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A RESPONSE OF YEAST CELLS TO HEAT STRESS: CELL VIABILITY AND THE STABILITY OF CYTOSKELETAL STRUCTURES

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A b s t r a c t

The cells of *Saccharomyces cerevisiae* were exposed to the effect of mild and lethal heat stress and their viability and changes in their microtubular and actin structures were studied.

An increase in cultivation temperature from 25 °C to 46 °C (lethal heat shock, LHS) resulted, during 10 min, in a rapid decrease in cell viability. However, a mild increase in incubation temperature from 25 °C to 37 °C or 41 °C (mild heat shock, MHS) induced cell tolerance to LHS and the cells were able to survive at 46 °C for up to 30 min. A mild heat shock increased the level of Hsp104 in cells, as demonstrated by Western blot analysis and indirect immunofluorescence microscopy.

In cell exposed to LHS, immunofluorescence microscopy showed rapid degradation of both cytoplasmic and nuclear microtubules, but the spindle pole bodies (SPB) remained preserved. On the other hand, MHS did not produce microtubule degradation. Microtubules remained stable after the LHS that had been preceded by cell cultivation at 37 °C. It is suggested that stress proteins induced by MHS were involved in maintaining microtubule stability. The actin cytoskeleton was very sensitive to heat shock and transfer of cells to both 37 °C and 41 °C resulted in disintegration of actin filaments and the spreading of actin dots from the bud to the whole cell surface. No stabilisation effect of stress proteins on actin structures was observed.

Resistance to LHS was also induced by an osmotic shock in the presence of 1M KCl in culture medium. However, the production of stress proteins in response to osmotic shock was generally slower than to MHS because the cells became resistant to LHS only after being exposed to 1M KCl for 3 h.

K e y w o r d s

Saccharomyces cerevisiae, heat shock, Hsp104, actin, microtubules

INTRODUCTION

Physical or chemical stressors induce direct or indirect structural changes in proteins, which results in protein aggregation and, subsequently, disturbed functioning of cell compartments. However, the impairment of cell structures need not always be irreversible. Low-intensity stressors have been found to increase the synthesis of one protein category, namely, heat-shock proteins (Hsp), that help the organism to acquire tolerance to stress induced by the initial agents *(1).* In the yeast *Saccharomyces cerevisiae*, great attention has been paid to the Hsp100/Clp protein family *(2),* of which Hsp104 has a key role in tolerance to

heat and other stressors *(3).* Hsp104, together with Hsp70 and Hsp40, compose a chaperone complex that facilitates reactivation of heat-damaged proteins *(4)* and thus assists in maintaining the essential processes in the cell under stress. An increased synthesis of heat-shock proteins can also be induced by hypertonic conditions, ethanol and many other stressors *(2,3).*

The objective of this study was to investigate responses of the cytoskeletal structures to stress factors in yeast cells. In Saccharomyces cerevisiae cells, the response of the cytoskeleton to hypertonic stress reported, in a previous study *(5),* was manifested by rapid disintegration of both actin cables and microtubules and their subsequent repolymerisation within several hours. However, another transfer of the cells to hypertonic medium was not followed by microtubule disintegration, which was accounted for by a protective action of Hsp. In this paper, we studied the effect of heat shock on the stability of microtubules and actin structures with the aim to find out the extent of Hsp production and the role of these proteins in the cytoskeleton response to heat stress. It appeared that stress proteins were involved in maintaining the stability of microtubules but not actin structures.

MATERIALS AND METHODS

Organisms and cultivation

Cells of Saccharomces CCU 21–4–59, were grown on agar MEB medium (2% malt extract broth, 2 % glucose). Cells incubated in liquid MEB medium at 25 °C were subjected to heat stress when the culture achieved OD(560 nm) equal to 1.0, which corresponded to a density of 10^7 cells/ml. Protein synthesis was inhibited by adding cycloheximide (Upjohn Comp.) at a final concentration of 20 μg/ml.

Stress induction

Mild heat stress was induced by heating a 10- ml cell suspension for 30 min at 37 °C and for another 30 min at 41 °C. Strong heat stress was induced by heating the suspension for 10 to 30 min at 46 °C.

Osmotic stress was induced by adding a KCl solution to a 10-ml cell culture to achieve a final concentration of 1M; the suspension was divided into two equal samples which were incubated at 25 °C for 1 and 3 h, respectively.

Cell viability assay

Both the control cells and the cells exposed to heat were, after dilution, seeded onto MEB agar in Petri dishes and their viability was assessed by counting colony forming units (CFU).

Western blot assay

A sample of proteins was obtained by either protoplast osmolysis or mechanical disintegration of cells frozen in liquid nitrogen. Proteins were

separated on 10 % SDS-polyacrylamide gel with 5 % focusing gel *(6),* subsequently blotted to a nitrocellulose membrane *(7)* and immunoassayed using the anti-Hsp104 polyclonal antibody SPA-1040 (StressGen) at a dilution of 1:2000, and a secondary antibody (Sigma, anti-rabbit IgG) conjugated with peroxidase. Hsp104 was detected by the ECL method (Amersham).

Cell fixation and permeabilisation

A culture of exponentially growing cells was fixed, at a 3:1 ratio, with a fixative containing 15 % formaldehyde, 100 mM KH_2PO_4 buffer (pH, 6.2), 0.5 mM $MgCl_2$ and 1mM EGTA *(8,9,10)* for 90 min at room temperature. The culture was rinsed with phosphate buffer and incubated with NovoZym 234 (1 mg/ml) in phosphate buffer for 20 min at room temperature. After subsequent rinsing with buffer, the cells were permeabilised with 1 % Triton X-100 for 3 min.

Immunofluorescence

Fluorescence staining. The permeabilised cells were incubated with 2 % bovine serum albumin (BSA) for 20 min and, after an addition of the primary antibody, allowed to stand overnight at 4 °C.

Hsp104 was labeled with the SPA-1040 antibody. Tubulin was stained with mouse monoclonal anti-tubulin antibody TAT 1 (*11*); SwAR/FIFC or SwAM/FITC (Institute of Sera and Vaccines, Prague) were used as secondary antibodies. The cells were washed free of the antibodies and mixed with a mounting medium (Vectashield, Vector, USA) with DAPI (4-6-diamino-2-phenylindole) at a final concentration of 1µg/ml *(9).*

The actin cytoskeleton was labeled by means of rhodamine-phalloidin (Molecular Probes) according to *Pringle et al (9).* The stained cells were viewed and photographed with a Leitz Laborlux S fluorescence microscope.

RESULTS

1. Induction of thermotolerance after mild heat stress (MHS)

The cells grew fast at 25 °C and also 37 °C was well tolerated. They slowed down growth at 41 įC and ceased growing at 46 °C. Their growth was arrested in the presence of cycloheximide (*Fig. 1*).

If the cells grown at 25 °C were transferred to a temperature of 47 °C or higher, their ability to form colonies decreased rapidly. A certain proportion of the cell population survived for 10 min but, after 20 min of cultivation at 46 °C, less than 1 % of the cells produced colonies (*Fig. 2*). Therefore, the temperature of 46 °C was chosen as a reference lethal heat stress factor (LHS).

If a culture grown at 25 °C was incubated first at 37 °C (30 min) and then at 41 °C (30 min) and subsequently transferred to 46 °C, the proportion of vital cells markedly increased and the cells survived cultivation at this temperature for

30 min (*Fig. 2*). Incubation at 37 and 41 °C was considered to produce mild heat stress (MHS), which apparently was the reason for an increase in tolerance to LHS at 46 °C.

Tolerance to LHS was also induced by 60 min of incubation at 37 °C, or 60 min at 41 °C. An increase in incubation temperature from 25 °C to 37 or 41 °C had, in itself, no damaging effect on the cells; on the contrary, the cell counts slightly increased during this incubation (*Fig. 1,2*). If, during MHS, protein synthesis was inhibited by cycloheximide, thermotolerance did not develop and the cells became sensitive to LHS again (*Fig. 2*).

2. Heat shock protein synthesis

The response of cells to heat stress is associated with the synthesis of heat shock proteins (*1*). Our experiments were designed to detect one of these proteins, Hsp104. While, in the lysate of control cells, only traces of this protein were found, in cells exposed to mild heat stress, the band corresponding to Hsp104 was much more apparent (*Fig. 3*). The increase of the Hsp104 in MHS cells was clearly demonstrated by immunofluorescence microscopy. The control cells showed only scattered fluorescent dots while the MHS-exposed cells had many fluorescent dots evenly distributed throughout the cytoplasm (*Fig. 4*) without apparent co-localisation with any of the organelles or structures. The number of fluorescent dots increased with the increasing duration of MHS exposure.

3. Changes in the cytoskeleton after heat shock exposure

Cells grown at 25 °C showed a characteristic array of short cytoplasmic microtubules emanating from the spindle pole body or displayed a strongly fluorescent spindle (*Fig. 5A*). Upon the transfer from 25 to 46 °C the cells lost their cytoplasmic and nuclear microtubules within 10 min. and a point fluorescence was seen only at regions of the spindle pole bodies (*Fig. 5C).* When cells were first exposed to MHS and then transferred to 46 °C, cytoplasmic microtubules were slightly shortened but nuclear microtubules remained unaffected (*Fig. 5E*). This may imply the role of MHS-induced stress proteins in maintaining the stability of microtubules.

The actin cytoskeleton in the control cells showed a typical asymmetric arrangement: a cluster of fluorescent patches in the bud and in, the cytoplasm, actin filaments converging at the site of budding (*Fig. 6A*). An increase in temperature up to 37 °C resulted in disruption of actin filaments, while actin patches remained accumulated in the bud. At 41 °C the patches were seen distributed along the whole surface of the cell. In cells transferred from 25 °C to 46 °C, actin structures are rapidly degraded and large aggregates of actin without distinct relation to any cell structure were observed (*Fig. 6C*). A similar degradation of actin structures was observed if the cells were first exposed to

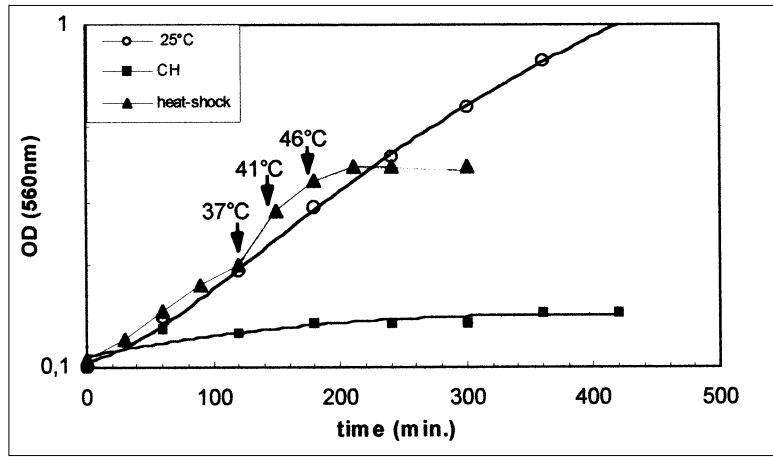

Fig. 1
Optical density of a *S.cerevisiae* culture grown at 25 °C, during a gradual increase in temperature and in the presence of cycloheximide.

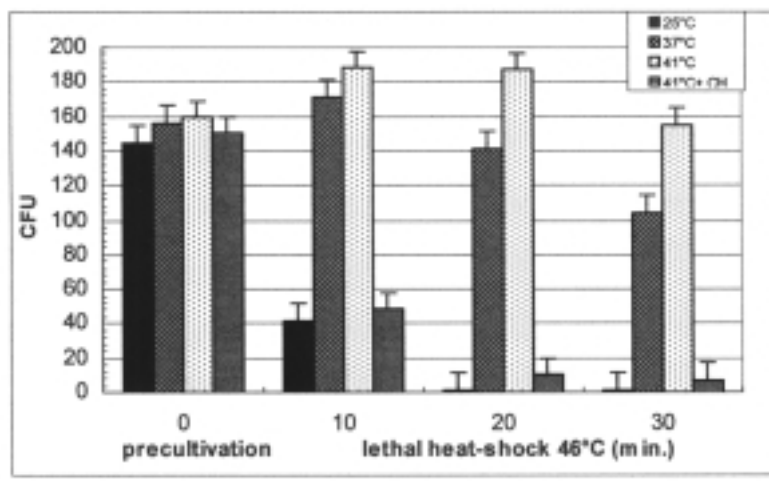

Fig. 2
Survival of cells after exposure to a lethal heat shock (LHS) in the control cells (25 °C), in cells exposed to a mild heat shock (MHS) and in cells incubated in the presence of cycloheximide. Standard deviation was determined at the 95 % level of significance.

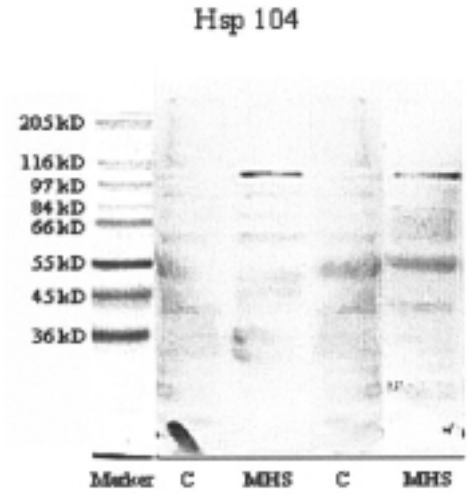

Fig. 3
Detection of Hsp104 protein by Western blot analysis in lysates of cells grown at 25 °C (**C**) and after exposure to mild heat shock (**MHS**).

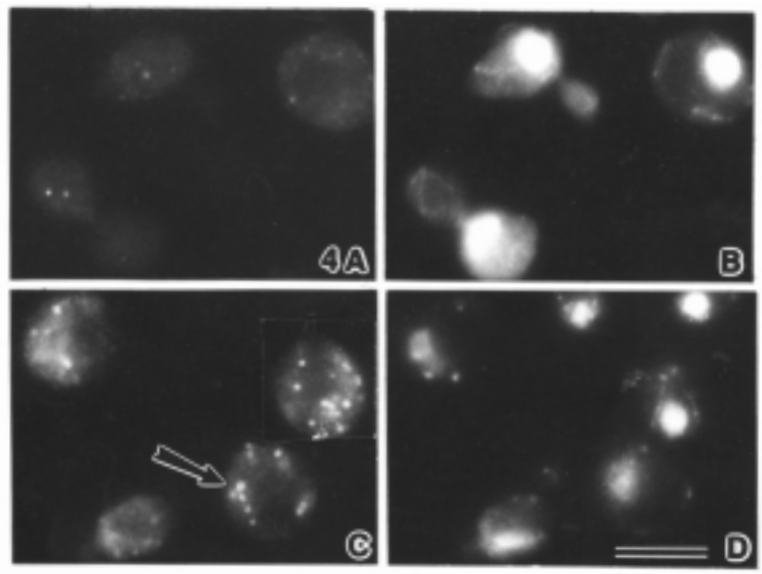

Fig. 4
Hsp104 protein visualised by immunofluorescence microscopy. Cells grown at 25 °C (**A**) and cells after exposure to MHS (**C**) show a great number of fluorescent dots (arrow). **B,D**, nuclei stained with DAPI.

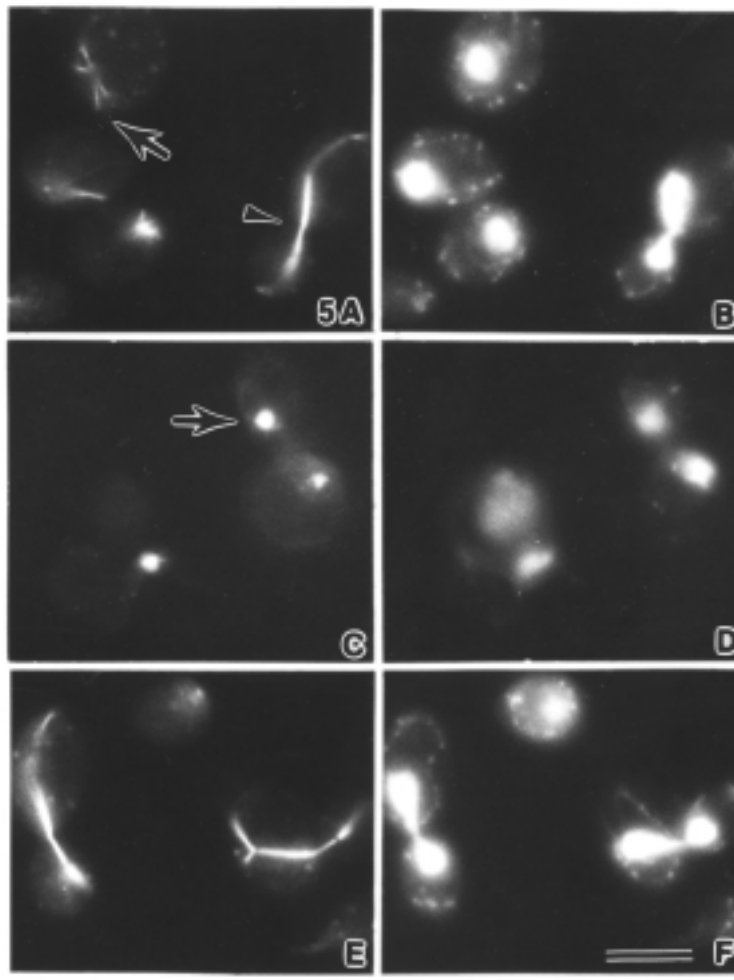

Fig. 5

Microtubules (MT) in *S. cerevisiae* cells. **A**, MTs in control cells (25 °C), note the short, astral MTs (arrow) and a spindle in a dividing cell (arrow head). **C**, MTs in cells after exposure to LHS (25 °C → 46 °C); only fluorescent SPBs are visible (arrow). **E,** MTs in cells after exposure to MHS and a subsequent LHS (25 °C → 37 °C, 41 °C → 46 °C). **B,C,F,** nuclei stained with DAPI.

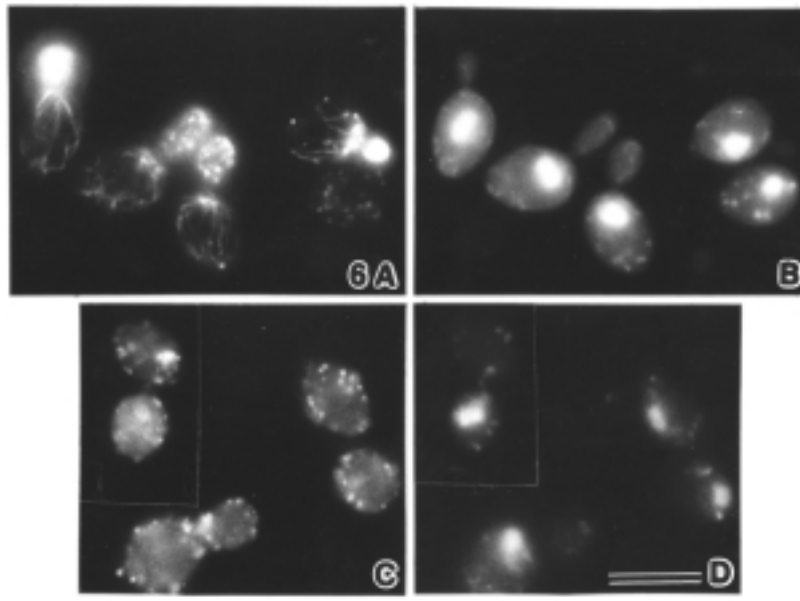

Fig. 6
Actin structures visualised by rhodamine-phalloidin staining. **A**, actin filaments and patches in control cells (25 °C). **C**, actin in cells after exposure to LHS (25 °C → 46 °C); actin patches are evenly distributed under the whole cell surface. **B,D**, nuclei visualised by DAPI staining.

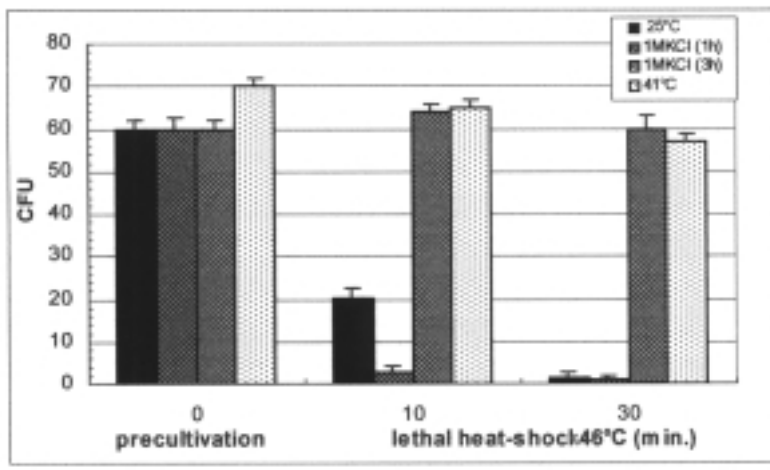

Fig. 7
Effect of prior conditioning by osmotic shock or MHS on survival of cells exposed to LHS.

MHS and then to LHS. Therefore, no effect of stress proteins on the stability of actin cytoskeleton was demonstrated.

4. Effect of osmotic stress on the induction of thermotolerance

Experiments were designed to find out whether stress proteins induced by other stressors could provide increased resistance of yeast cells to heat stress.

To a culture of cells grown at 25 °C the potassium chloride was added to a 1M concentration. Samples were taken at 1 h and 3 h of cultivation, and both were transferred to 46 °C. It appeared that the cells exposed to an osmotic shock for one hour were sensitive to LHS, as were the control cells. However, the cells exposed to an osmotic shock for 3 h were thermotolerant similarly to the MHS-exposed cells (*Fig. 7*).

The immunoblotting assay showed that, at 1 h of incubation with 1M KCl, the amount of Hsp104 present was low but increased markedly at 3 h of incubation *(Fig. 8).*

DISCUSSION

The results of our experiments with thermosensitivity and thermotolerance in *S. cerevisiae* were generally in agreement with the published data (*12,13*) with only a slight difference in the values of lethal temperature. This may have been a specific feature of the strain used. The rapid synthesis of Hsp104 was also in accordance with the findings published previously (*3*). The protein was distributed throughout the whole cytoplasm and its preferential location in the nucleus (*12*), was not confirmed. Findings in animal cells have shown Hsp co-localised with microtubules (*14*), but no such co-localisation was observed in our study. This can be explained by a small size of microtubular structures or by a small size of the yeast cell. The fact that osmotic shock resulted in an increased tolerance of yeast cells to temperature is a new finding that indicates that heat-shock proteins which are synthesised in response to osmotic stress are of the same category as the proteins produced in response to heat stress, and this in agreement with the literature data *(2,3).*

The biochemical consequences of heat and osmotic stress, including the identification of the STRE-genes in yeasts, have been well documented (*15*), but cytological correlates of the response of yeast cells to stress are, so far, poorly defined.

The effects of temperature on the changes in cytoskeletal components have been repeatedly reported, but with inconsistent results. The general idea is that eukaryotic cells respond to heat stress by disruption of their cytoskeletal elements (*16*). In some mammalian cells, an elevated growth temperature has led to actin disintegration but not to microtubule degradation (*17,18*). In other experiments, hyperthermia has induced a depolymerisation of actin, microtubules and

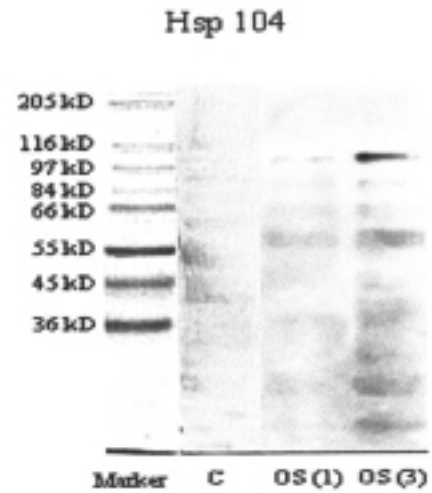

Fig. 8
Detection of Hsp104 by western blot analysis in cells subjected to osmotic shock. **C**, control cells (25 ¡C); **OS(1)**, cells incubated in the presence of 1M KCl for 1 h; **OS(3)**, cells grown in the presence of 1M KCl for 3 h.

intermediate, filaments, the latter was due to the disappearance of integrin (*19*). Heat stress has been reported to cause disruption of microtubules in plant cells, and the protective role of Hsps has not been seen (*20*). Our experiments showed that yeast cells behaved in a manner similar to other eukaryotic cells in that heat stress induced disintegration of both microtubules and actin structures, and the same response was induced by an osmotic shock (*5*).

A previous MHS, which had induced Hsp synthesis, maintained the stability of microtubules exposed to LHS. A similar prior conditioning, which facilitated microtubule stability, was also induced by osmotic stress.

Hsps seem to play an important role in the regulation of microtubule formation (*21*). In the absence of Hsps, abnormal, bent microtubules have been produced *(22)* or different types of microtubular dysfunction have occurred (*23,24*). It is probable that, under normal conditions, Hsp concentration in cells is low and exposure to any stress results in easy degradation of microtubules. However, an increase in Hsp concentration due to prior conditioning leads to a much higher stability of microtubules.

The resistance of microtubules to stress differs in relation to their type. Cytoplasmic microtubules seem to be most sensitive, nuclear microtubules are less sensitive and the most resistant microtubules are those related to the SPB. This difference in sensitivity may be due to the involvement of different microtubule-associated proteins or heat-shock proteins (*21*).

Yeast actin is known to be very sensitive to even mild stress (*9*). Actin cables disappear first; this is followed by the disappearance of the asymmetric arrangement of actin patches. We observed a similar response of actin structures regardless of whether the heat shock was mild or lethal. We failed to demonstrate any stabilizing effects of the heat-shock proteins synthesized during the prior heat conditioning on actin structures. *Slaninová et al.* (*5*) made similar findings in yeast cells exposed to osmotic shock. However, this actin sensitivity is apparently not a characteristic feature of cells in general because, in animal cells repeatedly exposed to heat shock, actin structures have not undergone depolymerisation (*18*).

Sanchez et al. (*3*) showed that, in *S. cerevisiae,* Hsp104 plays a role not only in thermotolerance but also in other kinds of stress. We confirmed this finding by demonstrating that an increase in thermotolerance could also be induced by osmotic stress. This implies either that stress signaling (*15,25,26*) may induce the production of a wide range of stress proteins or that these proteins may exert their protective roles in different kinds of stress.

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ODPOVĚĎ KVASINKOVÝCH BUNĚK NA TEPLOTNÍ ŠOK: ŽIVOTASCHOPNOST BUNĚK A STABILITA CYTOSKELETU

S o u h r n

Kvasinky *Saccharomyces cerevisiae* byly vystaveny účinku mírného a letálního teplotního stresu a byla sledována životaschopnost buněk a změny mikrotubulů a aktinových struktur.

Bylo zjištěno, že zvýšení inkubační teploty z 25 °C na 46 °C vede již během 10 min k rychlé ztrátě životaschopnosti (letální teplotní šok). Mírné zvýšení inkubační teploty z 25 °C na 37 °C nebo 41 °C (mírný teplotní šok) navodí toleranci buněk k letálnímu teplotnímu šoku a buňky v této teplotě přežívají až 30 min. Jak jsme prokázali Western blotting assay, mírný teplotní šok zvýší v buňkách hladinu stresového proteinu Hsp104. Tento protein byl zřetelně detekován také metodou nepřímé imunofluorescence v cytoplasmů buněk vystavených mírnému teplotnímu šoku.

Letální teplotní šok 46°C způsobuje v kontrolních buňkách rychlou degradaci cytoplasmatických a jaderných mikrotubulů. V cytoplasmě fluoreskují pouze organizační centra mikrotubulů (SPB). Mírný teplotní šok (37–41°C) mikrotubuly nedegraduje. Mikrotubuly zůstávají stabilní i po letálním teplotním šoku, kterému předcházela předkultivace buněk při 37°C. Stresové proteiny, které se vytváří při zvýšení inkubační teplotě, se tedy evidentně podílejí na udržování stability mikrotubulů. Aktinový cytoskelet je na teplotní šok velmi citlivý; pouhý přenos buněk do 37–41°C vede k rozpadu aktinových vláken a k dislokaci aktinových teček z rostoucích pupenů. Nepozorovali jsme žádné stabilizující účinek stresových proteinů na aktin.

Zjistili jsme, že resistenci buněk na teplotní šok 46°C navodí také osmotický šok (1M KCl). Stresové proteiny se zde tvoří zřejmě pomaleji, neboť termoresistence se objeví v buňkách až po 3 hodinách po aplikaci šoku.

REFERENCES

1. *Nover L.* Inducers of HSP synthesis: heat shock and chemical stressors. In: Heat Shock Response. ed L Nover, CRC Press, Florida 1991: 5–40.
2. *Sanchez Y, Lindquist SL.* Hsp104 required for induced thermotolerance. Science 1990; 248: 1112–1115.
3. *Sanchez Y, Taulien J, Borkovich KA, Lindquist S.* Hsp104 is required for tolerance to many forms of stress. EMBO Journal 1992; 11: 2357–2364.
4. *Glover JR, Lindquist S.* Hsp104, Hsp70, and Hsp40: A novel chaperone system that rescues previously aggregated proteins. Cell 1998; 94: 73–82.
5. *Slaninová I, Svoboda A, Farkaš V.* Cell wall and cytoskeleton reorganization as the response to hyperosmotic shock in *Saccharomyces cerevisiae*. Arch Microbiol 2000; 173: 245–252.
6. *Laemmli UK.* Cleavage of structural proteins during the assembly of the head of bacteriophage. Nature 1970; 227: 680–685.
7. *Towbin H, Staehelin T, and Gordon J.* Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA 1979; 90: 3644–3648.
8. *Hagan IM, Hyams JS.* The use of cell division cycle mutants to investigate the control of microtubule distribution in the fission yeast *Schizosaccharomyces pombe.* J. Cell Sci 1988; 89: 343–357.
9. *Pringle JR, Preston RA, Adams AE, Stearns T, Drubin DG, Haarer BK, Jones EW.* Fluorescence microscopy metods for yeast. In: Prescott DM (Ed) Methods in Cell Biology 31 (pp 357–343). Academic Press, New York 1989.
10. *Svoboda A, Bähler J, Kohli J.* Microtubule-driven nuclear movements and linear elements as meiosis-specific characteristics of the fission yeasts *Schizosaccharomyces versatilis* and *Schizosaccharomyces pombe*. Chromosoma 1995; 104: 203-214.
11. *Woods A, Sherwin T, Sasse R, MacRae TH, Baines AJ, Gull K.* Definition of individual components within the cytoskeleton of *Trypanosoma brucei* by a library of monoclonal antibodies. J. Cell Biol. 1989; 130: 687–700.
12. *Kawai R, Fujita K, Iwahashi H, Komatsu Y.* Direct evidence for the intracellular localization of Hsp 104 in *Saccharomyces cerevisiae* by immunoelectron microscopy. Cell stress and Chaperones 1999; 4(1): 46–53.
13. *Lindquist S, Kim G.* Heat-shock protein 104 expression is sufficient for thermotolerance in yeast. Proc Natl Acad Sci USA 1996; 93(11): 5301–5306.
14. *Hino M, Kurogi K, Okubo MA, Muratahori M, Hosoya H.* Small heat shock protein 27 (HSP27) associates with tubulin/microtubules in HeLa cells. Biochem Biophys Res Commun 2000; 271: 164–169.
15. *Moskvina E, Schuller C, Maurer CTC, Mager WH, Ruis H.* A search in the genome of *Saccharomyces cerevisiae* for genes regulated via stress response elements. Yeast 1998; 14: 1041–1050.
16. *Haskin CL, Athanasiou KA, Klebe R, Cameron IL.* A heat-shock-like response with cytoskeletal disruption occurs following hydrostatic-pressure in MG-63 osteosarcoma cells. Biochemistry and Cell Biology 1993; 71: 361–371.
17. *Han SI, Ha KS, Kang KI, Kim HD, Kang HS.* Heat-shock induced actin polymarization, SAPK/JNK activation, and heat-shock protein expression are mediated by genistein-sensitive tyrosine kinase(s) in K562 cells. Cell Biology International 2000; 24: 447–457.
18. *Kang YS, Ko YG, Seo JS.* Caveolin internalization by heat shock or hyperosmotic shock. Experimental Cell Research 2000; 255: 221–228.
19. *Huang SH, Yang KJ, Wu JC, Chang KJ, Wang SM.* Effects of hyperthermia on the cytoskeleton and focal adhesion proteins in a human thy carcinoma cell line. J Cell Biochem 1999; 75: 327–337.
20. *Smertenko A, Draber P, Viklický V, Opatrný Z.* Heat stress affects the organization of microtubules and cell division in *Nicotiana tabacum* cells. Plant Cell Environ 1997; 20: 1534–1542.
21. *Liang P, MacRae TM.* Molecular chaperones and the cytoskeleton. J Cell Sci 1997; 110: 1431–1440.
22. *Oka M, Nakai M, Endo T, Lim CR, Kimata Y, Kohno K.* Loss of hsp70-hsp40 chaperone activity causes abnormal nuclear distribution and aberrant microtubule formation in M-phase of *Saccharomyces cerevisiae*. J Biol Chem 1998; 273: 29727–29737.
23. *Ursic D, Sedbrook JC, Himmel KL, Culbertson MR.* The essential yeast Tcp1 protein affects actin and microtubules. Mol Biol Cell 1994; 5: 1065–1080.
24. *Brown CR, Doxsey SJ, Hongbrown LQ, Martin RL, Welch WJ.* Molecular chaperones and the centrosome – A role for TCP-1 in microtubule nucleation. J Biol Chem 1996; 271: 824–832.
25. *Ruis H, Schuller C.* Stress signaling in yeast. BioEssay 1995; 17: 959–965.
26. *Mager WH, Hohmann S.* Stress response mechanisms in the yeast *Saccharomyces cerevisiae*. In: Hohmann S., Mager WH (Eds) Yeast Stress Responses. Austin: R.G. Landes Company; 1997; 1–5.