JOURNAL OF BACTERIOLOGY, May 1990, p. 2798–2801
0021-9193/90/052798-04$02.00/0
Copyright © 1990, American Society for Microbiology
Vol. 172, No. 5

Development of Thermotolerance in *Neurospora crassa* by Heat Shock and Other Stresses Eliciting Peroxidase Induction

M. KAPOOR,* G. M. SREENIVASAN, N. GOEL, AND J. LEWIS

Cellular, Molecular, and Microbial Biology Division, Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada T2N 1N4

Received 26 July 1989/Accepted 5 February 1990

Hyperthermia, $CdCl_2$, sodium arsenite, and H_2O_2 led to the rapid appearance of high levels of peroxidase in *Neurospora crassa* cultures and induced tolerance toward normally lethal temperatures in 60-h-old colonies. Intracellular superoxide dismutase levels did not correlate with the development of thermotolerance.

One of the universal features of sublethal hyperthermal exposure is the rapid, transient synthesis of a specific array of proteins, termed heat shock proteins (HSPs) (23). In addition, a variety of treatments, such as oxidative stress, toxic metals, metabolic inhibitors, and ethanol, also stimulate the production of all or a subset of the HSPs in most organisms. Functions for the major HSPs have only recently started to emerge. In eucaryotes, the members of the HSP70 family are ATP-binding proteins which have been shown to function in the maintenance of nucleolar morphology following hyperthermal stress (24), dissociation of clathrin triskelions from coated vesicles (28), and unfolding of transmembrane proteins to facilitate translocation (3, 7). The heat shock GroEL protein of *Escherichia coli* participates as an active agent in the correct assembly of oligomeric proteins (10). The identities of some of the relatively minor HSPs as isoforms of glycolytic enzymes (enolase [16] and glyceraldehyde phosphate dehydrogenase [22]) in *Saccharomyces cerevisiae*, ubiquitin in chicken embryo fibroblasts (2), and protease in *E. coli* (25) lend credence to the view that HSPs encompass a variety of components involved in protection of essential functions uniquely threatened in specific organisms.

The development of thermotolerance as a consequence of heat shock has been documented for a wide range of organisms. The correlation between the synthesis of HSPs and the acquisition of thermotolerance is evident in studies with some systems (6, 15), while other reports do not support such a conclusion (20, 29). However, the identities of proteins necessary for the acquisition of thermotolerance and of the signal for the heat shock response remain elusive. Among the proposed candidates for the signaling mechanisms are the superoxide free radical (26), denatured proteins (1), and changes in Ca^{2+}-regulated intracellular processes (21). We have previously reported that one of the *Neurospora crassa* HSPs is a peroxidase that confers resistance towards toxic doses of H_2O_2; its induction parallels the development of thermotolerance (18, 19). In this communication, evidence for a strong correlation between the capacity of stress-inducing agents to enhance intracellular peroxidase levels and their effectiveness in eliciting thermotolerance is presented.

Wild-type *N. crassa* FGSC 262 mycelium was grown in Vogel minimal medium (30) as described previously (17). For plate cultures, conidial suspensions were plated on semisolid medium (minimal medium plus 1% sorbose plus 0.1% sucrose) and incubated at 30°C for 60 h. Heat shock was given by transfer to a humid incubator at 52°C (plate surface temperature of 48°C for at least 40 min). Replica plates were prepared by transfer of colonies to sterile Whatman no. 1 filter paper disks (Whatman, Inc., Clifton, N.J.) soaked in sterile H_2O. The filter paper was laid on the agar surface, the plates were incubated for another 12 to 24 h at 30°C, and finally the filter paper was placed on a fresh sorbose-agar plate. After 12 to 24 h, the filter paper was removed and percent survival was determined by colony counts. For chemical treatments, the 60-h-old colonies were layered with a sterile solution of the desired agent. After 1 to 2 h, the solution was poured out, and the plates were washed briefly with sterile H_2O and incubated for 1 h at 30 or 48°C before replica plating took place. Thermotolerance experiments were performed by transferring 60-h-old heat-shocked or control plates for 1 h to a humid incubator at 57°C (plate surface temperature of at least 51.5°C for 40 min). Crude extracts of lyophilized mycelium in 0.01 M phosphate buffer–0.1 M NaCl (pH 7.2) were assayed spectrophotometrically for peroxidase and superoxide dismutase activity (9, 14).

Fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) was employed to assess the relative membrane fluidity of normally grown and heat-shocked cells. Samples of the cultures were withdrawn and centrifuged at 3,000 × *g* for 10 min. To 1.5 g (wet weight) of the pellet, 4 μl of a freshly prepared 2×10^{-3} M DPH solution (in distilled tetrahydrofuran) was added. After these ingredients were mixed, 2.0 ml of 1% agar in phosphate-buffered saline (0.15 M NaCl–0.01 M phosphate buffer, pH 7.2) cooled to 40°C was added; after the mixture was rapidly vortexed, it was poured into 5-cm-diameter petri dishes. The solidified agar, sliced into strips of uniform size, was inserted in quartz cuvettes and overlaid with phosphate-buffered saline. A blank cuvette was prepared with the same amount of mycelial cells without DPH. The cuvette containing intact cells embedded in agar was incubated at room temperature for 15 min. Fluorescence polarization measurements were made with an Aminco SPF-500 spectrophotofluorometer (excitation and emission at 355 and 430 nm, respectively). Polarization anisotropy (*A*) is defined by the relationship $A = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$.

Heat shock treatment of 60-h-old colonies for 1 h at 48°C led to the acquisition of a high degree of tolerance toward the normally lethal temperature. In contrast, plates directly transferred to the lethal temperature exhibited virtually no surviving colonies. A considerable extent of colony survival

* Corresponding author.

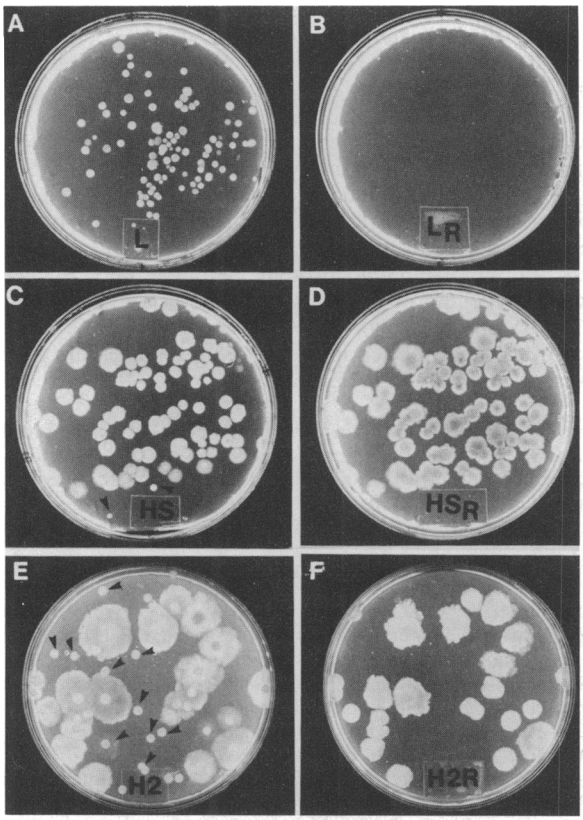

FIG. 1. Thermotolerance induction by heat shock and hydrogen peroxide treatment. (A) 60-h-old *N. crassa* colonies on a sorbose-agar plate after "lethal" shock (L); (B) replica plate (LR) prepared from plate in panel A; (C) 60-h-old colonies subjected to heat shock (HS) at 48°C for 1 h and "lethal" shock for 1 h; (D) replica (HSR) of panel C; (E) plate with 60-h-old colonies treated with 1 mM H_2O_2 (H2) for 45 min followed by "lethal" shock; (F) replica (H2R) of panel E. Arrowheads show some of the colonies killed by "lethal" shock.

was also witnessed in H_2O_2-treated plates (Fig. 1). Since H_2O_2 is an effective inducer of peroxidase (18), degradation of intracellular H_2O_2 by the latter may be a reasonable explanation for this effect. The accumulation of intracellular H_2O_2 or peroxides derived therefrom could account for the biological damage occasioned by heat shock. The implication of these results is that oxidative stress is a vital factor in cellular damage inflicted by heat shock; hence, its amelioration by peroxidase may constitute a major element in the development of thermotolerance. Exposure to sodium arsenite also permitted significant colony survival after lethal shock (Table 1). It is pertinent to note that treatment of *N. crassa* mycelium with arsenite and H_2O_2 elicits the synthesis of only a small subset of HSPs (17), suggesting that the presence of all of the HSPs is not essential for thermotolerance. Upon pretreatment with $CdCl_2$, >65% survival was witnessed (Table 1), but exposure of mycelium to 100 μM $CdCl_2$ does not stimulate detectable HSP synthesis (17).

A pronounced induction of peroxidase was recorded in heat-shocked cells, whereas in controls it was virtually undetectable (Table 2). $CdCl_2$, $MnCl_2$, and $CuCl_2$ promoted various levels of peroxidase in nonshocked cells. In cells subjected to heat shock and chemical stress simultaneously, Cd^{2+} and arsenite supported a high level of peroxidase, but Mn^{2+} and Cu^{2+} depressed the levels of peroxidase in heat-shocked cells. Considered together, these observations illustrate that treatments permitting high-level induction of peroxidase at the heat shock temperature coincide with those conferring thermotolerance. Furthermore, intracellular superoxide dismutase levels in stressed cultures do not correlate with thermotolerance (Table 2).

TABLE 1. Development of thermotolerance by 60-h-old *N. crassa* colonies subjected to various pretreatments

Pretreatment	Duration (min)	% Survival at lethal temp
None		5–10[a]
Heat shock	60	75–95[a]
Sodium arsenite (750 μM)	60	52
Sodium arsenite (400 μM)	60	50–60[a]
+ heat shock	60	
Hydrogen peroxide (1 mM)	45	45
Dithiothreitol (2 mM)	60	31
Cadmium chloride (50 μM)	120	69
Cadmium chloride (50 μM)	120	77
+ heat shock	60	

[a] Range established from results of several independent experiments. The remaining data are based on average colony counts from three plates.

Since cellular membranes have been recognized as possible targets of thermal damage, we decided to examine the overall state of *N. crassa* membranes after heat shock. A qualitative assessment of the extent of heat-induced changes in membrane fluidity (27) can be obtained from plots of fluorescence polarization anisotropy (*A*) of DPH as a function of temperature. A decrease in the value of *A* for heat-shocked cells relative to controls is indicative of increased fluidity of membranes of the latter (Fig. 2, left panel), illustrating one of the deleterious consequences of thermal stress. No clear lipid transitions were discernible, as these measurements entailed the use of whole cells, the data being reflective of the combined contribution from mitochondrial and plasma membranes. Phenyl ethyl alcohol (PEA), a perturbant of membrane structure, is known to alter membrane fluidity (11). The validity of the heat shock-induced fluidity changes of *N. crassa* membranes was confirmed by adding 50 and 100 μM PEA to the growth medium. Polarization anisotropy measurements with DPH showed greater fluidity of membranes in PEA-exposed cultures than in controls (Fig. 2).

These experiments demonstrate the close parallelism between the acquisition of thermotolerance and the induction of peroxidase in *N. crassa*. The specific treatments that led to the development of thermotolerance also elicited marked induction of peroxidase at heat shock temperatures, indicating that peroxidase is vital for protection from thermal

TABLE 2. Relative levels of superoxide dismutase and peroxidase activities in stressed and unstressed mycelium

Treatment	Relative activity of:			
	Peroxidase		Superoxide dismutase	
	Control	Heat shock	Control	Heat shock
No additions	1	340	1	0.6
Sodium arsenite (400 μM)	330	860	0.8	1.6
$CdCl_2$ (100 μM)	580	880	1.2	0.8
$MnCl_2$ (100 μM)	200	80	1.3	0.6
$CuCl_2$ (100 μM)	450	60	0.6	1.0

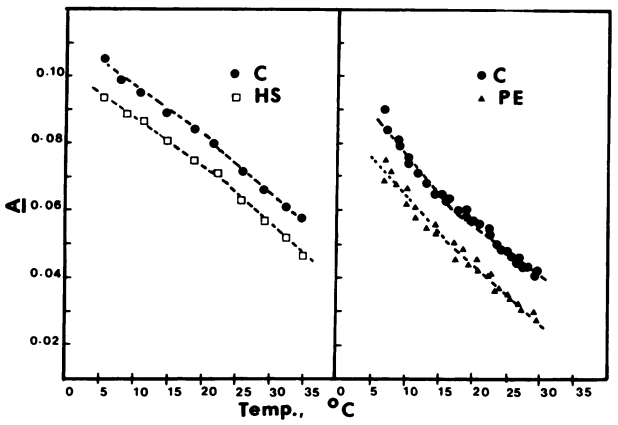

FIG. 2. Effect of heat shock and PEA on cellular membranes. Left panel, Polarization anisotropy (*A*) of DPH in mycelium grown at 28°C for 15 h (C) and in that grown for 14 h at 28°C and heat shocked for 1 h (HS) as a function of temperature. Right panel, Polarization anisotropy of DPH in mycelium grown for 15 h at 28°C in minimal medium plus 2% sucrose (C) and in 15-h-old mycelium grown in the presence of 100 μM PEA (PE). Temp., Temperature.

injury. A link between hyperthermia and oxidative stress has been discerned in eucaryotic as well as procaryotic systems (4, 26). For instance, bacteria are endowed with an adaptive mechanism toward H_2O_2 and a protective regulon under the control of the *oxyR* locus encompassing a number of genes (8). Exposure of *E. coli* cells to $O_2^{\cdot-}$-generating agents results in the induction of proteins specifically required for defense against oxidative damage (31).

Figure 3 presents a hypothetical scheme outlining the interrelations of heat shock, oxidative stress, and thermotolerance in *N. crassa*. It is proposed, in agreement with other workers (5, 26), that a primary consequence of hyperthermia is the acceleration of $O_2^{\cdot-}$ production. Employing a fluorometric procedure, we have obtained direct evidence for a substantial increase in $O_2^{\cdot-}$ levels in the growth medium of heat-shocked cells compared with nonshocked controls (unpublished data). The next step is envisioned as the heightened production of H_2O_2 catalyzed by the multiple isoforms of *N. crassa* superoxide dismutase (13). H_2O_2, a deleterious

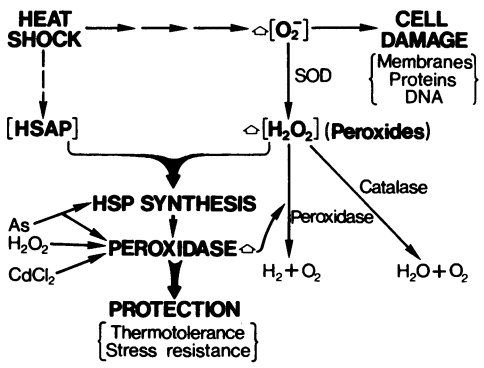

FIG. 3. Hypothetical scheme for the role of oxidative stress and peroxidase in the heat shock response and the development of thermotolerance in *N. crassa*. HSAP, Heat shock activator protein; SOD, superoxide dismutase.

agent, also generates ·OH, an even more potent species, via the Haber-Weiss reaction. Intracellular H_2O_2 is potentially more harmful than $O_2^{\cdot-}$ per se, since lipid peroxides derived from it are membrane-damaging agents, causing severe perturbation of the permeability properties (12). It is proposed that endogenously generated H_2O_2 (or a derivative) in conjunction with a transcriptional activator protein (HSAP) triggers the expression of heat shock genes to produce, among other proteins, high levels of peroxidase. Peroxidase constitutes a critical defense against thermal and oxidative stress. A DNA-binding protein demonstrated in heat-shocked mycelium only (17) is a possible candidate for the role of HSAP. This hypothesis is supported by the coincidence of the ability of various stresses to induce peroxidase as well as thermotolerance.

This work was supported in part by an operating grant from the Natural Sciences and Engineering Research Council of Canada (to M.K.) and by Alberta Heritage Foundation for Medical Research summer studentship awards to G.M.S. and N.G.

LITERATURE CITED

1. **Ananthan, J., A. L. Goldberg, and R. Voellmy.** 1986. Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. Science **232**:522–524.
2. **Bond, U., and M. J. Schlesinger.** 1985. Ubiquitin is a heat shock protein in chicken embryo fibroblasts. Mol. Cell. Biol. **5**: 949–956.
3. **Chirico, W. J., M. G. Waters, and G. Blobel.** 1988. 70k heat shock proteins stimulate protein translocation into microsomes. Nature (London) **332**:805–810.
4. **Christman, M. F., R. W. Morgan, F. S. Jacobson, and B. N. Ames.** 1985. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. Cell **41**:753–762.
5. **Courgeon, A.-M., E. Rollet, J. Becker, C. Maisonhaute, and M. Best-Belpomme.** 1988. Hydrogen peroxide (H_2O_2) induces actin and some heat shock proteins in *Drosophila* cells. Eur. J. Biochem. **171**:163–170.
6. **Craig, E. A., and K. Jacobsen.** 1984. Mutations of the heat inducible 70 kilodalton genes of yeast confer temperature sensitive growth. Cell **38**:841–849.
7. **Deshaies, R. J., B. D. Koch, M. Werner-Washburne, E. A. Craig, and R. Schekman.** 1988. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. Nature (London) **332**:800–805.
8. **Farr, S. B., D. Touati, and T. Kogoma.** 1988. Effects of oxygen stress on membrane functions in *Escherichia coli*: role of HPI catalase. J. Bacteriol. **170**:1837–1842.
9. **Flohé, L., and F. Ötting.** 1984. Superoxide dismutase assays. Methods Enzymol. **105**:93–104.
10. **Goloubinoff, P., A. A. Gatenby, and G. H. Lorimer.** 1989. GroE heat-shock proteins promote assembly of foreign prokaryotic ribulose bisphosphate carboxylase oligomers in *Escherichia coli*. Nature (London) **337**:44–47.
11. **Halegoua, S., and M. Inouye.** 1979. Translocation and assembly of outer membrane proteins of *Escherichia coli*. Selective accumulation of precursors and novel assembly intermediates caused by phenethyl alcohol. J. Mol. Biol. **130**:39–61.
12. **Halliwell, B., and J. M. C. Gutteridge.** 1984. Lipid peroxidation, oxygen radicals, transition metal ions and disease. Biochem. J. **219**:1–14.
13. **Henry, L. E. A., R. Cammack, J. P. Schwitzgnebel, J. M. Palmer, and D. O. Hall.** 1980. Intracellular localization, isolation and characterization of two distinct varieties of superoxide dismutase from *Neurospora crassa*. Biochem. J. **187**:321–328.
14. **Hoffman, P. S., N. R. Krieg, and R. M. Smibert.** 1979. Studies of the microaerophilic nature of *Campylobacter fetus* subsp. *jejuni*. I. Physiological aspects of enhanced aerotolerance. Can. J. Microbiol. **25**:1–7.

15. **Iida, H., and I. Yahara.** 1984. A heat shock-resistant mutant of *Saccharomyces cerevisiae* shows constitutive synthesis of two heat shock proteins and altered growth. J. Cell Biol. **99:** 1441–1450.
16. **Iida, H., and I. Yahara.** 1985. Yeast heat-shock protein Mr 48,000 is an isoprotein of enolase. Nature (London) **315:**688–690.
17. **Kapoor, M., and J. Lewis.** 1987. Alteration of the protein synthesis pattern in *Neurospora crassa* by hyperthermal and oxidative stress. Can. J. Microbiol. **33:**162–168.
18. **Kapoor, M., and J. Lewis.** 1987. Heat shock induces peroxidase activity in *Neurospora crassa* and confers tolerance toward oxidative stress. Biochem. Biophys. Res. Commun. **147:**904–910.
19. **Kapoor, M., and M. G. Sreenivasan.** 1988. The heat shock response of *Neurospora crassa*: stress-induced thermotolerance in relation to peroxidase and superoxide dismutase levels. Biochem. Biophys. Res. Commun. **156:**1097–1102.
20. **Landry, J., and P. Chrétien.** 1983. Relationship between hyperthermia-induced heat-shock proteins and thermotolerance in Morris hepatoma cells. Can J. Biochem. Cell Biol. **61:**428–437.
21. **Landry, J., P. Crete, S. Lamarche, and P. Chrétien.** 1988. Activation of Ca^{2+}-dependent processes during heat shock: role in thermoresistance. Radiat. Res. **113:**426–436.
22. **Lindquist, S.** 1986. The heat-shock response. Annu. Rev. Biochem. **55:**1151–1191.
23. **Nover, L. (ed).** 1984. Heat shock responses of eukaryotic cells. Springer-Verlag KG, Berlin.
24. **Pelham, H. R. B.** 1984. HSP70 accelerates the recovery of nucleolar morphology after heat shock. EMBO J. **3:**3095–3100.
25. **Phillips, T. A., R. A. VanBogelen, and F. C. Neidhardt.** 1984. *lon* gene product of *Escherichia coli* is a heat-shock protein. J. Bacteriol. **159:**283–287.
26. **Ropp, M., A. M. Courgeon, R. Calvayrac, and M. Best-Belpomme.** 1983. The possible role of the superoxide ion in the induction of heat-shock and specific proteins in aerobic *Drosophila* cells during return to normoxia after a period of anaerobiosis. Can J. Biochem. Cell Biol. **61:**456–461.
27. **Shinitzky, M., and Y. Barenholz.** 1978. Fluidity parameters of lipid regions determined by fluorescence polarization. Biochim. Biophys. Acta **515:**367–394.
28. **Ungewickell, E.** 1985. The 70-kD mammalian heat shock proteins are structurally and functionally related to the uncoating protein that releases clathrin triskelia from coated vesicles. EMBO J. **4:**3385–3391.
29. **VanBogelen, R. A., M. A. Acton, and F. C. Neidhardt.** 1987. Induction of the heat shock regulon does not produce thermotolerance in *Escherichia coli*. Genes Dev. **1:**525–531.
30. **Vogel, H. J.** 1956. A convenient growth medium for *Neurospora crassa* (N medium). Microb. Genet. Bull. **13:**42–43.
31. **Walkup, L. K. B., and T. Kogoma.** 1989. *Escherichia coli* proteins inducible by oxidative stress mediated by the superoxide radical. J. Bacteriol. **171:**1476–1484.