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Daily heat stress treatment rescues denervation-activated mitochondrial clearance and atrophy in skeletal muscle

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Key points

- Traumatic nerve injury or nerve disease leads to denervation and severe muscle atrophy. Recent evidence shows that mitochondrial loss could be a key mediator of skeletal muscle atrophy.
- Here, we show that daily heat stress treatment rescues denervation-induced loss of mitochondria and concomitant muscle atrophy.
- We also found that denervation-activated autophagy-dependent mitochondrial clearance (mitophagy) was suppressed by daily heat stress treatment. The molecular basis of this observation is explained by our results showing that heat stress treatment attenuates the increase of key proteins that regulate the tagging step for mitochondrial clearance and the intermediate step of autophagosome formation in denervated muscle.
- These findings contribute to the better understanding of mitochondrial quality control in denervated muscle from a translational perspective and provide a mechanism behind the attenuation of muscle wasting by heat stress.

Abstract Traumatic nerve injury or motor neuron disease leads to denervation and severe muscle atrophy. Recent evidence indicates that loss of mitochondria and the related reduction in oxidative capacity could be key mediators of skeletal muscle atrophy. As our previous study showed that heat stress increased the numbers of mitochondria in skeletal muscle, we evaluated whether heat stress treatment could have a beneficial impact on denervation-induced loss of mitochondria and subsequent muscle atrophy. Here, we report that daily heat stress treatment (mice placed in a chamber with a hot environment; 40°C, 30 min day^{-1}, for 7 days) rescues the following parameters: (i) muscle atrophy (decreased gastrocnemius muscle mass); (ii) loss of mitochondrial content (decreased levels of ubiquinol–cytochrome *c* reductase core protein II, cytochrome *c* oxidase subunits I and IV and voltage-dependent anion channel protein); and (iii) reduction in oxidative capacity (reduced maximal activities of citrate synthase and 3-hydroxyacyl-CoA dehydrogenase) in denervated muscle (produced by unilateral sciatic nerve transection). In order to gain a better understanding of the above mitochondrial adaptations, we also examined the effects of heat stress on autophagy-dependent mitochondrial clearance (mitophagy). Daily heat stress normalized denervation-activated induction of mitophagy (increased mitochondrial microtubule-associated protein 1A/1B-light chain3-II (LC3-II) with and without blocker of autophagosome clearance). The molecular basis of this observation was explained by the results that heat stress attenuated the denervation-induced increase in key proteins that regulate the following steps: (i) the tagging step of mitochondrial clearance (increased mitochondrial Parkin, ubiquitin-conjugated, P62/sequestosome 1 (P62/SQSTM1)); and (ii) the elongation step of autophagosome formation (increased Atg5–Atg12 conjugate and Atg16L). Overall, our results contribute to the better understanding of mitochondrial quality control and the mechanisms behind the attenuation of muscle wasting by heat stress in denervated skeletal muscle.

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Abbreviations ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; Atg, autophagy-related gene; COX, cytochrome *c* oxidase subunit; CS, citrate synthase; Drp1, dynamin-related protein 1; FAT/CD36, fatty acid translocase/cluster of differentiation 36; Fis1, mitochondrial fission 1; GLUT4, glucose transporter 4; 3-HAD, 3-hydroxyacyl-CoA dehydrogenase; 4-HNE, 4-hydroxy-2-nonenal; HSP, heat shock protein; LC3, microtubule-associated protein 1A/1B-light chain 3; Mfn2, mitofusin-2; mTORC1, mechanistic (mammalian) target of rapamycin complex 1; Opa1, Optic atrophy 1; PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1-α; ROS, reactive oxygen species; SQSTM1, sequestosome; TRAP-1, tumour necrosis factor receptor-associated protein-1; ULK1, unc-51-like kinase 1; UQCRC2, ubiquinol–cytochrome *c* reductase core protein II; USP30, ubiquitin-specific-processing protease 30; VDAC, voltage-dependent anion channel.

Introduction

Skeletal muscle constitutes 40% of total body mass, representing the largest tissue in the body. Skeletal muscle plays important roles not only in locomotion, but also in metabolic and endocrine organs, contributing to daily movement and improvement of whole-body metabolism. Skeletal muscle mass and function are regulated by motor innervation. Patients with denervation caused by conditions such as traumatic nerve injury, diabetic neuropathy, degenerative disc disease, amyotrophic lateral sclerosis and spinal muscular atrophy are forced into a vicious cycle of chronic muscle disuse myopathy, which includes muscle wasting, weakness and pain (Bongers *et al.* 2013). To establish effective therapies, researchers are actively exploring the cellular and molecular mechanisms involved in denervation-initiated myopathy using animal models.

Together with muscle wasting, denervation also induces mitochondrial toxicity, such as excessive accumulation of oxidative stress (Leary *et al.* 2012), loss of mitochondrial content (Lokireddy *et al.* 2012; Furuya *et al.* 2014) and a reduction of oxidative capacity (Wicks *et al.* 1991; Furuya *et al.* 2014) in skeletal muscle. Recent studies show that these mitochondrial toxicities could be key mediators of muscle wasting (Powers *et al.* 2012; Tryon *et al.* 2014); for example, skeletal muscle atrophy is suppressed by pharmacological scavenging of mitochondrial oxidative stress (Min *et al.* 2011; Talbert *et al.* 2013) or maintenance of mitochondrial content via genetic deletion of key proteins regulating mitochondrial degradation (mitophagy; Lokireddy *et al.* 2012; Furuya *et al.* 2014). Importantly, these studies suggest that conservation of healthy mitochondria can be an effective therapeutic target.

Recently, we have reported that heat stress treatment activates cellular signalling cascades associated with mitochondrial gene transcription and induces mitochondrial adaptations that include increased mitochondrial content and oxidative capacity in the skeletal muscle of mice (Tamura *et al.* 2014). Others recently reported that overexpression of heat shock protein (HSP) 72, a heat stress-inducible molecular chaperone, increases the number of mitochondria in mouse skeletal muscle (Henstridge *et al.* 2014). Furthermore, Naito *et al.* (2000) demonstrated that heat stress attenuates muscle atrophy in 'hindlimb-suspended' rats. These studies strongly suggest the possibility that heat stress treatment attenuates denervation-induced loss of mitochondrial content, the reduction of oxidative capacity and subsequent muscle atrophy. However, the following two important issues remain: (i) it is unknown whether heat stress treatment also rescues 'denervation-induced' muscle atrophy, because denervation leads to inimitable cellular responses/adaptations (Hornberger *et al.* 2001; Machida *et al.* 2012; Tang *et al.* 2014) and different efficacy with pharmacological intervention compared with other models of muscle disuse (MacDonald *et al.* 2014); and (ii) the impact of heat stress treatment on mitochondrial toxicity during muscle disuse including denervation is completely unknown.

The purpose of this study, therefore, was to examine whether daily heat stress treatment rescues denervation-induced mitochondrial loss and subsequent muscle atrophy. In order to gain a better understanding of mitochondrial adaptations, we also investigated the effects of daily heat stress treatment on cellular machinery regulating mitochondrial biogenesis and degradation in the denervated muscle.

Methods

Ethical approval

All experiments were approved by the Animal Experimental Committee of The University of Tokyo (no. 24-18). The authors have read, and all experiments complied with, the policies and regulations of *Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions* published by the Ministry of Education, Culture, Sports, Science and Technology, Japan (no. 71,

2006) and *The Journal of Physiology* written by Drummond (2009).

Experimental animals

Eight-week-old male ICR mice (CLEA Japan, Tokyo, Japan; $n = 110$, including preliminary experiments) were used throughout this study. Mice were individually housed (cage size 16.5 cm × 9.5 cm × 10.0 cm) on a 12 h–12 h light–dark cycle (dark 07.00–19.00 h) in an air-conditioned room (22°C). All mice had access to standard chow (MF; Oriental Yeast, Tokyo, Japan) and water *ad libitum*. Mice were divided into a non-heat stress group ($n = 6$) or heat stress group ($n = 6$–8). All mice underwent unilateral sciatic nerve transection surgery. We performed experiments independently as follows: acute core temperature measurements, whole muscle analysis, mitochondrial isolation and/or colchicine treatment. Twenty-four hours after the final heat stress and/or colchicine treatment, mice were anaesthetized using isoflurane [4% (v/v) induction, 3% (v/v) maintenance, flow rate 0.5 l min^{-1}] and killed by cervical dislocation. Given that some biochemical analyses require a large mass of muscle, the gastrocnemius muscles were collected and prepared for further analysis.

Sciatic nerve transection

We employed 10 days of unilateral sciatic nerve transection as surgical denervation, because previous studies showed that this model induced sufficient muscle atrophy and decreased the mitochondrial content in skeletal muscle (Brault *et al.* 2010; Kitaoka *et al.* 2014). Briefly, mice were anaesthetized using isoflurane [4% (v/v) induction, 3% (v/v) maintenance, flow rate 0.5 l min^{-1}]. A small incision was made in the posterior aspect of the left hindlimb to expose the sciatic nerve at the level of the femoral trochanter. A length of at least 5.0 mm of sciatic nerve was excised using small operating scissors. The skin was closed with surgical glue. The right hindlimb served as the sham-operated control.

Daily heat stress protocol

A previous study has shown that anaesthesia during heat stress impairs the thermoregulation of normal mice (Leon *et al.* 2005) and induces a non-physiological situation. Therefore, we employed whole-body heat stress without anaesthesia, as previously described (Tamura *et al.* 2014). Briefly, mice were exposed to a hot environment chamber (40°C, 30 min day^{-1}, from day 3 to 9 during the 10 day denervation period) without anaesthesia. We have previously reported that this heat stress method does not affect the spontaneous physical activity levels of mice during exposure and does not increase plasma corticosterone concentrations, a biomarker for systemic stress, beyond the levels of those induced by low-to-moderate intensity and volume treadmill running (Tamura *et al.* 2014). Mice in the non-heat stress group were exposed to a normal rearing environment chamber (22°C). Mice had no access to chow and water during exposure.

Mitophagic and autophagic flux

To evaluate mitophagic and autophagic flux, we used colchicine (C9754; Sigma-Aldrich, St Louis, MO, USA), which is a microtubule depolarizing agent, according to a previously reported protocol with minor modifications (Ju *et al.* 2010; Mofarrahi *et al.* 2013). Mice received I.P. injections of saline (without colchicine group) or colchicine (0.4 mg kg^{-1} day^{-1}; with colchicine group) after the final heat stress treatment (i.e. monitoring mitophagic and autophagic flux from day 9 to 10).

Rectal temperature measurement

Rectal temperature was measured after a single bout of heat stress. Immediately after a single heat stress treatment, mice were anaesthetized using isoflurane [4% (v/v) induction, 3% (v/v) maintenance, flow rate 0.5 l min^{-1}]. Rectal temperature was measured using a thermocouple (E52-CA20AY D = 3.2; Omron, Tokyo, Japan) connected to a data logger (GL200; Graphtec, Yokohama, Japan).

Mitochondrial enzyme activity

Maximal activities of citrate synthase (CS) and 3-hydroxyacyl-CoA dehydrogenase (3-HAD) were determined in whole muscle homogenates. Frozen and crushed gastrocnemius muscle specimens were homogenized in 100 (v/w) of 100 mM potassium phosphate buffer. Maximal CS and 3-HAD activities were measured spectrophotometrically using the methods of Srere (1969) and Bass *et al.* (1969), respectively.

Mitochondrial isolation

Mitochondria-enriched/associated fractions were isolated from gastrocnemius muscles using a previously described differential centrifugation method with minor modifications (Safdar *et al.* 2011). Briefly, gastrocnemius muscles were mildly homogenized using a polytron and Potter glass homogenizer in mitochondrial isolation buffer (67 mM sucrose, 50 mM Tris, 50 mM KCl, 10 mM EDTA and 0.2% (w/v) fatty acid-free bovine serum albumin, pH 7.4) containing protease inhibitors (complete protease

inhibitor cocktail; Roche Diagnostics, Tokyo, Japan). The homogenate was centrifuged at 700*g* for 15 min at 4°C. The supernatant was collected and centrifuged again at 12,000*g* for 20 min at 4°C. The mitochondrial pellet was resuspended in mitochondrial isolation buffer and centrifuged at 12,000*g* for 20 min at 4°C. The supernatant was removed and the mitochondrial pellet resuspended in lysis buffer for Western blot analysis. The protein concentration was measured using the Bradford method. We confirmed the purity of the mitochondria-enriched fraction by Western blotting using antibodies against glyceraldehyde 3-phosphate dehydrogenase (cytosolic marker) and cytochrome *c* oxidase subunit (COX) IV (mitochondrial marker; data not shown).

The protein expressions of Parkin, ubiquitin-conjugated protein, p62/sequestosome 1 (SQSTM1) and microtubule-associated protein 1A/1B-light chain 3-II (LC3-II) in mitochondria-enriched/associated fractions were measured using Western blotting to evaluate mitophagy in accordance with previous studies (O'Leary *et al.* 2013; Webster *et al.* 2013).

Western blot analysis

Muscle samples were homogenized as previously described (Hoshino *et al.* 2012) using lysis buffer [1% (v/v) Triton X-100, 50 mM Tris–HCl, 1 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 10 mM sodium 3-glycerol phosphate, 5 mM sodium pyrophosphate and 2 mM DTT; pH 7.5] containing 10 μg ml of pepstatin A, aprotinin and leupeptin, 1 mM sodium orthovanadate and 0.177 mg ml^{-1} phenylmethylsulfonyl fluoride. Sample proteins were measured using the Bradford method. Equal amounts (5–15 μg) of protein were separated using standard SDS-PAGE [7.5–17% (w/v) polyacrylamide gels] and transferred to a polyvinylidene difluoride membrane (Hybond-P; GE Healthcare Japan, Tokyo, Japan). Transfer of protein was confirmed by staining with Ponceau S (P7170-1L; Sigma-Aldrich). Membranes were blocked with 3–7.5% (w/v) bovine serum albumin in Tris-buffered saline containing 0.05% (v/v) Tween 20 (TBST) for 1 h and incubated overnight with the primary antibodies. After incubation, membranes were washed in TBST, incubated for 1 h at room temperature with secondary antibodies (A106PU or A102PT; American Qualex, San Clemente, CA, USA) and washed again in TBST. Chemiluminescent reagents (SuperSignal West Pico Chemiluminescent Substrate; Thermo Fisher Scientific, Waltham, MA, USA) were used to facilitate blot detection. Blots were scanned and quantified using ChemiDoc XRS (170-8071; Bio-Rad, Hercules, CA, USA) and Quantity One (170-9600, version 4.5.2, Windows; Bio-Rad). Blots of 4-hydroxy-2-nonenal (4-HNE) and ubiquitin conjugated were quantified the range between 25–150 kDa.

Primary antibodies for Western blotting

The following primary antibodies were purchased: HSP60 (ADI-SPA-806; Enzo Life Sciences, Tokyo, Japan), HSP72 (ADI-SPA-810-D; Enzo Life Sciences), COX IV (ab14744; Abcam, Cambridge, UK), MitoProfile Total OXPHOS Rodent WB Antibody Cocktail [ubiquinol–cytochrome *c* reductase core protein II (UQCRC2), COX I, ab110413; Abcam], voltage-dependent anion channel [VDAC, 4661S; Cell Signaling Technology (CST) Japan, Tokyo, Japan], peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α, 516557; Millipore, CA, USA), glucose transporter 4 (GLUT4, ab33780; Abcam), fatty acid translocase/cluster of differentiation 36 (FAT/CD36, ab137320; Abcam), Parkin (ab77924; Abcam), ubiquitin (3933; CST), p62/SQSTM1 [PM045; Medical & Biological Laboratories (MBL), Nagoya, Japan], LC3 (M152-3; MBL), mitofusin-2 (Mfn2, ab124773; Abcam), Optic atrophy 1 (Opa1, 612606; BD Transduction Laboratories, Tokyo, Japan), mitochondrial fission 1 (Fis1, ab96764; Abcam), dynamin-related protein 1 (Drp1, ab56788; Abcam), 4-HNE (ab48506; Abcam), ubiquitin-specific-processing protease 30 (USP30, SAB4503385; Sigma-Aldrich), p-AMPK T172 (2535; CST), AMPK (5832; CST), phosphorylated acetyl-CoA carboxylase (p-ACC S79, 3661; CST), ACC (3662; CST), p-Akt S473 (9271; CST), Akt (9272; CST), p-P70S6K T389 (9205; CST), P70S6K (9202; CST), p-ULK1 S555 (5869; CST), p-ULK1 S757 (6888; CST), ULK1 (8054; CST), Atg13 (M183-3MS; MBL), Beclin-1 (PD017MS; MBL), Atg14L (PD026MS; MBL), Atg5–Atg12 conjugate (M153-3MS; MBL) and Atg16L (M150-3MS; MBL).

Statistical analysis

All data are expressed as means ± SEM. Student's unpaired *t* test, two-way ANOVA (denervation × heat stress), or three-way ANOVA (denervation × heat stress × colchicine) were performed. If an interaction was observed, the Tukey–Kramer multiple-comparison test was performed. Statistical significance was defined as $P < 0.05$. A trend was defined as $P < 0.10$. Statistical analysis was performed using JMP (Version 9.0.1, Macintosh; SAS Institute, Cary, NC, USA).

Results

Validation and profile of experimental model

A single bout of heat stress treatment in this study increased rectal temperature (Fig. 1*A*). In addition, the protein content of HSP60 and HSP72, sensitive biomarkers for mitochondrial and cellular heat shock responses (Naito *et al.* 2000; Oishi *et al.* 2002), in the gastrocnemius muscle

were dramatically increased after 7 days heat stress treatment (Fig. 1*B–D*). These observations indicate that, in this study, heat stress sufficiently induced physiological and biochemical responses. We also confirmed that daily heat stress treatment did not affect body weight and total energy intake, which potentially initiate cellular adaptations in skeletal muscle (Fig. 1*E* and *F*). Denervation decreased gastrocnemius muscle mass by 38.2%. In contrast, daily heat stress treatment significantly increased sham-operated and denervated gastrocnemius muscle mass by 6.0 and 15.5%, respectively (Fig. 1*G*). Crucially, this result demonstrates that even if denervation results in inimitable cellular adaptations compared with other muscle disuse models, daily heat stress treatment is also effective on denervation-induced muscle atrophy. Moreover, these data indicate that further investigations on mitochondrial adaptations are appropriate.

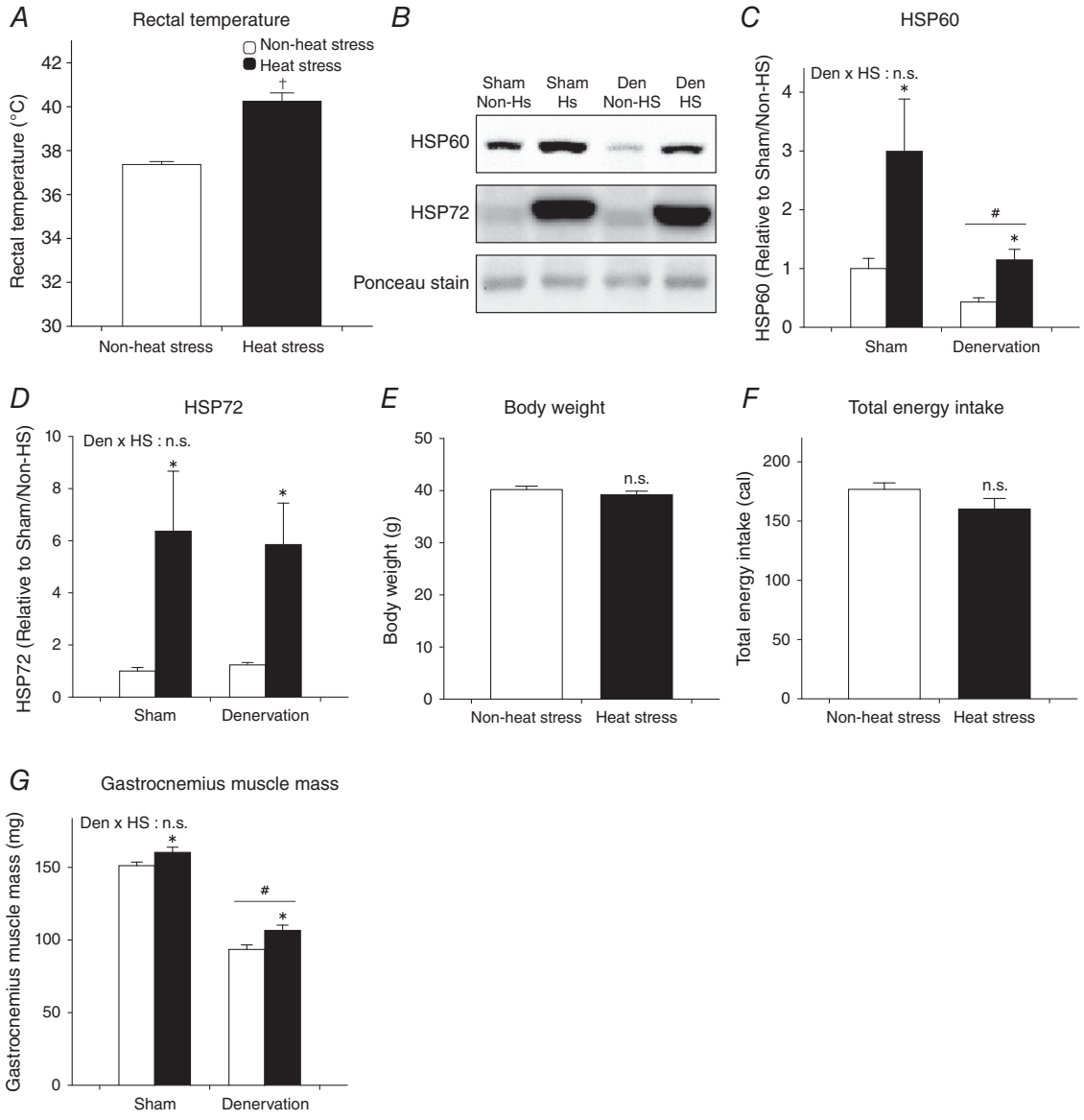

Figure 1. Physiological and biochemical responses/adaptations to heat stress
A, acute response to rectal temperature with a single bout of heat stress treatment. *B–G*, adaptation of daily heat stress treatment. Open columns, non-heat stress; filled columns, heat stress. Student's unpaired *t* test (*A*, *F* and *G*) or two-way ANOVA was performed (*C*, *D* and *E*). $^{\dagger}P < 0.05$ *vs.* non-heat stress. $^{\#}P < 0.05$, main effect of denervation. $^{*}P < 0.05$, main effect of heat stress. Abbreviation: HSP, heat shock protein, n.s., not significant. In this and all subsequent figures, data are shown as the means + SEM.

Daily heat stress treatment rescues loss and oxidative stress of mitochondria

In order to examine mitochondrial adaptations, we first examined the protein expression of mitochondrial enzymes and channels (UQCRC2, COX I, COX IV and VDAC) as biomarkers for mitochondrial content in the gastrocnemius muscle. Denervation dramatically decreased the protein content of these biomarker proteins (Fig. 2*A*–*E*). However, daily heat stress treatment rescued the loss of these mitochondrial proteins in denervated muscle (Fig. 2*A*–*E*). We also examined the maximal activity of mitochondrial enzymes (CS and 3-HAD) as biomarkers for maximal oxidative capacity in the gastrocnemius muscle. Like mitochondrial content, mitochondrial enzyme activities were reduced by denervation, but were maintained by heat stress (Fig. 2*F* and *G*). Dysfunctional mitochondria excessively produce reactive oxygen species (ROS), which accumulate and cause oxidative stress in mitochondria and the cell (Min *et al.* 2011; Muller *et al.* 2007). It is therefore regarded that the accumulation of ROS is an index of mitochondrial toxicity (Wei *et al.* 2009; Chaiswing *et al.* 2005). Denervation upregulated cellular and mitochondrial 4-HNE-conjugated protein, which is one biomarker of oxidative stress (Fig. 2*H*–*J*). In contrast, heat

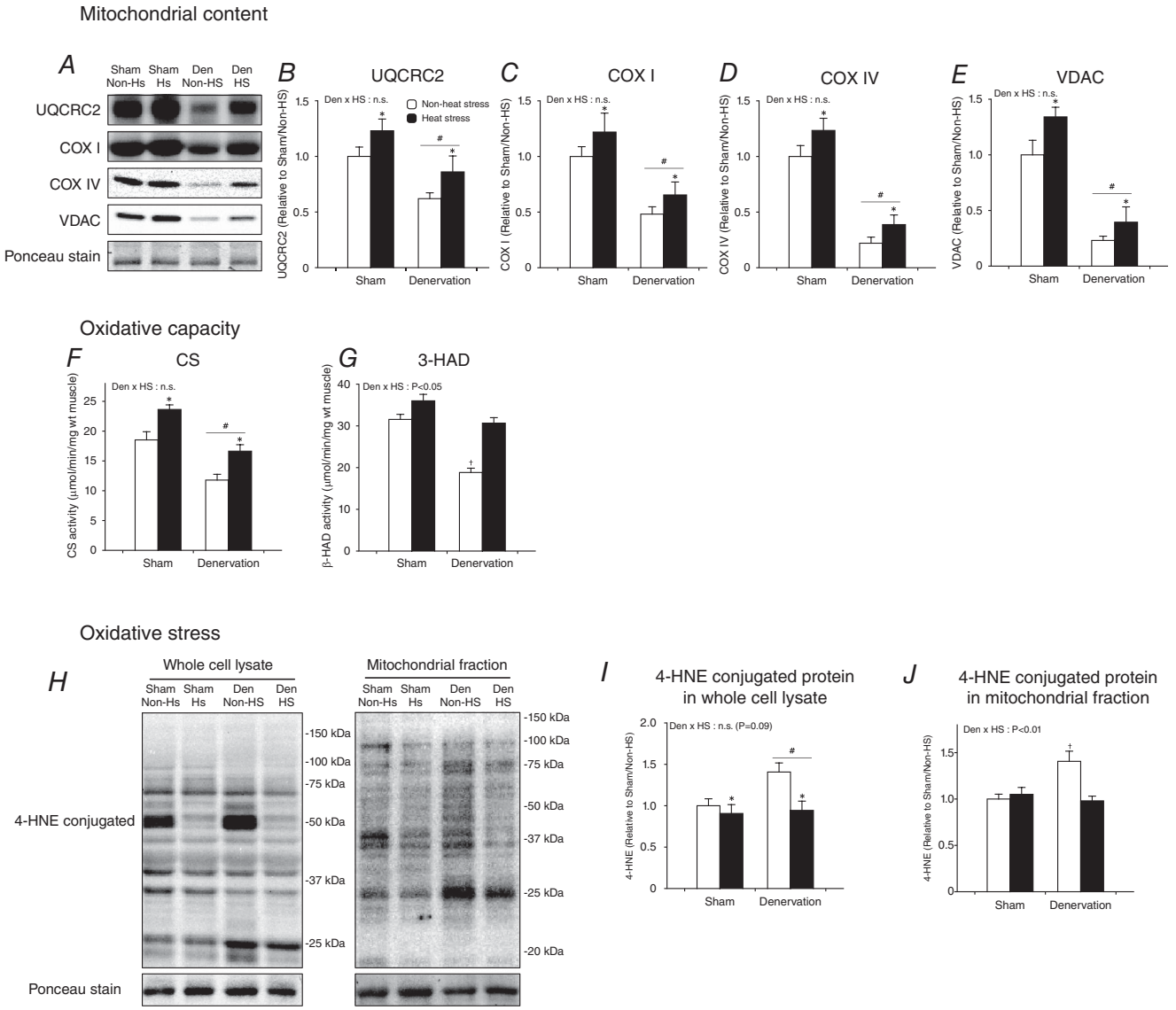

Figure 2. Daily heat stress treatment rescued denervation-induced loss of mitochondria
Open columns, non-heat stress; filled columns, heat stress. Two-way ANOVA was performed. $^{\#}P < 0.05$, main effect of denervation. $^{*}P < 0.05$, main effect of heat stress. $^{\dagger}P < 0.05$ *vs.* non-heat stress/sham. Abbreviation: UQCRC2,ubiquinolcytochrome c reductase core protein II; COX, cytochrome c oxidase subunit; VDAC, voltage-dependent anion channel; CS, citrate synthase; 3-HAD, 3-hydroxyacyl-CoA dehydrogenase; 4-hydroxy-2-nonenal; n.s., not significant.

stress normalized the upregulation of 4-HNE-conjugated protein in both whole-cell and mitochondrial fractions of denervated muscle (Fig. 2*H–J*).

Taken together, these results indicate that daily heat stress treatment rescued denervation-induced loss of mitochondrial content and oxidative capacity in the gastrocnemius muscle, supporting our hypothesis. Importantly, heat stress-rescued mitochondria did not show an unhealthy phenotype.

Daily heat stress treatment does not attenuate loss of PGC-1α

Next, we attempted to clarify the mechanisms underlying the maintenance of mitochondrial content and oxidative capacity by heat stress. Loss of mitochondria due to muscle disuse is mediated by a decrease in PGC-1α (Brault *et al.* 2010; Cannavino *et al.* 2014), a regulator of mitochondrial biogenesis and oxidative metabolism (Lin *et al.* 2002), and can be reversed by genetic and pharmacological PGC-1α overexpression (Sandri *et al.* 2006; Brault *et al.* 2010). Therefore, we examined our hypothesis that heat stress could rescue denervation-induced loss of PGC-1α protein. Contrary to our hypothesis, loss of PGC-1α due to denervation was not inhibited by heat stress (Fig. 3*A* and *B*). In order to validate this observation, we also evaluated the protein content of substrate transporters (glucose, GLUT4 and fatty acid, FAT/CD36) that are other targets of PGC-1α (Benton *et al.* 2008). Concomitant with the loss of mitochondria and PGC-1α, GLUT4 and FAT/CD36 were also decreased by denervation (Fig. 3*C* and *D*). However, heat stress did not attenuate the loss of GLUT4 and FAT/CD36 (Fig. 3*C* and *E*).

Importantly, these observations suggest that the countering impacts of heat stress on denervation-induced mitochondrial loss are most likely to be mediated by mitochondrial selective machinery rather than a PGC-1α-associated pathway.

Daily heat stress treatment normalizes mitophagy

Mitochondrial content and oxidative capacity are determined not only by biogenesis, but also by degradation. To control mitochondrial quality, damaged and dysfunctional mitochondria are degraded by mitochondrial selective autophagy (mitophagy). The process of Parkin-mediated mitophagy, the most actively studied mitophagy, involves the following steps.

i. Mitochondria are tagged for clearance when Parkin, a mitochondrial E3 ubiquitin ligase, is recruited from the cytosol to the membrane of dysfunctional mitochondria, which then ubiquitinates various mitochondrial outer-membrane proteins.
ii. Ubiquitin-conjugated mitochondria are recognized by P62/SQSTM1.
iii. Mitochondria adapted with poly-ubiquitin and P62/SQSTM1 are engulfed by double-membrane vesicles (autophagosomes) consisting of the biomarker protein LC3-II, and are finally degraded by lysosomes.

Recent studies show that denervation continuously activates mitophagy in skeletal muscle (Lokireddy *et al.* 2012; O'Leary *et al.* 2013). Therefore, to clarify the mechanisms involving the beneficial effects of heat stress on mitochondria in denervated muscle, we examined whether daily heat stress treatment normalizes denervation-activated mitophagy. As a result, denervation increased mitophagy-related proteins, including Parkin, ubiquitin-conjugated, P62/SQSTM1 and LC3-II, in the mitochondrial fraction (Fig. 4*A*, *B*, *D*, *E* and *F*), supporting previous reports. However, heat stress attenuated the denervation-induced upregulation of these mitophagy-related proteins without any change in USP30, which was recently identified as mitochondrial de-ubiquitinase in neurons (Bingol *et al.* 2014; Fig. 4*A* and *C*).

Although the presence of mitochondrial LC3-II is closely correlated with autophagosome-engulfed mitochondria and is widely used as a biomarker (O'Leary *et al.* 2013; Webster *et al.* 2013), the presence of mitochondrial LC3-II does not represent 'mitophagic flux'. Hence, it remains an important question whether heat stress suppresses induction of mitophagy or accelerates the final degradation of damaged mitochondria. To elucidate this issue, we used colchicine, which is a pharmacological inhibitor of autophagosome degradation (Ju *et al.* 2010; Mofarrahi *et al.* 2013). In conditions where autophagosome degradation was inhibited, heat stress treatment attenuated the upregulation of mitochondrial LC3-II by denervation (Fig. 4*G* and *H*). This observation indicates that heat stress attenuates the induction of mitophagy but does not accelerate the final clearance of damaged mitochondria.

Heat stress does not affect proteins associated with mitochondrial dynamics in denervated muscle

Mitochondria are highly dynamic organelles that undergo fusion and fission dependent on cellular conditions. Mitochondrial quality is tightly controlled by mitophagy and mitochondrial dynamics (Ashrafi *et al.* 2013; Iqbal *et al.* 2014). Previous studies show that mitochondrial fission events predate mitophagy (Ashrafi *et al.* 2013; Iqbal *et al.* 2014). In order to gain further understanding of the effects of heat stress on mitochondrial quality control, we next evaluated the expression of proteins that regulate mitochondrial dynamics. As a result, denervation decreased mitochondrial fusion-related proteins, such as

Mfn2 and Opa1 (Fig. 4*I–K*), but increased mitochondrial fission proteins, such as Fis1 with no change in Drp1 (Fig. 4*I*, L and *M*). These results demonstrate that denervation shifts mitochondria towards fission.

In sham-operated muscle, heat stress significantly increased Mfn2 and tended to increase Opa1, but did not affect proteins involved in the regulation of mitochondrial fission (Fig. 4*I–K*). Contrary to sham-operated muscle, in denervated muscle there was no significant effect of heat stress on proteins regulating mitochondrial dynamics. Interestingly, these results indicate that heat stress attenuates the induction of mitophagy and that the mitochondrial fission phenotype is sustained.

Heat stress suppresses denervation-activated induction of autophagy

Autophagy is excessively activated in catabolic situations, including denervation, contributing to progressive activation of mitophagy and muscle atrophy (Grumati & Bonald, 2012; Neel *et al.* 2013; Tyron *et al.* 2014). In order to gain a better understanding of the normalization of mitophagy induction and attenuation of muscle atrophy by daily heat stress, we examined whether heat stress also attenuates the activation of autophagy induction in denervated muscle. As for mitophagy, heat stress suppressed the increase in LC3-II, both with and without colchicine treatment (Fig. 5*A–D*). These results show that heat stress normalizes denervation-activated induction of autophagy. To identify the molecular mechanism, we also examined the effects of daily heat stress on the expression of key proteins that regulate each step of autophagy induction in denervated muscle. The regulating steps of autophagy induction are often categorized as follows: (i) the initiation step; (ii) the nucleation step; and (iii) the elongation step of autophagosome formation. Denervation significantly upregulated the active (phosphorylated) form and/or total expression of proteins controlling the initiation step (AMPK, AMPK functional readout ACC, ULK1 and Atg13), nucleation step (Atg14L and Beclin 1) and elongation step (Atg5-Atg12 conjugate and Atg16L) of autophagosome formation (Fig. 6*A–S*). In contrast, heat stress significantly attenuated the denervation-induced increase in protein

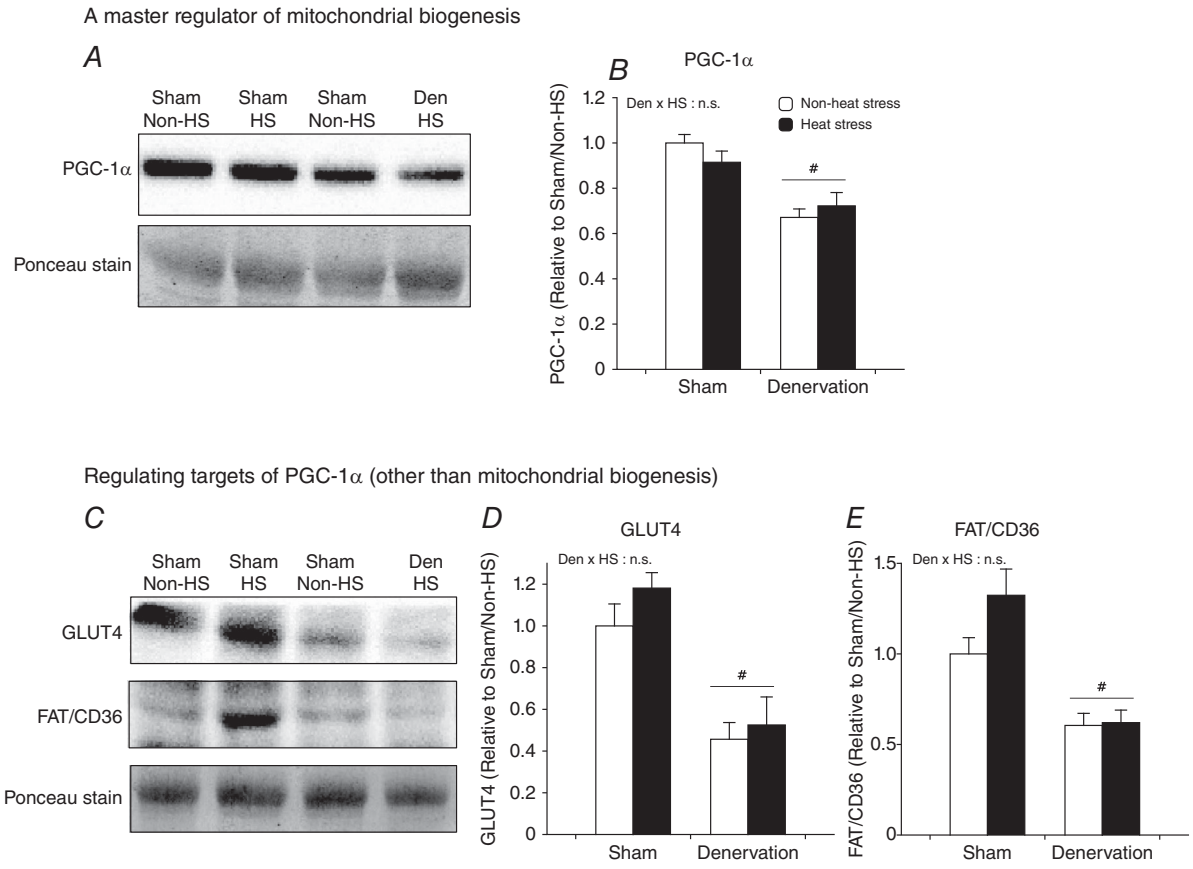

Figure 3. Daily heat stress treatment did not rescue peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α) levels
Open columns, non-heat stress; filled columns, heat stress. Two-way ANOVA was performed. $^{\#}P < 0.05$, main effect of denervation. Abbreviation: PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1-α; glucose trans- porter 4; FAT/CD36, fatty acid translocase/cluster of differentiation 36; n.s., not significant.

expression regulating the elongation step (Atg5–Atg12 conjugate and Atg16L), but did not change the expression of proteins associated with the initiation step [including the suppressing pathway, Akt/mechanistic (mammalian) target of rapamycin complex 1 (mTORC1) and p-ULK1 Ser757] and the nucleation step of autophagosome formation (Fig. 6*Q*–*S*). These observations indicate that daily heat stress normalizes autophagy induction via selective suppression of the elongation step of autophagosome formation.

Discussion

Key findings and translational significance

Traumatic nerve injury or motor neuron disease leads to denervation and subsequent severe myopathy, representing muscle atrophy (Tang *et al.* 2014). Unfortunately, to date, no effective therapy is established (Kanning *et al.* 2010). Here, we focused on heat stress treatment as a potential therapy, because it is a classic, simple and widely applicable therapy. However, the cellular and molecular responses and adaptations in skeletal muscle induced by heat stress treatment are poorly understood.

This study provides evidence that daily heat stress treatment rescues denervation-induced mitochondrial toxicity, such as loss of content, reduction of oxidative capacity, accumulation of oxidative stress and concomitant muscle atrophy. Furthermore, we also found that denervation-activated autophagy-dependent mitochondrial clearance (mitophagy) was suppressed by daily heat stress treatment. The molecular basis of this observation is explained by results showing that heat

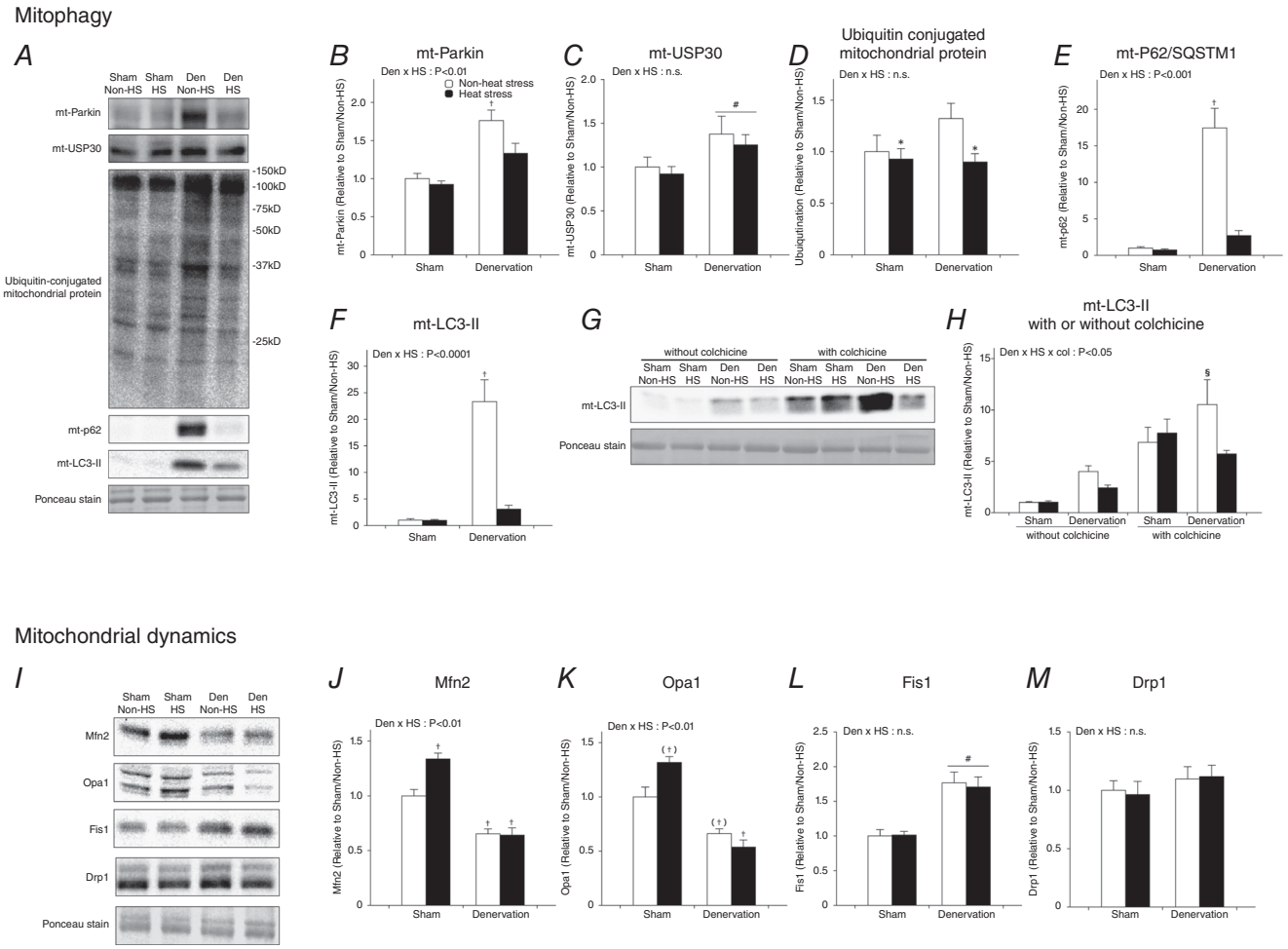

Figure 4. Daily heat stress treatment normalized continuously activated mitophagy induction
Open columns, non-heat stress; filled columns, heat stress. Two- or three-way ANOVA was performed. $^{\#}P < 0.05$, main effect of denervation. $^{*}P < 0.05$, main effect of heat stress. $^{\dagger}P < 0.05$ *vs.* non-heat stress/sham. $(^{\dagger})P = 0.05$ *vs.* non-heat stress/sham. $^{\S}P < 0.05$ *vs.* heat stress/denervation/colchicine. Abbreviation: USP, ubiquitin-specific-processing protease; SQSTM1, sequestosome 1; LC3, microtubule-associated protein 1A/1B-light chain 3; Mfn2, mitofusin-2; Opa1, Optic atrophy 1; Fis1, mitochondrial fission 1; Drp1, dynamin-related protein 1; n.s. not significant.

stress treatment attenuated the increase in key proteins regulating the tagging step for mitochondrial clearance and the elongation step of autophagosome formation in denervated muscle. These observations will contribute to our understanding of mitochondrial quality control in wasting skeletal muscle from a translational perspective and can explain the mechanisms behind the attenuation of muscle wasting by heat stress.

Unique effects of heat stress on mitochondrial quality control machinery

Mitochondrial quality control by mitophagy in skeletal muscle has been actively studied in recent years, because impairment to mitochondrial quality and its machinery can contribute to muscle disorders (Powers *et al.* 2012; Tryon *et al.* 2014). One of the major findings in this study is that denervation-activated mitophagic pathways were normalized by heat stress.

A recent study has shown that prolonged oxidative stress initiates further mitochondrial dysfunction, such as decreased membrane potential and impaired respiratory activity, which is followed by induction of mitophagy in skeletal muscle cells (Iqbal *et al.* 2014). In the present study, heat stress attenuated the denervation-induced accumulation of mitochondrial oxidative stress. Therefore, our results suggest that daily heat stress primarily conserves mitochondrial health, which consequently represses the necessity for mitochondrial clearance. The reason why muscle disuse increases mitochondrial ROS production remains unknown. Dysregulated mitochondrial protein import has been suggested to be one possible cause (Powers *et al.* 2012). Mitochondrial protein and transcriptional factors encoded by nuclear DNA are translated in the cytosol as precursor proteins. Precursor proteins are transported into mitochondria and refolded by HSP60 and HSP72 (Ljubicic *et al.* 2010). In the present study, denervation decreased HSP60, but heat stress normalized loss of HSP60 and increased HSP72 in denervated muscle. One could speculate that upregulation of HSP60 and HSP72 by heat stress may attenuate the dysfunction of mitochondrial protein import, folding and subsequent mitochondrial ROS production. This is further supported by a previous study in the hypothalamus demonstrating that decreased HSP60 protein by short hairpin RNA impairs mitochondrial respiration and induces oxidative stress (Kleinridders *et al.* 2013). Furthermore, a recent study demonstrated that HSP72 works interactively with

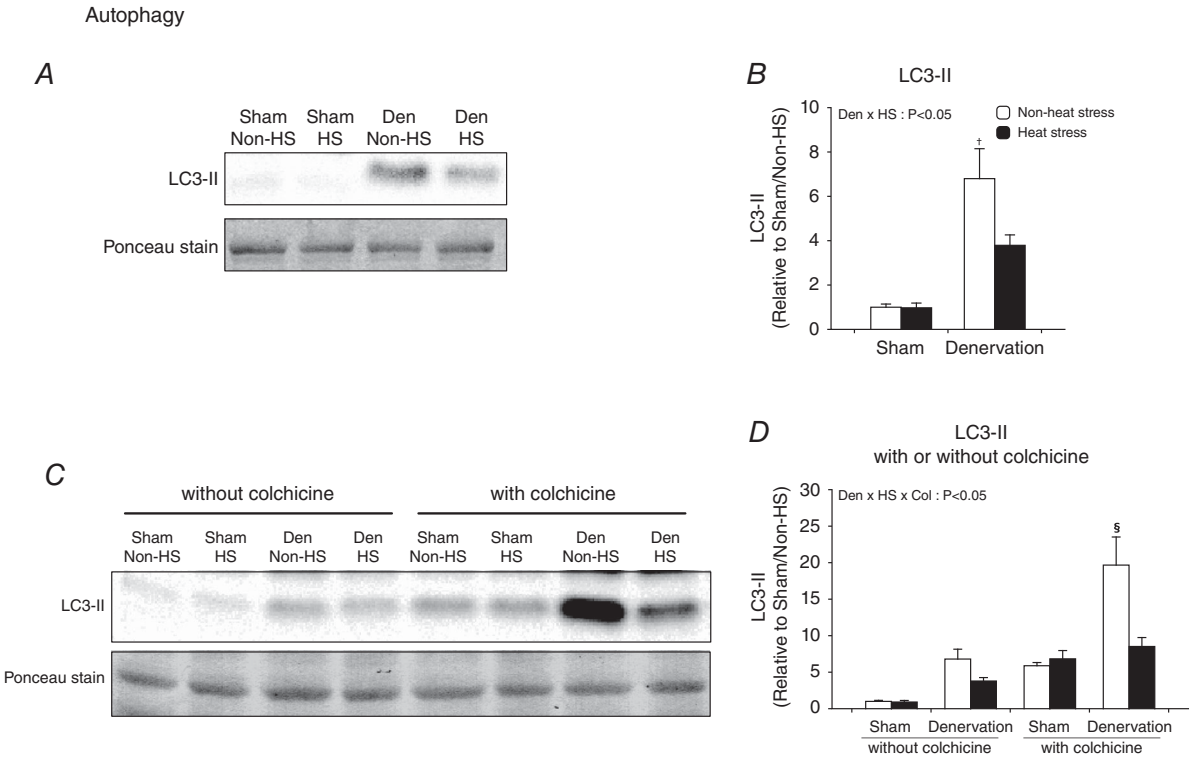

Figure 5. Daily heat stress treatment normalized continuously activated autophagy induction
Open columns, non-heat stress; filled columns, heat stress. Two- or three-way ANOVA was performed. $^{\#}P < 0.05$, main effect of denervation. $^{*}P < 0.05$, main effect of heat stress. $^{\dagger}P < 0.05$ *vs.* non-heat stress/sham. $^{\S}P < 0.05$ *vs.* heat stress/denervation/colchicine. Abbreviation: LC3, microtubule-associated protein 1A/1B-light chain 3; n.s. not significant.

Parkin as a mitochondrial stress sensor and most probably plays important roles in mitochondrial homeostasis in skeletal muscle (Drew *et al.* 2014). In contrast, previous studies show that HSP25 and TRAP-1 (a mitochondrial homologue of HSP90) can work against oxidative stress and mitochondrial cell death (Altieri *et al.* 2012; Lee *et al.* 2005). However, HSP25 and TRAP-1 were not changed by the heat stress treatment in this study (data not shown), suggesting that some but not all HSPs may be key players for the counter effects of heat stress on denervation-induced mitochondrial dysfunction in skeletal muscle. Therefore, our future research will be focused on the relationship between HSPs and mitochondrial health in muscle atrophying conditions.

A recent study identified that USP30, a mitochondrial de-ubiquitinase, inhibits Parkin-mediated mitophagy in neurons (Bingol *et al.* 2014). Therefore, USP30 may prevent mitochondrial ubiquitination. This is the first study to investigate USP30 expression with respect to induction of mitophagy in skeletal muscle. We expected that denervation would decrease USP30 expression in contrast to Parkin, because USP30 counteracts the action of Parkin. However, interestingly, denervation increased both Parkin and USP30. The physiological significance of these observations may be to prevent excessive mitochondrial ubiquitination. In contrast, heat stress suppressed the upregulation of Parkin only, suggesting that heat stress favours de-ubiquitination, thereby preventing mitochondrial clearance.

Dysfunctional mitochondria are removed by mitophagy and mitochondrial fission. A previous study demonstrated that continuous mitochondrial fission itself triggers muscle atrophy (Romanello *et al.* 2010). In agreement with this idea, in the present study, denervation also upregulated proteins related to mitochondrial fission and downregulated proteins related to mitochondrial fusion, concomitantly with induction of mitophagy and muscle atrophy. Interestingly, heat stress normalized

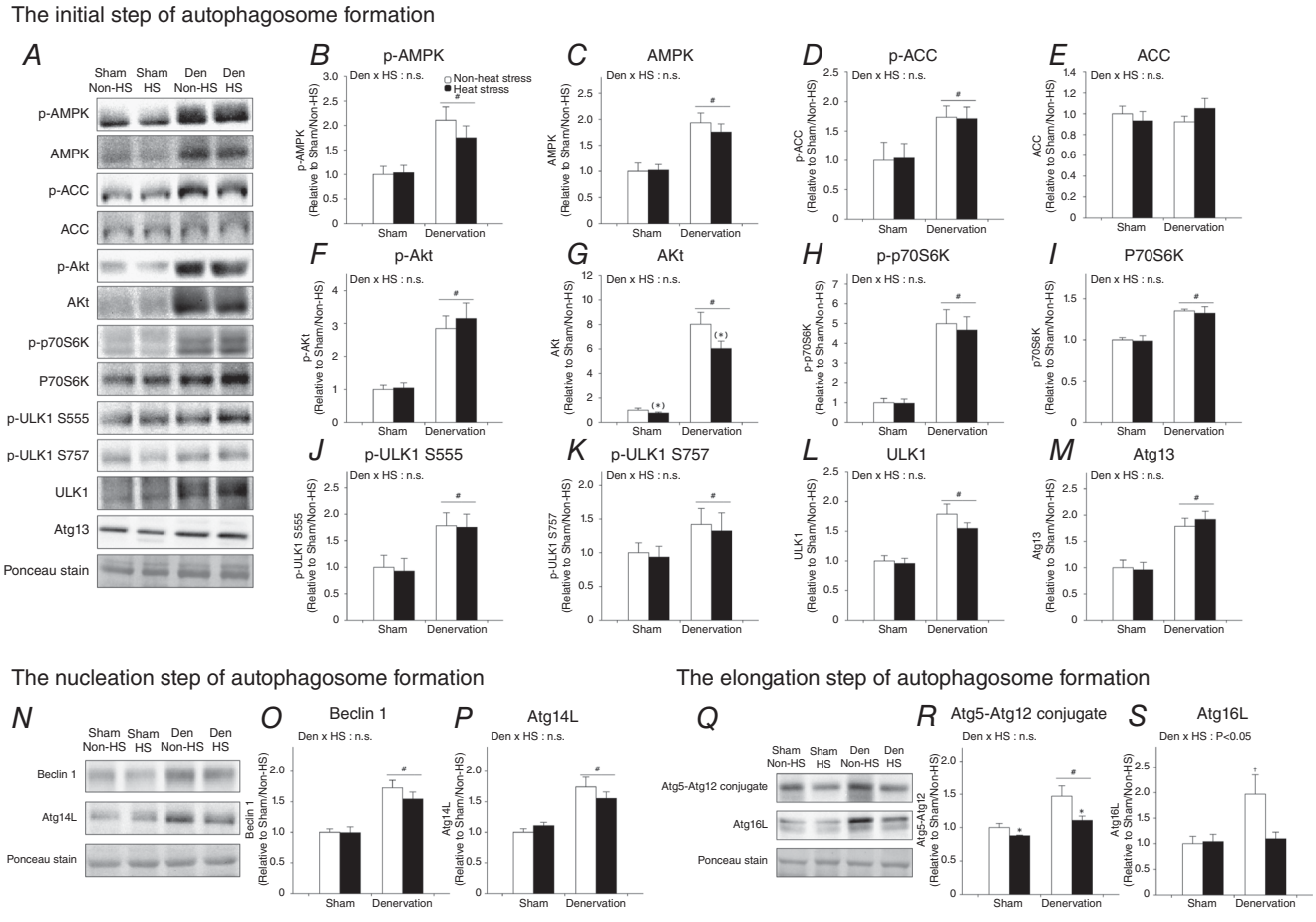

Figure 6. Daily heat stress treatment suppressed the increase in proteins regulating the elongation step of autophagosome formation
Open columns, non-heat stress; filled columns, heat stress. $^{\#}P < 0.05$, main effect of denervation. $^{*}P < 0.05$, main effect of heat stress. $(^{*})P = 0.06$ of main effect of heat stress. $^{\dagger}P < 0.05$ *vs.* non-heat stress/sham. $^{\S}P < 0.05$ *vs.* heat stress/denervation/colchicine. Abbreviation: AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; ULK1, unc-51-like kinase 1; Atg, autophagy-related gene; n.s. not significant.

mitophagy and attenuated muscle atrophy, but did not affect the protein content associated with the regulation of mitochondrial dynamics in denervated muscle. This is a unique case, in which mitochondrial fission did not always coincide with the status of mitophagy and muscle atrophy.

Daily heat stress also normalizes continuously activated autophagy

Skeletal muscle mass is determined by the net balance between protein synthesis and breakdown. Recently, we and others showed that heat stress treatment acutely activates mTORC1, a cellular signalling cascade associated with protein synthesis, in skeletal muscle (Yoshihara *et al.* 2013; Tamura *et al.* 2014). In contrast, the effects of heat stress on pathways related to protein breakdown remain unknown. Recently, researchers have been focusing on the autophagic signalling of proteolysis machineries. Autophagy is sometimes described as a 'double-edged sword', because both inhibition of requisite autophagy (Masiero & Sandri, 2014) and overstimulation of autophagy (Risson *et al.* 2009) can trigger mitochondrial toxicity and muscle wasting (Neel *et al.* 2013). Excessive autophagy is sometimes induced following physical inactivity, such as denervation (Ju *et al.* 2010; Lokireddy *et al.* 2012). Induction of autophagy is critically regulated by over 30 autophagy-related genes. In fact, denervation-activated autophagy increased various proteins associated with autophagy in this study. Interestingly, daily heat stress treatment suppressed denervation-activated induction of autophagy and the upregulation of proteins regulating the elongation step of autophagosome formation (Atg5–Atg12 conjugate and Atg16L). Atg5, Atg12 and Atg16L play a critical role in LC3 lipidation, the conversion of LC3-I to LC3-II and autophagosome formation. Recent studies demonstrate that inhibiting the increase in Atg5, Atg12 or Atg16L by small interfering RNA or short hairpin RNA suppresses the activation of autophagy in various cell types (Han *et al.* 2013; Wang *et al.* 2013; Nowag *et al.* 2014). Therefore, inhibiting the increase in Atg5, Atg12 and Atg16L by daily heat stress could contribute to less autophagy activation and less mitochondrial clearance and muscle atrophy. However, further studies on why daily heat stress attenuates only proteins regulating the elongation step, but not the initiation and nucleation steps of autophagosome formation are required.

Perspectives for translation

To evolve and translate our present understanding, further research is required. In the present study, we were preferentially interested in the mitochondrial content and its regulating machinery; therefore, we have limited functional information on mitochondria and muscle to report. Mitochondria play critical roles in various cellular processes, such as respiration, substrate oxidation, calcium handling, ion transport, ROS production and apoptosis, which determine muscle health and function. In future studies, we consider that multiple measurements and integrative interpretation of mitochondrial function will be very important subjects for improving our understanding the health and function of muscle as a locomotor, metabolic and endocrine organ.

Conclusion

This study provides evidence that daily heat stress treatment rescues denervation-induced mitochondrial toxicity, such as loss of content, reduction of oxidative capacity, accumulation of oxidative stress and concomitant muscle atrophy. Furthermore, we also found that denervation-activated autophagy-dependent mitochondrial clearance (mitophagy) was suppressed by daily heat stress treatment. The molecular basis of this observation is explained by results showing that heat stress treatment attenuated the increase in key proteins regulating the tagging step for mitochondrial clearance and the elongation step of autophagosome formation in denervated muscle.

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Additional information

Competing interests

None declared.

Author contributions

Y.T. designed the study, performed all experiments, interpreted results and wrote the first draft of the manuscript. Y.K. assisted with mitochondrial isolation experiments and interpreting the results and edited the manuscript. Y.M. assisted with animal experiments and edited the manuscript. D.H. assisted with designing experiments and interpreting results and edited the manuscript. H.H. assisted with designing the study and interpreting results and edited the manuscript. All authors read and approved the final manuscript. All experiments were performed at The University of Tokyo.

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