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Biochemical and Biophysical Research Communications 358 (2007) 331–335
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Geranylgeranylaceton induces heat shock protein 72 in skeletal muscle cells

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Received 4 April 2007
Available online 30 April 2007

Abstract

Effects of an antiulcer drug, geranylgeranylaceton (GGA), and/or heat-stress on 72 kDa heat shock protein (HSP72) expression and protein content in cultured skeletal muscle cells were studied. Mouse skeletal muscle cells (C_2C_{12}) were subjected to either 1) control (cultured at 37 °C without GGA), 2) GGA administration (10^{-11}–10^{-8} M), 3) heat-stress at 41 °C for 60 min, or 4) GGA administration combined with heat-stress. Expression of HSP72 was up-regulated by GGA administration. Heat-stress further enhanced the GGA-related up-regulation of HSP72. Administration of GGA caused an increase of muscular protein content as a dose-dependent manner. Protein synthesis was also stimulated by heat-stress alone in myotubes. It was suggested that GGA stimulates the differentiation of myoblasts and protein synthesis. These observations may also suggest that the administration of GGA could be one of the useful tools to gain muscular mass not only in athletes, but also in patients during rehabilitation.
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Keywords: Skeletal muscle cells; Heat shock protein; Antiulcer drug; Heat-stress; Muscle protein

Heat shock proteins (HSPs) are part of the tightly regulated systems for maintenance of cellular homeostasis during the normal cell growth and for survival in response to detrimental environmental stresses [1,2]. Among the several multiple member families of HSPs, a stress-inducible HSP70, namely 72 kDa HSP (HSP72), is one of the best-known endogenous factors protecting cell and tissue injury under pathophysiological conditions [3,4]. It has been well-reported that HSP72 in skeletal muscles is also up-regulated by physical exercises [5–10], as well as heat-stress [11–13]. However, the precise mechanism, as well as the physiological roles, for the up-regulation of skeletal muscles is not fully understood.

An antiulcer drug, geranylgeranylaceton (GGA) is known to be a non-toxic HSP72 inducer that protects cultured gastric mucosal cells against ethanol-induced injury and rat gastric mucosa against stress-associated ulcer [14]. It has been reported that GGA stimulates HSP72 expression in not only gastric mucosa [14] but also heart of rats [15]. However, there is no evidence regarding the effects of GGA on HSP72 expression in skeletal muscle cells. Therefore, in the present study, we investigated the effects of GGA on HSP72 expression in cultured skeletal

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doi:10.1016/j.bbrc.2007.04.129

muscle cells. To our knowledge, this is the first report showing that GGA-associated up-regulation of HSP72 in skeletal muscle cells.

Materials and methods

Myoblast cells (C_2C_{12}) of mouse were cultured on culture plates with a genetic type I collagen bound surface (Biocoat, Becton–Deckinson Labware, NJ). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Rockville, MD) supplemented with 10% heat-inactivated fetal calf serum in humidified atmosphere of 95% air and 5% CO_2 for proliferation.

Administration of GGA. The GGA was supplied by Eizai Co. Ltd. (Tokyo). The concentration of GGA, used, was between 10^{-11} and 10^{-8} M in conditioned culture medium. Stock solutions of GGA were prepared in dimethylsulfoxide (DMSO) and diluted to the final DMSO concentration at 0.2% in the medium. The DMSO alone was administered for the control group. Cultures were maintained for 3 days in the growth medium and 9 days in the differentiation medium. Medium was changed every 24 h. Images of myoblasts and myotubes were analyzed under a light microscopy, as described below.

Application of heat-stress. Generally, cells were kept in the incubation chamber maintained at 37 °C in humidified atmosphere with 95% air and 5% CO_2. The plates with the cells in the heat-stressed groups were transferred to other chamber in which the temperature was set at 41 °C and they were returned to the original chamber after 60 min. Our previous study showed that the temperature of the culture medium gradually increased and reached to 41 °C after ~45 min and was maintained stable thereafter in the chamber for heat-stress [16].

Experiment 1

Effects of 2-day GGA administration and/or heat-stress, 2-day prior to sampling, on the expression of HSP72. The C_2C_{12} myoblasts (6.0×10^4) were seeded in each 35 mm^2 culture plate and were maintained for 3 days in the growth medium, composed of DMEM with 4.5 g glucose/L and 10% heat-inactivated fetal bovine serum, as was described elsewhere [16]. Myoblasts were maintained at a subconfluent seeding density during 3 days after seeding, and the differentiation from myoblasts into myotubes was initiated in some plates by changing the growth medium to the differentiation medium consisting of DMEM with 1 g glucose/L and 2% horse serum. Then, the cells were maintained in the differentiation medium for 9 days.

During the 2nd and 3rd day in the growth medium or 8th and 9th day in the differentiation medium, GGA (10^{-8} M) were added to the conditioned medium. Some of the cells were subjected to heat-stress (41 °C) for 60 min 2 days before the sampling. The cells were collected following 2 days after the initiation of GGA administration or heat-stress. Since a greater effect of heat-stress than GGA administration on HSP72 expression in myotubes was observed (Fig. 1), an additional experiment was performed to investigate the combined effects of heat-stress and GGA administration (Fig. 2).

Experiment 2

Effects of continuous GGA administration or a single bout of heat-stress on the differentiation of C_2C_{12} cells. The C_2C_{12} myoblasts were seeded and maintained in the growth medium as in Experiment 1. Myoblasts were maintained at a subconfluent seeding density during 3 days after seeding, and the differentiation from myoblasts into myotubes was induced by changing the medium to the differentiation medium consisting of DMEM with 1 g glucose/L and 2% horse serum. The GGA (10^{-11}–10^{-8} M) was added to the conditioned medium throughout the 9 day culture. Heat-stress was applied to some cells, which were cultured without GGA. Further, none of the heat-stress or GGA administration was performed for the control group.

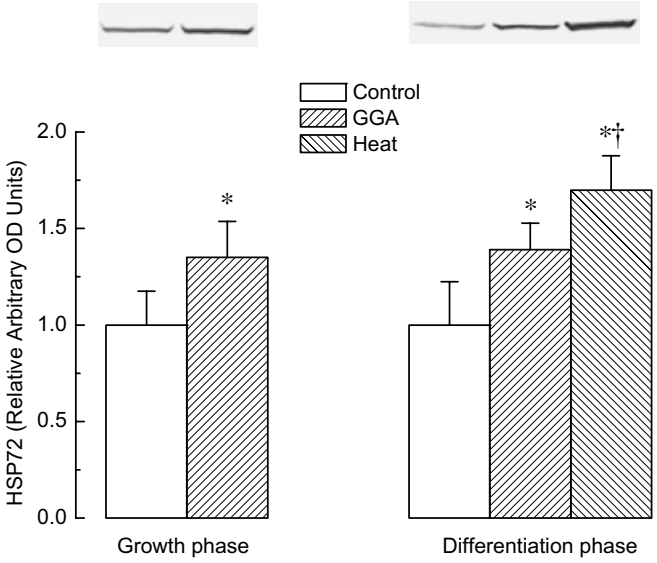

Fig. 1. Effects of geranylgeranylaceton (GGA) administration or heat-stress on the expression of 72 kDa heat shock protein (HSP72) during the growth (myoblasts) and differentiation (myotubes) phases. Representative patterns and the mean (±SEM) levels of HSP72 expression are shown. $n = 6$/group. Control: untreated control group, GGA: GGA-administrated group (10^{-8} M GGA was added to the culture medium for 2 days before sampling), heat: group with a bout of heat-stress (cells were exposed to 41 °C for 60 min and sampled after 2 days), OD: optical density, growth phase: myoblasts sampled after the 3-day culture in the growth medium with or without GGA administration, and differentiation phase: myotubes sampled after the 9-day culture in the differentiation medium with or without GGA administration or heat-stress. *,†Significantly different ($p < 0.05$) compared with the value of control and GGA groups, respectively.

Imaging analysis. The images of myoblasts and myotubes during the proliferation and the differentiation phases were taken by using a calibrated color imaging camera (DP12, Olympus, Tokyo) set up to a phase contrast light microscope (CK40, Olympus). The levels of the proliferation and the differentiation of myoblasts were checked in the multiple separate microscope fields on, at least, six separate culture plates.

Sample collection. The cells in each plate were rinsed with 1 mL of ice-cold phosphate-buffered saline (PBS) twice, and scraped into 0.2 mL of cell lysis reagent (CelLytic™-M, Sigma–Aldrich, St. Louis, MO). Then, the cells in the cell lysis reagent were sonicated to solubilize completely.

Western blotting and densitometry. Extracted samples in the cell lysis reagents were mixed 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} at a concentration of 2 mg protein/mL and boiled for 3 min, and equal amount of protein (20 μg) was loaded on each 10% SDS–polyacrylamide gel. The changes in the expressions of HSP72 (HSP70 inducible) were investigated. Following SDS–PAGE, proteins were transferred to PVDF membranes (0.2 μm pore size, Bio-Rad, Hercules, CA) at a constant voltage of 100 V for 1 h at 4 °C. The membranes were blocked for 1 h by using a blocking buffer {5% skim milk with 0.1% Tween 20 in Tris-buffered saline (TTBS), pH 7.5}. Then, the membranes were incubated for 1 h with a polyclonal antibody for HSP72 (SPA-812, StressGen, Victoria, BC) and then reacted with a secondary antibody (goat anti-mouse immunoglobulin G conjugated to alkaline phosphatase; Sigma–Aldrich) for 2 h. The membranes were subsequently reacted with bromochloroindolyl phosphate-nitro blue tetrazolium substrate. Quantification of the bands from the immunoblots was performed by using a computerized densitometry [11–13,16]. Standard curves were constructed in the preliminary experiment to ensure the linearity.

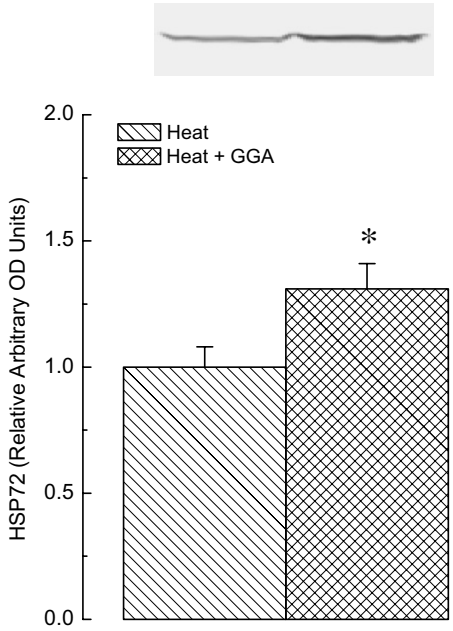

Fig. 2. Effects of heat-stress with or without 2-day GGA administration on the expression of HSP72 in myotubes sampled after the 9-day culture. Representative patterns and the mean (±SEM) levels of HSP72 expression are shown. $n = 6$/group. Heat + GGA: GGA (10^{-8} M)-administration with a bout of heat-stress (41 °C for 60 min). Other abbreviations and experimental protocol are the same as in Fig. 1. *Significantly different ($p < 0.05$) compared with the value of heat-stress alone group.

Protein concentrations. To evaluate the muscle protein content, the concentration of proteins in cell lysis reagent was determined by using the Bradford technique (Protein Assay kit, Bio-Rad, Hercules, CA). Protein concentrations in the cell lysis reagents were expressed as mg per mL.

Statistical analyses. All values were expressed as means ± SEM. Statistical significance was analyzed by using analysis of variance followed by Scheffé's post hoc test. The significance was accepted at $p < 0.05$.

Results

Experiment 1

Effects of GGA and/or heat-stress on the expression of HSP72

Administration of 10^{-8} M GGA induced an up-regulation of HSP72 expression in C_2C_{12} cells either in the growth medium or in the differentiation medium (Fig. 1). The levels of HSP72 expression in the growth and differentiation mediums with GGA were significantly higher than those without GGA ($p < 0.05$). The HSP72 expression induced by heat-stress, without GGA administration, was higher than that induced by GGA administration in the differentiation medium. The combination of GGA administration and heat-stress further up-regulated the HSP72 expression of myotubes (Fig. 2). The HSP72 expression induced by the combination of GGA and hest-stress significantly higher than that in heat-stress alone ($p < 0.05$). However, there was no significant difference in the protein contents between each group (data not shown).

Experiment 2

Effects of GGA and/or heat-stress on the muscular protein contents

The typical images of myotubes cultured for 9 days with or without GGA at various concentrations are shown in

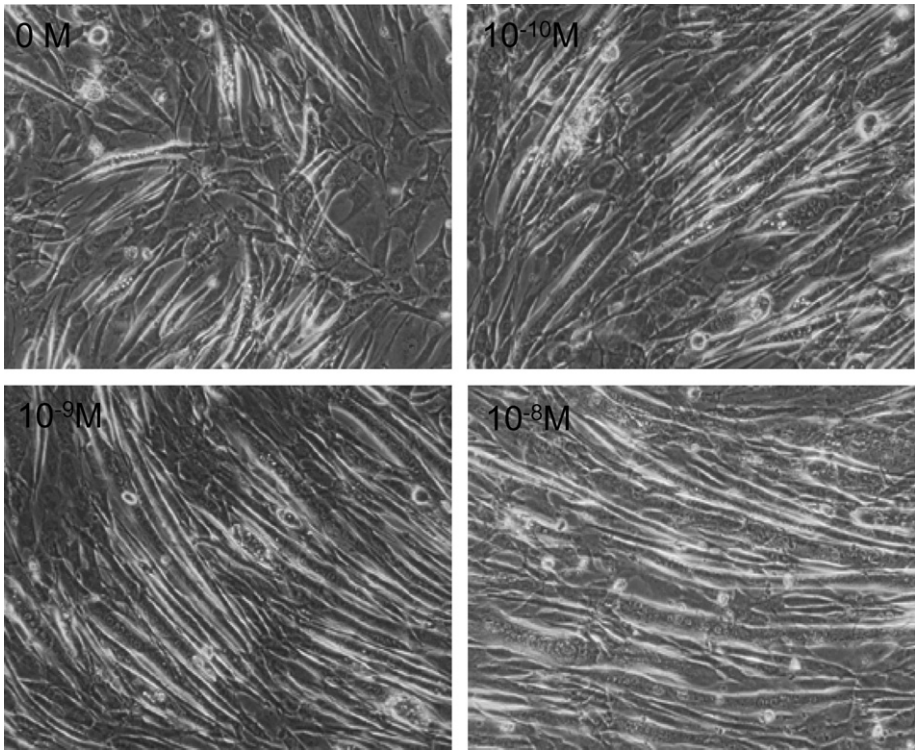

Fig. 3. Pictures showing the typical images of myotubes at the end of 9-day culture with or without geranylgeranylaceton at various concentrations.

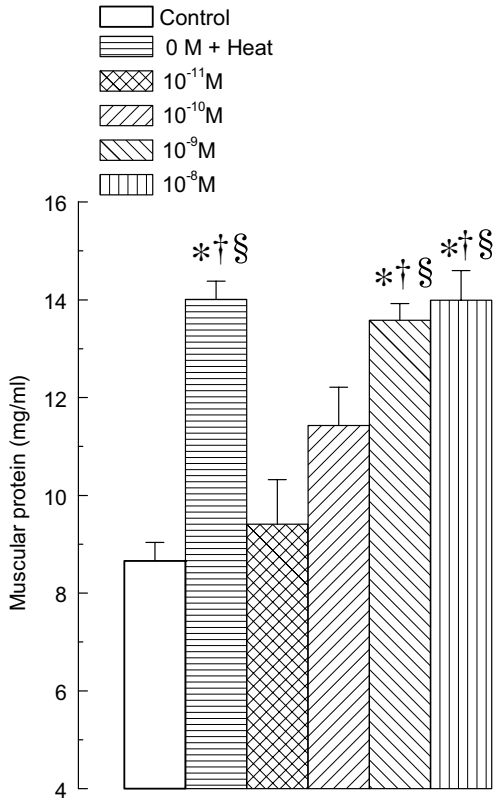

Fig. 4. Effects of continuous GGA administration or a bout of heat-stress on the protein contents of myotubes cultured in the differentiation medium. The GGA at the concentration of either 0, 10^{-11}, 10^{-10}, 10^{-9}, or 10^{-8} M was administered to the medium, when the differentiation was initiated after 3 days of culture in the growth medium. In one group, cultured without GGA, cells were exposed to heat (0 M + heat, 41 °C) for 60 min at the beginning of 9-day culture in the differentiation medium. The control group was cultured without GGA and heat-stress. Mean ± SEM. $n = 6$/group. Other abbreviations are the same as in Fig. 1. *,†,§Significantly different ($p < 0.05$) compared with the value of control, 10^{-11} M, and 10^{-10} M groups, respectively.

Fig. 3. Fig. 4 shows the effects of GGA or heat-stress on muscular protein contents in myotubes. Heat-stress alone significantly increased the protein content of muscle cells ($p < 0.05$). Further, administration of GGA caused an increase in muscular protein contents as a dose-dependent manner. Muscular protein contents of high dose (10^{-9}–10^{-8} M) of GGA were significantly higher than those of control and low dose (10^{-11}–10^{-10} M) of GGA ($p < 0.05$) and comparable to the heat-stressed condition.

Discussion

The present study clearly demonstrated that the expression of HSP72 in cultured mouse skeletal muscle cells (C_2C_{12}) was up-regulated by the administration of an antiulcer drug, GGA, and/or heat-stress. In addition, the administration of GGA or heat-stress facilitated the differentiation of myoblasts into myotubes, and the muscular protein content was increased as a dose-dependent manner. To our knowledge, this is the first report showing the GGA-associated up-regulation of HSP72 in cultured muscle cells and of the differentiation of myoblasts.

HSP72 expression

It is well known that HSP72, the inducible form of HSP70, is up-regulated by exposure to heat [9,10,16,17]. Up-regulation of HSP72 is induced by not only heat-stress but also many types of cellular stresses, such as the reduced peripheral blood circulation, oxygen radicals, and/or H^+ [9,18–21]. The HSP72 function as an important molecular chaperone [22,23]. Therefore, the cellular proteins could be stabilized following the up-regulation of HSP72. Overexpression of HSP72 is thought to be beneficial for the protection against the occlusion–reperfusion-induced cardiac injuries [24–26].

In skeletal muscles, it has been reported that the preconditioning by heat-stress, which increases the HSP72 expression, could attenuate the muscle atrophy induced by unloading [27]. It has been also shown that heat-stress also stimulated protein synthesis in skeletal muscles [13,16,28]. Further, application of heat-stress (41 °C for 60 min) with or without mechanical stretch facilitated the muscle hypertrophy [13,16,28] and the recovery of atrophied muscles [11]. The up-regulation of HSP72 induced by heat-stress, as well as mechanical stretch, may play one of the signals for muscle hypertrophy.

In the present study, the administration of GGA and/or the application of heat-stress induced the up-regulation of HSP72 expression in C_2C_{12} cells during both growing and differentiating phase. The HSP72 expression induced by the combination of GGA and hest-stress significantly higher than those induced by GGA or heat-stress alone ($p < 0.05$). However, the molecular mechanism responsible for the GGA-induced up-regulation of HSP72 is still unclear.

Differentiation

In the present study, the administration of GGA facilitated the differentiation of myoblasts, and the muscular protein content was increased as a dose-dependent manner. Heat-stress also caused an increase of muscular protein contents. This result is consistent with the previous study [16]. It is, however, unclear whether the increase in muscular protein is directly associated with the GGA-induced up-regulation of HSP72 or not. Kojima et al. [29] also reported that there was no direct relationship between the level of HSP72 expression and protein content in muscle, suggesting that the expression of HSP72may not be the key signal for protein synthesis.

Perspective

It was indicated that administration of an antiucler drug, GGA, as well as the heat-stress, to culture medium

increased the expression of HSP72 and protein content in muscle cells. It was suggested that GGA may activate the intracellular signals, which stimulates the differentiation of myoblasts and protein synthesis. These observations may also suggest that the administration of GGA could be one of the useful tools to gain muscular mass not only in athletes, but also in patients during rehabilitation.

Acknowledgments

This study was supported, in part, by Grants-in-Aid for Scientific Research (C, 17500444, K.G.; A, 18200042, T.Y.; S, 19100009, Y.O.) from Japan Society for the Promotion of Science, Ground-based Research Program for Space Utilization from Japan Space Forum (K.G.), and the Research Grant (16B-2) for Nervous and Mental Disorders from the Ministry of Health, Labor and Welfare (Y.O.).

References

[1] A.L. Fink, Chaperone-mediated protein folding, Physiol. Rev. 79 (1999) 425–449.
[2] F.U. Hartl, Molecular chaperones in cellular protein folding, Nature 381 (1996) 571–579.
[3] M. Jaattela, Heat shock proteins as cellular lifegurds, Ann. Med. 31 (1999) 261–271.
[4] G. Minowada, W.J. Welch, Clinical implications of the stress response, J. Clin. Invest. 95 (1995) 3–12.
[5] Y. Liu, S. Mayr, A. Opitz-Gress, C. Zeller, W. Lormes, S. Baur, M. Lehmann, J.M. Steinacker, Human skeletal muscle HSP70 response to training in highly trained rowers, J. Appl. Physiol. 86 (1999) 101–104.
[6] Y. Liu, W. Lormes, C. Baur, A. Opitz-Gress, D. Altenburg, M. Lehmann, J.M. Steinacker, Human skeletal muscle HSP70 response to physical training depends on exercise intensity, Int. J. Sports Med. 21 (2000) 351–355.
[7] M. Locke, E.G. Noble, B.G. Atkinson, Exercising mammals synthesize stress proteins, Am. J. Physiol. 258 (1990) C723–C729.
[8] A. Puntschart, M. Vogt, H.R. Widmer, H. Hoppeler, R. Billeter, Hsp70 expression in human skeletal muscle after exercise, Acta Physiol. Scand. 157 (1996) 411–417.
[9] D.C. Salo, C.M. Donovan, K.J.A. Davies, HSP70 and other possible heat shock or oxidative stress proteins are induced in skeletal muscle, heart, and liver during exercise, Free Radic. Biol. Med. 11 (1991) 239–246.
[10] R. Skidmore, J.A. Gutierrez, V. Guerriro, K.C. Kregal, HSP70 induction during exercise and heat stress in rays: role of internal temperature, Am. J. Physiol. 268 (1995) R92–R97.
[11] K. Goto, M. Honada, T. Kobayashi, K. Uehara, A. Kojima, T. Akema, T. Sugiura, S. Yamada, Y. Ohira, T. Yoshioka, Heat stress facilitates the recovery of atrophied soleus muscle in rat, Jpn. J. Physiol. 54 (2004) 285–293.
[12] T. Kobayashi, K. Goto, A. Kojima, T. Akema, K. Uehara, H. Aoki, T. Sugiura, Y. Ohira, T. Yoshioka, Possible role of calcineurin in heating-related increase of rat muscle mass, Biochem. Biophys. Res. Commun. 331 (2005) 1301–1309.
[13] K. Uehara, K. Goto, T. Kobayashi, A. Kojima, T. Akema, T. Sugiura, S. Yamada, Y. Ohira, T. Yoshioka, H. Aoki, Heat-stress enhances proliferative potential in rat soleus muscle, Jpn. J. Physiol. 54 (2004) 263–271.
[14] T. Hirakawa, K. Rokutana, T. Nikawa, K. Kishi, Geranylgeranylacetone induces heat shock proteins in cultured guinea pig gastric mucosal cells and rat gastric mucosa, Gastroenterology 111 (1996) 345–357.
[15] T. Ooie, N. Takahashi, T. Saikawa, T. Nawata, M. Arikawa, K. Yamanaka, M. Hara, T. Shimada, T. Sakata, Single oral dose of geranylgeranylaceton induces heat-shock protein 72 and renders protection against ischemia/reperfusion injury in rat heart, Circulation 104 (2001) 1837–1843.
[16] K. Goto, R. Okuyama, H. Sugiyama, M. Honda, T. Kobayashi, K. Uehara, T. Akema, T. Sugiura, S. Yamada, Y. Ohira, T. Yoshioka, Effects of heat stress and mechanical stretch on protein expression in cultured skeletal muscle cells, Pflügers Arch. 447 (2003) 247–253.
[17] E.A. Craig, B.D. Grambill, R.J. Nelson, Heat shock proteins: molecular chaperones of protein biogenesis, Microbiol. Rev. 57 (1993) 402–414.
[18] J.J. Cotto, R.I. Morimoto, Stress-induced activation of heat-shock response: cell and molecular biology of heat-shock factors, Biochem. Soc. Symp. 64 (1999) 105–118.
[19] G. Luo, X. Sun, E. Hungness, P.-O. Hasselgren, Heat shock protects L6 myotubes from catabolic effects of dexamethasone and prevents downregulation of NF-κB, Am. J. Physiol. 281 (2001) R1193–R1200.
[20] G. Weitzel, U. Pilatus, L. Rensing, Similar dose response of heat shock protein synthesis and intracellular pH change in yeast, Exp. Cell Res. 159 (1985) 252–256.
[21] H.R. Wong, Potential protective role of the heat shock response in sepsis, New Horiz. 6 (1998) 194–200.
[22] J.L. Kilgore, T.I. Musch, C.R. Ross, Physical activity, muscle, and the HSP70 response, Can. J. Appl. Physiol. 23 (1998) 245–260.
[23] J.B. Sass, E.S. Weinberg, P.H. Krone, Specific localization of zebrafish hsp90 alpha mRNA to myoD-expressing cells suggests a role for hsp90 alpha during normal muscle development, Mech. Dev. 54 (1996) 195–204.
[24] J. Jayakumar, K. Suzuki, I.A. Sammut, R.T. Smolenski, M. Khan, N. Latif, H. Abunasra, B. Murtuza, M. Amrani, M.H. Yacoub, Heat shock protein 70 gene transfection protects mitochondrial and ventricular function against ischemia–reperfusion injury, Circulation 104 (2001) I-303–I-307.
[25] D.S. Latchman, Heat shock proteins and cardiac protection, Cardiovasc. Res. 51 (2001) 637–646.
[26] S. Okubo, O. Wildner, M.R. Shah, J.C. Chelliah, M.L. Hess, R.C. Kukreja, Gene transfer of heat-shock protein 70 reduces infarct size in vivo after ischemia/reperfusion in the rabbit heart, Circulation 103 (2001) 877–881.
[27] H. Naito, S.K. Powers, H.A. Demirel, T. Sugiura, S.L. Dodd, J. Aoki, Heat stress attenuates skeletal muscle atrophy in hindlimb-unweighted rats, J. Appl. Physiol. 88 (2000) 359–363.
[28] T. Kobayashi, K. Uehara, K. Goto, A. Kojima, M. Honda, T. Akema, T. Sugiura, S. Yamada, Y. Ohira, T. Yoshioka, Muscular hypertrophy is induced by heat stress in rat skeletal muscles, St. Marianna Med. J. 31 (2003) 131–138.
[29] A. Kojima, K. Goto, S. Morioka, T. Naito, T. Akema, H. Fujiya, T. Sugiura, Y. Ohira, M. Beppu, H. Aoki, T. Yoshioka, Heat stress facilitates the regeneration of injured skeletal muscle in rats, J. Orthop. Sci. 12 (2007) 74–82.