was performed using the Kodak ID Image Analysis Software with local background subtraction. Local background subtraction removes the effect of background by only quantifying the signal value above the background, thus providing a far more accurate measure of signal intensity compared with ignoring background or global background subtraction. Equal protein loading was confirmed using Ponceau staining with subsequent optical density analysis.

HSP 32 (HSP32) content was measured using the Rat HO-1 ELISA kit (EKS 810, Stressgen).

Enzyme kinetics. All antioxidant enzymatic assays were performed in triplicate in microplates using a Spectramax 190 microplate reader (Molecular Devices, Downingtown, PA) using homogenate further diluted to 1:100 in PBS buffer. Glutathione peroxidase was determined by the method of Flohe and Gunzler (6); glutathione reductase activity was determined by the method of Carlberg and Mannervik (4); catalase activity was determined by the method of Aebi (1); and manganese SOD (MnSOD) and copper-zinc SOD (CuZnSOD) activity was determined simultaneously by the method of McCord and Fridovich (26).

Statistical analyses. With the exception of body mass, data was compared using a one-way ANOVA. Changes in body mass were compared using a multivariate ANOVA with repeated measures. ANOVAs yielding an F -test that was significantly different were compared using a Newman-Keuls post hoc test. Alpha was set at $P < 0.05$. Data are presented as means \pm SE unless otherwise noted.

RESULTS

Before our study interventions, body mass was similar across groups (Table 1). One week of immobilization resulted in an $\sim 10\%$ weight loss in all immobilized groups, relative to the initial mass, and this was also different from the Con group who tended to gain $\sim 7\%$ mass ($P > 0.05$). Reambulation in RC and RH treatments prevented further reductions in body mass but did not increase body mass. During the second week of this study, total body mass for the Con animals increased by an additional 6%, which was statistically greater than the initial mass for this group.

Muscle mass. When each animal was killed, soleus wet weight was determined immediately before freezing (Fig. 1). Immobilization resulted in a 35% reduction in muscle mass compared with Con. Whereas 1 wk of reloading increased mass $\sim 20\%$, it was not different from Im. When reloading was combined with heat, the result was a significantly larger muscle compared with the muscles of the Im group. Furthermore, animals receiving heat treatment during reloading experienced significantly greater hypertrophy compared with normothermic reloading. Muscle water content did not differ between groups, indicating the soleus mass differences were not a function of edema in reloaded animals (Table 2).

Oxidative damage. Oxidative damage was assessed by measurement of HNE and NT. Immobilization increased HNE products compared with Con (Fig. 2). Reloading further increased HNE products, because the RC group was significantly higher than Con and Im. Heating in conjunction with reloading abolished the rise in HNE to values similar to Con. The results of the NT assay were similar to the pattern of HNE change. Immobilization resulted in a significant increase in damage that was still present during reloading (Fig. 3). Reloading in combination with heat eliminated this

Table 3. Activities of predominant antioxidant enzymes in the Con, Im, RC, and RH groups

	Con	Im	RC	RH
Manganese superoxide dismutase	356 \pm 30	304 \pm 32	261 \pm 41	288 \pm 26
Copper-zinc superoxide dismutase	212 \pm 18	310 \pm 20*†	293 \pm 25*†	229 \pm 17
Catalase	2.20 \pm 0.07	2.67 \pm 0.04*†‡	2.44 \pm 0.09*	2.30 \pm 0.06
Glutathione peroxidase	7.20 \pm 0.43	6.77 \pm 0.37	8.04 \pm 0.41	6.96 \pm 0.33
Glutathione reductase	1,159 \pm 115	1,137 \pm 110	1,328 \pm 88	1,138 \pm 69

Values are means \pm SE given in units per gram of wet weight per minute. Manganese superoxide dismutase: $n = 9$ Con, 9 Im, 7 RC, 9 RH; copper-zinc superoxide dismutase: $n = 7$ Con, 7 Im, 7 RC, 9 RH; catalase: $n = 8$ Con, 8 Im, 8 RH, 9 RH; glutathione peroxidase: $n = 8$ Con, 8 Im, 8 RC, 8 RH; Glutathione reductase: $n = 9$ Con, 10 Im, 8 RC, 9 RH). *Different from Con, $P < 0.05$. †Different from RH, $P < 0.05$, ‡Different from RC, $P < 0.05$.

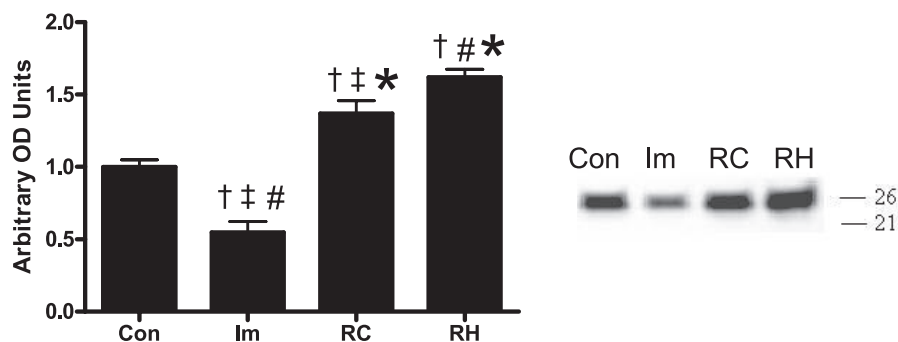


Fig. 4. Relative heat shock protein 25 content in the Con, Im, RC, and RH groups. Values are means \pm SE; $n = 10$ Con, 9 Im, 9 RC, 7 RH. Nos. at right are molecular weight. †Different from Con, $P < 0.05$. ‡Different from RH, $P < 0.05$. #Different from RC, $P < 0.05$. *Different from Im, $P < 0.05$.

damage, as NT was not different between the RH group and the Con group.

Antioxidant enzymes. Intermittent hyperthermia during reloading provides the cell with increased resistance to oxidant damage, which, may be due to changes in antioxidant enzyme activities. In this regard, both CuZn SOD and MnSOD activities were measured along with catalase and the glutathione-handling enzymes glutathione peroxidase and glutathione reductase (Table 3). MnSOD, glutathione peroxidase, and glutathione reductase activities did not differ between groups. Immobilization caused an increase in CuZnSOD activity, which was maintained during reloading. Reloading combined with heat, however, lowered this measure to Con levels. Catalase activity was significantly elevated with immobilization. Reloading lowered catalase activity; however, it was still elevated over Con values. Reloading with heat lowered catalase activity to Con levels. These data suggest that heating does not increase antioxidant protection by increasing antioxidant enzyme activity because antioxidant enzyme activity in RH was not increased above that of RC in any instance.

HSPs. In the present study, the endogenous antioxidant enzymes assessed were not responsible for attenuating oxidant damage during reloading, indicating that some other factor(s) must be responsible for this observation. HSP25 was significantly reduced following immobilization compared with all other groups (Fig. 4). Following 1 wk of reloading, HSP25 levels increased to above Con levels. Application of a heat treatment further elevated HSP25 levels by an additional 25%. HSP72 followed a very similar pattern in that there was a significant decline in HSP72 content following immobilization that was reversed with reloading (Fig. 5). Heating during reloading increased HSP72 by an additional 30% over reloading alone. In contrast, HSP32 was increased in Im (32%), and it returned to baseline with reloading (Fig. 6). Reloading with

heat, however, produced 37 and 25% increases in HSP32 levels compared with Con and RC, respectively. These observations were not different from Im, however. The content of all HSPs in RH were significantly higher than RC.

DISCUSSION

To return to a preatrophy functional level, reloading of the skeletal muscle is necessary. In this investigation, we observed an increased oxidative stress associated with muscle regrowth during reloading. To counter this phenomenon, we performed intermittent heating during reloading and observed potentiated muscle regrowth and reduced oxidant damage compared with the reloaded controls. In addition, intermittent heating caused elevated HSP25, HSP32, and HSP72 contents in reloaded soleus muscles. This is the first investigation to show that intermittent heating increases the rate of muscle regrowth during 1 wk of reloading. Moreover, we show for the first time that heating will attenuate oxidative damage during reloading. Given this association, and the known interaction between HSPs and oxidative damage, we suggest that HSPs may be playing a pivotal role in augmenting muscle regrowth in this system.

Despite the novel findings of this investigation, this is not the first to employ heat as a protective intervention in skeletal muscle. In previous work by our group and others, heat therapy protected muscle from atrophy and oxidant damage during disuse (30, 38). In addition, a single bout of controlled hyperthermia has been demonstrated to induce hypertrophy or increase protein synthesis (9, 15). Moreover, our finding of enhanced muscle mass in a heated reloaded group compared with a reloaded control group is supported by Goto et al. (8) who found increased muscle-to-body weight ratio following a single heat stress.

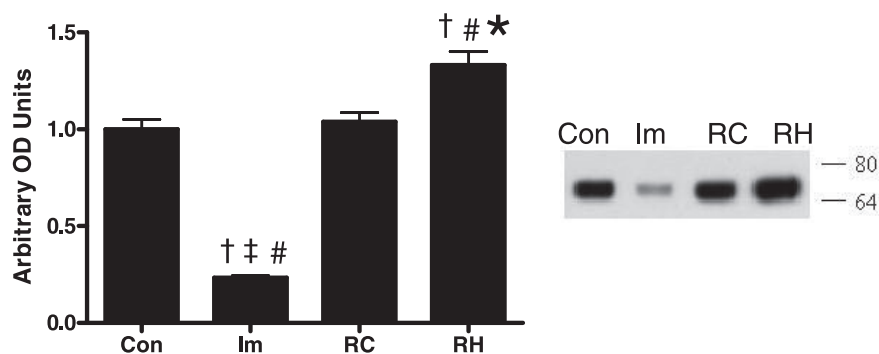


Fig. 5. Relative heat shock protein 72 content in the Con, Im, RC, and RH groups. Values are means \pm SE; $n = 8$ Con, 6 Im, 5 RC, 6 RH. Nos. at right are molecular weight. †Different from Con, $P < 0.05$. ‡Different from RH, $P < 0.05$. #Different from RC, $P < 0.05$. *Different from Im, $P < 0.05$.

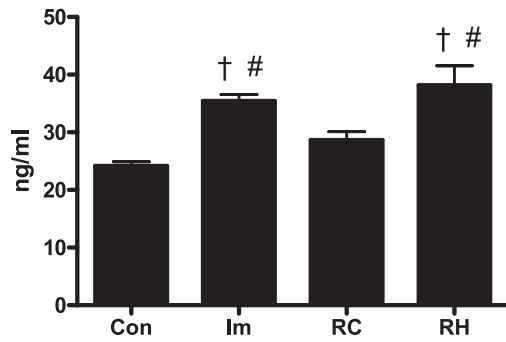


Fig. 6. Heat shock protein 32 content. Values are means \pm SE; $n = 6$ Con, 5 Im, 7 RC, 7 RH. †Different from Con, $P < 0.05$. #Different from RC, $P < 0.05$.

In this investigation, reloading resulted in oxidative damage in Im and RC animals as measured by NT and HNE (Figs. 2 and 3). Because oxidant damage was attenuated in heated-reloaded animals, we chose to assess the effect of heating on endogenous antioxidant enzymes thought to be most responsible for maintaining redox balance in stressed skeletal muscle (34). In this regard, CuZnSOD and MnSOD, catalase, glutathione peroxidase, and glutathione reductase activities were evaluated. Similar to previous works, MnSOD, glutathione reductase, and glutathione peroxidase activities did not change in immobilized or reloaded treatments (18, 38). In agreement with our previous work, CuZnSOD activity was increased in response to unloading and was also increased in response to reloading (38). SOD activity has been shown to increase in response to elevated $O_2^{\cdot-}$ content (3, 10). Because CuZnSOD is increased during reloading, it may indicate that $O_2^{\cdot-}$ is contributing to cytosolic oxidant stress. By contrast, MnSOD was not increased during reloading, and this may indicate that mitochondrial $O_2^{\cdot-}$ is not contributing to free radical damage in this model. In addition, catalase activity was increased in RC, whereas RH was similar to Con. Hence, oxidative stress attenuation in the heated group was not due to endogenous upregulation of these enzymes. Because these enzymes are likely not responsible for the oxidant stress attenuation in the RH group, alternative antioxidants must be considered.

Several investigations have demonstrated that heating appears to improve redox balance against a host of free radical generating conditions (7, 14, 16, 31, 36). In this investigation, HSP25 and HSP72 behaved similarly in that immobilization resulted in a reduction in expression, whereas reloading returned HSP expression to Con or above Con levels. Heating combined with reloading increased HSP expression by $\sim 25\%$ vs. RC 48 h following the last hyperthermic bout, representing the minimum hyperthermic-induced HSP expression, rather than peak hyperthermic-induced HSP expression. HSP25 can directly scavenge free radicals, and it is also known to support glutathione recycling (27, 36, 45). Alternatively, HSP72 can assist in the delivery of severely modified proteins to proteases for degradation so that they cannot form cytotoxic protein aggregates. In addition, cells overexpressing HSP72 are more resistant to death and membrane damage during H_2O_2 exposure (16). Further, Smolka et al. (40) demonstrated that HSP72 likely provided increased resistance to oxidative stress during exercise.

The finding of a reduction in HSP 72 expression with disuse and an increase with reloading is consistent with previous publications (8, 19, 30, 33). Other publications show that HSP 72 expression is not reduced by disuse and another shows that reloading does not induce recovery of HSP72 expression (8, 19, 32, 38, 39). Although changes in HSP25 during unloading are inconsistent, more consistency is found during reloading, including the present investigation, where an increase in HSP25 expression is shown (19, 38, 39). It is likely that effects of strain, gender, duration of disuse or reloading, model of disuse, or some yet to be identified factor are influencing HSP25 and HSP72 expression following disuse and reloading.

Previous work supports our finding of an increase in HSP32 expression following disuse (12). Because HSP32 is increased in response to free heme, it may indicate that myoglobin, cytochrome *c*, and hemoglobin contribute to the prooxidant intracellular environment (20–23). Elevated HSP32 activity ultimately results in the catabolism of heme and the production of the antioxidant bilirubin. In aggregate, these adaptations may function to attenuate oxidative damage found during reloading.

Alternate attempts to augment muscle regrowth following disuse have included exogenous supplementation of the antioxidant vitamin E (17). In that study, vitamin E supplementation improved reloading-induced muscle growth following 1 wk of immobilization. Similarly, we have shown that intermittent heating during 1 wk of reloading augments hypertrophy by 30%. Based on these results, elevated HSP content in the heated soleus seems to potentiate muscle mass regrowth at least partially through improved cellular redox status. Moreover, gene array studies reveal that heat stress consistently increases HSP expression, whereas endogenous antioxidant enzyme responses are inconsistent (35, 41, 42, 48). Whether HSPs other than HSP25, HSP32, and HSP72 are responsible for this protective effect is a matter for future study.

In summary, intermittent heating was shown to be an efficacious treatment in the augmentation of skeletal muscle regrowth following immobilization. These data support our hypothesis that compared with reloading alone, hyperthermia in conjunction with reloading significantly improves muscle mass reacquisition. Our findings suggest that HSP overexpression may increase muscle mass through attenuation of the local oxidative stress response. The significance of this finding is magnified by the discovery that the endogenous antioxidants generally thought to fortify cellular antioxidant levels were unaltered by heating. Further determination of the interaction between HSPs, redox balance, and pathways leading to protein synthesis will help clarify the precise physiology occurring in this model and help to refine this technique for therapeutic use. In addition, future experiments utilizing extended immobilization and hyperthermic reloading periods may better clarify whether intermittent hyperthermia directly influences regrowth kinetics or if it represents a more fundamental physiological adaptation.

GRANTS

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