Specific heat shock proteins protect microtubules during simulated ischemia in cardiac myocytes

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Bluhm, Wolfgang F., Jody L. Martin, Ruben Mestril, and Wolfgang H. Dillmann. Specific heat shock proteins protect microtubules during simulated ischemia in cardiac myocytes. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H2243–H2249, 1998.—The protective effects of heat shock proteins (HSPs) during myocardial ischemia are now well documented, but little is known about the mechanisms of protection and the specificity of different HSPs. Because cytoskeletal injury plays a crucial role in the pathogenesis of irreversible ischemic damage, we tested whether overexpression of specific HSPs protects the integrity of microtubules during simulated ischemia in rat neonatal cardiac myocytes. Overexpression of specific HSPs was achieved by adenovirus-mediated transgene expression. Damage was assessed by comparing control cells to cells that were subjected to a simulated ischemia protocol. Microtubular integrity was measured by indirect immunofluorescence, confocal microscopy, and image analysis. Within 14 h of simulated ischemia, microtubular integrity decreased significantly in uninfected myocytes (from 24.6 ± 1.2 to 13.2 ± 0.4) and in myocytes infected with a control virus that expressed no transgene (from 25.9 ± 1.8 to 13.1 ± 1.4). Microtubular integrity after ischemia was significantly better preserved in cells overexpressing constitutive Hsp70 (21.7 ± 1.6) or αB-crystallin (18.0 ± 2.7) but not in cells overexpressing inducible Hsp70 (11.5 ± 0.8) or Hsp27 (14.0 ± 2.2). We conclude that specific HSPs protect the microtubules during simulated cardiac ischemia.

Confocal microscopy; immunofluorescence; image analysis; cytoskeleton; tubulin

It is now well documented that heat shock proteins (HSPs) protect against myocardial ischemic damage. The cardioprotective effects of inducible heat shock protein 70 (Hsp70), the most markedly inducible HSP, have been shown in stably transfected myogenic cell lines (19), after adenovirus-mediated gene transfer in neonatal cardiomyocytes (20), and in the hearts of transgenic animals after global or regional ischemia (11, 17, 23, 24). Recently, protection during myocardial ischemia has also been shown for the small HSPs Hsp27 and αB-crystallin (18). However, little is known about the mechanisms of protection and the specificity of different HSPs. In light of the significance and prevalence of ischemic heart disease, elucidation of these mechanisms should be highly desirable.

There is strong evidence that cytoskeletal injury plays a crucial role in the pathogenesis of myocardial ischemic injury (1, 5, 6, 10, 14, 28). Microtubules are an important constituent of the cardiac cytoskeleton (25). Decreased tubulin staining after ischemia (1) and disruption of microtubules early in ischemia (12) or with reperfusion (27) have been shown. The constitutive heat shock protein 70 (Hsp70c) binds to tubulin (26) and may aid the assembly of microtubules (9) and their regrowth after heat shock (3, 4). Cytoskeletal associations have also been reported for Hsp27 (16) and αB-crystallin (2, 21), and, interestingly, αB-crystallin production is induced by agents that promote the disassembly of microtubules (15).

The aim of our study was to examine the microtubules as a potential structural target for the cardioprotective effects of HSPs during myocardial ischemia. Quantitative analysis of microtubular integrity was achieved by immunofluorescence, confocal microscopy, and image analysis. Ischemic damage was assessed by comparing control cells with cells that were subjected to simulated ischemia as previously described (19).

METHODS

Construction of adenoviral vectors. The rat constitutive hsp70c gene (22) was inserted into the E1 region of an adenoviral vector construct using the general strategy previously described (8). Briefly, the rat hsp70c was cloned into the multiple cloning site of the adenoviral shuttle plasmid pACCmVpLpASR (kindly provided by Dr. Robert D. Gerard, University of Texas, Southwestern Medical Center, Dallas, TX) (7). This plasmid contains the 5'-end of the adenovirus serotype 5 genome (map units 0–17), in which the E1 region has been substituted with the human cytomegalovirus enhancer-promoter followed by the multiple doning site from pAC19 and the polyadenylation region from SV40. The resulting plasmid was cotransfected with pJM17, a plasmid that contains the complete adenovirus 5 genome, into the human embryonic kidney cell line 293 using the calcium phosphate transfection method. Infectious viral particles containing the inserted hsp70c were generated by in vivo recombination in the 293 cells and were isolated as single plaques 10 to 20 days later. The isolated plaques were propagated in 293 cells for several passages to obtain high titer stocks. Viral particles were purified by CsCl purification.

To confirm specific transgene expression, cardiomyocytes were infected with the adenoviral construct for 48 h and then metabolically labeled with [35S]methionine for 4 h, after which total protein extracts were prepared. Labeled protein samples (5 × 105 counts·min⁻¹·lane⁻¹) were fractionated by 8% gradient SDS-PAGE, enhanced, dried, and exposed to X-ray film. Expression of hsp70c transgene did not induce the expression of other HSPs.

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Adenoviral constructs expressing Hsp70, Hsp27, and αB-crystallin were made as previously described (18), and transgene expression in neonatal myocytes was previously verified by Western blot analysis (18).

Preparation of cardiac myocytes. Neonatal cardiac myocytes were isolated by collagenase-pancreatin digestion from 1- to 2-day-old rats as previously described (13). The myocytes were plated at a density of ~200 cells/mm² on four-well chamber slides (LabTek) that were precoated with fibronectin. Increased levels of specific HSPs (αB-crystallin, Hsp27, Hsp70c, Hsp70l) were obtained by HSP transgene expression using a human adenovirus 5 vector. Cells were infected 1 day after isolation for 1 h in DMEM containing no serum. Cells were then kept for 2 days in DMEM with 2% fetal bovine serum and in an atmosphere containing 8% CO₂ at 37°C.

H9c2 cells (used only for evaluating the image analysis methods) were obtained from American Type Culture Collection, plated at subconfluent density in gelatin-coated four-well chamber slides, and grown to confluence.

Simulated ischemia. To subject cells to simulated ischemia, regular cell medium was replaced with normotonic but glucose-free and serum-free Hanks’ balanced salt solution (in mmol/l: 1.3 CaCl₂, 5 KCl, 0.3 KH₂PO₄, 0.8 MgSO₄, 0.3 Na₂HPO₄, 4 NaHCO₃, and 138 NaCl). The cells were then placed in an airtight jar in which an atmosphere of <0.2% O₂ and 5–8% CO₂ was established with the GasPak system (BBL Microbiology Systems), and ischemia was maintained for 14 h. Control (nonischemic) cells were left in regular DMEM under normal cell culture conditions.

Immunofluorescence. Cells were fixed with 100% methanol for 2 min. Cells were washed three times with PBS (in mmol/l: 137 NaCl, 2.7 KCl, 10 Na₂HPO₄ and 1.8 KH₂PO₄), followed by 20 min of blocking with 10% goat serum in PBS containing 3% BSA. Cells were washed again three times with PBS, followed by 1 h of incubation with monoclonal anti-tubulin antibody (T-5168, Sigma) at a dilution of 1:1,000 in PBS containing 3% BSA. After three further washes with PBS, cells were incubated for 1 h with a secondary FITC-conjugated anti-mouse antibody raised in goat (F-0257, Sigma) at a dilution of 1:100 in PBS containing 3% BSA. After three final washes with PBS, the slides were aspirated dry and sealed with coverslips using the Vectashield mounting medium (Vector Laboratories) to prevent rapid photobleaching.

Confocal microscopy and image analysis. Confocal microscopy was performed with a Bio-Rad MRC 1024 confocal microscope. The 488-nm wavelength line of the krypton-argon laser was used for excitation, and the emitted light was collected at 522 nm. Image-acquisition settings (laser intensity, iris size, gain) were strictly maintained for all images taken on the same day to allow quantitative, paired comparison data. Images were stored on rewritable 1.2-GB disks for later analysis.

The LaserSharp software of the confocal microscope was used for image analysis. Each image consists of 512 × 512 individual pixels with an intensity ranging from 0 to 255. Pixel intensity histograms (number of pixels versus pixel intensity) were obtained for each image. The mean pixel intensity would be the simplest measure of average staining intensity. However, it was found to be significantly affected by background staining. Instead, the width (i.e., the standard deviation) of the pixel intensity histogram (as calculated by the LaserSharp software) was found to be a reliable and sensitive measure of the integral of microtubules. This width is close to zero for uniform background staining (i.e., after complete loss of tubulin staining following prolonged ischemia) and increases with the number and staining intensity of intact tubulin filaments.

Study design. Three separate sets of experiments were conducted. The first set examined the protective effects of Hsp70c in comparison with uninfected cells or cells infected with a control virus expressing no transgene (SR−). The second set of experiments examined the protective effects of Hsp70l in comparison with SR− infected cells. The third set of experiments examined the protective effects of the small heat shock proteins (αB-crystallin, Hsp27) in comparison with SR− infected cells.

Two slides (4 wells each) were treated identically except that one slide was submitted to the simulated ischemia protocol after 2 days of transgene expression. After immunostaining for tubulin, 10 randomly chosen views from each well were taken at the confocal microscope with a ×20 objective (30–40 cells per view). Microtubular integrity was determined by image analysis as described in Confocal microscopy and image analysis for each view and averaged for all views. Experiments were performed in duplicate (2 pairs of slides) each day and repeated on three separate days (n = 3).

Statistical analysis. For each different experimental condition (uninfected or adenovirus infected, control or ischemic), all microscopic views obtained on 1 day (10 views each from 2 duplicate slides) were averaged to yield one data point. All data are presented as means ± SE (n = 3). Comparisons between multiple groups were performed by repeated-measures ANOVA, the equivalent of paired comparisons for multiple groups. Paired statistics were used because crucial parameters such as plating of myocytes or severity of ischemia were matched for all cells analyzed on any one day but were subject to day-to-day variation.

RESULTS

Microtubular integrity in cardiac myocytes was analyzed by using indirect immunofluorescent staining for tubulin, followed by confocal microscopy and quantitative image analysis. Simulated ischemia led to a disintegration of microtubules (Fig. 1A). Microtubular integrity was measured reliably by the width (i.e., standard deviation) of the pixel intensity histogram (Fig. 1B). This method was further validated by analyzing microtubular disintegration caused by colchicine treatment (Fig. 2).

Binding of Hsp70 to tubulin has been reported (26). To examine whether overexpression of Hsp70c protects the integrity of microtubules during simulated ischemia, we compared neonatal myocytes infected with adenovirus overexpressing Hsp70c with both uninfected myocytes and myocytes infected with an empty control adenovirus that expressed no transgene. Overexpression of Hsp70c was confirmed by Western blot analysis (Fig. 3). Infection with the control adenovirus had no effects on microtubular integrity either before or after ischemia (Fig. 4). Microtubular integrity decreased within 14 h of simulated ischemia from 24.6 ± 1.2 to 13.2 ± 0.4 in uninfected myocytes, and from 25.9 ± 1.8 to 13.1 ± 1.4 in myocytes infected with the empty control virus. However, in myocytes overexpressing Hsp70c, microtubular integrity was significantly better preserved after simulated ischemia (21.7 ± 1.6, P < 0.01). In nonischemic cells, overexpression of...
Hsp70c led to a slight but not significant increase in microtubular integrity (30.7 ± 2.6). Representative micrographs showing the protective effect of Hsp70c are shown in Fig. 5.

In contrast to the protective effects of the constitutive Hsp70c, overexpression of Hsp70i (see Fig. 3) did not preserve the integrity of microtubules during simulated ischemia (Fig. 6). To rule out the possibility of cytotoxic side effects of forced overexpression of Hsp70i, adenoviral infection was performed in this case with three different viral titers. There was no effect of Hsp70i overexpression on microtubular integrity before or after simulated ischemia at either concentration. Partial nuclear or perinuclear translocation of Hsp70i, but not Hsp70c, was observed with simulated ischemia (Fig. 7).

To examine whether microtubules are also protected by small HSPs, we compared neonatal myocytes overexpressing either Hsp27 or αB-crystallin with cells infected with the empty control virus (Fig. 8). In nonischemic cells, the microtubular integrity in cells overexpressing αB-crystallin (29.6 ± 2.2) or Hsp27 (30.9 ± 0.9) was not significantly different from cells infected with an empty control virus (31.6 ± 1.9). After simulated ischemia, microtubular integrity was significantly better preserved in cells overexpressing αB-crystallin than in cells infected with the control virus (18.0 ± 2.7 vs. 13.6 ± 3.0, \( P < 0.05 \)). In comparison, overexpression of Hsp27 did not confer protection on the microtubules (14.0 ± 2.2, \( P = 0.7 \)). Individual micrographs showing the effects of Hsp27 and αB-crystallin are shown in Fig. 9.

To rule out confounding differences in expression levels, we quantified protein levels for αB-crystallin (endogenous and forced overexpression) as well as the small HSPs Hsp27 (forced expression) and Hsp25 (its endogenous equivalent) by including known amounts of recombinant protein on Western blots. Both adenoviruses yielded almost identical amounts of overexpressed protein: overexpressed αB-crystallin amounted...
to 0.46% of total protein, and overexpressed Hsp27 amounted to 0.44% of total protein. On the other hand, endogenous αB-crystallin amounted to 0.5% of total protein, whereas endogenous Hsp25 amounted to only 0.073% of total protein. Therefore, infection with the αB-crystallin virus led to a roughly twofold overexpression [(0.46 + 0.5)/0.5], whereas infection with Hsp27 adenovirus led to an approximately sevenfold overexpression [(0.44 + 0.073)/0.073].

DISCUSSION

The objective of this study was to quantitatively assess the integrity of microtubules during simulated ischemia in rat neonatal cardiac myocytes and to evaluate possible protective effects of overexpressing specific HSPs. We developed a simple and reliable measure of microtubular integrity based on immunofluorescence, confocal microscopy, and image analysis. Microtubular integrity decreased during simulated ischemia and was significantly protected by overexpression of the HSPs Hsp70c and αB-crystallin but not by overexpression of the HSPs Hsp70i or Hsp27.

A number of prior studies have shown cardioprotective effects of HSPs during cardiac ischemia. Ischemic damage has been assessed by determining infarct size of the whole heart (11, 17, 23) or by measuring the release of enzymes indicative of cellular damage, such as creatine phosphokinase or lactate dehydrogenase, from cardiomyocytes or myogenic cell lines (18–20). These studies have been of great value in documenting cardioprotection mediated by HSPs. Still, however, little is known about the mechanisms of protection or the specificity of different HSPs in the protective process.

The methods used in this study were designed to examine a particular cellular structure, namely, the microtubular network, as a potential target for the protective effects of HSPs. Introducing an easy and reliable quantitative measure for the integrity of the microtubules was of particular importance because it allowed for an objective and unbiased assessment of...
ischemic cytoskeletal injury. Damage usually varied considerably within each well, and quantitation of microtubular integrity enabled averaging and statistical analysis. It also made it possible to objectively select representative pictures to show the effects of each HSP by matching the measure of integrity for the selected image to the statistical average of a large number of measurements.

Neonatal cardiac myocyte preparations contain certain amounts of nonmyocytes, predominantly fibroblasts. Immunostaining with fibronectin revealed very small numbers of fibroblasts (<5% in 3 consecutive primary cell cultures), and cells were maintained in medium containing only 2% serum to limit fibroblast proliferation. Furthermore, cells were plated at a rather low cell density (~200 cells/mm²), at which fibroblasts become readily apparent as dense colonies after several days in culture. On the rare occasion that such “clusters” were detected in the confocal images, such images were excluded from further analysis. Therefore, the results of our study can be primarily attributed to protective effects of HSPs on cardiac myocytes.

Cytoskeletal injury during myocardial ischemia is not limited to microtubules. Several other cytoskeletal proteins have been reported to be damaged during ischemia, such as actin, actinin, desmin, and vinculin (1, 6, 10, 28). However, several reports have suggested that tubulin may be a particularly sensitive and early indicator of irreversible damage during ischemia or reperfusion (10, 12, 27). Furthermore, an association of Hsp70c and tubulin was indicated by prior studies (3, 4, 9, 26). These considerations clearly identified tubulin...
as a promising candidate protein for protection by HSPs during myocardial ischemia. Finally, quantitative structural analysis of this particular protein was aided by excellent immunofluorescent staining patterns.

Our results offer clear evidence for the protection of microtubules by certain HSPs during simulated ischemia in neonatal cardiac myocytes. Specifically, we were able to show that Hsp70c, which has been reported to bind to tubulin (26), actually confers protection during an ischemic insult, although the major chaperonin required for tubulin folding is the cytosolic TCP-1 ring complex (29). Whereas the general cardioprotective effects of Hsp70i have been documented in prior studies (11, 18–20, 23–24), the present results document specific protection of microtubules by Hsp70c. The lack of microtubular protection by Hsp70i, despite its strong overall protective effects, may be related to its translocation into the nucleus, i.e., away from the microtubules. This lack of protection by Hsp70i also rules out the possibility that the different degrees of microtubular protection are only secondary to differences in overall protection as measured, e.g., by enzyme release or cell survival.

Lately, small HSPs have become of increasing interest. In particular, the protective effects of αB-crystallin have been shown only recently (18). Whereas there are some indications that αB-crystallin associates with desmin and actin (2, 21), we were able to demonstrate protection of tubulin by αB-crystallin. This finding gives further substance to the importance of αB-crystallin in the heart, which is otherwise most abundant in the lens of the eye.

In contrast to Hsp70c and αB-crystallin, overexpression of Hsp27 did not protect the microtubular network during simulated ischemia. This lack of protection of Hsp27 occurred despite similar absolute and greater relative overexpression in comparison with αB-crystallin. Although Hsp27 did not protect the microtubules, it may still offer protection for other cytoskeletal proteins such as actin-based microfilaments (16). Similarly, αB-crystallin may protect actin or desmin (2, 21) in addition to tubulin. Therefore, an extension of the methodology presented in this paper to other cytoskeletal proteins appears desirable and promising. However, the image analysis technique may have to be modified, because other proteins may present different cellular distributions, staining patterns, or manifestations of ischemic damage.

In conclusion, the results of our study identified the microtubules as a structural target for the cardioprotective effects of certain HSPs during simulated ischemia in vitro. In addition, it has identified differential speci-
References


