Protein synthesis is required for stabilization of hsp70 mRNA upon exposure to both hydrostatic pressurization and elevated temperature

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Abstract We have recently described that in chondrocytic cells high hydrostatic pressure (HP) causes a heat shock response via mRNA stabilization without a transcriptional activation of the hsp70 gene. In this study, we investigated whether this exceptional regulatory mechanism occurs more generally in different types of cells. Indeed, hsp70 mRNA and protein accumulated in HeLa, HaCat and MG-63 cells under 30 MPa HP, without DNA-binding of heat shock transcription factor 1 (HSF1) to the heat shock element of the hsp70 gene or formation of nuclear HSF1 granules, revealing a lack of transcriptional activation. Moreover, we observed that protein synthesis is needed for mRNA stabilization. Thus, high HP offers a model to study the mechanisms of hsp70 mRNA stabilization without HSF1-mediated induction of the heat shock gene response.

Key words: Heat shock protein; mRNA stabilization; Hydrostatic pressure

1. Introduction

Diverse chemical or physical stresses induce the expression of heat shock proteins (Hsps) [1], the regulation of which involves both transcriptional and posttranscriptional control levels [2]. The transcriptional regulation is mediated by heat shock transcription factor 1 (HSF1). When completely activated, HSF1 undergoes oligomerization from an inactive monomer to a trimer, translocates to the nucleus to bind to the promoter of heat shock genes and then becomes hyperphosphorylated [3,4]. Recent studies show that as a consequence of the classical stress stimuli, HSF1 forms nuclear granules with kinetics resembling that of the transcriptional induction of heat shock genes [5–8]. However, the intensity and kinetics are variable, depending on the presence of modulators or the cell type [9–14]. Posttranscriptional regulation, which involves mRNA stabilization and translation, has remained a less studied regulatory control mechanism of the heat shock response [14–16]. In this study, we show that Hsp70 accumulation in pressurized cells through mRNA stabilization is not specific for a chondrocytic cell line [14], but appears to be a more general, cell line-independent mechanism that requires protein synthesis. In addition, the formation of nuclear HSF1 granules was not involved in the posttranscriptional heat shock gene regulation in the presence of high hydrostatic pressure (HP).

2. Materials and methods

2.1. Cell culture and stress treatments

HeLa cervical carcinoma cells, human HaCat keratinocytes and MG-63 osteosarcoma cells were grown to confluency in a standard incubator in DMEM (Gibco) including 10% fetal calf serum, penicillin, streptomycin and glutamine. Before exposure to HP or elevated temperature, the medium was changed and 15 mM HEPES (pH 7.3) was added. For heat shock treatment, the plates were sealed with Parafilm and immersed in a 43°C water bath. The stability of mRNA was studied by adding actinomycin D (ActD; Sigma) to cultures at a final concentration of 10 μg/ml. Protein synthesis was blocked by the presence of 10 μg/ml cycloheximide (CHX; Sigma) in the medium. To expose the cells to HP, the culture dishes were completely filled with the medium and sealed with a covering plastic membrane and pressurized as described previously in detail [17].

2.2. Northern blotting

Total RNA (20–25 μg) isolated with Trizol reagent (Gibco) was separated on a 1% agarose/formaldehyde gel, transferred to a nylon membrane (Hybond-N, Amersham) and hybridized with [α-^{32}P]dCTP-labeled plasmids specific for human hsp90α [18], hsp70 [19], hsp27 (StressGen, SPD-910) and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [20]. A PhosphorImager™ (Molecular Dynamics) was used for the quantification of the levels of specific mRNAs.

2.3. Western blotting and confocal microscopy

Whole cell extracts (20 μg protein) were electrophoresed on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Millipore). For immunolocalization, monoclonal antibodies recognizing the inducible form of Hsp70 (StressGen, SPA-810) and the constitutive form of Hsc70 (heat shock protein cognate 70; StressGen, SPA-815) were used. For confocal immunofluorescence analysis of HSF1, adherent cells on cover slips were examined with the rabbit anti-human HSF1 antisera as shown recently by Holmberg et al. [8].

2.4. Gel mobility shift assay

Whole cell extracts for electrophoretic mobility shift assay were prepared as previously described [14,21]. [γ-^{32}P]dATP-labeled oligonucleotide corresponding to the proximal heat shock element of the human hsp70 promoter was used as a probe. The oligonucleotide was [γ-^{32}P]dATP-labeled with T4 polynucleotide kinase (Promega).

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Abbreviations: ActD, actinomycin D; CHX, cycloheximide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Hsc, heat shock protein cognate; HSF1, heat shock transcription factor 1; Hsp, heat shock protein; HP, hydrostatic pressure

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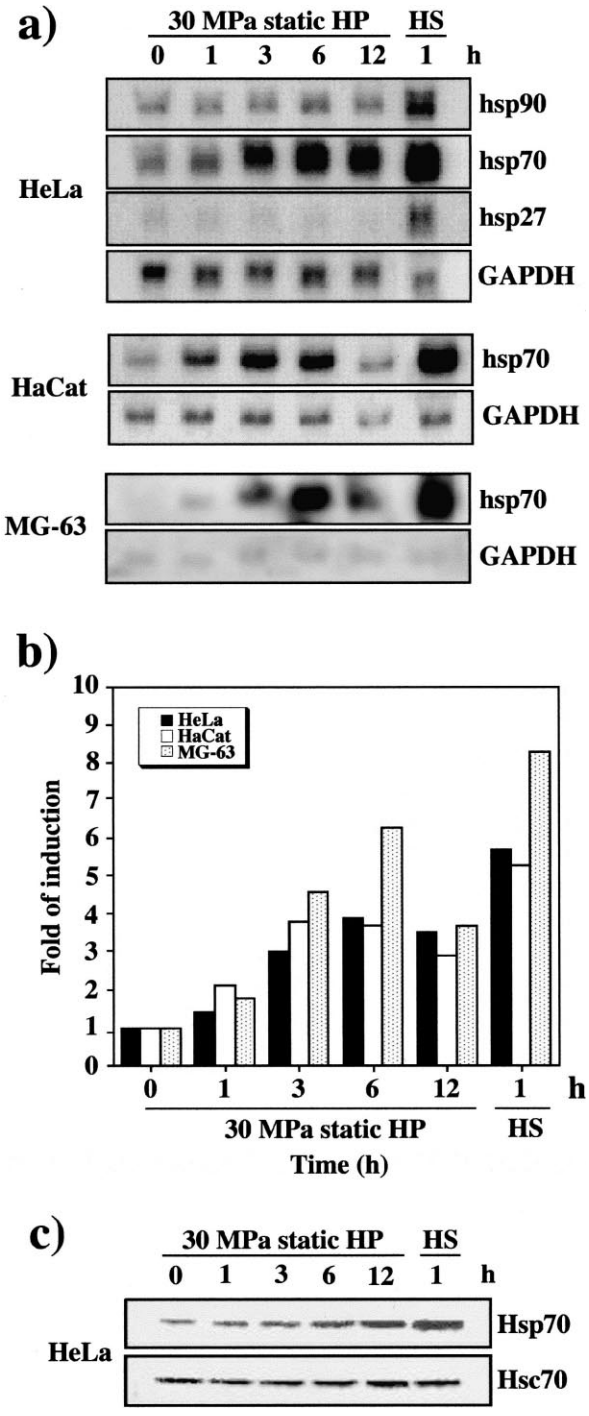

Fig. 1. The levels of hsp mRNAs were examined by Northern blot hybridization of untreated (0 h) HeLa, HaCat and MG-63 cells, cells exposed to static HP (30 MPa, 1–12 h), or to heat shock (HS, 43°C, 1 h). Northern blots were hybridized with [α-32P]dCTP-labeled cDNA probes for hsp90α, hsp70, hsp27 and GAPDH (a). Quantification of the hsp70 mRNA level in untreated cells (0 h) and those exposed to static 30 MPa HP (1–12 h) or to heat shock (HS, 43°C, 1 h) was performed with a PhosphorImager™, normalizing the values against respective GAPDH values (b). Whole cell extracts of untreated (0 h) HeLa cells, cells exposed to HP (30 MPa, 1–12 h) or to HS (43°C, 6 h) were analyzed by Western blotting using antibodies against Hsp70 and Hsc70 (c).
←

3. Results and discussion

In this study, we investigated whether different established cell lines exposed to high HP would induce heat shock response through hsp70 mRNA stabilization as was shown to take place in human immortalized T/C28a4 chondrocytic cells [14]. When HeLa cells were exposed to 30 MPa continuous HP for up to 12 h, the steady-state levels of hsp70 mRNA increased significantly (Fig. 1a,b), reaching the maximal four-fold induction level at 3–6 h (Fig. 1b). Heat shock (1 h at 43°C) led to approximately 5.5-fold induction (Fig. 1b). In contrast to increased hsp70 gene expression, hsp90α and hsp27 mRNA levels were not markedly affected by HP (Fig. 1a), while a clear increase was detected after heat shock treatment (Fig. 1a). In the HaCat and MG-63 cells, HP and elevated temperature exposures increased amounts of hsp70 mRNA corresponding to the intensity and kinetics of hsp70 mRNA response in the HeLa cells (Fig. 1a,b)

Continuous 30 MPa HP for up to 12 h or heat stress for 6 h at 43°C elevated Hsp70 protein levels both in the pressurized and in the heat-shocked HeLa (Fig. 1c), HaCat and MG-63 cells (data not shown), following consistently the amount of hsp70 mRNA (Fig. 1a,b). As expected, no alterations were noticed in Hsc70 levels (Fig. 1c). Continuous 10 MPa HP did not increase the amount of Hsp70 or Hsc70 proteins in any of the analyzed cell types, neither did the two intermittent HP loading exposures (0.5 Hz, 1 s on/1 s off) at the magnitudes of 10 or 30 MPa (data not shown).

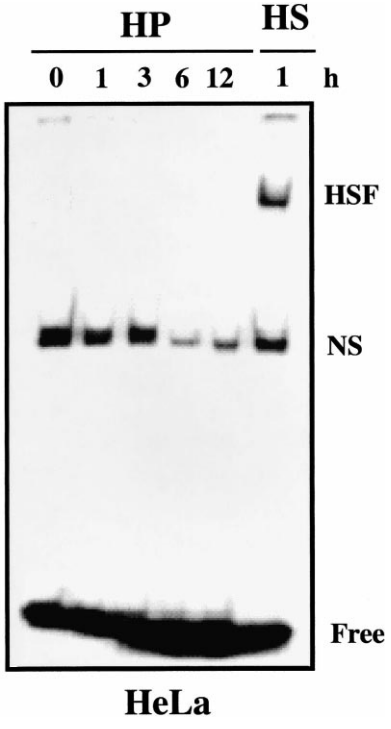

Fig. 2. The HP response occurs without activation of HSF. The HSF DNA-binding activity was analyzed by gel mobility shift assay in whole cell extracts from untreated HeLa cells (0 h), cells exposed to HP (30 MPa, 1–12 h) or to HS (43°C, 1 h). The binding of HSF to its radioactively-labeled target DNA probe (the band marked as HSF) is apparent only in heat-shocked cells. Non-specific binding activity to the DNA probe is marked as NS, and the migration position of the unbound (Free) probe is shown also.

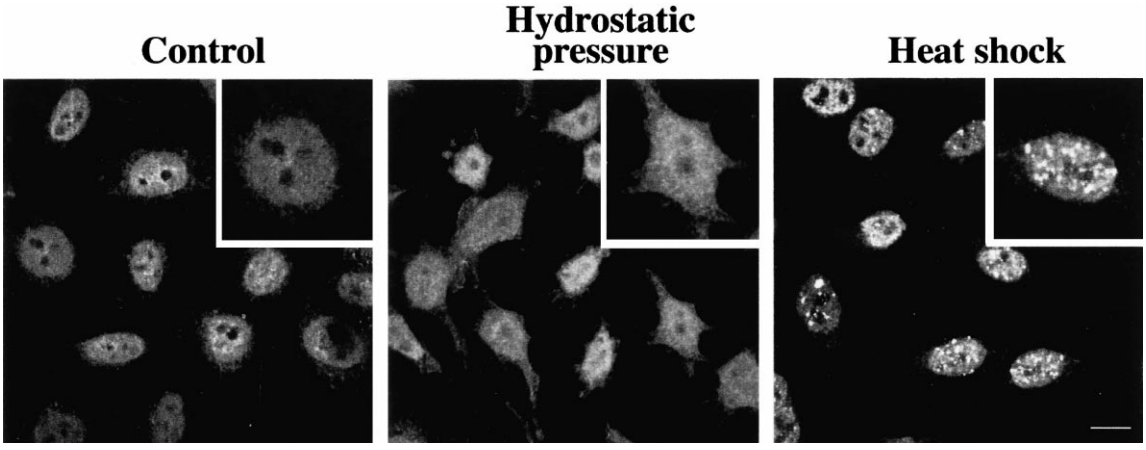

Fig. 3. Confocal microscopy analysis of HSF1. Untreated (control), HP-treated (30 MPa, 1 h), or HS-treated (43°C, 1 h) HeLa cells were analyzed by FITC-immunofluorescence with the rabbit anti-human HSF1 antisera. Granular appearance of HSF1, indicative of transcriptional activation, can be observed only in heat-shocked cells. The insets show at higher magnification representative cells after each treatment. Bar = 10 μm.

According to the electrophoretic mobility shift assay, no HSF1-binding to labeled double-stranded DNA probes containing the heat shock element was induced in HeLa cells exposed to high HP, whereas an induced DNA-binding was clearly detected in the heat-shocked cells (Fig. 2). To test the possibility that the conformation of the HSF1 structure would undergo a change under high HP, thereby inhibiting its DNA-binding capacity, we analyzed HSF1-binding to the heat shock element in untreated HeLa cells, in cells exposed to 43°C for 1 h and in cells simultaneously exposed both to 30 MPa continuous HP and heat shock at 43°C for 1 h. Simultaneous exposure to high HP and heat shock did not prevent the DNA-binding activity of HSF1 (data not shown),

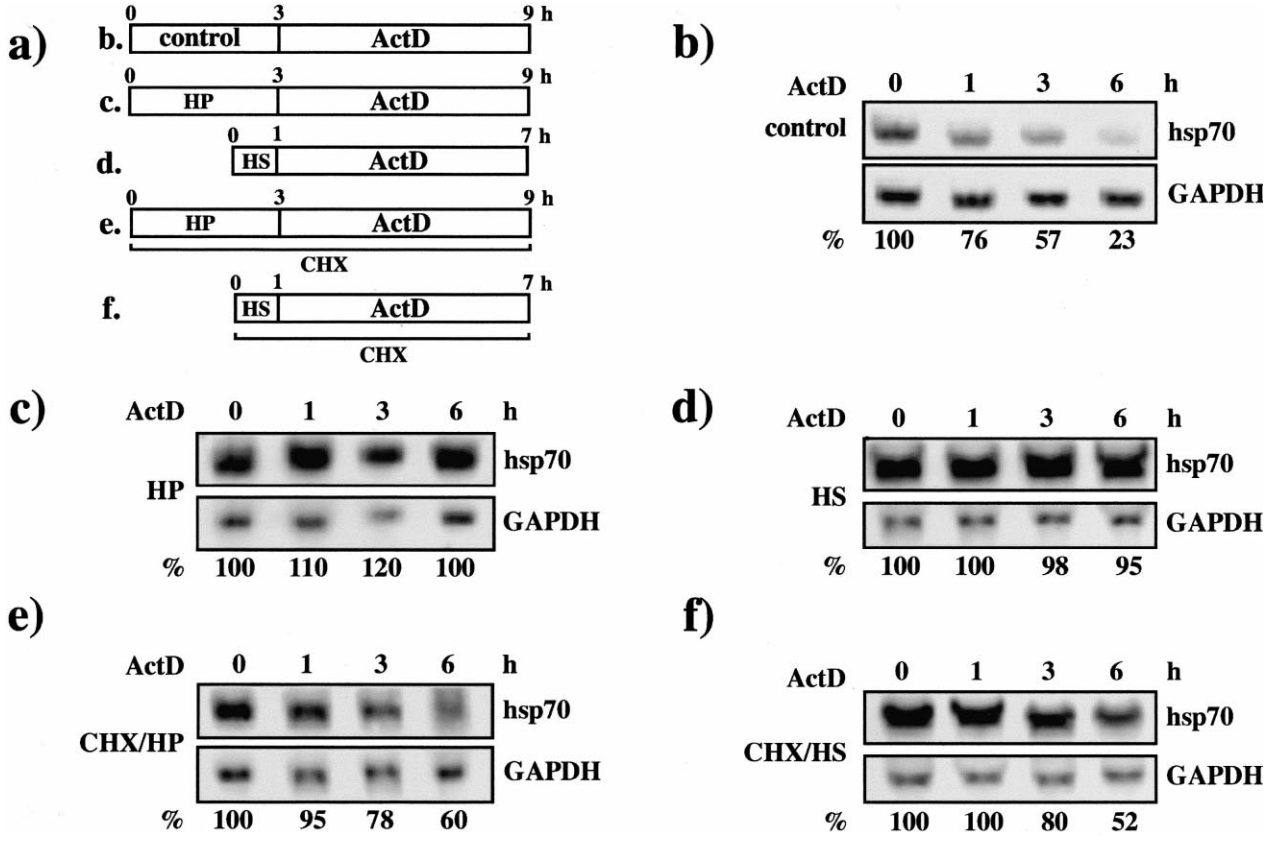

Fig. 4. Stabilization of hsp70 mRNA during HP and HS treatments, for details see the diagram of the treatments (a). Hsp70 mRNA stability was examined by Northern blot hybridization in untreated (control) (b), HP-treated (30 MPa, 3 h) (c) and HS-treated (43°C, 1 h) HeLa cells (d), which were further treated with ActD (10 μg/ml, 0–6 h). The effect of inhibited protein synthesis on hsp70 mRNA stability was studied in cells exposed to HP (e) or HS (f) in the presence of CHX (10 μg/ml) and followed by ActD treatment. Quantification of the levels of hsp70 mRNA was performed with a PhosphorImager™ and the results are presented below the blots as percentages, relative to the control (100%). The hsp70 mRNA values were normalized against the respective GAPDH values.

excluding the possibility that high HP would affect the ability of HSF1 to trimerize and to bind to DNA.

Recently, the formation of nuclear HSF1 granules has been indicated as a marker for HSF1 activation when cells are exposed to various forms of stress [5–8]. The granules appear transiently in various primary and transformed human cells in concert with transcriptional induction of the heat shock genes [5,6]. Therefore, immunolocalization of HSF1 was considered another way to further confirm that HSF1-mediated signaling was not involved in HP loading. HeLa cells were exposed to either 30 MPa static HP or to 43°C for 1 h and the HSF1 was visualized by immunofluorescence using an antibody specific for human HSF1 (Fig. 3). In the absence of stress, HeLa cells showed HSF1 staining both in the cytoplasm and the nucleus. The staining was similar to the pattern detected in cells pressurized with 30 MPa continuous HP (Fig. 3). However, in heat-shocked cells, HSF1 was found primarily in the nucleus as brightly stained granules (Fig. 3). In accordance with the analysis of HSF1 DNA-binding activity (Fig. 2), simultaneous treatment with high HP and HS induced the formation of nuclear HSF1 granules (data not shown). Our biochemical assays and analysis of HSF1 localization suggest that HSF1 DNA-binding activity is not required for the elevated expression of heat shock genes under 30 MPa continuous HP. Furthermore, HP does not interfere with heat-induced activation of HSF1.

Finally, we investigated whether stabilization of hsp70 mRNA would occur in the HeLa cells under high HP similarly to the pressurized human SV 40 immortalized chondrocytic cells [14]. In the beginning of the experiment (for experimental design, see Fig. 4a), HeLa cells were exposed to static 30 MPa HP for 3 h, or to elevated temperature (43°C) for 1 h, to allow the cells to establish the mRNA stabilizing property. Then ActD was added for up to 6 h to block the synthesis of novel RNA molecules. Quantitative analysis of hsp70 mRNA levels, relative to GAPDH, indicated that in the unstressed HeLa cells, hsp70 mRNA was reduced to about 20% of the control level during the follow-up period of 6 h (Fig. 4b). However, in the pressurized (Fig. 4c) and heat-shocked (Fig. 4d) cells the amount of hsp70 mRNA changed only slightly during the follow-up. This shows that both these treatments cause stabilization of hsp70 mRNA.

To examine whether protein synthesis affects the decay of hsp70 mRNA, CHX was present in a few cell cultures for the whole period of the experiment to block cellular protein synthesis. In the presence of CHX, hsp70 mRNA levels decreased by about 40% in the pressurized (Fig. 4e) and by 50% in the heat-shocked cells (Fig. 4f). Thus, it appears that the stabilization of hsp70 mRNA is a common mechanism to control heat shock gene expression upon exposure to high continuous HP, and it requires protein synthesis. Since blocking of the protein synthesis with CHX did not completely bring the hsp70 mRNA to the control level in the pressurized or the heat-shocked cells, the data suggest that there is a pre-existing pool of proteins capable of stabilizing hsp70 mRNA in stressful conditions.

Under certain conditions, the Hsp70 response is regulated posttranscriptionally via mRNA stabilization [13–15]. The response also leads to a remarkable block in protein synthesis during heat shock or under mechanical loading [15,22]. Continuous 30 MPa HP causes the Hsp70 response due to hsp70 mRNA stabilization. It appears that hsp70 mRNA half-life is affected by the inhibition of protein synthesis during pressurization and heat shock, suggesting that a pool of constitutively expressed cellular proteins together with newly synthesized proteins are involved in the regulation of hsp70 mRNA stability under stressful conditions. Hsp70 itself could be one candidate protein, as it has been indicated to control the stability of erythropoietin mRNA [23]. Moreover, Hsp70 has been suggested to be involved in the turnover of AU-rich mRNAs [24]. In this study, hsp70 mRNA levels decreased in concert with increased Hsp70 accumulation, but it remains to be clarified whether Hsp70 controls its own mRNA degradation during HP loading.

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