Early and delayed tolerance to simulated ischemia in heat-preconditioned endothelial cells: a role for HSP27

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Loktionova, Svetlana A., Olga P. Ilyinskaya, and Alexander E. Kabakov. Early and delayed tolerance to simulated ischemia in heat-preconditioned endothelial cells: a role for HSP27. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H2147–H2158, 1998.—An ischemia-mimicking metabolic disturbance in cultured endothelial cells from the human aorta or umbilical vein caused ATP depletion, a rise in cytosolic free Ca2+, fragmentation and aggregation of actin filaments, retraction of the cytoplasm, and disintegration of cell monolayer. Simultaneously, the constitutive heat shock protein 27 (HSP27) underwent dephosphorylation and formed granules inside cell nuclei. Prior heat shock (45°C, 10 min) in confluent cultures conferred two phases (early and delayed) of tolerance to simulated ischemia. Although heat preconditioning did not retard the ATP drop and the free Ca2+ overload within ischemia-stressed cells, each phase of the tolerance was manifested in longer preservation of normal cell morphology during the stress. Cells exhibiting the early tolerance within 3 h after heating altered the F-actin response to ischemic stress; no microfilament debris but, instead, translocation of F-actin to the tight submembranous layer was observed. In contrast, the delayed cytoprotection preserved the preexisting F-actin bundles under simulated ischemia; this happened only after 12- to 14-h post-heat shock recovery, elevating the intracellular HSP content, and was sensitive to blockers of HSP synthesis, cycloheximide and quercetin. The dephosphorylation and intranuclear granulation of HSP27 were markedly suppressed in both phases of the heat-induced tolerance. Without heat pretreatment, similar attenuation of the HSP27 dephosphorylation/granulation and the actin cytoskeleton stability during simulated ischemia were achieved by treating cells with the protein phosphatase inhibitors cantharidin or sodium orthovanadate. We suggest that prior heat shock ameliorates the F-actin response to ischemic stress by suppressing the HSP27 dephosphorylation/granulation; this prolongs a sojourn in the cytosol of phosphorylated HSP27, which protects microfilaments from the disruption and aggregation.

endothelium; adenosine 5′-triphosphate depletion; cytoskeleton; stress proteins; cross-tolerance

INTEGRITY of the filamentous (F)-actin structures in the vascular endothelium is necessary for its physiological functioning as a selectively permeable barrier (30, 32, 33, 40). Increased permeability of the endothelial barrier provokes edema that can result in severe injury of the adjacent tissue. Because the endothelial permeability becomes elevated under ischemia-reperfusion (34, 35), this is considered to be one of the causes of ischemia-associated tissue lesions and thrombosis. Such a dysfunction of the endothelium during acute ischemia appears to be caused by failing ATP synthesis when metabolic substrates and oxygen are not available. Similar interruption of energy metabolism and the ATP drop-evoked damage of the endothelium may result from some surgical procedures [e.g., transplantation or bypass perfusion of organs (18)]; these vascular disorders usually lead to complications and unfavorable outcome. ATP depletion in endothelial cultures does cause a rise in cytosolic free Ca2+ and collapse of F-actin that are accompanied by retraction of the cytoplasm and an increase in permeability of the cell monolayer (12, 13, 21, 38). Hence, in case of in vivo ischemia, a response of endothelial microfilaments to the decrease in cellular ATP may determine the state of blood vessels and capillaries and, sometimes, the persistence of all the involved tissues.

It is a well-known phenomenon that prior heat shock confers cytoprotection against damage induced by ischemia or ATP depletion in cardiac, nervous, and some other mammalian tissues and cell cultures (5, 16–18, 20, 29, 37). These and other examples of cellular adaptation to ischemic conditions hopefully enable discovery of novel ways to combat such fatal human pathologies as myocardial infarction and brain stroke. The molecular basis of the heat-induced tolerance to ischemia is not completely unraveled, but the role of heat shock proteins (HSP) in this phenomenon seems prominent. The ability of inducible HSP70 (HSP70i) to protect against ischemic injury was confirmed by experiments with the transfected cell lines and transgenic mice (reviewed in Refs. 18 and 29), although the precise mechanism of HSP70i-mediated anti-ischemic defense remains unclear. In contrast to HSP70i, the elevated levels of HSP90 or HSP60 alone did not preserve neuronal and heart-derived cells under simulated ischemia (1, 7, 11). With regard to the small HSPs, Martin et al. (26) showed that overexpression of either HSP27 or αB-crystallin in cultures of adult rat cardiomyocytes improves the cell survival after an ischemia-mimicking stress. However, the cellular machinery responsible for the protective effect was not explored. Earlier, it was speculated (16, 18) that HSP27 enables cells to escape actin aggregation under ischemic states, although no evidence has been obtained yet showing that HSP27 is involved in protection of the cytoskeleton within ischemia-stressed cells.

Our previous study (25) revealed that sustained ATP depletion in cultured human vascular endothelial cells causes dephosphorylation of cytosolic HSP27 and its assembly into Triton X-100-insoluble granules inside cell nuclei. HSP27 is known to regulate dynamics of microfilaments, with both the functional activity and oligomeric structure of HSP27 being dependent on its phosphorylation (4, 10, 19, 22, 28). Consequently, we suggested that the F-actin derangement (12, 13, 21, 38) and the changes in the HSP27 status (25) occurring in...
ATP-deprived endothelial cells are closely connected with each other. Examination of this suggestion was the main goal of the present work. To probe a link between the responses of endothelial HSP27 and actin to ischemic stress, we used mild heat shock (45°C for 10 min) before simulated ischemia because, first, heating does affect phosphorylation, localization, and supramolecular organization of HSP27 in vivo (3, 23, 25, 31, 41) and, second, heat preconditioning can induce tolerance of the cytoskeleton to a lack of cellular ATP (5, 17, 18). In addition, to suppress the HSP27 dephosphorylation in the ischemia-stressed cells, the protein phosphatase inhibitors cantharidin and sodium orthovanadate were used. We thereby demonstrated that heat shock in endothelial cultures confers early and delayed phases of the cytoresistance to simulated ischemia and that, in both cases, the protective effects seem to be associated with increased stability of F-actin and arrest of the HSP27 dephosphorylation/granulation during ATP depletion.

MATERIALS AND METHODS

Cell culture. Endothelial cells were isolated from thoracic segments of adult human aortas (fresh cadaveric material) or human umbilical veins as previously described (2) with the minor modification that 0.15% dispase was used instead of collagenase. The isolated cell suspension was diluted in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 µg/ml heparin, 50 U/ml penicillin, and 100 µg/ml streptomycin and then placed in gelatin-covered plastic dishes with a seeding density of 3.5 × 10⁴ cells/cm². The adherent cells were grown in the same medium at 37°C in a humidified atmosphere with 5% CO₂. Replicated cultures were obtained after trypsinization of the primary culture that reached confluence. The cultures were identified as homogeneous endothelial cell populations on account of their total positive immunostaining with antibodies to factor VIII-related antigen. The confluent cultures from passages 1–4 were used for the experiments.

In vitro simulated ischemia. To imitate ischemic insult, the adherent cells were incubated at 37°C in glucose-free DMEM in the presence of 3% FBS and 20 µM carboxyl cyanide m-chlorophenylhydrazone (CCCP), an uncoupler of oxidative phosphorylation in mitochondria. Such an exposure arrests both glycolytic and mitochondrial ATP generation in endothelial cells (25), which quite adequately mimics glucose starvation, hypoxia, and ATP depletion occurring at the cellular level under ischemia (12, 18, 38). The stress-induced cellular damage was monitored by phase-contrast microscopy on characteristic changes in morphology of endothelial monolayer.

ATP measurements. Determination of the ATP levels in cultured endothelial cells was performed as described previously (17) using the luciferin-luciferase assay adapted for adherent cells (25). Endothelial cells were grown to confluence on 35-mm culture dishes. To extract intracellular ATP, the incubation medium was removed and 300 µl of an ice-cooled solution containing 2.5% (wt/vol) trichloroacetic acid and 3 mM EDTA were added to each dish, and the cells were quickly scraped with a rubber policeman. The solution containing cellular debris was collected from the dishes and frozen for later assay. The ATP concentration was determined using an ATP assay kit in a Wallac 1251 luminometer (Turku, Finland) (17, 25).

Calcium determination. Concentrations of cytosolic free Ca²⁺ were measured using the fluorescent Ca²⁺ indicator fura 2 as previously described for porcine aortic endothelial cells (21). Confluent endothelial cultures on cover slips were loaded by the indicator as a result of 50-min incubation with 5 µM fura 2-acetoxyethyl ester (fura 2-AM) in the dark. After a threefold medium change, the cover slips were placed in a cuvette for fluorescence analysis in a Shimadzu spectrophotometer (Kyoto, Japan). Excitation was alternated between 340 and 380 nm, and the emitted fluorescence was collected at 510 nm. Calibration of fura 2 fluorescence and calculation of the cytosolic free Ca²⁺ concentration were carried out according to Kuhne et al. (21).

Tolerance-inducing cell treatments. For heat shocking the cells, hermetically sealed dishes with endothelial cultures were plunged into a thermostatic water bath at 45°C for 10 min, followed by return to normal conditions (instant change of growth medium and placement into a CO₂ incubator at 37°C). Thereafter, the heat preconditioned cells were subjected to the ischemia-mimicking insult immediately or at different time intervals after heating.

To inhibit protein phosphatases in vivo, 0.2 µM cantharidin or 100 µM sodium orthovanadate was added to the nonpreheated cells 20 min before simulated ischemia or directly to the glucose-free DMEM with CCCP for all periods of metabolic stress. The anti-ischemic cytoresistance induced by either prior heating or the protein phosphatase inhibitors was revealed on the persistence of normal cell morphology in the treated endothelial cultures after 2 h of simulated ischemia.

Fluorescence microscopy. Adherent endothelial cells were grown on gelatin-covered glass cover slips within 24-well plates. After experiment exposures, the cells were fixed and permeabilized by PBS (8 mM Na₂PO₄, 2 mM KH₂PO₄, 2 mM KCl, and 140 mM NaCl, pH 7.5) containing 3% paraformaldehyde and 0.2% Triton X-100 for 10 min. The fixing solution was then removed, and the cells were washed three times with PBS and then incubated for 20 min with PBS containing 1% BSA at room temperature. To reveal F-actin patterns, the fixed cell preparations were stained with phalloidin coupled to tetramethylrhodamine isothiocyanate (TRITC) for 30 min at room temperature in the dark. Intracellular HSP27 was visualized by immunofluorescent staining with anti-HSP27 rabbit antibodies and anti-rabbit IgG antibodies conjugated with Texas red (25). Cover slips carrying the stained cell samples were sealed in a drop of PBS-glycerol mixture (1:1) and then viewed and photographed on an Opton III microscope (Carl Zeiss, Oberkochen, Germany).

Electrophoresis and immunoblotting. The cells growing in 60-mm dishes were washed three times with PBS and then lysed into the sample buffer containing 20 mM Tris·HCl (pH 8.0), 4% SDS, 1% dithiothreitol, 4 mM EDTA, 10% (vol/vol) glycerol, 2 µM phenylmethylsulfonyl fluoride, and 40 µg/ml leupeptin. The lysates were then boiled in a water bath for 5 min. Aliquots of the samples were run in slabs with 4% stacking and 15% separating polyacrylamide gels under Laemmli conditions as previously described (17, 25). Electrophoretically separated proteins were transferred from the gel onto nitrocellulose membrane (0.45 µm), and nonspecific binding was blocked by incubating blots overnight in Tris-buffered saline (TBS; 25 mM Tris·HCl and 140 mM NaCl, pH 7.5) supplemented with 0.1% Tween 20 and 1% BSA. Blots were then incubated for 2 h with murine monoclonal anti-HSP70i or rabbit anti-HSP27 antibodies diluted 1:1,000 in 7.5% iodoacetamide-protease inhibitor cocktail (Sigma) and 3% milk in TBS. After fivefold washing with TBS-0.1% Tween 20, blots were incubated for 2 h with horseradish peroxidase-conjugated secondary antibodies diluted 1:3,000 in TBS-0.1% Tween 20. Blots were then developed using an ECL enhanced chemiluminescence kit (Amersham, Arlington Heights, IL) and exposed to X-ray film.
HSP band images, blots were exposed on X-ray film using the enhanced chemiluminescence (ECL) method (25). The relative changes in the HSP contents were assessed by scanning the antigen band tracks on an Ultroscan XL laser densitometer (LKB, Uppsala, Sweden) (17).

Analysis of HSP27 isoform spectra. To evaluate changes in the phosphorylation of endothelial HSP27, we assessed shifts in its isoelectric composition by isoelectric focusing (IEF) of the total cell lysates followed by immunoblotting with anti-HSP27 antibodies (14, 41). Confluent cultures on 60-mm dishes were lysed into IEF buffer containing 8 M urea, 5% β-mercaptoethanol, 2% Nonidet P-40, and 100 µM sodium orthovanadate. Aliquots of the cell lysates were loaded in tubes with 5% polyacrylamide gel containing 8 M urea, 1% Nonidet P-40, and 2% ampholines, pH 5–7, and fractionated under 500 V overnight and then under 1,000 V for 1 h. Electrotransfer of proteins from the gel rods onto nitrocellulose membrane was performed according to Zhou et al. (41). The blocking step and immunodetection of HSP27 isoforms in blots were carried out as described in Electrophoresis and immunoblotting for Western Blotting with anti-HSP27 antibodies.

Statistical analysis. The presented results were obtained from several independent experiments on endothelial cultures of the aortic and venous origin. Quantitative data of ATP and Ca2+ determinations and immunoblot scanning are given as means ± SE as analyzed using Student’s unpaired t-test. Differences with P < 0.05 were considered to be significant.

Materials. Dispase was from Boehringer Mannheim. RPMI 1640 medium, FBS, and other components of the growth medium were from HyClone (Cramlington, UK). Culture plastic was purchased from Costar (Cambridge, MA). The following reagents were from Sigma Chemical (St. Louis, MO): BSA, cantharidin, CCCP, cycloheximide, DMEM, goat anti-rabbit IgG, rabbit IgG-peroxidase, rabbit anti-mouse IgG-peroxidase, thornbin, and TRITC-phallodin conjugates. Histamine and quercetin were obtained from Serva (Heidelberg, Germany), the ATP assay kit from Calbiochem (La Jolla, CA), fura-2-AM from Molecular Probes (Eugene, OR), and sodium orthovanadate from Aldrich Chemical (Milwaukee, WI). Chemicals for electrophoresis and nitrocellulose were from Bio-Rad (Richmond, CA), and ampholines were from LKB. Rabbit antibodies to HSP27 were kindly provided by Prof. M. Gaestel (9). Murine monoclonal antibody C92F3A-5, specific for HSP70i, was purchased from StressGen Biotechnologies (Victoria, BC, Canada). Goat anti-rabbit IgG-Texas red conjugates were from Southern Biotechnology Associates (Birmingham, AL). Reagents and X-ray film for ECL were from Amersham (Arlington Heights, IL). Herbimycin A was from Gibco BRL (Gaithersburg, MD). SB-203580 was a gift from SmithKline Beecham. All other chemicals were of analytic grade.

RESULTS

Effects of simulated ischemia on endothelial cultures. After in vitro exposure to ischemia-like stress, endothelial cells lost ATP and cytosolic concentrations of free Ca2+ rose. Within 2.5 h of the exposure, the cellular ATP was reduced to 4–5% of its initial level (Fig. 1), and the levels of free cytosolic Ca2+ increased ~10-fold (Fig. 2). These biochemical perturbations were accompanied by visible changes for the worse in morphology, namely, formation of serrate edges and retraction of the cytoplasm that resulted in disturbance of a characteristic cobblestone-like structure intrinsic to intact endothelial monolayer. After 2.5–3 h of simulated ischemia, cell release from culture dish surface began (not shown). Such a deterioration of the endothelial cell morphology followed by disintegration of cell monolayer appears to be a result of the stress-induced collapse of the cytoskeleton. Indeed, disorganization of the F-actin framework in ATP-depleted endothelial cells from bovine and porcine arteries has been documented (12, 13, 21, 38). We also observed fragmentation of microfilaments and formation of F-actin aggregates in injured endothelial cells exposed to ischemia-like conditions in vitro. The intact cells contain numerous stress fibers extended across the cytoplasm (Figs. 3A, 5A, and 7A). The sustained (~2 h) ischemia-mimicking treatment results in shortening and clumping of the F-actin bundles and, eventually, full disappearance of the fibrillar pattern (Figs. 3C and 7C). The dissolution of the long F-actin bundles usually preceded the morphological changes that arose in endothelial cultures in response to simulated ischemia. Thus the state of stress fibers in cultured endothelial cells can reflect, as the in situ indicator, the degree of cellular injury.

While microfilaments undergo fragmentation/aggregation in ATP-depleted, Ca2+-overloaded endothelial cells, cytosolic HSP27 is dephosphorylated and concentrates into globular particles (granules) inside nuclei.
cytosolic Ca\textsuperscript{2+} shock followed by recovery overnight attenuates free Ca\textsuperscript{2+}), and triphosphorylated (\textsuperscript{a}isoform)

In vivo human HSP27 can comprise up to four major isoforms corresponding to nonphosphorylated (\textsuperscript{a}isoform), monophosphorylated (\textsuperscript{b}isoform), biphosphorylated (\textsuperscript{c}isoform), and triphosphorylated (\textsuperscript{d}isoform) HSP27 (14, 23). The three sites of the human HSP27 phosphorylation in vivo have been identified as Ser-15, Ser-78, and Ser-82 (23). In our model, the major isoform \textsuperscript{a} and all three phosphorylated (phospho-) isoforms of HSP27 are present in the samples of unstressed endothelial cells (Fig. 4, A and D, row 1). The HSP27 dephosphorylation resulting from simulated ischemia was revealed by IEF/immunoblotting on reduction of isoform \textsuperscript{b}, disappearance of isoforms \textsuperscript{c} and \textsuperscript{d}, and augmentation of isoform \textsuperscript{a} (Fig. 4A) that is in full agreement with previous data of two-dimensional blotting (25).

As immunofluorescence shows, in unstressed endothelial cells, constitutive HSP27 is diffusely distributed in the cytoplasm and the nucleus, and sometimes it forms heterogeneous clumps in the cytoplasm (Figs. 3B, 5B, and 7B). Simulated ischemia caused concentration of HSP27 into spheric granules inside the cell nuclei (Figs. 3D and 7D); it should be noted that, without ischemic stress, the nuclei of cultured endothelial cells never contained such granules.

Early heat-induced tolerance to simulated ischemia. Heat shock pretreatment (45°C for 10 min) of confluent endothelial cultures of aortic or venous origin rendered their morphology considerably more resistant to subsequent ischemia-mimicking stress. This tolerance was discontinuous and had two separated periods. The early protection is well marked within 3 h after heating and then interrupted until the opening of the "second (delayed) window" of the cytoprotection (see Delayed tolerance to simulated ischemia). Immediately after heat shock, the cultures displayed the cobblestone structure and intact stress fibers (Fig. 3E). Notably, these cells, being ischemia-stressed, kept normal morphology for a longer time, and, in cases of extremely sustained stress (\textgreek{theta}>2.5 h), less cell detachment from the adhesion substrate took place (not shown). Although not affecting the preexisting endothelial F-actin structures, heat shock nevertheless changed dramatically the actin response to the ischemia-like insult. If the latter was initiated immediately after heat shock or within the next 3 h, all stress fibers gradually disappeared and, instead, F-actin concentrated near the cellular edges, forming a continuous ring along the inner perimeter (Fig. 3G). The actin transition from stress fibers to the submembranous layer occurred without mass generation of short fragments and aggregates of F-actin in the perinuclear region. Such specific reorganization of actin in response to ischemic stress was a hallmark of the early tolerance following heat shock; apparently, this device enables the preheated cells to avoid detrimental F-actin debris and to retain the correct shape and area of the cytoplasm for a longer time in the course of simulated ischemia.

In the previous study (25), we demonstrated that severe heat shock (45°C for 20–30 min) causes transient association of HSP27 with stress fibers in preconfluent endothelial cells. As we show here, shorter heating (45°C for 10 min) did not influence the HSP27 distribution patterns within confluent endothelial cells (compare Fig. 3, B and F). At the same time, 10-min heat shock increased phosphorylation of HSP27, which is clearly seen as a shift in the isoform spectrum toward predominance of the phosphoisoforms (Fig. 4B). If the ischemia-like exposure began within 3 h after heat shock, both the dephosphorylation and intranuclear granulation of HSP27 were arrested. In contrast to the control cells, the preheated cells preserved biphosphorylated HSP27 (isoform \textsuperscript{c}) and the higher level of monophosphorylated HSP27 (isoform \textsuperscript{b}) after 2 h of simulated ischemia (compare Fig. 4, A and B, row 2). Under the same conditions, no HSP27-containing granules were observed in the nuclei of cells exhibiting the early anti-ischemic protection (Fig. 3H). Later, if the ischemic stress was induced during the break between the early and delayed tolerance, HSP27 underwent again the dephosphorylation/granulation that coincided with the appearance of microfilament debris in the perinuclear region. Inhibitors of the HSP synthesis, cycloheximide and quercetin (see Delayed tolerance to simulated ischemia), exerted no effects on induction of the early tolerance to simulated ischemia.

Heat shock is known to trigger a mitogen-activated protein (MAP) kinase cascade leading to HSP27 phosphorylation (3, 8, 10, 31), and the p38 MAP kinase/HSP27 pathway can mediate reorganization and protection of F-actin within stimulated or stressed cells (10, 14). Accordingly, we expected that the actin and HSP27
responses intrinsic to the early tolerance may be due to the transiently enhanced phosphorylation of HSP27. To examine this, we used the highly specific inhibitor of p38 MAP kinase, SB-203580, which at 25 µM blocked the p38 MAP kinase activity in oxidant-stressed endothelial cells, thus abolishing the intensification of HSP27 phosphorylation (14). In our model, 25 µM SB-203580 did prevent the increase in HSP27 phosphorylation after heating, but it did not reduce the three phosphoforms of HSP27 relative to their initial levels in the unstressed cells (compare Fig. 4, A and B). Such action of SB-203580 abolished neither the arrest of dephosphorylation (Fig. 4B, row 4) nor the suppression of intranuclear granulation of HSP27 (not shown) in endothelial cultures exposed to ischemic stress immediately after heat shock. Likewise, the actin redistribution and the preservation of normal morphology in the course of the early cytoprotection were not sensitive to SB-203580. Pretreatment of the cultures with thrombin or histamine, which enhances HSP27 phosphorylation in human endothelial cells (24), did not protect against the HSP27 dephosphorylation/granulation and the F-actin collapse after 2 h of simulated ischemia (not shown). On one hand, these findings suggest that prior hyperphosphorylation of endothelial HSP27 is not responsible for the actin rearrangement in response to early heat-induced cytoprotection. On the other hand, the HSP27 dephosphorylation within ischemia-stressed endothelial cells appears to be a fairly rapid process so that the transient increase in phosphorylated HSP27 dissipates before the critical stage of the stress, when F-actin is destroyed. Meanwhile, the duration of the early tolerance was restricted by the time limits in which the suppression of the HSP27 dephosphorylation/granulation was observed. Thus the early heat-induced cytoprotection seems to be linked to retardation of the HSP27 dephosphorylation at a level lower than some critical level during ischemic stress. If
Delayed tolerance to simulated ischemia. The delayed tolerance is acquired after 12–14 h of post-heat shock incubation of the cells in rich growth medium and then persists within 12–16 h. The second window of the cytoprotection was most pronounced from 16 to 24 h after heat preconditioning. Having reached the delayed phase of the protection, endothelial cells do not differ morphologically from the control (nontolerant) cells, and both their F-actin and HSP27 distribution patterns are identical to control patterns (see Fig. 5, A and B). During ischemic stress in these tolerant cells, the F-actin bundles (Fig. 5C) and normal morphology (not shown) were preserved without visible changes compared with the unstressed cells (Fig. 5A). The cells exhibiting the delayed tolerance preserved an almost unchanged profile of the HSP27 isoforms after 2 h of simulated ischemia (Fig. 4C). Herein, the availability of isoform d indicates that the stress-evoked dephosphorylation of HSP27 is more suppressed during the delayed cytoprotection than in the case of the early tolerance (compare Fig. 4, B and C). The arrest of intranuclear granulation of HSP27 was intrinsic to the delayed cytoprotection (Fig. 5D) as well as to the early tolerance (see Fig. 3H).

It is generally accepted that if tolerance to ischemia is induced by a short period of stress followed by a prolonged period of recovery, the cytoprotection, at least in part, is due to poststress HSP accumulation (5, 16–18, 20, 29, 37). In support of this point of view, immunoblotting shows powerful expression of HSP70i and an increase in the HSP27 content in heat preconditioned endothelial cells that have reached the delayed phase of the tolerance (Fig. 6A). Distinguishable bands of HSP70i had really showed up within 6–9 h after heat shock, i.e., in the interval between the early and delayed phases of the anti-ischemic protection (Fig. 6A). However, densitometric scanning revealed a 2.4 ± 0.4-fold increase in the HSP70i band intensity within the time limits of the delayed tolerance compared with that at 9 h of post-heat shock recovery (mean ± SE, n = 8; P < 0.05). In addition, we have found from analyses of blots a reliable 2.1 ± 0.3-fold elevation of the HSP27 level in the cultures exhibiting delayed tolerance to simulated ischemia (mean ± SE, n = 8; P < 0.05). Despite the increase in the HSP27 content in heat preconditioned endothelial cells, a ratio of its isoforms 18 h after heat shock seems to be the same as that of the control cells (compare Fig. 4, A and C). Importantly, an ultimate loss of the delayed tolerance 30–32 h after priming heat shock coincided with the disappearance of HSP70i and reduction of HSP27 to the initial level (see Fig. 6A). The suppression of the HSP27 dephosphorylation/granulation during simulated ischemia was also revealed only within the time limits of the delayed tolerance.

To determine whether the late cytoprotection following heat shock depends on the HSP induction, we examined the effects of cycloheximide and quercetin on
development of the delayed tolerance. Cycloheximide is an inhibitor of total protein synthesis in the cytosol, whereas quercetin selectively represses the HSP gene transcription. Both the former (17, 37) and the latter (36, 39) were previously shown to abolish the delayed cytoresistance mediated by inducible HSPs. We added either 30 µM quercetin or 50 µM cycloheximide (both at nontoxic concentrations) to heat-shocked endothelial cells for 9–12 h and then removed the inhibitors and incubated the cells in the usual growth medium for 6 h before simulated ischemia. This treatment did not lead to alterations in the actin network, HSP27 status, and morphology of the preheated cells (not shown); however, it completely blocked the HSP accumulation (Fig. 6B) and nullified the late microfilament resistance to simulated ischemia (Fig. 5E). Quercetin also abolished such characteristic signs of tolerance as preservation of normal morphology (not shown) and prevention of intranuclear granulation of HSP27 during the insult (Fig. 5F). No attenuation of HSP27 dephosphorylation in response to ischemic stress was observed in the cells if they were treated with 50 µM cycloheximide or 30 µM quercetin after priming heat shock (not shown). Thus, in contrast to the early post-heat shock tolerance, de novo synthesis and accumulation of inducible HSP(s) appear to be necessary for development of the delayed protection. Another preliminary conclusion is that heat preconditioning induces in endothelial cells the expression of a member(s) of the HSP family, which somehow ensures the inhibition of HSP27 dephosphorylation during ischemic stress.

Because HSP70 was suggested as a potential protector of the cytoskeleton against the ischemia-provoked collapse (5, 16–18, 29), we tried to examine whether the delayed protection of endothelial F-actin is due to the accumulation of HSP70 alone. For this purpose, we used herbimycin A; this reagent was shown to selectively induce HSP70 in cultured rat cardiomyocytes, which improved their survival after simulated ischemia (27). In our model, 16 h of pretreatment of endothelial cells with herbimycin A (1 µg/ml) also caused considerable accumulation of HSP70 without a rise in the content of HSP27 (Fig. 6B). However, such a selective overproduction of HSP70 did not confer a good protection of microfilaments and did not interfere with the HSP27 dephosphorylation/granulation in the

Fig. 5. Delayed effects of heat preconditioning on F-actin and HSP27 in ischemia-stressed endothelial cells and inhibitory action of quercetin. A and B: unstressed tolerant cells demonstrating intact microfilaments (A) and usual distribution of HSP27 (B). Fluorescence patterns do not markedly differ from those in unstressed control cells (see Fig. 3, A and B). C and D: delayed protection. Cells were exposed to simulated ischemia (glucose deprivation + 20 µM CCCP for 2 h) 18 h after priming heat shock (45°C, 10 min). Note stability of stress fibers (C) and full absence of granulated HSP27 in cell nuclei (D). E and F: abolishing effects of quercetin. Cells were incubated after heat shock in presence of 30 µM quercetin for 12 h and then for 6 h without reagent and were thereafter subjected to 2 h of ischemic stress. Note that quercetin completely nullifies both the stabilization of microfilaments (E) and suppression of intranuclear granulation of HSP27 (F) that were intrinsic to tolerant cells undergoing simulated ischemia. The same effects as with quercetin were observed with 50 µM cycloheximide (not shown). Original magnification, ×400.
free Ca²⁺ prevent depletion of ATP and an increase in cytosolic and F-actin stability during simulated ischemia (Figs. 3G with arrest of HSP27 dephosphorylation. The increased F-actin stability during simulated ischemia (Figs. 3G and 5C), and, as a probable consequence, longer preservation of normal morphology and integrity of endothelial monolayer (not shown) were clearly observed in both phases of the post-heat shock cytoprotection. Evidently, the protective effects of the heat pretreatment were not due to attenuation of the ATP drop and a burst of free Ca²⁺, because prior heating did not prevent depletion of ATP and an increase in cytosolic free Ca²⁺ within ischemia-stressed endothelial cells (Figs. 1 and 2). From the negative results obtained with SB-203580, thrombin, and histamine (see Early heat-induced tolerance to simulated ischemia), it seems unlikely that the early heat-induced tolerance to simulated ischemia is mediated by the heat-triggered hyper-phosphorylation of HSP27. In turn, the cells exhibiting the delayed protection are also not enriched by phosphorylated HSP27 compared with the nontolerant cells (see Fig. 4, A and C).

At the same time, prior heat shock strongly suppresses dephosphorylation and intranuclear granulation of HSP27 during the ischemia-like insult; this was found as a common event equally intrinsic to both phases of the discontinuous cytoprotection (Figs. 3–5). The ischemia-associated characteristic shifts in the HSP27 isoform spectrum, namely, the reduction of the phosphoisoforms (isoforms b–d) and the increase in the nonphosphorylated isoform a, were markedly impaired in the samples from the preheated cells (Fig. 4, B and C). Presumably, through the inhibition of dephosphorylation of HSP27, its intranuclear granulation under ischemic stress was also suppressed in heat-preconditioned (tolerant) endothelial cultures (Figs. 3H and 5D). The blockage of HSP27 dephosphorylation in vivo might beneficially influence F-actin, because the high level of phosphorylated HSP27 in cells ensures positive dynamics of microfilaments (3, 22, 28) and their resistance to heating (23), oxidants (15), and cytochalasin D (10, 23). To assess a role of HSP27 dephosphorylation in the context of simulated ischemia, we performed the latter in the presence of the cell-permeant protein phosphatase inhibitors sodium orthovanadate or cantharidin. In vivo, the phospho-serine residues in HSP27 are dephosphorylated by protein phosphatase 2A (6). Sodium orthovanadate is known mostly as a blocker of tyrosine protein phosphatases; however, this reagent at a concentration of 100 µM may also inhibit protein phosphatase 2A in living cells (A. Kabakov, unpublished data). The latter effect may be indirect, because protein phosphatase 2A is negatively regulated by a tyrosine protein kinase (6), whereas sodium orthovanadate is able to block dephosphorylation of a phosphotyrosine residue in the inactivated enzyme. Cantharidin is an inhibitor of protein phosphatases 1 and 2A that is known to suppress HSP27 dephosphorylation in vivo (6). Having chosen optimal, nontoxic concentrations for each inhibitor, we observed that 0.2 µM cantharidin (Figs. 4D and 7F) or 100 µM sodium orthovanadate (not shown) prevented both dephosphorylation and intranuclear granulation of HSP27 within the ischemia-stressed cells. Furthermore, the inhibitor treatment of the nonpreheated cells before and/or during ischemic stress virtually mimicked the delayed protective effects of heat preconditioning on F-actin and cell morphology (Fig. 7E). If development of the delayed tolerance in the preheated cells was blocked by cycloheximide or quercetin (see Delayed tolerance to simulated ischemia), the protein phosphatase inhibitors still protected these cells from simulated ischemia (not shown). In that case, the protective effect correlated with the inhibition of the dephosphorylation of residual HSP27 that still persists in the cells after the HSP induction is blocked (see Fig. 6). The protective action of the phosphatase inhibitors was not linked to longer maintenance of the ATP/Ca²⁺ homeostasis, because they do not slow down the decrease in ATP and rise in cytosolic free Ca²⁺ under simulated ischemia (V. Gabai and A. Kabakov, unpublished data). Therefore, it is the inhibition of dephosphorylation of HSP27 that arrests its intranuclear granulation within the ischemia-stressed tolerant cells. It seems likely that the longer preservation of somewhat phosphorylated HSP27 in the cytosol of ATP-
depleted, Ca$^{2+}$-overloaded cells is also essential for the F-actin protection against the collapse and aggregation during simulated ischemia. In support of this, we found fairly good correlations between 1) the inhibition of HSP27 dephosphorylation and 2) the absence of both microfilament debris in the cytoplasm and changes for the worse in cell morphology in the ischemia-stressed tolerant endothelial cultures (see Figs. 3, 4, 5, and 7).

**DISCUSSION**

Although the ability of preconditioned (tolerant) cells to preserve the cytoskeleton under ischemia or ATP depletion is discussed (5, 16–18, 29), this mechanism remains to be defined. A decline in the intracellular ATP-to-ADP ratio per se destabilizes F-actin (reviewed in Ref. 18). The ATP loss-provoked free Ca$^{2+}$ overload in the cytosol may lead to activation of an actin-severing protein, gelsolin, that was suggested to be responsible for F-actin fragmentation in energy-deprived endothelial cells (21). Some actin-regulating proteins (e.g., protein kinases, myosin, HSP27) are sensitive to ATP deficiency, and their dysfunction under ischemia should also cause disorganization of microfilaments. Accordingly, the F-actin protection observed after heat preconditioning might be due to attenuation of the intracellular ATP drop/Ca$^{2+}$ burst during simulated ischemia or, conversely, to maintenance of actin and accessory proteins in a stable, functionally suitable state despite the lack of ATP and surplus free Ca$^{2+}$ in the cytosol. The presented data show that neither the early nor the delayed tolerance abolishes ATP depletion and free Ca$^{2+}$ overload within ischemia-stressed endothelial cells (Figs. 1 and 2).

With regard to actin-regulating proteins, at least the ischemia-associated changes in the HSP27 status are considerably impaired in preheated endothelial cultures (Figs. 3–5), which may be connected with the improved response of F-actin to ischemic stress. Indeed, dephosphorylated HSP27 can inhibit actin polymerization (3, 4), and overexpression of the nonphosphorylatable mutant form of human HSP27 reduces cortical F-actin concentration within transfected cells (22, 28). In contrast, only a pool of phosphorylated HSP27 is involved in the microfilament protection.
against heat shock, oxidants, and cytochalasin D (10, 15, 23). Taken together, these data suggest that, if dephosphorylation of HSP27 in vivo prevails over its phosphorylation (e.g., under ATP depletion), inhibition of this process may be a beneficial factor for F-actin. Results of the present work show that, in ischemia-stressed endothelial cells, the arrest of HSP27 dephosphorylation correlates with the acquired F-actin resistance to the stress-provoked damage. Apparent attenuation of the ischemia-associated intranuclear granulation of HSP27 in the tolerant cells implies, on one hand, the suppression of the HSP27 dephosphorylation during the insult and, on the other hand, a longer sojourn of phosphorylated HSP27 in the cytosol compared with the nontolerant cells. It was suggested earlier that only a soluble, nonmultimeric form of phosphorylated HSP27 really contributes to a defense mechanism against cellular stresses (19, 23). The cytoprotective function of phosphorylated HSP27 appears to be realized via its capacity to stabilize microfilaments in stressed cells (3, 10, 15, 23). However, it is unknown how cytosolic HSP27 protects the actin cytoskeleton. We hypothesize that phosphorylated HSP27 specifically interacts with certain cytosolic proteins and that this leads somehow to stabilization of F-actin. One possible way may be inhibition of an actin-severing protein [presumably gelsolin (21)] that becomes activated in response to ischemia and executes the F-actin fragmentation. The ability of the preheated cells to maintain the cytosolic pool of phosphorylated HSP27 during ischemic insult may thereby protect F-actin from the disruption. Moreover, the availability of phosphorylated HSP27 in the cytosol may enable stressed cells to restore microfilaments immediately after the fragmentation; such an instant restoration, if it occurs, should postpone a total breakdown of the actin network. Apparently, nontolerant endothelial cells are unable to realize this mechanism, because under ischemic stress their cytosolic HSP27 rapidly loses phosphates and is transformed into an inactive form (intranuclear granules). Accordingly, exogenous inhibitors of the in vivo dephosphorylation of HSP27 may help to stabilize microfilaments during simulated ischemia (see Fig. 7E).

The facts that 1) SB-203580 failed to repeal the early anti-ischemic resistance of the preheated cells and 2) thrombin and histamine did not mimic the early effects of heat shock on the ischemia-stressed cells allow us to exclude the p38 MAP kinase/HSP27 pathway from the reactions of the cell relevant to the early cytoprotection in our model. The machinery translocating F-actin to cellular edges in response to simulated ischemia remains undiscovered for the present. Except for the p38 MAP kinase/HSP27 pathway, other stress-sensitive cellular mechanisms may modulate the organization of microfilaments. The suppression of ischemia-evoked HSP27 dephosphorylation/granulation at the early phase of the tolerance seems to be due to inhibition of protein phosphatase 2A. This may result from the transient thermal denaturation of the enzyme; otherwise, the heat shock response of the cell may include a regulatory inactivation of protein phosphatase 2A [e.g., via its tyrosine phosphorylation (6)]. Obviously, in cases of early tolerance following heat shock, the longer preservation of phosphorylated HSP27 by itself does not determine the submembranous localization of F-actin, because the suppression of HSP27 dephosphorylation by the protein phosphatase inhibitors did not induce the F-actin redistribution toward cellular edges (see Fig. 7E). Nonetheless, some threshold level of HSP27 phosphorylated at a particular site(s) may save endothelial F-actin from disorderly severing and aggregating, which are noxious for cell morphology. In favor of such supposition is the fact that the early post-heat shock cytoprotection continues only until the arrest of the HSP27 dephosphorylation/granulation takes place.

The positive correlation between the development of delayed tolerance and the HSP accumulation in heat-preconditioned endothelial cells and the abolishing effect of quercetin (Fig. 6B) suggests that the delayed anti-ischemic cytoprotection is mediated by the HSP induction. As heat shock stimulates the synthesis of a whole set of stress-inducible HSPs, it is unknown which HSP(s) confers the delayed protection of microfilaments. In previous studies, beneficial effects of heat pretreatment on ATP-depleted cell cultures were attributed to HSP70i rather than to other HSPs (17, 37). Reduced actin aggregation and prevention of the microfilament derangement were observed during ATP depletion in murine tumor cells (17) and proximal tubule cells (5) in which the HSP70 content increased after prior heat stress. A potential capacity of HSP70 to stabilize the cytoskeleton under ischemia in vivo is also discussed (16, 18, 29). In our experiments with the selective induction of HSP70i by herbimycin A (Fig. 6B), excess HSP70 prevented neither F-actin destruction nor HSP27 dephosphorylation/granulation in ischemia-stressed cells. Therefore, the increase in endothelial HSP70 alone is not responsible for the late antiischemic resistance following heat shock. At the same time, overexpression of HSP27 alone was shown to elevate the viability of rat cardiomyocytes under simulated ischemia (26), although the F-actin resistance and the HSP27 status were not analyzed in that system. In our model, we found an approximately twofold increase in the HSP27 content within preheated endothelial cells exhibiting the delayed tolerance, and this heat-induced surplus of HSP27 might confer some additional cytoprotection. For all that, a mechanism of the suppression of the HSP27 dephosphorylation/granulation at the late phase of the tolerance is unclear and worthy of separate investigations. This mechanism appears to be mediated by inducible HSPs, and perhaps accumulated chaperones (HSPs) are able somewhat to oppose the unbalanced protein phosphatase action in the course of ischemic stress. Further experiments with differential and combined overexpression of various HSPs could clarify this intriguing issue.

The data obtained tempt us to speculate about anti-ischemic strategies aimed at alleviation of myocardial
infarction and brain stroke. We believe that artificially elevated persistence of the vascular endothelium under ischemia might avert formations of thrombi and edema that in turn should improve the outcome of an acute ischemic attack. If ischemia-like conditions are created in the process of surgical treatment connected with bypass perfusion or transplantation of organs, it would be beneficial to adapt endothelial and other cells to the forthcoming stress beforehand. From our findings, one can see two potential ways to minimize ischemic injury: 1) inhibition of the HSP27 dephosphorylation during the insult and 2) heat preconditioning to increase the intracellular HSP content before the insult. Although the second way appears difficult to perform in organisms of patients, it might be used as the adapting pretreatment for an isolated, perfused donor organ before its implantation into a recipient. The first way can motivate development of new anti-ischemic drugs that would be able to arrest the HSP27 dephosphorylation in the affected tissues. Theoretically, cell-permanent and nontoxic inhibitors of protein phosphatase 2A will do for this purpose.

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