Atypical heat shock response and acquisition of thermotolerance in P388D1 cells

Deepu Oommen [a,b,*], Zoltan Giricz [a,c], Usha K. Srinivas [b], Afshin Samali [a]

[a] Apoptosis Research Centre, School of Natural Sciences, National University of Ireland, Galway, Ireland
[b] Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India
[c] Department of Pharmacology and Pharmacotherapy, Semmelweis University, Budapest, Hungary

ARTICLE INFO

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.

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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%

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