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Influence of Phosphorylation and Oligomerization on the Protective Role of the Small Heat Shock Protein 27 in Rat Adult Cardiomyocytes

JODY L. MARTIN,* EILEEN HICKEY,† LEE A. WEBER,† WOLFGANG H. DILLMANN,‡ AND RUBEN MESTRIL*[1]

*Department of Physiology, The Cardiovascular Institute, Loyola University, Chicago, IL
†Department of Biology, University of Nevada, Reno, NV
‡Department of Medicine, University of California, San Diego, CA

Recent reports have demonstrated that the heat shock proteins (hsp) and in particular the hsp70 confer protection against cardiac ischemic damage. More recently, we have shown that increased expression of another heat shock protein, the hsp27, through an adenovirus vector system protects adult cardiomyocytes against ischemic injury. This small heat shock protein undergoes phosphorylation when the cell is under stress. This has led many to speculate that phosphorylation of hsp27 is required for the protective role this protein plays in the cell. In order to investigate this possibility, we have mutated the serines that are the sites of phosphorylation on the hsp27, to glycines or alanines. These nonphosphorylatable mutants of hsp27 were cloned into adenoviral vectors and used to infect adult rat cardiomyocytes to assess their ability in protecting against ischemic injury. In addition, we used a specific inhibitor of p38 MAP kinase that is a key member of the kinase pathway responsible for phosphorylating the hsp27. Our present results show that the nonphosphorylated hsp27 forms larger oligomeric complexes than the phosphorylated hsp27. Interestingly, phosphorylation of hsp27 seems not to play a role in its ability to protect adult rat cardiomyocytes against ischemic damage.

Heat shock proteins Ischemia Cardiomyocytes p38 MAP kinase Cardioprotection

THE small heat shock protein (hsp), hsp25 in rodents and hsp27 in humans, is protective against thermal stress, chemotherapeutic agents, and oxidative damage. Recently, we demonstrated protection against ischemic damage associated with the overexpression of hsp27 in adult rat cardiac myocytes. Hsp27 is phosphorylated at serines 15, 78, and 82 in response to thermal, shear, and oxidative stress, in addition to mitogen and cytokine treatment. This phosphorylation is catalyzed by MAPKAP kinase 2/3 and/or the newly described PRAK kinase (15), which are both regulated by the p38 MAP kinase pathway. Recent in vivo evidence has linked the activation of this pathway with the signal transduction cascade associated with ischemic preconditioning (18). It has been implicated that the phosphorylation status of hsp27 is significant in a variety of pathophysiological roles via actin microfilament modulation and general chaperone function (14,17).

Another characteristic of the small heat shock proteins is their oligomeric-complexing property. Hsp27's native molecular mass ranges in size from 100 to 700 kDa. The size of this structure is dependent on the cell's physiology, and generally shifts down in size with an increase in the level of hsp27 phosphorylation. Therefore, as the kinases respond to stress so does the size of the hsp27 oligomeric complex.

[1]Address correspondence to Ruben Mestril, Ph.D., The Cardiovascular Institute, Loyola University Medical Center, 2160 South First Avenue, Bldg. 110, Room 5227, Maywood, IL 60153. Tel: (708) 327-2395; Fax: (708) 327-2849; E-mail: rmestri@luc.edu

Although modulation by phosphorylation of hsp27 and its structural organization has been described, its significance in hsp27-associated cellular protection is still controversial. Reduced thermal resistance and altered actin microfilament dynamics have been observed in Chinese hamster cell lines overexpressing nonphosphorylatable hsp27 (9). In vitro actin polymerization studies have also linked increasing phosphorylation with a decrease in hsp27 oligomer size and inhibition of actin addition to the barbed end (1). In contrast, Knauf and coworkers (6) demonstrated no change of in vitro chaperone and in vivo thermoresistance functions of the murine hsp25 with the phosphorylatable serines substituted to alanines. More recently, Mehlen and co-investigators (11) demonstrated that only large unphosphorylated hsp27 was able to protect NIH 3T3 cells against damage induced by TNF-α.

Therefore, we sought to examine the significance of the phosphorylation of hsp27 in adult cardiomyocytes. Recombinant adenoviral constructs expressing the wild-type hsp27 and two different nonphosphorylatable mutants of hsp27 were used to directly compare their ability to protect against ischemic damage and differences in oligomeric size.

MATERIALS AND METHODS

Primary Cell Culture

Ventricular myocytes from adult Sprague-Dawley rats (250–300 g) were prepared as previously described (3). Briefly, four rat hearts were perfused 45 min at 35°C with modified Krebs-Hensleit buffer containing 0.5 mg/ml collagenase (Boehringer Mannheim) and 25 μM Ca^{2+}. Following the removal of the atria, the ventricles were cut into several pieces and dispersed with gentle agitation and wide-bore tip pipetting. The cells were filtered twice through nylon mesh and washed 3 times at $50 \times g$ for 1 min. The cell pellet was suspended in 0.1% bovine serum albumin and Ca^{2+} added slowly to a final concentration of 1 mM. These preparations provided 5–10 million cells per heart with 80% viability based on rod-shaped morphology and trypan blue exclusion. One hundred thousand cells were plated on 35-mm culture dishes precoated with laminin (GIBCO BRL). After 1 h the media was changed to M199 supplemented with HEPES, taurine, creatinine, carnitine, and BSA. These cells were then infected with adenoviruses at an MOI of 1 to 10 for 1–2 h followed by a media change. Two days later, the cells were subjected to simulated ischemia. The p38 MAP kinase specific inhibitor, SB203580 (Calbiochem), was added to the cells (10 μM, final concentration) for 1 h prior to harvesting cells for native gel analysis or simulated ischemia treatment. The level of infection achieved was routinely checked by infecting adult cardiomyocytes with the adenoviral construct containing the bacterial β-galactosidase gene with a mammalian nuclear localization signal. Cardiomyocytes were fixed 48 h after infection with 1.25% glutaraldehyde and then reacted for 4 h with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

Construction of Recombinant Adenoviral Vectors

The bacterial LacZ preceded by a mammalian nuclear localization signal, the human hsp27 wild-type plus the mutated constructs Triple G (serines 15, 78, and 82 to glycines) and Triple A (serines 15, 78, and 82 to alanines) were cloned into the multiple cloning site of the adenoviral shuttle vector pACCMVpLpASR-(kindly provided by Dr. Robert D. Gerard). This plasmid contains the 5′ end of the adenovirus serotype 5 genome (map units 0 to 17) in which the E1 region has been replaced with the human CMV enhancer-promoter followed by the pUC19 multiple cloning site and the polyadenylation region from SV40. Adenoviral constructs were generated as previously described (12).

Simulated Ischemia and Enzyme Quantitation

Simulated ischemia was achieved by placing the cells in a hypotonic, balanced salt solution (in mmol/l: $CaCl_2$ 1.3, KCl 5, KH_2PO_4 0.3, $MgCl_2$ 0.5, $MgSO_4$ 0.4, NaCl 69, $NaHCO_2$ 4, and Na_2HPO_4 0.3) without glucose or serum. The plates were then placed in an airtight jar containing the oxygen consuming GasPak System (BBL Microbiology Systems), and flushed for 5 min with argon to rapidly achieve $< 0.2\%$ O_2. After 6 h, the dishes were removed from the chamber and both medium and cells were separately assayed for cytosolic enzyme content. After removing the medium, the cells were scraped into 1 ml of cold PBS and sonicated (Ultrasonic Homogenizer 4710, Cole-Parmer) for 15 s. Sonicated cells were then centrifuged at $12{,}000 \times g$ for 10 min. Lactate dehydrogenase (LDH) activity present in the medium and in the sonicated cells was quantitated using an LDH determination kit (Sigma). The amount of enzyme released in the nonstressed controls is subtracted as background from the value obtained from the ischemic cells. This value is then normalized to the amount released by cells infected with the control adenovirus (that contains no transgene). This is necessary to pool multiple primary cardiomyocyte experiments. Results shown are from four independent experiments and are expressed as mean ± SEM. The statistical analysis was carried out with a Student's *t*-

test, and results were interpreted to be significantly different when $p < 0.05$.

Protein Analysis

For denaturing SDS-PAGE, cells were harvested in solution B (20 mM NaCl, 20 mM Tris, pH 7.5, 0.1 mM EDTA) containing 1% Triton X-100, 0.5% deoxycholate, and 5 μM 2-mercaptoethanol. The samples were vortexed vigorously and placed on ice for 15 min prior to centrifugation for 15 min at $12{,}000 \times g$. The concentration of the supernatant was determined using a Bradford assay (BioRad). Protein extracts from metabolically labeled cells using [^{35}S]Translabel (ICN) were fractionated by 12% SDS-PAGE by loading equal amounts of trichloroacetic acid precipitable counts per lane. Gels were fixed, enhanced, dried, and exposed to X-ray film for 14 h at −70°C. For native gel analysis, cells were washed with ice-cold PBS and then harvested in 10 mM HEPES, pH 7.4, containing 0.2 mM PMSF, 0.5 mg/l leupeptin, and 0.7 mg/l pepstatin. These extracts were then sheared through a 27-gauge needle and their concentration determined by using the Bradford protein assay. Protein (20 μg per lane) was loaded onto a 4% polyacrylamide native gel, fractionated at 400 V/h, and then electrotransferred onto nitrocellulose for Western blot analysis. The antibody used was the monoclonal anti-human hsp27 (SPA-800, StressGen) and blot was developed using an Enhanced Chemiluminescence kit (Amersham).

RESULTS

Our previous studies have shown that adenoviral-mediated expression of the human hsp27 in rat adult cardiomyocytes leads to increased protection against ischemic injury (10). Several reports have shown that phosphorylation and dephosphorylation of the hsp27 alters its oligomeric status and potentially affects its function in the cell (1,5,9). Therefore, we were interested in investigating if phosphorylation and oligomerization status of the hsp27 have any influence on its ability to protect against ischemic damage. The human hsp27 is phosphorylated on serines 15, 78, and 82. We have previously generated two nonphosphorylatable mutants of the human hsp27. In one case the serines have been mutated into glycines, referred to here as Triple G, and in the other the serines have been mutated into alanines, referred to here as Triple A. We then introduced these two nonphosphorylatable mutants of the human hsp27 gene into adenoviral vectors.

As previously shown, rat adult cardiomyocytes are readily infectable with these adenoviral constructs (10). Nonetheless, to assess the level of infectability in our present experiments we routinely infected a dish of cardiomyocytes with an adenoviral construct containing the bacterial β-galactosidase gene with a mammalian nuclear localization signal. This adenoviral construct permits us to control for the amount of cardiomyocytes infected in each experiment. Cardiomyocytes are fixed and reacted with the β-galactosidase substrate X-gal. A representative example of the level of infection achieved is shown in Fig. 1. Dark blue staining is clearly observed in the nuclei of infected cardiomyocytes due to the nuclear localization of the adenoviral-infected β-galactosidase gene. The level of infection achieved was routinely more than 90% of the cardiomyocytes present in the culture dish.

To assess the level of expression from the wild-type and mutant human hsp27 adenoviral constructs, we infected cardiomyocytes with a multiplicity of infection (MOI) of 10 to 1. Forty-eight hours later, we metabolically labeled the cardiomyocytes using [^{35}S]Translabel that contains labeled methionine and cysteine, in media lacking both of these amino acids, for 4 h. Trichloroacetic acid-precipitable radioactive counts were determined for each of the protein extracts prepared from these infected cardiomyocytes. Equal amounts of counts for each protein sample

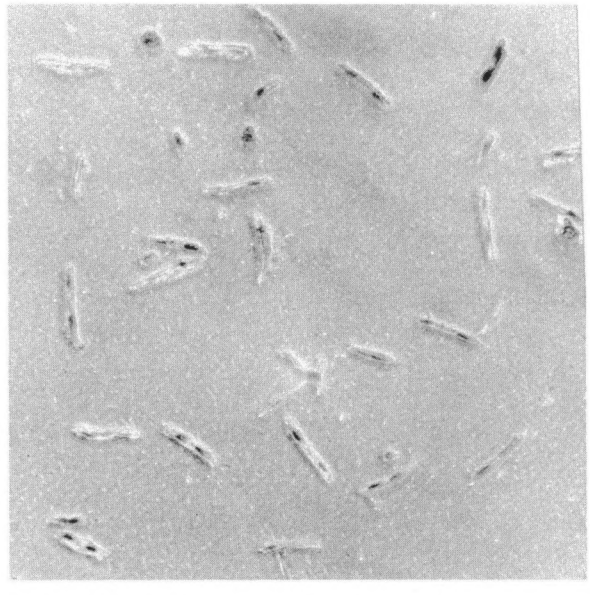

FIG. 1. Adenoviral-infected rat adult cardiomyocytes. Cardiomyocytes infected with an adenoviral construct containing the bacterial β-galactosidase gene with a mammalian nuclear localization signal. Cardiomyocytes were infected at a multiplicity of infection (MOI) of 10 to 1. After 48 h, cardiomyocytes were fixed and reacted with the β-galactosidase substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for 4 h. Intense dark blue staining is present in the nuclei of more than 90% of the cardiomyocytes.

were loaded and fractionated on a 12% denaturating polyacrylamide gel. Figure 2 shows an autoradiogram of a representative gel where one can observe the similar levels of expression achieved by our different adenoviral constructs (wthsp27, Triple A, and Triple G).

In order to determine how the level of phosphorylation affected the oligomeric structure of our adenoviral-expressed human mutant and wild-type hsp27, we prepared native protein extracts from infected cardiomyocytes with the different adenoviral constructs. In addition, we treated cardiomyocytes infected with the wild-type hsp27 with the p38 MAP kinase inhibitor, SB203580; this inhibitor has previously been shown to block the activation of MAPKAP kinase 2/3, which phosphorylates hsp27 (7). Protein extracts from these cardiomyocytes were fractionated on 4% native polyacrylamide gels. The native gels were transferred to nitrocellulose and reacted with a monoclonal antibody specific for the human hsp27. Blots were developed as described in Materials and Methods. Figure 3 shows a representative Western blot obtained using this approach. As can be observed, the wild-type hsp27 forms several different oligomeric structures of approximately 100 kDa, in both control and ischemia-treated cardiomyocytes. The nonphosphorylatable mutant hsp27 (mthsp27), in this case the Triple G mutant, forms a larger oligomeric structure of approximately 133 kDa, in control and ischemia-treated cardiomyocytes. Interestingly, in cardiomyocytes infected with the wild-type hsp27 and treated with the p38 MAP kinase inhibitor, hsp27 forms intermediate oligomeric structures of approximately 120 kDa. This confirms previous results that the phosphorylation status of the hsp27 affects its oligomeric structure.

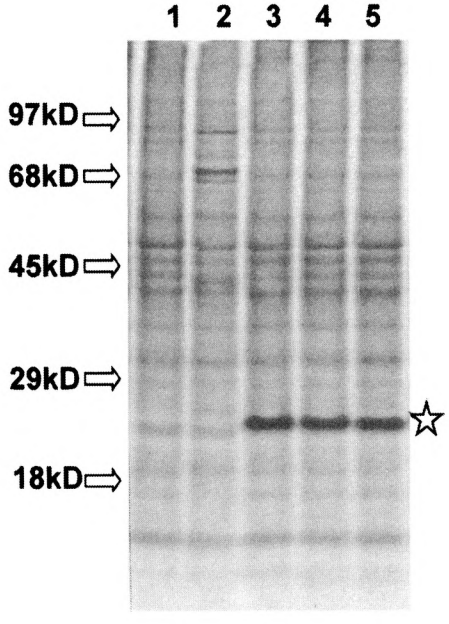

FIG. 2. Autoradiogram of metabolically labeled adenoviral-infected cardiomyocytes. Rat adult cardiomyocytes were either left untreated (lane 1), submitted to simulated ischemia (lane 2), infected with the adenoviral construct containing the Triple G mutated hsp27 (lane 3), the wild-type hsp27 (lane 4), or the Triple A mutated hsp27 (lane 5). Cardiomyocytes were metabolically labeled and fractionated on a 12% denaturing polyacrylamide gel, as described in Materials and Methods. The position of the molecular mass markers is shown on the left side of autoradiogram. The star on the right side of autoradiogram indicates position of the adenoviral expressed mutant and wild-type hsp27.

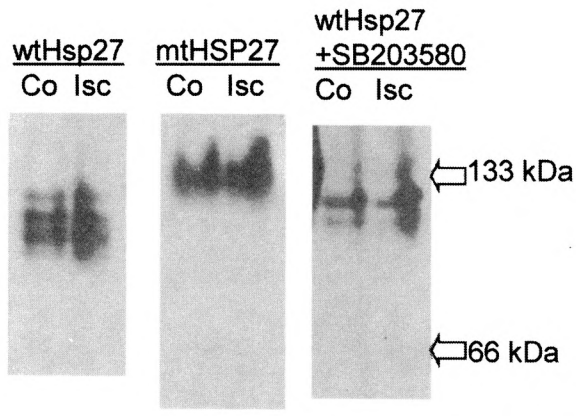

FIG. 3. Western blot analysis of native polyacrylamide gel. Rat adult cardiomyocytes infected with the adenoviral construct containing the wild-type hsp27 in the absence and presence of the p38 MAP kinase inhibitor, SB203580, and the Triple G mutant of the hsp27 were either left untreated (Co) or submitted to simulated ischemia (Isc). Protein extracts from these cardiomyocytes were fractionated on a 4% native polyacrylamide gel, blotted onto nitrocellulose, and reacted with a monoclonal antibody specific to the human hsp27, as described in Materials and Methods. The position of the molecular mass markers is shown on the right side of Western blot.

We then proceeded to determine if the changes in phosphorylation status and oligomeric structure affected the ability of human hsp27 to protect cardiomyocytes from ischemic injury. Initially, we infected rat adult cardiomyocytes with the adenoviral constructs, the wild-type and mutant hsp27s. Two days following infection, the cardiomyocytes were submitted to simulated ischemia and cellular damage was determined by measuring lactate dehydrogenase (LDH) release as described in Materials and Methods. Figure 4 summarizes the results of four independent experiments. As can be observed, all three adenoviral constructs expressing the mutants (Triple A and G) and wild-type hsp27 (hsp27) protect the cardiomyocytes against ischemia-induced damage compared to either noninfected cardiomyocytes (Noninf) or cardiomyocytes infected with our control adenoviral construct (Con).

In order to confirm that the lack of phosphorylation does not affect hsp27's ability to protect cardio-

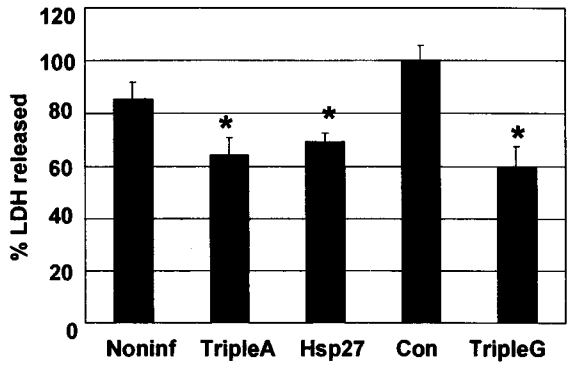

FIG. 4. Effects of mutant and wild-type hsp27 on enzyme release induced by simulated ischemia. Cardiomyocytes were either not infected (Noninf), infected with the control (Con), mutant Triple A, Triple G, or wild-type hsp27 (Hsp27) adenoviral constructs, 48 h prior to the simulated ischemia treatment. Data are a mean of four independent experiments. $*p < 0.01$ vs. control.

myocytes against ischemia-induced cellular damage, we performed a second series of experiments where cardiomyocytes infected with the wild-type hsp27 were treated with the p38 MAP kinase inhibitor. Figure 5 summarizes the results obtained in four independent experiments. These results show that protection of cardiomyocytes by the adenoviral-mediated expression of the hsp27 is not affected by the inhibition of phosphorylation by the p38 MAP kinase inhibitor, SB203580. The level of protection achieved by cardiomyocytes infected with the wild-type hsp27 in the absence or presence of the inhibitor was almost identical. Note also that the level of protection achieved by the nonphosphorylatable mutant Triple A was comparable to that of the wthsp27 in comparison to cardiomyocytes infected with the control adenoviral construct in the presence or absence of inhibitor.

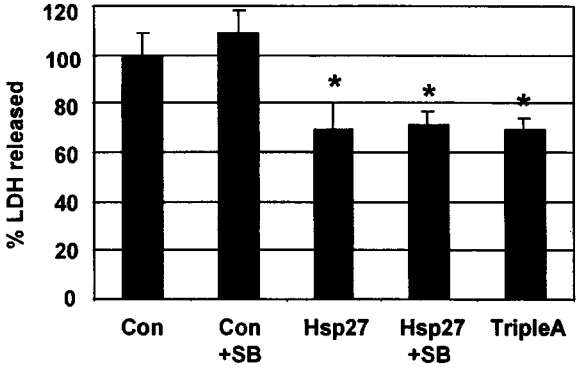

FIG. 5. Effects of p38 MAP kinase inhibition on enzyme release induced by simulated ischemia. Cardiomyocytes infected with the control (Con) and wild-type hsp27 adenoviral constructs were either left untreated or treated with the inhibitor SB203580 as described in Materials and Methods. The effect on enzyme release following simulated ischemia by the presence of the mutant Triple A was also included in this series of experiments. Data are a mean of four independent experiments. $*p < 0.05$ vs. control.

DISCUSSION

The hsp27 has been shown to be protective against a variety of noxious stresses including hyperthermia, osmotic stress, mitogens, and several cytotoxic agents. Hsp27s association with cytoskeletal structures and potential stabilization of these structures is believed to be the mechanism of how the hsp27 contributes to the cell's increased tolerance to stress. For example, the hsp27 has been shown to stabilize the actin filaments in smooth muscle cells (8,13), whereas the increased expression of the hsp27 in Chinese hamster lung cells confers resistance to F-actin fragmentation induced by H_2O_2 and menadione (4). Our recent results have shown that increased expression of the hsp27 in rat adult cardiomyocytes renders these cells resistant to ischemic damage (10).

The hsp27 is heavily phosphorylated during noxious stress to the cell. The kinases involved in phosphorylating the hsp27 are the mitogen-activated protein kinase-activated protein 2/3 (MAPKAP 2/3) and the p38-regulated/activated protein kinase (PRAK) (15). Both of these kinases are phosphorylated or activated by the p38 mitogen-activated protein kinase (p38 MAP kinase).

Recent studies have implicated that the activation of the p38 MAP kinase plays a key role during ischemic preconditioning. It is thought that the kinases activated by p38 MAP kinase, such as MAPKAP kinase 2/3, may be responsible for phosphorylating important substrates that are crucial for the cardioprotection observed during preconditioning (18). One of the candidate proteins activated by MAPKAP kinase 2/3 and important for cardioprotection is the heat shock protein, hsp27 (2,14). We were, therefore, interested in investigating the potential role that phosphorylation may play in activating the protective function of the hsp27 in cardiomyocytes. To achieve this goal we have used a recombinant adenoviral vector system.

The recombinant adenoviral vector system has the advantage of allowing us to achieve high levels of expression of the transgene delivered in primary cultures of rat adult cardiomyocytes (Fig. 1). We have now constructed adenoviral vectors that contain the unphosphorylatable mutants of the human hsp27 in which serines 15, 78, and 82 were replaced by alanines or glycines. Together with our adenoviral construct that contains the wild-type hsp27, we have used these constructs to investigate the influence of phosphorylation status and oligomeric structure on the ability of the hsp27 to protect cardiomyocytes

against ischemic damage. In addition, we have used the p38 MAP kinase-specific inhibitor, SB203580, in order to block activation of MAPKAP kinase 2/3 or PRAK and thus phosphorylation of the hsp27.

In the absence of phosphorylation either due to the lack of phosphorylatable serines or the action of the p38 MAP kinase inhibitor, the hsp27 tends to form larger oligomeric structures than when fully phosphorylated (Fig. 3). This finding is in agreement with previous studies that have also shown the same effect of phosphorylation on the oligomeric structure of the human hsp27 (11) and the rodent hsp25 (16).

We found that the lack of phosphorylation in the unphosphorylatable mutants of hsp27 (Triple A and Triple G) has no effect on the observed protective function of the hsp27 against ischemic-induced injury (Fig. 4). Both unphosphorylatable mutants are able to render the rat adult cardiomyocytes tolerant to simulated ischemia to the same level as the wild-type hsp27. In addition, inhibition of the phosphorylation of the wild-type hsp27 by using the p38 MAP kinase specific (SB203580) inhibitor did not diminish the protective ability of hsp27 against ischemic-induced damage (Fig. 5). Similar results have recently been obtained for the rodent hsp25 in protecting cells against TNF-α- and H_2O_2-induced cell death (16). These investigators found that a rodent hsp25 mutant with serines changed into alanines or the wild-type hsp25 in the presence of the p38 MAP kinase (SB203580) inhibitor forms large oligomeric forms (> 300 kDa). Nonetheless, these large oligomeric complexes of nonphosphorylated hsp25 are able to protect murine L929 fibrosarcoma cells against cell death induced by either H_2O_2 or TNF-α.

In summary, our present results demonstrate that the lack of phosphorylation of the human hsp27 that leads to a change in its oligomeric structure does not affect its ability to protect rat adult cardiomyocytes against ischemia-induced injury. These results make it improbable that the protective effect observed during ischemic preconditioning is due to increased phosphorylation of hsp27 by the ischemia-activated p38 MAP kinase cascade, because hsp27 phosphorylation status does not change its ability to protect the cardiomyocyte against ischemic injury.

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