Biochemical and Biophysical Research Communications 430 (2013) 236–240

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Atypical heat shock response and acquisition of thermotolerance in P388D1 cells

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ARTICLE INFO

Article history:
Received 15 October 2012
Available online 6 November 2012

Keywords:
Heat shock response
HSPs
P388D1
Thermotolerance

ABSTRACT

Reports of atypical heat shock response in some tumour cell lines emphasize the possibilities of alternate stress response mechanisms. We demonstrate here that P388D1, a mouse macrophage tumour cell line, failed to induce heat shock proteins (HSPs) in response to either heat stress (42 °C, 1 h) or to heavy metal stress induced by arsenic trioxide (5–20 μM). Heat shock transcriptional factor 1 (HSF1) that mediates transcriptional up regulation of HSPs during stress was found to be deficient in transactivation despite its binding to the promoter region of HSP genes. Interestingly, cells exhibited thermotolerance in the absence of induced HSPs. However, the tolerance was abrogated in cells treated with cycloheximide (250 ng/ml) suggested that thermotolerance was dependent on *de novo* protein synthesis.

1. Introduction

Cells respond to stressful conditions by inducing the synthesis of a family of proteins called stress proteins or heat shock proteins (HSPs). Heat shock proteins are highly conserved molecular chaperones. In higher eukaryotes, HSPs are broadly categorized according to their molecular mass, which includes small HSPs (sHSPs), HSP60, HSP70, HSP90 and HSP100 family [1]. Induction of HSPs is mediated through the activation of heat shock transcription factor 1 (HSF1) which binds to the conserved regulatory sequences called heat shock elements (HSEs) located in the promoter regions of inducible HSP genes. HSF1 exists as inactive monomers in normal cells. HSF1 is hyperphosphorylated, homotrimerizes and becomes transcriptionally competent upon stress [2,3].

The rapidly synthesized HSPs function to protect cells from harmful effects of not only heat, but also a diverse array of other stresses [4,5]. Cell lineage has a major influence on the cellular response to stress. Deficient transcriptional induction of HSP70 has been reported in erythroleukemia cell lines [6]. Heat-induced apoptosis due to the lack of HSP synthesis in rat histiocytoma BC8 has also been reported [7]. However, the mechanism by which different cells respond to hyperthermia is yet to be defined at large.

Thermotolerance is a phenomenon where cells continue to function normally at lethal temperature after a pre-exposure to mild hyperthermia. Thermotolerance is attributed to induced HSPs that maintain cellular homeostasis and the integrity of organelles which are otherwise compromised by elevated temperature. The development of thermotolerance is highly correlated with the increased expression of HSPs [8,9].

In the present study, we investigated the heat shock response of the mouse macrophage tumour cell lines, P388D1 and J774. P388D1 cells lacked typical heat shock response due to a defect in HSF1-mediated transactivation of HSP genes. In contrast, J774, another mouse macrophage cell line exhibited a typical heat shock response with induction of HSPs and thermotolerance. Despite the lack of induced HSPs, heat shock preconditioning rendered P388D1 cells resistant to lethal heat shock and acquisition of thermotolerance was dependent on *de novo* protein synthesis.

2. Materials and methods

2.1. Cell culture and heat shock conditions

Chemicals were purchased from Sigma–Aldrich unless otherwise mentioned. P388D1, PCC4 and J774 macrophage cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 U/ml) streptomycin (50 μg/ml) and 10% (v/v) fetal bovine serum. Exponentially growing cells (1×10^6) were heat shocked by submerging sealed polystyrene culture flasks in water baths set at 42 or 43.5 °C for 1 h. Following the heat shock, cells were allowed to recover at 37 °C for indicated times.

2.2. SDS–PAGE and western blotting

Cells were washed in ice-cold phosphate-buffered saline pH 7.4 and lysed in ice-cold lysis buffer (150 mM NaCl, 1 mM EDTA, 1%

Abbreviations: SDS–PAGE, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis; RT-PCR, reverse transcription polymerase chain reaction.

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0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.10.124

Nonidet P40, 50 mM Tris–HCl (pH 7.5), 10% glycerol) containing 1 mM dithiothreitol and protease inhibitor cocktail (Roche, Mannheim, Germany) and incubated at 4 °C for 15 min. Protein content was determined with Bradford reagent (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as a standard. Samples containing 20 μg of total protein were prepared with Laemlli's buffer and boiled for 3 min. Proteins were separated by 10% SDS–PAGE and transferred to a nitrocellulose membrane (Hybond C, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and incubated with antibodies against either HSP25, HSP70, HSP90 or Actin (Stressgen Biotechnologies, Victoria, BC). Bands were visualized by using BM Chemiluminescence Western Blotting Kit (Roche, Mannheim, Germany).

2.3. RNA isolation, cDNA synthesis and PCR amplification

Total RNA was isolated from cells using Trizol (Invitrogen, Carlsbad, CA). Two micrograms of RNA was reverse-transcribed and cDNA was amplified with gene specific primers as described previously [10]. Primers for amplification were: HSP70 (Forward 5′-GCC AAC AAG ATC ACC ATC ACC AAC-3′, Reverse 5′-CGT GCC CAA ACA GCT ATC AAG TG-3′), HSP27 (Forward 5′-ATG AGT GGT CGC AGT GGT TCAG-3′, Reverse 5′-GCA CCG AGA GAT GTA GCC ATG TTC-3′), and β-actin (Forward 5′-ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG-3′, Reverse 5′-CGT CAT CCT GCT TGC TGA TCE ACA TCT GC-3′).

2.4. Electrophoretic mobility shift assay (EMSA) and electrophoretic mobility super shift assay (EMSSA)

EMSA was performed as described earlier [11]. For EMSSA, nuclear extracts were incubated with anti-HSF1 or anti-HSF2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 20 min at room temperature prior to the addition of binding reaction mixture containing labeled HSEs. For cold competition, 25-fold excess unlabelled double-stranded HSEs were added to the binding mixture containing the labeled HSEs.

2.5. Luciferase reporter assay for HSF1 activation

HSP70 promoter region was released from HSP70 B-CAT reporter vector (Stressgen Biotechnologies, Victoria, BC) by digesting with restriction enzymes, Bgl II and Hind III (New England Biolabs, Ipswich, MA). The resulting 1.4 kb fragment containing HSP70 promoter region was cloned upstream to luciferase gene in PGL3-B basic vector (Promega, Madison, WI). Cells were plated at 8×10^4 cells per 35 mm dish and cultured for 24 h before transfection. Cells were transfected with 100 ng of β-galactosidase expression vector pSV110 and 200 ng of HSP70 luciferase reporter gene by lipofection using lipofectamine (Invitrogen, Carlsbad, CA). After 40 h, P388D1/PCC4 cells were subjected to heat shock. Luciferase and -galactosidase assays were performed according to the manufacturer's instructions (Promega, Madison, WI).

2.6. Propidium iodide (PI) exclusion method

Cells were trypsinized and re-suspended in phosphate-buffered saline, pH 7.4 supplemented with 50 μg/ml PI. PI positive cells were determined by flow cytometry (FACS Calibur, BD Biosciences, San Jose, CA) in FL2 channel.

3. Results and discussion

Exposure of mouse cell lines to 42 °C for 1 h was optimal for HSP induction and the maximum expression of HSPs was found after 6 h of recovery at 37 °C. Mouse embryonic carcinoma cell line, PCC4 that mounts a typical heat shock response under these conditions was employed as a positive control. HSP70 and HSP25 are the major heat stress-inducible HSPs. As shown in (Fig. 1 A), heat stressed PCC4 cells induced both HSP25 and HSP70 at protein level. Semi-quantitative PCR analysis of mRNA from heat stressed PCC4 cells also confirmed the transcriptional induction of HSP genes (Fig. 1 B). On the other hand, mouse macrophage tumour cell line, P388D1 failed to mount a typical heat shock response with induction of HSPs (Fig. 1A and B). J774, another mouse macrophage tumour cell line exhibited typical heat shock response with induction of both HSP25 and HSP70 under similar conditions as demonstrated by Western blot analysis (Fig. 1A). Interestingly, expression of HSP90 remained unchanged in heat stressed cells (Fig. 1A). The data suggest that P388D1 could not mount a typical heat shock response with induction of HSPs.

Heat shock-induced HSP synthesis is mediated by HSF1. HSF1 is regulated at multiple levels including trimerization, nuclear translocation, DNA binding, and transactivation [12]. Nuclear translocation and binding of HSF1 to HSEs were analyzed by electromobility shift assay (EMSA). Interestingly, nuclear extracts from heat stressed P388D1 cells retarded HSE oligonucleotides (Fig. 2A) and the retardation was competed away by the cold HSEs (Fig. 2A). Despite the absence of induced HSPs, HSF1–HSE complex was formed in heat stressed P388D1. The specific binding of HSF1 to HSEs was confirmed by super shift assay. As demonstrated in Fig. 2A, the complex was further super shifted by anti-HSF1 antibody. Neither anti-HSF2 antibody nor the rabbit serum was able to super shift the complex (Fig. 2A). Hence, HSF1 is partially activated in heat stressed P388D1 cells. This suggested that HSF1 is distinctly regulated at different levels as previously reported [12].

Next, we investigated whether the partially activated HSF1 can transactivate the expression of HSP genes. The functionality of HSF1 in heat stressed P388D1 cells was analyzed by luciferase reporter assay. P388D1 cells were transiently transfected with a recombinant plasmid, PGL3B, having luciferase gene driven by HSP70 promoter. HSP70 promoter has high affinity for HSF1. Despite the binding of HSF1 to HSEs, no significant induction of luciferase activity was detected upon heat stress in P388D1 cells (Fig. 2B). However, PCC4 cells that mount a typical heat shock response, exhibited a 50-fold induction in the luciferase activity (Fig. 2B). -Galactosidase gene under CMV promoter was employed as an internal control for transfection in all experiments (Fig. 2B). These results confirmed that HSE-bound HSF1 is deficient in transactivation during heat stress in P388D1 cells. Phosphorylation of HSF1 by kinases that promote either activation or inhibition has been reported [13–15]. Recently, it has been reported that mTORC1 activity is required for HSF1 activation during proteotoxic stress including heat shock [16]. Possibility of mutation in the functional domains of HSF1 was ruled out by sequencing HSF1 cDNA from P388D1 cells (data not shown). Thus, a defect in post-translational modification of HSF1 would probably explain the lack of heat shock response in P388D1 cells. Analyzing the aforementioned kinases upon heat shock would shed light into the atypical stress response in P388D1 cells.

It has been well documented that in addition to heat, a variety of stresses including heavy metals induce the expression of HSPs [1]. Hence, we determined whether exposure to heavy metal could trigger the synthesis of HSPs in P388D1 cells. Arsenic(III) oxide (ATO) has been reported to induce HSPs [17]. Interestingly, ATO could induce neither HSP70 nor HSP25 in P388D1 cells (Fig. 3). On the other hand, J774 cells exhibited an increased expression of both HSP70 and HSP25 at 5, 10 and 20 μM of ATO (Fig. 3). Taken together, the data confirmed that P388D1 could not mount the synthesis of HSPs in response to multiple stresses. Thus, P388D1 cells lack a typical HSF1 mediated stress response. Generally, as a

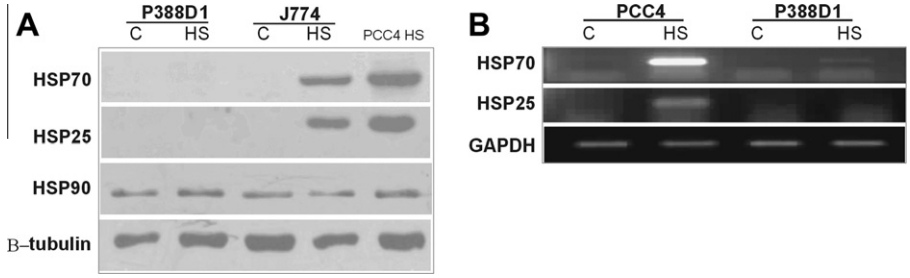

Fig. 1. Lack of HSP induction in heat-stressed P388D1 cells. (A) Western Blot analysis of HSP protein expression. Cells were exposed at 42 °C for 1 h followed by 6 h of recovery at 37 °C and were immunoblotted with HSP antibodies and (B) RT-PCR analysis of HSP mRNA. Cells were exposed to 42 °C for 1 h followed by recovery at 37 °C for 2 h. C-Control cells, HS-Heat stressed cells. J774 and PCC4 cells were used as positive controls for typical heat shock response.

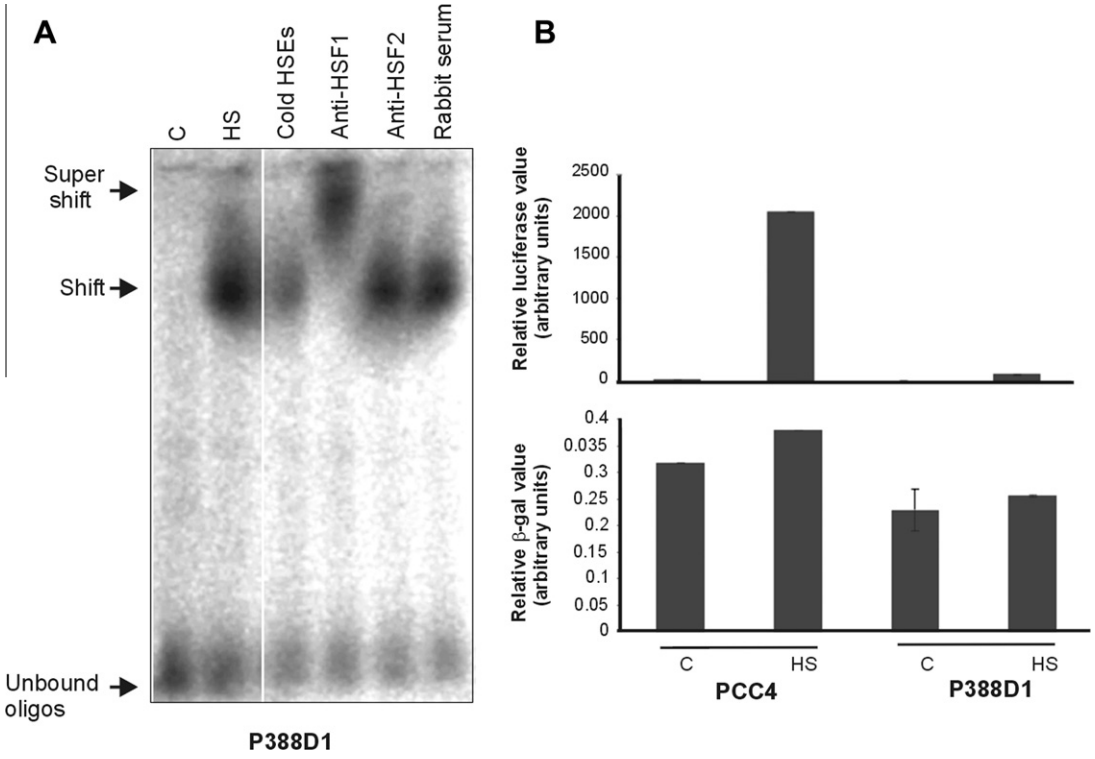

Fig. 2. Lack of HSF1 transactivation upon heat shock. (A) Electromobility shift analysis (EMSA). Nuclear extracts from heat stressed or control cells were incubated with labeled HSEs and analyzed on 4% native PAGE. For cold competition, 25-fold excess of unlabelled HSEs was added to the reaction mixture as described in materials and methods. Supershift analysis was done with HSF1 or HSF2 antibodies and (B) luciferase activity was assayed after heat stress (42 °C for 1 h) followed by 6 h of recovery at 37 °C. β galactosidase expression was determined as transfection control in all experiments. C-Control cells, HS-Heat stressed cells. PCC4 cells were used as a positive control for typical heat shock response. Bar represents mean value of two independent experiments ± standard deviation.

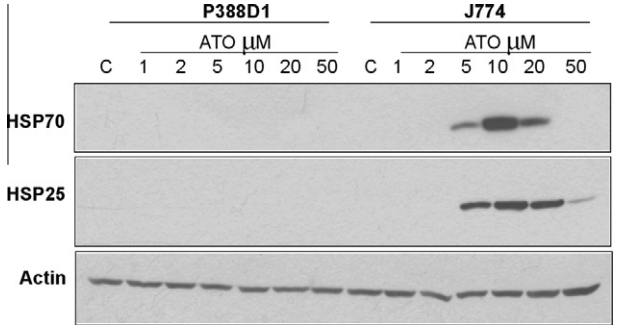

Fig. 3. P388D1 cells could not mount a typical stress response to arsenic (III) oxide. Cells were treated with increasing concentrations of ATO for 6 h and total cell lysate was made and Western Blot analysis was performed for HSP70 and HSP25. Actin was determined as loading control. Mouse macrophage cell line J774 which exhibited a typical heat shock response was used as positive control. C-Control.

part of survival mechanism, cells upregulate HSPs in response to stress. However, as previously mentioned, there are reports of cells exhibiting aberrant stress response [6,7]. Interestingly, we demonstrated for the first time that P388D1 could not activate the synthesis of HSPs under stress. HSPs have been heavily implicated in carcinogenesis [18]. Limited availability of nutrients and hypoxia due to the lack of proper vasculature activate the expression of HSPs in solid tumours [19]. These induced HSPs enhance tumour survival and progression to a more malignant phenotype which is resistant to various therapies [19]. In the context of our study, further investigations have to be done to determine whether the lack of typical stress response in P388D1 cells render them more sensitive to anticancer agents.

Pre-exposure to sub-lethal temperature enable cells to cope with the lethal stress by acquiring thermotolerance. We investigated whether pre-conditioning at 42 °C will render P388D1 cells tolerant to lethal heat shock at 43.5 °C. Strikingly, despite the ab-

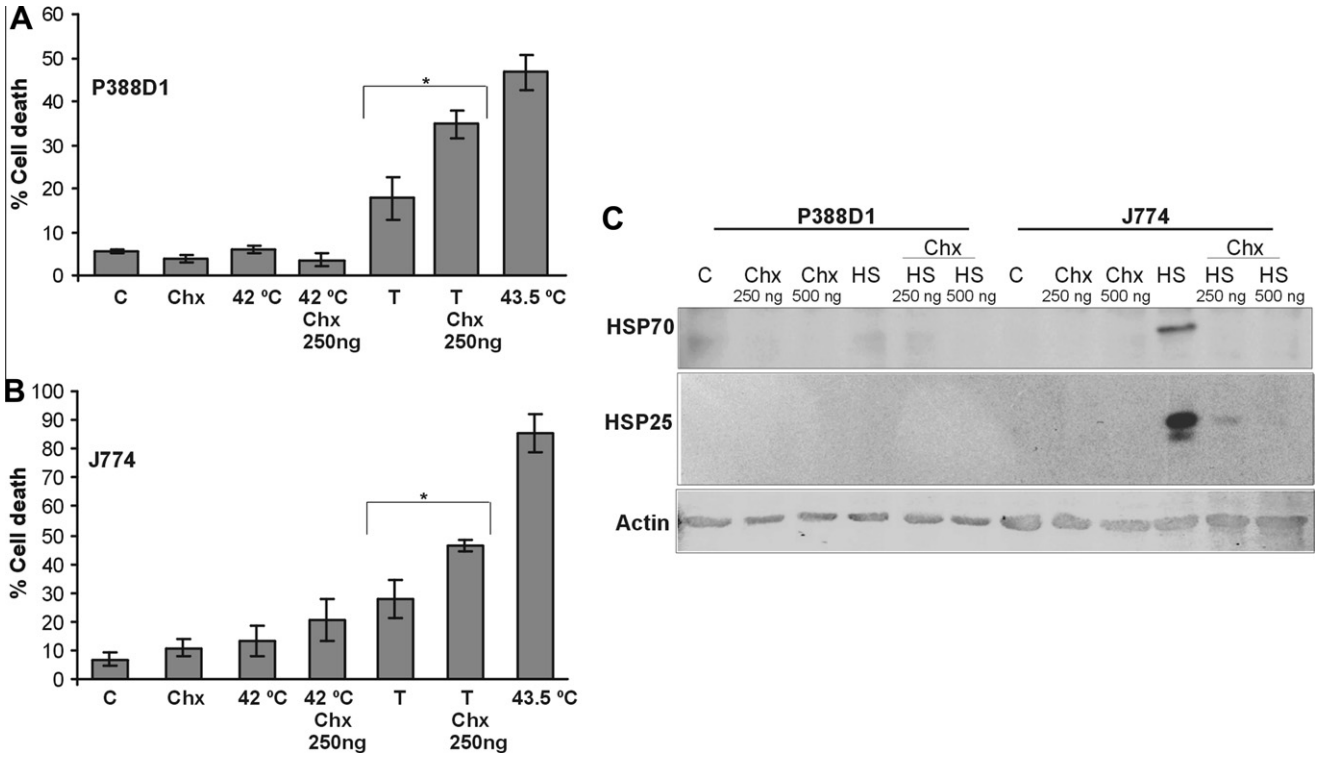

Fig. 4. Heat shock pre-conditioning and thermotolerance in P388D1 cells. (A & B) Cells were treated with 250 ng/ml cycloheximide for 1 h before subjected to heat shock. Cells treated with cycloheximide along with untreated cells were exposed at 42 °C for 1 h followed by 6 h of recovery at 37 °C. After 6 h of recovery, cells were exposed to lethal heat shock at 43.5 °C for 1 h and cell viability was determined by propidium iodide exclusion assay as described in materials and methods after 18 h of recovery at 37 °C. C-Control, Chx-Cycloheximide, T-Thermotolerant cells exposed at 43.5 °C. Bar represents mean value of three independent experiments ± standard deviation. * represents a significant difference ($p < 0.05$) in percentage cell death between T and T Chx in both cell lines and (C). Cells were treated with 250 ng/ml or 500 ng/ml of cycloheximide for 1 h and subjected to 42 °C for 1 h followed by 6 h of recovery at 37 °C. Total cell lysate was made and Western blot analysis was performed for HSP70 and HSP25. Actin was determined as loading control. Mouse macrophage cell line J774 which exhibited a typical heat shock response was used as positive control. HS-Heat-stressed.

sence of induced HSPs, P388D1 cells that were pre-heated at 42 °C exhibited a significant increase in cell viability when subjected to lethal heat shock (Fig. 4A). As predicted, J774 cells which mount a typical stress response was tolerant to lethal heat stress after heat shock pre-conditioning (Fig. 4B).

It has been reported that induced HSPs at sub-lethal temperature play a major role in imparting thermotolerance [8,9]. However, apparently, our study suggests that cells could perhaps acquire tolerance to otherwise lethal stress in the absence of induced HSPs. In order to determine whether *de novo* protein synthesis is required for thermotolerance, cells were treated with cycloheximide to inhibit protein synthesis. Treating cells with 250 ng/ml of cycloheximide completely abrogated the induction of both HSP70 and HSP25 in J774 cells (Fig. 4C). Strikingly, there was a significant reduction in viability of P388D1 cells that was pre-heated but treated with cycloheximide (Fig. 4A). This suggested that synthesis of proteins, other than HSPs is required for thermotolerance in P388D1 cells. As expected, tolerance was significantly reduced in J774 cells treated with cycloheximide (Fig. 4B). Studies from our lab have also shown (unpublished data) that heat shock activates a battery of genes in addition to HSPs. Future studies will be directed to identify the expression of these genes in P388D1 cells during heat stress. Findings from this study also revealed that the induction of HSPs as an essential part of heat shock response is not a universal phenomenon and stress response can be cell type specific.

References

[1] S. Lindquist, E.A. Craig, The heat-shock proteins, Annu. Rev. Genet. 22 (1988) 631–677.
[2] C. Wu, Heat shock transcription factors: structure and regulation, Annu. Rev. Cell Dev. Biol. 11 (1995) 441–469.
[3] H. Sakurai, Y. Enoki, Novel aspects of heat shock factors: DNA recognition, chromatin modulation and gene expression, FEBS J. 277 (2010) 4140–4149.
[4] A. Samali, S. Orrenius, Heat shock proteins: regulators of stress response and apoptosis, Cell Stress Chaperones. 3 (1998) 228–236.
[5] H.M. Beere, Death versus survival: functional interaction between the apoptotic and stress-inducible heat shock protein pathways, J. Clin. Invest. 115 (2005) 2633–2639.
[6] L. Aujame, The major heat-shock protein hsp 68 is not induced by stress in mouse erythroleukemia cell lines, Biochem. Cell Biol. 66 (1988) 691–701.
[7] A.S. Sreedhar, B.V. Pardhasaradhi, Z. Begum, A. Khar, U.K. Srinivas, Lack of heat shock response triggers programmed cell death in a rat histiocytic cell line, FEBS Lett. 456 (1999) 339–342.
[8] G.C. Li, Z. Werb, Correlation between synthesis of heat shock proteins and development of thermotolerance in chinese hamster fibroblasts, Proc. Natl. Acad. Sci. USA 79 (1982) 3218–3222.
[9] P. Chrétien, J. Landry, Enhanced constitutive expression of the 27-kDa heat shock proteins in heat-resistant variants from chinese hamster cells, J. Cell Physiol. 137 (1988) 157–166.
[10] S. Gupta, A. Deepti, S. Deegan, F. Lisbona, C. Hetz, A. Samali, HSP72 protects cells from ER stress-induced Apoptosis via enhancement of IRE1alpha-XBP1 signaling through a physical interaction., PLoS Biol. 8 (2010) e1000410.
[11] S.I. Han, S.Y. Oh, S.H. Woo, K.H. Kim, J.H. Kim, H.D. Kim, et al., Implication of a small GTPase Rac1 in the activation of c-Jun N-terminal kinase and heat shock factor in response to heat shock, J. Biol. Chem. 276 (2001) 1889–1895.
[12] R. Voellmy, On mechanisms that control heat shock transcription factor activity in metazoan cells, Cell Stress Chaperones 9 (2004) 122–133.
[13] B. Chu, F. Soncin, B.D. Price, M.A. Stevenson, S.K. Calderwood, Sequential phosphorylation by mitogen-activated protein kinase and glycogen synthase kinase 3 represses transcriptional activation by heat shock factor-1, J. Biol. Chem. 271 (1996) 30847–30857.
[14] R. Dai, W. Frejtag, B. He, Y. Zhang, N.F. Mivechi, C-Jun NH2-terminal kinase targeting and phosphorylation of heat shock factor-1 suppress its transcriptional activity, J. Biol. Chem. 275 (2000) 18210–18218.
[15] C.I. Holmberg, V. Hietakangas, A. Mikhailov, J.O. Rantanen, M. Kallio, A. Meinander, et al., Phosphorylation of serine 230 promotes inducible transcriptional activity of heat shock factor 1, EMBO J. 20 (2001) 3800–3810.

[16] S.-D. Chou, T. Prince, J. Gong, S.K. Calderwood, mTOR is essential for the proteotoxic stress response HSF1 activation and heat shock protein synthesis., PloS One. 7 (2012) e39679.
[17] Y.-H. Kim, E.-J. Park, S.T. Han, J.-W. Park, T.K. Kwon, Arsenic trioxide induces Hsp70 expression via reactive oxygen species and JNK pathway in MDA231 cells, Life Sci. 77 (2005) 2783–2793.
[18] D.R. Ciocca, A.P. Arrigo, S.K. Calderwood, Heat shock proteins and heat shock factor 1 in carcinogenesis and tumor development: an update. Arch. Toxicol., 2012.
[19] S.K. Calderwood, M.A. Khaleque, D.B. Sawyer, D.R. Ciocca, Heat shock proteins in cancer: chaperones of tumorigenesis, Trends Biochem. Sci. 31 (2006) 164–172.