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Research Article

Expression of heat shock protein 70 (hsp70) is stimulated during ischemia, but its proposed cytoprotective function during metabolic stress has remained conjectural. We introduced a human hsp70 gene into mouse 10T1/2 cells and assessed the susceptibility of these cells to injury in response to conditions that mimic ischemia. Transiently transfected cells, in the absence of stress, expressed human hsp70 to levels equal to or greater than those induced by heat shock, as assessed by RNAse protection, immunoblot, and immunohistochemical analyses. By comparison to cells transfected with a control plasmid, cells expressing the human hsp70 transgene were resistant to injury induced by glucose deprivation and inhibition of mitochondrial respiration. These results provide direct evidence for a cytoprotective function of hsp70 during metabolic stress.

Human Heat Shock Protein 70 (hsp70) Protects Murine Cells from Injury during Metabolic Stress

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Abstract

Expression of heat shock protein 70 (hsp70) is stimulated during ischemia, but its proposed cytoprotective function during metabolic stress has remained conjectural. We introduced a human hsp70 gene into mouse 10T1/2 cells and assessed the susceptibility of these cells to injury in response to conditions that mimic ischemia. Transiently transfected cells, in the absence of stress, expressed human hsp70 to levels equal to or greater than those induced by heat shock, as assessed by RNAse protection, immunoblot, and immunohistochemical analyses. By comparison to cells transfected with a control plasmid, cells expressing the human hsp70 transgene were resistant to injury induced by glucose deprivation and inhibition of mitochondrial respiration. These results provide direct evidence for a cytoprotective function of hsp70 during metabolic stress. (*J. Clin. Invest.* 1993. 92:503–508.) Key words: heat shock protein 70 • stress proteins • ischemia • cytoprotection

Introduction

The interval between the onset of coronary artery occlusion and the restoration of blood flow determines the extent of irreversible tissue injury (1) and is an important determinant of the clinical outcome of thrombolytic therapy for acute myocardial infarction (2). A variety of approaches to minimize cellular injury during myocardial ischemia have been proposed (3, 4), but the alacrity with which irreversible cytotoxicity follows coronary occlusion remains a major clinical problem. The proposed cytoprotective effects of major stress proteins during thermal stress (5–8), and the observation that expression of stress proteins is stimulated by ischemia (9–11), have suggested that these proteins also may have an important role in maintaining or restoring intracellular structures critical for cell survival following metabolic stress. Evidence for this putative cytoprotective function of heat shock protein 70 (hsp70)[1] or other stress proteins has, however, been largely circumstantial.

In previous investigations we have explored the intracellular signaling mechanisms that trigger expression of stress proteins during metabolic stress (12, 13). In the present study we address the functional significance of hsp70 in metabolically stressed cells. Our major hypothesis was that loading cells with high concentrations of hsp70 before the onset of a severe metabolic stress would extend the period of time before irreversible injury ensued. The results confirm this prediction, and have implications for the design of further investigations in which gene transfer strategies are used to limit ischemic injury in intact tissues of living animals.

Methods

Plasmid constructions. A 2.3-kb BamHI-HindIII fragment containing a full length human hsp70 cDNA (14) kindly provided by R. I. Morimoto was inserted downstream of the human β-actin promoter (15). The resulting construction, which also carries a neomycin resistance gene controlled by the SV40 early promoter, was termed pHβ-hsp70, and its identity was verified by restriction mapping and sequencing of the junctions. The same plasmid backbone (pHβ-SVneo) was used to place the firefly luciferase gene (16) under the control of the human β-actin promoter in the construction termed pHβ-luc. Plasmid pHβ-heat shock transcription factor contains a truncated version of heat shock transcription factor from *Saccharyomyces cerevisiae* that includes the DNA binding domain (17) but lacks sequences involved in transcriptional activation, placed 3′ to the β-actin promoter. The protein encoded by this plasmid binds specifically to the heat shock element in a mammalian hsp70 promoter (not shown), and we are currently testing the possibility that truncated forms of heat shock factor may function as dominant negative inhibitors of the heat shock response. In this study, plasmids pHβ-heat shock transcription factor and pHβ-SVneo (which carries no protein coding sequences downstream of the β-actin promoter) were employed only as negative controls.

Conditions for gene transfer and metabolic stress. Mouse 10T1/2 cells were propagated in DME plus 10% fetal bovine serum (growth medium). At 80% confluence, cells were trypsinized and suspended at 2×10^7 cells/ml. Plasmids were introduced by electroporation (320 mV, 960 μFD) of 10^7 cells with 60 μg of total input DNA, comprised of a mixture of 10 μg of pHβ-luc, 40 μg sonicated salmon sperm DNA, and 10 μg of either pHβ-SVneo or pHβ-hsp70.

After electroporation, cells were plated in 60-mm culture dishes containing growth medium with 5 mM butyric acid. On the next day

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1. *Abbreviation used in this paper:* hsp70, heat shock protein 70.

after transfection, cells either were placed in fresh growth medium (unstressed) or were shifted to glucose-free DME containing 2×10^{-4} M rotenone (metabolic stress) for 2–4 h. Previous experiments indicated that these conditions of metabolic stress induce expression of endogenous hsp70 and evoke severe intracellular acidosis and ATP depletion (13). After the 2–4-h period of severe substrate deprivation, the cells were harvested on the following day after maintenance overnight in medium containing 2×10^{-4} M rotenone but supplemented with glucose (Opti-MEM).

Cells stably transformed with pHβ-SVneo or pHβ-hsp70 were selected by growth in increasing concentrations of G418. To assess expression of mouse hsp70, 10T1/2 cells were heat shocked at 43°C for 30 min. More severe heat shock (44°C for 60 min) was uniformly lethal in cells that did not express the hsp70 transgene. To provide a positive control for detection of human hsp70 mRNA, HeLa cells were heat shocked at 43°C for 30 min.

Analysis of transgene expression. Expression of human hsp70 mRNA was examined by RNAse protection assays (Ambion Ribonuclease Protection Assay RPA II), using a 450-nucleotide antisense RNA transcribed in vitro as the probe. This probe has the advantage of including a segment complementary to mouse hsp70 mRNA, such that both human and mouse transcripts could be detected with the same probe, but distinguished on the basis of size of the region protected from nuclease (Fig. 1 *A*). A second probe protects a 250-nucleotide region of mouse β-actin mRNA (18) and provided an internal control for the integrity of RNA within each sample. Synthesis of human hsp70 protein was detected by immunoblot assays (19), and by an immunoperoxidase staining procedure (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA) using monoclonal antibody C922F3A-5 (StressGen Biotechnologies Corp., Sidney, Canada) directed against inducible forms of hsp70.

Cytotoxicity assays. Conventional assays of cytoxicity based on release of intracellular enzymes were unsuitable to assess the effects of transient expression of the hsp70 transgene, since only a minority of cells in the population take up the foreign DNA. To circumvent this limitation, we devised a method designed to assess cytotoxicity selectively in the population of cells that were successfully transfected. The assay was based on the determination of luciferase activity, expressed from a plasmid (pHβ-luc) cotransfected with either the hsp70 expression plasmid (pHβ-hsp70) or with the vector plasmid alone (pHβ-SVneo). Such cotransfection of heterologous plasmids results in simultaneous uptake of both plasmids in the majority of cells that are successfully transfected (20). Since luciferase is a nonsecreted, intracellular enzyme localized to peroxisomes, we reasoned that declines in the residual luciferase activity associated with adherent cells would reflect cellular injury. For simplicity, luciferase activity detected in extracts of adherent cells surviving metabolic stress, calculated as a percentage of the activity in unstressed cells, was chosen as the primary marker of cell viability. In this assay system, the retention of intracellular enzymes in the face of potentially lethal stress is sampled only in transfected cells, since nontransfected cells do not produce luciferase. In addition, the calculations are independent of the fraction of cells that are successfully transfected, so long as expression of luciferase in the absence of stress is consistent among sister cultures transfected in parallel within the same experiment.

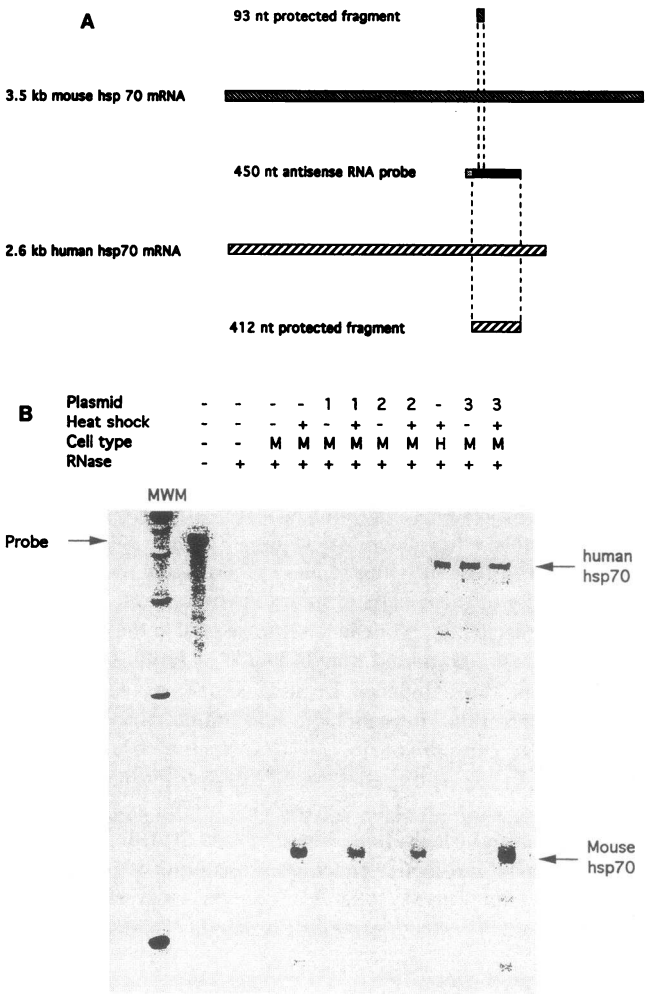


Figure 1. Expression of human and mouse hsp70 mRNA in mouse 10T1/2 cells. (*A*) Schematic representation of the antisense (complementary) RNA probe and the regions of human and mouse hsp70 protected from nuclease due to hybrid formation with the probe. (*B*) RNAse protection assay of total RNA extracted from mouse 10T1/2 cells (*M*) or human HeLa (*H*) cells after either heat shock (+) or unstressed (heat shock −) conditions. Cells were transfected 24 h previously by electroporation of plasmids bearing the human β-actin promoter linked either to no cDNA sequence (pHβ-SVneo; plasmid 1), a truncated form of yeast heat shock factor (pHβ-tHSF; plasmid 2), or human hsp70 cDNA under the control of the human β actin promoter (pHβ-hsp70; plasmid 3). Mock transfected cells are designated as plasmid −. Bands corresponding to full length probe, human hsp70 mRNA, and mouse hsp70 mRNA are identified by arrows.

Results

Human hsp70 is expressed in murine cells after introduction of pHβ-hsp70. RNAse protection assays (Fig. 1) demonstrated the presence of human hsp70 mRNA in cultures transiently transfected with pHβ-hsp70. As indicated in Fig. 1 *A*, the probe can distinguish between human and mouse hsp70 mRNA, and these assays provide definitive evidence for expression of the human transgene. Immunoblots probed with a monoclonal antibody prepared against human hsp70 indicated that transfected cells expressed elevated levels of hsp70 mRNA, even when unstressed (Fig. 2). This antibody, however, cross-reacts with mouse hsp70 so that the mouse and human proteins cannot be distinguished by this assay.

Within the total population of cells in these cultures, the human hsp70 transgene generated levels of hsp70 mRNA and protein approximately equal to the levels of endogenous mouse hsp70 induced by heat shock alone in these cells (Figs. 1 and 2). It is likely, however, that levels of human hsp70 mRNA and protein within individual cells expressing the transgene reached levels greater than those produced by heat shock, since only a minority of cells present within the entire population

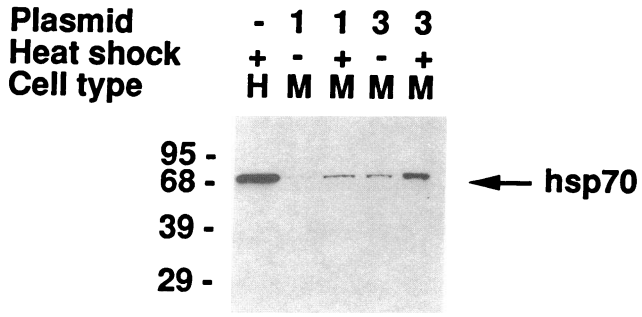


Figure 2. Immunoblot of hsp70 protein after transfection of mouse 10T1/2 cells. Soluble protein extracts were prepared from untransfected human cells (HeLa; *H*) or mouse (*M*) cells transfected either with vector alone (pHβ-SVneo; plasmid 1) or the hsp70 expression plasmid (pHβ-hsp70; plasmid 3). After transfection, cells were maintained either under unstressed, control conditions (heat shock −) or subjected to heat shock (+). The protein band representing hsp70 is indicated (*arrow*). Unlike the RNAse protection assays, this immunoblot technique does not distinguish between the mouse and human gene products, but the total pool of hsp70 is increased by tranfection of the human transgene. The position of migration of several molecular size markers is shown along the vertical axis.

take up and express foreign DNA. Histochemical staining of hsp70 in unstressed cells transfected with pHβ-hsp70 (Fig. 3) illustrates the selective expression of the transgene in a subset of cells within the entire population, and provides support for the above assertion.

The conclusion that unstressed cells expressing the transgene accumulate levels of hsp70 in excess of those produced by heat shock is buttressed further by the results shown in Fig. 4. No staining for hsp70 is detectable in unstressed cells transfected with the control plasmid (pHβ-SVneo) (Fig. 4 *A*) but all cells express hsp70 after heat shock (Fig. 4 *B*). Unstressed cells transfected with pHβ-hsp70 (Fig. 4 *C*) can express concentrations of hsp70 that appear to be considerably in excess of those induced by heat shock in cells lacking the hsp70 transgene (compare Fig. 4, *B* and *C*, which were performed in parallel and are shown at the same magnification and under the same staining conditions).

Transient expression of human hsp70 protects cells against injury induced by metabolic stress. Cells transfected with the control plasmid (pHβ-SVneo) exhibited increasing cytotoxicity with increasing durations of exposure to severe metabolic stress (Fig. 5). Cell injury was minimal after 2 h, but half of the luciferase activity within adherent cells was lost by 3 h and more than three-fourths was lost by 4 h of metabolic stress. With these longer durations of energy starvation, many cells became nonadherent, indicating irreversible cell injury.

In contrast, cells transfected with pHβ-hsp70 were relatively resistant to cell injury resulting from metabolic stress. The most striking difference between hsp70-expressing cells and control cells was observed at the 3-h time point: under

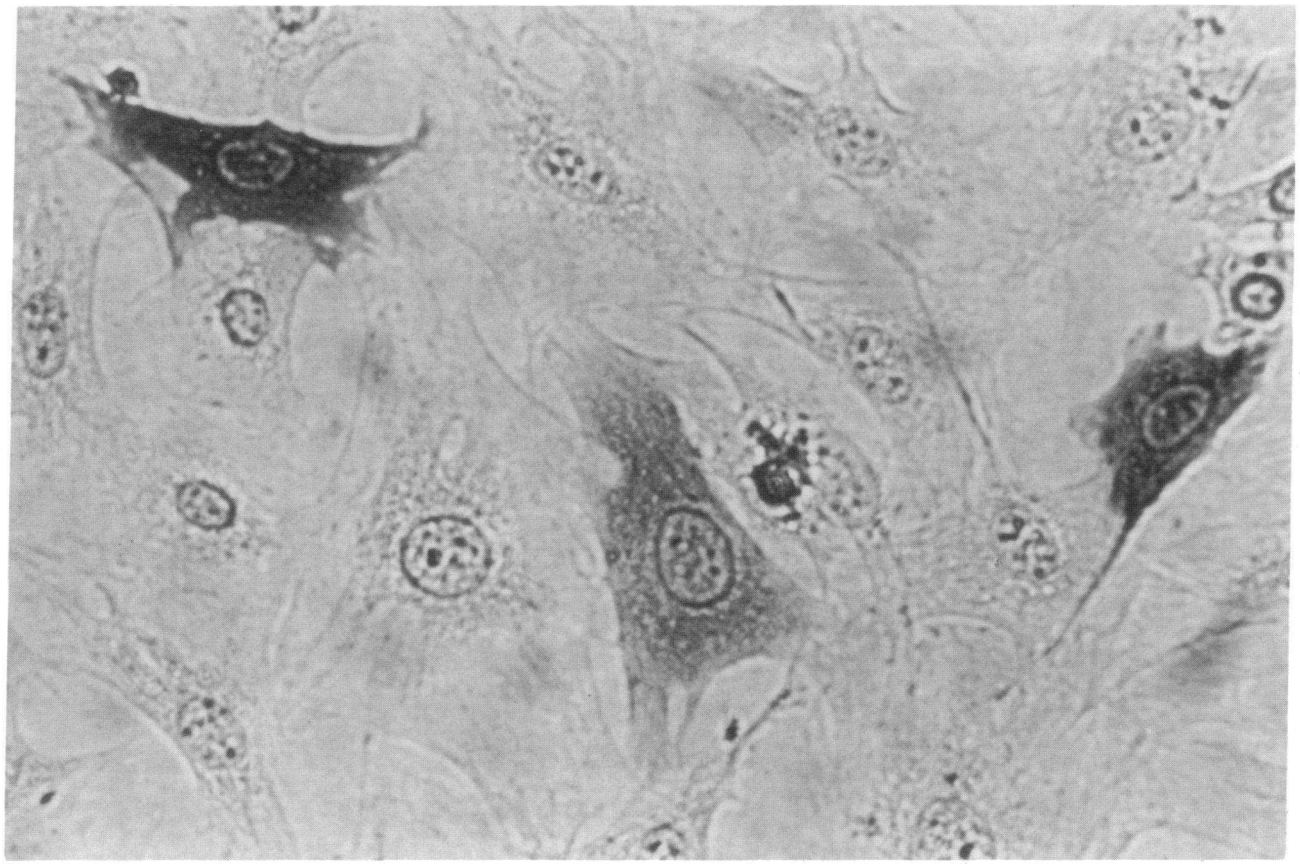
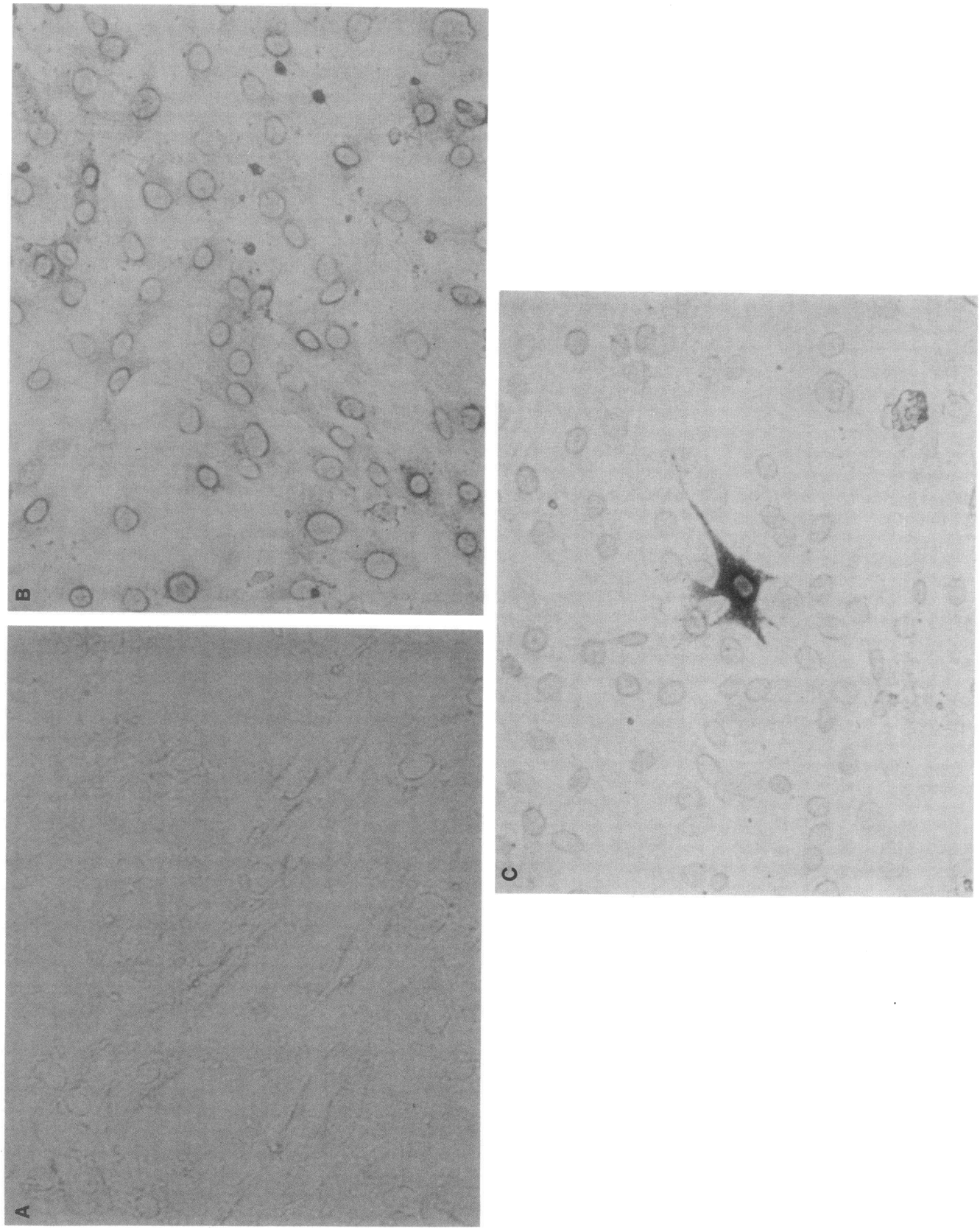


Figure 3. Immunohistochemical staining of hsp70 protein after transfection of mouse 10T1/2 cells with pHβ-hsp70. Darkly stained cells are expressing high concentrations of hsp70 in the absence of stress. Approximately 10% of cells stain positive with the monoclonal antibody directed against hsp70. ×1,000.


B
C
A

conditions in which only 53% of luciferase activity was retained in vector-transfected cells, virtually no cell injury was detected in the cells transfected with pHβ-hsp70. Hsp70-expressing cells remained susceptible to injury resulting from more prolonged metabolic stress (4 h), but such injury was less marked in cells transfected with pHβ-hsp70 (55 vs. 23% retention of luciferase activity).

Stable transformation of 10T1/2 cells with a human hsp70 transgene. Selection for the neomycin resistance marker carried in pHβ-hsp70 permitted us to derive several clonal lines with stable chromosomal integration of the human hsp70 transgene. Human hsp70 mRNA was detectable in these cells (not shown), but was less abundant than in transiently transfected cells. In addition, expression of hsp70 protein in these stably transformed cell lines, as assessed by immunoperoxidase staining (not shown), was absent or inconsistent. No densely staining cells resembling those depicted in Figs. 3 and 4 *C* were observed. These cells also exhibited altered growth characteristics with slower doubling times. These results suggest that chronic elevation of hsp70 may constrain proliferative growth of unstressed cells, and that mutations limiting constitutive expression of the hsp70 transgene confer a selective growth advantage.

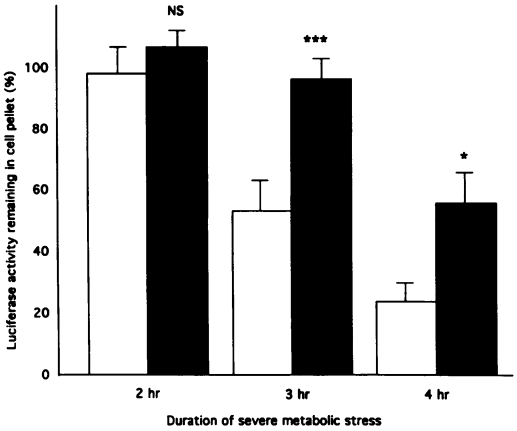


Figure 5. Effects of hsp70 transgene expression on cell injury induced by severe metabolic stress. Luciferase activity was measured in the cell pellet 24 h after cells were subjected to 2–4 h of severe metabolic stress and calculated as a percentage of the activity present in unstressed cells. Data are presented as mean values (±SE) from duplicate determinations in five independent experiments. Control cultures (*open bars*) transfected with pHβ-SVneo were more susceptible to cell injury induced by metabolic stress than cells transfected with pHβ-hsp70 that express the human transgene (*closed bars*). Differences between control and hsp-70 expressing cells were statistically significant (unpaired Student's *t* test) at both the 3 h (***$P < 0.005$) and 4 h (*$P < 0.025$) time points, but were not significant (NS) after 2 h of metabolic stress.

Discussion

The major finding of this study is that mammalian cells can be protected from cell injury resulting from severe metabolic stress by forced expression of hsp70. This study provides direct evidence that hsp70 exerts a cytoprotective function during metabolic stress resulting from glucose deprivation and blockade of mitochondrial respiration.

The molecular mechanisms of this cytoprotective effect of hsp70 remain to be elucidated, but may be related to the known functions of this class of proteins in normal, unstressed cells. Hsp70 and related proteins serve as molecular chaperones by binding to nascent polypeptides and by preventing inappropriate folding until proteins have been sorted into specific intracellular compartments or assembled into macromolecular complexes (5, 21–24). Protein denaturation appears to be a common denominator of many stress states, and is thought to unmask binding sites for hsp70 (25–27). Binding of hsp70 probably maintains proteins in a state from which they can be refolded into functional configurations after the stress condition has been relieved so that normal cellular processes can resume more rapidly. Apparently, the endogenous pool of hsp70 available for accomplishing these cytoprotective functions is inadequate to saturate all sites of possible beneficial action, since increasing this pool by forced overexpression results in additional cytoprotective effects.

Previous studies have noted that prior heat stress can limit myocardial injury following subsequent coronary occlusion (11, 28), suggesting that the abundance of hsp70 or other stress proteins is a determinant of cell survival during myocardial ischemia. Our current results support the hypothesis that hsp70 is cytoprotective during metabolic stresses encountered by ischemic cells, and demonstrate further that the endogenous defense mechanisms engaged by cells during energy deprivation do not constitute a biological limit. It is possible, at least under some conditions, to use genetic approaches to improve cell survival in the face of severe metabolic perturbations.

While transient elevations of hsp70 exert a cytoprotective function during metabolic stress, our attempts to derive cell lines that maintain persistently high concentrations of hsp70 were unsuccessful. It appears that chronic overexpression of hsp70 is accompanied by constraints on proliferative growth of cells under nonstressed conditions. While this study was in progress, similar results were reported from studies of unstressed insect cells stably transformed to overexpress hsp70 (29). It remains to be determined whether postmitotic cardiac myocytes or quiescent endothelial cells of the adult heart also would suffer deleterious effects from sustained elevations of hsp70.

In summary, transient expression of a human hsp70 transgene can extend the period of time required to compromise viability of mammalian cells subjected to metabolic stresses similar to those induced in tissues by deprivation of blood flow. The relevance of these findings to cell survival during authentic, rather than simulated, ischemia in intact tissues should be testable using transgenic animal models, and such studies represent a logical extension of this work.

Figure 4. Comparison of hsp70 protein abundance in mouse 10T1/2 cells after heat shock or transfection. No staining is detected in unstressed cells transfected with pHβ-SVneo (*A*), but endogenous hsp70 is induced in all cells by heat shock (*B*). Introduction of the human hsp70 transgene carried in pHβ-hsp70 generates an abundant pool of hsp70 in unstressed cells that have taken up the foreign DNA (*C*). ×400.

Acknowledgments

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