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Effect of Exogenous Stress on the Tissue-Cultured Mouse Lens Epithelial Cells

Mihir Bagchi,* Malkhan Katar, and H. Maisel

Department of Anatomy/Cell Biology, Wayne State University School of Medicine, Detroit, Michigan 48201

Abstract The effects of heat, oxidative and osmotic stress on heat shock proteins (HSP-70(I), HSC-70, and HSP-40 of tissue cultured mouse lens epithelial cells (αTN-4) were investigated. The effect of stress on the heat shock transcription factor (HSF-1), and a nuclear matrix protein (NM-60) of αTN-4 cells was also examined. Cells were exposed to heat (45°C), oxidative stress (50 mM H_20_2) and osmotic (600 mM medium) shock for 30 min, and then allowed to recover for 18 h in physiological medium. Control cells were maintained at 37°C in an isosmolar medium. Cellular proteins were isolated and fractionated by SDS–PAGE. Western blot was used to determine the levels of HSP and nuclear proteins. Heat stressed cells were also examined, by immunofluorescence, for the specific localization of NM-60 and HSF-1. The results revealed that both NM-60 and HSF-I were present in specific locations in normal and heat-stressed cell nuclei. Nuclei isolated immediately after stress showed localization of fluorescence near the nuclear membrane. When heat stressed cells were allowed to recuperate at 37°C, most of the fluorescence were relocated in discrete areas of the nucleus. These experiments showed that αTN-4 cells responded to stress by overexpression of HSP-70(I) and HSP-40. This increase was not present immediately after the end of the stress period, but clearly evident at 18 h of recovery in physiological medium. Immunofluorescent data suggest that heat stress induced the localization of NM-60 and HSF-1 near the nuclear membrane. J. Cell. Biochem. 86: 302–306, 2002. © 2002 Wiley-Liss, Inc.

Key words: heat shock; HSP-70(I); HSP-40; HSC-70; heat shock transcription factor; lens epithelial cells (αTN-4)

Optimal vision is highly dependent on the clarity of the ocular lens. It has been established that lens transparency is maintained by the supramolecular organization of its constituent proteins [Piatigorsky, 1993]. Preservation of this organization is maintained by the fidelity of native proteins. Quarternary protein structure depends on (a) proper folding of newly synthesized polypeptides, and (b) adequate protection from environmental or pathophysiological stress [Bukau et al., 2000]. Destabilization of native protein organization induces opacity of the lens [Hejtmancik and Piatigorski, 1994]. Thus a detailed understanding of how lens proteins retain their native structure for sustained periods of time is essential. Furthermore the lens, by its unique anatomical location, is continuously exposed to a stressful environment. The lens has no direct blood supply, thus essential biologically important molecules such as oxygen or amino acids are limited, while waste products such as toxins or inflammatory substances remain in close proximity for extended periods of time. Therefore, the lens needs continuous protection against microenvironmentally-induced stress. Without such protection lens proteins could degrade causing cataract.

Most animal and plant cells respond to stress by overexpressing heat shock proteins (HSP) [Morimoto, 1998; Mirkes et al., 1999; Leppa et al., 2001].

Therefore, in this study, we examined the levels of several HSP in response to exogenous stress applied to the lens. Both the constitutive and inducible forms of HSP-70, HSC-70, and HSP(I)-70 were analyzed. The experiments showed that environmental stress induced overexpression of HSP (HSP(I)-70 and HSP-40) and

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*Correspondence to: Mihir Bagchi, Ph.D., Department of Anatomy/Cell Biology, Wayne State University School of Medicine, Detroit, Michigan 48201.
E-mail: mbagchi@med.wayne.edu

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translocated heat shock transcription factor HSF-I to discrete areas of the nucleus, probably where heat shock protein genes are located [Cotto et al., 1997, Jolly et al., 1999].

MATERIALS AND METHODS

Confluent αTN-4 cells (obtained from Dr. P. Russel, NIH) were grown in DMEM, supplemented with 20% fetal bovine serum, in a humidified incubator at 37°C. The culture medium was kept at 300 mOsm and pH 7.2. Thermal, osmotic or oxidative stress on αTN-4 cells were produced by 30 min exposure to (a) 45°C, (b) culture in 600 mOsm Mannitol containing medium, and (c) culture in DMEM containing 50 mM hydrogen peroxide. Some stressed cells were allowed to recuperate after exposure to stress in normal culture medium at 37°C for 18 h. Total protein was isolated from parallel groups of cells immediately after exposure to stress. Both water soluble (WS) and water insoluble (WI) protein was isolated from normal and stressed cells [Bagchi et al., 2001]. The protein concentration in each fraction was determined by the Bradford Method.

Western Blot

Equal amounts of WI and WS protein cells were subjected to 10% SDS–PAGE. Duplicate sets of SDS–PAGE were run. One set was stained with Coomassie blue, whereas the other set was electrophorectically transferred to nitrocellulose paper. Specific HSP were identified by immunoblot analysis. Antibodies were obtained from Stressgen (Vancuver, B.C., Canada). Alkaline phosphatase conjugated secondary antibodies were employed for color development (Bio-Rad).

Immunofluorescence

The αTN-4 cells were grown on special chambered glass slides and exposed to heat shock (45°C). Some stressed cells were fixed immediately after heat shock in 4% paraformaldehyde, while other cells were allowed to recover in isosmolar DMEM at 37°C for 18 h before fixation in 4% paraformaldehyde. Nuclei from αTN-4 cells were isolated by the modified Penman technique [Bagchi et al., 2001]. The treated nuclei were incubated with polyclonal antibodies to the lens nuclear matrix protein NM-60 [Bagchi et al., 2001] or human HSF-1 (Stressgen) at 37°C for 1 h. Nuclei were then washed with PBS and treated with goat anti-rabbit IgG conjugated with fluorescein at 37°C for 1 h. The nuclei were counter stained with DAP1 (250 μg/ml) and observed under a Zeiss axiomat epifluorescence microscope. The digitized images were analyzed with Metamorph software.

RESULTS

Experiments were performed to examine the effect of heat stress on the levels of HSP-70(I), HSPC-70, and HSP-40 in tissue cultured αTN-4 cells. Levels were determined by immunoblot. Figure 1 shows the results of αTN-4 cells, which had been stressed at 45°C for 30 min and then allowed to recover at 37°C for 18 h. Heat stress produced a significant increase in the levels of HSP-70(I) and HSP-40 in both the WS and WI fractions. There was no change in the level of HSC-70 (C) nor in the levels of HSF-1 and NM-60 (Fig. 2) at 18 h after heat stress. The effect of heat on HSP was not immediate, since the levels of HSP-70(I) and HSP-40 were similar in control cells, and in stressed cells collected immediately after incubation at 45°C for 30 min (Fig. 3).

The effect of osmotic and oxidative stress on αTN-4 cells, which had been allowed to recover for 18 h in control DMEM is shown in Figure 4. Both osmotic and oxidative stress resulted in increased levels of HSP-70(I), but not of the

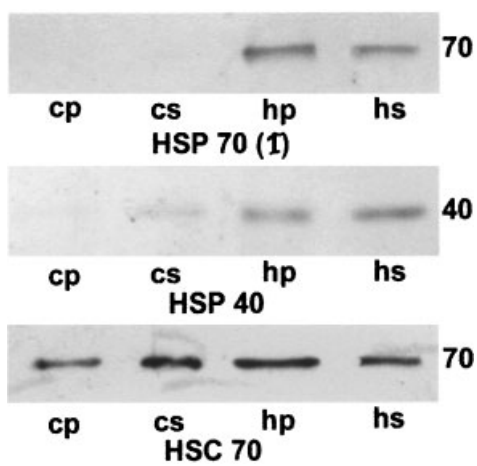

Fig. 1. Effect of heat shock on tissue-cultured αTN-4 cells. Heat-treated cells were incubated at 45°C for 30 min, then transferred to a 37°C incubator and allowed to recuperate for 18 h. Control cells were kept at 37°C. WS and WI cell fractions were fractionated by SDS–PAGE, transferred to nitrocellulose paper, and probed with antibodies to HSP-70(I), HSP-40, and HSC-70. cp, control WI pellet; cs, control WS fraction; hp, heat shocked cell WI pellet; hs, heat-shocked cell, soluble fraction.

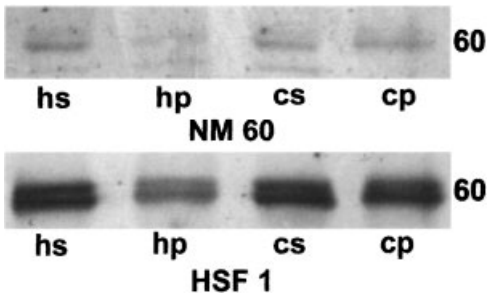

Fig. 2. Effect of heat shock on αTN-4 cells. Immunoblots were probed with NM-60 and HSF-1 antibodies. Heat-treated cells were allowed to recuperate at 37°C for 18 h, and shown as controls.

constitutive HSP-70. In contrast to increased levels of HSP-70(I), there was a marked decrease in the amount of NM-60 and HSF-1 as a result of oxidative and osmotic stress (Fig. 4).

Immunofluorescence microscopy was used for further analysis of the effect of heat stress on the nuclear localization of NM-60 and HSF-1 (Fig. 5). Control αTN-4 cells (ONM) showed discrete fluorescence in the nucleoplasm. However, there was discrete localization of NM-60 near the nuclear membrane in cells fixed immediately after heat stress. In some cases, there was a prominent fluorescent ring near the periphery of the nucleus. The nuclei of heat stressed αTN-4 cells after 18 h recovery showed a redistribution of NM-60 in the nucleoplasm similar to control cells. Figure 6 shows HSF-1 localization in the nuclei of αTN-4 cells. The nuclei of cells fixed immediately after heat stress showed ring like fluorescence near the nuclear membrane. However, when heat stressed cells were allowed to recover for 18 h, anti-HSF-1 binding was more diffuse throughout the nucleoplasm. Some nuclei still retained the ring-like fluorescence.

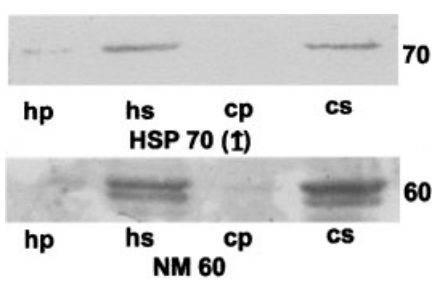

Fig. 3. Effect of heat shock on αTN-4 cells. WS and WI cellular proteins were isolated 30 min after heat shock. Control cells were maintained at 37°C. Immunoblots were probed with HSP-70(I) and NM-60 antibodies.

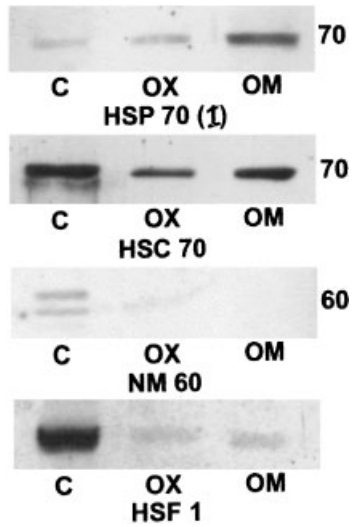

Fig. 4. Effect of oxidative and osmotic stress on αTN-4 cells. αTN-4 cells were exposed to either 50-mM H_2O_2 or hyperosmolar medium (600 mM) for 30 min and then transferred to control medium and further incubated for 18 h. Total cell proteins were isolated and probed for HSP-70(1), HSC-70, NM-60, and HSF-1. C, control cells. OX, exposed to H_2O_2-containing medium. OM, incubated in hyperosmolar medium.

DISCUSSION

A rapid increase in the synthesis of HSP, HSP-70(I), and HSP-40 is a common cellular response to environmental stress. [Collier and Schlesinger, 1986; Dejong et al., 1986; Dash et al., 1994; Liberek et al., 1995]. Such a response was also found in αTN-4 cells exposed to heat stress. The response was time dependent, since no elevation of HSP was found in cells at the end of 30 min at 45°C. It was, however, readily apparent at 18 h after heat stress. The elevation of both HSP-70(I) and HSP-40 is important, since they form a complex, which participates, in refolding damaged proteins, and in folding of newly-synthesized polypeptides [Liberek et al., 1995]. Indeed the complex of HSP-70(I) and HSP-40 is more effective as a foldase HSP than either of the proteins individually [Pierpaoli et al., 1997]. In these experiments, the level of the constitutive HSC-70 was not altered.

Since the induction of HSP-70(I) and HSP-40 expression in response to stress is primarily regulated by the heat shock transcription factor HSF-1 [Abravaya et al., 1991], we monitored the levels of HSF-1 in normal and heat stressed αTN-4 cells. The data showed that stress did not alter the level of HSF-1 immediately or even at

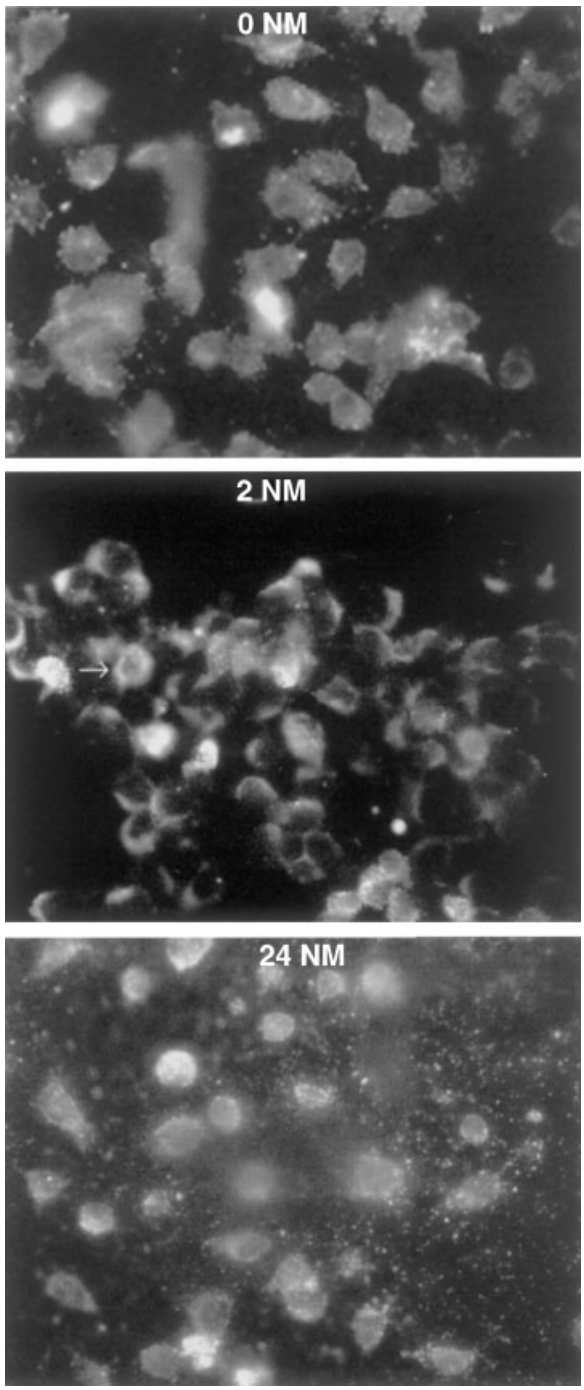

Fig. 5. Immunofluorescence produced by anti-NM-60 antibodies. ONM, control cells showing speckled fluorescence in the nucleus. 2 NM, 30 min after heat shock. Fluorescence is localized near the nuclear membrane. 24 NM, heat-treated cells were further incubated at 37°C for 24 h. Arrow shows localization of fluorescence near nuclear membrane.

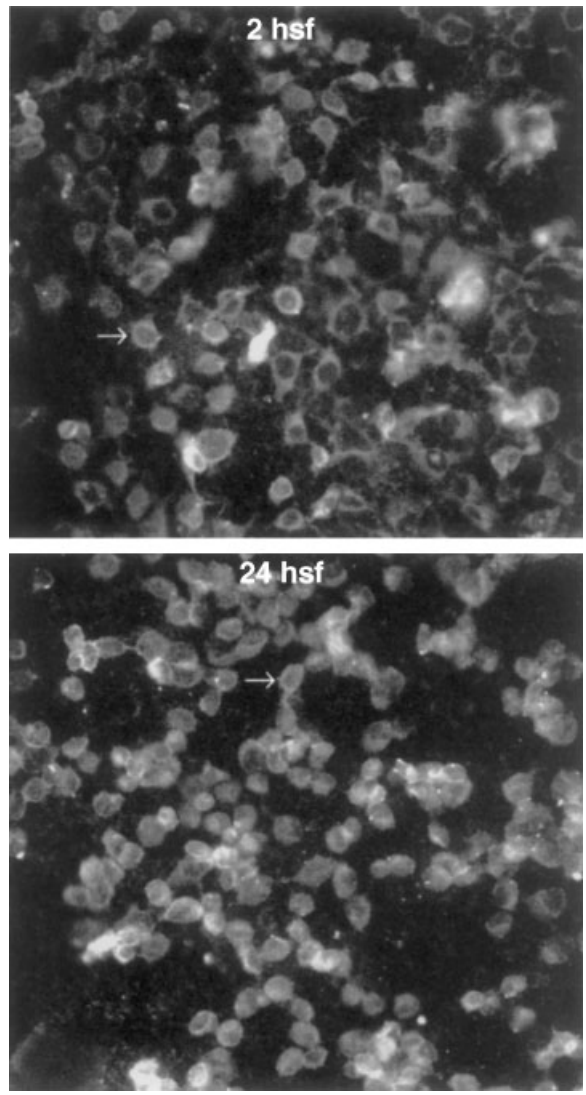

Fig. 6. Immunofluorescence with anti HSF-1 antibodies, after 30 min of heat shock (2 hsf) and 24 h of recovery at 37°C. Arrow indicates localization of fluorescence near nuclear envelopes.

18 h after stress. This finding is consistent with previous reports on prokaryotic and eukaryotic cells [Morimoto, 1998]. Although stress does not directly enhance the amount of HSF-1, it induces the migration of HSF-1 to specific areas of the nucleus [Cotto et al., 1997] where it initiates the transcription of HSP [Jolly et al., 1999]. Heat stress also did not affect the level of NM-60, a lens nuclear matrix-associated protein with significant sequence similarity to HSF-1 [Bagchi et al., 2001].

The results of osmotic and oxidative stress on levels of HSF-1 and NM-60 at 18 h of recovery differed from that of heat stress. Heat stress did not affect the level of HSF-1 and NM-60, whereas these were a marked decline in amount of these proteins 18 h after osmotic and oxidative stress. This could be due to

migration of NM-60 and HSF-1 within the nucleus and the formation of complexes with genetic material. Such complex formation could make the proteins unavailable for antibody binding. The level of HSP-70(I) increased under all forms of stress but HSP (C) remained unchanged.

The immunofluorescence data showed that NM-60 is sequestered in discrete areas of the nucleus. However, immediately after stress, it relocated to an area near nuclear membrane. When cells were allowed to recuperate NM-60 was again found in discrete areas throughout the nucleus, as in normal cells.

When αTN-4 cells were heat-stressed for 30 min, anti HSF-1 antibodies were localized near the nuclear membrane, similar to the location of NM-60. However, some fluorescence was still located near the nuclear membrane even at 18 h after recovery.

We propose that heat stress caused migration of NM-60 and HSF-1 from their discrete storage sites to areas near nuclear membrane. It is not known, whether the latter regions of the nucleus are actively transcribing HSPs. However, this relocation of HSP-1 and NM-60 in stressed αTN-4 cells is similar to that observed in other heat stressed cells [Cotto et al., 1997; Jolly et al., 1997; Mercier et al., 1997].

In conclusion, data presented here show that environmental stress induced an increase of HSP-70(1) and HSP-40, and this increase was temporal. Whether the increased synthesis is regulated at the transcriptional level or at the translational level is not known. However, HSF-1, the main regulatory transcription factor for HSP synthesis, relocated in the nucleus, but did not show any increase in total amount. Since, NM-60 protein behaved similarly in response to stress, as HSF-1, we propose that they are either the same protein or belong to the same transcription factor family. It is also can be suggested that, like many known transcription factors, HSF-1 is partitioned between nuclear matrix bound and free form [Bagchi et al., 1998]. Exogenous stress could induce free HSF-1 to bind with the nuclear matrix and initiate HSP mRNA synthesis.

ACKNOWLEDGMENT

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