The effect of short-term heat stress on protein synthesis signaling in isolated rat skeletal muscle

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Abstract Heat stress (HS) is a potent stimulus for activating glucose metabolism in skeletal muscles. However, the effect of short-term HS on protein turnover in skeletal muscles is unclear. This study aimed to investigate the effect of short-term HS on protein synthesis and protein degradation in skeletal muscles. The epitrochlearis muscle was isolated from male Sprague–Dawley rats weighing 150-160 grams (g) and incubated with or without HS at 42°C for 10 or 30 min in alpha minimum essential medium. HS for 30 min significantly decreased phosphorylation of 70-kDa ribosomal protein S6 kinase at Thr389 and 4E-binding protein 1 at Thr37/46. Correspondingly, HS for 30 min decreased the rate of protein synthesis. In contrast, HS had no effect on the expression of autophagy-related proteins, including microtubule-associated protein light chain 3 and p62, or on the mRNA expression of muscle-specific ubiquitin ligases, including muscle RING-finger 1 (MuRF1) and atrogin-1/MAFbx. These findings suggested that short-term HS for approximately 30 min is a physiologically relevant stimulus that suppresses protein synthesis signaling in skeletal muscles.

Keywords: hyperthermia, protein metabolism, autophagy, ubiquitin-proteasome system, exercise

Introduction

Exercise increases cellular energy demands in contracting skeletal muscles and therefore, results in switching off of ATP-consuming anabolic pathways and switching on of ATP-generating catabolic pathways. Skeletal muscle protein synthesis, a major anabolic process, is suppressed during resistance-type exercise1 and endurance-type exercise2). In contrast, skeletal muscle glucose utilization, a major catabolic process, is enhanced during resistance-type exercise3) and endurance-type exercise4). The ATP-dependent ubiquitin–proteasome system

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another key protein degradation pathway, which is primarily responsible for the degradation of myofibrillar proteins. Skeletal muscle protein degradation increases immediately after endurance-type exercise, which may be attributed to the AMPK-induced activation of autophagy and ubiquitin-proteasome systems. However, to the best of our knowledge, there are no current reports evaluating the effect of short-term HS on skeletal muscle protein degradation.

Therefore, the present study aimed to investigate the direct effect of short-term HS on protein synthesis and degradation in skeletal muscles. For this purpose, we examined changes in signaling pathways associated with protein synthesis and degradation in isolated rat skeletal muscles exposed to HS.

Material and Methods

Animals. Five-week old male Sprague-Dawley rats weighing 150-160 g were obtained from Shimizu Breeding Laboratories (Kyoto, Japan). Experimental protocols were approved by the Kyoto University Graduate School of Human and Environmental Studies and the Kyoto University Radioisotope Research Center (approval number: 28-A-1).

Muscle incubation. Muscles were exposed to HS as previously described. Rats were fasted overnight prior to experimentation. Immediately after cervical dislocation, the epitrochlearis muscle was dissected and preincubated for 60 min at 35°C in alpha minimum essential medium (αMEM) (Nacalai Tesque, Kyoto, Japan) supplemented with 0.01% bovine serum albumin (Sigma, St. Louis, MO, USA), 2.2 g/L NaHCO3, 5 mmol/L mannitol, 2.54 mol/L CaCl2, 10% fetal bovine serum (Biowest, Nuaillé, France), 50 μM/L insulin, and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). Muscles were then incubated for 10 or 30 min in fresh αMEM maintained at 35°C or 42°C. The buffers were continuously gassed with 95% O2 and 5% CO2. Buffer pH was measured using a pH meter (F72S, HORIBA, Ltd., Kyoto, Japan).

Protein synthesis assay. Protein synthesis was assayed as previously described, with modifications. After exposure to HS, the epitrochlearis muscles were incubated in Krebs–Ringer bicarbonate buffer (120 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, and 24.6 mM NaHCO3) containing 2 mM pyruvate, 0.5 mM phenylalanine, and 2 μCi/ml of [14C]phenylalanine for 10 min. After incubation, muscles were homogenized in 10% trichloroacetic acid (TCA) and centrifuged at 16,000 g for 40 min at 4°C. Denatured proteins were separated on polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked with nonfat dry milk and incubated with commercially available antibodies against actin (ab4968), microtubule-associated protein light chain 3 (LC3) (6277S), p62 (#5114), p70S6K Thr389 (#9205), p70S6K (#9202), and 4E-BP1 Thr37/46 (#9459) purchased from Cell Signaling Technology, Danvers, MA, USA. Some membranes were incubated with a signal enhancer (Can Get Signal Immunoreaction Enhancer Solution, Toyobo, Tokyo, Japan). The membranes were then incubated with corresponding secondary antibodies coupled to horseradish peroxidase and developed with enhanced chemiluminescence reagents. Protein signals were detected with ImageCapture G3 (Liponics, Tokyo, Japan) or WSE-6100 LuminoGraph (ATTO, Tokyo, Japan).

Real-time reverse transcription (RT)-PCR analysis. A separate set of muscle samples was subjected to RT-PCR analysis, which was performed as previously described, with modifications. Frozen muscle was homogenized in ice-cold buffer (1:40 wt/vol) [comprising 20 mmol/L Tris-HCl (pH 7.4), 1% Triton X-100, 50 mmol/L NaCl, 250 mmol/L sucrose, 50 mmol/L NaF, 5 mmol/L sodium pyrophosphate, 2 mmol/L dithiothreitol, 4 mg/L leupeptin, 50 mg/L trypsin inhibitor, 0.1 mmol/L benzamidine, 1 mmol/L Na3VO4, and 0.5 mmol/L phenylmethysulfonyl fluoride] and centrifuged at 16,000 g for 40 min at 4°C. Total RNA was extracted from frozen muscles using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands). RNA was reverse-transcribed into complementary DNA (cDNA) using PrimeScript RT Master Mix (Perfect Real Time) (Takara Bio, Otsu, Japan). Synthesized cDNA was subjected to real-time RT-PCR (Step One Real Time System, Applied Biosystems, Carlsbad, CA, USA) using SYBR Premix Ex Taq II (Takara Bio) and then analyzed using StepOne Software v2.3 (Applied Biosystems). The PCR cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and 60°C for 60 s. Amplification specificity was confirmed by melt-curve analysis. The primers were designed using the Takara Bio Perfect Real Time Support System (Takara Bio). Primers used were as follows: MuRF1 (gene name: Trim63), 5′-GACTCTGTGGTACTGATTGACAA-3′ (forward) and 5′-TTCTGCCTTCAAGGTGCGTA-3′ (reverse); atrogenin-1/muscle atrophy F-box (MAFbx) (gene name: Fbxo32), 5′-CAACATGTGGGTTATCGAATGG-3′ (forward) and 5′-TGATGTTTACTGTTGAC ACA-
CAGG-3’ (reverse); and ribosomal protein S18 (gene name: Rps18), 5’-TTGGTGAGGTCATGCTGCTTT-3’ (forward) and 5’-AAGTTTCAGCACATCCTGCGAGT-3’ (reverse). Rps18 cDNA was used as an internal standard for normalizing the amount of total RNA in each reaction.

**Statistical analysis.** Data are expressed as mean ± standard deviation (SD). Multiple means were analyzed using one-way ANOVA followed by post hoc comparison with Bonferroni’s t test. Differences in buffer pH were measured by corresponding two-way ANOVA. Differences between two means were determined using Student’s t test. P < 0.05 was considered statistically significant.

**Results**

**Effect of HS on protein synthesis.** Phosphorylation of p70S6K at residue Thr\(^{389}\) and 4E-BP1 at Thr\(^{37/46}\) can promote protein translation and synthesis\(^{19,20}\). To determine the effect of HS on protein synthesis, we measured the phosphorylated state of these proteins. We found that phosphorylation of p70S6K at Thr\(^{389}\) (Fig. 1A) and 4E-BP1 at Thr\(^{37/46}\) (Fig. 1B) significantly decreased after HS for 30 min. In addition to the changes in signaling, we also found that the rate of protein synthesis tended to decrease after HS for 30 min (P = 0.07) (Fig. 1C).

**Effect of HS on buffer pH.** Changes in extracellular pH can influence cellular signaling, as well as the rate of protein synthesis\(^{21,22}\). Therefore, we measured the differences in buffer pH during muscle incubation for up to 60 min. The pH of the heated buffer (42°C) was slightly but significantly higher than that of the non-heated buffer (35°C) throughout the entire incubation period (temperature, P < 0.05) (Fig. 2). In addition, buffer pH increased slightly but significantly over time in both groups (time, P < 0.05).

**Effect of HS on protein degradation.** LC3BII and p62 are generally used as markers to monitor autophagy\(^{23,24}\). MuRF1 and atrogin-1/MAFbx are the crucial ligases mediating protein degradation via the ubiquitin-proteasome system in skeletal muscles\(^{25}\). To identify the effect of HS on protein degradation, we measured the protein expression of LC3BII and p62, and mRNA expression of MuRF1 and atrogin-1/MAFbx in skeletal muscles exposed to HS. We found that HS had no effect on either the expression of LC3BII (Fig. 3A) and p62 (Fig. 3B) or the mRNA expression of MuRF1 (Trim63) (Fig. 3C) and atrogin-1/MAFbx (Fbxo32) (Fig. 3D).

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**Fig. 1** Short-term HS inhibits protein synthesis pathway in isolated skeletal muscle.

Isolated epitrochlearis muscle was preincubated in medium at 35°C, and then incubated in medium at 35°C (solid line) or at 42°C (broken line) for 0, 10, or 30 min. Muscle was assayed for phosphorylated p70S6K Thr\(^{389}\) (p-p70S6K) (A) and phosphorylated 4E-BP1 Thr\(^{37/46}\) (p-4E-BP1) (B). Protein synthesis rate (C) was assayed following incubation period. Values are mean ± SD; n = 5-7 (A), 6-11 (B), 6 (C). *P < 0.05 vs. 0 min.
Skeletal muscle protein synthesis is suppressed during resistance- or endurance-type exercise\(^1,2\). This reduction in protein synthesis is mediated by a decrease in initiation and elongation during mRNA translation, due to a reduction in the phosphorylation of 4E-BP1\(^26\). p70S6K does not appear to be involved in this reduction because phosphorylation of p70S6K increased or remain unchanged during resistance-\(^27\) and endurance-type exercise\(^28\), respectively. In the present study, we found that short-term (30 min) HS decreased the phosphorylation of 4E-BP1 at Thr\(^{37/46}\) (Fig. 1B) and of p70S6K at Thr\(^{389}\) (Fig. 1A), accompanied by a decrease in protein synthesis (Fig. 1C). The change of protein synthesis rate in the non-heated condition during incubation period was not examined in the present study. However, we believe that the decrease in protein synthesis rate was induced by HS per se, but not by the incubation procedure, because a previous study has shown that the protein synthesis rate was not decreased in the 30 min incubation of isolated rat skeletal muscle at 6.5\(^{ \text{pH} } \) 7.0\(^{ \text{pH} } \) 7.5\(^{ \text{pH} } \) 8.0\(^{ \text{pH} } \) 8.5

Fig. 2 Changes in buffer pH during incubation.
The incubation buffer was maintained at 35°C (solid line) or at 42°C (broken line), continuously gassed with 95% O\(_2\) and 5% CO\(_2\). The buffer pH was measured at 0, 10, 20, 30, 40, 50, and 60 min incubation periods. Two-way ANOVA revealed that the main effects of temperature and time were statistically significant (P < 0.05). Values are mean ± SD; n = 6.

![Graph showing pH changes over time](chart.png)

Fig. 3 Short-term HS does not affect protein degradation pathway in isolated skeletal muscle.
Isolated epitrochlearis muscle was preincubated in medium at 35°C, and then incubated in medium at 35°C (solid line) or at 42°C (broken line) for 0, 10, or 30 min. Muscle was assayed for the expression of LC3BII (A), p62 (B), Trim63 mRNA (C) and Fbxo32 mRNA (D). Values are mean ± SD; n = 6-10 (A), 5-10 (B), 5-6 (C), 5-7 (D).
Collectively, these results suggest that short-term HS suppresses skeletal muscle protein synthesis via signaling that is partly distinct from that of exercise.

In contrast to our findings, a previous study reported that HS for 30 min activated p70S6K in rat skeletal muscle in vivo. These contrasting results may be attributed to differences in the heat exposure process. In the present study, HS was conducted using an in vitro isolated muscle preparation, whereas the previous study used an in vivo preparation. An in vivo procedure cannot exclude some confounding variables such as humoral, neuronal, and/or hormonal factors. Therefore, HS-induced activation of p70S6K in vivo may be attributed to secondary effects.

We believe that our in vitro procedure enabled us to evaluate the direct effects of HS on signaling in an isolated system.

Protein synthesis is an energy-dependent process and is, therefore, influenced by muscle energy state. A previous study demonstrated that a decreased rate of protein synthesis, during muscle contraction, is associated with ATP and phosphocreatine levels as well as glycogen availability. The energy depletion triggers an activation of a cellular energy sensor AMPK. In fact, short-term HS has been shown to activate AMPK in skeletal muscle accompanied by decreased ATP, phosphocreatine, and glycogen levels. It is accepted that AMPK negatively regulates protein synthesis through inhibiting mTOR/p70S6K signaling in skeletal muscle. Therefore, short-term HS may inhibit protein synthesis signaling partly through AMPK activation.

HS-induced muscle energy depletion was possibly due to an increase in energy utilization because ATP utilization is dependent on muscle temperature. In a physiological condition, muscle blood flow is increased in response to HS. Considering that the muscle blood flow level is associated with ATP production, it is possible that the presence or absence of muscle blood flow affected the difference in results obtained from in vitro and in vivo experiments. However, further research is needed to elucidate the exact mechanism.

Previous studies have reported that extracellular pH can influence protein synthesis and mTOR signaling. Protein synthesis was shown to be stimulated by increasing pH (from 7.4 to 7.8) in perfused rat hearts. Furthermore, phosphorylation of p70S6K at Thr was shown to decrease in response to a reduction in pH (from 7.4 to 6.8) in carcinoma cells. In the present study, HS resulted in increased buffer pH (mean pH for 0 to 30 min incubation: 35°C = 7.68; 42°C = 7.77) (Fig. 2) which was accompanied by decreased phosphorylation of p70S6K at Thr (Fig. 1A) and tended to decrease protein synthesis (Fig. 1C). Considering the slight change of pH, it is thought that changes in extracellular pH don’t play a role in HS-induced alterations of protein synthesis signaling.

Some studies have reported that the rate of phenylalanine appearance, an index of protein degradation, did not change after resistance-type exercise, whereas it increased immediately after endurance-type exercise. Accordingly, autophagy and ubiquitin–proteasome systems were shown to be upregulated during endurance-type exercise in skeletal muscles. In the present study, however, we found that HS did not affect the expression of autophagy-associated proteins, LC3BII (Fig. 3A) and p62 (Fig. 3B), or the mRNA expression of ubiquitin ligases, MuRF1 (Trim63) (Fig. 3C) and atrogin-1/MAFbx (Fbxo32) (Fig. 3D). Therefore, it is suggested that short-term HS has no effect on protein degradation via the autophagy or the ubiquitin–proteasome systems. This is the first study to report the effect of HS on the acute response of protein degradation in skeletal muscles.

In conclusion, our findings demonstrated that short-term HS suppressed signal transduction of protein synthesis pathways, but has no effect on protein degradation in isolated rat skeletal muscles. These findings contribute to our understanding of the comparative molecular responses between HS and exercise.

**Conflicts of interest**

The authors declare that there is no conflict of interests regarding the publication of this article.

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