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ORIGINAL PAPER

Heat stress enhances mTOR signaling after resistance exercise in human skeletal muscle

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Abstract This study investigated the effect of heat stress (HS) on mammalian target of rapamycin (mTOR) signaling involved in translation initiation after resistance exercise in human skeletal muscle. Eight young male subjects performed four sets of six maximal repetitions of knee extension exercises, with or without HS, in a randomized crossover design. HS was applied to the belly of the vastus lateralis by using a microwave therapy unit prior to and during exercise. Muscle biopsies were taken from the vastus lateralis before, immediately after, and 1 h after exercise. HS significantly increased the phosphorylation of Akt/PKB, mTOR, and ribosomal protein S6 at 1 h after exercise ($P < 0.05$), and the 4E-BP1 phosphorylation level, which had initially decreased with exercise, had recovered by 1 h after exercise with HS. In addition, the phosphorylation of ribosomal S6 kinase 1 was significantly increased immediately after exercise with HS ($P < 0.05$). These results indicate that HS enhances mTOR signaling after resistance exercise in human skeletal muscle.

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Introduction

Skeletal muscle hypertrophy is characterized by an increase in muscle protein mass. A variety of interventions (e.g., resistance exercise, nutrition, and hormone therapy) has been designed to effectively enhance muscle growth. Recent studies have shown that heat stress (HS) is a potent stimulus of increased protein synthesis and muscle mass [1–3]. Uehara et al. [2] showed that a period of acute HS increased the mass of rat soleus muscle measured 7 days after exposure. Moreover, Selsby et al. [4] found that intermittent HS enhanced the re-growth of atrophied rat muscle after disuse. A common explanation for such phenomena is the effect of heat shock proteins (HSPs), particularly the inducible form of 70-kDa HSP (HSP72) induced by HS. HSPs play an important role in chaperoning nascent peptides during translation [5, 6], and increased HSP72 expression due to HS may facilitate muscle protein synthesis, thereby increasing skeletal muscle mass. In contrast to this concept, Frier and Locke [7] reported that HS prior to mechanical overloading did not result in hypertrophy in rat plantaris muscle, despite increased HSP72 expression. This finding suggests that enhanced protein synthesis following HS may result from mechanisms other than the effect of HSP72. However, the precise mechanism responsible for HS-related muscle hypertrophy has not been elucidated.

Recent studies suggest that the mammalian target of rapamycin (mTOR) signaling pathway has a key role in stimulating translation initiation, which is a regulatory step in protein synthesis [8–10]. In this pathway, a variety of

stimuli, including mechanical load, nutrition, and hormone signaling, converge at mTOR [11, 12]. Specifically, mTOR is directly or indirectly regulated by upstream protein kinase Akt/protein kinase B (PKB), tuberous sclerosis complex 2 (TSC2), and AMP-activated protein kinase (AMPK). The activation of mTOR results in phosphorylation of the downstream targets ribosomal protein S6 kinase 1 (S6K1), ribosomal protein S6, and eukaryotic initiation factor (eIF) 4E binding protein 1 (4E-BP1), leading to enhanced mRNA translation [9, 10]. Additionally, S6K1 activation decreases eukaryotic elongation factor 2 (eEF2) phosphorylation to promote translation elongation [9, 10]. Bodine [13] demonstrated that rapamycin, a specific mTOR inhibitor, blocked muscle hypertrophy following functional overload in rats, suggesting that the activation of mTOR and its downstream targets (S6K1 and 4E-BP1) is requisitely involved in regulating skeletal muscle mass. Considering the role of mTOR signaling in regulating protein synthesis, it is reasonable to speculate that HS-related muscle hypertrophy is facilitated through an mTOR signaling mechanism. In support of this, previous in vitro studies have shown that HS increases the phosphorylation of Akt in rat ventricular myocytes [14] and of S6K1 in fibroblasts [15]. However, it is not known whether HS stimulates mTOR signaling in skeletal muscle in vivo.

It is well established that components of the mTOR pathway are rapidly upregulated in rat [16–18] and human [19–21] skeletal muscle following resistance exercise. Kumar et al. [22] showed that S6K1 phosphorylation was related to the muscle protein synthesis rate after resistance exercise in human skeletal muscle. Furthermore, studies in rodent [16] and human [23] skeletal muscle have shown that S6K1 phosphorylation after resistance exercise is positively correlated with the degree of skeletal muscle hypertrophy after long-term resistance training. These results indicate that the activation of mTOR and its downstream targets (S6K1 and 4E-BP1) after resistance exercise is critically important in regulating skeletal muscle mass. Additional stimuli (e.g., exercise volume, blood flow restriction, and essential amino acid ingestion) during and after resistance exercise enhance the activation of mTOR signaling in skeletal muscle concomitant with an increase in the rate of muscle protein synthesis [24–27], leading to muscle hypertrophy. Recently, Goto et al. [28] demonstrated that the combination of low-intensity resistance exercise and HS caused an increase in muscle size compared with exercise alone in humans. Based on in vitro observations [14, 15], HS may enhance the activation of mTOR signaling and protein synthesis after resistance exercise in skeletal muscle, although the effect of HS in human skeletal muscle remains unclear.

The purpose of this study was to examine the effect of HS on mTOR signaling molecules after resistance exercise in human skeletal muscle. We hypothesized that resistance exercise with HS would enhance mTOR signaling after exercise. Recent studies have suggested that resistance exercise also increases the phosphorylation of mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinase (ERK1/2) and p38, which are involved in translation initiation and protein synthesis in human skeletal muscle [29–32]. Thus, we examined the phosphorylation of protein kinases, including those involved in the mTOR signaling pathway as well as the MAPKs ERK1/2 and p38.

Materials and methods

Subjects

Eight healthy young male volunteers (mean ± SD: age = 22.3 ± 0.7 years, mass = 66.8 ± 4.2 kg, height = 176.3 ± 5.0 cm, BMI = 21.5 ± 1.2 kg m^{-2}) who were recreationally active with no formal weight-lifting experience or regular weight-lifting activity during the previous year participated in this study. All subjects were informed of the procedures and potential risks involved in the investigation. This study was approved by the Juntendo University Human Ethics Committee and was conducted in accordance with the standards of the Declaration of Helsinki.

Study design

The study employed a randomized crossover design in which each subject completed two acute resistance exercise sessions (RE and HRE) separated by a 3-week recovery period. For the RE session, the subjects performed a one-leg knee extension exercise that consisted of isokinetic concentric contraction. The HRE session consisted of the same exercise performed using the contralateral leg, with the belly of the vastus lateralis exposed to HS using a microwave therapy unit. The subjects' legs were randomly assigned to either RE or HRE condition.

Preparatory test

Prior to the experiment, the subjects participated in a preparatory test that measured their isometric strength using a computer-interfaced dynamometer (Biodex System 3; Biodex Medical Systems, Inc., Shirley, NY). The test was performed to determine the one-leg maximal force that could be exerted by each individual and to familiarize the subjects with the exercise procedure. The appropriate

settings on the machine were documented for each subject. The test was performed 5 days before the actual exercise study.

Study control

The subjects were asked to refrain from vigorous physical activity, ingestion of alcohol, and taking a bath for 3 days prior to the experiment. They were also asked to consume the same meal (17.1% protein, 67.5% carbohydrate, and 15.4% fat) no later than 9:00 p.m. on the penultimate day of the experiment. Subjects were permitted to drink only water at any time.

Experimental protocol

The subjects reported to the laboratory in the morning after fasting overnight. The subjects rested in a supine position for 30 min, and then a baseline muscle biopsy (pre) was taken from the vastus lateralis of the leg not selected for exercise using a disposable biopsy instrument (14 gauge, Max Core; C.R. Bard, Covington, GA) under sterile conditions and local anesthesia (1% lidocaine) as described previously [33]. At 10 min after the first biopsy, the subjects were escorted to a Biodex leg extension machine. In HRE condition, the vastus lateralis of the contralateral leg was subjected to HS using a microwave therapy unit (Microtizer MT-SDi; Minato Medical Co. Ltd., Osaka, Japan) as described previously [34]. The device was set to a power of 150 W; the distance between the skin surface and applicator was approximately 10–15 cm. We previously showed that this method increases muscle temperature to 41.1 ± 1.3°C [34]. After 20 min of resting in a sitting position on the Biodex with or without HS, the subjects performed four sets of six repetitions of one-legged knee extension exercises controlled at an angular speed of 30°/s. This isokinetic exercise protocol, controlled using the Biodex System 3, is able to add maximal load at all angles of the knee joint and measure the torque at all angles during exercise. The peak torque during exercise was determined using the Biodex System 3. The rest period between sets was 2 min. In HRE condition, HS to the vastus lateralis was continued during the knee extension exercises and intervals between the sets. After exercise, the subjects moved immediately to a bed, and a second biopsy (post) was obtained from the exercised leg within 5 min of stopping. At 1 h after exercise, a third muscle biopsy (1-h post) was obtained from the same incision used in the second biopsy, but with the needle inserted at a different angle. During all surgical procedures, the subjects remained in a supine position in a hospital bed. All muscle tissue was immediately frozen in liquid nitrogen and stored at −80°C until analysis.

Protein extraction

Approximately 10–15 mg of frozen muscle tissue was homogenized (1:9, w/v) in ice-cold buffer (50 mM HEPES, 10 mM EDTA, 4 mM EGTA, 50 mM β-glycerophosphate, 25 mM NaF, 5 mM Na_3VO_4, and 1% Triton X-100, pH 7.4) with phosphatase inhibitors (PhosSTOP tablet; Roche Diagnostics Corp., Indianapolis, IN) and protease inhibitors (Complete tablet; Roche Diagnostics). The homogenates were rotated for 30 min at 4°C and then clarified by centrifugation at 10,000×g for 10 min at 4°C. The supernatant was stored at −80°C. The total protein concentration of the supernatant was determined using a bicinchoninic acid protein assay (Thermo Fisher Scientific Inc., Waltham, MA) with bovine serum albumin as the standard. The residual pellet was analyzed for myosin heavy chain (MHC) as described below.

Western blot analysis

Samples containing 15 or 30 μg of total protein were electrophoresed in a 10 or 15% SDS polyacrylamide gel at 150 V for 60 min. After electrophoresis, the gels were incubated in transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol) for 15 min to equilibrate for optimal transfer. The proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories) at 100 V for 1 h on ice. After transfer, the membranes were blocked in Tris-buffered saline with 0.1% Tween 20 (T-TBS) containing 5% skim milk (for detection of eEF2, 4E-BP1, AMPK, p38, and ERK1/2) or PVDF blocking reagent (for detection of Akt, mTOR, S6K1, and S6; Toyobo Co. Ltd., Osaka, Japan) for 1 h each. The membranes were then incubated with commercially available phospho-specific primary antibodies in T-TBS containing 5% skim milk (eEF2, 4E-BP1, AMPK, p38, and ERK1/2) or primary antibody solution (NKB-201; Toyobo Co.; Akt, mTOR, S6K1, and S6) for 2 h at room temperature. The membranes were subsequently washed using T-TBS and incubated with rabbit horseradish peroxidase-conjugated secondary antibodies in T-TBS containing 5% skim milk (eEF2, 4E-BP1, AMPK, p38, and ERK1/2) or secondary antibody solution (NKB-301; Toyobo Co.; Akt, mTOR, S6K1, and S6) for 1 h at room temperature. After three washes in T-TBS, the phosphorylated proteins were visualized using enhanced chemiluminescence reagent (ECL plus; GE Healthcare) and an image analysis apparatus (Light Capture; ATTO, Bunkyo-ku, Japan). Each band was quantified by computerized densitometry using specialized software (CS Analyzer 3.0; ATTO).

To determine the total levels of each protein, the membranes were incubated in Restore Western blot stripping buffer (Thermo Fisher Scientific Inc.) and re-probed

with appropriate antibodies by as described above. Each Western blot contained a molecular weight marker or a commercially available loading control. All samples were transferred onto the same membrane, and the phosphorylation of each protein was expressed relative to the total amount of that protein. The data were analyzed as the fold change in phosphorylation compared with the pre-exercise level for each condition.

Antibodies

The primary antibodies used to detect phospho-Akt (Ser473; 1:2,000), phospho-mTOR (Ser2448; 1:2,000), phospho-S6K1 (Thr389; 1:500), phospho-S6K1 (Thr421/Ser424; 1:1,000), phospho-S6 (Ser235/236; 1:500), phospho-4E-BP1 (Thr37/46; 1:2,000), phospho-AMPKα (Thr172; 1:2,000), phospho-eEF2 (Thr56; 1:2,000), phospho-p38 (Thr180/Tyr182; 1:2,000), phospho-ERK1/2 (Thr202/Tyr204; 1:2,000), total Akt (1:2,000), total mTOR (1:2,000), total S6K1 (1:1,000), total S6 (1:1,000), total 4E-BP1 (1:2,000), total AMPKα (1:2,000), total eEF2 (1:2,000), total p38 (1:2,000), and total ERK1/2 (1:2,000), as well as anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:10,000) were purchased from Cell Signaling Technology (Beverly, MA).

SDS-PAGE analysis of MHC isoforms

The insoluble sediment after homogenization was suspended in a sufficient volume of SDS sample buffer (30% glycerol, 5% β-mercaptoethanol, 2.3% SDS, 0.05% bromophenol blue, and 62.5 mM Tris-HCl, pH 6.8) and boiled at 95°C for 15 min. The MHC composition was determined using glycerol SDS-PAGE according to previously described methods [35] with modifications. Briefly, the prepared protein samples were electrophoresed at 60 V and 8°C until the tracking dye exited the stacking gel and completely entered the separating gel. Then, the voltage was increased to 150 V, and electrophoresis was continued for 18 h at 8°C. Gels were stained with Coomassie brilliant blue (Biosafe G250; Bio-Rad Laboratories), followed by repeated rinses with water. The relative contents of MHC isoforms I, IIa, and IIx were determined using a calibrated densitometer (GS800; Bio-Rad Laboratories) and analytical software (Quantity One; Bio-Rad Laboratories).

Statistical analysis

The data are presented as mean ± SEM. To compare the levels of kinase phosphorylation at different time points for the two exercise conditions (RE and HRE), two-way (exercise condition and time) repeated-measures analysis of variance (ANOVA) was applied. When a significant overall effect was indicated, a Bonferroni post-hoc test was performed. To compare muscle strength and relative MHC isoforms between the two exercise conditions, a paired Student's t test was used. A probability level of $P < 0.05$ was considered to indicate statistical significance. All statistical analyses were performed using a statistical software package (Prism 5.0; GraphPad Software Inc., La Jolla, CA).

Results

Muscle strength and MHC composition

Maximal isometric strengths in RE and HRE legs were 241.8 ± 19.0 and 258.3 ± 27.5 Nm, respectively. The percentages of MHC I, IIa, and IIx in the RE versus HRE leg were 41.0 ± 2.0 versus 41.3 ± 2.4, 38.4 ± 2.4 versus 39.4 ± 2.1, and 20.6 ± 2.7 versus 20.1 ± 2.7, respectively. There were no differences in muscle strength or fiber composition between RE and HRE conditions.

Resistance exercise

All subjects completed four sets of six repetitions on a Biodex leg extension machine under RE and HRE conditions. The average peak torque for each of the four sets of six maximal isokinetic concentric contractions was similar between RE and HRE conditions (first set: 207.3 ± 14.3 vs. 213.6 ± 13.4 Nm; second set: 207.5 ± 15.7 vs. 213.5 ± 14.4 Nm; third set: 203.4 ± 16.2 vs. 211.9 ± 13.6 Nm; fourth set: 204.2 ± 15.5 vs. 213.4 ± 13.7 Nm, respectively).

Upstream mTOR regulators

Akt phosphorylation at Ser473 under RE conditions did not change significantly at any time point, whereas HS significantly increased Akt phosphorylation at 1 h post-resistance exercise under HRE condition ($P < 0.05$; Fig. 1a). AMPKα phosphorylation at Thr172 did not significantly change at any time point under RE or HRE conditions (Fig. 1b).

mTOR signaling

The phosphorylation of mTOR at Ser2448 did not change significantly at any time point under RE conditions, whereas HS significantly increased mTOR phosphorylation at 1 h post-exercise under HRE conditions ($P < 0.05$; Fig. 2a). The phosphorylation of 4E-BP1 at Thr37/46 significantly decreased immediately after exercise under RE and HRE conditions, but elevated phosphorylation levels

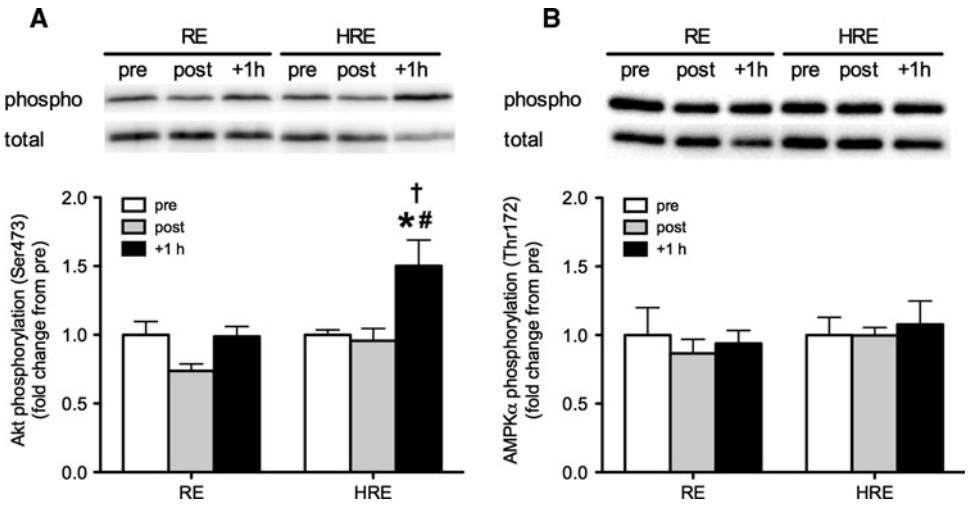

Fig. 1 Phosphorylation of Akt at Ser473 (**a**) and AMPKα at Thr172 (**b**) in human skeletal muscle before (pre), immediately after (post), and 1 h after (+1 h) resistance exercise performed under two different conditions (RE and HRE). Representative immunoblots are shown above each graph. All values are expressed relative to the pre values for each condition. The data are expressed as mean ± SEM ($n = 8$). $^{*}P < 0.05$ versus pre; $^{\#}P < 0.05$ versus post; $^{\dagger}P < 0.05$ versus RE

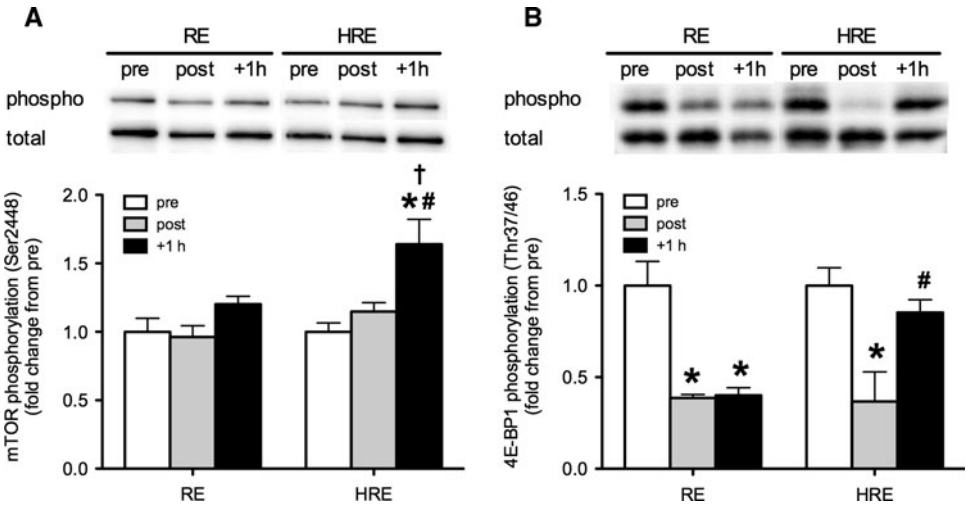

Fig. 2 Phosphorylation of mTOR at Ser2448 (**a**) and 4E-BP1 at Thr37/46 (**b**) in human skeletal muscle before (pre), immediately after (post), and 1 h after (+1 h) resistance exercise performed under two different conditions (RE and HRE). Representative immunoblots are shown above each graph. All values are expressed relative to the pre values for each condition. The data are expressed as mean ± SEM ($n = 8$). $^{*}P < 0.05$ versus pre; $^{\#}P < 0.05$ versus post; $^{\dagger}P < 0.05$ versus RE

were detected at 1 h post-exercise compared with immediately after under HRE conditions ($P < 0.05$; Fig. 2b).

S6K1 phosphorylation at Ser424/Thr421 was significantly increased both immediately after and at 1 h post-exercise as compared with pre-exercise levels under both RE and HRE conditions, and was significantly increased 1 h post-exercise compared with immediately post-exercise under both RE and HRE conditions ($P < 0.05$; Fig. 3a). S6K1 phosphorylation at Thr389 was significantly increased 1 h post-exercise as compared with pre-exercise under RE conditions, whereas HS significantly increased S6K1 phosphorylation at Thr389 immediately post-exercise, and the phosphorylation level remained elevated at 1 h post-exercise under HRE conditions ($P < 0.05$; Fig. 3b). S6 phosphorylation at Ser235/236 was significantly increased at 1 h post-resistance exercise under both RE and HRE conditions, but this phosphorylation level was significantly higher under HRE conditions ($P < 0.05$; Fig. 3c). The phosphorylation of eEF2 at Thr56 did not change significantly at any time point under RE or HRE conditions (Fig. 3d).

MAPK

ERK1/2 phosphorylation at Thr202/Tyr204 was significantly elevated at 1 h post-exercise as compared with before and immediately after exercise under RE conditions ($P < 0.05$; Fig. 4a), and was significantly increased at 1 h post-exercise as compared with pre-exercise under HRE conditions ($P < 0.05$; Fig. 4a). The phosphorylation of p38 at Thr180/Tyr182 was significantly increased both immediately after and at 1 h after exercise as compared with the pre-exercise level. Furthermore, p38 phosphorylation immediately post-exercise was significantly higher under HRE than under RE conditions ($P < 0.05$; Fig. 4b). The phosphorylation of p38 under HRE conditions was significantly decreased 1 h post-exercise as compared with immediately post-exercise ($P < 0.05$; Fig. 4b).

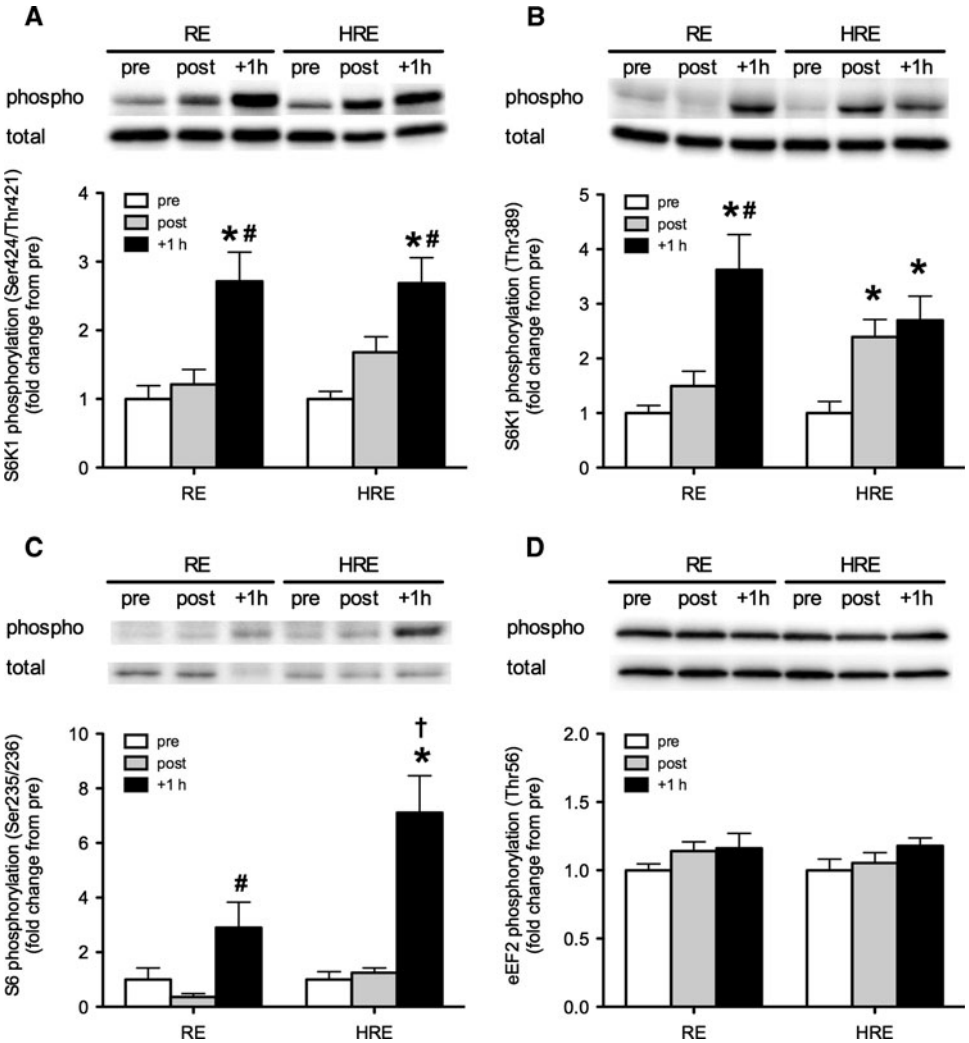

Fig. 3 Phosphorylation of S6K1 at Ser424/421 (**a**) and at Thr389 (**b**), S6 at Ser235/236 (**c**), and eEF2 at Thr56 (**d**) in human skeletal muscle before (pre), immediately after (post), and 1 h after (+1 h) resistance exercise performed under two different conditions (RE and HRE). Representative immunoblots are shown above each graph. All values are expressed relative to the pre values for each condition. The data are expressed as mean ± SEM ($n = 8$). $^{*}P < 0.05$ versus pre; $^{\#}P < 0.05$ versus post; $^{\dagger}P < 0.05$ versus RE

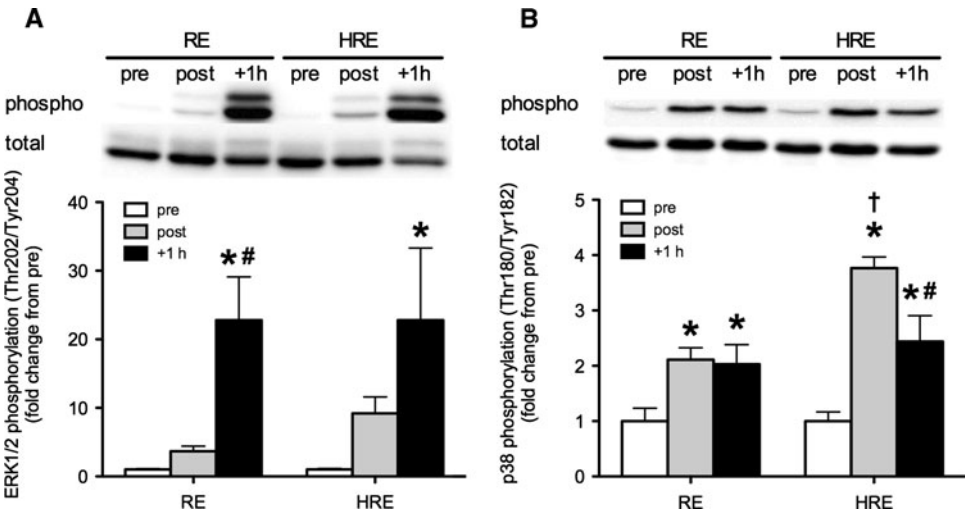

Fig. 4 Phosphorylation of ERK1/2 at Thr202/Tyr204 (**a**) and p38 at Thr180/182 (**b**) in human skeletal muscle before (pre), immediately after (post), and 1 h after (+1 h) resistance exercise performed under two different conditions (RE and HRE). Representative immunoblots are shown above each graph. All values are expressed relative to the pre values for each condition. The data are expressed as mean ± SEM ($n = 8$). $^{*}P < 0.05$ versus pre; $^{\#}P < 0.05$ versus post; $^{\dagger}P < 0.05$ versus RE

Discussion

The purpose of this study was to determine the effect of HS on the mTOR signaling pathway after resistance exercise in human skeletal muscle. The novel finding of this study was that HS enhanced the phosphorylation of molecules involved in mTOR signaling after resistance exercise in human skeletal muscle. Specifically, S6 phosphorylation at

Ser235/236 was increased, and the decreased 4E-BP1 (Thr37/46) phosphorylation level had returned to the baseline level by 1 h after resistance exercise with HS. Our results indicate the possibility that the combination of resistance exercise with HS, compared with resistance exercise alone, can promote translation initiation and protein synthesis.

HS in this study was applied to the belly of the vastus lateralis in accordance with the methods of a previous study [34]. As the physiological characteristics of the subjects in the present study were almost identical to those of the subjects in the previous study [34], it was expect that HS in this study would similarly increase the muscle temperature of the vastus lateralis from 35.0 ± 0.8 to 41.1 ± 1.3°C, as previously described [34]. The resistance exercise protocol employed in the present study contributed little to muscle temperature elevation because of the short total exercise duration (~72 s). Nosaka et al. [36] reported that 12 maximal eccentric contractions of the forearm flexors caused muscle temperature to rise less than 1°C. Furthermore, Ferguson et al. [37] demonstrated muscle temperature elevations of less than 2°C with dynamic knee extensor exercises for 10 min. Although we did not measure the muscle temperature change due to exercise, it seems clear that the muscle temperature change induced by HS was much higher than that induced by resistance exercise alone. Additionally, the average peak torque during maximal isokinetic concentric contraction was similar between RE and HRE conditions, suggesting no warm-up effect of HS to improve neuromuscular coordination and thereby increase mechanical stimulus.

Resistance exercise is known to upregulate mTOR signaling; the contractile mode also appears to have an impact on the phosphorylation status of mTOR signaling. Eliasson et al. [38] showed that maximal eccentric contractions led to significant increases in S6K1 and S6 phosphorylation levels, whereas maximal concentric contractions did not result in any significant changes. However, in the present study, the maximal isokinetic concentric contractions during the knee extension exercise significantly increased the phosphorylation of S6K1 and S6, whereas 4E-BP1 phosphorylation decreased and remained low during the recovery period, consistent with previous results [38–41]. On the other hand, there was no effect of resistance exercise alone on mTOR, Akt, AMPK, or eEF2 phosphorylation. These disparate findings may be attributable to differences in the exercise protocols (e.g., volume, number of repetitions, and intensity), the subject's training status, and/or the timing of muscle biopsy. In any case, our results indicate that maximal isokinetic concentric contractions can activate the phosphorylation of S6K1 and S6 in human skeletal muscle.

In the present study, the combination of HS and resistance exercise significantly increased Akt and mTOR phosphorylation at 1 h post-exercise in human skeletal muscle. Akt phosphorylation was shown to be temperature sensitive in an in vitro study [42], and Wei et al. [14] reported that heat shock caused a significant increase in Akt phosphorylation at Ser473 in rat ventricular myocytes. Although a comparison between in vitro and in vivo models is difficult, it may be suggested that an elevation in muscle temperature by HS has a small effect on Akt phosphorylation in human skeletal muscle. Alternatively, the higher phosphorylation level may be due to increased muscle blood flow accompanying the elevation in muscle temperature. Recently, Fujita et al. [43] showed that an improvement in blood flow enhanced the rate of muscle protein synthesis as well as Akt and mTOR phosphorylation in response to insulin infusion in older individuals, suggesting the possibility that increased blood flow may enhance the sensitivity to anabolic signaling. Another possibility is that nitric oxide, which can be mediated by blood flow and HS [44], may activate Akt phosphorylation [45]. In the present study, therefore, it is speculated that an increase in blood flow induced by HS prior to resistance exercise enhanced Akt and mTOR phosphorylation in response to anabolic signaling (e.g., insulin, IGF-1, and GH) and nitric oxide post-exercise.

mTOR signaling to its downstream effector S6K1 plays a key role in stimulating translation initiation and protein synthesis [11, 12]. Several studies have shown that the Thr389 of S6K1 is rapamycin sensitive [13, 46], suggesting that only mTOR phosphorylates S6K1 at Thr389. However, in the present study, it is noteworthy that resistance exercise with HS stimulated S6K1 phosphorylation at Thr389 immediately after exercise, without mTOR phosphorylation. Although the effect of HS on mTOR signaling in skeletal muscle remains unclear in vivo, previous in vitro studies have demonstrated heat shock-induced S6K1 phosphorylation [15, 47]. These findings suggest that S6K1 phosphorylation after HS is regulated by an mTOR-independent mechanism in skeletal muscle. Activated S6K1 phosphorylates and activates S6, a 40S ribosomal protein, thereby enhancing the translation of 5′TOP mRNA [46]. Our data are the first to indicate that HS increased the phosphorylation of S6 at 1 h after resistance exercise compared with resistance exercise alone. This suggests that an increase in S6 phosphorylation by HS may enhance translation initiation and protein synthesis. Although we did not observe a link between S6K1 and S6 at 1 h after resistance exercise with HS, some studies have demonstrated resistance exercise-induced S6K1 and S6 phosphorylation at different time points post-exercise [39, 40]. Thus, it is possible that S6K and S6 phosphorylation did not increase simultaneously in the present study.

Alternatively, the increased phosphorylation of S6 after resistance exercise with HS might have been due to p90 ribosomal protein S6 kinase polypeptide 1 (RSK1) via MAPKs. Some studies have reported that RSK1 can also phosphorylate S6 at Ser235/236 [48, 49], leading to enhanced translation initiation. Further investigation is needed to clarify the mechanisms by which resistance exercise with HS activates S6K1 and S6 phosphorylation in human skeletal muscle.

The hyperphosphorylation of 4E-BP1 by mTOR promotes the formation of the multiprotein eIF4E-G scaffolding complex (eIF4F), which binds the 40S ribosomal subunit to mRNA [9]. Many studies have shown that resistance exercise decreases 4E-BP1 phosphorylation post-exercise [19, 26, 40, 50]. Dreyer et al. [19] have suggested that reduced 4E-BP1 phosphorylation may contribute to the inhibition of muscle protein synthesis during resistance exercise. We also found that 4E-BP1 phosphorylation decreased following resistance exercise. However, 4E-BP1 phosphorylation returned to baseline sooner after resistance exercise with HS than without HS, indicating that protein synthesis might have been less inhibited with HS. Moreover, this occurred concomitantly with the elevation in mTOR phosphorylation, indicating a strong relationship between mTOR and 4E-BP1.

AMPK is often referred to as a sensor of cellular energy status, and an increased AMP/ATP ratio leads to a rise in AMPK activity [51]. In addition, AMPK activation leads to eEF2 phosphorylation at Thr56 via the phosphorylation-induced activation of eEF2K [52, 53]. Previously, AMPK activity has been shown to increase immediately following resistance exercise [19, 21, 54]. However, we did not observe any change in AMPK phosphorylation after four sets of six isokinetic maximal concentric contractions, in agreement with Tannerstedt et al. [20], who also found no change in AMPK phosphorylation after four sets of six maximal eccentric contractions. The differences in these results may be explained by differences in the duration of exercise, as the total exercise time was much shorter in the present study (4 sets × 6 repetitions) than in previous studies (e.g., 8 or 10 sets × 10 repetitions) [19, 21, 54]. Thus, exercise duration or number of repetitions may affect AMPK activity after exercise. Moreover, in the present study, HS for 30 min did not change the AMPK or eEF2 phosphorylation level immediately after resistance exercise. Our data suggest that an HS-induced increase in muscle temperature may have no effect on the energetic stress response.

We also examined the phosphorylation of MAPKs, including p38 and ERK1/2. The levels of phosphorylated ERK1/2 and p38 have been shown to increase after resistance exercise in human skeletal muscle [30–32, 55]. We also found increased phosphorylation of ERK1/2 and p38 after resistance exercise. Deldicque et al. [32] proposed the existence of crosstalk between MAPK (ERK1/2 and p38) and S6K1. However, in the present study, p38 phosphorylation did not occur concomitantly with S6K1 phosphorylation at Ser424/421, but compared with exercise alone, HS increased p38 phosphorylation immediately after resistance exercise. Bijur et al. [56] showed in vitro that p38 phosphorylation gradually increased during heat shock in a time-dependent manner. Hence, it is possible that the duration of HS and subsequent muscle temperature elevation influences the phosphorylation status of p38.

HSPs, which are important for chaperoning nascent peptides during translation [5, 6], have been investigated in several studies as a possible mechanism of HS-related muscle hypertrophy [1–3]. Previous studies have shown that HSP protein levels increased in skeletal muscle at 4 h [57] and later [33, 58] after HS. Although we did not measure HSP expression in this study, the levels of HSPs most likely did not increase within 1 h after HS, based on previous data [33, 57, 58], and thus HS-induced HSP expression probably did not play a large part in skeletal muscle protein synthesis, at least within 1 h after HS.

This study has several limitations. First, we did not study the effect of only HS on mTOR signaling in human skeletal muscle. This might have helped to clarify the effect of HS and resistance exercise on the signaling molecules. Second, we did not measure blood flow or other potential physiological changes secondary to resistance exercise and HS; therefore, we could not clarify the mechanism by which mTOR signaling molecules are activated. Nevertheless, the change in mTOR signaling molecules after resistance exercise with HS, as demonstrated in this study, may partly explain previous results showing that the combination of muscle contraction and HS can result in increased muscle protein content [1] and muscle mass [28]. Further study is needed to resolve these limitations and clarify the relationship to muscle adaptation.

In summary, our present findings indicate that the combination of HS and resistance exercise, compared with resistance exercise alone, enhances mTOR signaling molecule phosphorylation in human skeletal muscle, which is believed to enhance the rate of protein synthesis in human skeletal muscle [22, 24]. These findings help to explain previous results related to HS-induced muscle hypertrophy [1, 28]. Although we expect that resistance exercise with HS will be a useful tool for maintaining and improving muscle mass, future studies should focus on the regulation of mTOR signaling molecules and the rate of protein synthesis in human skeletal muscle.

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Conflict of interest The authors declare no conflict of interest.

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