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Distinct effects of heat shock and ATP depletion on distribution and isoform patterns of human Hsp27 in endothelial cells

Svetlana A. Loktionova[a], Olga P. Ilyinskaya[a], Vladimir L. Gabai[b], Alexander E. Kabakov[b,*]

[a] *Cardiology Research Center, Russian Academy of Medical Sciences, Moscow 121552, Russian Federation*
[b] *Medical Radiology Research Center, Russian Academy of Medical Sciences, Obninsk 249020, Kaluga Region, Russian Federation*

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Abstract To study the cytoprotective capacity of Hsp27 under various cellular stresses, we compared the effects of heating and energy deprivation on its distribution and isoform composition. Cultured endothelial cells from human aorta or umbilical vein were subjected to heat shock (45°C) and ATP-depleting metabolic stress (CCCP or rotenone in a glucose-free medium). Both exposures led to the translocation of Hsp27 into the Triton X-100-insoluble cellular fraction, whereas the immunofluorescent Hsp27 pattern was characteristic for each stress employed. Heating (5–30 min) caused unexpected association of Hsp27 with thick bundles of actin microfilaments (stress fibers). ATP depletion within 30–120 min resulted in the appearance of Hsp27-containing compact granules in the nucleus. The insolubilization and relocalization of Hsp27 were reversible in both cases. The stress-induced shifts in the Hsp27 isoform spectrum indicate an increase in phosphorylation of Hsp27 in heat-shocked cells and its dephosphorylation in ATP-depleted cells. We suggest that these stresses diversely affect the phosphorylation status of endothelial Hsp27, thus altering its localization, supramolecular organization and functional activity toward actin.

Key words: Stress protein; Hsp27; Actin; Heat shock; ATP depletion; Vascular endothelial cell

1. Introduction

The small or low-molecular-mass (27-kDa) stress protein is a member of the family of heat shock proteins (Hsp) whose synthesis is stimulated by heating or other environmental stresses [1,2]. This protein (Hsp27) performs important biological functions in both stressed and unstressed cells. In particular, mitotic activity of Ehrlich tumor cells was shown to correlate inversely with the levels of Hsp27 and overexpression of the *Hsp27* gene in the cells led to suppression of proliferation [3] that suggests an involvement of the small stress protein in regulation of cell growth. Moreover, Hsp27 is known to be an actin barbed-end capping protein which inhibits actin polymerization and causes disassembly of F-actin [4,5]. In mammalian cells, the constitutively expressed Hsp27 is phosphorylated in response to serum, thrombin, various growth factors, hormones, cytokines, and inducers of differentiation [1,2,6,7]. Microfilament dynamics in activated cells is clearly regulated via Hsp27 phosphorylation [2,8] and therefore the small stress protein is a component of the signal transduction pathway from external stimuli to the actin skeleton.

At the same time, overexpressed Hsp27 seems to confer cell resistance to heat shock [9–12], oxidative stress [13–15], and a number of cytotoxic agents including cytochalasin D [10,12], tumor necrosis factor [13] and some anticancer drugs [1]. The defense mechanisms mediated by Hsp27 remain to be clarified. On the one hand, it was suggested that cytoprotection against heating [10,12], oxidants [14,15] and cytochalasin D [10,12] can be directly due to stabilization of microfilaments by excess Hsp27. The protective capacity of Hsp27 toward the actin framework appears to be dependent on phosphorylation of Hsp27 by stress-activated protein kinase(s) [2,12,14]. On the other hand, heat shock-induced translocation of Hsp27 to cell nuclei [2,16] and its involvement in post-heat shock reparative disintegration of the intranuclear aggregated protein [11] have also been shown. In a cell-free system, Hsp27 acts as an ATP-independent molecular chaperone which prevents protein aggregation at high temperatures and promotes enzyme reactivation after denaturation with urea [17,18]. However, except for the actin skeleton, no direct targets for in vivo chaperoning by Hsp27 were identified. Likewise, it is not quite understood whether the mechanisms of Hsp27-mediated cytoprotection are common under different stresses. Almost no data are available on the behaviour of the small stress proteins in ATP-depleted cells, while a possible protective role for Hsp27 during energy starvation has already been discussed [19,20].

The purposes of the present work were (i) to investigate how Hsp27 responds to the depletion of cellular ATP and (ii) to compare the effects of heating and ATP deprivation on the state of Hsp27 in the exposed cells. We have chosen human vascular endothelium as an object for the study because, firstly, Hsp27 is known to be a mediator of the signaling pathway under activation of endothelial cell functions [7] and, secondly, endothelial cells (EC) can in vivo suffer from ATP depletion during ischemia.

2. Materials and methods

2.1. Cells, stressful exposures and recovery

EC derived from human aorta or umbilical vein were isolated and cultured as described [21]. Exponentially growing cell cultures of the first passage were used in all experiments.

Heat shock treatment was performed by heating EC in a thermostatic water bath at 45°C. Depletion of ATP was achieved by incubating the cells in glucose-free Dulbecco's modified Eagle's minimal essential medium (Sigma) in the presence of 3% fetal bovine serum and 20 μM CCCP, an uncoupler of oxidative phosphorylation, or 20 μM rotenone, a respiratory inhibitor. To restore normal ATP level the treated cells were washed and plated in growth medium (with D-glucose). Cellular ATP was measured by luciferin/luciferase assay [22].

*Corresponding author. Fax: (7) (095) 956 1440.
E-mail: indep@mrrc.obninsk.su

Abbreviations: Hsp, heat shock protein; EC, endothelial cells; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; SDS, sodium dodecyl sulfate; ECL, enhanced chemiluminiscence

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2.2. Cell fractionation with Triton X-100

Growing in 35 mm plastic dishes, preconfluent EC were lysed with 0.3 ml of ice-cold phosphate-buffered saline (PBS, pH 7.4) containing 1% (w/v) Triton X-100 and 1 mM phenylmethylsulphonyl fluoride. The lysates were promptly centrifuged at 12000×*g* for 10 min at 4°C [23], and the pellets were then washed with the lysing buffer and resedimented. The first supernatants (Triton-soluble cellular fraction) and the washed pellets (Triton-insoluble cellular fraction) were prepared for Laemmli electrophoresis under reducing conditions as described [23].

2.3. Immunofluorescence staining

EC adhering to coverslips were fixed and permeabilized with a mixture of 3.7% formaldehyde and 0.1% Triton X-100 for 10 min. Thereafter the cell preparations were washed with PBS and exposed to 1% bovine serum albumin for 30 min. Incubation with anti-Hsp27 rabbit antibodies (kindly provided by Dr. M. Gaestel [24]) continued for 60 min. Anti-rabbit Ig antibodies coupled to Texas Red (South Biotech. Assoc., Inc.) and phalloidin-FITC conjugates (Sigma) were used for fluorescence labeling. The fluorescence patterns were analyzed on an Opton III microscope (Karl Zeiss, Germany).

2.4. Gel electrophoresis and immunoblotting

Cell proteins were subjected to SDS electrophoresis in a Laemmli system with 4% stacking and 15% separating polyacrylamide gels. Western blotting was performed with antibodies to Hsp27 [24] and secondary antibodies coupled to horseradish peroxidase (Sigma) using the enhanced chemiluminescence (ECL) method [23].

Two-dimensional electrophoresis was carried out according to Argo and Welch [25] by isoelectric focusing of the samples (total cell lysates in 8 M urea with 1% Nonidet P40, 2% β-mercaptoethanol and 100 μM sodium orthovanadate) at pH 5–7 in the first dimension, followed by SDS electrophoresis in the second dimension. The separated isoforms of Hsp27 were identified by immunoblotting with ECL [26].

3. Results

To examine the effects of heat shock and ATP depletion on the subcellular distribution of constitutively expressed Hsp27 we used Triton X-100 extraction of EC exposed in vitro to high temperature (45°C) or metabolic stress mimicking ischemia (incubation with CCCP or rotenone in the absence of glucose). While ATP fluctuations in heat-shocked EC were insignificant, severe but reversible depletion of cellular ATP took place under metabolic stress (Fig. 1). The dynamics of the stress-induced Hsp27 redistribution between the Triton-soluble and Triton-insoluble cellular fractions is represented in Fig. 2. Accumulation of Hsp27 in the pellets and its disappearance from the supernatants took place upon both heating and ATP deprivation. This decrease in solubility is reversible, since resolubilization of Hsp27 was observed during cell recovery following stresses (Fig. 2). In the case of metabolic stress but not heat shock, the solubility of Hsp27 depended on the level of cellular ATP (see Figs. 1 and 2). Such a relationship between cellular energetic status and the amount of Triton-extractable Hsp27 is not intrinsic to EC only, since similar results were obtained earlier on HeLa cells (Kabakov and Bensaude, unpublished data).

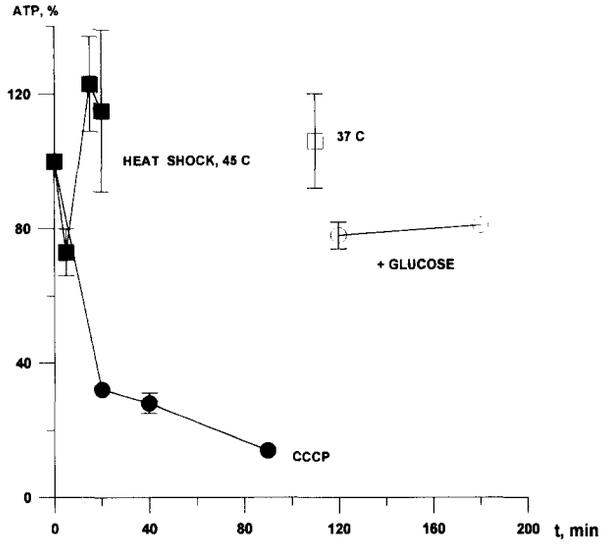

Fig. 1. ATP levels in EC undergoing heating at 45°C (■) or incubation with 20 μM CCCP in the absence of glucose (●) with subsequent recovery at 37°C (□) or in glucose-supplemented rich medium (○). 3×10^5 cells were taken for each measurement. The average ATP level in control (unstressed) EC was 2.8 nM per 10^5 cells. The data are means ± S.E.M. of three determinations; error bars are shown when they are greater than the size of the symbols.

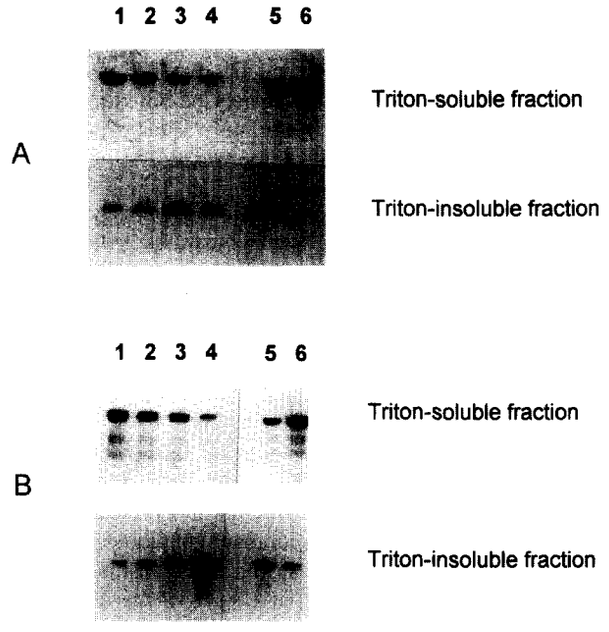

Fig. 2. ECL-revealed immunoblots demonstrating reversible insolubilization of Hsp27 in heat-shocked and ATP-depleted EC. Sample aliquots from equal numbers of cells (3×10^5) were loaded in each well. A: (1) unstressed EC; (2–4) 5, 10 and 15 min heating at 45°C; (5,6) 1.5 and 2.5 h recovery at 37°C. B: (1) unstressed EC; (2–4) 20 min, 40 min and 1.5 h incubation with 20 μM CCCP in the absence of glucose; (5,6) 0.5 and 1.5 h recovery in rich medium.

A decrease in Triton solubility of a cellular protein usually suggests its multioligomerization, formation of large co-aggregates with other proteins, or association with such poorly soluble structures as the cytoskeleton, chromatin and nuclear matrix. To examine these possibilities, we studied the stress-induced relocalization of Hsp27 within EC. In most of the unstressed EC, Hsp27 is diffusely distributed in nuclei and the perinuclear region, and is sometimes present in the cytoplasm in heterogeneous clumps (Fig. 3a). Both stresses cause antigen redistribution: Hsp27 decorates the cytoskeleton upon heat shock (Fig. 3b) and is concentrated into compact spherical granules inside the nucleus during ATP-depleting treatments (Fig. 3c,d).

The cytoskeletal structures trapping Hsp27 in heat-shocked EC appear to be microfilaments, namely thick F-actin bundles or so-called 'stress fibers'. This was directly confirmed by

double-label staining in which the fibrillar patterns coincided after the antibody and FITC-phalloidin labeling (not presented). The heat-induced binding of Hsp27 to F-actin occurred very rapidly and this effect may be noted after 5 min of heating when Hsp27 decorates only short fragments of stress fibers. The maximum of the small Hsp association with microfilament bundles occurred at 15–30 min of thermal exposure (Fig. 3b); more prolonged heating destroyed the actin skeleton in EC. During the recovery at 37°C, gradual dissociation of Hsp27 from stress fibers was observed and no Hsp27 bound to F-actin bundles was found from 2 h of post-heat shock. There is a good temporary correspondence between the alterations in the solubility of Hsp27 and its subcellular relocalization during heat shock and recovery (see Fig. 2).

An analogous coincidence has been established between the insolubilization of Hsp27 and its granulation in EC nuclei under ATP deprivation. Indeed, the fall in ATP caused intranuclear assembly of Hsp27 in compact conglomerates which should be less extractable with Triton. Most distinctly, round particles were seen in EC nuclei at 1.5–2 h of the ATP-depleting treatments (Fig. 3c,d), coinciding with the maximal insolubilization of Hsp27 (see Fig. 2). Further (>2 h) incubation of EC with CCCP in the absence of glucose led to dramatic changes in cell morphology and the beginning of cell detachment. Usually, 5–14 large granules were distinguished inside the nucleus after long-term ATP deprivation. The intranuclear granules were morphologically distinct from the cytoplasmic clumps of Hsp27 observed in some unstressed EC (Fig. 3a). The stress-induced granules have a similar size which has not yet been determined but the average diameter of a granule's fluorescent image appears to be about 1/3 of a nucleolar diameter. We do not know whether the granules consist of Hsp27 alone or if they include other proteins as well; however, the granules are not stained with FITC-phalloidin and antibody to Hsp70. Granule formation during CCCP treatment is not drug-specific, since the same effect was achieved with another blocker of mitochondrial ATP generation, rotenone (Fig. 3d). The presence of 10 mM D-glucose in the CCCP-containing medium prevented ATP depletion in EC and fully abolished

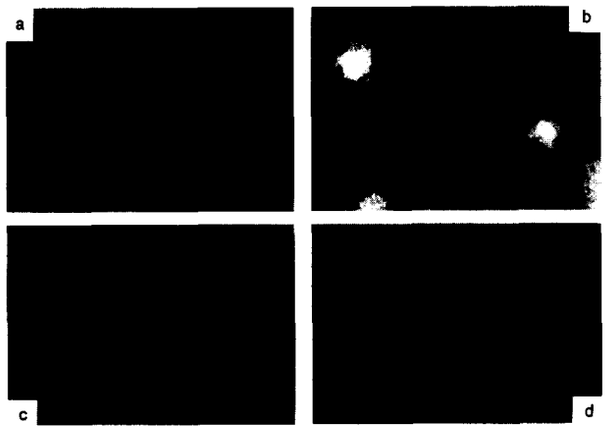

Fig. 3. Indirect immunofluorescence staining showing distribution of Hsp27 in unstressed, heat-shocked and ATP-depleted EC. (a) Unstressed EC; (b) EC heated at 45°C for 15 min; (c) EC incubated with 20 μM CCCP in glucose-free medium for 2 h; (d) EC exposed for 2 h to 20 μM rotenone without glucose. Magnification: (a,c,d) ×500; (b) ×300.

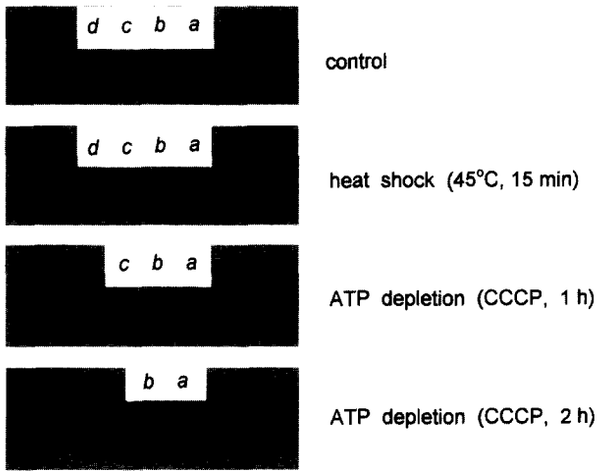

Fig. 4. Diversity in Hsp27 isoform patterns visualized by two-dimensional immunoblotting with ECL. The antigen material from equal numbers (3×10^5) of unstressed and stressed EC was immunodetected in each blot.

the above changes in solubility and localization of Hsp27. The cell recovery in a rich medium resulted in the rapid restoration of the ATP level (see Fig. 1) and the complete dissipation of intranuclear Hsp27-containing granules within 1 h of the recovery period (not shown). The granule disintegration coincided with Hsp27 resolubilization during recovery.

In many studies, alterations in the biological activity of Hsp27, its localization and supramolecular organization were associated with changes in its phosphorylation status [2,8,12,14,26–30]. Endothelial Hsp27 can comprise up to four isoforms: one unphosphorylated basic *a* isoform and three more acidic phospho-isoforms *b*, *c* and *d* [6]. As the isoform ratio closely reflects the degree of Hsp27 phosphorylation [6,25,26,28], we analyzed the stress-provoked shifts in the spectrum of Hsp27 isoforms in EC. Fig. 4 shows a diminished spot of *a* isoform and a marked increase in spots of the phospho-isoforms *c* and *d* after 15 min heat shock. ATP depletion exerted the reverse effect and therefore resulted in a decrease in the phospho-isoform tracks. Hsp27 from EC devoid of ATP for a long time (2 h) was immunodetected as a major spot of the unphosphorylated *a* isoform and a minor spot of the *b* isoform; the tracks of the other two phospho-isoforms are indistinguishable (Fig. 4). It should be noted that both the isoform profile and subcellular relocalization of Hsp27 were stress-specific.

All the above effects were reproduced several times on the cell cultures derived from various samples of human aortas and umbilical veins. No marked differences in the described phenomena were observed between EC of arterial and venous origins.

4. Discussion

Apparently, Hsp27 insolubilization in heat-shocked and ATP-depleted EC is due to different causes, namely, binding of Hsp27 to stress fibers in the former and its intranuclear granulation in the latter. It seems unexpected that Hsp27 associates with stress fibers in heat-shocked EC, since the constitutive Hsp27 was previously shown to migrate from the cytoplasm into the nucleus upon heat shock [2,16]. Furthermore, the fibrillar pattern presented in Fig. 3b is typical for

the distribution of any F-actin-binding protein rather than Hsp27 which in fact possesses actin barbed-end capping and depolymerizing activities [4,5].

The altered isoform spectrum (Fig. 4) suggests additional phosphorylation of Hsp27 in heat-shocked EC that could be performed by stress-activated protein kinase(s) [2,14]. The ability of Hsp27 to protect actin microfilaments from destruction by heating, oxidants and cytochalasin D [10,12,14,15] as well as the beneficial role of Hsp27 phosphorylation in this protection [12,15] have been established. What is not yet clear is the mechanism of Hsp27-mediated maintenance of microfilament integrity. Indeed, it is difficult to imagine how this protein interfering in vitro with actin polymerization and disassembling F-actin [4,5] can in vivo stabilize the actin skeleton during microfilament-disrupting exposures. To resolve this discrepancy Arrigo and Landry [2] have suggested that when actin-bound Hsp27 undergoes phosphorylation, it dissociates from actin, thus uncovering the barbed end for elongation. This supposition was in part supported by Benndorf et al. [28] who have shown that the phosphorylated Hsp27 does not inhibit actin polymerization. However, the disappearance of the inhibiting activity per se cannot explain the protection of the actin skeleton.

Taking into account our own observations, we hypothesize that levels of Hsp27 phosphorylation above the basal value not only abolish the inhibition of actin polymerization but may also confer a novel F-actin-binding activity which enables Hsp27 to stabilize preexisting microfilaments during stress. Since earlier association of hyperphosphorylated Hsp27 with the actin skeleton has been documented for thrombin-stimulated human platelets [29] and leukemic HL-60 cells treated with phorbol ester myristate [30], a similar mechanism might mediate decoration of endothelial stress fibers by Hsp27 overphosphorylated as a result of heat shock. Many diseases being in an acute phase can elevate human body temperature extremely (above 40°C); herein, heat-induced Hsp27 phosphorylation may serve for the protection of microfilaments from thermal injury. Obviously, such a response is not intrinsic to all types of cells and, for instance, Lavoie et al. [12] have found neither a correlation between the insolubilization and phosphorylation of Hsp27 nor its binding to the cytoskeleton in heat-shocked Chinese hamster cell lines.

Another of our findings, the intranuclear granulation of Hsp27 in ATP-depleted EC, may also be connected with the stress-induced changes in Hsp27 phosphorylation, since both the localization and oligomeric structure of the small Hsp appear to be regulated via its phosphorylation/dephosphorylation [2,8,12,14,26–30]. Actually, the long-term metabolic stress employed sharply decreases the ATP/ADP ratio in EC which may inhibit protein kinases and/or activate phosphatases. This proposed shift in cellular kinase/phosphatase machinery might explain the Hsp27 dephosphorylation during ATP depletion, however, the intranuclear aggregation of the dephosphorylated Hsp27 into large insoluble particles remains to be defined. A homolog of Hsp27, cardiac α-crystallin, was also shown to be insolubilized in an ischemic rat heart [31] but was explained by the protein aggregation at low pH owing to ischemic acidification of the cytosol. Before our study, Hsp27-containing granules had already been found in the cytoplasm of various unstressed and heat-shocked cells [2,28,32]. Furthermore, similar round particles were isolated from stationary Ehrlich tumor cells and characterized as multimeric complexes consisting of the non-phosphorylated small Hsp [28]. It is unknown whether the granules formed in nuclei of ATP-depleted EC possess the same features as the particles described in the above work [28], although their globular shape and lack of phosphorylated constituents coincide. Importantly, the in vivo dissociation of an aggregated form of Hsp27 as a result of its phosphorylation can occur after triggering cellular protein kinase cascades [27]. Perhaps an analogous mechanism carries out the granule dissolution under post-stress EC recovery when some protein kinases may be reactivated following ATP restoration.

The biological significance of the reversible accumulation and granulation of Hsp27 in endothelial nuclei upon ATP depletion/replenishment is not quite clear. It seems likely that a similar situation occurs in vivo during ischemia/reperfusion. Hereof, we might speculate about special microfilament protection from an expected destructive influence of the dephosphorylated Hsp27 on F-actin; such protection seems to be achieved by removal of Hsp27 from the cytoplasm (nuclear compartmentalization) and/or by transforming Hsp27 to an inactive form (granules). Indeed, non-phosphorylated monomers of the small Hsp inhibit actin polymerization, whereas their multimeric complexes do not [28]. An increase in a labile F-actin pool observed in ATP-depleted bovine EC by Hinshaw et al. [33] may also be related to the stress-induced 'inactivation' of Hsp27 according to the above reason. At the same time, possible harmful consequences of Hsp27 aggregation cannot be excluded. Such well-known effects of ATP depletion as cell retraction, blebbing and increased macromolecule permeability of endothelial monolayer may at least in part be caused by a defect in actin regulation owing to the compartmentalization and granulation of Hsp27. In any event, we consider fluctuations in the level of cellular ATP as one of the factors modulating the status and functional activity of the small Hsp.

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