Role of 72-kDa Heat Shock Protein in Heat-stimulated Regeneration of Injured Muscle in Rat

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Summary

The regeneration of injured muscles is facilitated by intermittent heat stress. The 72-kDa heat shock protein (HSP72), the level of which is increased by heat stress, is likely involved in this effect, but the precise mechanism remains unclear. This study was conducted to investigate the localization and role(s) of HSP72 in the regenerating muscles in heat-stressed rats using immunohistochemistry. Heat stress was applied by immersion of the rat lower body into hot water (42C, 30 min, every other day) following injection of bupivacaine into the soleus muscles. After 1 week, we found that HSP72 was expressed at high levels not only in the surviving myofibers but also in the blood vessels of the regenerating muscles in heated rats. In addition, leukocytes, possibly granulocytes, expressing cluster of differentiation 43 within the blood capillaries surrounding the regenerating myofibers also highly expressed HSP72. In contrast, marked expression of HSP72 was not observed in the intact or regenerating muscles without heat stress. These results suggest that heat-stress-induced HSP72 within the myofibers, blood vessels, and circulating leukocytes may play important roles in enhancing regeneration of injured muscles by heat stress. Our findings would be useful to investigate cell-specific role(s) of HSP72 during skeletal muscle regeneration. (J Histochem Cytochem 67: 791–799, 2019)

Introduction

Skeletal muscles are important tissues for locomotion and metabolic regulation of the body. Skeletal muscle tissues have a remarkable ability to regenerate from injury.[1–7] In previous studies focusing on the regeneration process, intramuscular injection of cardiotoxin[1,7] or bupivacaine[2–5] has been well adopted to induce damage to rodent myofibers. It was reported that regenerating fibers, such as myotubes with centrally located myonuclei, were observed 3 to 5 days after bupivacaine injection[3,7] and the volume and histopathological image of the injured muscles were recovered during 21 to 60 days following injury.[3,5]

Satellite cells, which are tissue resident multipotent cells, are important contributors to repair damaged myofibers.[8,9] Satellite cells locate between the plasma membrane of myofibers and the basal lamina. Following
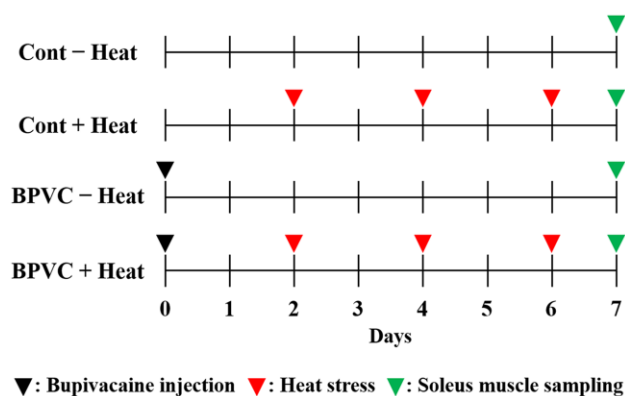
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Figure 1. The experimental design. For each group, timings of intramuscular injection of bupivacaine, heat stress, and muscle sampling are shown. Cont: normal control group, BPVC: muscle damaged group by injection of bupivacaine. Cont and BPVC groups were further separated into two groups with (+ Heat) or without application of heat stress (− Heat). The number of rats in each group was five.

skeletal muscle injury, satellite cells proliferate and form myotubes or fuse with damaged myofibers. In addition, critical roles of immune cells to guide and progress skeletal muscle regeneration via phagocytosis and secretion of cytokines have been reported.[10–12]

Heat stress has been shown to facilitate the regeneration of skeletal muscles from injury, and a concomitant increase in the 72-kDa heat shock protein (HSP72) content and activation of satellite cells were observed in heat-stressed regenerating muscles.[1,4,6] Although HSP72 may play important roles in the repair of injured skeletal muscles, the mechanism is unclear. Thus, this study was performed to investigate the localization and role(s) of HSP72 in the regenerating soleus muscles in heat-stressed rats using immunohistochemistry.

Materials and Methods

All experimental and animal care procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23). The study was also approved by the Animal Use Committee of Osaka University.

Experimental Animals and Design

Adult male Wistar rats were randomly divided [five rats in each group; initial body weight, 286 ± 8 (mean ± standard error of the mean) g] into four groups: (1) normal control without application of heat stress (Cont − Heat), (2) heat-stressed normal control (Cont + Heat), (3) bupivacaine-injected without heat application (BPVC − Heat), and (4) bupivacaine-injected plus heat stress (BPVC + Heat), as is shown in Fig. 1. All rats

were housed individually in an animal room with the temperature and humidity maintained at 22 ± 1C and ~60%, respectively, throughout the study, and food and water were supplied ad libitum.

Under anesthesia with intraperitoneal injection of sodium pentobarbital (5 mg/100 g body weight), 0.5 ml of bupivacaine (Marcain, Fujisawa Yakuhin Kogyo, Osaka, Japan) was injected into the soleus muscles of the bupivacaine-treated groups bilaterally using a disposable syringe with a 26-gauge needle as previously reported.[2] Heat stress was applied to conscious rats in the Cont + Heat and BPVC + Heat groups at 48 hr after injecting bupivacaine and every other day thereafter for 1 week, with the stress applied a total of three times. The lower half of the rat body was immersed in hot water maintained at 42 ± 1C for 30 min as reported previously by Garramone et al.[13] with slight modifications. The core rectal and hindlimb muscle temperatures were elevated to ~42C after ~5 min of immersion and were then maintained at a constant temperature. Heat stress had no adverse effects on the skin and the rats showed no overt signs of distress. The rats in Cont − Heat and BPVC − Heat groups were maintained in their cages under the stated conditions and were not subjected to heat treatments.

One week after the different treatments, both soleus muscles were removed from each rat in the four groups under anesthesia with intraperitoneal injection of sodium pentobarbital (5 mg/100 g body weight). The muscles in the Cont + Heat and BPVC + Heat groups were collected at 24 hr after the final bout of heat stress. The muscles were stretched gently to in vivo optimum length on a cork, and then pinned and quickly frozen in isopentane cooled with liquid nitrogen.

To identify cell populations expressing HSP72 within the blood circulation, an additional experiment was performed in the BPVC + Heat group (*n*=5). The rats were injected bupivacaine and heat-stressed as described above. One week after the treatment, transcardial perfusion with ice-cooled 0.1 M PBS was performed subsequently to blood sampling under anesthesia. And then both soleus muscles were removed and frozen. Approximately 5 ml of collected blood samples were centrifuged (2500 × g, 15 min, 23C) following clotting at room temperature (23C) for 30 min, and cells in the buffy coat were collected. And then, smears of the cells were made on 3-amino propylethoxysilane-coated glass slides and stored at –80C.

Immunostaining

Blinded analyses were performed for all parameters. Serial transverse-sections of frozen soleus (10- or 5-μm thick) were cut in a cryostat at −20C and mounted

onto 3-amino propylethoxysilane-coated glass slides. The muscle transverse-sections and smears of the cells in the buffy coat of blood were fixed with 4% para-formaldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 min and incubated with 0.1 M PBS containing 10% normal donkey serum and 1% Triton X-100 for 1 hr at room temperature to block nonspecific staining.[14]

Various multiple immunostaining techniques such as single, double, or triple staining were used to determine the subcellular localization of HSP72, M-cadherin, dystrophin, laminin, isolectin B4 (IB4), and cluster of differentiation 43 (CD43). The following primary antibodies were used in this study: mouse monoclonal anti-HSP72 (1:100, SPA-810, StressGen, Victoria, British Columbia, Canada), goat polyclonal anti-M-cadherin (1:50, SC6470, Santa Cruz Biotechnology, Dallas, TX), rabbit polyclonal anti-dystrophin (1:200, RB-9024-P, Thermo Fisher Scientific, Waltham, MA), rabbit polyclonal anti-laminin (1:3000, L9393, Sigma, St. Louis, MO), and mouse monoclonal anti-CD43 (1:100, MCA54G, Serotec, Oxford). The following secondary antibodies were used to detect the primary antibodies: Alexa Fluor 568-labeled donkey anti-rabbit IgG (1:400, Molecular Probes, Eugene, OR) and Alexa Fluor 488-labeled donkey anti-mouse IgG (1:400, Molecular Probes). Blood vessels in the soleus muscle sections were detected by using fluorescein-labeled griffonia simplicifolia lectin I (IB4, 1:50; FL-1201, Vector Laboratories, Burlingame, CA).

For triple fluorescence immunostaining, muscle transverse-sections were simultaneously reacted with two types of primary antibodies diluted in 0.1 M PBS containing 5% normal donkey serum and 0.3% Triton X-100 for 16 to 48 hr at 4C. After the reaction, the sections were washed with 0.1 M PBS and then simultaneously incubated with two different secondary antibodies diluted in 0.1 M PBS containing 5% normal donkey serum and 0.1% Triton X-100 overnight at 4C. The sections were mounted in Vectashield mounting medium with 4′,6-diamidino-2-phenyliodate (DAPI, Vector Laboratories).

Single immunostaining was performed by using the avidin–biotin complex (ABC) method. Transverse-sections of soleus muscles and smears of the cells in the buffy coat of blood were incubated with a primary antibody for 16 to 48 hr at 4C. The sections were then washed with 0.1 M PBS and reacted with a secondary antibody [Biotin-conjugated donkey anti-mouse IgG (1:400, 715-065-151, Jackson ImmunoResearch Laboratories, West Grove, PA)] diluted in 0.1 M PBS containing 5% normal donkey serum and 0.1% Triton X-100 for 1 hr at room temperature. The immunoreactive products were visualized by using a Vectastain Elite ABC kit (Vector Laboratories) and diaminobenzidine.

The muscle sections and smears were stained with hematoxylin to visualize the nuclei.

Results

Intermittent Heat Stress Apparently Facilitated Muscle Regeneration

Regeneration of the soleus muscles for 1 week after intramuscular bupivacaine injection was apparently facilitated by intermittent heat stress. By comparison with muscle transverse-sections in Cont – Heat and Cont + Heat groups (Fig. 2A-a), it was obvious that myofibers in bupivacaine-injected muscles (Fig. 2A-b and c) were smaller than in muscles that had not been injected with bupivacaine (Fig. 2A-a), indicating that they had undergone degeneration and regeneration. One week after the bupivacaine injection, many regenerating myofibers were noted. However, larger myofibers were observed in the BPVC + Heat group (Fig. 2A-b) compared with that in the BPVC – Heat group (Fig. 2A-c).

The Heat-stress-related Increase in HSP72 Expression Was Found in the Myofibers, Blood Vessels, and Circulating Cells, but not in Satellite Cells and Myonuclei

The positive effects of heat stress on muscle regeneration were closely associated with increased expression of HSP72. Intact soleus muscles in Cont – Heat rats showed low HSP72 levels in the myofibers (Fig. 2B-a). However, strong immunoreactivities of cytoplasmic HSP72 were observed in the intact myofibers in the Cont + Heat group (asterisks in Fig. 2B-b) and surviving myofibers in the BPVC + Heat groups (asterisks in Fig. 2B-c). Prominent HSP72 expressions were also observed in the blood vessels of the Cont + Heat and BPVC + Heat groups (arrows in Fig. 2B-b–d). Furthermore, the HSP72 expression was detected around the regenerating myofibers in the BPVC + Heat group (arrowheads in Fig. 2B-e). In contrast, these characteristic expressions of HSP72 were not observed in the BPVC – Heat group (Fig. 2B-f and g).

The intense HSP72 expression (arrowheads in Fig. 3A-a) was not detected in M-cadherin-positive satellite cells in the BPVC + Heat group (arrows in Fig. 3A-a). The HSP72 expression was detected outside of the dystrophin-positive plasma membranes of myofibers (arrows in Fig. 3A-b). Furthermore, the HSP72 expression was observed within the laminin-positive small circles around the myofibers and colocalized with DAPI-positive nuclei (arrowheads in Fig. 3A-c–f).
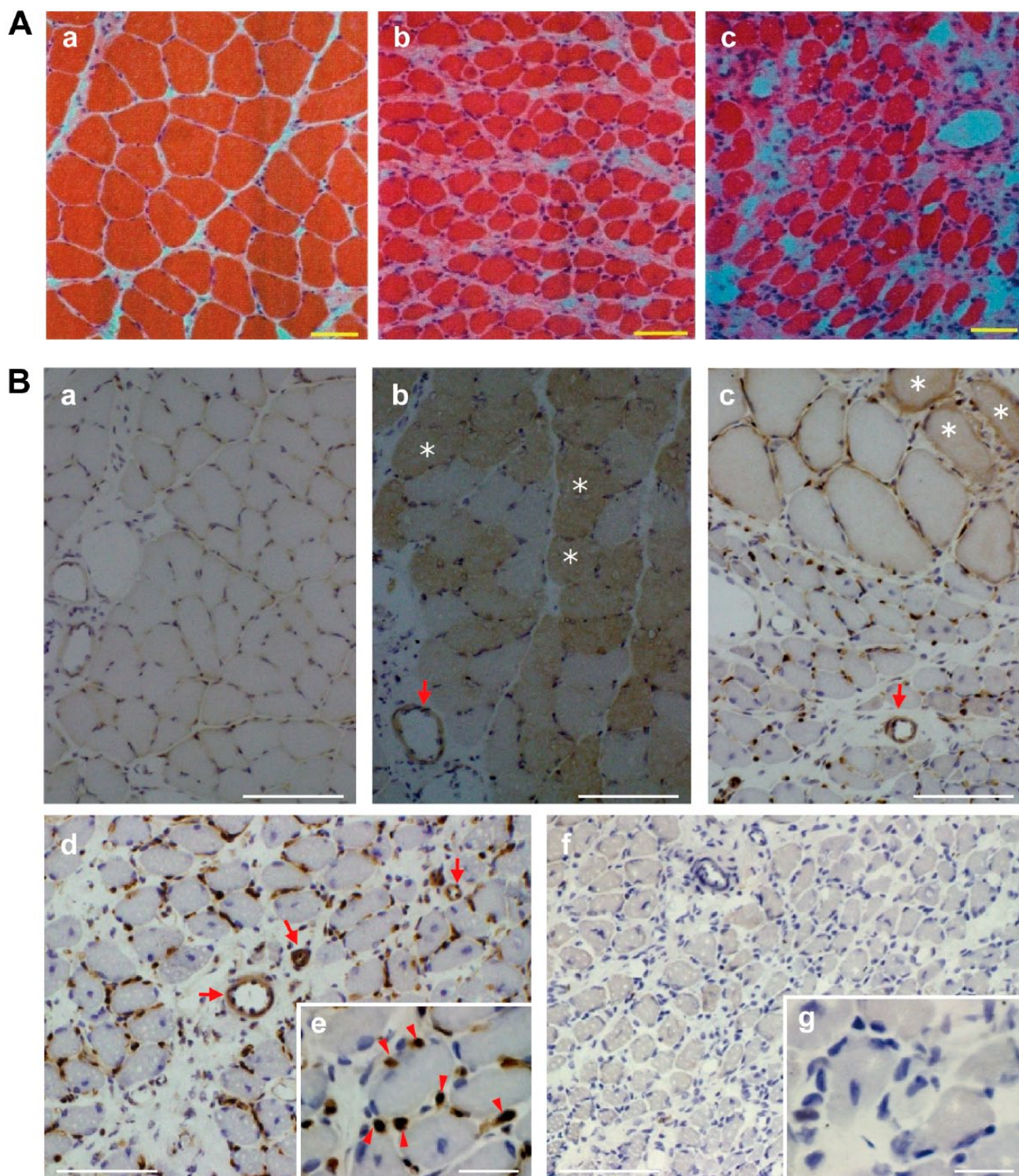
Figure 2. (A) Typical characteristics of soleus muscle fibers 1 week after intramuscular injection of bupivacaine with or without heat stress. a: Cont − Heat, b: BPVC + Heat, and c: BPVC − Heat. Bars in a–c = 50 μm. (B) Photomicrographs showing the localization of 72-kDa heat shock protein (HSP72) in soleus muscles in the Cont − Heat (a), Cont + Heat (b), BPVC + Heat (c–e), and BPVC − Heat (f and g) groups. e and g are enlarged images of an area in d and f, respectively. Asterisks in b and c, arrows in b–d, and arrowheads in e indicate the enhanced immunoreactivies of HSP72 in myofibers, blood vessels, and cells around the myofibers, respectively. Bars in a–d and f = 100 μm, while those in e and g = 30 μm. See Fig. 1 for the abbreviation of each group.
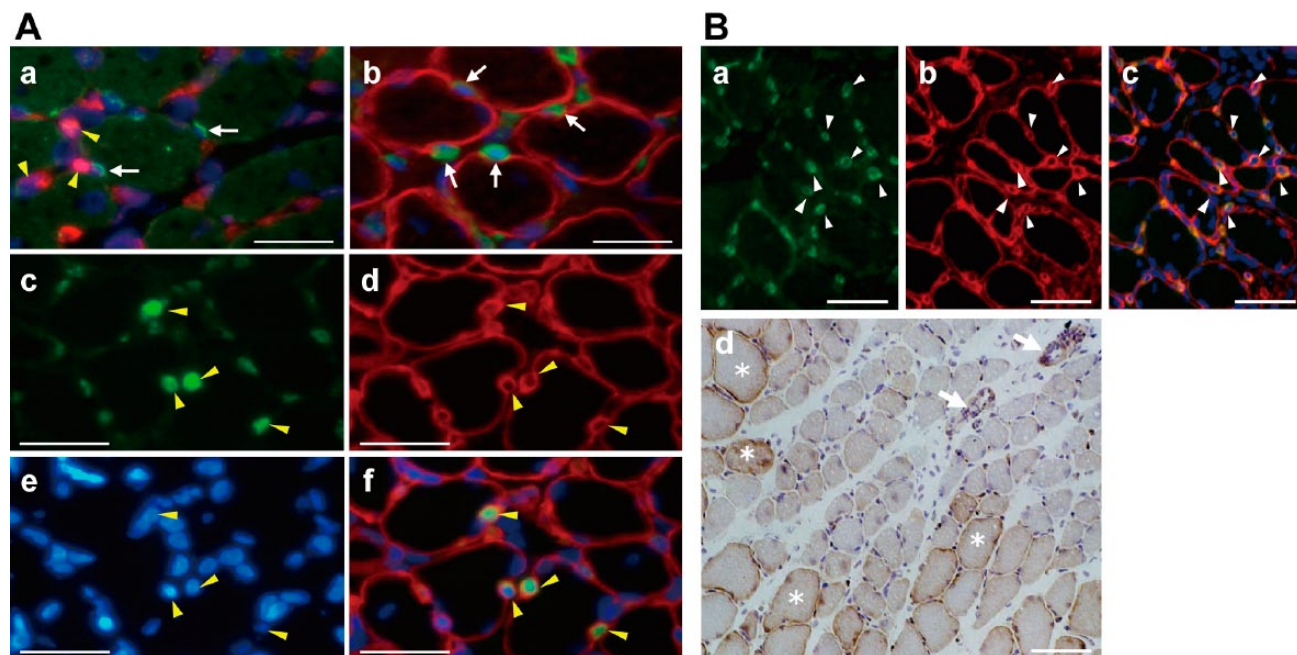
Figure 3. (A) Photomicrographs showing the localization of 72-kDa heat shock protein (HSP72)-expressing cells around the regenerating myofibers in the BPVC + Heat group. a: Localization images of HSP72 (red), M-cadherin (green), and nuclei (blue) are merged. Arrows and arrowheads indicate M-cadherin-positive satellite cells and HSP72-expressing cells, respectively. b: Localization images of HSP72 (green), dystrophin (red), and nuclei (blue) are merged. Arrows indicate HSP72-expressing cells outside the myofibers. Staining was also performed to detect the localization of HSP72 (green, c), laminin (red, d), and nuclei (blue, e), and the images were merged (f). Arrowheads indicate HSP72-positive cells within laminin-positive small circles around the myofibers. Bars in a–f = 30 μm. (B) Photomicrographs showing isolectin B4 (IB4)-positive blood capillaries and the effects of transcardial perfusion with PBS on HSP72 immunoreactivities in the BPVC + Heat group. Localization of IB4 (green, a) and laminin (red, b) is shown, and the images and 4′,6-diamidino-2-phenylindole–positive nuclear image (blue) were merged (c). Arrowheads indicate IB4-positive endothelial cells of the blood capillaries within the laminin-positive small circles around the myofibers. d: Photomicrograph showing immunoreactivities of HSP72 in the muscle transverse-section from the BPVC + Heat group after transcardial perfusion with PBS. Asterisks and arrows indicate HSP72-positive myofibers and blood vessels, respectively. Bars in a–d = 50 μm. See Figs. 1 and 2 for other abbreviations.

Laminin-positive small circles around the myofibers expressed IB4, which is a marker of endothelial cells in the blood capillaries (arrowheads in Fig. 3B-a–c). In addition, transcardial perfusion with PBS before muscle sampling resulted in drastic disappearance of HSP72 expression from the blood capillaries (Fig. 3B-d), although enhanced HSP72 expressions were still detected in the cytoplasm of regenerating myofibers (asterisks) and walls of the blood vessels (arrows in Fig. 3B-d).

Heat Stress Increased HSP72 Expression in Circulating Leukocytes, Possibly Granulocytes

The expression of CD43 was detected around regenerating myofibers in the BPVC + Heat group (arrowheads in Fig. 4A-a and b). CD43, which is also known as leukosialin, is a 95-kDa heavily glycosylated glycoprotein expressed in all leukocytes except for B lymphocytes. The expression of CD43 was colocalized with DAPI staining and was detected within the laminin-positive

blood capillaries (arrowheads in Fig. 4A-c–e). In addition, immunostaining of the adjacent serial transverse-sections of the soleus muscles in the BPVC + Heat group indicated the presence of cells expressing both CD43 and HSP72 (arrow in Fig. 4B-a and c) in laminin-positive blood capillaries (arrow in Fig. 4B-b and d). Furthermore, HSP72-positive leukocytes with multi-core (Fig. 4B-e), cyclic (Fig. 4B-f), and elliptical nuclei (Fig. 4B-g) were observed in smears of the cells in the buffy coat of the blood collected from the BPVC + Heat group. Judging from the pathological characteristics of nuclei, these leukocytes may be granulocytes.

Discussion

It has been reported that intermittent heat stress facilitates the regeneration of skeletal muscles following injury caused by intramuscular injection of cardiotoxin[1,7] or bupivacaine.[2–5] HSP72 was previously predicted to be induced in satellite cells and myofibers in heated regenerating muscles, as enhanced expression
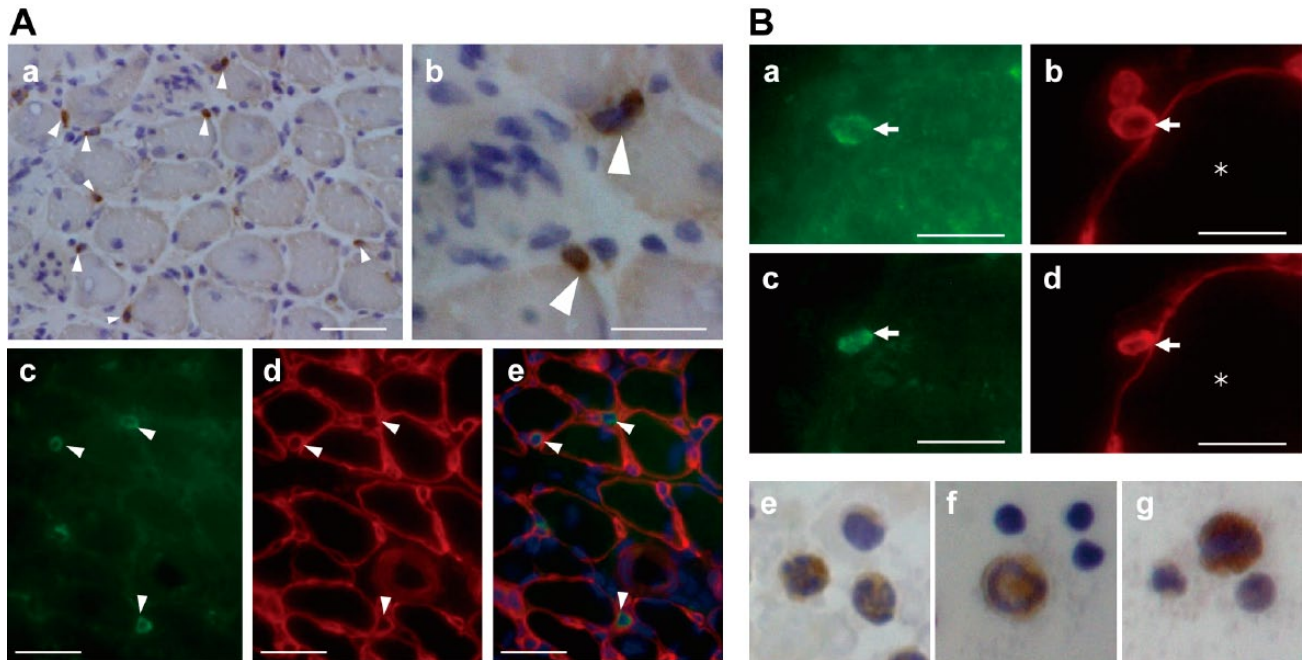
Figure 4. (A) Photomicrographs showing the localization of cluster of differentiation 43 (CD43)-positive leukocytes in the regenerating area of the BPVC + Heat group (a and b). b: Enlarged image of an area in a. Localization of CD43 (green, c), laminin (red, d), and nuclei (blue, d) is shown, and the images were merged (e). Arrowheads indicate CD43-positive leukocytes within laminin-positive blood capillaries around the myofibers. Bars in a and c–e = 50 μm, while that in b = 20 μm. (B) Leukocytes expressing 72-kDa heat shock protein (HSP72) in blood capillaries. Photomicrographs showing the localization of CD43 (green, a) and laminin (red, b) as well as HSP72 (green, c) and laminin (red, d) in adjacent serial transverse-sections. Arrows indicate a CD43 and HSP72-double-positive leukocyte in a laminin-positive blood capillary around a myofiber (asterisk, b and d). Bars = 20 μm. HSP72-positive (brown) leukocytes with multi-core (e), cyclic (f), and elliptical nuclei (g) are shown. See Figs. 1 and 2 for other abbreviations.

Table 1. Expression Patterns of HSP72 in Several Cells and Tissues.

Experimental Groups	Cytoplasm of Myofiber	Satellite Cell	Cytoplasm/Myonuclei of Myofiber	Blood Vessel	Leukocyte (CD43+)
Cont − Heat	+	/	/	−	−
Cont + Heat	++	/	/	++	−
BPVC − Heat	+	−	−	−	−
BPVC + Heat	++	−	−	++	++

−: lack of significant expression, +: weak expression, ++: intense expression, and /: not examined. Cont − Heat, Cont + Heat, BPVC − Heat, BPVC + Heat, and CD43 indicate normal control without heat stress, heat-stressed normal control, bupivacaine-injected without heat stress, bupivacaine-injected plus heat-stressed, and cluster of differentiation 43, respectively.

of HSP72 and activation of satellite cells were observed in these muscles.[1,4] However, in the present study, the heat-stress-related increase in HSP72 levels was found in the surviving myofibers, blood vessels, and circulating cells, but not in satellite cells and myonuclei, in regenerating rat soleus muscles. Our results also indicate that CD43-positive leukocytes, possibly granulocytes, express HSP72 in circulating blood. The expression patterns of HSP72 in several cells and/or tissues of the Cont – Heat, Cont + Heat, BPVC – Heat, and BPVC + Heat groups are summarized in Table 1.

HSP72 is essential for maintaining cellular homeostasis and cell survival, and increased levels of intracellular HSP72 mediate the protection of cells against environmental stress factors.[15] Therefore, HSP72, which was induced in myofibers in the BPVC + Heat group, may protect myofibers from harmful stresses caused by injury. In addition to myofibers, enhanced expression of HSP72 was observed in the blood vessels of the Heat and BPVC + Heat groups (Figs. 2B-b–d and 3B-d). Increased HSP72 expression has also been reported in the smooth muscle cells of both

arterial and venous vessels in trained skeletal muscles and vascular muscle cells in rat hearts exposed to whole-body heat stress.[1,16] In addition, both endothelial cells and smooth muscle cells in the placental microvessels express HSP72.[17] Based on our results and those of previous studies, the blood vessels (i.e., perhaps both endothelial cells and smooth muscle cells) may upregulate HSP72 in heat-stressed regenerating skeletal muscles. Increased HSP72 expression in the blood vessels may protect against further damage to the blood vessels and thus accelerate microvasculature remodeling in regenerating skeletal muscles.

Enhanced HSP72 expression within the blood capillaries of heat-stressed regenerating muscle was dramatically eliminated by transcardial perfusion with PBS before muscle sampling (Fig. 3B-d), although HSP72 was still detected in the cytoplasm of surviving myofibers (asterisks) and wall of blood vessels (arrows in Fig. 3B-d). Cells expressing both CD43 and HSP72 (arrow in Fig. 4B-a and c) were observed within the laminin-positive blood capillaries (arrow in Fig. 4B-b and d). Moreover, HSP72-positive leukocytes with multi-core (Fig. 4B-e), cyclic (Fig. 4B-f), and elliptical nuclei (Fig. 4B-g) were observed in the blood of the BPVC + Heat group. Although further classification of leukocytes expressing HSP72 should be performed, these leukocytes may be granulocytes based on their pathological characteristics of nuclei. These results indicate that heat stress increased HSP72 expression not only in the surviving myofibers and blood vessels but also in circulating CD43-positive leukocytes, possibly granulocytes, in regenerating muscles. Several studies have shown that various cell types express HSP72 in the circulation. For instance, HSP72 was detected in the peripheral circulation of healthy individuals,[18,19] and heat stress increased the HSP72 content in human peripheral blood cells.[20] Furthermore, in advanced atherosclerotic lesions, monocytes and macrophages were found to overexpress HSP72.[21] These findings support our result that HSP72 expression in circulating CD43-positive leukocytes, possibly granulocytes, increased in the soleus muscles of the BPVC + Heat group.

It is well-known that HSP72 is mainly located in the cytoplasm of mammalian cells during normal growth conditions, but translocates from the cytoplasm to the nucleus following heat stress where it interacts with various nuclear proteins.[22–25] In the present study, various nuclear HSP72 expressions were observed in numerous cell types including CD43-positive leukocytes in the regenerating soleus muscles following heat stress. However, nuclear expression of HSP72 was not observed in the BPVC – Heat group. These findings suggest that nuclear HSP72-expressing leukocytes may play important role(s) in facilitating the repair of injured myofibers. However, the critical roles of HSP72 translocated in nuclei and mechanism of how HSP72-expressing leukocytes are involved in muscle regeneration remain unclear. We speculated that cytokines secreted from these cells could be involved in beneficial effects of heat stress in skeletal muscle regeneration.

In addition to localizing in cells, HSP72 can be released from various cell types, such as into the blood circulation and cultured cell medium, where it is known as extracellular HSP72 (eHSP72).[26,27] eHSP72 can serve as a cytokine and binds with high affinity to peripheral blood monocytes, macrophages, natural killer cells, B lymphocytes, dendritic cells, and mast cells.[28–30] eHSP72 can also stimulate the production of pro-inflammatory cytokines and chemokines in monocytes and macrophages as well as activate the phagocytosis and chemotaxis of neutrophils.[31–33] Binding of eHSP72 to specific cells occurs through Toll-like receptor (TLR)-2 and TLR-4 in a CD14-dependent manner[34]; after binding, eHsp72 activates specific intracellular signaling pathways, such as the phosphoinositide 3-kinase pathway and nuclear factor kappa-light-chain-enhancer of activated B cells pathway, which regulate protein synthesis and mitochondrial biogenesis in myotubes, respectively.[31,35,36]

Although the origin of heat-stress-related HSP72 and effects of eHSP72 were not evaluated, HSP72 in the myofibers, blood vessels, and leukocytes, as detected in the present study, may have included eHSP72. eHSP72 may have been released into the regenerating area of muscles from HSP72-expressing cells to contribute to the regeneration of injured muscles by activating specific intracellular signaling pathways in the myofibers, blood vessels, and leukocytes in autocrine and/or paracrine manners.

In conclusion, the present study showed that intermittent heat stress following muscle injury increases HSP72 levels in the surviving myofibers, blood vessels, and CD43-positive leukocytes, possibly granulocytes, in regenerating rat soleus muscles. HSP72 in these cells and tissues may contribute to facilitating the repair of injured muscles by enhancing the protection of surviving myofibers, regrowth of myotubes, and reconstitution of the microvasculature in a concerted manner during skeletal muscle regeneration. Furthermore, circulating leukocytes expressing HSP72 in heat-stressed regenerating muscles may be an important factor that enhances the regenerative capacity. Although further classification of leukocytes expressing HSP72 is required, our findings would be useful to investigate cell-specific role(s) of HSP72 in regenerating skeletal muscles in the future.

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