Further study on the role of HSP70 on Ca\(^{2+}\) homeostasis in rat ventricular myocytes subjected to simulated ischemia

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Liu, Jing, Kenneth W. L. Kam, Gudrun H. Borchert, Gennadi M. Kravtsov, Heather J. Ballard, and Tak Ming Wong. Further study on the role of HSP70 on Ca\(^{2+}\) homeostasis in rat ventricular myocytes subjected to simulated ischemia. Am J Physiol Cell Physiol 290: C583–C591, 2006. First published October 5, 2005; doi:10.1152/ajpcell.00145.2005.—We hypothesized that activation of heat shock protein 70 (HSP70) by preconditioning, which is known to confer delayed cardioprotection, attenuates the impaired handling of Ca\(^{2+}\) at multiple sites. To test the hypothesis, we determined the following: 1) the Ca\(^{2+}\) transients induced by electrical stimulation and caffeine, which provide the overall picture of Ca\(^{2+}\) homeostasis; 2) expression of RyR, SERCA, and NCX; and 3) Ca\(^{2+}\) fluxes via NCX by the use of 45Ca\(^{2+}\) in the rat ventricular myocyte. We found that UP increased the activity of RyR, SERCA, and NCX and the expression of RyR and SERCA. These effects led to increases in the release of Ca\(^{2+}\) from the sarcoplasmic reticulum via RyR and in the removal of Ca\(^{2+}\) from the cytoplasm by reuptake of Ca\(^{2+}\) to the SR via SERCA and by extrusion of Ca\(^{2+}\) out of the cell via NCX. UP also reduced mitochondrial Ca\(^{2+}\) accumulation. All of the effects of UP were either abolished or significantly attenuated by blockade of HSP70 synthesis with a selective antisense oligonucleotide. The results are evidence that activation of HSP70 by preconditioning improves the ischemia-impaired Ca\(^{2+}\) homeostasis at multiple sites in the heart, which may be responsible, at least partly, for attenuated Ca\(^{2+}\) overload, improved recovery in contractile function, and cardioprotection.

intracellular Ca\(^{2+}\), k-opioid receptor; Na\(^{+}/Ca\(^{2+}\) exchanger; ryanodine receptor; sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase

HEAT SHOCK PROTEIN 70 (HSP70), the inducible form of a 70-kDa stress protein, mediates delayed cardioprotection resulting from ischemic preconditioning or k-opioid receptor (k-OR) stimulation with the k-OR agonist, U50488H (UP) (13, 29). More important, this cardioprotection is accompanied by attenuation of the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) overload (13), which is believed to be a precipitating cause of myocardial injury upon ischemia and reperfusion. The mechanisms responsible for Ca\(^{2+}\) handling leading to alterations in [Ca\(^{2+}\)]\(_i\) are not known.

It is well known that Ca\(^{2+}\) homeostasis within the myocyte is exquisitely controlled by regulatory proteins in sarcosomal and sarcoplasmic reticulum (SR) membranes. Ca\(^{2+}\) enters the cell via the L-type Ca\(^{2+}\) channel when the sarcosomal membrane is depolarized. Entry of Ca\(^{2+}\) triggers further release of Ca\(^{2+}\) via the ryanodine receptor (RyR) of the SR, leading to a sudden increase in [Ca\(^{2+}\)]\(_i\), known as a [Ca\(^{2+}\)]\(_i\) transient (8). The elevated [Ca\(^{2+}\)]\(_i\), which leads to contraction, is removed primarily by the SR by the Ca\(^{2+}\)-ATPase (SERCA) and out of the cell by the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX). Some Ca\(^{2+}\) released from the SR is transferred to mitochondria, another reservoir of Ca\(^{2+}\), via a coupling of RyR and closely apposed mitochondrial membrane (9). Thus both [Ca\(^{2+}\)]\(_i\) and mitochondrial Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{mit}\)) change in parallel in response to alterations in Ca\(^{2+}\) handling by the SR and pathological situations. It has been shown that upon myocardial ischemia and reperfusion, overload of both [Ca\(^{2+}\)]\(_i\) and [Ca\(^{2+}\)]\(_{mit}\) occur and attenuation of [Ca\(^{2+}\)]\(_{mit}\), but not [Ca\(^{2+}\)]\(_i\), overload is responsible for improved recovery in contractile functions (14).

We therefore hypothesized that activation of HSP70 by preconditioning, which confers delayed cardioprotection, may restore [Ca\(^{2+}\)]\(_i\) homeostasis by restoring the activities taking place in sarcosomal and SR membranes. To test this hypothesis, we investigated the Ca\(^{2+}\) handling in isolated ventricular myocytes preconditioned with U50488H, followed by blockade of HSP70 synthesis with a selective antisense oligonucleotide and subsequently subjected to simulated ischemia. We focused on Ca\(^{2+}\) release from the SR and removal of Ca\(^{2+}\) from the cytoplasm back to the SR and out of the myocyte via the NCX. We used three approaches, namely, measurement of the Ca\(^{2+}\) transients induced by electrical stimulation or caffeine, which provide information on overall dynamic changes in Ca\(^{2+}\) homeostasis; expression of proteins that handle Ca\(^{2+}\) fluxes across sarcosomal and SR membranes; and actual Ca\(^{2+}\) fluxes across the NCX. We also measured the changes in [Ca\(^{2+}\)]\(_{mit}\). Results showed that activation of HSP70 by preconditioning attenuated the impaired Ca\(^{2+}\) handling at multiple sites, namely, RyR, SERCA, and NCX, which may be responsible, at least partly, for restoring Ca\(^{2+}\) homeostasis to normal.

MATERIALS AND METHODS

Isolation of ventricular myocytes and experimental protocol. Ventricular myocytes were isolated from the hearts of male Sprague-Dawley rats (200–250 g body wt) using a collagenase method described previously (27). After isolation, the rats were allowed to stabilize for at least 30 min before experiments. The procedure described in our previous study (13) was adopted. As shown in Fig. 1, the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
myocytes were first subjected to 30-min pretreatment with a selective k-OR agonist, 30 µM U50488H or normal Krebs solution (vehicle pretreatment; VP) for 30 min. After incubation in culture medium for 20 h, with or without the presence of AS or sense oligonucleotides (10 µM) of HSP70, myocytes were then subjected to severe metabolic inhibition and anoxia (MeI/A) for 10 min by incubation in glucose-free Krebs solution containing 10 mM 2-deoxy-D-glucose (2-DOG), an inhibitor of glycolysis, and 10 mM sodium dithionite (Na2S2O4), an oxygen scavenger (10). Finally, the myocytes were transferred back to normal Krebs solution for 10-min reperfusion. A 20-h incubation period was adopted based on our previous studies, in which it was shown that delayed cardioprotection was most marked at 20 h after preconditioning (27), when there was also increased expression of HSP70 (29).

The study protocol was approved by the Committee on the Use of Experimental Animals for Teaching and Research of The University of Hong Kong.

Measurement of [Ca2+]i transients in the single myocyte. [Ca2+]i transients were measured using a spectrofluorometric method with fura-2 AM as the Ca2+ indicator. Ventricular myocytes were incubated with 5 µM fura-2 AM for 30 min. Fluorescent signals obtained at 340-nm and 380-nm excitation wavelengths were recorded and stored in the computer for data processing and analysis. To measure electrically induced [Ca2+]i transients (E[Ca2+]i), myocytes were electrically stimulated at 0.2 Hz, whereas the caffeine-induced [Ca2+]i transients (C[Ca2+]i) were recorded by applying 10 mM caffeine directly to the ventricular myocyte. The amplitude of E[Ca2+]i, and C[Ca2+]i, was determined as the difference between the resting and the peak [Ca2+]i levels; the time for 50% decay of the transients (t50) was used to quantify the decay of both transients.

Plasma membrane purification and NCX assay. By following the procedures described previously (21) with some modifications, we sonicated cells using three 15-s bursts in ice-cold lysis buffer (0.6 M sucrose, 10 mM imidazole-HCl, pH 7.0). The homogenate was centrifuged at 1,000 g for 5 min. The first supernatant was then centrifuged at 12,000 g for 30 min. The 12,000 g supernatant was diluted in the solution (pH 7.4) containing 160 mM NaCl, 20 mM HEPES-Tris, 0.25 M sucrose, and 1 mg/ml leupeptin. The solution was kept at 4°C for 30 min. Afterward, 5 µl of the vesicle suspension was placed on the side of a polystyrene Eppendorf tube containing 95 µl of K+ reaction medium: 160 mM KCl, 0.1 mM CaCl2, 0.2 mM EGTA, 2 µM valinomycin, and 20 mM HEPES-Tris (pH 7.4). The free [Ca2+]i in the medium was 50 µM as derived from calculation with the computer program Eq-Cal for Windows (Biosoft, 1996) for Ca2+-EGTA buffer. The Ca2+ influx was stopped by diluting the reaction mixture after 2, 5, or 10 s with 5 ml of ice-cold incubation medium (160 mM KCl, 2 mM LaCl3) to return K+ to 140 mM. Na+ dependent-specific Ca2+ uptake was defined as the total Ca2+ uptake minus unspecific Ca2+ uptake in solution B, which contained 0.2 mM EGTA, 0.1 mM CaCl2, 10 µM 45CaCl2, and 2 µM valinomycin, i.e., a solution in which no Na+ gradient existed across the membrane. All samples were filtered under vacuum, and filters (GF/F; Whatman) were washed twice with 6 ml of 140 mM KCl and 0.1 mM LaCl3. The protein content of each sample was determined using a kit obtained from Bio-Rad and BSA as a standard.

PAGE and Western blot analysis. To detect the expression of RyR and SERCA, SR vesicles were obtained using a method adopted previously (21). Briefly, cells were sonicated on ice in an extraction medium containing (in mM) 15 Tris-HCl, 10 NaHCO3, 5 NaN3, 250 sucrose, and 1 EDTA (pH 7.0). The homogenate was centrifuged for
5 min at 3,000 g to remove cellular debris. The supernatant was further centrifuged at 48,000 g for 75 min. Then the pellet was suspended in a mixture of 0.6 mM KCl and 20 mM Tris/HCl (pH 7.0) and centrifuged at 48,000 g for 60 min. The final pellet was rehomogenized in 250 mM sucrose and 40 mM imidazole-HCl and stored at 70°C. All solutions contained three protease inhibitors: 1 mg/ml aprotinin, 1 mM PMSF, and 1 mg/ml leupeptin.

For the measurement of NCX, purification of plasma membrane vesicles was carried out as described above. The pellet representing the sarcolemma-enriched fraction was dissolved in the lysis buffer (0.6 M sucrose and 10 mM imidazole-HCl, pH 7.0) and stored at −70°C. All solutions contained three protease inhibitors: 1 mg/ml aprotinin, 1 mM PMSF, and 1 mg/ml leupeptin.

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Fig. 2. Effects of UP on the amplitude (A), time to peak (B), and time to 50% decay (C) of electrically induced intracellular [Ca2+] transients (EiCa2+) in single ventricular myocyte subjected to 10-min MeI/A and 10-min reperfusion upon blockade of HSP70 with selective AS (left) or AS2 (right) oligonucleotides. Measurements were analyzed at the ends of MeI/A treatment and reperfusion. Values are means ± SE; n = 8–12 total cells obtained from 5–6 rats. *P < 0.05, **P < 0.01 vs. VP group; #P < 0.05, ##P < 0.01 vs. corresponding groups without AS or AS2 of HSP70.

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Time-lapse recording of [Ca2+]m. To monitor [Ca2+]m, single myocytes loaded with rhod-2 fluorescent probe were scanned with a laser-scanning confocal microscope (Fluoview FV300; Olympus). After 20 h in culture, the cells were incubated with 10 μM rhod-2 AM, which was reduced by sodium borohydride in advance for 2.5 h in 37°C incubator as described previously with some modifications (18). This method facilitates the selective accumulation of rhod-2 in the mitochondrial matrix.

The Ca2+-sensitive fluorescent indicator rhod-2 was excited at 543 nm, and the time-lapse confocal images (1,024 × 1,024 pixels) were sampled every 1 min. Experiments were analyzed with the use of Fluoview software (version 4.2; Olympus). [Ca2+]m was represented by the measured rhod-2 fluorescence intensity as a percentage of the intensity at the beginning of the experiment.
Blockade of synthesis of HSP70 with a selective AS oligonucleotides. The phosphorothioate AS (TGT TTT CTT GGC CAT) and sense oligonucleotides (ATG GCC AAG AAA ACA) to HSP70 were synthesized from sequences complementary to the initiation codon and four downstream codons of rat HSP70 mRNA (Life Tech). HSP70 synthesis has been demonstrated to be blocked after incubation with this AS at 10 μM in the studies of Kim et al. (11) and by our laboratory (13, 29). However, there were some limitations regarding this AS sequence, such as the relatively low melting temperature (36°C, Oligonucleotide Properties Calculator) and possible unspecific matches. To confirm that HSP70 was indeed targeted, we used another phosphorothioate AS oligonucleotide (5'-CAC CTT GCC GTG CTG GAA-3'; AS2) with much higher melting temperature (53°C), which may lower the likelihood of unspecific matches (4, 5). The S oligonucleotide (5'-TTC CAG CAC GGC AAG GTG-3'; S2) was used as the control. We obtained the same results as we did with AS of Kim et al. (11, 13, 29) in HSP70 expression and E[Ca\(^{2+}\)]i responses (Figs. 1A and 2). Moreover, neither of the two AS oligonucleotides abolished the enhanced expression of HSC70 (the constitutive form of a 70-kDa stress protein), confirming their selectivity to HSP70 (Fig. 1B).

Drugs and chemicals. U50488H, 2-DOG, Na\(_2\)S\(_2\)O\(_4\), and fura-2 AM were purchased from Sigma. \(^{45}\)CaCl\(_2\) was purchased from Amersham. Drug concentrations of U50488H, 2-DOG, and Na\(_2\)S\(_2\)O\(_4\) used were based on previous studies (27, 29). Rhod-2 AM was purchased from Molecular Probes, goat anti-SERCA2 antibody (sc-8094) was from Santa Cruz Biotechnology, mouse anti-RyR antibody (MA3-925) was from Affinity BioReagents, mouse anti-NCX1 antibody (ab6495), mouse anti-GAPDH antibody (ab9482) was from Abcam, and mouse anti-HSP70 antibody (SPA-810) and rat anti-HSC70 antibody (SPA-815) were purchased from StressGen.

Statistical analysis. All data are expressed as means ± SE. One-way ANOVA, followed by Newman-Keuls multiple-comparison tests, were used to assess differences between the mean values within the same study. A difference of \(P < 0.05\) was considered significant.

RESULTS

Effects of UP on expression of HSP70 in ventricular myocytes subjected to Mel/A. As shown in Fig. 1A, UP increased the expression of HSP70, an effect attenuated by the AS\(_2\). This finding is in agreement with our previous observation regarding AS, which also attenuated the increased expression of HSP70 induced by UP (13).
UP also enhanced the expression of HSC70, which is in agreement with our previous findings (29). Neither AS nor AS2 of HSP70 had any effect on HSC70 expression (Fig. 1B).

**Effects of UP on amplitude, time to peak, and t50 of E[Ca2+]i in single ventricular myocyte subjected to MeI/A upon blockade of HSP70.** The amplitude of E[Ca2+]i, representing the release of Ca2+ during excitation-contraction coupling and shown to be directly correlated with contraction (28), was markedly reduced after MeI/A and reperfusion. In agreement with our previous study, the reductions were restored by pretreatment with 30 μM U50488H, which was shown previously to confer delayed cardioprotection and enhance the expression of HSP70 (13, 29). Administration of either AS or AS2 to HSP70 during the incubation period abolished the effect of UP (Fig. 2A) as was also shown in the previous study (13).

Time to peak of E[Ca2+]i, represents the rate of Ca2+ release from the SR, mainly via RyR. As shown in Fig. 2B, after 10 min of MeI/A, the time to peak of E[Ca2+]i was delayed by 35% compared with the time taken before treatment. UP significantly shortened the prolongation of the time to peak to 19%, indicating improved function of the RyR with preconditioning. However, the ameliorating effect of UP was completely abolished by either AS or AS2 to HSP70. On the other hand, coinubcation with S or S2 oligonucleotides had a similar effect on UP. Similarly, after 10 min of reperfusion, the time to peak of E[Ca2+]i in UP was also significantly less than that in VP, and this effect was reversed by either AS or AS2 to HSP70.

The decay of E[Ca2+]i is mainly determined by Ca2+ uptake via SERCA, which is responsible for the removal of ~90% Ca2+ from the cytoplasm (2). We therefore measured the value of t50 as an indicator of SERCA activity. As shown in Fig. 2C, after 10-min MeI/A and 10-min reperfusion, the t50 of E[Ca2+]i was increased to 137% and 123% of the control, respectively. However, UP restored the increased t50 to 109% and 104%, respectively. UP lost its attenuating effect in the presence of either AS or AS2 of HSP70. AS of HSP70 had no effect on any of these parameters in the AS group without preconditioning.

**Effects of UP on recovery of E[Ca2+]i after caffeine administration in single ventricular myocyte subjected to MeI/A upon blockade of HSP70.** To further investigate the uptake of Ca2+ by SERCA, the gradual recovery of E[Ca2+]i after caffeine administration was also measured (10, 24). As shown in the typical traces in Fig. 3A, the amplitude of E[Ca2+]i after caffeine administration was also decreased (10, 24). However, the decay of E[Ca2+]i immediately after the caffeine (10 μM) application, after which it gradually recovered. Recovery in UP was quicker than that in VP after both MeI/A and reperfusion. Group results showed that the 50th second (Fig. 3B), the amplitude of E[Ca2+]i in UP was 80% of its control, whereas the corresponding value in VP was 64% after 10 min of MeI/A.

After 10-min reperfusion, the values were 84% and 68% in the UP and VP groups, respectively. The differences were significant (P < 0.01 and P < 0.05, respectively). AS of HSP70 reversed the effect of UP to the level of the VP group.

**Effects of UP on the amplitude and decay of C[Ca2+]i in single ventricular myocyte subjected to MeI/A upon blockade of HSP70.** The amplitude of C[Ca2+]i, an indication of Ca2+ content in SR, was also significantly decreased after MeI/A and reperfusion. UP attenuated this decrease, but the effect of UP was abolished in the presence of AS to HSP70 (Figs. 3A and 4A).

Because caffeine keeps the RyR open during its application, the decay of C[Ca2+]i, depends only on Ca2+ extrusion, which occurs mainly through NCX (22). Therefore the t50 of C[Ca2+]i, decay, which represents the activity of NCX, was determined. The decay of C[Ca2+]i after MeI/A and reperfusion was significantly prolonged in UP, and the effect was abolished by AS of HSP70 (Fig. 3A and 4B). AS of HSP70 had no effect on these two parameters as shown in AS group without preconditioning.

**Effects of UP on expression of RyR, SERCA, and NCX in ventricular myocytes subjected to MeI/A upon blockade of HSP70.** The expression of both RyR (Fig. 5A) and SERCA (Fig. 5B) in UP was enhanced significantly compared with that in the VP group after MeI/A and reperfusion. Moreover, after blocking HSP70 synthesis with AS, the expression of both proteins was returned to the same level as that in the VP group (Fig. 5, A and B). In contrast, there was no difference in NCX expression between groups (Fig. 5C), indicating that this was not affected by UP.
Effects of UP on NCX activity in ventricular myocytes subjected to Mel/A upon blockade of HSP70. In view of the discrepancy in NCX activity determined on the basis of the decay of C[Ca^{2+}], as well as in its expression as determined using Western blot analysis, we further determined the NCX activity by measuring the ^{45}Ca^{2+} fluxes via NCX. The NCX activity in UP was nearly twice that in control group after Mel/A and reperfusion. However, when the synthesis of HSP70 was inhibited, the NCX activity was partially restored toward that in the VP group (Fig. 6).

**DISCUSSION**

Our previous study showed that preconditioning with simulated ischemia or α-OR stimulation, which confers delayed...
HSP70 attenuates the [Ca\textsuperscript{2+}] overload (6), which is believed to precipitate cardiac injury (23). Blockade of the synthesis of HSP70 abolishes the attenuating effects on cardiac injury and [Ca\textsuperscript{2+}] overload, indicating an important role of HSP70 in cardioprotection and Ca\textsuperscript{2+} homeostasis (13). We hypothesized that activation of HSP70 restored Ca\textsuperscript{2+} homeostasis by restoring the actions at multiple sites involved in Ca\textsuperscript{2+} handling. We therefore further delineated the relationship between HSP70 and Ca\textsuperscript{2+} handling, with particular attention to RyR and SERCA in SR and NCX in sarclemma. We determined the C[Ca\textsuperscript{2+}], and E[Ca\textsuperscript{2+}], which provide information about overall dynamic changes in Ca\textsuperscript{2+} handling; expression of Ca\textsuperscript{2+} regulatory proteins, RyR, SERCA, and NCX; and Ca\textsuperscript{2+} fluxes across NCX. We found that preconditioning with a \kappa-OR agonist, U50488H, which was shown previously to confer delayed cardioprotection and attenuate [Ca\textsuperscript{2+}] overload in the rat ventricular myocyte (13), increased the expression and activities of RyR and SERCA as well as the activity of NCX in the ventricular myocyte. All of these changes were abolished or attenuated after blockade of the synthesis of HSP70 with a selective AS, indicating that these changes, which restore the Ca\textsuperscript{2+} homeostasis impaired by simulated ischemia, are due in part to activation of HSP70. The results not only confirm our previous finding that activation of HSP70 attenuates the [Ca\textsuperscript{2+}] overload induced by simulated ischemia but also, more importantly, provide information regarding the effects of HSP70 on Ca\textsuperscript{2+} handling at multiple sites, namely, RyR, SERCA, and NCX, which is responsible in part for the attenuation of Ca\textsuperscript{2+} overload, improved recovery in contractile functions, and cardioprotection.

Like [Ca\textsuperscript{2+}], the [Ca\textsuperscript{2+}]\textsubscript{m} in the ventricular myocyte was reduced after UP and the effect was also abolished by blockade of HSP70 synthesis. The similar responses in [Ca\textsuperscript{2+}], and [Ca\textsuperscript{2+}]\textsubscript{m} are expected as Ca\textsuperscript{2+} from SR is released to the cytoplasm via RyR as well as transferred to mitochondria via local coupling between closely apposed regions of the SR and mitochondria (9). Because attenuation in overload of [Ca\textsuperscript{2+}]\textsubscript{m}, but not [Ca\textsuperscript{2+}]\textsubscript{im}, was shown to be responsible for posts ischemic recovery in contractile function (14), the cardioprotective effect of UP against simulated ischemia is most likely due to attenuated [Ca\textsuperscript{2+}]\textsubscript{m} overload, and the loss of protection after blockade of HSP70 synthesis is due to failure to attenuate [Ca\textsuperscript{2+}]\textsubscript{im} overload.

In the present study, we found that the amplitude and the time to peak of the E[Ca\textsuperscript{2+}], were increased in the UP group, which was accompanied by an increased expression of RyR. Furthermore, the amplitude of the C[Ca\textsuperscript{2+}], was also increased, reflecting an increased Ca\textsuperscript{2+} content in the SR. These observations indicate that the release of Ca\textsuperscript{2+} from the SR was faster and greater, which is most likely due to increased activity of RyR and increased availability of Ca\textsuperscript{2+} from the SR.

We also found in the present study that the decay of the E[Ca\textsuperscript{2+}], was shorter after preconditioning with a \kappa-OR agonist and that the recovery in amplitude of E[Ca\textsuperscript{2+}], after depletion of Ca\textsuperscript{2+} from the SR by caffeine was faster. These observations indicate a faster uptake of Ca\textsuperscript{2+} by the SR after preconditioning. This is most likely due to increased SERCA activity as reflected by the increased expression of the protein.

The NCX activity was also increased after preconditioning as indicated by a shorter decay time of the C[Ca\textsuperscript{2+}], and a
greater NCX activity determined in the ⁴⁵Ca²⁺ flux study. Interestingly, the increased activity was not accompanied by an increased expression of NCX, suggesting that the increased activity is not secondary to increased expression of the protein.

The present study has shown that preconditioning with k-OR stimulation increased the release of Ca²⁺ from SR via the RyR. It also sped up the rate of removal of Ca²⁺ from cytoplasm by increasing uptake via SERCA and removal via NCX. In a previous study (10), we also observed similar changes in Ca²⁺ handling in the rat ventricular myocyte immediately after the addition of UP, which confers early cardioprotection. The increased release of Ca²⁺ from the SR, as reflected by an increased amplitude of E[Ca²⁺], is responsible for improved recovery in contractile function. The efficient removal of Ca²⁺ from cytoplasm prevents [Ca²⁺], overload, thus protecting the heart.

In normal conditions, HSP70 family members function as molecular chaperones by assisting in folding and assembly of newly synthesized proteins and by transporting these proteins to various organelles. Upon stress such as heat stress, which induces cell death, the inducible form HSP70 is activated and protects cells from apoptosis (16, 17). There is now evidence that CHOP-induced apoptosis is mediated by translocation of Bax, a proapoptotic member of the Bcl family, from the cytosol to the mitochondria (7). Pairing with its cochaperone, DnaJ, which is known to regulate its function (12), HSP70 interacts with Bax and prevents its translocation to mitochondria, thus inhibiting apoptosis (7). It has also been shown that in HeLa cells, induction of Bax increases endoplasmic reticulum Ca²⁺ loading and [Ca²⁺]ₘ level (3), an upstream signal for cytochrome c release in some forms of apoptosis (19, 20). In our previous (13) and present studies, we found that [Ca²⁺]ₘ overload during simulated ischemia and reperfusion was significantly attenuated by UP, which increased HSP70 expression. In the present study, we also found that the [Ca²⁺]ₘ overload during reperfusion was significantly attenuated by UP. Blockade of synthesis of the protein with selective AS abolished the effects of UP, indicating that HSP70 is responsible for the cytosolic and mitochondrial responses. Therefore, interaction of HSP70 with Bax may be responsible for the actions of UP on Ca²⁺ homeostasis. In the present study, we showed that UP reversed the effects of simulated ischemia on expression and activity of RyR and SERCA, suggesting that Bax may affect the RyR and SERCA. In the present study, we also observed that UP affected the NCX activity. There is no evidence, however, suggesting a potential link with Bax. Further study is needed to confirm that Bax affects cytosolic and mitochondrial Ca²⁺ by affecting RyR and SERCA and to delineate the relationship between Bax and NCX.

Mitochondrial HSP70, a member of HSP70 family located in mitochondria, is a constitutive HSP with a 75-kDa molecular mass, whereas HSP70 is an inducible HSP of 70 kDa, and peptide mapping indicates that they are unique polypeptides (15). Basic Local Alignment Search Tool analysis does not show that the AS oligonucleotides used in our study match this protein. Therefore, it is unlikely that the AS oligonucleotides used in our study target mitochondrial HSP70. On the other hand, the mitochondrial HSP70 has been shown to play an important role in translocating cytosolic precursor proteins across the two mitochondrial membranes (26). It has been shown that HSP70 interacts with Bax and prevents its translocation to mitochondria. Whether mitochondrial HSP70 is involved in this process warrants further study.

Vitadello and co-workers (25) found that selective increase in glucose-regulated protein (GRP94), a member of the HSP90 family, protects cardiomyocytes against injury induced by ischemia or [Ca²⁺], overload counteracting [Ca²⁺], elevations. The finding indicates that in addition to HSP70, other heat shock proteins are also involved in Ca²⁺ homeostasis. More interesting and more important is that in human neuroblastoma cells, A-23187 (a Ca²⁺ ionophore) induces cell injury and increases the expression of GRP94, and overexpression of the protein suppresses A-23187-induced injury and stabilizes Ca²⁺ homeostasis (1). This study suggests a causal relationship between attenuation of [Ca²⁺], overload and cardiac protection upon increased expression/activation of heat shock proteins.

In conclusion, the present study has shown that activation of HSP70, which confers delayed cardioprotection, increased the release of Ca²⁺ via RyR and sped up the uptake of Ca²⁺ via SERCA and removal of Ca²⁺ via NCX in the rat cardiomyocytes. These actions restored Ca²⁺ homeostasis and attenuated [Ca²⁺], overload. The study has also provided evidence for the first time that activation of HSP70 attenuated [Ca²⁺]ₘ overload.

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in rat ventricular myocytes subjected to metabolic inhibition and anoxia. 


