ARTICLE
Journal of Cellular Biochemistry 114:2024–2031 (2013)

Journal of Cellular Biochemistry

Protective Induction of Hsp70 in Heat-Stressed Primary Myoblasts: Involvement of MAPKs

Daiva Bironaite,[1]* Ulf Brunk,[2] and Algirdas Venalis[1]

[1]*Department of Stem Cell Biology, State Research Institute Centre for Innovative Medicine, LT01102, Vilnius, Lithuania*

[2]*Department of Pharmacology, Linkoping University, SE-581 83, Linkoping, Sweden*

ABSTRACT

The involvement of extracellular signal-regulated kinases 1 and 2 (ERK1,2), stress kinase p38 and c-Jun NH_2-terminal kinases 1 and 2 (JNK1,2) on Hsp70-upregulation following mild heat shock, and resulting cell protection, was studied on rabbit primary myoblasts. Cells subjected to heat stress (42°C; 60 min) showed a significantly enhanced amount of heat-shock-induced protein 70 (Hsp70), correlating with sustained phosphorylation of MAP kinases ERK1,2, inhibition of p38 and JNK1,2 activation. Induced Hsp70 did not autocrinally suppress activation of transcription factor c-Jun, suggesting involvement of the latter in the protection of myoblasts following heat shock. The inhibition of stress kinases p38, JNK1,2, and MEK1,2 by SP600125, SB203580, and UO126, respectively, established the involvement of JNK1,2 and p38 as upstream, and ERK1,2 as downstream targets of Hsp70 induction. Moreover, the effect of the MEK1,2 inhibitor UO126 revealed a new pathway of c-Jun activation by ERK1,2 in myogenic heat-stressed stem cells. The presented data show that transient activation of JNK1, JNK2, and p38 is necessary for Hsp70 induction and ensuing cell protection. In conclusion, affecting myogenic stem cell protective mechanisms might be a useful strategy in improving stem cell survival and their expanded application in therapy. J. Cell. Biochem. 114: 2024–2031, 2013.

KEY WORDS: STEM CELLS; SIGNALING; MAPK; HSP; HEAT STRESS

Degenerative and chronic inflammatory diseases are often related to increased stressful affects and depressed defense systems. Cells in general are prepared to cope with various forms of stress by upregulating numerous defense systems and have many sophisticated ways to improve survival. Stem cell therapy is presently an exciting and promising strategy for the treatment of many diseases. Unfortunately, transplanted stem cells face various stresses, in particular ischemia-reperfusion, resulting in poor survival of transplanted myoblasts [Qu et al., 1998]. Increased expression of heat-stress-induced proteins (Hsp(s)) is one way that cells fight stress and prevent damage. Therefore, clarification of signaling mechanisms responsible for regulation of stem cells' defense following stress is of major interest.

Heat shock proteins (Hsp(s)) are a family of highly homologous chaperone proteins that are constantly expressed or induced by various chemical and physical stresses. They are coded for by distinct genes and named according to their approximate molecular mass that is Hsp110, Hsp90, Hsp70, Hsp60, and small heat shock proteins (sHsps). The Hsp70 group is the most prominent one and includes stress-induced protein 70 (Hsp70, iHsp70, and Hsp72), the constitutively expressed form (Hsc70 and Hsp73) as well as the glucose-regulated protein 78. The exact roles of the individual members of the Hsp70 family are not clearly delineated. Historically, the main protective role of Hsp(s) is considered related to chaperonic functions, including normal protein folding, correction of miss-folded proteins and regulation of degradation [Hartl and Hayer-Hartl, 2002; Dreiseidler et al., 2012]. Additionally, it was proposed that Hsp(s) might play a significant role in cell protection, inhibition of apoptosis, cytokine production and stimulation of phagocytosis [Asea et al., 2000; Seki et al., 2012]. It is also known, that Hsp70 participates in the regulation of myocardial apoptosis following ischemia-reperfusion, neurodegenerative and other chronic diseases

Abbreviations: ERK, extracellular signal-regulated kinase; Hsp, heat shock protein; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase.

The authors have no competing or other conflict of interests.

Grant sponsor: The Lithuanian State Science and Studies Foundation; Grant numbers: T-65/07, U-04001, C-07023.

*Correspondence to: Daiva Bironaite, Department of Stem Cell Biology, State Research Institute Centre for Innovative Medicine, Zygimatu 9, LT01102 Vilnius, Lithuania. E-mail: daibironai@gmail.com; d.bironaite@imcentras.lt

Manuscript Received: 1 October 2012; Manuscript Accepted: 12 March 2013

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 28 March 2013

DOI 10.1002/jcb.24550 • © 2013 Wiley Periodicals, Inc.

such as diabetes, neuropathy, and epilepsy [Currie et al., 1988; Yenari et al., 1998; Suzuki et al., 2000; Sittler et al., 2001; Kurthy et al., 2002]. However, the regulation of Hsp70-induction and function in stem cells is still obscure.

The mitogen-activated protein kinase (MAPK)-cascade is one of the most ancient and evolutionarily conserved signaling pathways, stretching from unicellular organisms to humans [Widmann et al., 1999]. Three major groups of MAPKs are known: extracellular signal-regulated protein kinase 1 and 2 (ERK1,2) and ERK5 (Big MAPK, BMK), p38 (α, β, γ, and δ isophorms) known as stress-activated protein kinase 2 (SAPK2), and c-Jun N-terminal kinases JNK1, JNK2, and JNK3 (SAPK-γ, SAPK-α, and SAPK-β, respectively) [Rubinfeld and Seger, 2005; Bogoyevitch et al., 2010; Kyriakis and Avruch, 2012a]. Most signals that activate the ERK pathways are related to membrane perturbations, receptor activation or ion channel stimulation with subsequent regulation of cell proliferation and transformation processes [Rane, 1999; Naor et al., 2000; Rangarajan et al., 2004; Schigapova et al., 2005; McKay and Morrison, 2007]. Stress kinases JNKs and p38 mostly respond to various toxic environmental stimuli, such as calcium ions, heat stress and reactive oxygen species (ROS), influencing cell death and survival mechanisms [Volloch et al., 2000; Takeda et al., 2004; Matsuzawa et al., 2005]. However, one of the major consequences of MAPKs activation is their involvement in the transcriptional regulation of cell fate under normal and stressful conditions [Kyriakis and Avruch, 2012b]. Despite the fact that MAP kinases are well investigated and found to regulate many cellular activities, new functions of MAPKs are still found.

A number of studies suggest that MAPKs may have both pro- and anti-survival functions and are cell type, stress origin, and intensity dependent. Considering the critical role of MAPKs in cell survival, the objective of this study was to evaluate the role of ERK1,2, JNK1,2, and p38 in the induction of Hsp70 by mild heat shock with ensuing protection of myogenic stem cells. The ability to regulate Hsp70 induction would be useful in stem cell therapy. Therefore, learning more about the regulation of stem cell pro-death and pro-survival molecular mechanisms would be of great importance in the treatment of many diseases.

MATERIALS AND METHODS

CHEMICALS

Monoclonal antibodies against inducible (Hsp70) and constitutive (Hsc70) forms of Hsp70 were purchased from Stressgen Biotechnologies Corporation (Canada). Monoclonal antibodies against the phosphorylated forms of ERK1,2, p38, and JNK were obtained from Cell Signaling Technology (Beverly, MA). Polyclonal antibodies for the detection of total JNK, p38, and ERK1,2 were from Cell Signaling Technology (Beverly, MA), while monoclonal antibodies against phosphorylated and total c-Jun were from BD Biosciences (San Jose, CA). Secondary anti-mouse and anti-rabbit antibodies were from New England Biolabs (UK) and the inhibitors U0126, SP600125, and SB203580 from Sigma Chemical Co. (St Louis, MO). Stock solutions (20 mM) of all inhibitors were prepared in dimethylsulfoxide (DMSO). The final concentration of DMSO in growth medium never exceeded 0.2%.

ISOLATION AND IDENTIFICATION OF MYOGENIC STEM CELLS

Rabbit myogenic stem cells were isolated and identified as previously described [Bukelskiene et al., 2006]. California rabbits weighing 2.0–2.5 kg received good care according to the "Law on care, welfare and use of animals" of the Lithuanian Republic. License for the use of laboratory animals in stem cell research (No. 0121, 2004-07-09) was received from the Lithuanian Food and Veterinary office. A longitudinal incision was made intramuscularly over the projection of the tibialis anterior muscle. The incision was done under sterile conditions and general anesthesia (50 mg/kg of ketamin and 5 mg/kg diazepam). A pierce of skeletal muscle tissue (0.3 cm^3) was placed on a plate with cold Hank's salt solution (Sigma) and minced with scissors. The minced tissue was exposed to digestive solution containing 0.125% trypsin–EDTA (Sigma), 1 mg/ml collagenase type V (Sigma), and 0.3 mg/ml hyaluronidase (Sigma) in PBS as described in Sirmenis et al. [1999]. The cell mass was washed with Iscove's modified Dulbeco medium (IMDM) (Sigma), supplemented with 10% fetal calf serum (FCS) (Sigma). The number of cells was counted using a hematocytometer, and cell viability was evaluated by the trypan blue exclusion test.

Cells were grown in 25 cm^2 polystyrene culture flasks containing IMDM growth medium supplemented with FCS (10%), penicillin (100 U/ml), and streptomycin (100 µg/ml). Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2, passaged twice a week and detached by a 0.25% (w/v) trypsin/EDTA solution (Gibco, Grand Island, NY).

INDUCTION OF HSP70 PROTEIN AND ASSESSMENT OF CELL VIABILITY AND APOPTOSIS

To allow 70–80% confluence, cells were seeded in 12-well plates at a concentration of 75×10^3/ml 24 h before the heat-shock experiment. Media was changed and cells were subjected to 42°C for 15–60 min in a water bath and than allowed to recover for various periods of time at standard culture conditions. The number of viable cells before and after heat stress was determined by the trypan blue dye exclusion test (0.4% trypan blue in phosphate buffer [80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl]) as well as by MTT (see below).

Briefly, cell suspensions were mixed 1:1 with trypan blue dye solution and cell viability was evaluated under a light microscope. The method is based on the penetration of trypan blue dye into nonviable cells, coloring them blue. Cell viability before and after heating at 42°C for 60, 120, and 180 min was 98 ± 2%, 67 ± 2%, and 60 ± 3%, respectively.

The MTT test was performed as described by Mosmann [1983]. The MTT assay is based on the activity of preserved mitochondrial succinate dehydrogenase and is broadly used for the estimation of cell proliferation and viability. Briefly, cells were grown in 24-well plates, the incubation media was replaced with 0.5 ml of a tetrazolium dye (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide, MTT) solution at an MMT concentration of 0.2 mg/ml in PBS. Cells were incubated for 0.5 h at 37°C and then washed with PBS. Reduced MTT dye (formazan) within cells was extracted with ethanol. Formazan absorption was monitored at 570 nm. Living cells were colored purple and the extraction solution had high absorption values.

Cell apoptosis was measured using fluorescent dye 4′-6-diamidino-2-phenylindole (DAPI). DAPI is known to form fluorescent

complexes with double-stranded DNA. When DAPI binds to DNA, its fluorescence enhances. Harvested cells were washed by PBS and suspended in PBS containing 0.1% Triton X-100 and kept on ice for 10 min, then spinned down and resuspended in 4% PFA, containing 10 µg/ml DAPI (Sigma). A drop of cell suspension was then placed in a hemocytometer and observed under a fluorescence microscope (Olympus). Viable cell nuclei were glowing bright and homogenously, whereas apoptotic cell nuclei had condensed chromatin or fragmented nuclei. Three hundreds cells were counted and apoptosis was expressed as percentage of not homogenously stained nuclei.

ISOLATION OF NUCLEI

Following heat stress and subsequent recovery, cells were harvested and washed twice with cold PBS. Nuclear extracts were prepared using a nuclei isolation kit (Nuclei EZ) according to the manufacturer recommendations (Sigma-Aldrich Corp., St. Louis, MO). The protein contents of the isolated nuclei fraction and cytoplasm were assayed by the Bradford method. The cytoplasmic and nuclear proteins were assayed using anti-Hsp70 antibody.

IMMMUNOBLOTTING

After exposure to heat stress, cells were rinsed and lysed in ice-cold lysis buffer (10 mM Tris–HCl pH 7.4, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 2 mM Na_3VO_4 with 0.1% BSA and 1% Triton X-100), supplemented with protease inhibitors (20 µg/ml aprotinin and 1 mM PMSF). Equal amounts of protein, estimated by the Bradford method, were separated by SDS–PAGE on 10% acrylamide gels. Separated proteins from the gel were transferred to a PVDF membrane (ROTH, Germany) and blocked with blocking buffer (5% low fat milk in TBST [4 mM Tris–HCl, 1 mM Tris base, 0.1% Tween 20, and 154 mM NaCl]) for 1 h at room temperature (RT). The membranes were then washed with TBST for 3×5 min, initially probed with primary antibodies in 5% low fat milk in TBST for 24 h at 4°C and again with secondary antibodies in the same solution for 1 h at RT. After incubation, membranes were washed for 3×5 min in TBST. Proteins were visualized by the Amersham ECL system. Quantification of western blots was carried out using the "ImageJ" program. Amounts of proteins were expressed as immunoblot band volume (relative optical density of the band multiplied with its area).

IMMUNOCYTOCHEMISTRY

For immunocytochemical detection of Hsp(s), myogenic stem cells were seeded on glass cover slips in 12-well plates and grown in complete media for 24 h. Cells were then subjected to heat stress as described above, allowed to recover for various periods of time at standard culture conditions, washed with PBS and fixed in 4% formaldehyde, freshly prepared form paraformaldehyde, permeabilized with 0.1% Triton 100 solution and incubated overnight with anti-Hsp70 antibodies (SPA-810; Stressgen Biotechnologies). Alexa Fluor 488-conjugated goat anti-mouse IgG antibodies (Molecular Probes, Inc.) were used as secondary antibodies. Cells were observed under a Nikon C1 confocal scanning microscope with controlled light exposure. Confocal images were obtained using the immersion objective PlanApo-VC (magnification 60×, NA 1.4) and the EZ-C1 program. Alexa Fluor-conjugated secondary antibodies were detected through a 522-nm filter using an Ar laser (e.g., 488 nm). Obtained images were analyzed with the Bitplane programme Imaris 5.0.1. The concentrations of primary and secondary antibodies were optimized to obtain maximal fluorescence specificity.

STATISTICAL ANALYSIS

Data are presented as means ± SD from at least three independent experiments. Statistical analyses were performed using Student *t*-test in the SigmaPlot 2001 program. Differences were considered statistically significant at $^{*}P < 0.05$.

RESULTS

Data of immunocytochemistry and immunoblotting presented in Figure 1 show heat-shock-induced synthesis of Hsp70 protein in rabbit myogenic stem cells. Hsp70 expression started to increase already 15 min following the initiation of heat stress, while maximal effect was observed after 60 min of such stress (Fig. 1A). Control cells did not show any expression of Hsp70. Cell viability before and after a heat stress period of 60 min or less was never below 95%.

Temperature and time during and following heat stress were found to be highly important for the degree of Hsp70-upregulation. The largest upregulation of Hsp70 was found 8 h following a 60-min 42°C heat-chock (Fig. 1B and C). Nuclear Hsp70 was also maximally

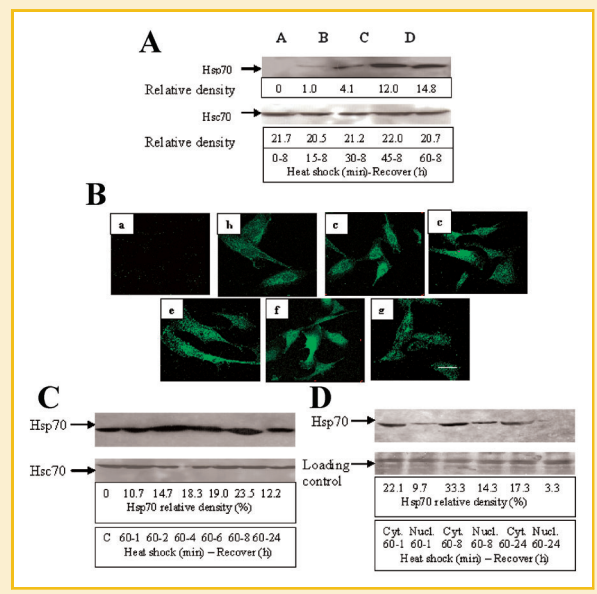

Fig. 1. Upregulation of Hsp70 in myogenic stem cells by heat stress (15–60 min; 42°C; various periods of recovery). A: Selection of the most effective heat shock periods at 42°C (performed as described in the Material and Methods Section) in combination with 8 h of recovery at standard culture conditions. B: Time course (1–24 h) of Hsp70 induction following heat stress. a—control cells. Recovery periods: b—2 h; c—4 h; d—6 h; e—8 h; f—24 h. Maximum Hsp70 expression was observed 8 h after heat stress. Isotype controls were used for each immunocytochemical experiment; scale bar: 50 µm. C: Time course of Hsp70-induction following heat stress shown by immunoblotting. D: Hsp70 expression in cytosol and nuclei at 1, 8, and 24 h of recovery. Quantitative immunoblotting was performed using the ImageJ program. Data shown are representative of at least three independent experiments.

increased at 8 h after heat stress (Fig. 1B and D). Hsp70 expression started to decrease in both cytosol and nucleus 24 h after heat stress (Fig. 1B, C, and D). Based on these test results, the bulk of the remaining experiments were performed using stress conditions giving maximum of Hsp70 expression that is heat shock at 42°C for 60 min followed by 8 h of recovery. Cell recovery periods longer than 24 h resulted in decreasing Hsp70 expression that was not significant for our study and data are therefore not included.

The MAPK subfamily ERK is known to mediate cell survival signals in response to various stimuli. Our data demonstrate that our chosen heat stress conditions (42°C for 60 min with 8 h recovery) activated ERK1,2 in myogenic stem cells, whereas total ERK1,2 remained stable (Fig. 2A and B). The activation and total amount of ERK2 in both control- and heat-stressed cells was higher than that of ERK1 (Fig. 2A and B). The induction of Hsp70 (Fig. 1A) and phosphorylation of ERK1,2 kinases (Fig. 2A) correlated with the activation of transcription factor c-Jun and its total upregulation (Fig. 2C and D).

Stress kinases JNK1 and JNK2 were activated by 15–30 min of heat stress, whereas 45–60 min stress started to suppress both JNK1,2 activation and the total amount of protein (Fig. 2E and F). Similar heat stress-induced inhibition was observed when p38 activation was analyzed (Fig. 2G). Suppression of JNK1,2, p38 activation and total amount of JNK1,2 showed an opposite correlation with maximal Hsp70 upregulation (Figs. 1A and 2E, F, and G). The total amount of p38 remained stable (Fig. 2H). Surprisingly, phosphorylation and total amount of the transcription factor c-Jun was not inhibited similar to JNK1,2, suggesting another way of c-Jun activation (Fig. 2C and D). The activation of ERK1,2 and c-Jun and inhibition of JNK and p38 in heat-stressed myogenic stem cells demonstrate their pro-survival and pro-death functions, respectively.

In the next set of experiments, we further explored the involvement of MAPKs in the induction of Hsp70. Inhibition of stress kinases, JNKs and p38, by the specific inhibitors SP600125 (20 μM) and SB239063 (20 μM), respectively, demonstrated involvement of JNK1, JNK2, and p38 as upstream targets in the initiation of Hsp70 synthesis by heat stress (Fig. 3A). The MEK1,2 inhibitor U0126 only slightly suppressed expression of Hsp70 (Fig. 3A). Surprisingly, the JNK and p38 inhibitors, SP600125 (20 μM) and SB239063 (20 μM), respectively, partly inhibited activation of ERK1,2, revealing ERK1,2 to be a downstream target of Hsp70 synthesis (Fig. 3A). The efficiency of inhibitors SP600125 and SB2035800 was confirmed by the resulting phosphorylation of c-Jun Ser 63, 73, and p38 Tyr 182, respectively (Fig. 3B). Moreover, exposure of cells to the MEK1,2 inhibitor U0126 after heat-shock-suppressed c-Jun-activation, confirming the interaction between ERK1,2 and c-Jun (Fig. 3B). Exposure of cells to various concentrations of quercetin, an inhibitor of heat shock factor 1 (HSF1), right after completed heat shock, inhibited ERK1,2 and c-Jun activation, thus additionally revealing cross-interaction between these signaling pathways (Fig. 3C). The mode of expression of total c-Jun was not changed by quercetin (Fig. 3C).

Inhibitors of stress kinases JNKs and p38, SP600125 and SB2035800, respectively, significantly inhibited cell viability at 8 and 24 h following heat stress. The strongest effect on cell viability was obtained by 200 μM of quercetin, whereas MEK1,2 inhibitor U0126 had only minor effect (Fig. 4). Applied specific MAPK

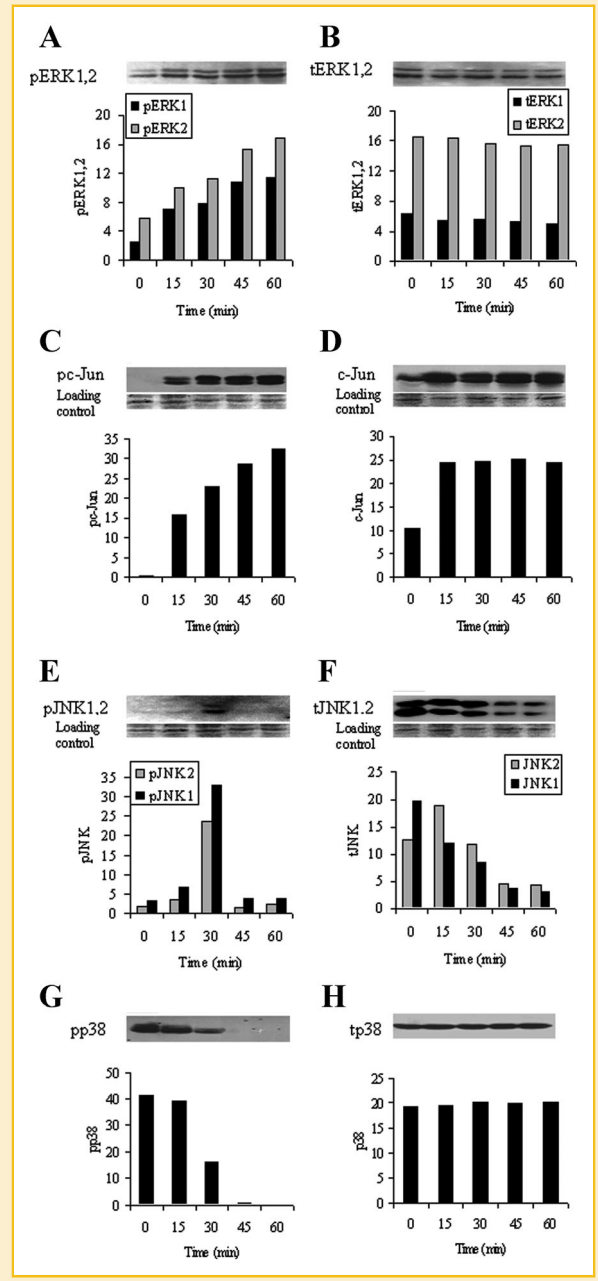

Fig. 2. Activation of MAPKs and transcription factor c-Jun by heat stress (60 min; 42°C; 8 h of recovery). A: Activation of ERK1,2; (B) expression of total ERK1,2; (C) activation of c-Jun; (D) expression of total c-Jun; (E) activation of JNK1 and JNK2; (F) expression of total JNK1 and JNK2; (G) activation of p38; (H) expression of total p38. The immunoblots were evaluated using the ImageJ program and expressed as relative density (%).

inhibitors showed direct correlation between inhibition of Hsp70 and induced suppression of cell viability. The apoptotic mode of cell death was shown by intercalation of fluorescent dye DAPI into pycnotic and fragmented DNA (Fig. 4B).

The scheme presented in Figure 5 summarizes the results of our study. More detailed mechanisms of apoptosis and additional ways of signal transfer regulating differentiation and pro- and anti-cell

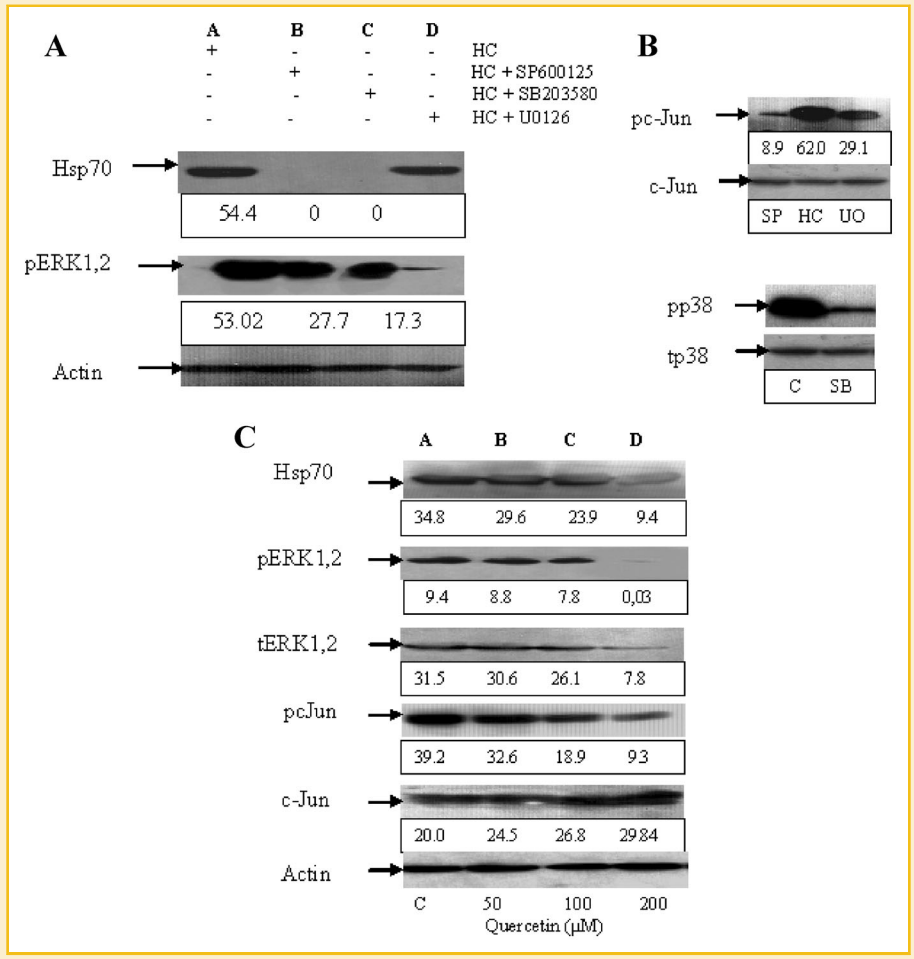

Fig. 3. Involvement of MAPKs in Hsp70 expression following heat stress (60 min; 42°C; 8 h of recovery) evaluated by specific MAPKs inhibitors. A: Result of cell exposure to the JNK1,2, MEK1 and p38 inhibitors SP600125, UO126, and SB203580, respectively, on Hsp70 induction and ERK1 phosphorylation; (B) result of exposure to the JNK1,2 inhibitor SP600125 and the MEK1 inhibitor UO126 on the activation of c-Jun. The efficiency of inhibitor SB203580 was demonstrated by inhibition of p38 activation; (C) effect of quercetin on induction of ERK1,2 and c-Jun, both being downstream targets of Hsp70. SP—SP600125, an inhibitor of JNK1,2; SB—SB203580, an inhibitor of p38; UO—UO126, an inhibitor of MEKs. Cells were exposed to 20 μM of inhibitors directly after heat stress. The immunoblots were evaluated using the ImageJ program and expressed as relative density (%).

survival mechanisms in stressed myogenic stem cells are under investigation.

DISCUSSION

Stem cells engrafted into a damaged organ can be subjected to various stress factors and conditions, the most obvious one being oxidative stress due to post-ischemic reperfusion, which is a major problem and explains the often low viability of transplanted cells. Stem-cell adaptation to stressful conditions usually starts with the initiation of stress-signaling pathways that play a central role in the development of cardiac and other muscle, dysfunctions [Molkentin and Dorn, 2001]. Hsp proteins with their chaperonic functions are strongly involved in cell protection under stressful conditions. Recently, broad cytoprotective functions of Hsp(s), particularly the Hsp70 family, have been connected to signaling pathways including MAPKs and Akt/PKB, stabilization of mitochondrial and lysosomal membranes or binding lysosomal iron in a non-redox-active form [Volloch et al., 2000; Nylandsted et al., 2004; Banerjee Mustafi et al., 2009; Kurz and Brunk, 2009; Berndt et al., 2010]. By working together, three branches of MAPKs, JNK1,2, p38, and ERK1,2 not only regulate cell growth and differentiation but also cell survival under various growth conditions [Schaeffe and Weber, 1999]. For this reason, we investigated the involvement of MAPKs in the induction of Hsp70 and the mechanism of protection of myogenic stem cells after heat stress.

A vast number of literature reports point out that ERK1,2 activation protects cells, although it is less than clear in which way this happens. Some studies have demonstrated that the protective role of ERK1,2 in cardiomyocytes is connected to the inhibition of Cox-2 [Adderley and Fitzgerald, 1999], while other ones indicate that the protection is connected to FADD-like interleukin 1β-converting enzyme-expression or formation of mitochondrial PKCepsilon-ERK modules [Yeh et al., 1998; Baines et al., 2002]. The supposed

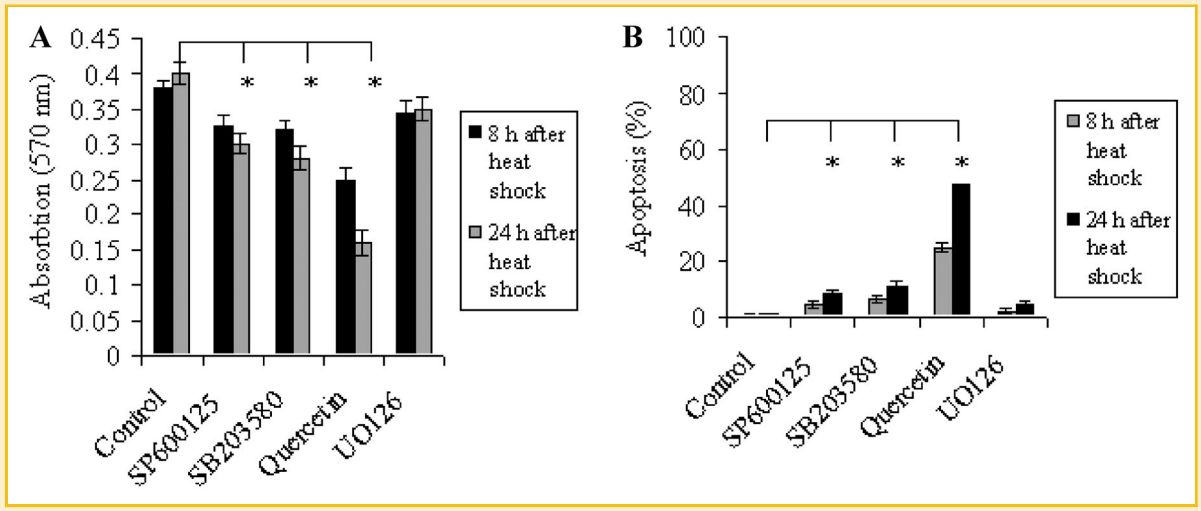

Fig. 4. Influence of specific MAPKs and Hsp70 inhibitors on the viability of myogenic stem cells. A: Cell viability after exposure to specific inhibitors; (B) induction of apoptosis by specific inhibitors: SP—SP600125, an inhibitor of JNK1,2; SB—SB203580, an inhibitor of p38; UO—UO126, an inhibitor of MEKs; Q—quercetin, an inhibitor of HSF1. Cells were exposed for 8 or 24 h to 20 μM of the inhibitors, starting right after heat shock. Cell viability and apoptosis were measured by MTT reduction and DAPI intercalation assays, respectively. Results are represented as means ± SD of three independent experiments. $^{*}P$ indicates significant differences from control cells ($P < 0.05$).

mechanisms behind the interaction of transcription factors c-Jun and ERK1,2 and their involvement in the regulation of cell functioning are still vague and often controversial. c-Jun, a component of AP-1 transcription factors, influences expression of many genes through forming hetero- or homo-dimers and binding to DNA [Raivich, 2008]. c-Jun activity can be regulated by post-translational modifications, including phosphorylation [Raivich, 2008]. Phosphorylation of Ser63/73 in c-Jun is mostly involved in enhanced c-Jun expression and might be a potential substrate not only for the JNK1,2 but also for ERK1,2 [Pulverer et al., 1991; Deng et al., 2012]. Other studies showed that only purified JNKs, but not ERK, activate c-Jun in vitro (Minden et al., 1994). The involvement of ERK1,2 and JNK1,2 in phosphorylation of c-Jun Ser 63/73 has been shown in mice fibroblasts and microglia cells [Morton et al., 2003; Deng et al., 2012]. However, data of this study demonstrate the involvement of the ERK1,2 signaling pathway in c-Jun Ser 63 and 73 phosphorylation and it being a downstream effect of Hsp70 induction in heat-shocked myogenic stem cells, thereby supporting viability of heat-stressed myogenic stem cells.

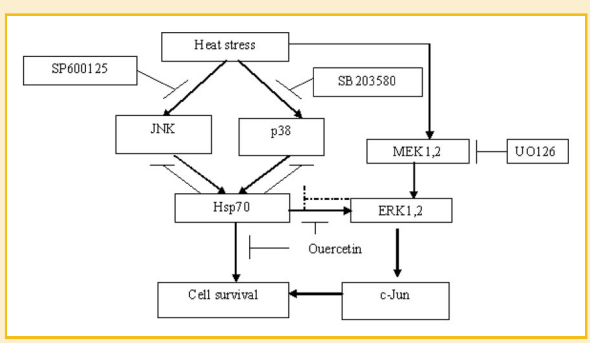

Fig. 5. Scheme over proposed participation of MAPKs in induction of Hsp70 and ensuing protection of myogenic stem cells as a result of mild heat stress.

The molecular mechanisms behind JNK and c-Jun interaction were investigated using v-Jun that has an N-terminal deletion of 27 amino acids affecting Ser 63/73 phosphorylation [Nishimura and Vogt, 1988]. The binding of c-Jun to JNK depends not only on the delta but also on the docking domain, a conserved LXL motif, and regions outside of the c-Jun [Hibi et al., 1993; Kallunki et al., 1996; Sharrocks et al., 2000]. All these binding peculiarities might explain the different interaction of ERK and JNK with c-Jun and might also explain why various JNK isophorms have different influence on c-Jun that is JNK2 has a higher binding affinity to c-Jun, whereas JNK1 has a higher phosphorylation capacity [Gupta et al., 1994; Kallunki et al., 1996]. Possibly, c-Jun transcription is directly stimulated by its own product, thereby creating a positive cell regulatory and protective loop [Angel et al., 1988]. Nevertheless, our results demonstrate that transient phosphorylation of JNK1,2 by heat stress, though mainly affecting JNK1, induces Hsp70 and activates c-Jun. Moreover, the suppression of JNK1,2 activity by induced Hsp70 did not affect c-Jun activation, affirming that other signaling mechanisms than the JNK pathway are also involved in c-Jun activation through phosphorylation of Ser 63 and 73.

Also p38 kinase is suggested a proximal effector of apoptosis in many cell types. The phosphorylation of p38 kinase by various kinds of stresses occurs at the Tyr and Thr residues with subsequent induction cell death [Segreto et al., 2011]. On the other hand, it has been shown that activated p38 might have a protective role as well [Sun et al., 2006]. Although p38 and JNK1,2 are activated by similar stimuli, it was shown that p38 does not activate c-Jun neither in vivo nor in vitro [Raingeaud et al., 1996]. Our data revealed that activation of Tyr 182 on p38 α and β is required for induction of Hsp70. The p38 inhibitor SB2035800 also suppressed activation of ERK1,2, being a downstream target of Hsp70 induction. The presence of active stress

kinases in control cells suggests that under normal growth conditions, some pro-death pathways are not necessarily dangerous but may be in balance with pro-survival pathways that are disturbed under stressful conditions.

In conclusion, data of the present study for the first time show interaction between ERK1,2 and c-Jun and their participation in protection of heat-stressed myogenic stem cells. Additionally, following mild heat shock, transient activation of the stress kinases JNK1, JNK2, and p38 is linked to induction of Hsp70 as an upstream target with ensuing protection of myogenic stem cells. We also demonstrate that cell protection by ERK1,2 is carried out trough activation of transcription factor c-Jun, not trough induction of Hsp70. Drugs suppressing the SAPKs JNK1,2 and p38 should be used with care, since under stressful situations they may harm stem cells. Studies of the regulation of stem cell survival and death mechanisms under stressful situations may allow us to find new ways to prevent several degenerative diseases and, perhaps, slow down aging. Eventually, the purposeful stimulation of stem cell survival mechanisms would increase efficiency of therapeutical cellular transplantation.

ACKNOWLEDGMENTS

The authors want to thank to Dr. M. Valius, Dr. A. Kalvelyte, and Mrs. D. Jakutiene for excellent technical assistance.

REFERENCES

Adderley SR, Fitzgerald DJ. 1999. Oxidative damage of cardiomyocytes is limited by extracellular regulated kinases 1/2-mediated induction of cyclooxygenase-2. J Biol Chem 274:5038–5046.

Angel P, Hattori K, Smeal T, Karin M. 1988. The jun proto-oncogene is positively autoregulated by its product, Jun/AP-1. Cell 55(5):875–885.

Asea A, Kraeft SK, Kurt-Jones EA, Stevenson MA, Chen LB, Finberg RW, Koo GC, Calderwood SK. 2000. HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. Nat Med 6:435–442.

Baines CP, Zhang J, Wang GW, Zheng YT, Xiu JX, Cardwell EM, Bolli R, Ping P. 2002. Mitochondrial PKCe and MAPK form signaling modules in the murine heart: enhanced mitochondrial PKCe–MAPK interactions and differential MAPK activation in PKCe-induced cardioprotection. Circ Res 90:390–397.

Banerjee Mustafı S, Chakraborty PK, Dey RS, Raha S. 2009. Heat stress upregulates chaperone heat shock protein 70 and antioxidant manganese superoxide dismutase through reactive oxygen species (ROS), p38MAPK, and Akt. Cell Stress Chaperones 14(6):579–589.

Berndt C, Kurz T, Selenius M, Fernandes AP, Edgren MR, Brunk UT. 2010. Chelation of lysosomal iron protects against ionizing radiation. Biochem J 432(2):295–301.

Bogoyevitch MA, Ngoei KRW, Zhao TT, Yeap YYC, Ng DCH. 2010. c-Jun N-terminal kinase (JNK) signaling: recent advances and challenges. Biochim Biophys Acta 1804(3):463–475.

Bukelskiene V, Baltriukiene D, Bironaite D, Imbrasaite A, Širmenis R, Balciunas M, Žurauskas E, Kalvelyte A. 2006. Muscle-derived primary stem cell lines for heart repair. Semin Cardiol 11:1–7.

Currie RW, Karmazyn M, Klock M, Mailer K. 1988. Heat shock response is associated with enhanced postischemic ventricular recovery. Circ Res 63:543–549.

Deng Z, Sui G, Rosa PM, Zhao W. 2012. Radiation-induced c-Jun activation depends on MEK1-ERK1/2 signaling pathway in microglial cells. PLoS One 7(5):e36739.

Dreiseidler M, Dick N, Höhfeld J. 2012. Analysis of chaperone-assisted ubiquitylation. Methods Mol Biol 832:473–487.

Gupta S, Barrett T, Whitmarsh AJ, Cavanagh J, Sluss HK, Derijard B, Davis RJ. 1994. Selective interaction of JNK protein kinase isoforms with transcription factors. EMBO J 15:2760–2770.

Hartl FU, Hayer-Hartl M. 2002. Molecular chaperones in the cytosol: from nascent chain to folded protein. Science 295:1852–1858.

Hibi M, Lin A, Smeal T, Minden A, Karin M. 1993. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Dev 7(11):2135–2148.

Kallunki T, Deng T, Hibi M, Karin M. 1996. c-Jun can recruit JNK to phosphorylate dimerization partners via specific docking interactions. Cell 87(5):929–939.

Kurthy M, Mogyorosi T, Nagy K, Kukorelli T, Jednakovitis A, Talosi L, Biro K. 2002. Effect of BRX-220 against periferal neuropathy and insulin resistance in diabetic rat models. Ann N Y Acad Sci 967:482–489.

Kurz T, Brunk UT. 2009. Autophagy of HSP70 and chelation of lysosomal iron in a non-redox-active form. Autophagy 5:93–95.

Kyriakis JM, Avruch J. 2012. Mammalian MAPK signal transduction pathways activated by stress and inflammation: a 10-year update. Physiol Rev 92(2):689–737.

Kyriakis JM, Avruch J. 2012. Mammalian MAPK signal transduction pathways activated by stress and inflammation: a 10-year update. Physiol Rev 92: 689–737.

Matsuzawa A, Saegusa K, Noguchi T, Sadamitsu C, Nishitoh H, Nagai S, Koyasu S, Matsumoto K, Takeda K, Ichijo H. 2005. ROS-dependent activation of the TRAF6-ASK1-p38 pathway is selectively required for TLR4-mediated innate immunity. Nat Immunol 6:587–592.

McKay MM, Morrison DK. 2007. Integrating signals from RTKs to ERK/MAPK. Oncogene 26:3113–3121.

Minden A, Lin A, Smeal T, Dérijard B, Cobb M, Davis R, Karin M. 1994. c-Jun N-terminal phosphorylation correlates with activation of the JNK subgroup but not the ERK subgroup of mitogen-activated protein kinases. Mol Cell Biol 14(10):6683–6688.

Molkentin JD, Dorn GW, II. 2001. Cytoplasmic signaling pathways that regulates cardiac hypertrophy. Annu Rev Physiol 63:391–426.

Morton S, Davis RJ, McLaren A, Cohen P. 2003. A reinvestigation of the multisite phosphorylation of the transcription factor c-Jun. EMBO J 22(15):3876–3886.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65(1/2):55–63.

Naor Z, Benard O, Seger R. 2000. Activation of MAPK cascades by G-protein-coupled receptors: the case of gonadotropin-releasing hormone receptor. Trends Endocrinol Metab 11:91–99.

Nishimura T, Vogt PK. 1988. The avian cellular homolog of the oncogene jun. Oncogene 3(6):659–663.

Nylandsted J, Gyrd-Hansen M, Danielewicz A, Fehrenbacher N, Lademann U, Høyer-Hansen M, Weber E, Multhoff G, Rohde M, Jäättelä M. 2004. Heat shock protein 70 promotes cell survival by inhibiting lysosomal membrane permeabilization. J Exp Med 200(4):425–435.

Pulverer BJ, Kyriakis JM, Avruch J, Nikolakaki E, Woodgett JR. 1991. Phosphorylation of c-jun mediated by MAP kinases. Nature 353(6345):670–674.

Qu Z, Balkir L, van Deutecom JC, Robbins PD, Pruchnic R, Huard J. 1998. Development of approaches to improve cell survival in myoblast transfer therapy. J Cell Biol 142(5):1257–1267.

Raingeaud J, Whitmarsh AJ, Barrett T, Dérijard B, Davis RJ. 1996. MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. Mol Cell Biol 16(3):1247–1255.

Raivich G. 2008. c-Jun expression, activation and function in neural cell death, inflammation and repair. J Neurochem 107(4):898–906.

Rane SG. 1999. Ion channels as physiological effectors for growth factor receptor and Ras/ERK signaling pathways. Adv Second Messenger Phosphoprotein Res 33:107–127.

Rangarajan A, Hong SJ, Gifford A, Weinberg RA. 2004. Species and cell type-specific requirements for cellular transformation. Cancer Cell 6:171–183.

Rubinfeld H, Seger R. 2005. The ERK cascade: a prototype of MAPK signaling. Mol Biotechnol 31:151–174.

Schaeffe HJ, Weber MJ. 1999. Mitogen-activated protein kinases: specific messages from ubiquitous messengers. Mol Cell Biol 19:2435–2444.

Schigapova N, Torok Z, Balagh G, Goloubinoff P, Vigh L, Horvath I. 2005. Membrane fluidarization triggers membrane remodeling which affects the thermotolerance in *Escherichia coli*. Biochem Biophys Res Commun 328:1216–1223.

Segreto HR, Oshima CT, Franco MF, Silva MR, Egami MI, Teixeira VP, Segreto RA. 2011. Phosphorylation cytoplasmic localization of MAPK p38 during apoptosis signaling in bone marrow granulocytes of mice irradiated in vivo the role of amifostine in reducing these effects. Acta S Histochem 113(3):300–307.

Seki T, Yoshino KI, Tanaka S, Dohi E, Onji T, Yamamoto K, Hide I, Paulson HL, Saito N, Sakai N. 2012. Establishment of a novel fluorescence-based method to evaluate chaperone-mediated autophagy in a single neuron. PLoS ONE 7(2): e31232.

Sharrocks AD, Yang SH, Galanis A. 2000. Docking domains and substrate-specificity determination for MAP kinases. Trends Biochem Sci 25 (9):448–453.

Sirmenis R, Bukelskiene V, Domkus V, Sirvydis V. 1999. Cellular cardiomyoplasty: isolation and cultivation of skeletal musclesatellite cells. Acta Med Lituanica 6:178–181.

Sittler A, Lurz R, Lueder G, Priller J, Lehrach H, Hayer-Hartl MK, Hartl FU, Wankler EE. 2001. Geldanamycin activates a heat shock response and inhibits hunting tin aggregation in a cell culture model of Huntington′s disease. Hum Mol Genet 10:1307–1315.

Sun XC, Li WB, Li QJ, Zhang M, Xian XH, Qi J, Jin RL, Li SQ. 2006. Limb ischemic preconditioning induces brain ischemic tolerance via p38 MAPK. Brain Res 1084(1):165–174.

Suzuki K, Sawa Y, Kagisaki K, Taketani S, Ichikawa H, Kaneda Y, Matsuda H. 2000. Reduction in myocardial apoptosis associated with over expression of heat shock protein 70. Basic Res Cardiol 95:397–403.

Takeda K, Matsuzawa A, Nishitoh H, Tobiume K, Kishida S, Ninomiya-Tsuji J, Matsumoto K, Ichijo H. 2004. Involvement of ASK1 in Ca^{2+}-induced p38 MAP kinase activation. EMBO Rep 5:161–166.

Volloch V, Gabai VL, Rits S, Force T, Sherman MY. 2000. HSP72 can protect cells from heat-induced apoptosis by accelerating the inactivation of stress kinase JNK. Cell Stress Chaperones 5(2):139–147.

Widmann C, Gibson S, Jarpe MB, Johnson GL. 1999. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev 79:143–180.

Yeh JH, Hsu SC, Han SH, Lai MZ. 1998. Mitogen-activated protein kinase kinase antagonized fas-associated death domain protein-mediated apoptosis by induced FLICE-inhibitory protein expression. J Exp Med 188:1795–1802.

Yenari MA, Fink SL, Sun GH, Chang LK, Patel MK, Kunis DM, Onley D, Ho DY, Sapolsky RM, Steinberg GK. 1998. Gene therapy with Hsp72 is neuroprotective in rat models of streoke and epilepsy. Ann Neurol 44:584–591.