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A possible role of NF-κB and HSP72 in skeletal muscle hypertrophy induced by heat stress in rats

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Abstract. Effects of heat stress on phosphorylated nuclear factor-κB (phospho-NF-κB) and tumor necrosis factor α (TNFα) contents in skeletal muscles were studied. Male Wistar rats (7-week-old) were randomly assigned to control and heat-stressed groups. Rats in heat-stressed group were exposed to heat stress (42°C for 60 min) in an incubator without anesthesia. Soleus muscles were dissected and weighted 1, 3, and 7 days after the heat exposure. Significant increases in the wet weight and protein content of soleus were observed 7 days following the exposure ($p < 0.05$). Heat stress also induced the up-regulation of heat shock protein 72 (HSP72), IκBα (inhibitor of NF-κB) and the increase in the relative population of Pax7-positive satellite cells to total muscle nuclei before the increase in muscle mass. The content levels of phospho-NF-κB and TNFα were significantly decreased 1 and 3 days after heat stress, respectively ($p < 0.05$). A negative correlation between HSP72 and phospho-NF-κB contents was observed 1 day after the heat exposure. These observations suggest that the decrease in NF-κB signaling may play a part of a role in heat stress-associated muscle hypertrophy.

Key words: Heat shock protein — Nuclear factor-κB — Tumor necrosis factor α — Satellite cell — Muscle protein

Abbreviations: NF-κB, nuclear factor-κB; TNFα, tumor necrosis factor α; HSP72, heat shock protein 72; MSCs, muscle satellite cells.

Introduction

Skeletal muscle exhibits a large plasticity in response to changes in extracellular stimuli such as mechanical stretch (Goto et al. 2003) and loading (Goldspink 1999). It has been generally accepted that the increase in mechanical loading, which is induced by stretch and exercise, is a typical stimulus for muscle hypertrophy (Goldspink 1999). Muscle fibers undergoing hypertrophy appear to require an external source of new nuclei to maintain a constant myonucleus-to-fiber volume ratio, so-called myonuclear domain (Allen et al. 1979). The source of these nuclei is attributed to skeletal muscle stem cells, muscle satellite cells (MSCs), located between the sarcolemma and the basal lamina of myofiber (Mauro 1961). It has been proposed that MSCs play a critical role in muscle hypertrophy (Hawke et al. 2001). Recent evidence also shows that mechanical load-

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ing results in an increased number and mitotic activity of MSCs (Smith et al. 2001).

Heat stress is considered to be one of hypertrophic stimuli on skeletal muscle in animals (Goto et al. 2003; Uehara et al. 2004; Kobayashi et al. 2005) as well as humans (Goto et al. 2007). Heat shock protein 72 (HSP72), which is up-regulated by heat stress, has the functions for repairing of denatured proteins, maintaining protein folding, and protecting of the cellular functions (Latchman 2001). Regardless it is unclear that heat stress itself and/or the up-regulated HSP72 might induce these effects on skeletal muscle mass, the exposure to heat stress induces the increase in muscular protein content (Kobayashi et al. 2005). Although heat stress could activate MSCs (Kojima et al. 2007), the detail for molecular mechanisms responsible for heat stress-associated muscle hypertrophy remain unclear.

It has been reported that the up-regulation of HSP72 would inhibit the activation of nuclear factor-κB (NF-κB), a major pleiotropic transcription factor modulating inflammation (Shi et al. 2006). It has been also reported that NF-κB plays an important role for skeletal myogenesis (Wang et al. 2007) and atrophy (Cai et al. 2004). Inhibition of NF-κB is considered to be one of intracellular signals which stimulate the differentiation of MSCs (Ardite et al. 2004). It is well known that tumor necrosis factor α (TNFα), which is one of inflammatory cytokines, is synthesized by NF-κB activation. TNFα activates NF-κB themselves, initiating an autoregulatory feedback loop of NF-κB signaling (Ghosh et al. 1998). Therefore, heat stress-associated muscle hypertrophy might be induced by the inhibition of NF-κB via the up-regulation of HSP72 contents in skeletal cells. However, it has been no report about the effects of heat stress on NF-κB and TNFα in skeletal muscles. In the present study, we investigated the effects of heat stress on NF-κB and TNFα contents in skeletal muscles and elucidate the roles of NF-κB and TNFα in heat stress-associated muscle hypertrophy.

Materials and Methods

Animals and grouping

All experimental procedures were conducted in accordance with the Japanese and American Physiological Society Guide for the Care and Use of Laboratory Animals. The study was also approved by the Animal Use Committee of Toyohashi SOZO University. Male Wistar rats, aged 7 weeks, were randomly assigned to control (Con) and heat-stressed (HS) groups ($n = 15$ in each group). Two or three rats were housed in a home cage 26 × 38 cm and 20 cm height in a clean room controlled at approximately 23°C and at 55% humidity with a 12/12 hours light-dark cycle. Solid diet and water were provided *ad libitum*.

Exposure to heat stress

HS group in the home cage with diet and water was exposed to environmental heat stress (42°C for 60 min) in a heating chamber without anesthesia. Heating protocol in the present study caused an increase in colonic temperature up to 41°C and induced HSP72 expression in rat skeletal muscle (Uehara et al. 2004; Kobayashi et al. 2005; Kojima et al. 2007). In a pilot study, the changes of colonic temperature was determined by using a digital thermo meter (PTC-201, Unique Medical, Tokyo, Japan) equipped with a wired thermo-sensor (PTW-300, Unique Medical). After approximately 30–45 min after starting of heat stress, the colonic temperature increased up to 41°C. At the end of heating, the colonic temperature ranged from 41–42°C. Therefore, the core temperature of rats was maintained at least 15 min. During heating, rats freely access to diet or water in the cage. After heating, all rats were quickly returned to the room, as explained above.

Sampling

Soleus muscles were dissected from both hindlimbs under anesthesia with *i.p.* injection of sodium pentobarbital (50 mg/kg) 1, 3 and 7 days after heating ($n = 5$ *per* group at each stage). Soleus was trimmed of excess fat and connective tissues, weighted, frozen in liquid nitrogen, and stored at −80°C until analyses. No abnormalities of moving and motion of rats were observed during as well as after heating. In the present study, no rats died.

Muscle dry weight and protein content

Left soleus muscles were cross-sectionally cut into halves at the midbelly region. Half of the frozen left muscle were homogenized in 1 ml of tissue lysis reagent (CelLyticTM-MT, Sigma-Aldrich, St. Louis, MO, USA) and completely solubilized by alkaline treatment with 2 N NaOH at 37°C for 1 h. Protein concentration of the tissue lysate was determined by using Protein assay kit (Bio-Rad, Hercules, CA, USA) and bovine serum albumin (Sigma) as the standard. Total protein content in whole muscle was then calculated.

Immunohistochemical analyses

Frozen right soleus muscles were cut cross-sectionally into halves. Serial transverse cryosections (8 μm thick) of the midbelly region of the proximal side were sliced at −20°C and mounted on glass slides. The slides were air dried and stained to analyze the histological stages and the profiles of Pax7-positive nuclei (MSCs) by the standard immunohistochemical stain method. Monoclonal anti-Pax7 antibody (undiluted tissue culture supernatant of hybridoma cells obtained from the Developmental Studies Hybridoma

Bank, Iowa, IA, USA) was used for the detection of MSCs (Hawke et al. 2001). To determine the number of Pax7-positive MSCs in the muscle, immunohistochemical analyses were performed by using the techniques described earlier (Kojima et al. 2007). Cross sections were fixed with 4% paraformaldehyde for 15 min, and then were post-fixed in ice-cold methanol for 15 min. After washing three times with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (TPBS), sections were blocked for 30 min by using a blocking reagent (1% Roche blocking reagent, Roche Diagnostic, Penzberg, Germany). Then, samples were incubated with the primary antibodies for Pax7 and rabbit polyclonal anti-laminin (Z0097, DakoCytomation, Glostrup) overnight at 4°C. After washing with TPBS, sections were also incubated with the second antibodies for Cy3-conjugated anti-mouse IgG (Jackson Immuno Research, West Grove, PA, USA; diluted 1 : 100 in PBS) and with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Sigma; diluted 1 : 200 in PBS) for 1 h at room temperature. After washing with TPBS, nuclei were stained by 15 min incubation in a solution of 2,4-diamidino-2-phenylindole dihydrochloride *n*-hydrate (Dapi, 1 μg/ml; Sigma) in TPBS at room temperature prior to the final wash. A cover glass was then placed above the section covering Vector Shield (Vector Laboratories, Burlingame, CA, USA).

Imaging analysis

The images of muscle sections were incorporated into a personal computer (DP-BSW version 02.02, Olympus) by using a microscope (IX81 with DP70, Olympus). The percentage of Pax7-positive nuclei located within the laminin-positive basal membrane relative to the total number of Dapi-positive nuclei in the whole transverse section of approximately 2000 muscle fibers in each animal was calculated.

Western blotting and densitometry

The other half of the left soleus muscles were homogenized in 1.5 ml isolation buffer of tissue lysis reagent (CelLyticTM-MT, Sigma-Aldrich) with 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 μg/ml leupeptin with glass homogenizer, sonicated and centrifuged at 12 000 rpm (4°C for 10 min), then the supernatant was collected. A part of the supernatant was solubilized in sodium-dodecylsulfate (SDS) sample buffer: 30% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2.3% (w/v) SDS, 62.5 mM Tris-HCl, 0.05% (w/v) bromophenol blue, (pH 6.8), and 1 mg of protein was loaded on a 10% SDS-polyacrylamide gel. Following SDS-PAGE, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (0.2 μm pore size, Bio-Rad) at a constant voltage of 100 V for 60 min at 4°C. The membranes were blocked for 1 h using a blocking buffer: 5% skim milk with 0.1% Tween 20 in Tris-buffered saline (TTBS), pH 7.5. The membranes were incubated for 1 h with a polyclonal antibody (HSP72, SPA-812, StressGen, Victoria, BC; NF-κB p65, KAS-TF110, StressGen; Phospho-NF-κB p65 (phospho-NF-κB), 3037, Cell Signaling Technology, Beverly, MA; IκBα, 9242, Cell Signaling Technology; β-actin, 4967, Cell Signaling Technology) and then reacted with a secondary antibody (goat anti-rabbit IgG conjugate to alkaline phosphatase, Sigma) for 1 h. The membranes were subsequently reacted with bromochloroindolyl phosphate-nitroblue tetrazolium substrate. The bands from immunoblots were quantified using computerized densitometry (Goto et al. 2003; Uehara et al. 2004; Kobayashi et al. 2005). Standard curves were constructed in the preliminary experiment to ensure the linearity.

Enzyme-linked immunosorbent assay

To determine the level of TNFα content in the supernatant of homogenates, enzyme-linked immunosorbent assay (ELISA) was performed. The level of TNFα content was determined by ELISA with a commercially available ELISA kit (Endogen, Rockford, IL, USA) according to the manufacturer's instructions. All samples were measured in duplicate.

Statistical analysis

All values were expressed at means ± SEM. Statistical significance was analyzed by using one-way analysis of variance followed by Tukey-Kramer test. The muscle wet weight relative to body weight and the muscle protein content relative to body weight 7 days after heat exposure were analyzed by the unpaired *t*-test. The relationship between HSP72 and phospho-NF-κB contents was analyzed by the Pearson correlation coefficient. The significance level was accepted at $p < 0.05$.

Results

The body weights in Con and HS groups significantly increased during experimental period (Fig. 1A, $p < 0.05$). However, there was no significant difference in the body weight between both groups. The absolute muscle wet weights in the both groups gradually increased during the experimental period but heat stress caused a further increase. The absolute muscle wet weight in HS group at Day 7 was significantly higher than the age-matched control level (Fig. 1B, $p < 0.05$). And the wet weight relative to body weight in HS group at Day 7 was significantly higher than that in Con group (Fig. 2A, $p < 0.05$). The total protein content of whole muscle in HS group was greater than that in Con group at Day 7 (Fig. 1C, $p < 0.05$); the muscle

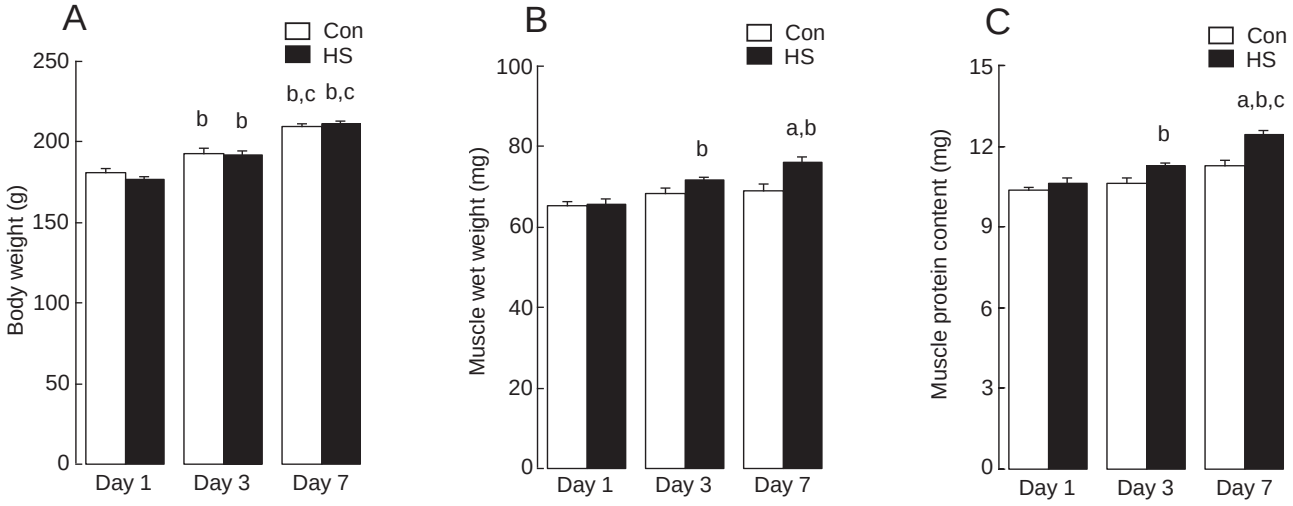

Figure 1. Effects of heat stress on the body weight (A), the absolute muscle wet weight (B) and the total muscle protein content (C) of soleus muscle. Values are means ± SEM. $n = 5$ *per* group each day. Con, control group; HS, heat-stressed group; [a, b, c] $p < 0.05$ *vs.* the age-matched control, Day 1 and 3 in each group, respectively.

protein content relative to body weight in HS group at Day 7 was also significantly greater than that of Con group (Fig. 2B, $p < 0.05$).

Using double immunostaining for anti-Pax7 and anti-laminin with additional nuclear staining (Dapi) of cryosections, Pax7-postive nuclei were counted in each muscle sample. The application of heat stress increased the relative population of Pax7-positive nuclei to total muscle nuclei in soleus muscle. The mean level of Pax7-postive nuclei in HS group was significantly higher than that in Con group at Day 3 (Fig. 3, $p < 0.05$). However, the level in HS group returned toward the control level 7 days after heating and no significant difference was observed between two groups. The distribution of Pax7-postive nuclei in the Con group unchanged throughout the experimental period.

HSP72 content in soleus was up-regulated by the exposure to heat stress. The level of HSP72 content in HS group was significantly higher than that in controls 1 day after heating (Fig. 4A, $p < 0.05$). On the other hand, the content of phospho-NF-κB at Day 1 was decreased following heat stress (Fig. 4B, $p < 0.05$). However, there was no significant difference in phospho-NF-κB between two groups at Day 3 and 7. Significant increase in IκBα (inhibitor of NF-κB) content was observed at Day 1 (Fig. 4C, $p < 0.05$). The mean value of TNFα content in HS group was lower than that in controls at Day 3 (Fig. 4D, $p < 0.05$). A negative correlation between the content levels of HSP72 and phospho-NF-κB 1 day after heating was observed (Fig. 5A, $p < 0.05$). At Day 3 and 7, however, there was no correlation between HSP72 and phospho-NF-κB contents (Figs. 5B and C).

Discussion

The present study demonstrated that phospho-NF-κB and TNFα contents in soleus muscle were decreased by the application of heat stress. A negative interrelationship between phospho-NF-κB and HSP72 contents in soleus muscle was also observed following heating. On the other hand, the population of Pax7-positive MSCs was induced by heat stress before the significant increase in muscle mass of soleus.

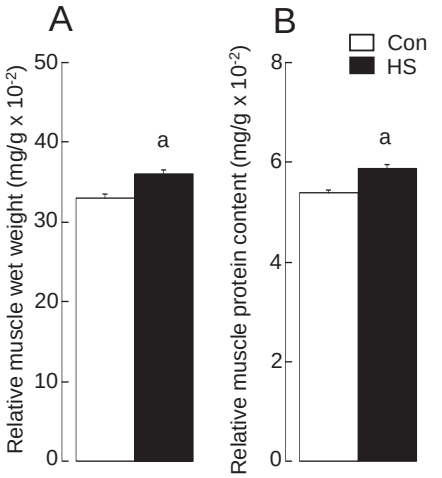

Figure 2. The muscle wet weight relative to body weight (A) and the muscle protein content relative to body weight (B) of soleus muscle 7 days after heat exposure. Values are means ± SEM. $n = 5$ *per* group. Con, control group; HS, heat-stressed group; [a] $p < 0.05$ *vs.* the control.

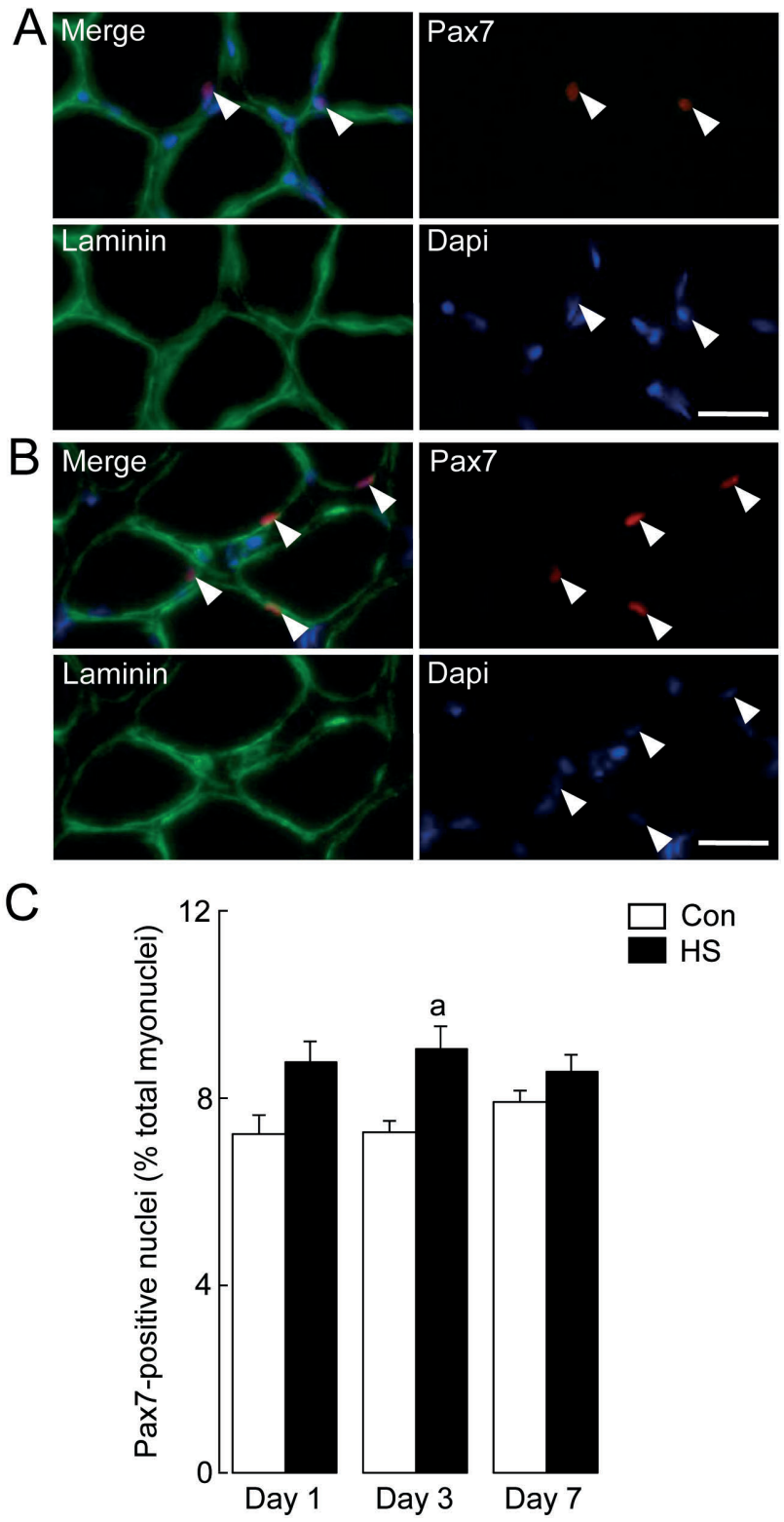

Figure 3. Pax7-positive nuclei in soleus muscle. **A.** control, Day 3. **B.** heat-stressed, Day 3. Transverse sections of soleus muscle were immunostained with anti-Pax7 (Red), anti-laminin (Green) and Dapi (blue). The Pax7-positive nuclei are indicated by arrow heads. Scale bars: 25 μm. **C.** Effect of heat exposure on the relative population of Pax7-positive nuclei to total muscle nuclei in soleus. Values are means ± SEM. $n = 5$ *per* group each day. Con, control group; HS, heat-stressed group; [a] $p < 0.05$ *vs.* the age-matched control.

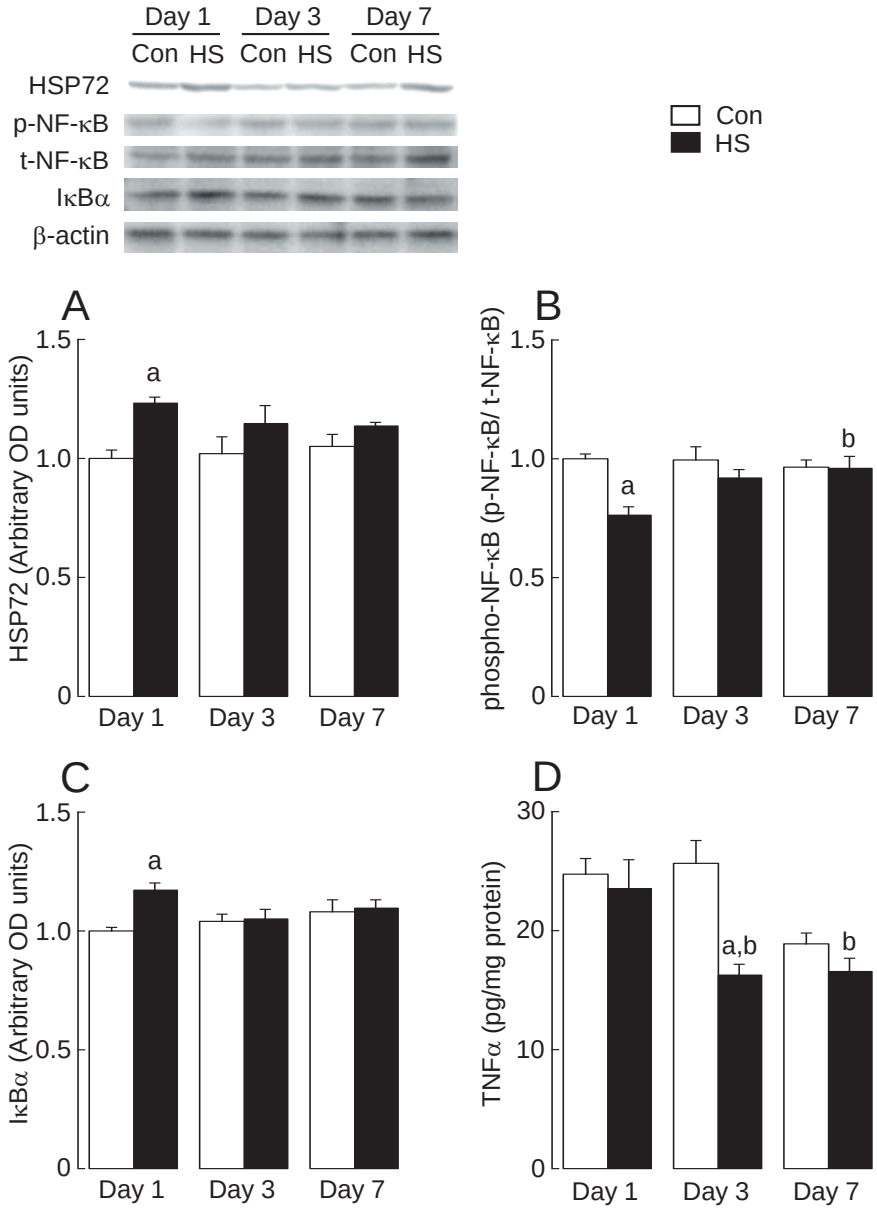

Figure 4. The content levels of HSP72 (A), phospho-NF-κB (B), IκBα (C) and TNFα (D) in soleus. p-NF-κB, phosphorylated nuclear factor-κB; t-NF-κB, total nuclear factor-κB. Values are means ± SEM. $n = 5$ *per* group each day. Con, control group; HS, heat-stressed group; [a, b] $p < 0.05$ *vs.* the age-matched control and Day 1 in each group, respectively.

Muscle hypertrophy and MSCs

The present study confirmed that heat stress could cause an increase in muscle mass in growing rats. The wet weight and the protein content of soleus increased 7 days after heating compared to the age-matched control. These results are consistent with the previous study that heat stress is one of hypertrophic stimuli for skeletal muscle (Uehara et al. 2004; Kobayashi et al. 2005). It has been generally accepted that MSCs are responsible for the postnatal growth and regrowth, as well as the regeneration, of skeletal muscle (Hawke et al. 2001; Mourkioti and Rosenthal 2005). Paired-box gene Pax7 is the specific indicator for both quiescent and mitotic active and proliferating MSCs (Hawke et al. 2001; Morgan and Partridge 2003; Reimann et al. 2004). Pax7 expression is essential at the control of the differentiation to MSCs (Seale et al. 2000). Previous studies reported that heat stress enhanced the proliferative and differentiative potential of skeletal mus-

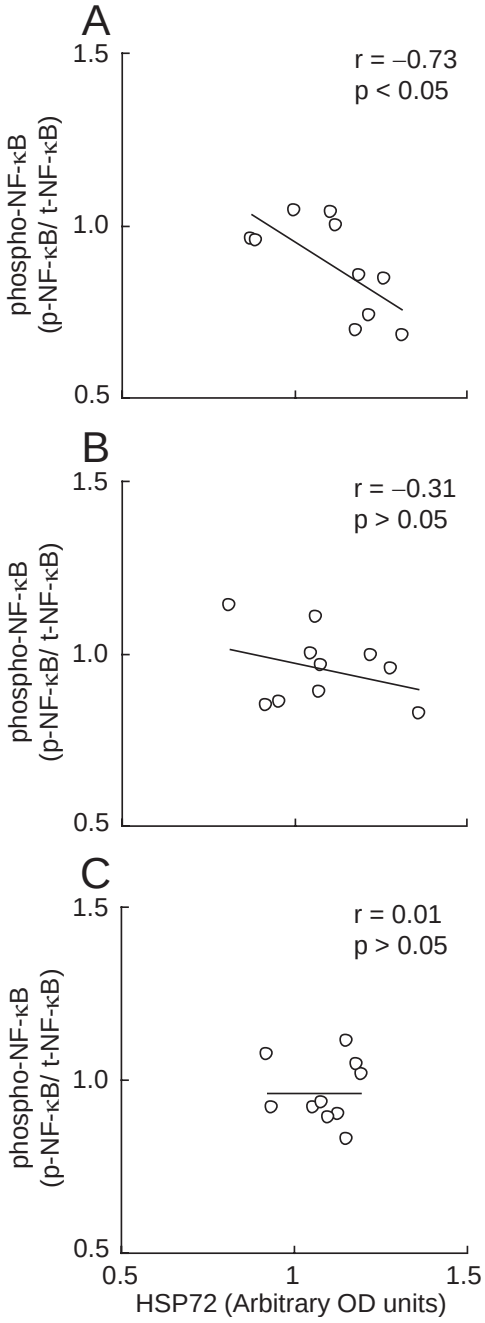

Figure 5. Relationship between HSP72 and phospho-NF-κB contents at Day 1 (A), Day 3 (B), Day 7 (C) in soleus.

cle and induced muscle hypertrophy in rat skeletal muscle (Goto et al. 2003; Uehara et al. 2004; Kojima et al. 2007). In the present study, a significant increase in the relative number of Pax7-positive nuclei was observed 3 days after heat stress. Therefore, it is strongly suggested that heat stress could stimulate the proliferation of MSCs. On the other hand, the muscle protein content in HS group increased at Day 7. Thus, heat stress may stimulate the proliferation and the differentiation of MSCs and thereafter muscle hypertrophy in growing animals might occur. Recently, heat stress-associated muscle hypertrophy has been observed in aged mice (Ohno et al., unpublished observations).

Possible roles of NF-κB-related signaling in heat stress-associated muscle hypertrophy

NF-κB acts as a negative regulator of late-stage muscle differentiation and inhibits skeletal myogenesis (Wang et al. 2007), and TNFα suppresses MSC differentiation (Degens 2007). In addition, the inhibition of NF-κB activation contributes to the differentiation of MSCs (Ardite et al. 2004). However, there was no report concerning about the effects of heat stress on the content levels of phospho-NF-κB and TNFα in skeletal muscle. Previous studies reported that exercise training inhibited NF-κB activity in skeletal muscle nuclei (Durham et al. 2004), but the increase in NF-κB activity was caused by unloading hypokinesia (Hunter et al. 2002; Durham et al. 2004). In the present study, the increases in the population of Pax7-positive MSCs and the muscle weight of soleus were observed following the decrease in the phospho-NF-κB content. These observations suggested that heat stress-associated prevention of NF-κB signaling might contribute to the gain of muscle mass.

The activity of NF-κB is primarily regulated by interaction with inhibitory IκB proteins. Inactive NF-κB is sequestered in the cytoplasm by IκBα protein (Brockman et al. 1995). Upon receiving a multitude of extracellular signals, IκBα protein is phosphorylated, ubiquitinated, and targeted to the proteasome for degradation (Yaron et al. 1998). NF-κB is dissociated from IκBα inhibitory proteins and then translocated to the nucleus leading to transcriptional activity. In the present study, heat stress significantly increased the IκBα content and decreased the content of phospho-NF-κB at Day 1 (Figs. 4B and C). This phenomenon agrees with the report that the heat stress up-regulated the expression of IκBα, and then resulted in the inhibition of NF-κB activity (Wong et al. 1999). Therefore, heat-stress might inhibit an intracellular signals involving NF-κB.

Heat stress as well as various cellular stresses up-regulate the expression of HSP72, the inducible form of HSP70 (Salo et al. 1991; Craig et al. 1993; Goto et al. 2003; Uehara et al. 2004; Kobayashi et al. 2005). On the other hand, the up-regulation of HSP72 prevents NF-κB activation and translocation (Meldrum et al. 2003; Shi et al. 2006). The present study demonstrated that heat stress induced the up-regulation of HSP72 and the down-regulation of phospho-NF-κB content in soleus muscle 1 day after heat exposure. A significant negative correlation between HSP72 and phospho-NF-κB contents 1 day after heating was observed (Fig. 5). Therefore, heat stress-associated decreases in the

level of phospho-NF-κB content might be caused by the up-regulation of HSP72.

In the present study, heat stress also caused a decrease of TNFα in soleus at Day 3. This decrease in TNFα was observed following an increase of HSP72 content and a decrease of phospho-NF-κB content. Up-regulation of HSP72 prevents TNFα gene transcription (Meldrum et al. 2003) and NF-κB activation synthesizes TNFα (Ghosh et al. 1998). Therefore, the inhibition of TNFα by heat stress might be caused by the up-regulation of HSP72 and/or the down-regulation of phospho-NF-κB content.

It is speculated that the application of heat stress to skeletal muscles may activate the intracellular signals, which stimulates to the protein synthesis and/or modifies the protein degradation. However, NF-κB responds to many signals and regulates many genes that act for the development of innate and adaptive immunity (Kumar et al. 2004) therefore the inhibition of NF-κB caused by heat stress could have a negative effect on these immune responses. Further study would be needed to elucidate the effect of heat stress on the immune system.

Heat stress-associated muscle hypertrophy

It has been generally accepted that the increase in mechanical loading, which is induced by stretch and exercise, is a typical stimulus for muscle hypertrophy (Goldspink 1999). Molecular mechanisms for mechanical loading-associated muscle hypertrophy are still unclear. Molecular responses to mechanical loading and stretch might be different from those to heat stress. It has been suggested that the combination of heat stress with other types of hypertrophic stimuli, such as exercise training, could enhance muscle hypertrophy. Light-intensity-exercise (less than 50% repetition maximum (RM)) combined with heat-stress induced an increase in the maximum isometric torque and cross-sectional area (CSA) in biceps brachii muscle of human subjects (Goto et al. 2007). The observations in this study suggest that the application of heat stress to skeletal muscles may be a useful tool for promotion of increase in muscle mass in patients during rehabilitation.

Perspective

The present study strongly suggested that heat stress might be one of hypertrophic stimuli to induce the intracellular signals involving in inhibitions of NF-κB and TNFα in growing animals.

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