Phosphatase Inhibitors Prevent HSP27 Dephosphorylation, Destruction of Stress Fibrils, and Morphological Changes in Endothelial Cells during ATP Depletion

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 132, No. 9, pp. 350-353, September, 2001
Original article submitted January 22, 2001

Pretreatment with phosphatase inhibitors did not affect the decrease in ATP content in endothelial cells, but inhibited HSP27 dephosphorylation and redistribution, damages to actin cytoskeleton, and morphological changes in cells. Our results suggest that inhibition of stress-induced HSP27 dephosphorylation protects cells from ischemia-induced damages.

Key Words: *endothelium; ischemia; HSP27; actin; phosphatases*

Ischemia is an impairment (or arrest) of blood supply to the whole organ or its part, which is followed by exhaustion of oxygen and substrate pools for oxidative phosphorylation and glycolysis. These changes lead to depletion of intracellular ATP stores. ATP is a key regulator of actin cytoskeleton necessary for microfilament formation and activity of protein kinases controlling actin-binding proteins [3,6]. Exhaustion of ATP pool in cells during ischemia shifts the kinase-phosphatase balance [10] and induces microfilament destruction [6,9,10,12]. Heat shock protein HSP27 can modulate actin polymerization/depolymerization. Changes in phosphorylation and structural organization of this protein affect the resistance of microfilaments to damages induced by heat shock, oxidative stress, and cytochalasin D [7,11]. Human cells contain nonphosphorylated and mono-, bi-, and triphosphorylated HSP27 [1,11,12]. HSP27 phosphorylation by three serine residues is catalyzed by MAPKAP kinase 2/3 [13,14] and its dephosphorylation is catalyzed by phosphatase 2A [4]. Our previous studies showed that ATP depletion in human endothelial cells (EC) leads to dephosphorylation and redistribution of HSP27 from the cytoplasm into the nucleus with the formation of large detergent-insoluble granules [12]. In heat-shock-preconditioned EC the inhibition of stress-induced dephosphorylation and redistribution of HSP27 correlated with stabilization of the actin cytoskeleton [1]. Here we analyzed the relationship between HSP27 isoforms and stability of actin microfilaments under conditions of ATP depletion.

MATERIALS AND METHODS

Experiments were performed on cultured EC (passages 0-2) from human aorta or umbilical vein [2]. The cells were grown on cover glasses or plastic. ATP depletion in cells was induced by incubation in a glucose-free DMEM containing 20 μM m-chlorophenyl hydrazone carbonyl cyanide (uncoupler of oxidative phosphorylation) or 20 μM rotenone (inhibitor of mitochondrial respiration). ATP content in EC was measured by the luciferin-luciferase method for adherent cells [8]. The cells were fixed with a mixture of 3.7% formaldehyde and 0.1% Triton X-100. Immunofluorescence assay was performed using antibodies (AB) against HSP27 gifted by M. Gaestel and second AB conjugated to Texas red (South Biotech). Phalloidin conjugated to tetramethylrhodamine isothiocyanate (Sigma) was used for identification of F-actin. SDS electrophoresis was performed in Laemmli system consisting of 4% concentrating and 15% separating gels. Immunoblotting was performed by the ECL method using anti-HSP27

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0007-4888/01/1323-0914$25.00

AB. Isoelectric focusing and immunoblotting with AB to HSP27 were performed to estimate the isoform composition of HSP27 [15]. The cells were lysed in 8 M urea containing 1% nonidet P40, 2% β-mercaptoethanol, and 100 μM sodium orthovanadate (Na_3VO_4). Isoelectric focusing was performed at pH 5-7, proteins were transferred into a nitrocellulose filter, and HSP27 isoforms were detected by immunoblotting.

Phosphatase inhibitors were used in concentrations nontoxic for human EC: 100 μM Na_3VO_4 (tyrosine phosphatase inhibitor; phosphatase 2A activity is also regulated via phosphorylation by tyrosine residues), 1 μM okadaic acid (phosphatase 1 and 2A inhibitor), 0.2 μM cantharidin (specific inhibitor of phosphatase 2A). The inhibitors were added to culture media 20 min before ischemic stress.

RESULTS

Phosphatase inhibitors (Na_3VO_4, okadaic acid, and cantharidin) did not affect the decrease in intracellular ATP content, but inhibited dephosphorylation and redistribution of HSP27. The isoform composition of HSP27 changed during deenergization (Fig. 1, *1*, *2*). ATP depletion in cells pretreated with Na_3VO_4, okadaic acid (Fig. 1, *3*), or cantharidin (Fig. 1, *4*) was less pronounced. After 2-h depletion of ATP bi- and triphosphorylated d- and c-isoforms disappeared, the content of monophosphorylated b-isoform decreased, while the content of nonphosphorylated a-isoform increased. In cells pretreated with phosphatase inhibitors the isoform composition of HSP27 after 2-h deenergization remained unchanged. Fractionation of EC with nonionic detergent Triton X-100 revealed HSP27 redistribution during ischemic stress from detergent-soluble to detergent-insoluble fraction (Fig. 2, *a*). In cells pretreated with okadaic acid (Fig. 2, *b*), Na_3VO_4, or cantharidin HSP27 redistribution was less pronounced.

These findings were confirmed by immunofluorescence data on HSP27 localization in EC (Fig. 3). In nonstressed cells HSP27 was diffusely localized in the nucleus and cytoplasm and formed small cytoplasmic aggregates (Fig. 3, *a*). Two-hour depletion of ATP caused redistribution of HSP27 from the cytoplasm into the nucleus, where this protein formed large spherical granules (Fig. 3, *c*). Pretreatment with okadaic acid (Fig. 3, *e*), Na_3VO_4, or cantharidin inhibited stress-induced redistribution of HSP27 in cells.

Inhibition of HSP27 dephosphorylation and redistribution in deenergized cells correlated with preservation of their actin cytoskeleton and normal morphology (Fig. 3). In native EC the actin cytoskeleton is presented by microfilament bundles (stress fibrils, Fig. 3, *b*). ATP depletion was accompanied by destruction of these structures (Fig. 3, *d*), retraction of the cytoplasm, and formation of spherical cells. Pretreatment with okadaic acid (Fig. 3, *f*), Na_3VO_4, or cantharidin prevented destruction of stress fibrils and morphological changes in cells during ischemic stress.

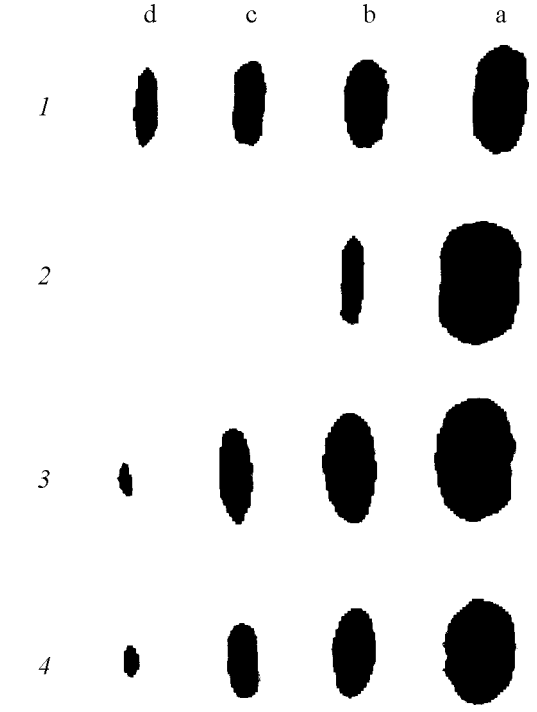

Fig. 1. Isoform composition of HSP27 in control EC (*1*), after 2-h ATP depletion (*2*), and after 2-h ATP depletion preceded by 20-min pretreatment with okadaic acid (*3*) or cantharidin (*4*).

Thus, phosphatase inhibitors decrease the degree of HSP27 dephosphorylation and redistribution, destruction of the actin cytoskeleton, and morphological changes in EC induced by ATP depletion. Our results

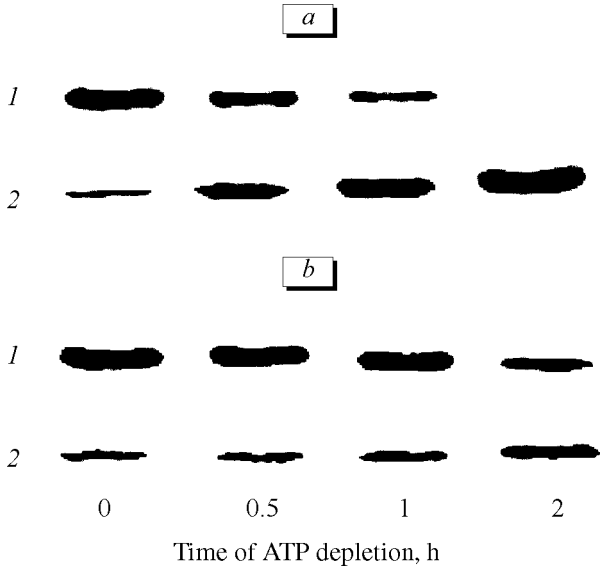

Fig. 2. HSP27 in Triton-soluble (*1*) and Triton-insoluble fractions (*2*): endothelial cells not pretreated (*a*) and pretreated with okadaic acid (*b*).

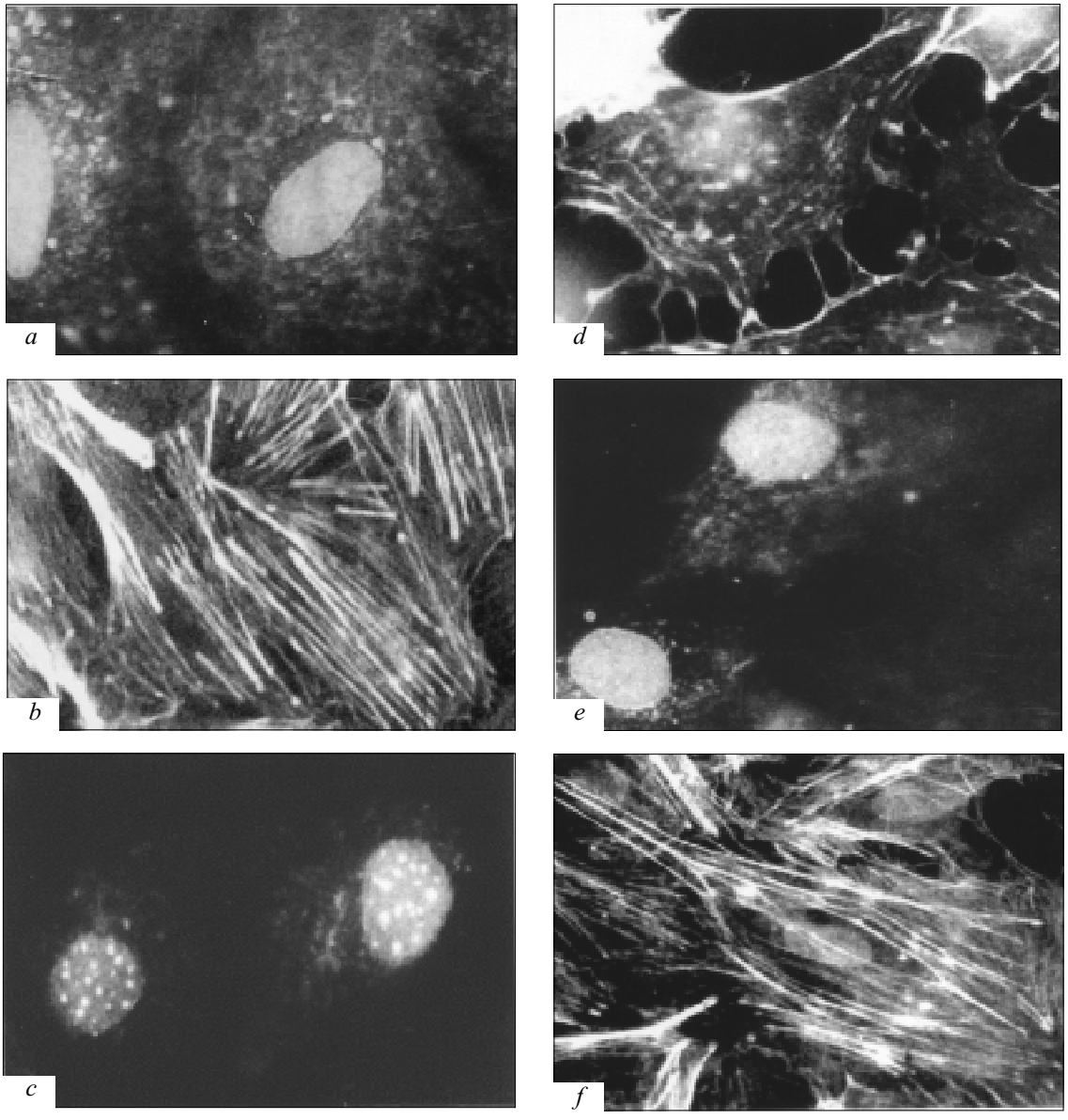

Fig. 3. Immunofluorescence assay of HSP27 (*a*, *c*, *e*) and visualization of F-actin (*b*, *d*, *f*) in endothelial cells: before (*a*, *b*) and after 2-h ATP depletion of cells (*c*, *d*) and after 2-h ATP depletion and pretreatment with okadaic acid (*e*, *f*).

and published data on the involvement of phosphorylated HSP27 in microfilament protection from damages induced by heat shock, oxidative stress, and cytochalasin D [7,11] suggest that the inhibition of HSP27 dephosphorylation by phosphatase inhibitors preserves the integrity of EC actin cytoskeleton during ATP depletion. These data should be taken into account during elaboration of new drugs for the therapy of ischemic damages.

This work was supported by the Russian Foundation for basic Research (grant No. 99-04-48119a).

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