Activation of heat-shock transcription factor 1 by hypertonic shock in 3T3 cells

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The exposure of 3T3 cells to a medium made hypertonic by the addition of NaCl induced activation of a heat-shock transcription factor (HSF). This activation, as monitored by gel-mobility-shift assays, occurred within 10 min of hypertonic shock and was dose-dependent in relation to the osmotic strength of the medium up to 0.7 osM. Competition analysis indicated that the effect of hypertonic shock on HSF binding activity was specific. The magnitude of the heat-shock element (HSE)–HSF binding induced by incubating the cells in a 0.7 osM medium was comparable in intensity and time course with that induced by a 44 °C heat shock. Following removal of the stressors, the decrease in HSF–HSE binding was more rapid in hypertonicity-shocked than in heat-shocked cells. Treatment of the cells with cycloheximide did not inhibit HSF–HSE binding, indicating that the activation was independent of new protein synthesis. By using a

specifically directed polyclonal serum, HSF1 was identified as the transcription factor involved in the hypertonicity-induced activation. HSF was also activated when a membrane-impermeable osmolyte such as sucrose was used to increase the osmolarity of the medium. However, no HSF–HSE binding was observed after addition of glycerol (a freely membrane-permeable osmolyte) in excess. There was a temporal relationship between the hypertonicity-induced volume decrease, the increase in the intracellular K⁺ concentration and the induction of HSF–HSE binding. In contrast, an increase in the intracellular Na⁺ concentration was not required to induce HSF–HSE binding. However, unlike the heat-shock response, the activation of HSF by hypertonic shock did not lead to elongation of the RNA transcript of heat-shock protein 70.

INTRODUCTION

Cells adapt to hypertonicity by a variety of biophysical [cell shrinkage and a subsequent regulatory volume increase (RVI)], biochemical (opening/closing of ion channels; increased transport or synthesis of compatible osmolytes; enzyme activation) and genetic (induction of selected gene expression) mechanisms [1,2]. In differentiated cell models, such as kidney cell lines, there is increased expression of those genes whose products are known to protect the cells against the hypertonic stress. For example, hypertonicity leads to the accumulation of compatible osmolytes [3] by enhancing the expression of enzymes involved in their synthesis (e.g. sorbitol and aldose reductase), or of the transporter involved in their uptake (e.g. betaine and its transporter) [1]. Interestingly, a tonicity-sensitive element that mediates increased transcription of the betaine transporter gene in response to hypertonic treatment has recently been characterized in kidney cells [4]. Numerous other genes whose expression is increased by hypertonicity have also been identified. They include genes for heat-shock proteins (HSPs; molecular chaperons [5,6]), immediate-early genes (encoding transcription factors [5]) and other genes (encoding metabolic enzymes, adhesion molecules, etc. [1,2]). How the induction of these different genes occurs during hypertonic stress and whether their expression is related to the adaptation process is not well understood.

HSP gene regulation by heat shock is well documented at all levels of expression, and it has been regarded as a paradigm for inducible genomic responses [7,8], whereas HSP induction by hypertonicity has been evaluated at the mRNA level and from the synthesis and accumulation of specific proteins [5,6,9,10]. Moreover, HSP induction by hypertonicity has been examined in long-term experiments, whereas HSP induction by hyperthermia

is usually analysed during heat shock or cell recovery following a heat shock of short duration. To compare the HSP response to osmotic stress with that induced by heat shock, we analysed HSP70 gene activation and transcription in 3T3 cells after exposure to hypertonic shock or heat shock of identical duration and similar intensity. We report here that a short exposure of 3T3 cells to a hypertonic medium activated the binding of a heat-shock transcription factor (HSF) to a heat-shock element (HSE) in a dose-response manner. Comparable binding to that attained during heat shock was observed which was quickly and fully reversible, although there was no ensuing transcription of the HSP70 gene. The possible involvement of cell shrinkage and the consequent increase in intracellular ionic strength in the induction of HSF activation was also investigated.

EXPERIMENTAL

Materials

[γ -³²P]ATP, [α -³²P]UTP and 3-*O*-methyl-D-[1-³H]glucose were obtained from Amersham International, Amersham, Bucks., U.K. Prestained protein molecular mass standards were obtained from Gibco BRL, Eggenstein, Germany. A monoclonal antibody directed against the human inducible 72 kDa HSP (C92F3A5) was generously donated by Dr. William J. Welch (University of California, San Francisco, CA, U.S.A.). A probe for the human HSP70 gene (pH 2.3) was kindly provided by Dr. Richard I. Morimoto (University of Illinois, Evanston, IL, U.S.A.) and was obtained from Dr. Luisa Schiaffonati (University of Milan, Italy). A probe for β -actin was obtained from Dr. Jeannette A. M. Maier (University of Milan, Italy). A double-stranded synthetic consensus HSE sequence (upper strand 5'-CTCGAA-

Abbreviations used: HSE, heat-shock element; HSF, heat-shock transcription factor; HSP, heat-shock protein; [K⁺]_i and [Na⁺]_i, intracellular concentrations of K⁺ and Na⁺ respectively; RVI, regulatory volume increase.

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GCTTCTAGAACGTTCTAG-3') was obtained from PRIMM (Milan, Italy). Media, fetal calf serum and antibiotics for culturing the cells were purchased from Gibco (Grand Island, NY, U.S.A.). Disposable plastics for laboratory use were obtained from Costar (Broadway, Cambridge, MA, U.S.A.). Reagents of analytical grade were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Reagents for electrophoresis and blotting were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

Cell cultures

Balb/c 3T3 cells (clone A31) were kept in culture for up to 2 months and then discarded; fresh cultures were started again from frozen stocks. The cells were maintained in minimum essential medium containing penicillin (100 units/ml) and streptomycin (100 µg/ml) supplemented with 10% (v/v) fetal calf serum. All cultures were kept in an incubator at 37 °C in a water-saturated 5% CO₂ atmosphere in air and were passaged twice per week. They were regularly checked for mycoplasma contamination with a mycoplasma detection kit (Boehringer, Mannheim, Germany). For the experiments, cells were seeded at a density of about 10⁴ cells/cm² and then grown for 3 days. At 12 h before the start of the experiments, the culture medium was changed to avoid nutrient starvation. During the experiments, cell densities were measured as µg of protein/cm². Protein content was determined by the dye fixation method (Bio-Rad) using BSA as standard [11].

Stress conditions

Cells were either exposed or not for 30 min to heat shock or hypertonic shock (shock phase), except when otherwise stated. During shock, cells were incubated in a water bath in a water-saturated 5% CO₂ atmosphere in air. In some experiments, after shock the monolayers were transferred to an iso-osmotic medium and kept in an incubator at 37 °C in a water-saturated 5% CO₂ in air for the indicated times (recovery phase).

Culture media of altered hypertonicity

Minimal essential medium was adjusted to the desired Na⁺ concentration by addition of NaCl in excess. Normal medium contained 143 mM Na⁺, 116 mM of which was derived from NaCl and the remainder from other components (sodium bicarbonate and sodium phosphate). Media were adjusted to the desired osmolarity by the addition of NaCl for most experiments, but sucrose and glycerol were also used where indicated in the Results section. Media with low Na⁺ concentrations (15 mM; derived from fetal calf serum) were obtained by omitting NaCl and replacing sodium bicarbonate with choline bicarbonate. The media were then brought to the desired final osmolarity by adding sucrose. The correct final concentration of the modified medium was checked with a vapour pressure osmometer (Wescor).

Determination of cell volume and intracellular univalent cation content

Intracellular volumes were estimated by measurement of the equilibration of 3-*O*-methyl-D-[1-³H]glucose, as described previously [12] using the method of Kletzien et al. [13]. Intracellular Na⁺ and K⁺ concentrations ([Na⁺]_i and [K⁺]_i, respectively) were estimated using the procedure described by Owen and Villereal [14], with slight modifications [15].

Western blotting

Immunoblotting analysis was performed as previously described in detail [10,16], according to the method of Burnette [17].

Gel-mobility-shift assay

For the gel-mobility-shift assay, a whole-cell extract (20 µg of protein) was incubated with a ³²P-labelled HSE oligonucleotide as previously described in detail [18]. To determine amounts of HSF1 and HSF2 in the total HSF-binding activity (super gel-mobility-shift assay), anti-HSF1 and -HSF2 sera were added to whole-cell extracts during the binding reaction. The complexes were then electrophoresed on a 0.5% agarose/2.5% acrylamide (30:0.8, acrylamide/bisacrylamide) gel made up in 1×TBE buffer (0.09% Tris/borate, 0.002 M EDTA).

Nuclear run-on transcription assay

Medium was removed from two tissue culture plates (81 cm² each) of Balb/c 3T3 cells, and the cells were washed three times with ice-cold PBS, scraped off into PBS and pelleted at 500 *g* for 5 min. The cell pellet (10⁷ cells) was resuspended in 1 ml of Nonidet P40 lysis buffer [10 mM Tris/HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.5% (v/v) Nonidet P40], incubated for 5 min on ice and centrifuged at 500 *g* for 5 min. The nuclear pellet was washed once with 2 ml of Nonidet P40 lysis buffer and centrifuged at 500 *g*. The supernatant was discarded, and the nuclei were resuspended in 100 µl of 'freezing buffer' [50 mM Tris/HCl, pH 8.3, 40% (v/v) glycerol, 5 mM MgCl₂, 0.1 mM EDTA] and frozen in liquid N₂. The nuclei were thawed for the run-on transcription assay and mixed with 100 µl of reaction buffer {10 mM Tris/HCl, pH 8, 5 mM MgCl₂, 300 mM KCl, 0.5 mM each of ATP, CTP and GTP, and 100 µCi of [α -³²P]UTP (3000 Ci/mmol)} and incubated at 30 °C for 30 min. The ³²P-labelled RNA was isolated as described above and treated with a final concentration of 0.2 M NaOH for 10 min on ice. The solution was neutralized by the addition of Hepes to a final concentration of 0.24 M and RNA was precipitated with ethanol after addition of carrier tRNA. After centrifugation at 12000 *g* for 30 min, the pellet containing the ³²P-labelled RNA was resuspended in TE buffer (10 mM Tris/HCl, pH 7.5, 1 mM EDTA) and hybridized to DNA immobilized on nylon filters in 50% formamide, 7% SDS, 0.12 M Na₂HPO₄, pH 7.2, 0.25 M NaCl and 1 mM EDTA at 43 °C for 24 h. Each filter was hybridized with the same number of c.p.m. of ³²P-labelled RNA [(5–10) × 10⁶ c.p.m./ml]. After hybridization, the filters were washed at room temperature for 15 min in each of the following solutions: 2×SSC (1×SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7) containing 0.1% SDS, 0.5×SSC/0.1% SDS and 0.1×SSC/0.1% SDS, at room temperature. The filters were then incubated at 37 °C in 0.2×SSC with 10 µg/ml RNase A for 30 min, air-dried and exposed. For binding to nylon filters, DNA was denatured by incubation with 0.4 M NaOH/10 mM EDTA at 100 °C for 10 min. The DNA was then spotted on to nylon membranes using the Bio-Rad dot-blot apparatus; 5 µg of DNA was applied per blot.

RESULTS

Similar effects of hypertonic shock and heat shock on HSF activation

Electrophoretic gel-mobility-shift assays were carried out with total cell extracts from cultures exposed to heat shock or hypertonic shock and a HSE from the inducible human HSP70

Figure 1 Gel-mobility-shift analysis of HSF–HSE binding induced by incubating 3T3 cells in media of increasing osmolarity

The HSF binding activity was analysed in cell extracts of 3T3 cells heat shocked for 30 min at 44 °C (Hs) or exposed to different hypertonic media (from 0.3 to 0.7 osM) for 30 min. Whole-cell extracts were prepared for binding and gel-mobility-shift assays as described in the Experimental section. For the competition experiment, cell extracts were mixed with a 200 × molar excess of unlabelled HSE prior to the addition of [³²P]HSE. The position of the specific HSF–HSE complex is indicated by the arrowhead. The lane marked / did not contain cell extract.

gene [18–20]. As shown in Figure 1, a 30 min heat shock strongly activated HSF–DNA binding. A 30 min hypertonic shock induced a dose-dependent activation of HSF, with maximal HSE binding observed at 0.7 osM and with an intensity comparable with that induced by the 44 °C heat shock. Also, competition analysis with unlabelled HSE oligonucleotide in excess indicated that hypertonic shock- or heat shock-activated HSF had the same DNA sequence specificity. The kinetics of HSF–HSE binding induced by heat shock and hypertonic shock were comparable, and both processes appeared to be reversible. However, in hypertonic shock-treated cells, binding completely disappeared during the first 15 min after removing the stress, whereas it took at least 2 h in heat shock-treated cells (results not shown).

At least two HSFs (HSF1 and HSF2) from mammalian cells have been identified and cloned [21,22], but only HSF1 was activated in response to either heat shock or hypertonic shock, as shown by the use of polyclonal antisera specifically directed against HSF1 or HSF2 (results not shown). Similarly, treatment of the cells with cycloheximide, at a concentration (50 µg/ml) that inhibits over 95% of new polypeptide synthesis, had no effect on HSF–HSE binding induced by either heat shock or hypertonic shock (results not shown). This observation is in agreement with previous reports indicating that HSE binding of heat-inducible HSF is not blocked by cycloheximide [23] and that the normal pattern of HSP70 mRNA turnover after heat shock is not dependent on new protein synthesis [24–26].

Run-on experiments indicate a lack of HSP70 gene transcription in hypertonicity-exposed cells

We examined the rates of transcription of the HSP70 and actin genes in nuclei isolated from 3T3 cells exposed to heat shock or hypertonic shock. Actin was chosen as an indicator of constitutive gene transcription for comparison with transcription of an

Figure 2 Nuclear run-on analysis of HSP70 and β -actin gene transcription

Nuclei were isolated from 3T3 cells heat-shocked at 44 °C for 30 min (HS) or exposed to 0.7 osM for 30 min (HyS 30 min) and then incubated in iso-osmotic medium for 3 h of recovery (HyS 3hR). Run-on transcripts were labelled and isolated as described in the Experimental section. Labelled RNA was hybridized to nylon filters containing plasmids encoding the human HSP70 gene (pH 2.3) or β -actin gene. A vector plasmid (pBR322) was used as a non-specific hybridization control.

Figure 3 (A) Gel-mobility-shift analysis of the HSF–HSE complex induced by 0.7 osM NaCl, sucrose or glycerol shocks, and (B) accumulation of inducible HSP70 during recovery from heat shock or hypertonic shock

(A) The HSF binding activity was analysed in cell extracts of 3T3 cells heat-shocked for 30 min at 44 °C (HS) or exposed for 30 min to 0.7 osM hypertonic media obtained by the addition of NaCl, sucrose (Sucr) or glycerol (Glyc) in excess. The position of the specific HSF–HSE complex is indicated by the arrowhead. C represents untreated control cells, and the lane marked / did not contain cell extract. (B) Cells were heat-shocked at 44 °C for 30 min or exposed for 30 min to 0.7 osM media obtained by addition of NaCl, sucrose or glycerol in excess. The cells were then incubated in iso-osmotic medium at 37 °C for 6 h (recovery phase). C represents untreated control cells. Cell proteins were extracted, separated by SDS/PAGE, blotted on to nitrocellulose and treated with a monoclonal antibody directed against inducible HSP70.

inducible gene such as that of HSP70. Figure 2 shows the hybridization of labelled nascent RNA chains to dot-blotted cloned DNA of these genes. As expected, transcription of the HSP70 gene was induced in cells exposed to heat shock. In contrast, 0.7 osM hypertonic shock, which permitted comparable transcription of the actin gene to that observed during heat shock, did not induce transcription of the HSP70 gene. A similar result was obtained with 0.5 osM hypertonic shock.

Influence of different osmolytes on HSF activation

We then investigated the signal involved in HSF activation during hypertonic shock. As shown in Figure 3(A), after in-

Table 1 Effect of cell incubation in media made hypertonic by the addition of different osmolytes on ion content and cell volume

3T3 cells were incubated for 30 min in isotonic medium at 44 °C or in hypertonic (0.7 osM) media obtained by addition of NaCl, sucrose or glycerol in excess. At the end of this period, the cells were incubated in physiological conditions for 30 min (recovery phase) where indicated. The univalent cation contents were converted into concentrations by dividing them by the corresponding values of cell volume recorded in the same sample. The values shown are means $\pm$ S.D. of six independent determinations.

Conditions	[Na ⁺] _i (mM)	[K ⁺] _i (mM)	Cell volume (μ l/mg of protein)
Control	15 $\pm$ 1	266 $\pm$ 4	5.36 $\pm$ 0.42
Heat shock, 44 °C	15 $\pm$ 1	214 $\pm$ 9	7.35 $\pm$ 0.50
Heat shock + recovery	15 $\pm$ 1	255 $\pm$ 8	6.06 $\pm$ 0.25
0.7 osM (NaCl)	66 $\pm$ 5	468 $\pm$ 8	3.96 $\pm$ 0.27
0.7 osM (NaCl) + recovery	17 $\pm$ 1	255 $\pm$ 9	6.18 $\pm$ 0.17
0.7 osM (Sucrose)	47 $\pm$ 4	521 $\pm$ 15	2.99 $\pm$ 0.19
0.7 osM (Sucrose) + recovery	18 $\pm$ 1	243 $\pm$ 11	6.94 $\pm$ 0.62
0.7 osM (Glycerol)	19 $\pm$ 1	263 $\pm$ 11	5.23 $\pm$ 0.21
0.7 osM (Glycerol) + recovery	16 $\pm$ 1	267 $\pm$ 7	5.21 $\pm$ 0.21

cubation of cells in a medium made hypertonic by the addition of sucrose (a neutral, membrane-impermeable osmolyte), we observed a similar induction of DNA binding activity to that obtained on addition of an electrolyte such as NaCl. However, despite the high level of HSF activation observed under the hypertonic conditions obtained by addition of excess sucrose to the incubation medium, HSP70 RNA transcript (results not shown) and HSP70 protein (Figure 3B) were not synthesized or accumulated. Interestingly, addition of a freely membrane-permeable solute such as 0.4 M glycerol to the culture medium (0.7 osM, final osmolarity) did not activate HSF–HSE binding activity (Figure 3A).

Effects of heat shock and hypertonic shock on cell volume

When animal cells are immersed in a hypertonic medium, they immediately shrink as water leaves them by osmotic flow. This leads to an increase in cellular salt concentration and osmotic strength. Cells subsequently modulate their internal osmotic strength to keep their volume constant by activating membrane transport antiporters and symporters for ions [27] and compatible osmolytes [28], i.e. a RVI takes place [29,30].

The immediate cell shrinkage and the consequent increase in the osmotic strength of the intracellular milieu, which must occur in cells suddenly incubated in hypertonic medium, might be the initial stimuli in the pathway leading to HSF activation. To gain further insight into this possibility, we measured cell volume and intracellular univalent ionic content during hypertonic shock of the cells. As shown in Tables 1 and 2, the volume of cells incubated in a medium made hypertonic (0.7 osM) by the addition of a membrane-impermeable or a poorly permeable osmolyte (such as sucrose or NaCl respectively), but not of an almost freely membrane-permeable osmolyte (such as glycerol), shrank by 25–40% with respect to cells incubated in iso-osmotic medium. However, at the end of hypertonic treatment, on re-incubation in iso-osmotic medium the cells quickly regained their previous volume (see Table 1). In this context, it is interesting to note that the volume of cells incubated at 44 °C was significantly greater than that of control cells. The possible role of this volume change in HSF activation in cells exposed to heat shock merits further investigation. Technical limitations (measurement of cell volume by analysing the distribution of methylglucose at equi-

Table 2 Effect of cell incubation in hypertonic media with low Na⁺ content on ion content and cell volume

3T3 cells were incubated for 30 min in isotonic or hypertonic media obtained by the addition of sucrose (with low Na⁺ content). The univalent cation contents have been converted into concentrations by dividing them by the corresponding values of cell volume recorded in the same sample. The values shown are mean $\pm$ S.D. of six independent determinations.

Final medium osmolarity (osM)	[Na ⁺] _i (mM)	[K ⁺] _i (mM)	Volume decrease (%)
0.3	24 $\pm$ 3	240 $\pm$ 22	
0.3 (low Na ⁺)	12 $\pm$ 2	263 $\pm$ 11	
0.5 (low Na ⁺)	15 $\pm$ 2	369 $\pm$ 20	22
0.7 (low Na ⁺)	34 $\pm$ 5	399 $\pm$ 25	44

librium, i.e. after 30 min) prevented us observing the immediate cell shrinkage presumed to occur on incubation of cells in hypertonic media. In an attempt to determine the volume reduction at early time points, we measured the distribution of labelled methylglucose after 10 min of hypertonic treatment in cells preincubated with the tracer up to the equilibrium distribution. Under these conditions, there was a significant reduction (–36%) in cell volume (results not shown), indicative of an early volume decrease in response to a sudden increase in the extracellular osmolarity.

Effects of hypertonic shock on univalent cation content

The univalent cation content ([Na⁺]_i and [K⁺]_i) roughly reflected the extra osmotic strength added to the medium. As expected and shown in Tables 1 and 2, the increment in [K⁺]_i was correlated with the volume decrease; in contrast, the significant increase in [Na⁺]_i observed after 0.7 osM treatment cannot be explained solely by a volume reduction. Increased Na⁺ entry, caused by the higher extra–intra sodium gradient, may also be involved. Taken together, these results suggest that the marked alteration in ion content during hypertonic treatment, the most significant consequence of cell shrinkage, may be involved in the induction of HSF activation.

Table 1 also indicates that the 0.7 osM medium obtained by the addition of excess sucrose, which induced strong HSF activation (see Figure 3A), led to a comparable or greater cell shrinkage and increase in [K⁺]_i than those induced by the 0.7 osM medium produced by addition of excess NaCl. In contrast, there was significantly smaller increase in [Na⁺]_i on sucrose addition. Since this result suggested a relatively modest involvement of [Na⁺]_i in the generation of the signal for HSF activation, we examined the effect of a medium made up with sucrose in excess and negligible Na⁺ (increasing addition of sucrose to a medium with low Na⁺ content). Figure 4 shows that, under these conditions, HSF activation was apparent even at 0.4 osM, reaching a peak around 0.5 osM. The corresponding values for ion content and volume of cells incubated in media of increasing osmolarity (obtained by adding excess sucrose) and with low Na⁺ content were then determined. As shown in Table 2, at the higher medium osmolarity tested we observed a similar increase in [K⁺]_i to that obtained by incubating cells in 0.7 osM medium made up by NaCl in excess (cf. Table 1). However, under the same conditions [Na⁺]_i was not significantly higher than control levels. Cell volume was correlated with the osmotic strength of the extracellular medium, irrespective of the combination of sucrose and NaCl used to increase osmolarity.

Figure 4 Gel-mobility-shift analysis of HSF–HSE binding induced by hypertonic medium with low Na^+ content

The HSF binding activity was analysed in cell extracts of 3T3 cells heat-shocked for 30 min at 44 °C (HS) or exposed for 30 min to media with a low Na^+ (15 mM) content; the final osmolarity (from 0.3 to 0.7 osM) of the medium was obtained by addition of sucrose in excess. The position of the specific HSF–HSE complex is indicated by the arrowhead. The lane marked / did not contain cell extract.

DISCUSSION

The responses to heat shock and hypertonicity stress are thought to be related [5,6]. For example, continuous cell exposure to hypertonicity induced by extracellular NaCl addition increases the expression of HSP70 [5,6,9,10]. Increased expression of the HSP70 gene is presumably triggered by denaturation of cell proteins induced by hyperthermia, and there is evidence that denatured or abnormal proteins are responsible for the induction of HSP synthesis [31]. To our knowledge, induction of HSP70 gene expression by hypertonic treatment has been most extensively evaluated in cells of kidney origin, i.e. cells that have to cope with marked alterations in salt concentration and osmotic strength [2]. All reports on this topic have evaluated HSP70 gene expression at the mRNA level and/or from HSP70 protein accumulation [5,6,10]. The relationship between the signalling pathway and transcription factor(s) involved and those induced by heat shock are unclear. Cohen and co-workers [5] have suggested a role for cell volume decreases and increased ion levels in the induction of HSP70 gene expression. Similarly, Sheikh-Hamad and co-workers [6] hypothesized that, following the initial shrinkage of cells exposed to high-NaCl medium, the subsequent rise in intracellular ion content denatures proteins, possibly altering interactions between HSF and chaperons. This would activate HSF, thereby inducing transcription of the HSP70 gene. However, there is as yet no experimental evidence for this plausible mechanism.

Our study demonstrates that, in 3T3 cells (i.e. mammalian cells not originating from kidney), hypertonic shock (0.7 osM) induces binding of HSF1 to HSE, with a timing and intensity paralleling those produced by heat shock (44 °C). The binding produced by either hypertonic shock or heat shock was independent of new protein synthesis, and was fully reversible on removal of the applied stress. However, the time course of attenuation of HSF1–HSE binding after removal of hypertonic shock was more rapid than that observed in cells recovering from heat shock: a decrease in binding was immediate following re-incubation of cells in iso-osmotic conditions. This result, indicating different

temporal characteristics in the recovery from the intracellular disturbance, suggests a different signalling pathway or at least a difference in the persistence of the signal involved in HSF activation in response to the different stresses.

Under the hypertonic conditions produced by addition of an impermeable (sucrose) or a poorly permeable (NaCl) osmolyte to the medium, a marked and early decrease in cell volume is likely to occur. This might well be the initial step in the pathway leading to HSE activation. This was supported by the lack of both a volume decrease and HSE–HSF binding in cells exposed to a medium made hypertonic by the addition of glycerol. Since glycerol is almost freely permeable through the membrane, it is likely to penetrate the cell rapidly. In this case, no osmotic gradient is established across the membrane and there is no cell shrinkage. This suggests that the increased and persistent osmotic pressure on the extracellular side of the plasma membrane, rather than the osmotic strength *per se*, acts as a trigger for HSF activation. Interestingly, neuronal volume has been found to be altered more by exposure to hyperosmolar conditions resulting from impermeable or poorly permeable osmolytes than to those resulting from glycerol [32]. However, it should be borne in mind that glycerol, in common with other polyols, may well protect proteins from denaturation [33].

Cells incubated in a medium made hypertonic by an excess of impermeable (e.g. sucrose) or poorly permeable (e.g. NaCl) solutes will behave as osmometers and decrease in volume, leading to an increase in intracellular ion levels (mainly $[\text{K}^+]_i$). Entry of ions and nutrients with a concomitant RVI should then take place ([29,30]; R. Alfieri, P. G. Petronini, S. Urbani and A. F. Borghetti, unpublished work). The rapid HSF activation accompanied by cell shrinkage that is observed in 3T3 cells exposed to an abrupt change in osmolarity is concomitant with increases in $[\text{Na}^+]_i$ and $[\text{K}^+]_i$. However, the HSF activation observed in cells incubated in medium made hypertonic with sucrose but low in sodium excludes a significant role for $[\text{Na}^+]_i$ in the signalling pathway (see Figure 4 and Table 2). Volume decreases and the rise in $[\text{K}^+]_i$ remain as possible candidates in the signalling pathway for HSF activation induced by hypertonic shock.

In spite of the high levels of HSF binding that occurred on hypertonic shock (0.7 osM), we did not observe any HSP70 RNA transcripts either during the shock or in the recovery phase. This was not attributed to an overall inhibition of transcription, since uridine incorporation into RNA during hypertonic shock still took place at around half the control rate (R. Alfieri, P. G. Petronini, S. Urbani and A. F. Borghetti, unpublished work; cf. [26]). Moreover, the fact that the actin gene was still transcribed during hypertonic shock suggests that hypertonicity inhibits the transcription of a few selective genes, including the HSP70 gene. It should be noted that inhibition of RNA synthesis was observed throughout the hypertonic shock, but uridine incorporation returned immediately to the control rate on return to normal osmolarity (results not shown). However, either before or simultaneous with the return to normal transcription rates, the cell volume and ion content quickly returned to control values.

Consideration of these observations suggested the following scheme of events in heat- or hypertonicity-shocked 3T3 cells. Activation and transcription of the HSP70 gene is induced during heat shock and in the recovery phase, since protein denaturation is immediate, intense and largely irreversible on exposure to 44 °C heat shock; denaturation of proteins by high ionic (mainly $[\text{K}^+]_i$) strength should also occur early during hypertonic shock; however, it is promptly reversible once the ionic environment returns to normal. We found that cell volume

and ion content quickly returned to control values on removal of the hypertonic stress. We thus assumed that the signals for activation disappeared rapidly during the recovery phase. In addition, the rapid attenuation of HSF–HSE binding observed during the recovery phase fits this scheme and can account for the absence of HSP70 RNA transcripts, despite the previous HSF activation, in cells recovering from hypertonic shock. However, we do not have a good explanation for the lack of transcription of the inducible HSP70 gene during hypertonic shock, despite the high HSF activation, significant RNA synthesis and specific transcription of a constitutive gene (actin). The multistep nature of HSP gene transcription and the rather inconsistent relationship between HSF activation and HSP gene transcription should not be overlooked [7]. Our present results are consistent with recent findings that certain agents and factors such as salicylate [34–37], arsenite [38], oxidative injury [39], alkaline shift [18] and hypo-osmotic stress [40] induce HSF–HSE binding but do not elicit transcription of the HSP70 gene.

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