Stressful preconditioning and HSP70 overexpression attenuate proteotoxicity of cellular ATP depletion

ALEXANDER E. KABAKOV,[1] KARINA R. BUDAGOVA,[1]
DAVID S. LATCHMAN,[2] AND HARM H. KAMPINGA[3]
[1]*Medical Radiology Research Center, Obninsk 249020, Russia;* [2]*Institute of Child Health, University College London, London WC1N 1EH, United Kingdom; and* [3]*Department of Radiation and Cell Stress Biology, University of Groningen, 9713 AV Groningen, The Netherlands*

Kabakov, Alexander E., Karina R. Budagova, David S. Latchman, and Harm H. Kampinga. Stressful preconditioning and HSP70 overexpression attenuate proteotoxicity of cellular ATP depletion. *Am J Physiol Cell Physiol* 283: C521–C534, 2002. First published March 27, 2002; 10.1152/ ajpcell.00503.2001.—Rat H9c2 myoblasts were preconditioned by heat or metabolic stress followed by recovery under normal conditions. Cells were then subjected to severe ATP depletion, and stress-associated proteotoxicity was assessed on *1*) the increase in a Triton X-100-insoluble component of total cellular protein and *2*) the rate of inactivation and insolubilization of transfected luciferase with cytoplasmic or nuclear localization. Both heat and metabolic preconditioning elevated the intracellular heat shock protein 70 (HSP70) level and reduced cell death after sustained ATP depletion without affecting the rate and extent of ATP decrease. Each preconditioning attenuated the stress-induced insolubility among total cellular protein as well as the inactivation and insolubilization of cytoplasmic and nuclear luciferase. Transient overexpression of human HSP70 in cells also attenuated both the cytotoxic and proteotoxic effects of ATP depletion. Quercetin, a blocker of stress-responsive HSP expression, abolished the effects of stressful preconditioning but did not influence the effects of overexpressed HSP70. Analyses of the cellular fractions revealed that both the stress-preconditioned and HSP70-overexpressing cells retain the soluble pool of HSP70 longer during ATP depletion. Larger amounts of other proteins coimmunoprecipitated with excess HSP70 compared with control cells deprived of ATP. This is the first demonstration of positive correlation between chaperone activity within cells and their viability in the context of ischemia-like stress.

chaperone; protein aggregation; metabolic stress; ischemic tolerance

AT THE CELLULAR LEVEL, failing energy metabolism and ATP depletion are the earliest cell-damaging factors of ischemic insults. In vivo, severe depletion of ATP is a proteotoxic stress that leads to dysfunction, destabilization, and aggregation of many cellular proteins, including enzymes, ion pumps, and constituents of cytoskeletal and contractile structures (1, 9, 17, 27). Sustained lack of ATP is obviously lethal for the cell.

On the contrary, a transient (reversible) drop in cellular ATP can confer tolerance to the next energy-depriving exposure, with heat shock proteins (HSPs) being involved in such an adaptive response (reviewed in Ref. 17). The HSP-involving cellular response appears to contribute to delayed ischemic tolerance (the second window of protection) found after heat or ischemic preconditioning in the myocardium. Like high temperature, cellular ATP depletion activates the heat shock transcription factor 1 (HSF1) that afterward induces HSP expression in the recovering cells (4, 36). Most HSPs are molecular chaperones stabilizing protein molecules under heat shock conditions in vitro and in vivo, and the same chaperone activity may protect HSP-enriched cells in the case of other proteotoxic stresses, e.g., ischemia or ATP-depleting treatments with inhibitors of energy metabolism. Despite the numerous reports describing HSP70-mediated cytoprotection against ischemic injury (for a review, see Refs. 5, 17, and 19), the precise mechanism of this phenomenon remains to be determined. Previously, suppression of ATP depletion-provoked protein insolubilization was found in heat-preconditioned ascites tumor cells (7, 16). Although it was speculated in those reports that such an effect of the preconditioning is due to stress-inducible HSPs (e.g., HSP70), no serious evidence was provided. Likewise, it has never been studied whether excess HSP70 in cells attenuates the proteotoxic effects of ischemia-associated ATP depletion, i.e., protects intracellular proteins from denaturation and aggregation during the stress. So far, it is also unknown how the cell viability under ischemia-like (or energy-depleting) conditions correlates with the in vivo chaperone activity of intracellular HSP70.

These two latter issues were addressed in the present study. We hypothesized that excess HSP70 in stress-preconditioned or HSP70-overexpressing cells can preserve cellular proteins from the ATP depletion-induced aggregation leading to cell death. To examine this hypothesis on an ischemia-relevant model, we used a rat embryonic heart-derived H9c2 line of myo-

Address for reprint requests and other correspondence: A. E. Kabakov, Medical Radiology Research Center, 4 Korolev St., Obninsk 249020, Russia (E-mail: aekabakov@hotmail.com).

blasts that retain some features of cardiac cells and can acquire tolerance to simulated ischemia (23) and thermal or oxidative stress (10, 34) after HSP-inducing heat pretreatments. Besides heat preconditioning, we also employed metabolic preconditioning to cause the transient (reversible) ATP depletion as an alternative HSP-inducing stress that to some extent mimics ischemic preconditioning in vivo. To evaluate contribution of the HSP induction, we treated the stress-preconditioned cells with quercetin, an inhibitor of HSF1 (13, 26). In parallel, to increase intracellular HSP70 by an HSF1-independent method, we overexpressed human inducible HSP70 in H9c2 cells using plasmid- or virus-based vectors. It was previously found that ATP depletion in cells renders many cellular proteins less extractable with a Triton X-100-containing buffer (14, 15). Such insolubilization of cellular proteins is due to their aggregation and correlates with the intensity of cell death under ATP-depleting stress (14, 17). Therefore, the stress-induced increase in the detergent insolubility of cellular proteins was quantified here to assess how the stressful preconditioning and HSP70 overexpression affect the proteotoxic impact within the energy-deprived cells.

In addition to monitoring of the total protein aggregation in the ATP-depleted myoblasts, we explored in them the catalytic activity and the solubility of a reporter enzyme, firefly (*Photinus pyralis*) luciferase, transiently expressed following transfection. This thermolabile enzyme is used as an in situ probe, allowing us to study protein denaturation, aggregation, and refolding in heat- or chemically stressed cells (2, 24, 25, 28, 29). Furthermore, firefly luciferase, when expressed in mammalian fibroblasts, aggregates and loses activity during cellular ATP depletion (27) and, thus, is a sensitive marker of the stress-associated proteotoxicity. We therefore introduced firefly luciferase into H9c2 myoblasts to evaluate effects of the preconditioning and the HSP70 overexpression on the proteotoxicity of ischemia-mimicking ATP depletion. Plasmids expressing recombinant forms of luciferase with either cytoplasmic or nuclear localization (25) were used to separately probe the situation within cytoplasmic and nuclear compartments of the ATP-depleted cells. To elucidate the role of inducible HSP70 alone, H9c2 cells were cotransfected with luciferase and human HSP70 simultaneously as previously employed in heat shock studies on other cell lines (24, 29).

We demonstrate here that pretreatments leading to HSP70 accumulation in the cells can reduce the protein aggregation resulting from cellular ATP loss. This attenuation of the ATP depletion-associated proteotoxicity seems to be HSP70 mediated and correlates with the elevated cell resistance to prolonged energy deprivation. Improved formation of nonaggregable (soluble) complexes between excess HSP70 and some damaged or instable proteins in the cytosol and nucleosol is suggested as a probable cause of the attenuated protein aggregation under ATP depletion in the HSP70-enriched cells. Analogous mechanisms may contribute to the phenomenon of delayed ischemic tolerance aris-

ing in the stress-preconditioned heart when the level of cardiac HSP70 is transiently increased.

MATERIALS AND METHODS

Cells. The embryonic rat heart-derived H9c2 line of myoblasts was obtained from American Type Culture Collection (CRL-146; ATCC, Rockville, MD). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with sodium bicarbonate and 10% fetal bovine serum (GIBCO BRL) in a humidified atmosphere of 5% CO_2 in air at 37°C. The preconfluent myoblast cultures were used for the experiments.

Stressful treatments and recovery. For heat preconditioning, dishes or tubes containing the adherent cells were plunged into a thermostatic water bath at 43°C for 30 min and then returned into a CO_2 incubator at 37°C. For preconditioning by metabolic (ATP-depleting) stress, the cells were exposed to 3 h of incubation at 37°C with glucose-free DMEM containing 3% fetal bovine serum and 20 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and then this medium was removed and the cells were washed twice with normal DMEM and added to the full growth medium. Recovery periods following either priming stress continued for 16–18 h before the challenging ATP depletion. Quercetin was added at a 30 μM concentration to some samples of the cells before the stressful preconditioning (21, 22); this drug was present in the incubation medium during the priming stress and the next 10 h, and then the drug-containing medium was replaced by the usual growth medium. To perform the challenging ATP depletion, we placed the cells in the CCCP-containing medium supplemented with 10 mM 2-deoxyglucose and incubated them at 37°C in a CO_2 incubator (21, 22).

Plasmids and transfection. Plasmid pRSVLL/V encoding cytoplasmic localized firefly luciferase (cyt-luciferase) was kindly provided by Dr. S. Subramani (University of California, San Diego, CA). Construction of plasmid pRSVnlsLL/V encoding firefly luciferase fused to a nuclear localization sequence (nuc-luciferase) has been described previously (25). For in situ control of the transfection efficiency and the compartment-specific localization of the expressed products, plasmid constructs encoding cyt- or nuc-luciferase fused at their COOH termini to enhanced green fluorescent protein (EGFP) were used (constructed by Dr. E. A. A. Nollen, University of Groningen, Groningen, The Netherlands). Distribution of the EGFP-luciferases and the diamidinophenylindole (DAPI)-labeled cell nuclei was viewed on an Opton III fluorescence microscope (Karl Zeiss, Oberkochen, Germany). Plasmid pCMV70 with fragment encoding cDNA for human inducible HSP70 was constructed as previously described (24).

The preconfluent cell cultures growing in six-well plates were transiently transfected with pRSVLL/V or pRSVnlsLL/V to express cyt- or nuc-luciferase, respectively. In a part of the experiments, the cells were cotransfected with one of the EGFP-luciferase-encoding plasmids and pCMV70 to express luciferase and human inducible HSP70 simultaneously (24, 29); the coexpression of both products in the same cells was confirmed by double-label fluorescence analysis on a flow cytometer. The transfection procedure was performed with Opti-MEM (GIBCO BRL) and GenePORTER transfection reagent (Gene Therapy Systems, San Diego, CA) according to the manufacturer's protocol. In all cases, 1 μg of the plasmid DNA was carried in each well; herein the DNA quantity was equalized using pSP64 (Promega). Then, 24 h after transfection, cell culture tubes or 35-mm dishes (Nunc) were seeded with the treated cells at a density of 5×10^4 cells per tube or

3×10^5 cells per dish. Another 24 h later, these cultures were taken for experiments with the challenging ATP depletion.

Infection of the cells with virus-based vectors. Human inducible HSP70 cDNA, or a gene encoding the green fluorescent protein (GFP), under the control of the cytomegalovirus promoter, were inserted into the HSV-1 genome as previously described (37). Cell culture dishes (35 mm) with equal numbers of the cells, in the state of preconfluent monolayer, were washed twice with sterile phosphate-buffered saline (PBS) from the growth medium, and then PBS was replaced with 1.5 ml of serum-free DMEM. The virus-based vectors expressing either HSP70 or GFP were added in the dishes at concentration of 30 plaque-forming units/ml and mixed into the serum-free medium. The cells were in contact with the viruses for 1 h in a humidified atmosphere with 5% CO_2 at 37°C. After that exposure, the virus-containing medium was removed and replaced with normal growth medium for 24 h before to the ATP-depleting treatments (6). The efficiency of infection and expression of the products were tested by flow cytometry and Western blotting.

Measurements of luciferase activity and cellular ATP. The luciferase transfectants adherent in tubes were instantly cooled in ice, briefly washed with ice-cold PBS, and then lysed in 0.5 ml of ice-cold *buffer A* [25 mM H_3PO_4-Tris, pH 7.8, 10 mM $MgCl_2$, 1 mM EDTA, 1% (vol/vol) Triton X-100, and 15% (vol/vol) glycerol] containing 0.5% 2-mercaptoethanol (24, 25). The lysates were frozen and kept at $-20°C$ until determination of luciferase activity. The latter was measured in a luminometer for 10 s after addition of 0.1 ml of *buffer A* containing substrates [1.25 mM ATP and 87 μg/ml luciferin (Sigma)] to 0.15 ml of the cell lysates (24).

Relative levels of cellular ATP were determined by the same measurement of the ATP-dependent luciferase/luciferin reaction while exogenous luciferin and luciferase but not ATP were added to the cell lysates (27). The light emission was measured in a luminometer during the 10 s after the cell lysate aliquots (0.15 ml) were mixed with 0.1 ml of *buffer A* containing 5 μg/ml luciferase (Boehringer Mannheim), 0.3 mM luciferin, 0.3 mM AMP, and 0.5% 2-mercaptoethanol. The endogenous ATP level in untreated cells was considered to be 100%. Care was taken to use the same amount of cells (10^5) in the same volume of the lysing buffer (27).

Cell fractionation with Triton X-100. The ATP depletion-induced protein aggregation was assessed upon the increase in the cellular protein insolubility (14, 15) by using the method adapted for adherent cells (24, 27). Cell culture dishes (35 mm) with equal numbers of the cells were washed twice with PBS and then lysed and scraped in 0.3 ml of the ice-cold *buffer A* devoid of glycerol. The lysates were centrifuged at 12,000 *g* for 15 min at 4°C, which divided them into pellets (Triton X-100-insoluble fractions) and supernatants (Triton X-100-soluble fractions) (24, 27). The pellets were dissolved in 60 μl of 6 M urea, and the protein contents there were determined using the BCA (bicinchoninic acid) kit (Sigma) (7).

For studying the stress-induced changes in the luciferase (in)solubility, the pellets and the supernatants obtained from the cell lysates were dissolved in a Laemmli sample buffer, boiled for 5 min, and then frozen until analysis by electrophoresis and Western blotting.

SDS gel electrophoresis and Western blot analysis. The samples prepared from the total cell lysates or cellular fractions were run by electrophoresis in a Laemmli system with SDS-10% polyacrylamide gel under reducing conditions. The separated proteins were electrotransferred from the gel slabs onto 0.45-μm nitrocellulose membrane (Bio-Rad). For integral detection of the endogenous HSC70 (heat shock cognate protein 70)/HSP70, monoclonal antibody N27F3–4 (Stress-Gen) was used in a 1:2,000 dilution. The human inducible HSP70 was detected in the cell lysates with monoclonal antibody C92F3A-5 (StressGen) diluted 1:1,500. Cyt- and nuc-luciferases were detected with rabbit polyclonal antibodies (Promega) in a 1:1,500 dilution. Anti-mouse Ig- and anti-rabbit Ig-peroxidase conjugates and an ECL (enhanced chemiluminescence) kit were employed to develop the antigen band tracks on X-ray film (all from Amersham). The track images were digitized by means of a flatbed scanner (Mustek 600 II CD). The digitized images were then quantitatively analyzed using the NIH Image software.

Determination of cell death and survival. Cell death during the challenging ATP depletion was quantified by counting the number of dead cells unable to exclude the dye trypan blue as a percentage of the total number of cells. In the case of EGFP-luciferase/HSP70 cotransfection, the percentage of cells killed by ATP depletion was counted with the use of the fluorescence microscope after staining with acridine orange (AO; 3 μg/ml) and propidium iodide (PI; 10 μg/ml), as described previously (8). At least 10 microscopic fields per plate (300–500 cells) were counted, and the recounting was repeated 3–4 times.

Colony survival assays were performed after the treated and control cells were trypsinized. The cell suspensions, when collected and counted, were serially diluted and replated, in triplicate, in 10-cm^2 culture dishes and stood at 37°C for 7–9 days (23). The cell cultures were then fixed with 70% ethanol and stained with 0.5% crystal violet. The number of separate colonies grown (colonies with a minimum of 50 cells) was counted. Cell survival was determined as the ratio of colonies formed by the initially plated cells and normalized to the plating efficiency (23).

Metabolic ^{35}S labeling and immunoprecipitation. Equal numbers of the cells growing in 60-mm culture dishes were preconditioned by heat or metabolic stress as described in *Stressful treatments and recovery*. Seven hours after the start of the preconditioning, the treated and control cells were incubated for eight hours at 37°C in methionine-free DMEM (Flow Laboratories) supplemented with 5% normal DMEM, 10% fetal bovine serum, and 40 μCi/ml [^{35}S]methionine (produced with a specific activity of 950 mCi/mmol by Institute of Physical Energetics, Obninsk, Russia). The label-containing medium was then harvested, and the cells were washed three times with PBS and three times with the full growth medium, followed by 3 h of incubation under normal growth conditions. These radiolabeled cells were extracted with 0.6 ml of the ice-cold glycerol-free *buffer A* containing a protease inhibitor cocktail (Sigma), and then the Triton X-100-soluble fractions were obtained according to the technique described in *Cell fractionation with Triton X-100*.

The freshly prepared Triton X-100-soluble fractions were used for immunoprecipitation experiments. Each sample (0.6 ml) of the cell extracts plunged in ice was at first preincubated for 30 min with 150 μl of protein A-Sepharose (Pharmacia) to minimize a nonspecific binding. After sedimentation and removal of the Sepharose pellets, 5 μl of affinity-purified rabbit polyclonal anti-HSP70/HSC70 antibodies (produced in our laboratory against bovine HSP70/HSC70) were injected into each supernatant; in some samples, 1 mM ATP or ADP (Sigma) was added along with the antibodies. After 1 h of incubation in ice, immune complexes were caught with 150 μl of the protein A-Sepharose within 30 min. The sedimented Sepharose pellets were thoroughly washed with the ice-cold *buffer A* and then dissipated into 0.25 ml of a Laemmli sample buffer and boiled for 5 min. Aliquots of the eluted material were run by electrophoresis as described

earlier. Tracks of ^{35}S-labeled polypeptides were revealed by autoradiography from the dried polyacrylamide gels onto Hyperfilm-MP (Amersham).

Without radiolabeling, the same protocol of immunoprecipitation was used for probing the extracts from the transfectants expressing cyt- or nuc-luciferase. The precipitated material was analyzed by electrophoresis and Western blotting.

Statistical analysis. All quantitative results are expressed as means ± SE of 4–6 separate experiments. Statistical differences between compared groups of the cells were analyzed using ANOVA (a multiway analysis of variance or covariance). ANOVA significance was applied when $P < 0.05$ and confirmed with the *F*-test.

RESULTS

Elevated resistance to ATP depletion-induced cell death in the stress-preconditioned and HSP70-overexpressing cells. Fluctuations in the ATP levels in H9c2 cells during and after heat preconditioning (43°C for 30 min) were insignificant (data not shown). Preconditioning of the cells by metabolic stress (glucose starvation + uncoupling of oxidative phosphorylation with CCCP) caused severe but reversible decrease in cellular ATP (Fig. 1*A*). Both preconditioning treatments followed by a 16- to 18-h period of recovery resulted in an approximately fourfold increase in the intracellular content of HSC70/HSP70 (Fig. 2*A*, Table 1). Probably, the content of other HSPs also increases in the cells following either stressful preconditioning, but their expression was not analyzed in the present study.

H9c2 cells infected with the virus-based vectors expressing HSP70 or GFP were then analyzed in a flow cytometer. The analysis revealed the high (~90–95%) efficiency of the infection with even distribution of either overexpressed product among the cell populations (data not shown). Similarly to both the stressful pretreatments, the virus vector-encoding HSP70 resulted in an ~4.5-fold increase in the level of HSP70 in the infected cells vs. the cells of the control group infected with the GFP-expressing virus (Fig. 2*B*, Table 1).

After 16–18 h of recovery from the priming (heat or metabolic) stress or 24 h after the virus infection, the cells were subjected to the challenging ATP depletion that led to a sharp decrease in the cellular ATP level to 4–5% from the initial level during the first hour of the energy-depleting exposure; later, the ATP level became yet lower (Fig. 1*B*). Those ATP-deprived cells remained adherent, although their morphology changed for the worse. The number of trypan blue-stained (dead) cells at 4 h of the ATP-depleting stress did not yet exceed a percentage of spontaneous cell death (1–2%) in control. Meanwhile, at 5 h and later, cell death among the stressed myoblasts was readily detectable (Fig. 3). Results of double staining with PI and AO revealed no condensation of the chromatin in the dead myoblasts (not shown), suggesting that ATP-depleted H9c2 cells die via necrosis rather than apoptosis.

Neither the stressful pretreatments nor the virus vector-induced HSP70 or GFP overexpression attenuated ATP depletion in the cells undergoing the chal-
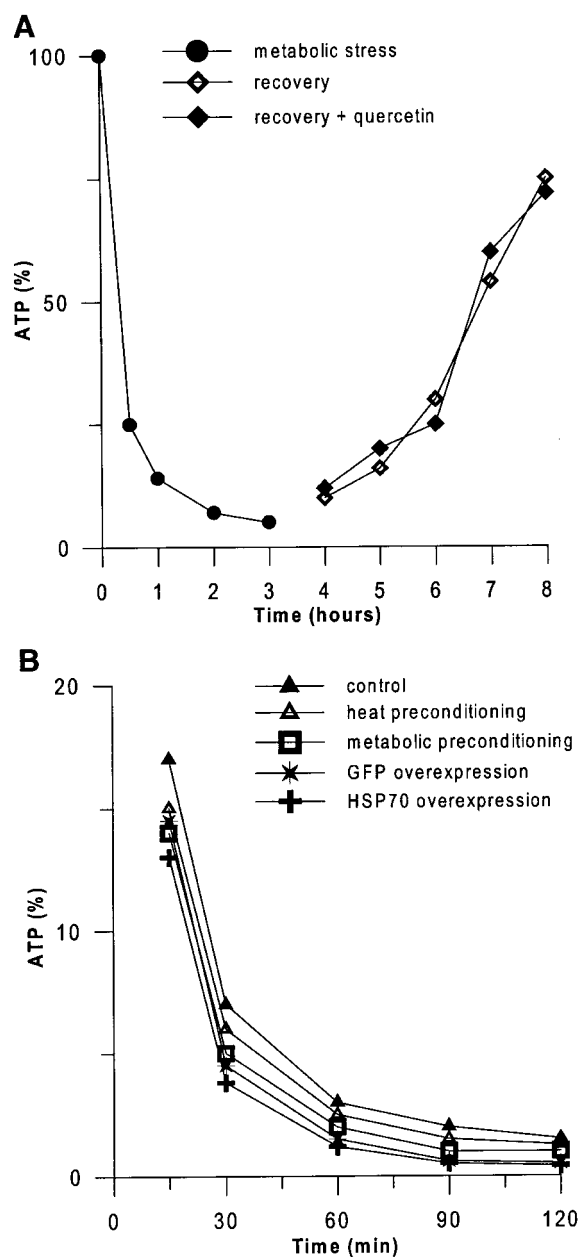

Fig. 1. Relative ATP content in H9c2 cells subjected to metabolic preconditioning (*A*) or challenging ATP depletion (*B*). Note that 30 μM quercetin does not affect the ATP replenishment following metabolic stress (*A*) and the delayed stressful preconditioning. The infection with virus-based vectors expressing green fluorescent protein (GFP) or heat shock protein 70 (HSP70) does not attenuate the decline of cellular ATP during the challenging ATP depletion (*B*).

lenging stress (Fig. 1*B*). However, Fig. 3 shows that the lethality following sustained (5–7 h) ATP depletion was 2 to 3.5 times lower in groups of the stress-preconditioned or HSP70-overexpressing cells than in control (the nonpreconditioned cells or the GFP-overexpressing cells, respectively). Likewise, both the stressful preconditioning and HSP70 overexpression significantly increased the poststress colony formation by the cells deprived of ATP for a long period (Fig. 4, 6 and
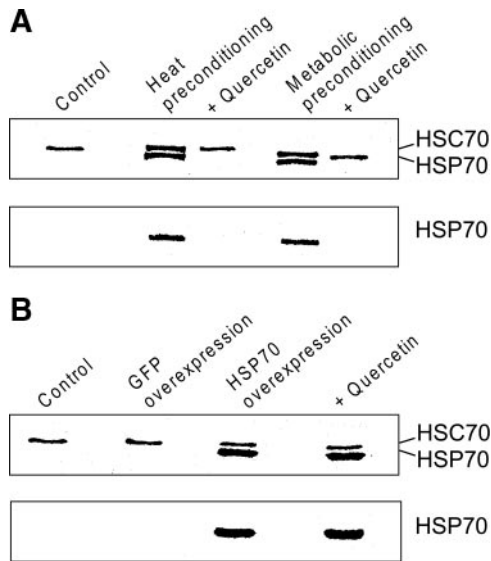
Fig. 2. Accumulation of HSP70 in H9c2 cells following delayed stressful preconditioning or virus infection and the effects of quercetin. Cells were subjected to heat or metabolic preconditioning (*A*) or were infected with the virus-based vectors (*B*) without or with 30 μM quercetin, and then, after an 18-h recovery period, cells were lysed as described in MATERIALS AND METHODS. Aliquots of the lysates from equal amounts of the untreated (control) and preconditioned cells were loaded in each well and analyzed by Western blotting with enhanced chemiluminescence (ECL) by using antibody recognizing both heat shock cognate protein 70 (HSC70) and HSP70 (*top blots*) or antibody with specificity to HSP70 (*bottom blots*).

7 h). In the case of stressful pretreatments, the acquired tolerance to ATP depletion was clearly observed between ∼16 and 30 h after the preconditioning; later, the cytoprotective effect declined and eventually disappeared while the level of endogenous HSP70 returned to baseline (data not shown).

To examine whether the cytoprotection conferred by delayed preconditioning is mediated by accumulated HSP(s), we used quercetin, a well-known suppressor of the stress-responsive HSF1 activation and HSP induction (13, 26). Previously, quercetin was shown to abolish the heat preconditioning-induced stabilization of F-actin in ATP-depleted endothelial cells, which suggested the protective role for stress-induced HSPs (21, 22). In the present study, quercetin, when added to H9c2 cells, did not affect normal morphology (not shown) and restoration of the ATP level following the metabolic preconditioning (Fig. 1*A*). Before the challenging ATP depletion, the H9c2 cultures exposed to quercetin for 11–13 h displayed no differences in morphology or cell density compared with control, but quercetin given between the preconditioning and challenging treatments completely reversed the protective effects against cell death (Figs. 3 and 4). This loss of cytoprotection was paralleled by a clear inhibition of the preconditioning-induced rise in intracellular HSP70. In contrast, quercetin treatment of the cells before the HSP70-encoding virus infection prevented neither the HSP70 accumulation nor the cytoprotective effects,

indicating that quercetin is not acting by directly blocking cytoprotection (see Figs. 2–4, Table 1).

Reduced aggregation of endogenous cellular proteins during ATP depletion in the tolerant (stress-preconditioned or HSP70-overexpressing) cells. Severe depletion of cellular ATP evokes massive damage and aggregation of proteins within the stressed cells as revealed by the accumulation of many initially soluble proteins in the Triton X-100-insoluble cellular fraction (1, 14–16, 27). Here, the stress-induced aggregation of endogenous proteins was quantified by comparing the relative content of protein in the Triton X-100-insoluble fractions from the ATP-depleted cells with that from the unstressed ones (7, 16). The basal level of total Triton X-100-insoluble protein in the cells following the delayed stressful preconditioning or virus infection, or the quercetin treatment, was the same as in the nonpretreated control cells (not shown). The data presented in Table 2 demonstrate that the detergent-insoluble protein component does increase during cellular ATP depletion. The extent of this insolubilization was significantly reduced in the stress-preconditioned and HSP70-overexpressing cells compared with control (the nonpreconditioned cells and the GFP-overexpressing cells, respectively).

Although not affecting the protein insolubility in the nonpreconditioned or GFP-overexpressing cells, the quercetin treatment fully prevented the attenuation of protein insolubilization during ATP depletion in the stress-preconditioned cells (Table 2). Contrary to that finding, the attenuation of ATP depletion-induced protein aggregation in the HSP70-overexpressing cells was indifferent to quercetin (Table 2). Such effects of

Table 1. *Effects of delayed stressful preconditioning and virus infection (without or with quercetin) on the relative content of HSC70/HSP70 in H9c2 cells*

Pretreatments	Relative HSC70/HSP70 Content in Cells, %
Heat preconditioning	392 ± 48*
Heat preconditioning + quercetin	94 ± 11
Metabolic preconditioning	385 ± 41*
Metabolic preconditioning + quercetin	111 ± 13
GFP overexpression	106 ± 10
HSP70 overexpression	444 ± 36*
HSP70 overexpression + quercetin	440 ± 42*

Relative HSC70/HSP70 content was assessed as the integral level of heat shock cognate protein 70 (HSC70) + heat shock protein 70 (HSP70) in the preconditioned or virus-infected cells vs. the level of HSC70 alone in cells of the control group. All data presented are means ± SE of 5 independent experiments. The relative HSC70/HSP70 content was determined 18 h after stressful preconditioning or 24 h after virus infection by scanning of blots as described in MATERIALS AND METHODS. The average content of HSC70 determined for nonpreconditioned H9c2 cells (control group) was considered to be 100%. The stressful preconditioning and the first 10 h of recovery period were performed in the presence of 30 μM quercetin as described in MATERIALS AND METHODS. The cells were incubated in the presence of 30 μM quercetin during the 1-h period of the virus infection and the next 10 h after removal of the virus-containing medium. *Significant difference from control, $P < 0.01$.

Fig. 3. Effects of delayed stressful preconditioning and virus infection (without or with quercetin) on the ability of H9c2 cells to exclude trypan blue dye during challenging ATP depletion. Cells were subjected to heat or metabolic preconditioning in the absence or presence of 30 μM quercetin, and then, after an 18-h recovery period, cells were exposed to challenging ATP depletion as described in MATERIALS AND METHODS. The percentage of dead cells at 5, 6, and 7 h of the ATP-depleting treatment was counted using a trypan blue exclusion test. Data presented are means ± SE of 5 independent experiments (*$P < 0.05$).
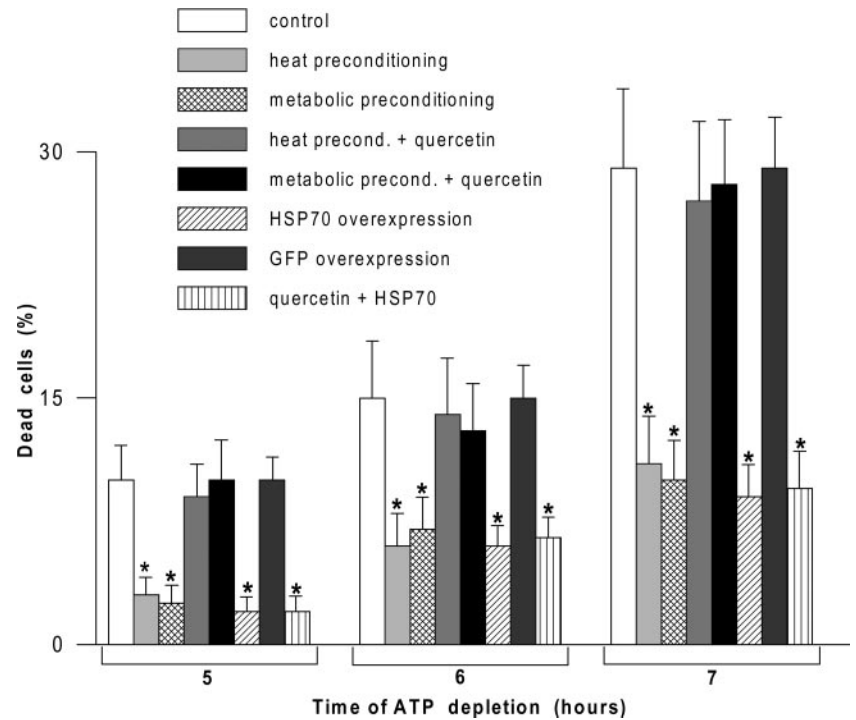
quercetin suggest an involvement of inducible HSP(s) in protection against protein aggregating within ATP-depleted cells. Also, an apparent relationship is seen among the intracellular HSP70 content, the level of the aggregation of endogenous proteins, and cell survival in the context of severe ATP depletion (see Tables 1 and 2 and Figs. 2–4).

Stressful preconditioning and HSP70 overexpression diminish the rate of inactivation and extent of insolubility of cyt- and nuc-luciferase in the tolerant ATP-

Fig. 4. Effects of delayed stressful preconditioning and virus infection (without or with quercetin) on the ability of H9c2 cells to form colonies after sustained ATP depletion. The numbers of surviving colonies for each group were normalized to the untreated cells: nonpreconditioned (control) cells exposed to 6-h ATP depletion were taken as 100% of possible surviving colonies. Colony survival in the groups of preconditioned cells is expressed as a percentage of that in the nonpreconditioned (control) cells. Data presented are means ± SE of 4 separate experiments (*$P < 0.05$).
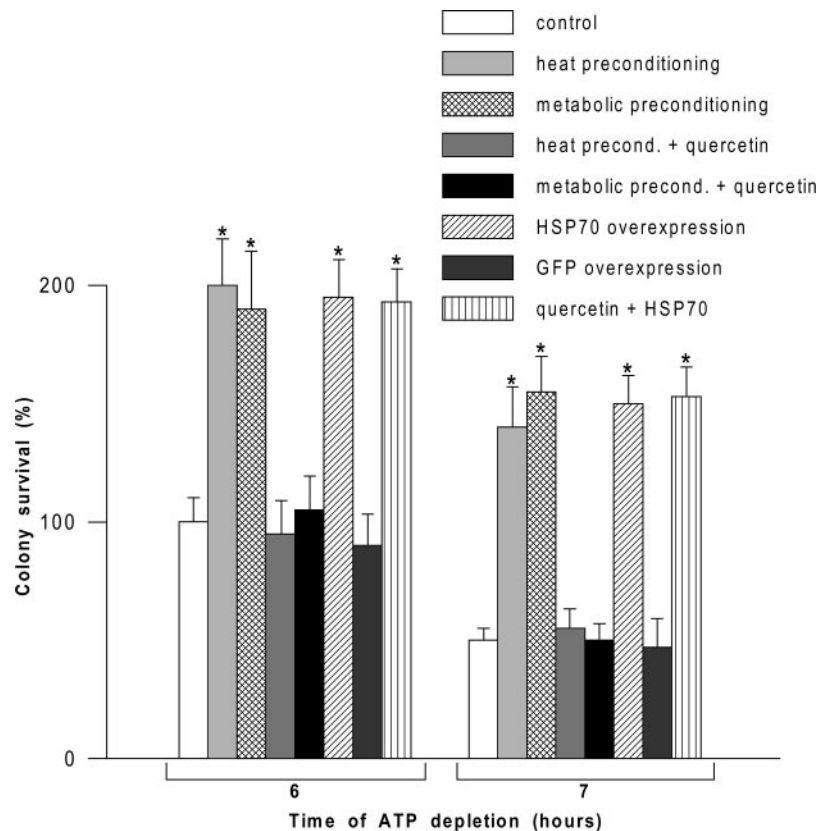
Table 2. *Effects of delayed stressful preconditioning and virus infection (without or with quercetin) on total protein insolubilization in ATP-depleted H9c2 cells*

Samples	Triton X-100-Insoluble Protein in Cells Exposed to ATP Depletion, %	
	2 h	4 h
Nonpreconditioned cells	157 ± 14*	186 ± 21*
+ Quercetin	155 ± 14*	188 ± 24*
Heat-preconditioned cells	118 ± 10†	140 ± 16†
+ Quercetin	152 ± 16*	183 ± 20*
Metabolically preconditioned cells	116 ± 12†	138 ± 15†
+ Quercetin	149 ± 13*	181 ± 23*
GFP-overexpressing cells	155 ± 15*	185 ± 22*
+ Quercetin	153 ± 14*	184 ± 21*
HSP70-overexpressing cells	112 ± 11†	136 ± 14†
+ Quercetin	115 ± 12†	141 ± 20†

All data presented are means $\pm$ SE of 5 independent experiments. The protein content in the Triton X-100-insoluble cellular fractions was determined as described in MATERIALS AND METHODS. For each type of cell pretreatment, the percentage of the insoluble protein in the ATP-depleted cells was counted vs. the basal level of the insoluble protein in the non-ATP- depleted cells. The basal levels of the insoluble protein in the non-ATP-depleted cells were the same in the different experimental groups and were considered to be 100%. The stressful preconditioning and the first 10 h of the 18-h recovery period were performed in the presence of 30 μM quercetin as described in MATERIALS AND METHODS. The cells were incubated in the presence of 30 μM quercetin during the 1-h period of the virus infection and the next 10 h after removal of the virus-containing medium. GFP, green fluorescent protein. *Significant difference from the basal level of the insoluble protein $P < 0.05$. †Significant difference from the values marked by asterisk (*) in the same column, $P < 0.05$.

depleted cells. Firefly luciferase is an ATP-dependent reporter enzyme that, when expressed in mammalian cells, is inactivated and insolubilized during heat or chemical stress (2, 24, 25, 28, 29) or depletion of cellular ATP (27). For probing of the situation within the cytoplasm and the nucleus separately, we transfected the cells with plasmid constructs encoding luciferases engineered for expression in either the cytoplasm (cyt-luciferase) or the nucleus (nuc-luciferase) (25). This specific compartmentalization of the expressed products and the transfection efficiency were checked by microscopic analysis of the distribution of EGFP-cyt- and EGFP-nuc-luciferases in the cells with DAPI-labeled nuclei (Fig. 5). The transfection usually yielded up to 20–25% positive cells (see Fig. 5), and its procedure did not affect the time course of the subsequent challenging ATP depletion (not shown).

We analyzed the catalytic activity of each form of luciferase in the cells lysed at different time points of in vivo ATP depletion (Fig. 6). Exogenous ATP and luciferin, as substrates, were added in nonlimiting concentrations to the cell lysates for measuring the enzymatic activity (see MATERIALS AND METHODS). In this case, the decrease in luciferase activity observed during cellular ATP depletion reflects the enzyme inactivation resulting from the stress (17, 27). Figure 6 demonstrates a gradual decline of the levels of catalytically active cyt- and nuc-luciferases within ATP-depleted H9c2 cells. As was found for heat shock (25), the nuc-luciferase is more sensitive to inactivation by in vivo ATP depletion than the cyt-luciferase. No luciferase activity was detected in supernatants over the ATP-depleted cells within 4 h of the challenging stress; this means that there is no efflux of the enzyme from the stressed cells. Importantly, the rate of ATP depletion-induced inactivation of luciferase was considerably attenuated in the stress-preconditioned cells (Fig. 6).

In vivo, under heat stress (24, 25, 28) and ATP depletion (27), the insolubilization of luciferase occurs in parallel with its inactivation. The data on the enzyme (in)solubility are presented in Fig. 7. Before the stress, either form of luciferase was cofractionated with the Triton X-100-soluble cellular material (Fig. 7, 0 h). During in vivo ATP depletion, cyt- and nuc-luciferases accumulated into the Triton X-100-insoluble fraction (Fig. 7, 2 and 3 h), which is consistent with the course of the stress-induced inactivation of these forms of the enzyme (see Fig. 6). The insolubilization was markedly attenuated in the stress-preconditioned cells (Fig. 7). Both modes of the stressful preconditioning similarly retarded the inactivation and insolubilization of both forms of luciferase during cellular ATP depletion.

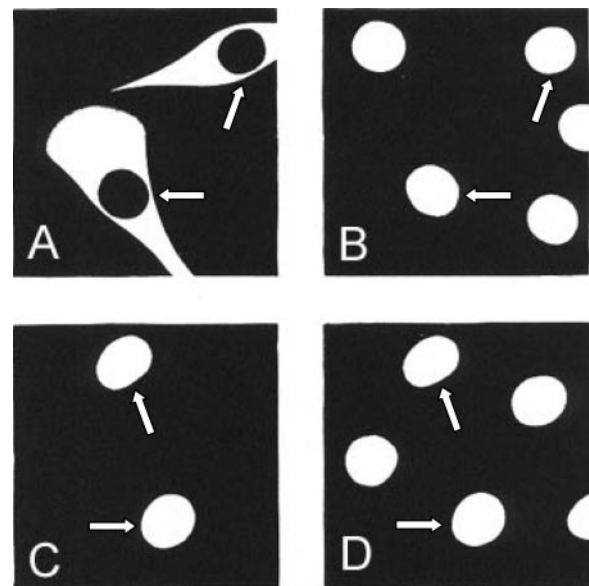Similarly to findings reported in the two previous sections, quercetin abolished the protective effects of

Fig. 5. Double-label fluorescence images showing expression of enhanced GFP (EGFP)-cytoplasmic (cyt)-luciferase and EGFP-nuclear (nuc)-luciferase in the preparations of diamidinophenylindole (DAPI)-stained H9c2 cells. *A* and *B*: EGFP fluorescence of cyt-luciferase distributed in the cytoplasm of 2 transfectants (*A*) among the cell population stained with DAPI for total labeling of the nuclei (*B*). *C* and *D*: EGFP fluorescence of nuc-luciferase localized to the nuclei of 2 transfectants (*C*) among the cell population stained with DAPI for total labeling of the nuclei (*D*). Arrows denote locations of the same nuclei in the EGFP- and DAPI-stained cells (transfectants) in couples of the double-labeled images. Original magnification, ×500.

Fig. 6. Effects of delayed stressful preconditioning (without or with quercetin) and HSP70 overexpression on the rate of inactivation of cyt- (*A*) and nuc-luciferase (*B*) in ATP-depleted H9c2 cells. The control and stress-preconditioned transfectants were subjected to 4 h of ATP depletion, and aliquots of the ATP-depleted cells were lysed by the hour. The catalytic capability of cyt- and nuc-luciferase was then measured in the lysates with addition of exogenous ATP and luciferin (see MATERIALS AND METHODS). Note the retarded inactivation of both forms of luciferase during ATP depletion in the stress-preconditioned or HSP70-overexpressing transfectants and the abolishing effects of quercetin in the groups with stressful preconditioning. Data presented are means $\pm$ SE of 6 independent experiments (*$P <$ 0.05).
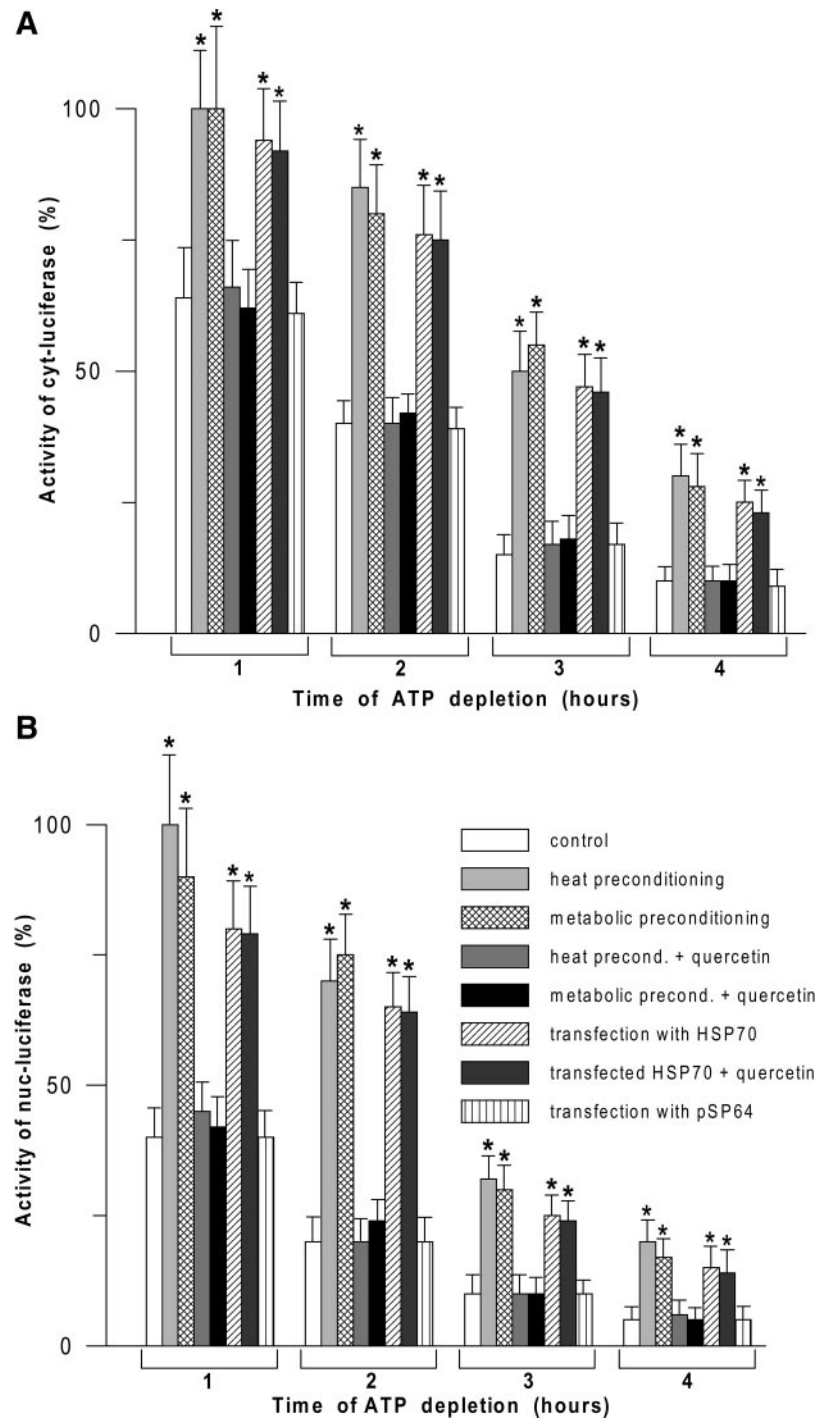
stressful preconditioning on the catalytic capacity and solubility of cyt- and nuc-luciferases in ATP-depleted H9c2 cells (Figs. 6 and 7). This allows us to suggest inverse correlation between the level of stress-induced HSP(s) (or the in situ chaperone activity) and the proteotoxic impact of ATP depletion within the cytoplasm and the nucleus.

Because overexpressed HSP70 alone was shown to reduce the total protein aggregation in ATP-depleted H9c2 cells (see Table 2), we examined whether excess HSP70, by itself, can attenuate the local proteotoxic effects of ATP depletion occurring in different cellular compartments. For this purpose, H9c2 cells were co-transfected with human inducible HSP70 and either EGFP-cyt- or nuc-luciferase as described in the previous heat shock studies (24, 29). The fact of coexpression of HSP70 and EGFP-luciferase in the same transfectants was confirmed by double-label fluorescence analysis in a flow cytometer: subpopulations of the EGFP-positive cells and the cells intensively stained with the specific antibody C92F3A-5 did coincide (not shown). The relative summarized content of HSC70/HSP70 in
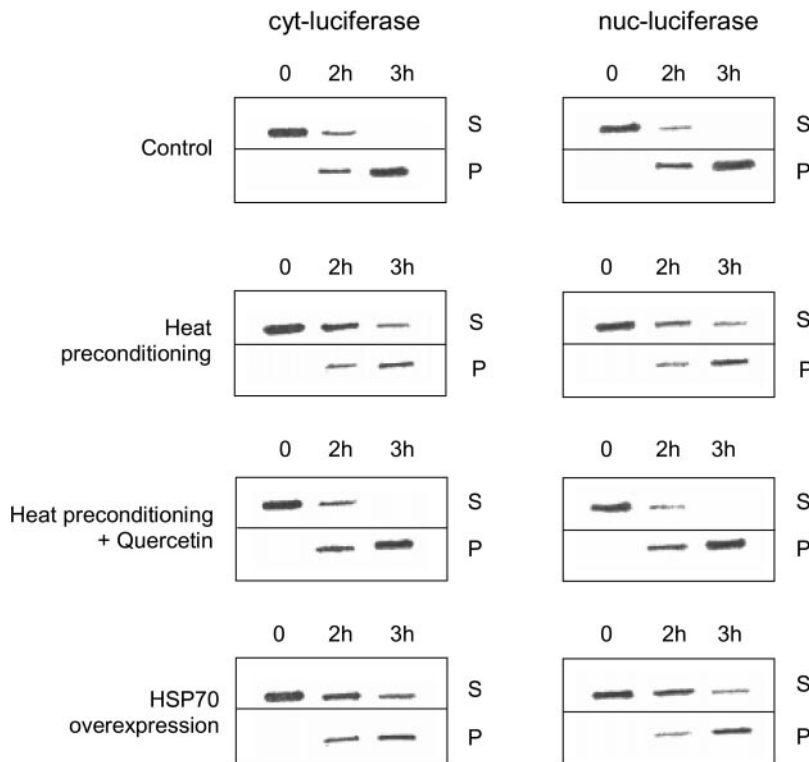
Fig. 7. Effects of delayed heat preconditioning (without or with quercetin) and HSP70 overexpression on insolubilization of cyt- (*left*) and nuc-luciferase (*right*) in ATP-depleted H9c2 cells. The non-ATP-depleted transfectants (0 h) and transfectants exposed to 2 or 3 h of ATP depletion were fractionated with the Triton X-100-containing buffer as described in MATERIALS AND METHODS. After the cellular fractions were run in electrophoresis, cyt- and nuc-luciferase were immunodetected in the supernatants (S) comprising Triton X-100-soluble cellular material and in the Triton X-100-insoluble pellets (P) by Western blotting with ECL. Note the attenuated insolubilization of both forms of luciferase during ATP depletion in the heat-preconditioned transfectants and transfectants overexpressing HSP70, and the abolishing effects of quercetin toward the heat preconditioning. Although very similar results including the abolishing effects of quercetin were obtained with metabolic preconditioning, no influence of quercetin was found in the case of HSP70 overexpression (not shown).

the EGFP-positive cells (cotransfectants) was five to six times greater than in the EGFP-negative cells (established on the relative intensity of fluorescence after staining with the antibody N27F3–4). In agreement with previous findings obtained on Chinese hamster ovary cells (30), we found that HSP70 overexpression reduced the rate of inactivation of cytoplasmic luciferase in ATP-depleted H9c2 myoblasts (Fig. 6*A*). Also, HSP70 retarded substantially the nuclear luciferase inactivation due to cellular ATP depletion (Fig. 6*B*). Furthermore, the data presented in Fig. 7 demonstrate that overexpressed HSP70 clearly protects cyt- and nuc-luciferase from insolubilization in the ATP-depleted cotransfectants. All these effects of overexpressed HSP70 alone were comparable with those of the stressful preconditioning. In contrast to the situation with stressful pretreatments (see Figs. 6 and 7), quercetin did not prevent the HSP70 overexpression-mediated protection of luciferase during ATP depletion in the cotransfectants (Fig. 6). This also supports the hypothesis that excess HSP(s) can attenuate the proteotoxicity of ATP depletion and that at least HSP70 seems to be involved in such a protective mechanism effactually acting within both cytoplasmic and nuclear compartments of ATP-depleted cells.

Finally, using the PI staining, we assessed the viability of the cotransfectants in the context of sustained (5–7 h) ATP-depleting stress (Table 3). The data presented in Table 3 clearly demonstrate that the cotransfectants expressing EGFP-cyt-luciferase and human HSP70 are more resistant to the ATP depletion-induced necrosis than the cells cotransfected with EGFP-cyt-luciferase and pSP64 or the nontransfected (EGFP negative) cells; the intensity of cell death was similar to that determined by trypan blue exclusion test (see Fig. 3). Taking into account that within these ATP-depleted cotransfectants cyt-luciferase is more slowly inactivated and insolubilized (Figs. 6 and 7), we have concluded that just the HSP70-overexpressing cells are able to withstand both the proteotoxicity and the cytotoxicity of severe ATP depletion. As in the case of virus vector-based HSP70 overexpression (see Figs. 3 and 4),

Table 3. *Elevated resistance to ATP depletion-induced cell death among the transfectants overexpressing HSP70*

Type of Cells Counted After ATP-Depleting Treatment	Percentage of Dead (PI Stained) Cells After ATP Depletion		
	5 h	6 h	7 h
EGFP-cyt-luciferase/HSP70 cotransfectants	2.4 ± 0.5	4.2 ± 0.7	8.5 ± 0.8
+ Quercetin	2.5 ± 0.5	4.4 ± 0.6	8.2 ± 0.9
EGFP-cyt-luciferase/pSP64 cotransfectants	11.2 ± 3.0	16.1 ± 3.8	32.6 ± 4.7
Nontransfected (EGFP negative) cells	10.5 ± 3.1	15.4 ± 3.5	30.9 ± 4.2

Cells were subjected to sustained ATP depletion, and cell death was quantified by using propidium iodide (PI) staining as described in MATERIALS AND METHODS. All data presented are means ± SE of 4 independent experiments. The average percentage of spontaneous cell death in the normal H9c2 culture was determined to be 1.5 ± 0.5%. After transfection, the cells were incubated in the presence of 30 μM quercetin for 10 h to mimic the drug administration used for the experiments with stressful preconditioning (see legends for Tables 1 and 2). EGFP, enhanced GFP; cyt, cytoplasmic; nuc, nuclear.

quercetin did not abolish the cytoprotective effect of HSP70 overexpressed following the plasmid transfection (Table 3).

Tolerant (stress-preconditioned or HSP70-overexpressing) cells, when depleted of ATP, have larger amounts of soluble HSP70 and its complexes with other proteins. To prove the direct involvement of HSP70 in the described effects, we compared the behavior of this chaperone during ATP depletion in the control and tolerant cells. It was previously shown in various cell lines that a major part of HSC70/HSP70 is insolubilized in response to ATP depletion (14, 15, 27), but this effect was never explored in cells rendered tolerant. Figure 8 demonstrates that, whereas H9c2 myoblasts are deprived of ATP, HSC70/HSP70 also accumulates into the Triton X-100-insoluble cellular fraction. There were not significant differences in the levels of the insolubilized HSP70 between the control and tolerant cells (Fig. 8). However, compared with control, both the stress-preconditioned and HSP70-overexpressing cells contain much more HSP70 in the Triton X-100-soluble fractions isolated at different times of the ATP-depleting exposure. In other words, the tolerant cells, being HSP70-enriched before ATP depletion, retain the soluble pool of this chaperone longer during sustained ATP depletion (Fig. 8).

We hypothesized that the soluble HSP70 still present in the ATP-depleted cells is not a pool of the free chaperone but that, on the contrary, most of the soluble HSP70 (if not all) can be in complexes with other proteins, e.g., stress-sensitive proteins having a tendency to aggregate. Such proteins, if they are in the complexes with HSP70, may thereby be preserved from the aggregation (insolubilization) resulting from cellular ATP depletion. To check this hypothesis, we carried out immunoprecipitation with anti-HSC70/HSP70 antibodies from the Triton X-100-soluble fractions isolated from the control and tolerant cells undergoing ATP depletion. Taking into account that a part of cytosolic HSC70/HSP70 in ATP-depleted cells can be stably bound to nascent polypeptide chains (3), we then incubated the cells preincubated with [^{35}S]methionine in the label-free medium for a long (3 h) period; this step resulted in a situation in which exclusively mature proteins contained ^{35}S and therefore could be detected by autoradiography. Figure 9*A* shows that before ATP depletion, all the patterns of the radiolabeled immunoprecipitates exhibit major bands of HSP70 and/or HSC70 with only few minor concomitants (see *lane 1* for each group). When the soluble fractions were isolated from the cells subjected to 2 h of ATP depletion, a number of mature proteins with different molecular mass coprecipitated with HSC70/HSP70 (Fig. 9*A*, *lane 2* for each group); these proteins are not yet identified. It is clearly shown that, compared with control, the samples derived from the tolerant (stress preconditioned or HSP70 overexpressing) cells yield larger amounts of HSC70/HSP70 and the coprecipitating protein material (compare *lane 2* for each group in Fig. 9*A*). Importantly, exogenous ATP being added to the extracts from the ATP-depleted cells
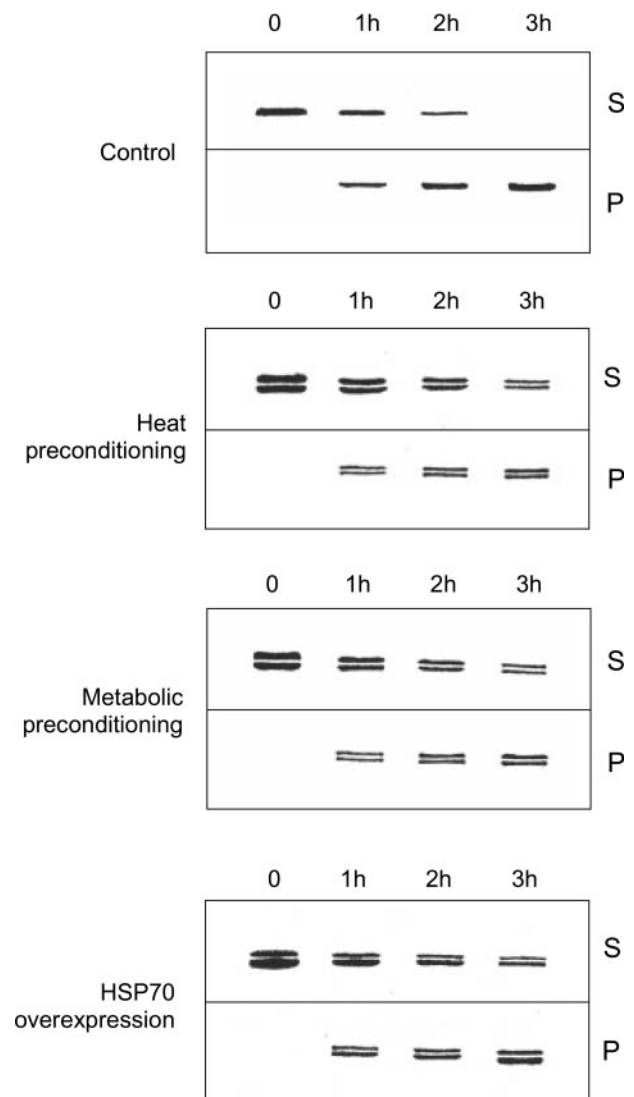

Fig. 8. Effects of delayed stressful preconditioning and HSP70 overexpression on the stress-induced redistribution of HSC70/HSP70 between the Triton X-100-soluble and -insoluble cellular fractions during ATP depletion in H9c2 cells. Equal numbers of the non-ATP-depleted cells (0 h) and the cells subjected to 1, 2, or 3 h of ATP depletion were fractionated with the Triton X-100-containing buffer as described in MATERIALS AND METHODS. After the cellular fractions were run in electrophoresis, HSC70/HSP70 was immunodetected in the supernatants (S) comprising Triton X-100-soluble cellular material and in the insoluble pellets (P) by using Western blotting with ECL along with the monoclonal antibody N27F3–4. Note the longer sojourn of HSC70/HSP70 in the soluble cellular fractions of the tolerant (stress preconditioned or HSP70 overexpressing) cells deprived of ATP. Besides these blots, very similar results were obtained in 4 independent experiments.

evoked a full disappearance of all the protein bands except HSC70/HSP70 (see *lane 3* for each group in Fig. 9*A*), whereas exogenous ADP (negative control) did not have any effects (not shown).

When the transfectants expressing cyt- or nuc-luciferase were probed in analogous experiments, both forms of the enzyme as well as HSC70/HSP70 were detected in the immunoprecipitates from the Triton X-100-soluble fractions of the ATP-depleted cells (Fig.
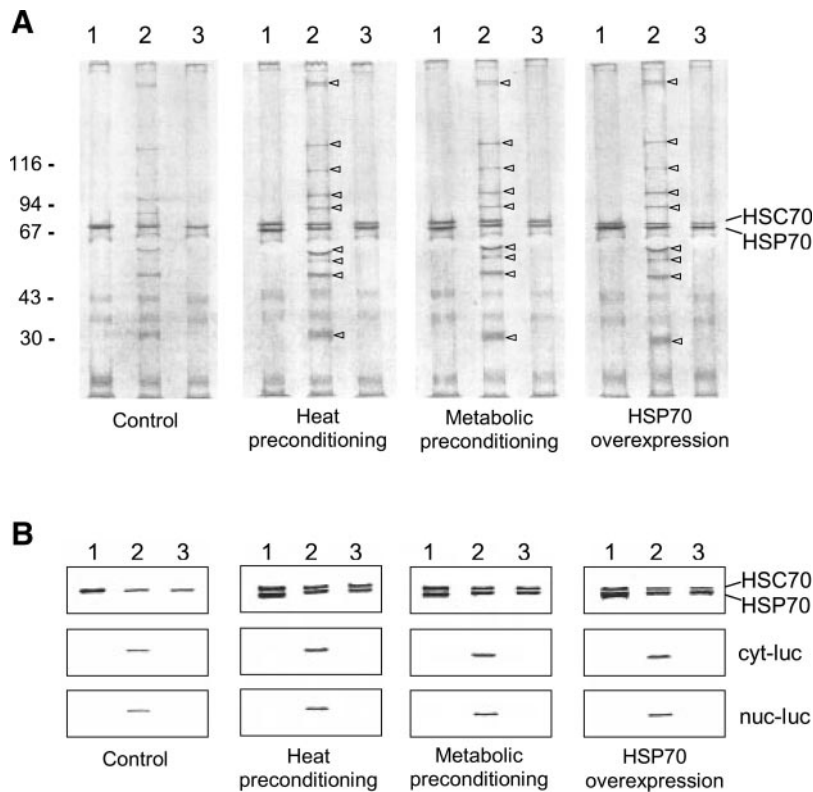
Fig. 9. Immunoprecipitation patterns showing the effects of delayed stressful preconditioning, HSP70 overexpression and exogenous ATP on formation of soluble complexes between HSC70/HSP70 and other proteins in ATP-depleted H9c2 cells. The Triton X-100-soluble fractions isolated from equal numbers of the non-ATP-depleted cells (*lane 1*) and the cells depleted of ATP for 2 h (*lanes 2* and *3*) were used for immunprecipitation with the anti-HSC70/HSP70 antibodies. The patterns in *lane 3* for each group are derived from the soluble fractions of the ATP-depleted cells in which immuno-precipitation was performed in the presence of exogenous ATP (1 mM). After electrophoresis was run, the immunoprecipitate patterns were visualized by either ^{35}S autoradiography (*A*) or Western blotting with ECL (*B*) as described in MATERIALS AND METHODS. Note the more abundant protein material coprecipitated with excess HSC70/HSP70 from the soluble fractions of the tolerant (stress preconditioned or HSP70 overexpressing) cells deprived of ATP (*A* and *B*, *lane 2* for each group). In *A*, arrows mark protein bands whose intensities increased in the patterns of the tolerant cells (see *lane 2* for each group); the same bands disappeared if immunoprecipitation was performed in the presence of exogenous ATP (see *lane 3* for each group). Autoradiographs and blots shown were obtained in 1 of 4 separate experiments with very similar results; luc, luciferase.

9*B*, *lane 2* for each group). In the samples from the tolerant cells, more intensive bands of HSC70/HSP70 and luciferases developed (Fig. 9*B*, *lane 2* for each group); scanning followed by quantitative analyses of the blot images revealed the 2.5- to 3.5-fold increase in the luciferase band intensities compared with the control samples. Meanwhile, no visible luciferase bands were found when exogenous ATP (but not ADP) was present in the cell extracts (see *lane 3* for each group in Fig. 9*B*).

These results are in full agreement with the data from the previous experiments. Indeed, the fact that the Triton X-100-soluble fractions from the tolerant cells deprived of ATP contain the increased amounts of HSP70 and its complexes with other proteins (see Figs. 8 and 9*A*) well correlates with the delayed protein insolubilization during ATP depletion in the tolerant cells (the data of Table 2). In turn, the more abundant yield of cyt- and nuc-luciferase coprecipitated with excess HSC70/HSP70 from the soluble fractions of the tolerant cells depleted of ATP (Fig. 9*B*) suggests the improved complex formation between the chaperone and the stress-sensitive enzymes in the cytosol and nucleosol of the tolerant (HSP70 enriched) cells undergoing ATP depletion. Also, this finding can nicely explain the protective effects of the stressful preconditioning and HSP70 overexpression on inactivation and insolubilization of luciferases during cellular ATP depletion (see Figs. 6 and 7).

As for the effect of exogenous ATP (Fig. 9, *A* and *B*, *lane 3* for each group), this finding implies that in vivo HSP70 interacts with the ATP depletion-sensitive proteins in an ATP-dependent manner and that it is cellular ATP depletion that promotes the chaperone binding to instable or damaged proteins in the stressed cells.

DISCUSSION

Preconditioning of H9c2 cells by nonlethal heat or metabolic stress transiently enhances the HSP70 expression. In parallel, cell resistance to the cytotoxic and proteotoxic effects of the challenging ATP depletion is transiently acquired, thus suggesting a casual link between these events. Here we discuss molecular mechanisms of the in vivo ATP depletion-associated proteotoxicity, its link to the stress-induced cell death, and the role of HSP70 in protection from the protein aggregation in energy-deprived cells.

In 1994, Nguyen and Bensaude (27) described two reporter enzymes, *Eschericia coli* β-galactosidase and firefly luciferase, that, both being Triton X-100-soluble in unstressed cells, are insolubilized during ATP depletion. Although those foreign enzymes were artificially expressed in mammalian cells, their insolubilization appears to reflect the in situ response of many endogenous cellular proteins to the stress. The data of Table 2 reveal that rather a large percentage of total cellular protein lost solubility because of ATP depletion. Such reaction implies at least two processes that may occur in parallel: some initially soluble proteins *1*) form aggregates that precipitate in Triton X-100-containing solutions and/or *2*) undergo translocation to the detergent-resistant cellular compartments such as the

cytoskeleton, chromatin, and nuclear matrix. Probably, both the former and latter are caused by structural changes in some instable protein molecules resulting from ATP depletion per se or its harmful consequences (e.g., ionic imbalance including acidosis). These structural changes promote undesirable intermolecular interactions leading to aggregation and abnormal translocation of a number of affected intracellular proteins. This may result in dysfunction of the involved proteins through the loss of their native conformations and through the inability of aggregated or abnormally localized proteins to interact with their natural substrates and cofactors.

The question arises, which intracellular proteins aggregate and are insolubilized in response to ATP depletion? Among mature cytosolic proteins, this feature is seen for the 68-kDa double-stranded RNA-dependent protein kinase (17, 27) and a major glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (1). The latter, being an abundant cytosolic enzyme in unstressed cardiomyocytes, associates with myofibrils as a result of ischemia-provoked ATP depletion (1). Within the nuclear compartment, proteins of the chromatin and/or nuclear matrix may coaggregate with the stress-sensitive nucleosolic enzymes; otherwise, the latter may aggregate with each other without sticking to the originally insoluble structures. As for nonenzymatic proteins, the cytosolic pools of cytoskeletal proteins such as G-actin, myosin, vinculin, and α-actinin can also supply material for aggregating in ATP-depleted cells (9, 12, 14–17). Despite the fact that the preexisting cytoskeleton undergoes disintegration in ATP-depleted cells, its debris remains insoluble and may entrap surrounding proteins, thus aggravating the total aggregation (17).

Importantly, the proteotoxic effects of ATP depletion correlate with its cytotoxicity as indicated by comparison of the data of Tables 2 and 3 and Figs. 3, 4, 6, and 7. The same suggestion came from the previous studies performed on murine tumor cells (7, 14, 16). Quercetin completely abolished both the HSP70-inducing and the cytoprotective and anti-proteotoxic effects of the stressful preconditioning, suggesting that the inducible HSP(s) can alleviate injury of ATP-depleted cells by reducing the protein aggregation. Although quercetin could exert other effects besides the HSF1 inhibition, we found that, in our model, this drug did not affect the time course of in vivo ATP depletion/replenishment, the basal level of the insoluble cellular protein, or the cell viability and protein aggregation under the challenging ATP depletion in the cells of control groups (non-stress-preconditioned or GFP-overexpressing H9c2 cells). While the HSF1-independent (i.e., plasmid or viral vector induced) overexpression of inducible HSP70 was already sufficient to moderate the cytotoxic and proteotoxic influence of ATP depletion in H2c9 cells, the quercetin treatment was not able to prevent these HSP70 overexpression-mediated effects. Taken together, these results allow us to attribute the cytoprotective and anti-proteotoxic effects of the stressful preconditioning to stress-induced HSP(s) (e.g., HSP70).

Although other inducible HSPs besides HSP70 can also be involved in cellular defense/repair from injurious consequences of ATP depletion, HSP70 appears to play a major role in the attenuation of the ATP depletion-induced protein aggregation revealed after the stressful preconditioning. Such a conclusion follows from the experiments with overexpression of HSP70 alone, which yielded both the intracellular HSP70 accumulation and the impairment of the ATP depletion-associated proteotoxicity quite comparable with those in the stress-preconditioned cells.

Intriguingly, HSP70 is known as an ATP-dependent chaperone, and, at first glance, it seems unclear how it could combat the proteotoxicity in the case of lack of ATP. However, previous heat shock studies (20, 33) showed that overexpression of the deletion mutant of human HSP70 devoid of the ATP-binding domain confers thermoresistance and reduced intranuclear protein aggregation in heat-shocked Rat-1 cells. Because the inability of the mutant to bind and hydrolyze ATP did not yet abolish its protective effects, the authors suggested that the mutant is still able to form complexes with heat-denatured cellular proteins, thus reducing protein aggregation during heat shock (20, 33). This heat shock model with the deletion mutant of HSP70 rather closely mimics the present situation with ATP depletion in HSP70-enriched H9c2 cells. Taking into account that HSP70 binds ADP much stronger than ATP (11, 31, 32) and the ATP/ADP ratio obviously declines in energy-deprived cells (15, 18), it seems likely that under severe depletion of ATP in vivo, most (if not all) HSC70/HSP70 molecules are in the "ADP state." The latter is characterized as having a slower on-rate but also slower off-rate of substrate than HSP70 in the "ATP state." Therefore, excess HSP70 in the ADP state within the ATP-depleted tolerant cells may form stable complexes with proteins affected by the stress. In favor of this idea, we find *1*) the longer sojourn of excess HSP70 in the soluble fractions of the tolerant cells undergoing ATP depletion, *2*) the larger amounts of soluble protein material coimmunoprecipitating with excess HSP70 from the tolerant cells deprived of ATP, and *3*) the dissociating effect of exogenous ATP on the soluble HSP70-protein complexes formed for lack of cellular ATP (see Figs. 8 and 9). Apparently, the proteins bound to HSP70 are thereby protected from aggregation with each other and/or sticking to the insoluble cytoskeletal/nuclear structures or debris despite the fact that the chaperone cannot undergo an ATP-ADP cycle in the ATP-deprived cells. As for the stress-damaged proteins, their stable association with HSP70 may retain them in a soluble, folding-competent state that should save these proteins from the aggregation during cellular ATP depletion. Besides the stress-damaged mature proteins, the preexisting pool of nascent polypeptide chains can recruit some part of HSP70 in ATP-depleted cells (3), and the chains bound to the chaperone may be better preserved from the stress-induced aggregation. Very similar mechanisms appear to act in the luciferase model: during ATP depletion, the stress-sensitive en-

zyme can be either involved in the aggregation cascade (inactivation and insolubilization) or trapped by HSP70 (protection). We suppose that during ATP depletion in the HSP70-enriched cells, the large part of inactivated luciferase still remains in a folding-competent state being bound to HSP70, and then, under cell lysis into the ATP-containing buffer, the instant ATP-mediated dissociation of the enzyme-chaperone complexes occurs, with released luciferase being detected as catalytically active in the assay. The immunoprecipitation experiments demonstrating the improved HSP70-luciferase complex formation in the tolerant cells depleted of ATP as well as the complex-disrupting effect of exogenous ATP (Fig. 9*B*) provide strong arguments in support of such a supposition.

Although the protection of two model enzymes within the HSP70-enriched cells cannot be directly linked to what happens with the cells' vital proteins, our data do indicate that excess HSP70 can diminish the ATP depletion-induced protein aggregation in both the cytoplasm and the nucleus. Moreover, evident correlation is observed between the cell viability under the challenging ATP depletion and the in situ chaperone potential of HSP70 toward cyt- and nuc-luciferase during the stress (see Figs. 6 and 7 and Table 3). Extrapolating the luciferase data to endogenous proteins of the ATP-depleted cells (see Table 2), we suggest that HSP70 can protect stress-sensitive cytoplasmic and nuclear proteins that otherwise are involved in the detrimental aggregation leading to necrotic cell death. The fact of partial migration of cytosolic HSP70 into the nuclei of ischemia-stressed rat cardiomyocytes (35) supports the suggestion that nuclear proteins can also become the chaperone targets under the energy-depriving stress. It seems likely that in the case of ischemia in vivo, excess HSP70 would also be able to minimize the proteotoxic impact of cellular ATP depletion, thereby allowing HSP70-enriched cells to better tolerate an acute phase of ischemic insults. Such speculations generate an additional reason for development of clinically applicable ways enabling to increase the HSP70 level in ischemia-attacked tissues of patients.

REFERENCES

1. **Barbato R, Menaro R, Dainese P, Carafoli E, Schiaffino S, and DiLisa F.** Binding of cytosolic proteins to myofibrils in ischemic rat hearts. *Circ Res* 78: 821–828, 1996.
2. **Beaucamp N, Harding TC, Geddes BJ, Williams J, and Uney JB.** Overexpression of hsp70i facilitates reactivation of intracellular proteins in neurones and protects them from denaturing stress. *FEBS Lett* 441: 215–219, 1998.
3. **Beckmann RP, Lovett M, and Welch WJ.** Examining the function of hsp 70 in cells subjected to metabolic stress. *J Cell Biol* 117: 1137–1150, 1992.
4. **Benjamin IJ, Horie S, Greenberg ML, Alpern RJ, and Williams RS.** Induction of stress proteins in cultured myogenic cells. Molecular signal for the activation of heat shock transcription factor during ischemia. *J Clin Invest* 89: 1685–1689, 1992.
5. **Benjamin IJ and McMillan DR.** Stress (heat shock) proteins: molecular chaperones in cardiovascular biology and disease. *Circ Res* 83: 117–132, 1998.
6. **Brar BK, Stephanou A, Wagstaff MJD, Coffin RS, Marber MS, Engelmann G, and Latchman DS.** Heat shock proteins delivered with a virus vector can protect cardiac cells against apoptosis as well as against thermal or hypoxic stress. *J Mol Cell Cardiol* 31: 135–146, 1999.
7. **Gabai VL and Kabakov AE.** Rise in heat-shock protein level confers tolerance to energy deprivation. *FEBS Lett* 327: 247–250, 1993.
8. **Gabai VL, Meriin AB, Yaglom JA, Wei JY, Mosser DD, and Sherman MY.** Suppression of stress kinase JNK is involved in HSP72-mediated protection of myogenic cells from transient energy deprivation. *J Biol Chem* 275: 38088–38094, 2000.
9. **Ganote C and Armstrong S.** Ischaemia and the myocyte cytoskeleton: review and speculation. *Cardiovasc Res* 27: 1387–1403, 1993.
10. **Heads RJ, Yellon DM, and Latchman DS.** Differential cytoprotection against heat stress or hypoxia following expression of specific stress protein genes in myogenic cells. *J Mol Cell Cardiol* 27: 1669–1678, 1995.
11. **Hightower LE and Sadis SE.** Interaction of hsc70 and hsp70 with unfolded proteins and peptides. In: *The Biology of Heat Shock Proteins and Molecular Chaperones*, edited by Morimoto RI, Tissieres A, and Georgopoulos C. New York: Cold Spring Harbor Laboratory, 1994, p. 179–207.
12. **Hinshaw DB, Armstrong BC, Burger JM, Beels TF, and Hyslop PA.** ATP and microfilaments in cellular oxidant injury. *Am J Pathol* 132: 479–488, 1988.
13. **Hokosawa N, Hirayoshi K, Kudo H, Takeshi H, Aoike A, Kawai K, and Nagata K.** Inhibition of the activation of heat shock factor in vivo and in vitro by flavonoids. *Mol Cell Biol* 12: 3490–3498, 1992.
14. **Kabakov AE and Gabai VL.** Protein aggregation as primary and characteristic cell reaction to various stresses. *Experientia* 49: 706–710, 1993.
15. **Kabakov AE and Gabai VL.** Stress-induced insolubilization of certain proteins in ascites tumor cells. *Arch Biochem Biophys* 309: 247–253, 1994.
16. **Kabakov AE and Gabai VL.** Heat shock-induced accumulation of 70-kD stress protein (HSP70) can protect ATP-depleted tumor cells from necrosis. *Exp Cell Res* 217: 15–21, 1995.
17. **Kabakov AE and Gabai VL.** *Heat Shock Proteins and Cytoprotection: ATP-Deprived Mammalian Cells*. Austin, TX: Landes, 1997. (Molecular Biology Intelligence Unit Series)
18. **Kabakov AE, Molotkov AO, Budagova KR, Makarova YuM, Mosin AF, and Gabai VL.** Adaptation of Ehrlich ascites carcinoma cells to energy deprivation in vivo can be associated with heat shock protein accumulation. *J Cell Physiol* 165: 1–6, 1995.
19. **Latchman DS.** Heat shock proteins: protective effect and potential therapeutic use. *Int J Mol Med* 2: 375–381, 1998.
20. **Li GC, Li L, Liu RY, Rehman M, and Lee WMF.** Hsp70 protects cells from thermal stress even after deletion of its ATP-binding domain. *Proc Natl Acad Sci USA* 89: 2036–2040, 1992.
21. **Loktionova SA, Ilyinskaya OP, and Kabakov AE.** Early and delayed tolerance to simulated ischemia in heat-preconditioned endothelial cells: a role for HSP27. *Am J Physiol Heart Circ Physiol* 275: H2147–H2158, 1998.
22. **Loktionova SA and Kabakov AE.** Protein phosphatase inhibitors and heat preconditioning prevent Hsp27 dephosphorylation, F-actin disruption and deterioration of morphology in ATP-depleted endothelial cells. *FEBS Lett* 433: 294–300, 1998.
23. **Mestril R, Chi SH, Sayen MR, O'Reilly K, and Dillmann WH.** Expression of inducible stress protein 70 in rat heart myogenic cells confers protection against simulated ischemia-induced injury. *J Clin Invest* 93: 759–767, 1994.
24. **Michels AA, Kanon B, Konings AWT, Ohtsuka K, Bensaude O, and Kampinga HH.** Hsp70 and Hsp40 chaperone

activities in the cytoplasm and the nucleus of mammalian cells. *J Biol Chem* 272: 33283–33289, 1997.

25. **Michels AA, Nguyen VT, Konings AWT, Kampinga HH, and Bensaude O.** Thermostability of a nuclear-targeted luciferase expressed in mammalian cells. Destabilizing influence of the intranuclear microenvironment. *Eur J Biochem* 234: 382–389, 1995.

26. **Nagata K.** Regulation of thermotolerance and ischemic tolerance. In: *Stress-Inducible Cellular Responses*, edited by Feige U, Morimoto RI, Yahara I, and Polla B. Basel: Birkhauser, 1996, p. 467–481.

27. **Nguyen VT and Bensaude O.** Increased thermal aggregation of proteins in ATP-depleted mammalian cells. *Eur J Biochem* 220: 239–246, 1994.

28. **Nguyen VT, Morange M, and Bensaude O.** Protein denaturation during heat shock and related stress. *Escherichia coli* β-galactosidase and *Photinus pyralis* luciferase inactivation in mouse cells. *J Biol Chem* 264: 10487–10492, 1989.

29. **Nollen EAA, Brunsting JF, Roelofsen H, Weber LA, and Kampinga HH.** In vivo chaperone activity of heat shock protein 70 and thermotolerance. *Mol Cell Biol* 19: 2069–2079, 1999.

30. **Nollen EAA, Kabakov AE, Brunsting JF, Kanon B, Hohfeld J, and Kampinga HH.** Modulation of in vivo HSP70 chaperone activity by Hip and Bag-1. *J Biol Chem* 276: 4677–4682, 2001.

31. **Palleros DR, Fink AL, and Welch WJ.** Interaction of hsp70 with unfolded proteins: effects of temperature and nucleotides on the kinetics of binding. *Proc Natl Acad Sci USA* 88: 5719–5723, 1991.

32. **Palleros DR, Reid KL, Shi L, Welch WJ, and Fink AL.** ATP-induced protein-hsp70 complex dissociation requires K^+ and does not involve ATP hydrolysis. Analogy to G proteins. *Nature* 365: 664–666, 1993.

33. **Stege GJJ, Li L, Kampinga HH, Konings AWT, and Li GC.** Importance of ATP-binding domain and nucleolar localization domain of hsp70 in the protection of nuclear proteins against heat-induced aggregation. *Exp Cell Res* 214: 279–284, 1994.

34. **Su CY, Chong KY, Owen OE, Dillmann WH, Chang C, and Lai CC.** Constitutive and inducible hsp70s are involved in oxidative resistance evoked by heat shock or ethanol. *J Mol Cell Cardiol* 30: 587–598, 1998.

35. **Tuijl MJM, van Bergen en Henegouwen PMP, van Wijk R, and Werkleij AJ.** The isolated rat-neonatal cardiomyocyte used in an vitro model for "ischemia." II. Induction of the 68 kDa heat shock protein. *Biochim Biophys Acta* 1091: 278–284, 1991.

36. **Van Why SK, Mann AS, Thulin G, Zhu XH, Kashgarian M, and Siegel NJ.** Activation of heat-shock transcription factor by graded reductions in renal ATP, in vivo, in rats. *J Clin Invest* 94: 1518–1523, 1994.

37. **Wagstaff MJD, Collaco-Moraes Y, Smith J, de Belleroche JS, Coffin RS, and Latchman DS.** Protection of neuronal cells from apoptosis by Hsp27 delivered with a Herpes Simplex Virus-based vector. *J Biol Chem* 274: 5061–5069, 1999.