Effect of betaine on HSP70 expression and cell survival during adaptation to osmotic stress

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Induced expression of the HSP70 gene in 3T3 and SV-3T3 cells was monitored by measurements of the synthesis of HSP70 and of the cellular contents of both HSP70 and its mRNA. The presence of betaine (*N*-trimethylglycine) at concentrations of 2.5–25 mM decreased the induction of HSP70 gene expression caused by incubation of 3T3 and SV-3T3 cells in hypertonic (0.5 osM) medium. This effect was accompanied by an enhancement of SV-3T3 cell adaptation, assayed by colony formation, to the hyperosmotic conditions. In contrast, the presence of betaine did not affect HSP70 gene expression induced in these cells by heat shock. After 6 h incubation with 25 mM betaine under hypertonic (0.5 osM) conditions the intracellular concentration of betaine in SV-3T3 cells was about 195 mM, compared with about 70 mM under isotonic (0.3 osM) conditions. Hence, with this concentration of extracellular betaine, the marked increase in the accumulation of betaine within the cells presumably counteracts the imposed osmotic pressure and eliminates the signal that otherwise initiates increased expression of the HSP70 gene.

INTRODUCTION

A series of studies of the effects of hyperosmotic conditions on cultures of chick-embryo fibroblasts, 3T3 and transformed 3T3 cells has revealed a variety of responses that may be summarized as follows. (*a*) General protein synthesis decreases (Petronini et al., 1986). (*b*) Cell proliferation decreases (Petronini et al., 1992). (*c*) The activity of amino acid transport system A increases (Tramacere et al., 1984). (*d*) There is induced synthesis of a protein that is either identical with or closely homologous to a 70 kDa heat-shock protein (HSP70) (Petronini et al., 1993). There are, however, some notable differences between untransformed 3T3 cells and transformed 3T3 cells in their responses to hyperosmotic stress. For example, 3T3 cells, in contrast with SV-3T3 cells, adapt to hyperosmolar conditions, and this adaptation is characterized by partial reversal of the responses noted above (Silvotti et al., 1991). Moreover, the synthesis of the inducible stress protein can be detected in 3T3 cells after 4–6 h exposure to hypertonicity, but only after 8–10 h in SV-3T3 cells (Petronini et al., 1993). Thus it appears that the induced synthesis of the stress protein is an important factor in these cells' responses and eventual adaptation to hypertonic conditions.

In another study with SV-3T3 cells we examined the effects of the presence of some 'compatible solutes' (Yancey et al., 1982; Yancey and Burg, 1990; Garcia-Perez and Burg, 1991) on the cells' adaptive responses to hyperosmotic stress. This revealed that the addition of one such solute, betaine (*N*-trimethylglycine), to the hypertonic incubation medium dramatically counteracts the usual induction of amino acid transport activity and the decreases in cell proliferation and general protein synthesis (Petronini et al., 1992). No direct measurement of the uptake of betaine by the cells was possible then, because of the unavailability of labelled betaine; but it seemed unlikely that the cells could accumulate a sufficiently high concentration of betaine to counteract the imposed osmotic pressure. Hence we considered the possibility that betaine might act by stimulating the induction of the HSP70 stress protein in SV-3T3 cells, so that they adapt more rapidly and behave more like untransformed cells. We have examined this possibility by monitoring the effects of added betaine on the expression of the HSP70 gene induced both by hypertonic incubation and by heat shock in 3T3 and SV-3T3 cells. In addition, having eventually developed a method of preparing radiolabelled betaine, we have measured its uptake by the cells under isotonic and hypertonic conditions.

EXPERIMENTAL

Materials

α-[^{32}P]dCTP, L-[4,5-^{3}H]leucine, L-[^{35}S]methionine, [^{14}C]methylated protein mixture, 3-*O*-methyl-D-[1-^{3}H]glucose and [*methyl*-^{14}C]choline chloride were obtained from Amersham International, Amersham, Bucks., U.K. Pre-stained protein molecular-mass standards were obtained from GIBCO BRL, Eggenstein, Germany. Monoclonal antibody directed against human inducible 72 kDa heat-shock protein (C92F3A5) was generously given by Dr. William J. Welch (San Francisco). Plasmid pH2.3 containing the human HSP70 gene was kindly provided by Dr. Richard I. Morimoto (Evanston) and was obtained through Dr. Luisa Schiaffonati (Milan). Media, fetal-calf serum and antibiotics for culturing the cells were purchased from GIBCO, Grand Island, New York, NY, U.S.A. Choline oxidase was obtained from Sigma Chemical Co., Poole, Dorset, U.K. Reagents for electrophoresis and blotting analysis were obtained from Bio-Rad Laboratories, Richmond CA, U.S.A.

Cell cultures

Balb/c 3T3 cells (clone A 31) and simian virus 40 (SV40)-transformed Balb/c 3T3 cells (clone SV-3T3) 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 Dulbecco's modified Eagle medium (D-MEM) containing penicillin (100 units/ml) and streptomycin (100 μg/ml) supplemented with 5% fetal-calf serum for transformed cells and 10% fetal-calf serum for 3T3 cells. All cultures were kept in an incubator at 37 °C in a water-saturated atmosphere of 5% CO_2 in air, and

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were passaged twice a week. They were regularly checked for mycoplasma contamination with the use of a mycoplasma detection kit (Boehringer, Mannheim, Germany). During experiments all cells, 3T3 and SV-3T3, were incubated in media containing 10% fetal-calf serum. For experiments, cells were seeded at a density of about 10000 cells/cm^2 for 3T3 cells and 40000 cells/cm^2 for SV-3T3 cells, and then grown for 2 days before the start of incubations. During experiments cell densities were measured in terms of μg of protein/cm^2 and the values for SV-3T3 cells were generally 4–5 times those for 3T3 cells.

Culture media of altered hypertonicity

Minimal essential medium was adjusted to the desired Na^+ concentration by addition of 1.5 M NaCl. The final osmolarity of the modified medium was checked with a vapour-pressure osmometer (Wescor). Normal medium contained 143 mM Na^+, 116 mM derived from NaCl, and the remainder from other components ($NaHCO_3$ and sodium phosphate).

Cell counting and determination of cell survival

Cells were detached from the substratum with trypsin, and appropriate dilutions of the resulting suspension were counted in a Bürker haemocytometer as described in detail elsewhere (Piedimonte et al., 1982). Cell survival was determined by cell viability and colony formation. After hyperosmotic treatment the cells were removed from the plates with trypsin, counted, and their viability was determined by Trypan Blue exclusion (Hunt, 1987). Colony formation by viable cells was then determined by seeding them at a density of 400 cells per 27 cm^2 in dishes containing 5 ml of culture medium. After 5–6 days incubation, the cells were fixed with 95% ethanol, stained with 0.1% Crystal Violet and counted.

PAGE

Cells were extracted in a solution containing 10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris/HCl (pH 7.4), 0.1% SDS, 0.1% Triton X-100, 10 μg/ml 4-aminophenylmethanesulphonyl fluoride, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin and 0.5 mM EDTA. The extracts were sonicated for about 1 min with a probe sonicator and then freeze-dried. Samples for one-dimensional PAGE were dissolved in a solution containing 2% SDS, 62.5 mM Tris/HCl (pH 7.4), 5% 2-mercaptoethanol, 0.002% Bromophenol Blue and 20% glycerol. The solution was heated to 100 °C for 2–3 min. Samples for two-dimensional PAGE were dissolved in a solution containing 9.5 M urea, 5% 2-mercaptoethanol, 2% Nonidet P-40, 1.5% SDS and 2% ampholytes (Bio-Lyte ampholine 1.8%, pH 5–7, 0.2% pH 3–10). One-dimensional PAGE was carried out essentially as described by Laemmli (1970) with 5–15% (w/v) linear gradient gels (cross-linker = 1:38), a discontinuous buffer system and a constant current of 35 mM for about 5 h. For two-dimensional gels, proteins were focused in the first dimension for 14–15 h at 400 V, followed by 2 h at 800 V. For the second dimension, 10%-polyacrylamide gels were used.

To label cell proteins for PAGE, methionine-free culture medium was supplemented with 1 μM unlabelled methionine and 100 μCi/ml [^{35}S]methionine (1115 Ci/mmol). Labelling was followed by addition of 1 mM unlabelled methionine for a 5 min chase period. The cell layer was washed gently with 3 × 2 ml of cold phosphate buffer solution and then extracted as described above. Samples for two-dimensional PAGE contained about 1.5×10^6 c.p.m. Autoradiography was performed with Kodak X-Omat film and 3 weeks exposure.

Western blotting

Protein concentration was determined by a dye-fixation method (Bio-Rad) with BSA as standard (Bradford, 1976). Immunoblotting analysis was performed essentially as described by Burnette (1981). Briefly, cell proteins separated by SDS/PAGE were electrophoretically transferred (30 V, overnight at 4 °C) from the slab gel to a nitrocellulose sheet in a Trans-Blot Cell (Bio-Rad) filled with a solution containing 25 mM Tris, 192 mM glycine (pH 8.3) and 20% methanol. Non-specific protein binding was blocked by incubating the sheets for 1 h in a solution containing 3% gelatin in 20 mM Tris/HCl (pH 7.6) and 500 mM NaCl. The saturated transfer membrane was washed twice (5 min per wash) with a solution containing 20 mM Tris/HCl (pH 7.5), 500 mM NaCl and 0.05% Tween-20 (solution W). The blot was then incubated for 4 h at laboratory temperature (about 25 °C) with a 1:5000 dilution of the antibody in solution W containing 1% gelatin, and then washed twice as above. This was followed by a 1 h incubation with a 1:3000 dilution (in solution W plus 1% gelatin) of goat anti-mouse IgG conjugated to alkaline phosphatase. The blot was finally washed twice with solution W and once with solution W minus the Tween. Detection of the antigen–antibody complex was carried out by incubating the sheet in a solution containing 0.015% Nitro Blue Tetrazolium, 0.7% *NN*-dimethylformamide, 0.03% 5-bromo-4-chloro-3-indolyl phosphate, 1 mM $MgCl_2$ and 100 mM $NaHCO_3$, pH 9.8. The development was stopped by immersing the sheet in distilled water for 10 min. Some blots were quantified with the use of a computing laser densitometer (Molecular Dynamics). The range of densities for which the method is reliable was established by trials with different concentrations of standard protein.

Northern blotting

Total RNA was extracted from cultured cells by the guanidium/caesium trifluoroacetate method (Okayama et al., 1987) with a RNA extraction kit (Pharmacia). RNA samples (10 μg) were fractionated by 1.2%-agarose-gel electrophoresis and transferred to nylon filters. The quality and quantity of RNA blotted on membranes was checked by u.v. absorption. Plasmid pH 2.3 containing the human HSP70 gene (Hunt and Morimoto, 1985) was nick-translated (Amersham kit N.5000) with α-[^{32}P]dCTP (3000 Ci/mmol). For hybridization the membranes were incubated in a solution containing 50% formamide, 7% SDS, 0.25 M NaH_2PO_4, 0.25 M NaCl and 1 mM EDTA. Afterwards they were washed according to the following routine, each solution being buffered at pH 7 and each wash lasting 10 min: five times at room temperature in 2 × SSC solution (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7) containing 0.1% SDS; once with 1 × SSC containing 0.1% SDS at 42 °C; twice with 0.1 × SSC at 42 °C. Autoradiographs were obtained from exposures of 12–24 h, with intensifying screens, at −80 °C.

Preparation of [^{14}C]betaine

[^{14}C]Betaine was prepared from [^{14}C]choline by enzymic oxidation. A mixture of 0.03 ml of choline oxidase solution (10 units dissolved in 0.5 M Tris/HCl, pH 8 at 20 °C) with 0.25 ml (50 μCi, 910 nmol) of [^{14}C]choline chloride was incubated at 37 °C for 2.5 h. The solution was then applied to a small column (total volume 2 ml) of CM-Sephadex G-25 that had been washed first with about 10 mM ammonia solution and then with water. The column was eluted with about 10 mM ammonia solution and the eluate collected as 0.22 ml fractions in Eppendorf tubes. The oxidase (apparent from its yellow colour) was eluted in fractions 4 and 5, together with a trace of betaine. Then approx. 86% of

the initial radioactivity was eluted as [^{14}C]betaine in fractions 7–13. These fractions were frozen and then concentrated by centrifugation under reduced pressure in an Hetovac (type VR-1). The final yield was 41 μCi of [^{14}C]betaine.

T.l.c. on silica gel was used to develop and monitor this procedure. Both choline and betaine were detected initially with the use of Dragendorff's reagent, and a clear separation (R_F = 0.1–0.2 for choline and R_F = 0.6–0.7 for betaine) was obtained with a developing solution composed of 0.155 M NaCl/methanol/conc. ammonia (10:10:1, by vol.). Approx. 98% of [^{14}C]choline co-migrated with unlabelled choline. After prolonged incubation with choline oxidase, t.l.c. analysis of the complete reaction mixture showed only two peaks of radioactivity, a small one remaining at R_F 0.1–0.2 and the other, containing about 96% of the label, at R_F 0.6–0.7. The same t.l.c. analysis was used to check the performance of the ion-exchange column and the content of cell extracts after incubation with the labelled betaine.

Betaine uptake and cell volume

Betaine uptake by the cells was monitored with the use of [^{14}C]betaine added to the incubation media with the appropriate concentration of unlabelled betaine and subsequently measured by scintillation counting of acid extracts of the washed cells. Intracellular volumes were estimated at the same time by measurement of the equilibration of 3-*O*-methyl-D-[1-^{3}H]glucose, as described previously (Petronini et al., 1992).

RESULTS

Effect of betaine on cellular accumulation of inducible HSP70

Since the monoclonal antibody detects the presence of induced HSP70 in 3T3 cells, but not in SV-3T3 cells, after only 6 h exposure to hypertonic conditions (Petronini et al., 1993) the effect of adding betaine was tested first under these conditions. No effect was observed with SV-3T3 cells, there being no detectable induced HSP70 in the presence or absence of betaine. Unexpectedly, however, with 3T3 cells the addition of 25 mM betaine to the hyperosmotic medium produced exactly the opposite effect from that postulated: it drastically decreased the amount of induced HSP70 detected by the antibody. This test was therefore repeated with the use of a range of betaine concentrations and longer exposure of the cells to hypertonic conditions. The results (Figure 1) show that a betaine concentration of only a few mM is sufficient to cause a significant decrease in the amount of induced HSP70, in both 3T3 and SV-3T3 cells, and higher concentrations of betaine progressively decrease the HSP70 to the limit of detection (Table 1).

Corroboration of this finding was provided by the more direct approach of monitoring protein synthesis in terms of the incorporation of L-[^{35}S]methionine. Analysis by two-dimensional PAGE of the polypeptides synthesized in 3T3 cells during incubation in hypertonic medium, with and without the addition of betaine, showed a very marked and specific decrease in labelling of inducible HSP70 when betaine was present (Figure 2).

HSP70 gene transcription

To examine the possibility that betaine affects HSP70 gene expression at the transcriptional level, we used a human genomic probe that recognizes two transcripts, corresponding to the inducible and constitutive isoforms of HSP70 (Colotta et al., 1990; Petronini et al., 1993). The results in Figure 3(a) show that the presence of 25 mM betaine during a 4 h incubation of 3T3 cells in hypertonic medium completely prevented the marked induction of the 2.7 kb transcript that occurred in the absence of betaine (hyperosmotic conditions also caused a parallel but smaller increase in the 'constitutive' 2.4 kb transcript, again abolished by the presence of betaine). Figure 3(b) shows not only similar results with SV-3T3 cells, but also that even 2.5 mM betaine caused some decrease in the induction of the 2.7 kb transcript.

Effect of betaine on colony formation by SV-3T3 cells exposed to osmotic stress

The poor ability of SV-3T3 cells, by comparison with 3T3 cells, to adapt to hyperosmotic stress was demonstrated previously by

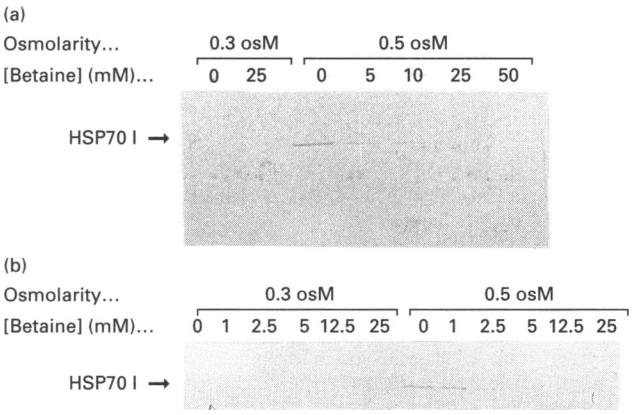

Figure 1 Accumulation of inducible HSP70 in cells during hypertonic incubations

Control cells were incubated in isotonic medium (0.3 osM) and test cells in hypertonic medium (0.5 osM) in the presence of the indicated concentrations of betaine. Cell proteins were then extracted, separated by SDS/PAGE, blotted on to nitrocellulose and made to react with monoclonal antibody directed against the inducible isoform of HSP70 (HSP70 I). The Western blots of the test cells were scanned with a computing densitometer (Molecular Dynamics) to quantify the results (see Table 1). (**a**) 3T3 cells after 16 h incubations; (**b**) SV-3T3 cells after 24 h incubations.

Table 1 Densitometer measurements of Western blots

The Western blots illustrated in Figure 1 were scanned with a computing densitometer (Molecular Dynamics) to provide a quantitative estimation of the relative intensities of the bands.

Cells	Betaine concn. (mM)	Relative intensity (arbitrary units)
(a) 3T3	0	100
	5	40
	10	25
	25	13
	50	10
(b) SV-3T3	0	100
	1	108
	2.5	51
	5	0
	12.5	0
	25	0

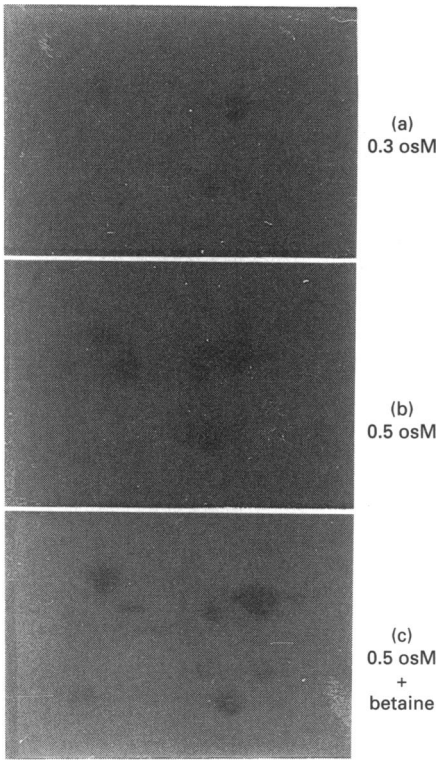

Figure 2 Induction of HSP70 synthesis during hypertonic incubation of cells

3T3 cells were incubated for 6 h in the presence of L-[^{35}S]methionine in (**a**) isotonic medium (0.3 osM), (**b**) hypertonic medium (0.5 osM), or (**c**) hypertonic medium also containing 25 mM betaine. After a 5 min chase period, proteins were extracted from the cells and separated by two-dimensional PAGE, and those labelled were detected by autoradiography, as described in the text. Only the zone containing the inducible HSP70 is shown.

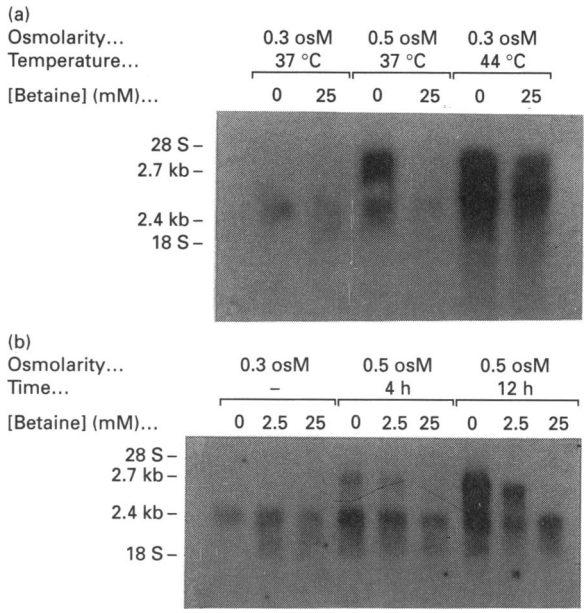

Figure 3 Expression of mRNA for HSP70

(**a**) 3T3 cells were incubated either for 4 h in isotonic or hypertonic media, or for 0.5 h at 44 °C followed by 3.5 h at 37 °C in isotonic medium. Each condition was duplicated with the addition of 25 mM betaine, as indicated. (**b**) SV-3T3 cells were incubated for 4 h or 12 h in isotonic or hypertonic media containing 0, 2.5 or 25 mM betaine, as indicated. Total cellular RNA was then extracted and analysed for the detection of HSP70 mRNA by Northern blotting as described in the text.

Table 2 Enhancement of betaine of cell adaptation of osmotic stress

SV-3T3 cells were grown for 2 days under isotonic conditions in complete culture medium. Then they were incubated for a further 24 h either under the same conditions or in hypertonic medium (0.5 osM) in the presence and absence of betaine, as indicated. Viable cells were identified as described in the text, and 400 cells from both control and hypertonic samples were seeded and left to grow for 5 days. Colony formation by these cells was evaluated as described in the text. Results from two independent experiments are given.

		No. of colonies formed by cells from:		
Expt.	Addition	Isotonic medium	Hypertonic medium	(% of control)
A	None	174	0	0
	Betaine (2.5 mM)	170	50	29
	Betaine (25 mM)	188	99	53
B	None	190	4	2
	Betaine (2.5 mM)	200	80	40
	Betaine (25 mM)	192	110	57

monitoring cell viability and plating efficiency (Petronini et al., 1993). The results in Table 2, which expresses cell survival in terms of ability to form colonies, confirm this finding and show further that the presence of betaine during the hypertonic incubation significantly increased cell survival.

Response to heat shock

Cells were incubated with betaine during exposure to heat shock (44 °C for 30 min) to see if it also modulates HSP70 gene expression under these conditions. Cellular accumulation of inducible HSP70 was monitored with the use of the monoclonal antibody (as in Figure 1) and gene transcription was assayed by Northern blotting (as in Figure 3). Although the response to the heat shock was clearly demonstrated by both Northern (Figure 3a) and Western (Figure 4) blotting, no convincing effect of betaine (25 mM) was detected.

Uptake of betaine by SV-3T3 cells

Figure 5 shows that betaine was not only taken up by SV-3T3 cells but also accumulated, so that its intracellular concentration was higher than that in the medium under both isotonic and hypertonic conditions. The extent of the accumulation, however, was much greater under the latter conditions, the cellular concentration reaching about 195 mM when the external concentration was 25 mM. Analysis of the labelled material in the cell extracts by t.l.c. revealed only a single species that co-migrated with the original [^{14}C]betaine.

When the cells were incubated for 5 min under isotonic conditions with 1 mM betaine, the uptake was 24 ± 2 (mean $\pm$ S.D.) nmol/5 min per mg of protein. Repetition with Li^+ substituted for most of the Na^+ in the medium gave only 4.0 ± 0.6 nmol/5 min per mg of protein. Hence it is most likely that at least 80% of betaine uptake is Na^+-dependent.

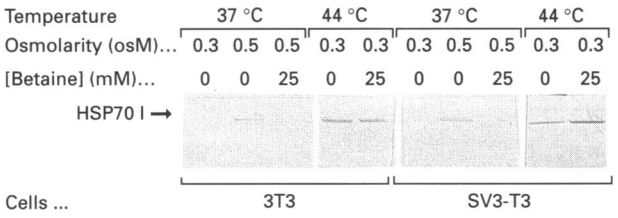

Figure 4 Lack of effect of betaine on HSP70 induction by thermal stress

For heat shock, the cells were incubated at 44 °C for 0.5 h and then at 37 °C for 15.5 h. All other cells were incubated for 16 h under the indicated conditions. Cellular proteins were then extracted, separated by SDS/PAGE, blotted on nitrocellulose and made to react with monoclonal antibody directed against the inducible isoform of HSP70 (HSP70 I).

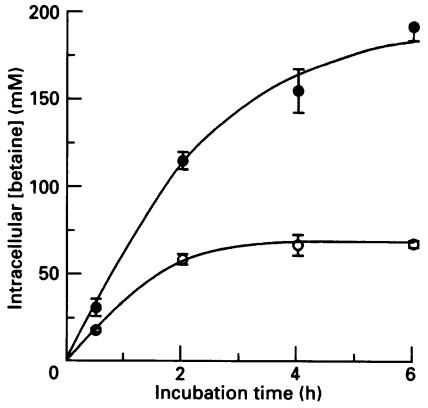

Figure 5 Uptake and accumulation of betaine by SV-3T3 cells

SV-3T3 cells were incubated for 6 h in the presence of 25 mM betaine in isotonic (0.3 osM) and hypertonic (0.5 osM) media. At the indicated times, both betaine uptake and cell volumes were measured as described in the text. Mean values (±S.D.) from three independent measurements are given. Key: ○, control isotonic medium; ●, test hypertonic medium.

DISCUSSION

Induced expression of the HSP70 gene in 3T3 and SV-3T3 cells occurs in response not only to heat shock but also to hypertonic stress (Petronini et al., 1993). This common response to two different primary stress signals indicates that the (as yet unknown) intracellular messages triggered by these signals must eventually converge. Since the results described here show that the presence of betaine decreases the induction of HSP70 gene expression normally resulting from hypertonic stress, but not that caused by heat shock, it is clear that betaine must act before the convergence of the messages. The finding that SV-3T3 cells can accumulate betaine to such high concentrations during incubation under hypertonic conditions supports an obvious explanation, that uptake and accumulation of betaine simply counteracts the imposed osmotic pressure and thus eliminates the primary stress signal. The uptake of betaine clearly occurs via an active transport system and the most likely source of energy is the concentration gradient of Na^+ ions. The marked increase in the uptake and accumulation of betaine by cells incubated under hypertonic conditions shows that hyperosmotic stress also stimulates this transport process in some way.

These observations and this interpretation partially parallel those made by others working with Madin–Darby canine kidney (MDCK) cells. HSP70 gene expression is similarly induced in MDCK cells in response to hyperosmotic stress (Cohen et al., 1991) and these cells accumulate betaine via a Na^+-dependent transporter that is osmotically regulated (Nakanishi et al., 1990; Garcia-Perez and Burg, 1991) and has recently been cloned (Yamauchi et al., 1992). The use of MDCK cells for this work stems from investigations of kidney function and the fact that hypertonicity in the renal medulla is fundamental to its normal function. Thus some kidney cells need to adapt to hypertonicity; but it is not obvious that cells from other tissues should also require this ability, and Cohen et al. (1991) noted that it remained "to be determined whether HSP70 induction by hyperosmotic stress is characteristic of other eukaryotic cells". Our findings suggest that all of these responses may well be a more widespread characteristic of eukaryotic cells.

The detailed properties of the betaine transport system in SV-3T3 cells remain to be established before the above interpretation can be accepted to account fully for all the observations. In addition to more thorough checks of its source of energy and investigation of the nature of its stimulation under hypertonic conditions, there is a need to examine the quantitative aspects in more detail. For example, although the cellular concentration of betaine attained after 6 h of incubation in the presence of 25 mM extracellular betaine was sufficient to counteract completely the imposed osmotic pressure, the situation with lower external concentrations of betaine has yet to be studied. At present, therefore, it is not certain that the effects of betaine detected when its extracellular concentration was only 2.5–5 mM are explicable in terms of an intracellular concentration sufficient to balance the imposed osmotic pressure. There is, however, another possibility worth noting. As pointed out by Cohen et al. (1991), a potential role for the HSP70 protein in the response to hyperosmotic stress is that of maintaining protein conformation in the presence of raised intracellular ion concentrations that accompany initial cell shrinkage. Since some compatible solutes have been shown to act in this fashion *in vitro* (see Yancey et al., 1982), it seems possible that intracellular betaine might also have such a protective role, in addition to that of increasing cellular osmolarity.

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