Amino Acids (2001) 20: 363–380

Amino
Acids

The effect of heat shock on amino acid transport and cell volume in 3T3 cells

P. G. Petronini, A. E. Caccamo, R. R. Alfieri, M. A. Bonelli, and **A. F. Borghetti**

Dipartimento di Medicina Sperimentale, Sezione di Patologia Molecolare ed Immunologia, Università degli Studi di Parma, Italy

Accepted July 27, 2000

Summary. In 3T3 cells temperatures higher than physiological stimulated amino acid transport activity in a dose-dependent manner up to 44°C. However, the temperature increase did not induce widespread transport increase of all other nutrients tested. The activities of both amino acid transport systems A and ASC were enhanced within a few minutes following cell exposure to increased temperature. The maintenance of this effect required continuous exposure of the cells to hyperthermia. Kinetic analysis indicated that the stimulation of the activity of transport System A occurred through a mechanism affecting Vmax rather than Km. The continuous presence of cycloheximide did not prevent the transport changes induced by hyperthermia. These results suggest that the increased amino acid uptake reflects an activation or relocation of existing amino acid transport proteins. During the hyperthermic treatment, the content of ninhydrin-positive substances (NPS), mostly amino acids, increased within the cells and the accumulation of these compatible osmolytes was parallelled by an increase in cell volume. The withdrawal of amino acids from the culture medium immediately before and during the shock phase counteracted the increase and reduced the NPS content but did not prevent the increase in amino acid transport, the cell swelling and the induction of the heat shock response.

Keywords: Amino acids – Amino acid transport – Cell volume – Heat shock – HSP70

Introduction

Cells respond to elevated temperature by increasing expression of genes coding for heat shock proteins (HSPs). The same proteins are also elicited by a wide range of noxious stimuli including nutrient starvation, hypoxia, hypertonicity, heavy metals, arsenite, amino acid analogs, ethanol (Hightower, 1991). The expression of heat shock genes has been widely

studied and the elucidation of their transcriptional regulation, evaluated as a paradigm for inducible genomic responses, has provided many insights into the molecular mechanisms of cellular adaptation to stress (Morimoto et al., 1992). However, few studies have been devoted to the early biophysical and biochemical events that occur immediately after the cells have been exposed to the stress and that precede or accompany the HSF activation. For instance, possible changes in membrane integrity and/or function have received scant attention (Welch, 1990). In spite of the importance of small nutrient (e.g. amino acids, glucose) availability and, hence, their transport regulation for the survival of mammalian cells, the transport of nutrients in cells exposed to an adverse environment has been little studied. Wallack and coworkers have investigated the modulation of amino acid transport by hyperthermia, although they reported conflicting results for thymocytes (transport stimulation by cell exposure to a 0.5 h heat shock) (Lin et al., 1978) and for a human T-cell line (transport impairment by cell exposure to hyperthermia lasting more than 1 hr) (Kwock et al., 1978). Another group reported an early increase of hexose transport in heat-shocked BHK cells and they concluded that stimulation of sugar uptake was a general response of stressed cells, in common with the induction of stress proteins (Warren et al., 1986). It has been suggested that some amino acids have a protective role in cells exposed to hyperthermia (Vidair and Dewey, 1987). Deprivation of CHO cells of amino acids has been shown to sensitize them to killing by heat, and that specific amino acids, including non-metabolizable analogs, reverse heat sensitivity. In addition, the particular role of glutamine on the heat shock response has been studied recently in detail. By the measurement of free amino acid, a correlation was noted between levels of glutamine and extent of HSPs expression level in Drosophila Kc cells, heat-shocked in the presence or absence of this amino acid (Sanders and Kon, 1991). Furthermore, glutamine availability has been found to lead to a marked elevation in the steady-state level of HSP70 mRNA and a dose-dependent increase in the production of HSPs in kidney cells (Nissim et al., 1993).

Moreover the addition of phenylalanine to cells starved of all other amino acids led to the classic heat shock induction of HSP72. Co-inclusion of alanine prevented the HSP72 induction by phenylalanine but not that caused by heat stress (Plakidou-Dymock and McGivan, 1994).

Cell volume is an important biophysical parameter related to the osmotic characteristics of the environment (Hoffmann and Dunham, 1995). Complex mechanisms are involved in the maintenance of constant volume on exposure of mammalian cells to osmotic changes. Alterations in the uptake of ions and compatible organic osmolytes (mostly amino acids) have been described (Yancey et al., 1982). We have recently reported that cell volume increases significantly during the heat shock response (Alfieri et al., 1996). Assuming that a cell volume increase is associated with an increased uptake of adequate solutes, and in view of the contradictory results on changes of amino acid transport during heat shock (Lin et al., 1978; Kwock et al., 1978; Warren et al., 1986), we were prompted to study in detail the relationship between amino acid transport and cell volume during the heat shock response. We were

particularly interested to find out whether amino acid availability and changes of amino acid transport activity and cell volume and related to the induction of HSP70 gene expression by heat shock.

Materials and methods

Chemicals

α-[^{32}P]dCTP, γ-[^{32}P]ATP, 3-O-methyl-D-[1-^{3}H]glucose, L-[U-^{14}C]glutamine, [^{3}H]thymidine, L-[5-^{3}H]proline, L-[4,5-^{3}H]leucine and [1,4-^{14}C]putrescine were obtained from Amersham International, Amersham, Bucks., 2-[1-^{14}C]methylaminoisobutyric acid (MeAIB) was obtained from Dupont/New England Nuclear (Boston, MA). [^{14}C]betaine was prepared from [^{14}C]choline by enzymic oxidation exactly as described previously (Petronini et al., 1993). Choline oxidase and betaine were bought from Sigma Chemical Co., Poole, Dorset, UK. The probe for human HSP70 gene (pH 2.3) was kindly provided by Dr. Richard I. Morimoto (Evanston, IL, USA), and was obtained from Dr. Luisa Schiaffonati (Milan, Italy). Probe for 28S rRNA was obtained from Dr. Lorenza Tacchini (University of Milan, Italy). A double-stranded synthetic consensus HSE (upper strand 5′-CTCGAAGCTTCTAGAACGTTCTAG-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.).

Cell cultures

Balb/c 3T3 cells (clone A31) were kept in culture for up to 2 months; fresh cultures were started again from frozen stocks. The cells were maintained in minimum essential medium (MEM) containing penicillin (100 units/ml) and streptomycin (100μg/ml) supplemented with 10% (v/v) fetal calf serum (FCS). All cultures were kept in an incubator at 37°C in a water-saturated 5% CO_2 atmosphere in air and were passaged twice per week. They were regularly checked for mycoplasmic contamination with a mycoplasma detection kit (Boehringer, Mannheim, Germany). For the experiments, cells were seeded at a density of about 10^4 cells/cm^2 and then grown for 3 days. Twelve hours before the start of the experiments, the culture medium was changed to avoid nutrient starvation.

Stress conditions

Cells were either exposed or not for 30 min at 44°C (heat shock), except when otherwise stated. During heat shock, cells were incubated in a water bath in a water-saturated 5% CO_2 atmosphere in air. In some experiments, after heat shock the monolayers were transferred to fresh medium and kept in an incubator at 37°C in a water-saturated 5% CO_2 in air for the indicated time (recovery phase).

Determination of cell volume

Intracellular volumes were estimated by measurement of the steady-state distribution of 3-O-methyl-D-[1-^{3}H]glucose as described previously (Tramacere et al., 1984a) using the method of Kletzien et al. (1975).

Uptake of radioactive nutrients

The following procedure was used to measure the rates of uptake of nutrients: after incubation in the appropriate medium, cell monolayers were washed for 1 min with Earle's balanced salt solution containing 0.1% glucose (EBSSG) and immediately incubated for the desired time in EBSSG containing the labelled substrate at 37°C (control) and 44°C (test). When necessary, Na^+ in the medium was replaced by choline ions. The incubations were stopped by quickly washing the cells with cold EBSSG, after which they were extracted with 5% trichloroacetic acid (TCA). Radioactivity in samples of the acid extracts was measured by scintillation counting. Cell protein, precipitated by TCA, was dissolved in 0.2 N NaOH, and its concentration was determined by a dye-fixation method (Bio-Rad) using bovine serum albumin as standard (Bradford, 1976).

Ninhydrin-positive substances (NPS)

The intracellular content of NPS was measured by the method of Law and Turner (1987) and was expressed as nmol/mg of protein.

Gel-mobility-shift assay

For the gel-mobility-shift assay, a whole-cell extract (20 μg of protein) was incubated with a ^{32}P-labelled HSE oligonucleotide as previously described in detail (Petronini et al., 1995).

Northern blotting

Total RNA was extracted from cultured cells using the Ultraspec RNA Isolation System from Biotecx (Chomczyski et al., 1987). RNA samples (10 μg) were fractionated by electrophoresis through 1% agarose gels and the separated bands transferred to nylon filters. The quality and quantity of RNA blotted on the membranes were checked by U.V. absorption. Plasmid pH 2.3 containing a fragment of the human HSP70 gene and 28S rRNA probe were nick-translated and used. Hybridization, washing and autoradiography were then carried out as described elsewhere (Petronini et al., 1993).

Results

Effect of hyperthermia on amino acid transport activity

The exposure of 3T3 cells to hyperthermia up to 44°C directly affected the initial rate of uptake of L-proline and betaine (NNN-trimethylglycine) (Table 1). In contrast, the uptake of another neutral amino acid as L-leucine as well as the uptake of other nutrients such as 3-O-methyl-D-glucose (a glucose analogue), putrescine and thymidine were not significantly affected during hyperthermia (see Table 1). In 3T3 cells we have previously shown that L-proline is a preferential substrate of transport system A (Borghetti et al., 1980). Moreover, the uptake of betaine by fibroblasts occurs via the Na^+-dependent A system for amino acid transport, rather than by a system more specific for betaine (Petronini et al., 1994). In fibroblasts, it has been reported that transport system ASC represents the primary mediation for L-glutamine

Table 1. Effect of hyperthermia on uptake of different nutrients

	Nutrient uptake (nmol/min per mg of protein)		
	Control, 37°C	Heat Shock, 44°C	Increase (%)
L-Leucine	12.290 ± 1.390	14.930 ± 2.130	21
L-Proline	1.280 ± 0.130	2.510 ± 0.380*	96
Betaine	0.970 ± 0.120	2.000 ± 0.570*	106
L-Thymidine	1.330 ± 0.190	1.230 ± 0.150	–
3-O-methyl-D-glucose	0.420 ± 0.050	0.470 ± 0.030	12
Putrescine	0.052 ± 0.006	0.057 ± 0.005	10

3T3 cells were incubated for 15 min in culture medium at 37°C or at 44°C. After this period, the cells were washed for 1 min with EBSSG. Initial rates of nutrients uptake were measured by incubating the cell monolayers for 1 min at 37°C or at 44°C in EBSSG. Thymidine and putrescine were present at 0.01 mM; the other substrates at 0.1 mM. The values shown are the means of four independent determinations. The experiment, repeated three times, yielded similar results. *Significantly different ($p < 0.01$) from respective controls.

entry (Dall'Asta et al., 1990). As there is considerable overlap in substrate specificity between the three main systems of mediation for neutral amino acids (systems A, ASC and L) (Guidotti et al., 1978), we performed an operational discrimination between these transport systems in 3T3 cells. The measurement of amino acid transport rate in the presence and absence of sodium ions and of adequate levels of methyl-aminoisobutyric acid (MeAIB), the best characterizing substrate of system A, allowed us to identify the transport systems involved and to evaluate the contribution of a specific system of mediation to the total uptake of a single substrate (Christensen, 1975). Table 2 presents the results of the discrimination analysis for L-proline and L-glutamine transport measured at 37°C and 44°C. It can be seen that in control cells L-proline was primarily taken up by a Na^+-dependent MeAIB-inhibitable mediation (system A). The residual uptake of L-proline by these cells occurred through a Na^+-independent transport system (presumed to be system L), whereas no contribution from the Na^+-dependent, MeAIB-non inhibitable route (system ASC) was observed. It is of note that hyperthermia increased the activity of both systems A and L. However, whereas the contribution of the A system to the L-proline total uptake was more relevant at 44°C than at 37°C, the Na^+-independent component of L-proline total uptake (system L) was significantly reduced at 44°C. It can be seen from the data presented in Table 2 that L-glutamine was taken up at 37°C only by the ASC system and by a Na^+-independent route. Hyperthermia induced a significant increase in L-glutamine uptake by system ASC and a novel albeit small utilization of system A, but had no significant influence on its uptake by system L. Taken together, these results indicate that the activities of both transport systems A and ASC are preferentially enhanced by exposure of 3T3 cells to heat.

Table 2. Effect of hyperthermia on the influx of L-proline and L-glutamine by specific systems of mediation. A discrimination analysis

Amino acid	Transport component	Amino acid uptake (nmol/min per mg of protein)	
		Control, 37°C	Heat Shock, 44°C
L-Proline	Na^+-dependent and MeAIB sensitive	1.25 (59)	3.19 (71)*
	Na^+-dependent and MeAIB-insensitive	0.00 (0)	0.00 (0)
	Na^+-independent	0.86 (41)	1.32 (29)*
L-Glutamine	Na^+-dependent and MeAIB-sensitive	0.00 (0)	4.20 (10)
	Na^+-dependent and MeAIB-insensitive	13.06 (47)	22.54 (53)*
	Na^+-independent	14.56 (53)	15.90 (37)

3T3 cells were incubated for 15 min in culture medium at 37°C or at 44°C. After this period, the cells were washed for 1 min with EBSSG and incubated for 1 min in Na^+-containing or in Na^+-free EBSSG, where choline replaced the cation in the sodium salts of the EBSSG mixture, in the presence of labelled 0.1 mM L-proline or 1 mM L-glutamine. The Na^+-dependent transport components were discriminated as MeAIB-inhibitable (system A) and MeAIB-non-inhabitable (system ASC) by adding the inhibitor model substrate at 10 mM final concentration. The values shown are the means of four independent determinations. The amino acid uptake by each transport component, as a percentage of total uptake, is shown in the brackets. *Significantly different ($p < 0.01$) from respective controls.

Time-course and dose-response of hyperthermic treatment on transport system A

The non-metabolizable analog MeAIB has been shown to be a good characterizing substrate of transport system A in several cell types (Guidotti et al., 1978). Figure 1 shows that incubation of 3T3 cells at 44°C resulted in a progressive increase in the initial rate of MeAIB uptake; furthermore, increases in uptake were detected as early as 2 min after exposing the cells to hyperthermia; MeAIB uptake continued to increase subsequently reaching a maximum after 15 min. Later on the heat-stimulated MeAIB entry decreased, and returned to control values when the cells were transferred and incubated for 5 min at 37°C. The hyperthermic treatment also stimulated L-proline uptake in a temperature-dependent manner up to 42–44°C and the transport stimulation was still present but with reduced intensity at 45°C (result not shown).

Initial rate kinetics

Initial velocities of L-proline transport were measured over a range of substrate concentrations (from 0.1 to 2.5 mM) in 3T3 cells exposed for 15 min

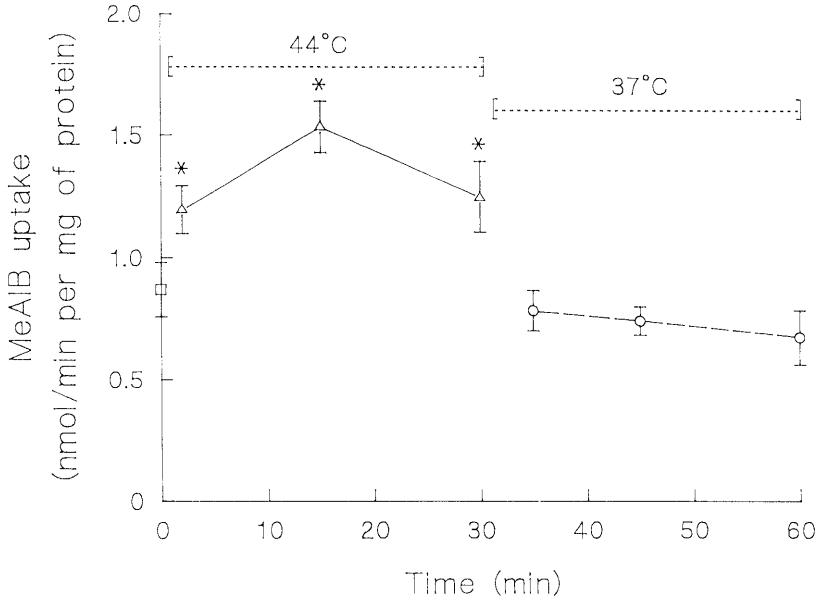

Fig. 1. Time-dependent changes in MeAIB transport during hyperthermic treatment (heat shock) and on return to physiological temperature (recovery). 3T3 cells were incubated at 37°C (□) or at 44°C (△) for the indicated intervals. Cells exposed for 30 min at 44°C were subsequently incubated for up to 30 min at 37°C (○). Initial rates (1 min) of uptake of 0.1 mM MeAIB were measured as described in Materials and methods. The values are shown with the mean ± S.D. for six independent determinations. * Significantly different ($p < 0.01$) from respective controls

to increased (44°C) or physiological (37°C) temperatures. The data were analyzed using the Fig-P curve-fitter programme (Biosoft) in terms of a saturable, Na^+-dependent component of influx (system A) plus a non-saturable, Na^+-independent component. The results are shown in Fig. 2. For control cells incubated at 37°C, the best-fit equation for the saturable component corresponded to values of 0.9 mM for *Km*, and 15.3 nmol of L-proline/min per mg of protein for *Vmax*. For cells incubated at 44°C the corresponding values were 0.72 mM for *Km* and 23.4 nmol/min per mg for *Vmax*. These results suggest that the stimulation of amino acid transport system A following hyperthermic treatment of 3T3 cells occurred via a mechanism which slightly affects the affinity of the carrier for substrate but significantly increases its capacity ($p < 0.01$).

Effect of cycloheximide on heat shock-induced amino acid uptake

The finding that exposing 3T3 cells at 44°C promotes L-proline influx by accelerating *Vmax* rather than by altering the *Km* of transport activity, is consistent with an increased availability (or synthesis) of active transport molecules at the cell membrane. We therefore examined whether the hyperthermia-induced changes in amino acid transport required protein

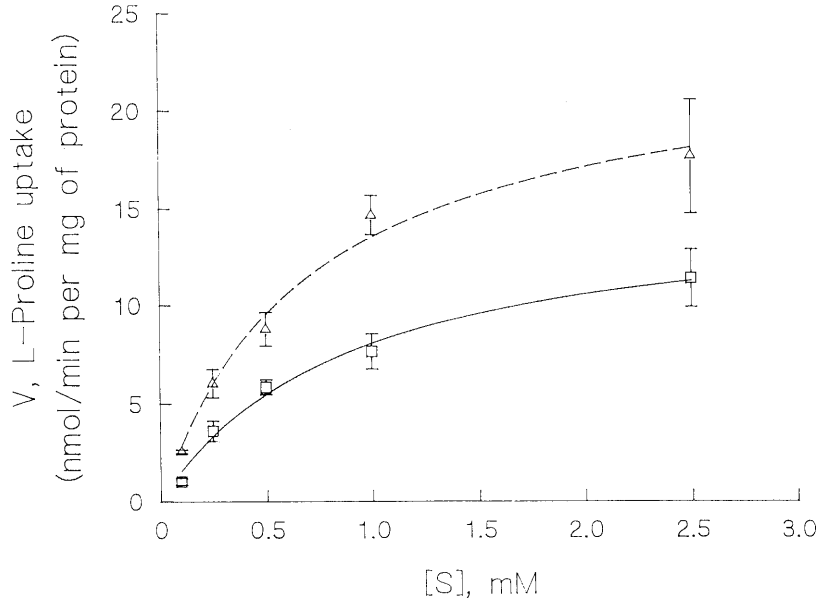

Fig. 2. Effect of the hyperthermic treatment on the kinetic parameters of L-proline uptake. Initial rates (1 min) of L-proline uptake were determined in control (□, 37°C) and 30 min-treated (△, 44°C) cell monolayers in the absence or presence of Na^+ ions as described in the caption of Table 1. Mean values of Na^+-dependent influx (±S.D.) from three measurements are given for each L-proline concentration tested. The curves are drawn with the use of the Fig-P curve-fitter program (Biosoft)

synthesis. The presence of 10 μg/ml cycloheximide, a concentration known to lead to a more than 90% inhibition in protein synthesis in 3T3 cells, did not prevent the heat shock-induced increase in MeAIB transport activity (data not shown). This would tend to rule out a requirement for a newly synthesized transporter component for the stimulation of system A.

Time-course of MeAIB uptake

Control and heat-exposed cells took up MeAIB, the characterizing substrate of system A, in an almost linear way throughout the 30 min period of incubation. However, in cells exposed to hyperthermia, MeAIB accumulation was markedly enhanced with respect to the control cells (see Fig. 3). This result obtained with an analogous and non-metabolizable amino acid suggests that natural amino acids could be accumulated in cells during exposure to heat.

NPS content and cell volume during heat treatment

The effect of incubating 3T3 cells at 44°C on the intracellular level of ninhydrin-positive soluble molecules [mainly amino acids, (Law and Turner,

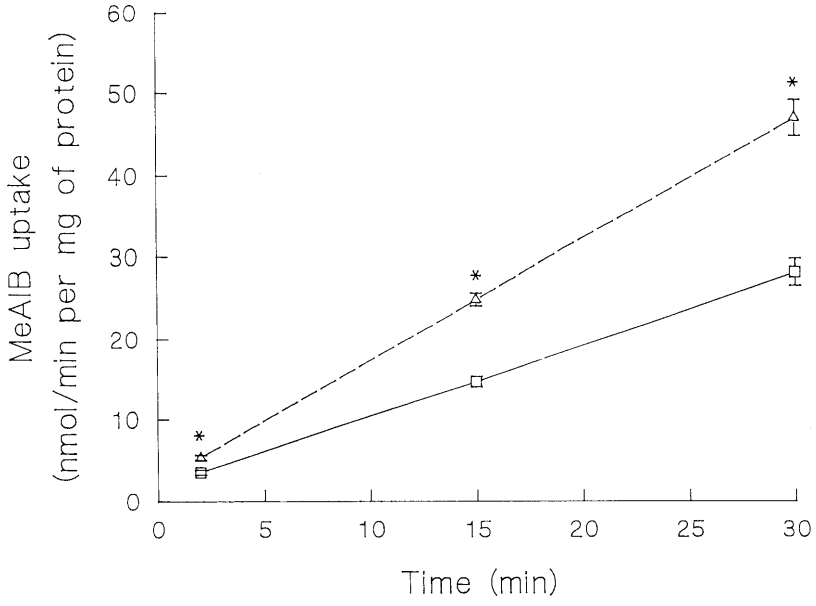

Fig. 3. Uptake and accumulation of MeAIB in control and hyperthermic-treated cells. 3T3 cells were incubated for the indicated times at 37°C (□) or at 44°C (△) in the presence of 1 mM radioactive MeAIB in complete culture media. Incubation was terminated as described in Materials and Methods. Mean values (±S.D.) from six independent measurements are given. * Significantly different ($p < 0.01$) from respective controls

1987)] was determined throughout the heating period. As shown in Fig. 4A, intracellular NPS content altered little during the first 5 min of cell exposure to 44°C. However, a progressive increase in NPS level was observed thereafter. After 30 min of hyperthermic treatment, NPS levels were more than 40% higher than control. As shown in Table 3, at the end of the hyperthermic treatment and on reincubation at 37°C, NPS content soon returned to control levels.

Since uptake and accumulation of small solutes (ions and compatible osmolytes including amino acids) are permissive to adjustments in cell volume on alteration in osmotic conditions [regulatory volume increase or decrease following cell incubation in hypertonic or hypotonic medium, respectively (Hoffmann and Dunham, 1995)] and in view of the increased transport activity and accumulation of some amino acids observed in cells exposed to heat, we evaluated the changes in cell volume in 3T3 cells during exposure to hyperthermia. Confirming our preliminary observation (cf Table 1 of Alfieri et al., 1996), Table 3 shows that the volume of cells incubated in medium at 44°C for 30 min was significantly greater than that of control cells. However, on reincubating at physiological temperature, the heat-shocked cells quickly regained their previous volume. In an attempt to determine cell swelling at early time points of hyperthermic treatment, we measured the distribution of the volume tracer (labelled methylglucose) in cells preincubated with the tracer up to its equilibrium distribution. Under these conditions, we observed

Table 3. Effect of hyperthermia on cell volume and NPS content

	Control, 37°C	Heat shock, 44°C	Heat shock + recovery
Cell volume (μl per mg of protein)	7.71 ± 0.64	9.87 ± 0.62*	7.14 ± 0.74
NPS content (μmol per mg of protein)	0.71 ± 0.08	1.10 ± 0.05*	0.74 ± 0.11

3T3 cells were incubated for 30 min in culture medium at 37°C (Control) or at 44°C (Heat shock). At the end of this period, the cells were incubated at physiological temperature for 30 min (recovery phase) where indicated. Cell volume and NPS content were determined as described in Materials and methods. The values shown are means ± S.D. of six independent determinations. *Significantly different ($p < 0.01$) from respective controls.

Table 4. Effect of amino acid depletion on proline transport, NPS content and cell volume during hyperthermia

		Control, 37°C	Heat shock, 44°C
L-Proline uptake (nmol/min/mg of protein)	+aa	1.42 ± 0.11	2.01 ± 0.11*
	−aa	1.70 ± 0.14	2.35 ± 0.08*
NPS content (μmol per mg of protein)	+aa	0.48 ± 0.03	0.87 ± 0.02*
	−aa	0.28 ± 0.01	0.24 ± 0.04
Cell volume (μl per mg of protein)	+aa	8.60 ± 0.80	12.5 ± 1.03*
	−aa	8.30 ± 0.80	11.2 ± 0.82*

For amino acid depletion (−aa condition), 3T3 cells were preincubated for 30 min at 37°C in an amino acid free medium. At the end of this period cell monolayers were incubated for 30 min at 37°C (Control) or at 44°C (Heat shock) in the presence or absence of amino acids (+aa or −aa, respectively). At the end of this period 0.1 mM L-proline uptake (1 min), NPS content and cell volume were determined as described in Materials and methods. The values shown are the means ± S.D. of six independent determinations. The experiment, repeated three times, yielded similar results. *Significantly different ($p < 0.01$) from respective controls.

a significant increase in volume after 15 min of incubation at 44°C with a maximum after 30 min of hyperthermia (see Fig. 4B).

Effect of depletion of amino acids on proline transport, NPS content and cell volume during heat shock

By removing amino acids from the culture medium immediately before and during heat shock, we tried to correlate in a more direct way amino acid availability, transport and accumulation with the heat-dependent cell volume increase. As shown in Table 4, the increased activity of L-proline transport induced by hyperthermia was not affected when cells were incubated in an amino acid-free medium. NPS content, however, was markedly reduced both

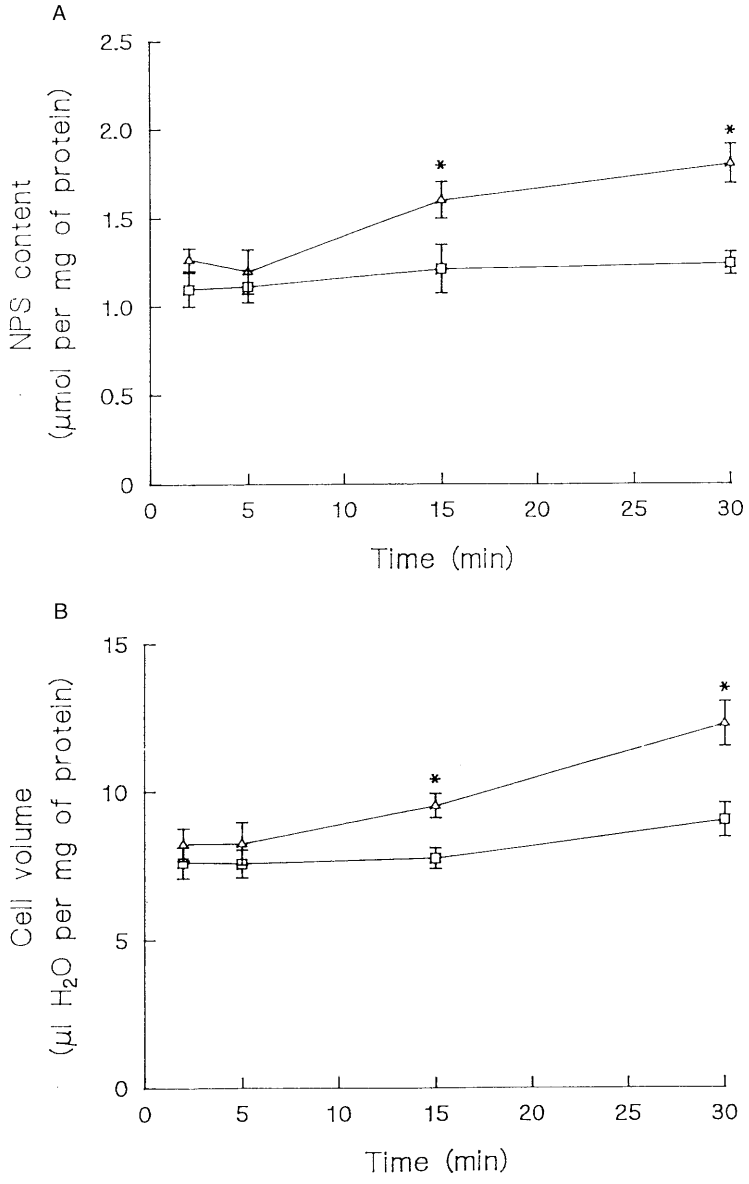

Fig. 4. Kinetics of NPS content and cell volume in 3T3 cells exposed to hyperthermic treatment. **A** Cells were incubated in complete culture medium at 37°C (□) or at 44°C (△). At the times indicated, NPS content was assayed as described in Materials and methods. Values shown are means ± S.D. of six independent determinations. **B** Cells were preincubated at 37°C with labeled 3-O-methyl-D-glucose up to its equilibrium distribution (30 min) and then further incubated in the continuous presence of the tracer at 37°C (□) or at 44°C (△) for the indicated periods. Values shown are means ± S.D. of six independent determinations. * Significantly different ($p < 0.01$) from respective controls

in control and heat-exposed cells. Cell volume was simultaneously recorded in sister cultures: as presented in the same table, 3T3 cells though deprived of amino acids remain significantly more swollen when exposed to heat than control cells.

Effect of amino acid starvation on HSF activation and HSP70 mRNA expression

There are contradictory results in the literature whether amino acid deprivation over a period of hours can affect the expression of stress proteins (Sanders and Kon, 1991; Nissim et al., 1993; Plakidou-Dymock and McGivan, 1994). Thus, we addressed the question whether amino acid availability before and during heat shock could affect the induction of HSP70 gene expression by heat shock in 3T3 cells. Two steps of gene expression has been then analyzed: HSF activation and HSP70 mRNA induction.

Electrophoretic gel-mobility-shift assays were carried out with total cell extracts from cultures exposed to heat shock and a HSE from the inducible human HSP70 gene (Alfieri et al., 1996). Northern blotting analysis was carried out with a human genomic probe that recognizes two transcripts in these cells, a constitutive one of about 2.4 Kb and an inducible transcript of about 2.7 Kb (Petronini et al., 1996; Colotta et al., 1990). As shown in Fig. 5, a 30 min heat shock strongly activated HSF-DNA binding, led to the appearance of the 2.7 Kb inducible HSP70 gene transcript and a definite increase of the constitutive one. However, when cells were incubated in an amino acid-free medium 30 min before and during the heat phase, HSF-DNA binding (see Fig. 5A) as well as the induced HSP70 mRNA expression (see Fig. 5B) were not significantly affected. Moreover, the same Fig. 5B also shows that the basal expression of the constitutive form of HSP70 mRNA was not affected by amino acid deprivation in cells not exposed to high temperature.

Discussion

Hyperthermia is the classical inducer of the heat shock response, which includes HSF activation, HSP70 gene transcription, HSP70 mRNA translation and HSP70 protein accumulation. We report here for the first time that during the early phase of a hyperthermic treatment 3T3 cells exhibit an increase in the activity of specific amino acid transporters (System A and ASC) as well as a consequent accumulation of NPS (mainly amino acids). There was also a heat dependent, concomitant increase in cell volume. The transport activity of neutral amino acid, increased at early (less than 5 min) time points of heat treatment and reached a maximum after 15–30 min. However, it immediately returned to control values on return of the cell incubation to physiological temperature. The possibility that amino acid transport change may be only a trivial effect of temperature increase, as expected for most chemical reactions, was soon ruled out because the heat

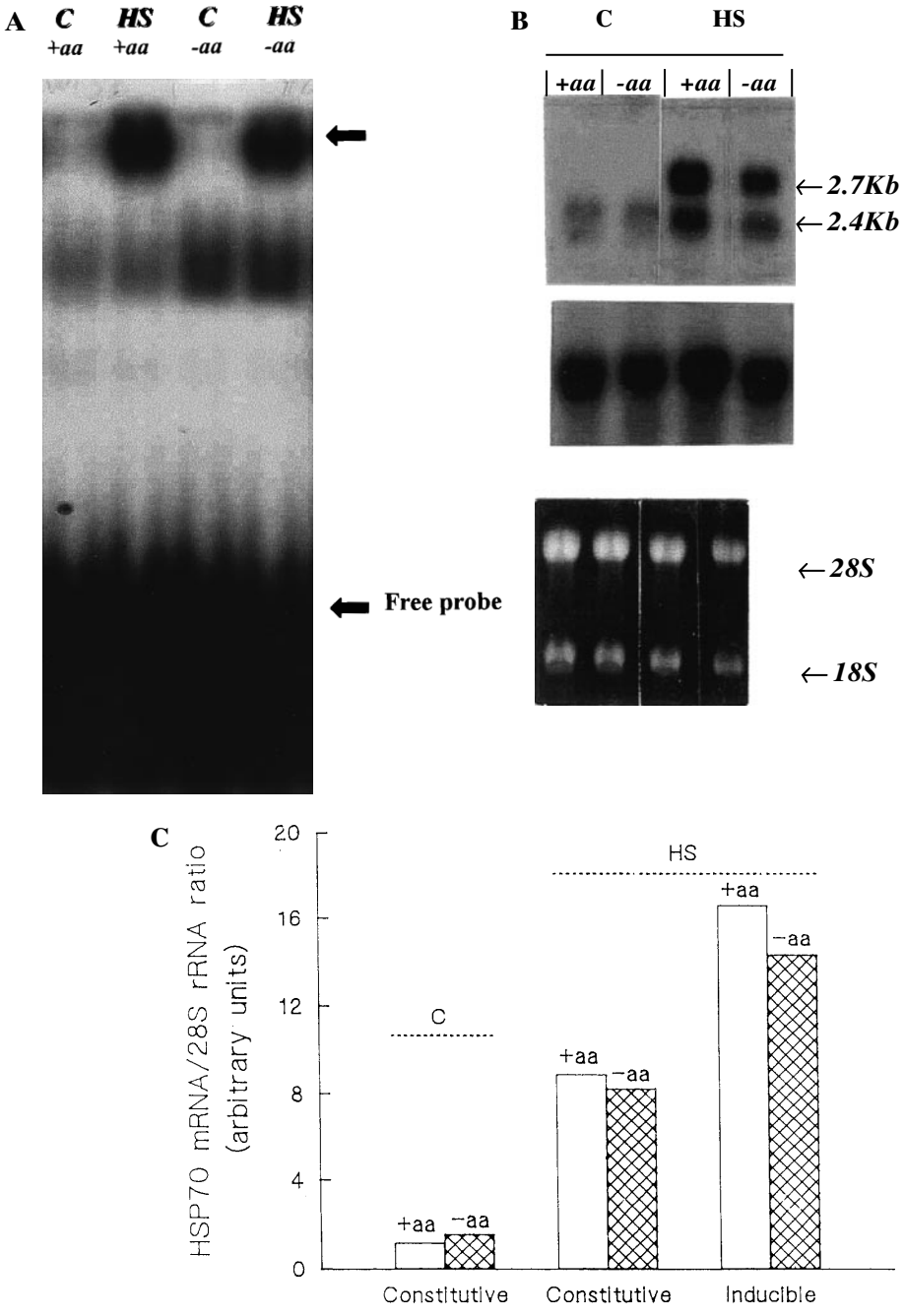

Fig. 5. HSF-HSE binding and HSP70 mRNA expression. For amino acid depletion (−aa condition), 3T3 cells were preincubated for 30 min at 37°C in an amino acid free medium. Then the cells were incubated at 37°C (*C*) or at 44°C (*HS*) for 30 min in the presence (+*aa*) or absence (−*aa*) of amino acids. **A** Whole-cell extracts were prepared for binding and gel-mobility-shift assay as described in Materials and methods. The position of the specific HSF-HSE complex is indicated by the arrowhead. **B** Total cellular RNA was extracted at the end of the shock period and analyzed for the presence of mRNA for HSP70 or for the 28S rRNA by Northern blotting, as described in Material and methods. **C** The levels of HSP70 mRNA were normalized to the levels of 28S rRNA and the data are presented in the histogram

treatment did not induce widespread up-regulation of the activity of all nutrient transporters tested.

An early increased hexose uptake has also been observed in BHK and CEF cells immediately following exposure to hyperthermia (Warren et al., 1986). These authors suggested that the increased hexose uptake, being protein synthesis-independent, reflected activation or relocation of existing protein transporters. They also regarded the increased hexose uptake as another indicator of the response to heat shock like that of HSP synthesis. It should be noted that their results documented for the first time an alteration in membrane transport associated with cellular stress. However, they did not report any increase in amino acid uptake during the heat shock. The apparent discrepancies between our observation of an increase in amino acid transport in 3T3 cells and those noted in BHK and CEF cells (Warren et al., 1986) can be accounted for by different temperatures chosen for measurement of amino acid transport activity.

Since two or three distinct transport systems may be involved in the uptake of a single amino acid (Guidotti et al., 1978), studies on specific amino acid transport must attempt to determine the exact systems involved in each case. By discrimination analysis, we inferred that there was a significant alteration in the transport activity of the Na^+-dependent, MeAIB-inhibitable component (system A) of L-proline uptake during heat shock. Interestingly, system A is thought to be where widely different factors or environmental stimuli converge to regulate amino acid transport in eukaryotic cells (Guidotti et al., 1978; McGivan and Pastor-Anglada, 1994). Since the activity of system L is known to be modulated by transtimulation, an increase in the Na^+-independent component (presumably system L) of L-proline transport after the hyperthermic treatment in undepleted cells could be ascribed to a different internal level of exchangeable amino acids (Guidotti et al., 1978; Tramacere et al., 1984b). L-glutamine enters fibroblasts mainly by the Na^+-dependent systems ASC and A and by a Na^+-independent route usually identified as system L. The relative contribution of these systems to the total saturable uptake of L-glutamine depends on the concentration of the amino acid and the nutritional state of the cell (Dall'Asta et al., 1990). It should be borne in mind that in most tissue culture media the concentration of L-glutamine is 5- to 20-fold greater than that of other amino acids (Eagle, 1955a,b; Eagle et al., 1956) and that L-glutamine is involved in cell volume control as a compatible osmolyte (Gazzola et al., 1991; Dall'Asta et al., 1994). During hyperthermia there was a faster uptake of this amino acid, and the predominant transport agency thus appeared to by system ASC.

Analysis of the kinetic parameters of amino acid transport system A suggests that there was either an increase in the number of functional carriers or a higher mobility of the substrate-carrier complex in the cell membrane. The fact that the stimulation of amino acid transport activity was not prevented by protein synthesis inhibitors and that normal transport activity was restored on return to physiological temperature, indicated that hyperthermia led to an increase in the mobility of the substrate-carrier complex or a faster relocation of existing protein transporters in the cell

membrane. That these hyperthermic effects on amino acid transport are not related to particular characteristics of 3T3 cells was indicated by similar observations in SV40-transformed 3T3 cells and WI-38 human fibroblasts (our unpublished data).

Taken together, our results indicate that during a hyperthermic treatment up to 44°C and concomitant with a cell volume increase there is an enhanced entry and accumulation of neutral amino acids mediated by the Na^+-dependent systems A and ASC. This is consistent with the pivotal role ascribed to internal levels of amino acids in cell volume control in cultured fibroblasts (Gazzola et al., 1991). It is well known that during heat shock there is accumulation of denatured, unfolded and mis malfolded proteins within the cells (Morimoto et al., 1992). It has recently been proposed that cellular osmolytes (amino acids and derivatives, carbohydrates and methylamines) act as chemical chaperones in counteracting proteotoxic environments (Welch and Brown, 1996). There are only few studies on the possible mechanism of the compatible role and/or stabilizing effect of neutral amino acids on proteins. *In vitro* studies showed that neutral amino acids such as proline, alanine and serine can stabilize lysosome structure against thermal denaturation (Arakawa and Timasheff, 1985). In this respect, cellular heat sensitivity has been shown to be modulated by specific amino acids: an increase in the intracellular concentrations of a few neutral amino acids enables CHO cells to become resistant to hyperthermia (Vidair and Dewey, 1987).

These evidences suggest that neutral amino acids may be involved in stress response in vivo. Some indirect evidence in support of this are the findings that system A transport may be coordinately regulated with heat shock proteins in the mutants of CHO cells (Jones et al., 1994). In a previous study (Alfieri et al., 1996), we showed that the volume of 3T3 cells incubated at 44°C was significantly greater than that of control cells, although there were no changes in ion content. In contrast to the intracellular accumulation of inorganic ions that can induce adverse effects on the structure and function of proteins (Kwon and Handler, 1995), the early accumulation of "compatible osmolytes" that we observed, precedes the synthesis of HSPs, which occurs later on, and may serve to protect the cells until newly synthesized molecular chaperones can participate in the unfolding and folding of fragile proteins (Cohen et al., 1991).

Although the molecular events that lead to the transcription and translation of the stress proteins have been actively investigated, relatively little is known about the biophysical, biochemical phenomena and nutritional requirements occuring during the stress response and their possible role in the mechanism of HSPs induction. For instance, availability, transport and intracellular accumulation of compatible osmolytes as well as associated cell volume changes occurring during the heat shock response were not actively pursued. Studies on cultured Drosophila Kc cells have indicated that L-glutamine supplement may enhance and support the expression of genes encoding stress proteins (Sanders and Kon, 1991). Another report (Nissim et al., 1993) highlights the influence of L-glutamine on HSP expression and cell

survival. Moreover, the classic heat shock induction of HSP72 was found to be mimicked without prior heat shock, by phenylalanine addition to cells simultaneously deprived of all other amino acids (Plakidou-Dymock and McGivan, 1994). However, in this report we present evidence that the withdrawal of all amino acids from the culture medium did not affect the basal level of HSP70 mRNA and its induction by heat shock. Similarly, the heat shock-induced increase of amino acid transport activity was not affected by amino acid deprivation. This culture condition, however, markedly reduced, as expected, the intracellular NPS content in both control and heat-shocked cells. However, it should be noted that, despite of the known connection between compatible osmolyte accumulation and osmotic cell volume regulation (Hoffmann and Dunham, 1995; Gazzola et al., 1991; Dall'Asta et al., 1994), the heat dependent cell volume increase was not significantly affected by deprivation of all amino acids. Furthermore, and in some contrast with the results cited above on the positive influence of specific amino acids on the heat shock response (Sanders and Kon, 1991; Nissim et al., 1993; Plakidou-Dymock and McGivan, 1994), our results also indicate that amino acid availability and their intracellular accumulation during heat shock is not required for a full HSP70 gene expression in 3T3 cells. However, HSF activation and HSP70 mRNA expression appear to be temporally and tightly associated with the heat-dependent induction of amino acid transport activity and cell volume increase, phenomena which early occur during the heat shock response. Further investigation will be required to confirm or exclude whether nutrient uptake increase and volume change are early signals causally involved in triggering the heat shock response.

Acknowledgment

Investigation supported by grants from MURST and CNR, Rome.

References

Alfieri R, Petronini PG, Urbani S, Borghetti AF (1996) Activation of heat-shock transcription factor 1 by hypertonic shock in 3T3 cells. Biochem J 319: 601–606

Arakawa T, Timasheff SN (1985) The stabilization of proteins by osmolytes. Biophys J 47: 411–414

Borghetti AF, Piedimonte G, Tramacere M, Severini A, Ghiringhelli P, Guidotti GG (1980) Cell density and amino acid transport in 3T3, SV3T3, and SV3T3 revertant cells. J Cell Physiol 105: 39–49

Bradford RH (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–258

Chomczyski P, Sacchi N (1987) Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159

Christensen HN (1975) Biological transport, 2nd edn. In: Benjamin WA (ed) Inc Reading, Massachusetts

Cohen DM, Wasserman JC, Gullans SR (1991) Immediate early gene and HSP70 expression in hyperosmotic stress in MDCK cells. Am J Physiol 261: C594–C601

Colotta F, Polentarutti N, Staffico M, Fincato G, Mantovani A (1990) Heat shock induces the transcriptional activation of c-fos protooncogene. Biochim Biophys Res Comm 168: 1013–1019

Dall'Asta V, Rossi PA, Bussolati O, Guidotti GG, Gazzola GC (1990) The transport of L-glutamine into cultured human fibroblasts. Biochem Biophys Acta 1052: 106–112

Dall'Asta V, Rossi PA, Bussolati O, Gazzola GC (1994) Response of human fibroblasts to hypertonic stress. J Biol Chem 269: 1–7

Eagle H (1955a) The specific amino acid requirements of mammalian cell (strain L) in tissue culture. J Biol Chem 214: 839–853

Eagle H (1955b) Nutrition needs of mammalian cells in tissue culture. Science 122: 501–504

Eagle H, Oyama VI, Levy M, Horton CL, Fleischman R (1956) The growth response of mammalian cells in tissue culture to L-glutamine and L-glutamic acid. J Biol Chem 218: 607–617

Gazzola GC, Dall'Asta V, Nucci FA, Rossi PA, Bussolati O, Hoffmann EK, Guidotti GG (1991) Role of amino acid transport system A in the control of cell volume in cultured human fibroblasts. Cell Physiol Biochem 1: 131–142

Guidotti GG, Borghetti AF, Gazzola GC (1978) The regulation of amino acid transport in animal cells. Biochim Biophys Acta 515: 329–366

Hightower LE (1991) Heat shock, stress proteins, chaperones, and proteotoxicity. Cell 66: 191–197

Hoffmann EK, Dunham PB (1995) Membrane mechanism and intracellular signalling in cell volume regulation. Int Rev Cytol 161: 173–262

Jones M, Gupta RS, Englesberg E (1994) Enhancement in amount of P1 (hsp60) in mutants of Chinese hamster ovary (CHO-K1) cells exhibiting increases in the A system of amino acid transport. Proc Natl Acad Sci USA 91: 858–862

Kletzien RF, Pariza MW, Becker JE, Pottler VR (1975) A method using 3-O methyl-D-glucose and phloretin for the determination of intracellular water space of cells in monolayer culture. Anal Biochem 68: 537–544

Kwock L, Lin P-S, Hefter K, Wallack DFH (1978) Impairment of Na^+-dependent amino acid transport in a cultured human T-cell line by hyperthermia and irradiation. Cancer Res 38: 83–87

Kwon HM, Handler JS (1995) Cell volume regulated transporters of compatible osmolytes. Curr Opin Cell Biol 7: 465–471

Law RO, Turner DPJ (1987) Are ninhydrin-positive substances volume-regulatory osmolytes in rat renal papillary cells? J Physiol 386: 45–61

Lin P-S, Kwock L, Hefter K, Wallack DFH (1978) Modification of rat thymocyte membrane properties by hyperthermia and ionizing radiation. Int J Radiat Biol 33: 371–382

McGivan JD, Pastor-Anglada M (1994) Regulatory and molecular aspects of mammalian amino acid transport. Biochem J 299: 321–334

Morimoto RI, Sarge KD, Abravaya K (1992) Transcriptional regulation of heat shock genes. J Biol Chem 267: 21987–21990

Nissim I, States B, Hardy M, Pleasure J, Nissim I (1993) Effect of glutamine on heat shock-induced mRNA and stress proteins. J Cell Physiol 157: 313–318

Petronini PG, Alfieri R, De Angelis E, Campanini C, Borghetti AF, Wheeler KP (1993) Different HSP70 expression and cell survival during adaptive responses of 3T3 and transformed 3T3 cells to osmotic stress. Br J Cancer 67: 493–499

Petronini PG, De Angelis E, Borghetti AF, Wheeler KP (1994) Osmotically inducible uptake of betaine via amino acid transport system A in SV-3T3 cells. Biochem J 300: 45–50

Petronini PG, Alfieri R, Campanini C, Borghetti AF (1995) Effect of an alkaline shift on induction of the heat shock response in human fibroblasts. J Cell Physiol 162: 322–329

Plakidou-Dymock S, McGivan JD (1994) Amino acid deprivation-induced stress response in the bovine renal epithelial cell line NBL-1: induction of HSP 70 by phenylalanine. Biochim Biophys Acta 1224 2: 189–197

Sanders MM, Kon C (1991) Glutamine is a powerful effector of heat shock protein expression in Drosophila Kc cells. J Cell Physiol 146: 180–190

Tramacere MR, Petronini PG, Severini A, Borghetti AF (1984a) Osmoregulation of amino acid transport activity in cultured fibroblasts. Exp Cell Res 151: 70–79

Tramacere MR, Petronini PG, Borghetti AF (1984b) Effect of hyperosmolarity on the activity of amino acid transport system L in avian fibroblasts. J Cell Physiol 121: 81–86

Vidair CA, Dewey WCM (1987) Modulation of cellular heat sensitivity by specific amino acids. J Cell Physiol 131: 267–275

Warren AP, James MH, Menzies DE, Windell CC, Whitaker-Dowling PA, Pasternak CA (1986) Stress induces an increased hexose uptake in cultured cells. J Cell Physiol 128: 383–388

Welch WJ (1990) The mammalian stress response: cell physiology and biochemistry of stress proteins. In: Stress proteins in biology and medicine. Cold Spring Harbor Laboratory Press, pp 223–278

Welch WJ, Brown CR (1996) Influence of molecular and chemical chaperones on protein folding. Cell Stress Chaperones 1: 109–115

Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN (1982) Living with water stress: evolution of osmolyte systems. Science 217: 1214–1222

Authors' address: Dr. Angelo F. Borghetti, Dipartimento di Medicina Sperimentale, Sezione di Patologia Molecolare ed Immunologia, Università degli Studi di Parma, Via Volturno, 39, I-43100 Parma, Italy, Fax +39-521-903742, E-mail: borghet@unipr.it

Received June 30, 1999