Volume 327, number 3, 247–250 FEBS 12751 August 1993

Rise in heat-shock protein level confers tolerance to energy deprivation

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Received 2 June 1993

Heat shock (44°C for 10 min) or ATP depletion by an uncoupler (CCCP for 20 min) is shown to result in stimulation of hsp68/70 synthesis in Ehrlich tumor cells. After 3 h of recovery, the cells become thermotolerant. Surprisingly, repeated ATP depletion caused by CCCP or rotenone (a respiratory inhibitor) treatment, had a much lower effect on cell viability. Both induction of tolerance to energy deprivation and hsp68/70 synthesis were totally suppressed by cycloheximide, an inhibitor of cytosolic protein synthesis. In tolerant cells, rotenone still induced ATP depletion; however, protein aggregation (the rise in Triton-insoluble proteins) was inhibited in these cells. It is suggested that cellular chaperones (e.g. hsp70) are involved in the protection of ischemic cells from necrosis, preventing protein aggregation under ATP deficiency.

Heat-shock protein; Ischemic cell death; Protein aggregation; Ehrlich ascite carcinoma

1. INTRODUCTION

Heat-shock proteins (hsp) are necessary both for normal cellular functions (translation, protein folding, assembly and translocation) and for protection of cells from various stressing influences and agents, e.g. high temperature, UV-radiation, adriamycin, SH-reagents, tumor necrosis factor; the same transient stresses activate the hsp synthesis [1–4]. Apparently, hsp can be induced by protein-damaging factors; on the other hand, hsp can confer resistance to these agents. Why hsp can protect cells from such different kinds of stresses is not fully understood but it is believed that hsp70 and other hsp can act as chaperones, preventing stress-induced protein aggregation and restoring normal protein folding, at least in the case of heat-treated cells [2,5].

Previously we have found that ATP depletion in EL-4 tumor cells induced by rotenone and an uncoupler (2,4-dinitrophenol) led to aggregation of some cytoskeletal proteins (e.g. actin, vinculin) [6]; ischemia and various ATP-depleting agents could also induce hsp synthesis [7–10]. The role of hsp in protection of cells from ischemia was recently suggested [2,11] but no clear evidence was obtained. Below we show that induction of hsp synthesis provides protection of Ehrlich ascites carcinoma (EAC) cells from ischemic necrosis and suggest a possible mechanism of this protection.

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CHX, cycloheximide; EAC, Ehrlich ascites carcinoma; HBSS, Hanks' balanced salt solution; hsp, heat-shock proteins; TISP, Triton-insoluble protein.

2. MATERIALS AND METHODS

EAC cells (log phase) were taken from peritoneal cavities of mice (5–6 days after inoculation of 10^7 cells/mouse). After washing the cells with HBSS, they were treated either with CCCP (2 μM) for 20 min in the same medium or heated for 10 min in DMEM and then incubated for 3 h in DMEM (with or without CHX, 50 μM). A second energy deprivation (2 μM rotenone, or 2 μM of CCCP, or 2 μg/ml of oligomycin) and heat treatment of these cells were performed in HBSS. Their viability was determined by Trypan blue staining.

ATP measurements were done using a lucefirin–luceferase assay kit (Calbiochem, USA) [6].

Nuclear–cytoskeletal protein (Triton-insoluble protein, TISP) fraction was obtained by adding extraction buffer (2% Triton X-100; 50 mM Tris-HCl; 10 mM EDTA; 2 mM PMSF (pH 7.5)) to an equal volume of cell suspension as described [6]; the pellet was used for protein determination (BCA reagent, Pearse) and for polyacrylamide gel-electrophoresis [12] in 5–15% gradient gels. Immunoblotting was performed according to the method of Towbin et al. [13] with monoclonal antibodies to hsp68 (inducible) and hsp68/70 (both inducible and constitutive), kindly provided by Prof. W.J. Welch (University of California, San Francisco). The relative amount of each protein was assesed by measuring the intensity of Coomassie R250- or peroxidase-stained bands on an Ultroscan XL laser densitometer (LKB).

The data presented are means ± S.E.M. of 3–6 independent experiments.

3. RESULTS

Brief heat shock (44°C, 10 min) was shown to result in an increase in the EAC cell hsp68 level (Fig. 1) and an about 2-fold increase in the hsp70 level (not shown) measured after 3 h recovery. The cells were more resistant to a second more prolonged heat treatment (Table I), demonstrating the well-known phenomenon of thermotolerance. The same effect on hsp induction and thermoresistance was observed when the cells were treated with an uncoupler, CCCP, for 20 min (Fig. 1, Table I) which transiently decreased cellular ATP levels to 4–5% of the initial value (not shown). When the

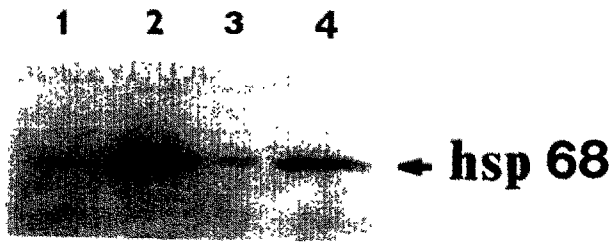

Fig. 1. Western-blot analysis of EAC cells with monoclonal antibody to hsp68 (inducible form). (Lane 1) control cells; (lane 2) heat-shocked cells (44°C, 10 min) after a 3 h recovery period; (lane 3) the same as (2), but CHX (50 μM) was present during recovery; (lane 4) cells recovered after treatment with 2 μM of CCCP (transient ATP depletion).

thermotolerant cells were subjected to a second, more prolonged ATP depletion (with the respiratory inhibitor, rotenone), suprisingly, their death was significantly diminished compared with control cells; moreover, both heat- shock and CCCP induced tolerance to rotenone (Fig. 2). The resistance to rotenone was not specific, since the pretreated cells were also resistant to another ATP-depleting agents, CCCP, and oligomycin, an inhibitor of mitochondrial H^+-ATPase (not shown). The toxic effect of these agents on EAC cells was totally prevented by glucose (10 mM), which activated glycolysis and maintained a high cellular ATP level, as we previously found in EL-4 tumor cells [6].

To determine whether hsp synthesis is necessary for development of tolerance to rotenone, the cells were incubated during recovery with an inhibitor of cytosolic protein synthesis, CHX. This drug fully suppressed hsp68/70 synthesis (Fig. 1), development of thermotolerance (Table I) and tolerance to rotenone (Fig. 2B). The same effect as with CHX was observed if EAC cells were incubated during recovery in HBSS with glucose (i.e. in a medium without amino acids; not shown). CHX alone effected neither control cell viability (Fig. 2B) nor cell sensitivity to rotenone (not shown). If rotenone was added to the cells immediately after heat shock, their death was accelerated rather than prevented (Table I). Thus recovery period with hsp synthesis was necessary for induction of tolerance to energy deprivation.

One possible explanation of the observed effect of tolerance to rotenone may be a prevention of ATP loss in tolerant cells. From studying the time–course of ATP depletion, we found some inhibition of the ATP decrease in tolerant cells after 15 min of incubation with rotenone. The effect disappeared after 0.5–1 h incubation (Table II) when cell death occurred (Fig. 2). Apparently, the tolerant cells had slightly lower rates of ATP consumption and/or higher levels of endogenous glycolytic substrate, but this was not the main reason of the tolerance to rotenone-induced energy deprivation. In accordance with this reasoning, heat shock (0.5–1 h) had no significant effect on cellular ATP levels (not shown) but resulted in higher cell killing than rotenone in the tolerant cells (Table I, Fig. 2).

Another possibility may be the following. Hsp were supposed to be responsible for prevention of heat-induced protein aggregation [2,5,14,15]. As we found previously, different stresses, including ATP depletion, resulted in a significant rise in the TISP fraction in tumor cells; this rise indicated protein aggregation and correlated well with cell damage and death [16]. Here we have found that the heat-induced increase in TISP was significantly lower in tolerant cells, but not in cells treated with CHX during recovery (Table I), demonstrating excellent correlation ($r = 0.96$) between the rise in TISPs and cell death after the 1 h incubation. When the rise in TISP was studied in ATP-depleted cells, we also observed suppression of protein aggregation in the tolerant cells (Table II). In comparison with heat shock, ATP depletion had a lesser effect on the rise in TISP since, as we suggested previously, it did not result in protein denaturation but instead rendered (aggregated) cytoskeletal proteins insoluble [16]. Here, the effect of the treatment was especially evident when electrophoresis of TISP fractions was carried out (Fig. 3). As one can see, rotenone-induced aggregation of the major cytoskeletal proteins, actin and the 57 kDa protein of intermediate filaments, was inhibited in the tolerant, but not in the control and CHX-treated cells (Fig. 3; see the legend for data on densitometric scanning). Thus, the protective role of hsp under ATP depletion consists of prevention of aggregation of some proteins (e.g. actin and 57 kDa protein).

Table I

Effect of various treatments on death and protein aggregation (rise in TISP) in heat-shocked EAC cells

Conditions	Cell death (%)		Rise in TISP (%)
	1 h	2 h	1 h
HS 30 min	20 ± 3	20 ± 3	45 ± 2
HS 1 h	39 ± 2	49 ± 2	89 ± 7
HS 10 min, rec., HS 1 h	23 ± 4*	36 ± 2*	67 ± 1*
CCCP, rec., HS 1 h	18 ± 2*	20 ± 6*	61 ± 6*
+CHX during recovery			
HS 10 min, rec., HS 1 h	35 ± 3	56 ± 3	85 ± 6
CCCP, rec., HS 1 h	45 ± 4	44 ± 7	100 ± 11
HS 30 min + rot. (without recovery)	52 ± 8	56 ± 3	116 ± 9

*$P < 0.05$ compared to controls (heat shock (HS) 1 h) by t-test. Cell death was determined by Trypan blue staining, and rise in TISP was determined as described in section 2. TISP in control cells was 36 ± 2 $\mu g/10^6$ cells and was taken as 100%. Heat shock (HS) = 44°C; CCCP = 2 μM for 20 min; CHX = 50 μM; rotenone (rot.) = 2 μM; recovery (rec.) = 3 h.

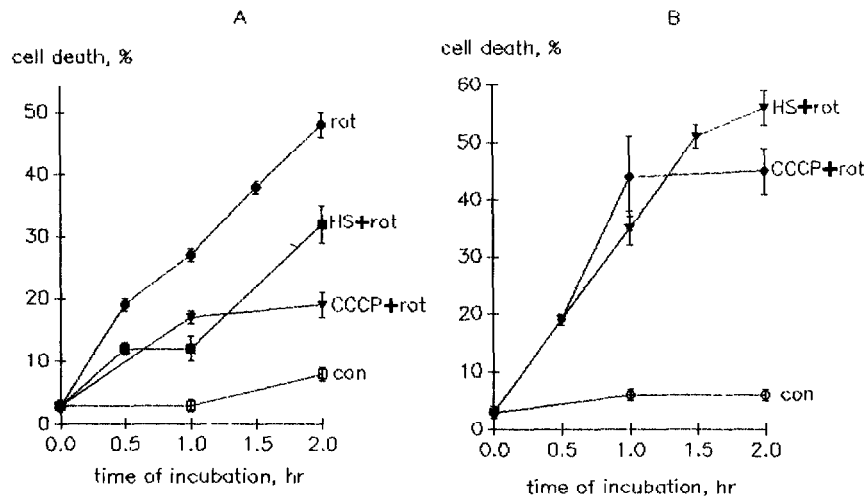

Fig. 2. Effect of various treatments on rotenone-induced death of EAC cells. (A) rot = effect of 2 μM rotenone on control cells; con = control cells incubated without rotenone; HS + rot = cells were heat-shocked (44°C, 10 min), allowed to recover for 3 h and then rotenone was added; CCCP + rot = the cells were treated with 2 μM CCCP for 20 min, allowed to recover for 3 h and then rotenone was added. (B) The same as A but 50 μM of CHX was present during recovery.

4. DISCUSSION

During the past decade, many functions of hsp in cellular physiology, under both normal and pathological conditions, have been described (see section 1). In this report we show another important function of these proteins, namely protection of cells from energy deprivation. The following results confirm this conclusion: (i) both heat shock and CCCP resulted in hsp68/70 induction and protection from energy deprivation; (ii) induction of hsp68/70 and tolerance to ATP loss was prevented by CHX; (iii) aggregation of proteins in ATP-depleted cells was inhibited after tolerance acquisition; (iv) conversely, when protein aggregation and a decrease in the free (soluble) form of hsp68/70 were caused by heat shock, the cells became more sensitive to rotenone provided it was added immediately after heat shock.

Some data of previous works also support our finding. First of all, induction of hsp synthesis and thermotolerance can be observed not only after brief heat shock, but also after in vivo ischemia and in vitro energy deprivation (anoxia, uncouplers, rotenone) [7–10]. Moreover, in vivo mild hyperthermia renders brain and heart more resistant to ischemia–reperfusion injuries [11,17], Although these results suggested a role for hsp in protection of organs and cells from ischemia [2,11,17], no evidence for this was obtained and possible mechanisms of such protection were not discussed. Especially, it was not clear how hsp68/70 can protect cells from energy deprivation if ATP is necessary for its disaggregating action in vitro and in vivo [7,14]. When rat fibroblasts over-expressed hsp70 without the ATP-binding domain, however, the cells acquired the same thermoresistance as the cells over-expressing intact hsp70 [18]. Possibly, ATP is not needed for prevention of aggregation by hsp70 but may accelerate its disaggregating action. In addition, hsp70 has a very low K_m for ATP (1–2 μM) [19]. According to our data (Table II), even after 1 h of incubation with rotenone, the ATP

Table II

Effect of rotenone on ATP levels and aggregation of proteins (rise in TISP) in control and tolerant cells

Conditions	ATP (% of initial)			Rise in TISP (%)
	15 min	30 min	60 min	60 min
Rotenone	4.5 ± 0.1	3.6 ± 0.5	3.3 ± 0.2	42 ± 4
HS 10 min, rec., rot.	15.7 ± 3.0*	4.3 ± 0.7	1.4 ± 0.4	24 ± 3*
CCCP, rec., rot.	9.3 ± 2.2	3.8 ± 1.6	2.1 ± 1.0	23 ± 5*
+CHX during rec.				
HS 10 min, rec., rot.	8.0 ± 2.3	3.8 ± 0.6	2.4 ± 0.9	43 ± 2
CCCP, rec., rot.	1.5 ± 0.1	1.2 ± 0.3	1.1 ± 0.1	41 ± 2

*$P < 0.05$ compared to effect of rotenone in control cells. Rotenone (rot.) = 2 μM; heat shock (HS) = 44°C; CCCP = 2 μM; CHX = 50 μM; recovery (rec.) = 3 h. The initial ATP level was 26 ± 2 nM/10^6 cells and did not change during 1 h of incubation without rotenone.

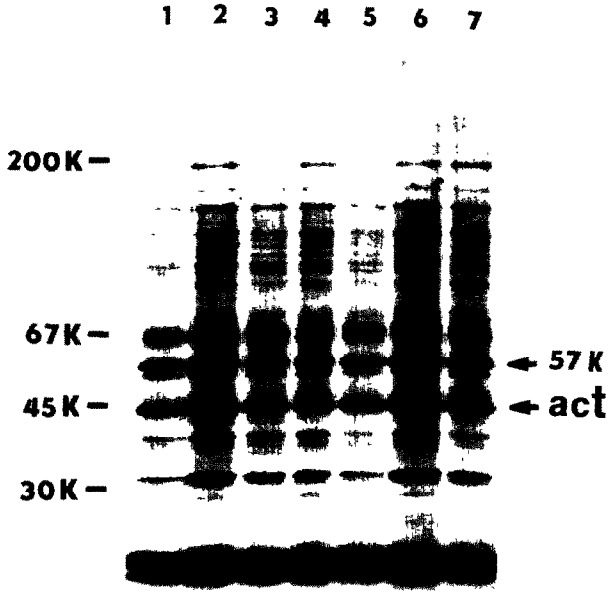

Fig. 3. Coomassie-stained gel showing TISP from EAC cells. The TISP fractions were obtained from equal numbers (10^6) of control (lanes 1,3,5) and ATP-depleted (lanes 2,4,6,7) cells. (Lane 1) TISP fraction from (lane 1) control cells; (lane 2) rotenone-treated control cells; (lane 3) cells allowed 3 h to recover after heat shock (44°C, 10 min); (lane 4) as lane 3, but rotenone was added after recovery; (lanes 5,6) as lanes 3,4, but CHX (50 μM) was present during recovery; (lane 7) rotenone-treated cells preincubated with CCCP (2 μM, 20 min) and allowed to recover for 3 h (conditioning by transient ATP depletion). Rotenone, 2 μM for 1 h. The position of actin (43 kDa) and the major protein of intermediate filaments are denoted by arrows on the right. The increases in actin band intensity (compared with the control, lane 1) were 58% (lane 2), 29% (lane 3), 34% (lane 4), 0% (lane 5), 51% (lane 6), 0% (lane 7); and the increases in the 57 kDa band intensity were 62% (lane 2), 33% (lane 3), 25% (lane 4), 3% (lane 5), 80% (lane 6), 11% (lane 7).

level was approx. 1–3% of the initial one (about 3–5 mM), i.e. 30–150 μM; this may be sufficient for the disaggregating function of hsp70.

We have not yet studied induction of other hsp (hsp90 and hsp27) in EAC cells, but they may also contribute to the observed effect of tolerance to ATP deprivation. Apart from hsp70, these proteins do not possess ATPase activity, although they render cells resistant to heat shock [2,5,20]. Hsp90 was shown to have disaggregating action in vitro and ATP did not accelerate this function [21], however, further studies, for example, transfection with different hsp, are necessary to evaluate the relative contribution of various stress proteins in protection of cells from energy deprivation.

In the course of this work, we also found that stationary EAC cells (8 days after inoculation) spontaneously expressed hsp68 in vivo and were resitant both to heat shock and to ATP depletion (unpublished data). Recently activation of hsp27 synthesis in stationary EAC cells was observed [20]; in solid tumors, the heat shock transcription factor was spontaneously activated [22]. Since hypoxia and nutrient deprivation are usual features of many tumors, this may stimulate hsp synthesis and render them resistant both to ischemia (as we demonstrated in this work) or hyperthermia, which is now widely used in anticancer therapy. In addition, the phenomenon described above for tumor cells is, apparently, important for normal cells and organs and may have a clinical significance (e.g. in ischemic states).

REFERENCES

[1] Subjeck, J.R. and Shyy, T.-T. (1986) Am. J. Physiol. 250, C1–C17.
[2] Welch, W.J. (1992) Physiol. Rev. 72, 1063–1081.
[3] Jaattela, M., Wissing, D., Bauer, P.A. and Li, G.C. (1992) EMBO J. 11, 3507–3512.
[4] Suzuki, K. and Watanabe, M. (1992) Biochem. Biophys. Res. Commun. 186, 1257–1264.
[5] Hightower, L.E. (1991) Cell 66, 191–197.
[6] Gabai, V.L., Kabakov, A.E. and Mosin, A.F. (1992) Tissue Cell 24, 171–177.
[7] Beckman, R.P., Lovett, M. and Welch, W.J. (1992) J. Cell Biol. 117, 1137–1150.
[8] Currie, R.W. (1987) J. Mol. Cell Cardiol. 19, 795–808.
[9] Haveman, J., Li, G.C., Mak, J.Y. and Kipp, J.B.A. (1986) Int. J. Radiat. Biol. 50, 51–64.
[10] Tuyl, M.J.M., van Bergen en Henegouwen, P.M.P., van Wijk, R. and Verkleij, A.J. (1991) Biochim. Biophys. Acta 1091, 278–284.
[11] Dwyer, B.E. and Nishimura, R.N. (1992) Brain Pathol. 2, 245–251.
[12] Laemmli, U.K. (1970) Nature 227, 680–685.
[13] Towbin, H.T., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[14] Skowyra, D., Georgopoulos, C. and Zylicz, M. (1990) Cell 62, 939–944.
[15] Pinto, M., Morange, M. and Bensaude, O. (1991) J. Biol. Chem. 266, 13941–13946.
[16] Kabakov, A.E. and Gabai, V.L. (1993) Experientia (in press).
[17] Karmazyn, M., Masler, K. and Currie, R.W. (1990) Am. J. Physiol. 259, H421–H431.
[18] Li, G.C., Li, L., Liu, R.Y., Rehman, M. and Lee, W.M.F. (1992) Proc. Natl. Acad. Sci. USA 89, 2036–2040.
[19] Sadis, S. and Hightower, L.E. (1992) Biochemistry 31, 9406–9412.
[20] Knauf, U., Bielka, H. and Gaestel, M. (1992) FEBS Lett. 309, 297–302.
[21] Wiech, H., Buchner, J., Zimmerman, R. and Jakob, U. (1992) Nature, 358, 169–170.
[22] Giaccia, A.J., Ager, E.A., Koong, A., Terris, D.J., Minchinton, A.I., Hahn, G.M. and Brown, J.M. (1992) Int. J. Radiat. Oncol. Biol. Phys. 23, 891–897.