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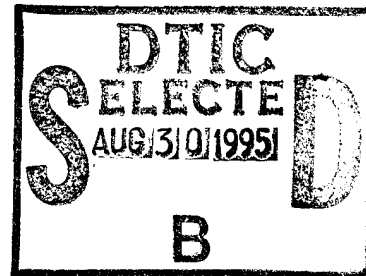
TITLE: SKELETAL MUSCLE ISCHEMIA AND HEAT SHOCK PROTEINS

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13. ABSTRACT (Maximum 200 words) Blood loss resulting in decreased organ perfusion and subsequent ischemic injury of cardiac and skeletal muscle presents a significant problem for the soldier in combat. Recent findings have indicated that different forms of noxious stress including exposure to increased temperature, noxious chemical agents, and ischemia lead to increased expression of heat shock proteins (hsp) which have a protective effect against injury induced by noxious stimuli. We will determine in this proposal if a rat skeletal muscle derived permanent cell line, L6 cells, expressing increased amounts of hsp70 will show protection against damage induced by simulated ischemia. To generate L6 cells which permanently overexpress the inducible hsp70 proteins, cells will be transfected with a neomycin resistance gene and the inducible hsp70 gene. Stable lines will be selected by growing L6 cells in the presence of neomycin. Cells which have the neomycin resistance gene and the hsp70 gene incorporated into their DNA will survive. Such stably transfected L6 cell lines will then be exposed to simulated ischemia consisting of hypoxia, absence of glucose, low toxicity, and resultant ischemic damage will be determined by quantitating cell viability measured in colony formation assays, the inhibition of protein synthesis and the release of cytoplasmic enzymes like creatine kinase. These studies will determine if inducible hsp70 exerts a protective effect against ischemia mediated muscle injury. Demonstrating a protective effect of hsp70 protein will make it a useful agent to reduce ischemic muscle damage in soldiers exposed to muscle injury in combat.

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INTRODUCTION

This research effort is directed at subobjective 2 as defined in the recent shock trauma mediators review memo from the U.S. Army Institute of Surgical Research. Specifically, we aimed at the prevention of secondary damage after hemorrhage by temporizing fundamental physiological or biochemical processes leading to cell death and organ failure. For this purpose, increased expression of the inducible heat shock 70 (hsp70i) will be employed. Hsp70i works as a chaperone attaching to short hydrophobic peptide sequences as they are exposed in ischemic cells in which proteins undergo denaturation. By the association of hsp70 with such protein sequences further protein aggregation and cell damage is prevented. This leads to a faster recovery of cell function after the ischemic or hemorrhage episodes. Our results which are further summarized in the body indicate that overexpression of hsp70 in the heart of transgenic mice leads to marked protection against cardiac ischemic injury (1). In preliminary studies in collaboration with Dr. P. Lyden, UCSD and Dr. B. Nishimura, UCLA, we have found that the hsp70 transgene is also expressed in CNS-neurons and exerts a protective effect against ischemic damage in the brain as judged by behavioral scores in these transgenic mice. These transgenic mice were also made available to Dr. Dave at the Walter Reed Army Research Institute for studies on brain ischemia. We could also show in preliminary experiments that the tyrosine kinase inhibitor, Herbimycin A, induces the expression of the inducible hsp70 gene in cardiac myocytes. If a tyrosine kinase inhibitor-like compound can also exert a protective effect against noxious cell damage, such compounds could be used in the future for multi-organ protection. Furthermore, the inducible hsp70 gene has been cloned by us into a replication deficient human adenovirus 5 vector and is used to infect cardiac myocytes in cell culture resulting in high levels of transgene expression and protection against ischemic injury. The details of this work are described below.

BODY

In a recent study which was published in the Journal of Clinical Investigation 1995;95:1446-1456 (1), we constructed a transgenic mouse line overexpressing the inducible heat shock protein 70 (hsp70) in heart, skeletal muscle and brain. A human cytomegalovirus enhancer chicken β -actin promoter drove expression of the rat inducible hsp70 transgene in cardiac muscle, skeletal muscle and brain. Heterozygous transgenic mice had very high levels of the hsp70 transgene protein expressed in their hearts. To determine if high levels of expression of hsp70 mediated a protective effect against ischemic injury, we proceeded in the following manner. Isolated perfused hearts were used in a Landgendorf type of set-up and subjected to 20 mins of warm 37°C, 0-flow ischemia and up to 120 mins of reflow while contractile recovery and creatine kinase efflux were measured. Myocardial infarction was determined by triphenyl-tetrazolium staining and was expressed as infarct size as a percent the bed of risk. In transgene positive mice compared with transgene negative mice, the zone of infarction was reduced by 40%; contractile function at 30 mins of reflow was doubled; and the efflux of creatine kinase was reduced by 50%. Our findings suggest for the first time, therefore, that increased myocardial expression of the inducible hsp70 results in protection of the heart against ischemic injury. These studies point to a potential therapeutic usefulness of increased hsp70 gene expression. As indicated above in preliminary studies, we also found that in these transgenic mice increased expression of the hsp70

transgene in the brain leads to a protective effect against CNS ischemic damage. In order to further pursue potential protective effects of hsp70, we cloned the rat hsp70 transgene into an E1 deleted replication deficient human adenovirus 5 vector. Infection of muscle cells with this virus leads to high levels of expression of the transgene protein (see Fig. 1). Furthermore, very recent data indicate that infection of neonatal myocytes with the adenovirus expressing the hsp70 transgene leads to marked protection against ischemic injury as measured by creatine kinase release. In contrast, infecting myocytes with an adenovirus coding for β -galactosidase does not alter the ischemic injury in those cells in comparison to native neonatal myocytes not infected with an adenovirus. Furthermore in recent experiments, we exposed neonatal myocytes to the tyrosine kinase inhibitor, Herbimycin A. Exposure of neonatal myocytes to Herbimycin A leads to a significant induction of hsp70 mRNA levels in these myocytes (see Fig. 2). More recent data indicate that increased hsp70 mRNA levels lead to increased amounts of protein. We are currently in the process of determining if increased hsp70 expression leads to a protective effect in neonatal myocytes. Should it be possible to develop tyrosine kinase inhibitor derivatives which by themselves have no cytotoxic effect but markedly induce hsp70 mRNA levels, such compounds could be used for expression of hsps in multiple organs leading to multi-organ protection for the soldier in a combat situation.

CONCLUSION

In summary, we have obtained convincing evidence that increased expression of the inducible hsp70 in the heart leads to a significant protection against ischemic injury. Furthermore, a protective effect is also apparent in preliminary data protecting the brain against ischemic injury in transgenic mice expressing increased levels of hsp70. To harness the protective effect of hsp70 in the future for the soldier in a combat situation, we have cloned the hsp70 into adenovirus vectors which lead to a strong expression of the transgene are able to transmit a protective effect in cell culture. A future gene therapy-type of approach using viral vectors which are currently under development may be available to the soldier in a combat situation. In addition, we are pursuing a pharmacological approach using tyrosine kinase inhibitors to increase the expression of the endogenous hsp70 and in this way mediate a protective effect.

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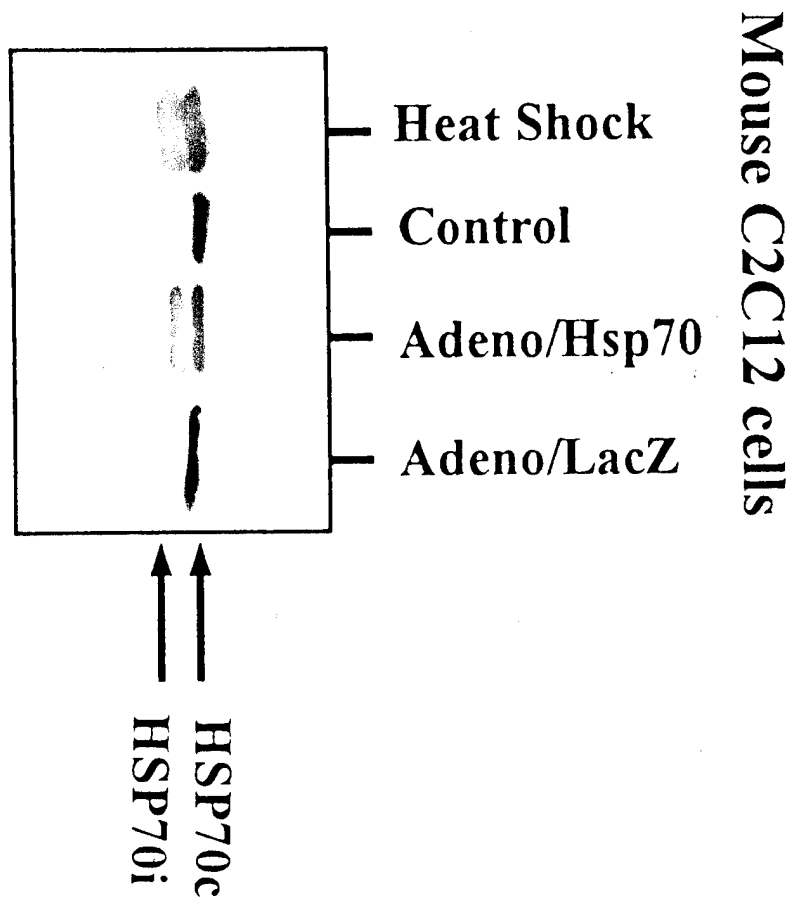


FIGURE 1: *Expression of Rat Inducible hsp70 Transgene Encoded by Adenovirus Vectors in Mouse C2C12 Cells:* Cells of the mouse skeletal muscle line, C2C1212 (106 cells/plate), were maintained under different conditions and proteins were prepared for Western blotting and probed with antibodies recognizing the constitutive (hsp70c) and inducible (hsp70i) protein. In heat shocked cells (lane 1) and cells infected with human adenovirus 5 encoding for hsp70i (lane 3), the hsp70i protein is present. In C2C12 cells maintained under control conditions (lane 2) or in cells infected with adenovirus coding for LacZ (lane 4), only hsp70c is detectable.

Rat Neonatal Cardiomyocytes

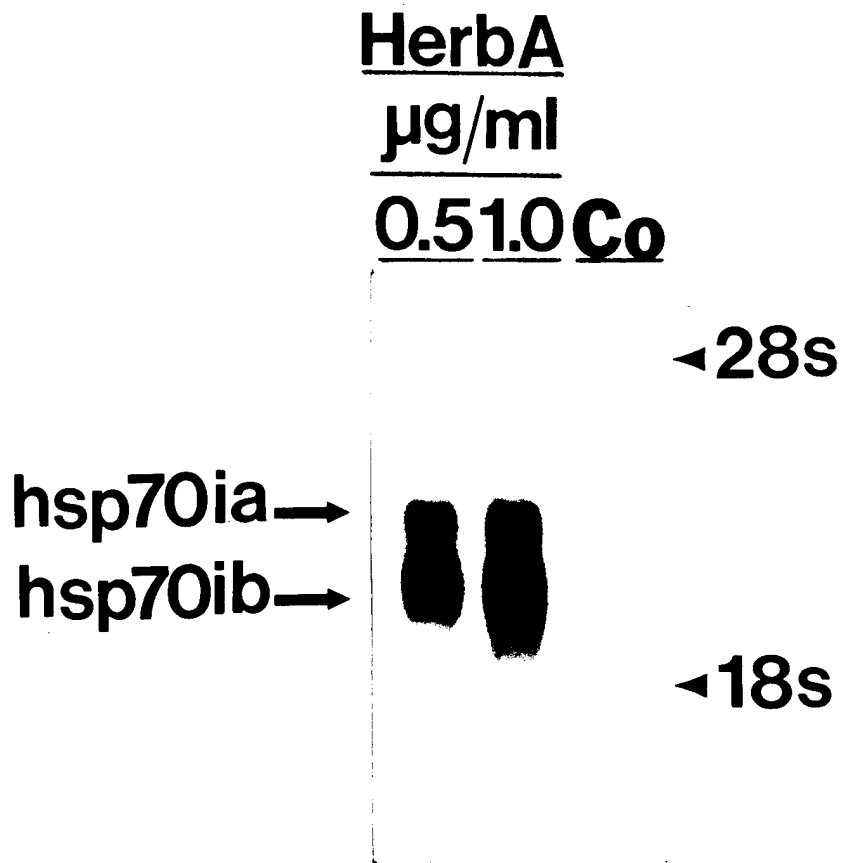


FIGURE 2: *Induction of hsp70i mRNA in Rat Neonatal Cardiac Myocytes by Herbimycin A:* Rat neonatal myocytes 1×10^6 per plate were incubated with Herbimycin A at $0.5 \mu\text{g}$ Herbimycin A per ml or $1 \mu\text{g}$ Herbimycin A per ml or without Herbimycin A. RNA was prepared and a Northern blot was probed with cDNA coding for hsp70. The two inducible hsp70 species, hsp70ia and hsp70ib, are formed at significant amounts in Herbimycin A treated but in control myocytes. The position of 28S and 18S RNA is indicated.

Overexpression of the Rat Inducible 70-kD Heat Stress Protein in a Transgenic Mouse Increases the Resistance of the Heart to Ischemic Injury

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Abstract

Myocardial protection and changes in gene expression follow whole body heat stress. Circumstantial evidence suggests that an inducible 70-kD heat shock protein (hsp70i), increased markedly by whole body heat stress, contributes to the protection. Transgenic mouse lines were constructed with a cytomegalovirus enhancer and β -actin promoter driving rat hsp70i expression in heterozygote animals. Unstressed, transgene positive mice expressed higher levels of myocardial hsp70i than transgene negative mice after whole body heat stress. This high level of expression occurred without apparent detrimental effect. The hearts harvested from transgene positive mice and transgene negative littermates were Langendorff perfused and subjected to 20 min of warm (37°C) zero-flow ischemia and up to 120 min of reflow while contractile recovery and creatine kinase efflux were measured. Myocardial infarction was demarcated by triphenyltetrazolium. In transgene positive compared with transgene negative hearts, the zone of infarction was reduced by 40%, contractile function at 30 min of reflow was doubled, and efflux of creatine kinase was reduced by ~50%. Our findings suggest for the first time that increased myocardial hsp70i expression results in protection of the heart against ischemic injury and that the antiischemic properties of hsp70i have possible therapeutic relevance. (*J. Clin. Invest.* 1995. 95:1446-1456.) Key words: myocardial infarction • myocardial protection • heat shock proteins

Introduction

A number of independent investigators have shown that 24 h after whole body temperature elevation to 42°C for 15 min the heart shows enhanced protection against ischemic injury (1-7). This whole body heat stress procedure has been shown to reduce infarct size in vivo (1-3) and in vitro (4) and to enhance

postischemic contractile function in vitro (5-7). 24 h after heat stress a change must therefore occur within the heart that gives rise to this protection. The exact nature of this change is at present uncertain (8, 9). However, a number of lines of evidence suggest that alterations in myocardial stress proteins and/or antioxidant enzymes are of particular importance (8, 9).

Among the myocardial stress proteins increased after heat stress, an inducible member of the hsp70 family (hsp70i)¹ shows marked changes (1-7), and its possible involvement in myocardial protection has been shown by a number of studies. In a papillary muscle model, hsp70i concentration correlated with resistance to substrate deprivation (10), similarly in vivo infarct size was negatively correlated with myocardial hsp70i content (11). Unfortunately both these studies (10, 11) provide only circumstantial evidence to link hsp70i to protection. For example, hsp70i content is likely to be related to the severity of the heat stress procedure and thus co-correlate with other thermally induced changes within the myocardium (10). Other evidence linking hsp70i to myocardial protection has been derived from in vitro studies that demonstrate that an embryonal heart-derived cell line becomes resistant to simulated ischemia after stable transfection with an hsp70i-encoding plasmid (12, 13). However, such studies are not directly relevant to true ischemia in the whole heart.

The antioxidant enzyme catalase is also increased within the myocardium 24 h after whole body heat stress (5). Catalase is relevant to protection since it may be capable of reducing the free-radical injury associated with myocardial ischemia/reperfusion (14). In addition, inhibiting catalase can at least partially abolish post-heat stress protection when contractility is used as the endpoint of injury (15), although results are more difficult to interpret when other endpoints are considered (7, 16).

To overcome these difficulties, attempts have been made to induce myocardial hsp70i more specifically by nonthermal stress. For example, short sublethal episodes of cardiac ischemia both increase myocardial hsp70i (2, 17) and result in cardiac protection (2, 18), but increases also occur in a 60-kD stress protein (2) and in another myocardial antioxidant enzyme, superoxide dismutase (19).

At present it is therefore uncertain which of the changes observed within the myocardium after thermal and other stresses is responsible for protection. This knowledge is a prerequisite to targeted interventions designed to trigger the benefits but avoid the abuse associated with whole body heat stress.

M. S. Marber and R. Mestri contributed equally to this manuscript.

This work was presented in part at the 67th Scientific Session of the American Heart Association in Dallas on 14-17 November 1994 (1994. *Circulation*. 90[Suppl. I]:I-536).

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1. Abbreviations used in this paper: CK, creatine kinase; CMV, cytomegalovirus; hsp70c, constitutive member of the 70-kD heat stress protein family; hsp70i, inducible member of the 70-kD heat stress protein family; mhsp70i, mouse inducible heat stress protein 70; rhsp70i, rat inducible heat stress protein 70.

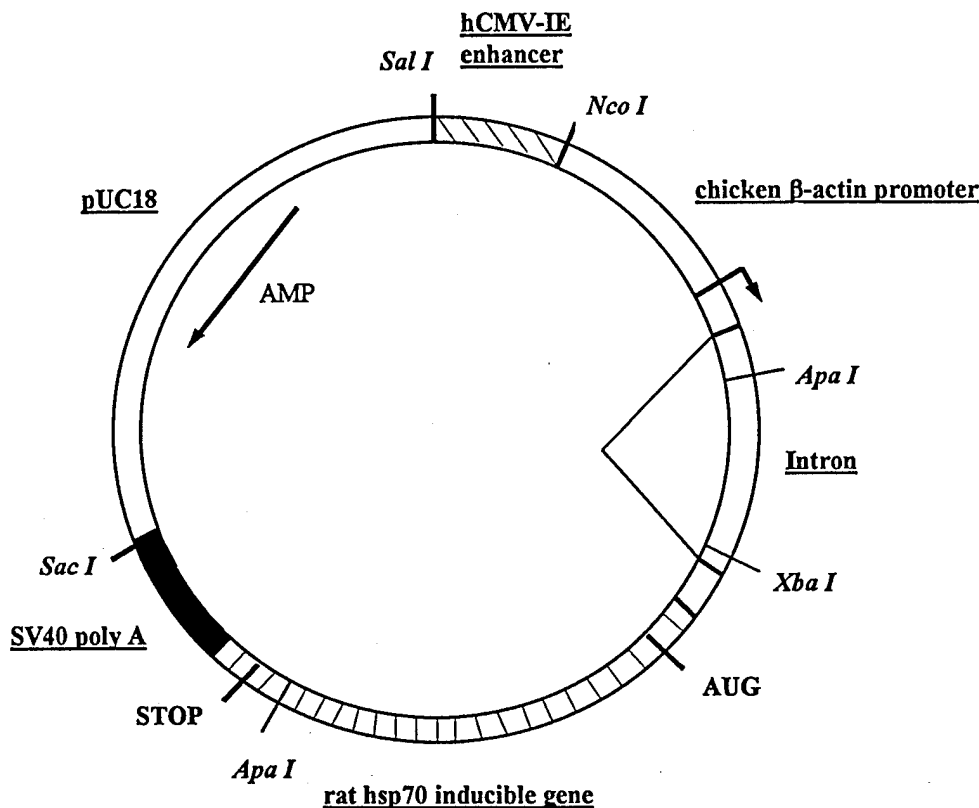


Figure 1. Map of the hCMV β -actin/rhsp70i transgene. The coding region of the rat inducible 70-kD heat shock protein gene (rhsp70i) is under the control of the human CMV immediate-early (hCMV-IE) enhancer and chicken β -actin promoter with first intron. The rhsp70i coding region is followed by the SV40 polyadenylation signal. The SalI to SacI fragment was used to generate transgenic mice. The 3.5-kb fragment generated by ApaI digestion of genomic DNA from the tails of transgenic mice was detected on Southern blots by a probe prepared from the ApaI to XbaI fragment.

Our aim was to determine which component of the myocardial response to stress is likely to be responsible for ischemic protection. In light of the evidence discussed above, together with a wealth of information regarding the protective role of the hsp70 family in a variety of cell types against various injuries (for review see reference 20), we felt that hsp70i was the candidate most likely responsible for protection. We examined this hypothesis by producing a transgenic mouse line overexpressing hsp70i within the heart and determining the resistance of these hearts to ischemia.

Methods

The development of the transgenic mouse line. Transgenic mice were generated using a chimeric transgene consisting of a rat inducible hsp70 (rhsp70i) gene (21) inserted into the vector pCAGGS (22). The rhsp70i amino acid sequence is 95% identical to that of the human hsp70i and 97% identical to that of the mouse hsp70i (21). The pCAGGS construct places the rhsp70i gene under the control of the human cytomegalovirus immediate early enhancer (hCMV-IE) and chicken β -actin promoter with first intron (22) (Fig. 1). The chimeric transgene was cut out of the plasmid by SalI and SacI digestion, purified, and used to generate transgenic mice (23).

In brief, the male pronuclei of fertilized eggs from hyperovulated B6 \times SJL mice were injected with 1–2 μ l of DNA solution at a concentration of 2 μ g/ml, equivalent to 200–400 copies of the transgene. Injected eggs were transferred into the oviduct of pseudopregnant CD1 mice. 20 injected eggs were implanted per mouse, and litters were delivered after 19–20 d of gestation.

When mice were 3 wk old, genomic DNA was isolated from 1-cm tail clips and subjected to Southern blotting as follows. The ApaI digests of genomic DNA were separated by electrophoresis, blotted onto a nylon membrane, and hybridized with a [32 P]-labeled probe. The probe used

was the ApaI to XbaI fragment from the hCMV- β -actin/rhsp70i transgene construct (Fig. 1) and contained the chicken β -actin promoter and intron. If the chimeric transgene was present in the genome, ApaI digestion would result in a 3.5-kb fragment which would specifically hybridize with the probe.

Founder mice, that had integrated the transgene, were bred with mice of the same strain (B6 \times SJL). The resultant litters were analyzed by Southern blotting of tail clips as described above. Transgene positive mice (heterozygotes) and transgene negative littermates were then used for the experiments described below.

The level of expression of the transgene at the transcript and protein level was examined by Northern and Western blotting using protocols that we have described previously (10, 12). Transgene negative littermates served as negative controls, and positive controls were obtained by heat stressing mice to 42°C for 15 min using a technique described elsewhere (24). The possible influence that expression of the chimeric rhsp70i transgene could have on the expression of other heat shock proteins was examined by stripping and rehybridizing Northern membranes with probes for hsp60, hsp90, and hsp27.

The Langendorff perfused mouse heart. Transgene positive and transgene negative mice were anesthetized by intraperitoneal injection of ketamine (Aveco Co, Fort Dodge, IA) 150 mg/kg and xylazine (Lloyd Laboratories, Shenandoah, IA) 24 mg/kg coadministered with 10.8 ml/kg of normal saline and 100 IU of heparin. Once the mouse was deeply anesthetized, the heart was removed by sternotomy and trimmed under a dissecting microscope while in iced Tyrode's solution. The aorta was then cannulated, with a 20G phalanged stainless steel cannula, under the Tyrode's solution to prevent air embolization of the coronary circulation. Once the aorta had been tightly secured using 4/0 silk suture, the cannula was transferred to the perfusion rig. During transfer the cannula was continuously perfused through a side arm with Tyrode's solution under low pressure to ensure that no air entered the cannula or connectors. Side arm perfusion was stopped once the cannula was rigidly attached to the perfusion rig and retrograde perfusion at 80 mmHg had commenced.

The heart was perfused with modified Tyrode's solution of the following composition: NaCl 118.0 mM, NaHCO₃ 24.0 mM, KCl 4.0 mM, NaH₂PO₄ 1.0 mM, CaCl₂ 2.5 mM, MgCl₂ 1.2 mM, di-sodium EDTA 0.5 mM, sodium pyruvate 2.0 mM, glucose 10.0 mM, prefiltered to 0.22 μ m and equilibrated with 95% O₂/5% CO₂.

Under direct vision a small incision was made in the pulmonary artery immediately above the right ventricular outflow, to allow free drainage of coronary effluent. Through this incision a micro-thermocouple (type K) connected to a digital thermometer (Physitemp Bat-12; Sensortek, Clifton, NJ) was introduced retrogradely into the right ventricle. 4/0 silk suture on a round bodied needle was passed through the apex of the heart to attach the apex to a light weight, rigid coupling rod, which in turn was attached to a force transducer (Statham, Gould Inc., Cleveland, OH). The tension of the suture was adjusted to generate a resting (diastolic) force of \sim 1 g.

The temperature of the heart (sensed in the right ventricle) was maintained at 37 \pm 0.2°C by warming the perfusion fluid by means of warm water circulating (Lauda T1, Messgerate, Germany) in the jacketing of a bubble trap and heat exchanger. During ischemia, the temperature of the heart was maintained by lowering the heart and the lower portion of the coupling rod into an organ bath containing modified Tyrode's solution at 37°C. The frequency response of the force transducer, coupling rod, and mounting gantry was flat to at least 50 Hz.

At appropriate time points during the experiment, the force developed by the heart was recorded at fast paper speed (100 mm/s, Gould Mark 200 recorder; Gould Inc.), and coronary effluent was collected over 30 s. These timed collections were weighed to measure coronary flow and then frozen for the subsequent analysis of creatine kinase (CK) activity.

The Langendorff perfusion protocol. The operator was unaware of the transgene status of the mice at the time of Langendorff perfusion.

After commencing retrograde aortic perfusion at a pressure of 80 mmHg, hearts were allowed to stabilize, and after \sim 20 min baseline contractility and coronary flow measurements were made. Hearts were then lowered into the organ bath, and coronary flow was stopped for a period of 20 min. After this period, flow was recommenced at 80 mmHg. At predetermined time points, collections of coronary effluent and records of heart contraction at fast paper speed were made to generate profiles during reperfusion, contractile recovery, heart rate, coronary flow, and CK efflux. At the end of the reperfusion period, the zone of myocardial infarction within each heart was measured as described below.

The assessment of the amount of infarction. At the end of the experimental procedure, the heart was again lowered into the organ bath, and a 10% (wt/vol) solution of triphenyltetrazolium in phosphate buffer (Na₂HPO₄ 88 mM, NaH₂PO₄ 1.8 mM) was infused into the coronary vasculature through the sidearm of the aortic cannula. Once the heart had become discolored (tetrazolium stains the viable myocardium deep red) it was removed from the aortic cannula, blotted dry, weighed, and frozen at -70° C.

At a later date the hearts, while still frozen, were sliced into sections \sim 0.8 mm thick in a plane perpendicular to their long axis and approximately parallel to the atrioventricular groove. Sections were then fixed in 2% paraformaldehyde overnight. The following day, slices were orientated caudal surface upward and compressed, together with a calibration grid, between two Plexiglas plates separated by 0.57-mm spacers. Heart slices were then illuminated, and a magnified video image was digitized (QuickCapture Frame Grabber Board; Data Translation Inc., Marlboro, MA and NIH Image v1.5; National Institutes of Health, Bethesda, MD). For each slice, the area of infarction and the total area of the slice were planimetered (see Discussion), and area was expressed in arbitrary units, calculated by Simpson's rule. These units were then converted to square millimeters by normalizing to the area of the calibration grid at the same magnification. Slices that contained atrial or valvular tissue were excluded from the analysis. The areas of infarction and the overall areas of the individual slices from each heart were then summed. In this manner a total area of infarction and a total area at risk of infarction were derived for each heart. These values were then

multiplied by slice thickness (0.57 mm) to generate volume of infarction and volume at risk of infarction for each heart.

The measurement of CK efflux. The CK concentration in timed aliquots of the coronary effluent was measured spectrophotometrically using a commercial kit (catalogue No. 45-UV; Sigma Immunochemicals, St. Louis, MO). CK was then expressed as activity leaked per min per gram wet weight of heart.

The measurement of myocardial catalase. Catalase is a ubiquitous tissue enzyme that catalyzes the conversion of H₂O₂ to H₂O and O₂. In the heart, it may be capable of attenuating free-radical injury by preventing the conversion of H₂O₂ to more reactive species (14).

Transgene positive and transgene negative mice were anesthetized, hearts were removed, and aortas were cannulated and retrogradely perfused with Tyrode's solution as described above. Once the blood had been washed out of the coronary circulation and the left and right ventricular cavities, hearts were removed from the perfusion rig, weighed, and homogenized for the analysis of catalase using a modification of a previously described method (25).

In brief, 100 mg of heart tissue was Dounce homogenized in 1 ml of isotonic sodium phosphate buffer with 1% ethanol. After centrifugation and the addition of 1% Triton X-100, supernatants were diluted 10-fold in phosphate buffer with 1% ethanol. Two 0.25-ml aliquots were then taken. To one aliquot (test), 2.5 ml of 6 mM H₂O₂ in potassium phosphate buffer was added, and the decomposition of H₂O₂ was allowed to proceed for exactly 3 min after which the reaction was terminated by the addition of 0.5 ml of 6 N H₂SO₄. To the other aliquot (blank), H₂SO₄ was added before H₂O₂. All the above reactions were performed at 4°C. The remaining H₂O₂ in both test and blank was then quenched by the addition of 3.5 ml of 0.01 M KMnO₄, and absorption at 480 nm was recorded to derive Abs_{test} and Abs_{blank}, respectively. The absorbance at 480 nm of 3.5 ml of 0.01 KMnO₄ in 2.75 ml of potassium phosphate buffer with 0.5 ml 6 N H₂SO₄ was also recorded to derive Abs_{std}. Catalase activity in the heart was then defined as $\log[(\text{Abs}_{\text{std}} - \text{Abs}_{\text{blank}}) \div (\text{Abs}_{\text{std}} - \text{Abs}_{\text{rest}})] \times (400 \times 2.3 \div 180)$ U/g wet weight.

Statistical analysis. Results are expressed as means with standard errors determined by conventional methods. Statistical comparisons were performed between transgene positive and negative hearts at individual time points by using the Student's two-tailed, unpaired *t* test. The effect of the transgene was examined between baseline and the 30-min time point by two-way analysis of variance. All analyses were performed using the Statview v4.0 statistical package (Abacus Concepts Inc., Berkeley, CA). A probability value \leq 0.05 was considered significant, and a value $0.1 \leq P \leq 0.05$ was marginally significant.

Results

Experimental exclusions and group sizes. In one transgene positive and one transgene negative heart, coronary flows were very high and on close inspection tears were evident in the aortic root below the level of the coronary tie necessitating exclusion. One transgene negative experiment was also excluded because of intractable arrhythmias during the stabilization period. All of these exclusions occurred at the time of experimentation and no data were gathered. One unblinded transgene negative experiment was therefore performed to ensure equal group size. Each group consisted of 15 experiments.

Two durations of reperfusion were used. In the first set of experiments ($n = 7$ for each group) reperfusion was for 30 min. However, because of a theoretical possibility that this short reflow period would not be of adequate duration for the washout of the enzymatic cofactor giving rise to the tetrazolium stain (26), more experiments ($n = 8$ for each group) were performed with a 120-min period of reperfusion. Therefore the data comprise of information from 15 hearts in each group, apart from the 60-, 90-, and 120-min reperfusion time points where data are derived from 8 hearts in each group.

The size of the infarction zone was similar within hearts from the same group, whether reperfused for 30 or 120 min, therefore all available hearts were used in the final analysis.

In three transgene negative hearts, infarct size could not be determined. In one experiment, the heart came off the aortic cannula during tetrazolium infusion and could not be resuspended. In two other (consecutive) experiments, there was no tetrazolium staining implying either complete infarction or failure of staining. Since these hearts had some contractile function, a fault in the staining technique was assumed.

A further 18 mice were used for characterization studies. Five transgene negative and three transgene positive hearts were used to measure catalase. The hearts of a further 10 mice were used for the preparation of protein and RNA.

Characterization of hearts from transgenic mice. The hearts from transgene positive and transgene negative mice (designated on the basis of Southern analysis of genomic DNA obtained from tail clips) were analyzed by Northern and Western blotting.

Western blots were probed with a polyclonal antibody (27) that recognizes both constitutive and inducible forms of hsp70 and with a monoclonal antibody (C92F3A-5; StressGen Biotechnologies Corp., Victoria, British Columbia) that only recognizes the inducible form of hsp70. As can be seen in Fig. 2, the hearts from transgene positive mice have appreciable hsp70i immunoreactivity, and the amount of constitutive hsp70 (hsp70c) protein (Fig. 2 A) does not appear to be significantly altered by the expression of the transgene. At approximately equal protein loading, the myocardial hsp70i immunoreactivity in transgene positive mice is much greater than that seen after heat stress. In contrast to our findings in larger rodents (2, 6, 10), whole body heat stress in the mouse appears to be a relatively poor stimulus for the induction of hsp70 within the heart compared with other organs. This finding is in keeping with previous reports (24). A possible explanation for this low level of induction is that the blood returning to the left heart has been cooled by room air during passage through the lungs and is therefore able to reduce myocardial temperature. In addition, the larger surface area/body weight ration in the mouse compared with larger rodents results in more rapid equilibration of core to environmental temperature. Thus, for a heat shock procedure where core temperature is elevated to 42°C for 15 min, the duration of time that core temperature is greater than basal temperature is much shorter for the mouse than for larger species.

Fig. 3 shows the Northern blot of cardiac (A) and skeletal muscle (B) RNA from a transgene positive mouse, a transgene negative mouse, and a transgene negative mouse 8 h after whole body heat stress. After heat stress the endogenous mouse hsp70i mRNAs are barely perceptible in cardiac tissue, but the previously described A and B forms (21) are clearly seen in skeletal muscle. The disparate levels of mRNA induction in heart and skeletal muscle are in keeping with the weak induction of myocardial hsp70i protein described above (Fig. 2). The chimeric transgene (containing the rhsp70i B form) is transcribed into an mRNA of a unique size due to the addition of chimeric hybrid sequences derived from the chicken β -actin gene before the translation start site and from SV40 after the translational stop site. The resultant chimeric transcript has a size of 2.6 kb and migrates between the mRNAs for the two endogenous mouse hsp70i transcripts with sizes of 2.7 and 2.5 kb. In sum-

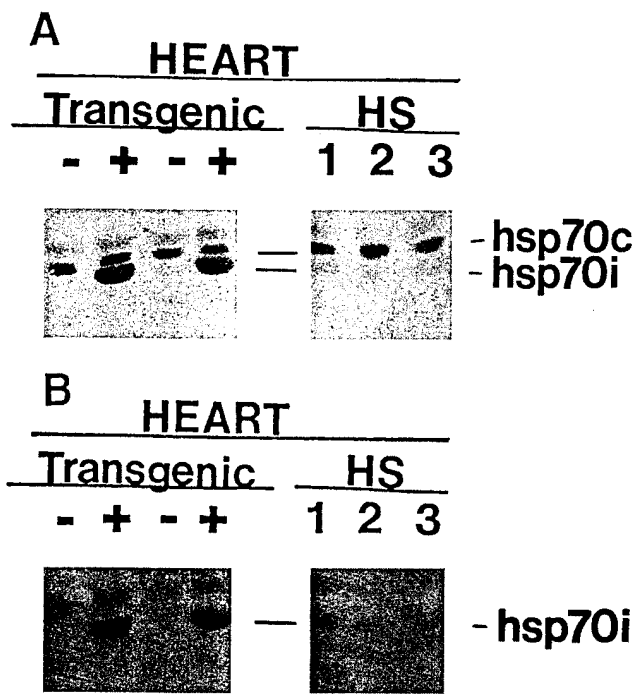


Figure 2. Western blot of samples prepared from hearts harvested from transgene positive and transgene negative mice after whole body heat stress. The samples are probed with a polyclonal (A) and a monoclonal (B) antibody recognizing hsp70. In both panels, (lanes from left to right) - denotes transgene negative; + denotes transgene positive; and 1, 2, and 3 are samples from transgene negative mice harvested 8–24 h after heat shock. (A) The primary polyclonal antibody recognizes constitutive (hsp70c) and inducible (hsp70i) forms of the 70-kD heat shock protein. hsp70c runs above hsp70i. A strong hsp70i signal is seen in the hearts of transgene positive mice and no signal is seen in transgene negative littermates. The signal is present, but weak, in hearts harvested from transgene negative mice after heat stress (HS). For a discussion of the reasons for the weak hsp70i signal after heat stress, see text. (B) The primary monoclonal antibody recognizes only the hsp70i band, the upper band is a nonspecific signal. The immunoreactive staining confirms that the pattern seen with the polyclonal antibody is indeed due to hsp70i rather than a degradation product of hsp70c.

mary, the novel chimeric rhsp70i RNA is the transcript responsible for the excess hsp70i immunoreactivity seen in Fig. 2.

In the heart (Fig. 3 A) the level of mRNA for hsp70c is not altered by overexpression of rhsp70i protein or by the presence of abundant transgenic mRNA.

Analysis of RNA from transgene negative and transgene positive hearts showed no differences in the level of expression of the hsp27, hsp60, and hsp90 heat shock protein genes (data not shown).

The catalase activity within the myocardium was unaltered by the presence and expression of the transgene. The activity in transgene positive myocardium was 1.45 ± 0.47 U/g wet wt ($n = 3$) and in transgene negative myocardium was 1.53 ± 0.38 U/g wet wt ($n = 5$).

Baseline characteristics of the Langendorff hearts. Despite high levels of rhsp70i expression in skeletal muscle, brain, and heart, transgene positive mice appeared normal. The average body weight, heart weight, and heart performance were similar in littermates with an without the transgene (see Table I). Since basal characteristics were similar between groups, contractile

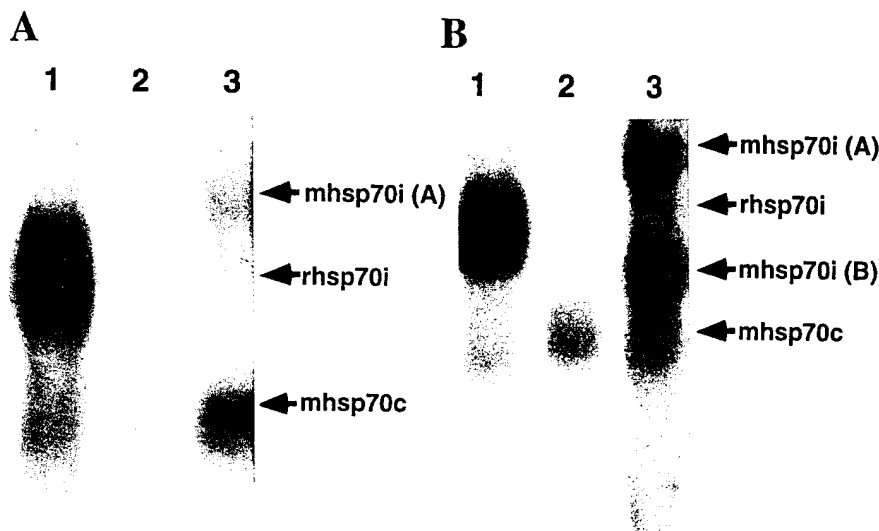


Figure 3. RNA analysis of hearts (A) and skeletal muscles (B) harvested from a transgene positive mouse, a transgene negative mouse, and a transgene negative mouse 8 h after whole body heat stress. In both A (heart) and B (skeletal muscle), lane 1 is prepared from a transgene positive mouse, lane 2 a transgene negative mouse, and lane 3 a transgene negative mouse 8 h after heat stress. A shows the transcript for the rat inducible 70-kD heat shock protein (rhsp70i), seen only in transgene positive hearts, runs above that for the mouse constitutive heat shock protein 70 (mhsp70c) and below that for the mouse inducible heat shock protein 70 (mhsp70i). The unique size of the rhsp70i transgene transcript is due in part to the additional sequences before the transcriptional start and after the transcriptional stop codons (see Fig. 1). B is prepared from skeletal muscle where the heat shock

response is more marked and has been included to show the signals of both forms (A and B) of mhsp70i. The size of the chimeric transgene remains unique. The possible reasons for the poor signal after heat stress in cardiac compared with skeletal muscle are discussed in the text.

data for each individual experiment were expressed as a percentage of the baseline value.

Developed force at baseline tended to be higher in transgene positive hearts, however these hearts also tended to be slightly larger so that myocardial tension, if measured, would have been similar.

The performance of the Langendorff heart. Initial experiments were performed on the hearts from transgene negative mice with 30 and then 25 min of no-flow ischemia. Contractile recovery in these experiments was below 5%, so the ischemic time was shortened to 20 min. This duration of ischemia is classically thought to result in only minimal irreversible cardiac injury or necrosis (28). However, in view of the very poor contractile recovery, we felt that the mouse heart was unusually susceptible to infarction, possibly because of high intrinsic heart rates and therefore metabolic rates.

Contractility in the isolated heart was measured as the difference between the systolic and diastolic force generated at the apical force transducer as the heart attempted to shorten between the apical force transducer and aortic cannula. As shown in Fig.

Table 1. Baseline Characteristics of Transgene Positive and Transgene Negative Mice

Characteristic	Transgene status	
	+	-
Body wt (g)	26.7±1.2	25.9±1.1
Heart wt (mg)*	147.4±7.3	135.3±9.0
Baseline flow (ml/min)	3.82±0.22	3.77±0.17
Baseline developed force (g)	2.72±0.29	2.61±0.37
Baseline diastolic force (g)	1.06±0.09	1.14±0.10

* Heart wet weight was measured at the end of the experimental protocol after 20 min of global ischemia and up to 120 min of reperfusion. Developed force was peak systolic force minus diastolic force. Diastolic force was measured at end diastole. $n = 15$ for each group.

4, although contractility in both groups was severely reduced after 20 min of zero-flow ischemia, hearts from transgene positive mice had better postischemic recovery. Most hearts showed a paradoxical hypercontractile phase during the first 2 min of

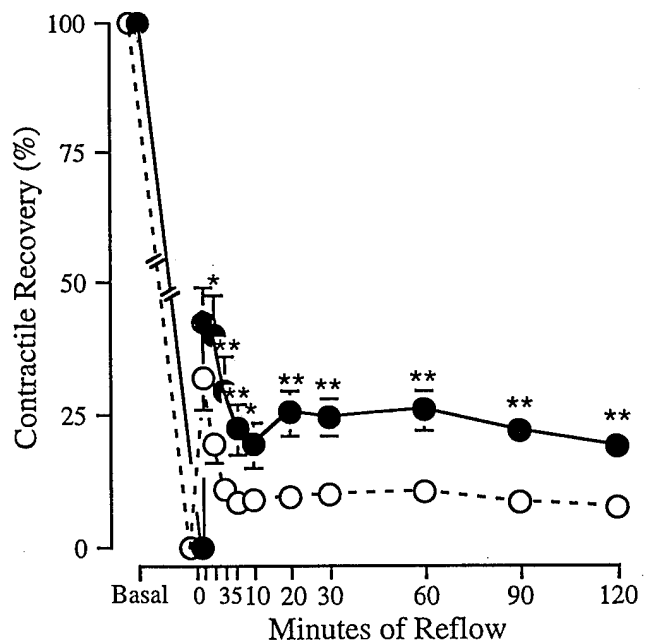


Figure 4. Contractile recovery of the isolated mouse heart after 20 min of global ischemia. Force between an apical force transducer and the aortic cannula was measured during each contraction. Contractility was defined as developed force calculated by subtracting diastolic force from peak systolic force. At each time point, contractility during reflow was expressed as a percentage of basal contractility. \circ , transgene negative hearts; \bullet , transgene positive hearts. Bars represent one standard error of mean, * $P \leq 0.05$, ** $P \leq 0.01$, two-way analysis of variance, $P = 0.01$ for the effect of group. Basal to 30 min reflow, $n = 15$ for each group; 60–90 min of reflow, $n = 8$ for each group.

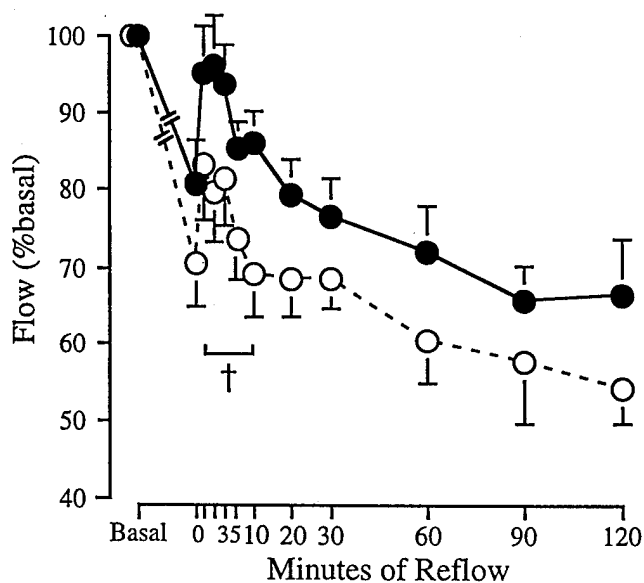


Figure 5. Changes in coronary flow after 20 min of global ischemia. Coronary flow was measured by collecting and weighing the coronary effluent. Flow at each time point was expressed as a percentage of baseline flow. ○, transgene negative hearts; ●, transgene positive hearts. Bars represent one standard error of mean. There was no significant difference between groups at each time point, two way analysis of variance $P = 0.046$ for the effect of group. Between 2 and 10 min of reflow the differences between the groups were marginally significant, $0.08 > P > 0.05$. Basal to 30 min reflow, $n = 15$ for each group; 60–90 min of reflow, $n = 8$ for each group.

reperfusion which subsequently decayed, so that developed force was fairly constant between 5 and 120 min of reperfusion (see Fig. 4). Postischemic developed force in transgene positive hearts was approximately twice that of hearts from transgene negative littermates (at the 30-min time point, transgene positive $24.3 \pm 3.7\%$, $n = 15$ compared with transgene negative $9.6 \pm 2.1\%$, $n = 15$, $P = 0.002$).

The better contractile recovery was associated with higher postischemic coronary flows in the hearts from transgene positive mice (see Fig. 5). Since coronary perfusion pressure was constant, coronary flow reflects coronary vascular resistance. It is likely that the lower coronary flow in transgene negative hearts is secondary to no reflow within the larger areas of infarction seen in these hearts (see below). In addition diastolic force and therefore tension tended to be higher in these hearts (data not shown), and this would also have acted to impede flow. The cause for the gradual reduction in coronary flow seen in both groups during the course of reperfusion is not known, but this phenomenon is often seen in the postischemic Langendorff heart (29).

The heart rates between experimental groups were similar, though variable in individual preparations (Fig. 6). Hearts were not paced, but rates did not differ significantly at baseline or during the first 180 s of ischemia. Beyond 180 s contractile amplitude was insufficient to measure rate. Small differences in baseline heart rate between groups are unlikely to account for large differences in postischemic contractile recovery. In our experiments basal heart rate did not correlate with either contractile recovery at 30 min or normalized infarct size (data not shown). It is therefore unlikely that a fast heart rate before

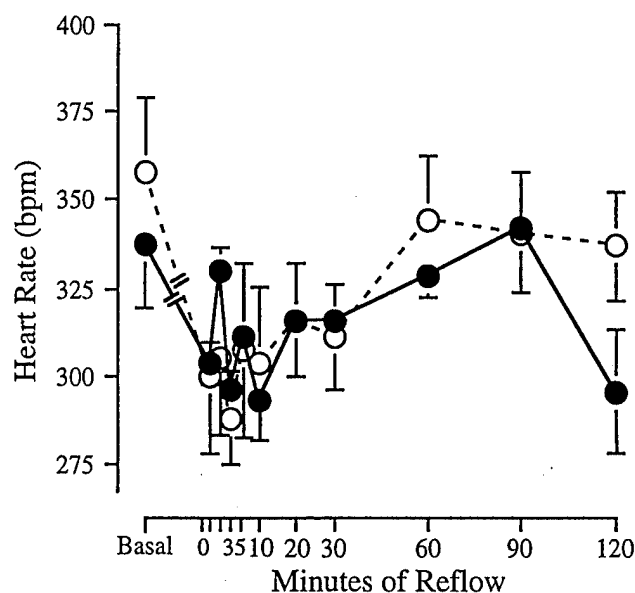


Figure 6. Heart rate before and during reflow after 20 min of global ischemia. Hearts were not paced. The slight difference in heart rates at baseline did not alter postischemic performance (see Results). ○, transgene negative hearts; ●, transgene positive hearts. Bars represent one standard error of mean. There was no significant difference between groups at each time point, two way analysis of variance $P = 0.91$ for the effect of group. Basal to 30 min reflow, $n = 15$ for each group; 60–90 min of reflow, $n = 8$ for each group.

ischemia increased the metabolic demands of the myocardium and sensitized the heart to ischemic injury (28). Moreover, when the transgene positive experiments with the three slowest initial heart rates were excluded, mean basal heart rates for transgene positive and transgene negative groups became identical (355 ± 357 bpm, respectively), but contractility at 30 min ($21.7 \pm 3.4\%$, $n = 12$ and $9.6 \pm 2.1\%$, $n = 15$, $P \leq 0.01$, respectively) and normalized infarct size ($27.6 \pm 2.7\%$, $n = 12$ and $45.1 \pm 3.6\%$, $n = 12$, $P \leq 0.01$, respectively) continued to indicate a significant reduction in the extent of ischemic injury in transgene positive hearts.

The efflux of CK. The efflux of CK during reperfusion is shown in Fig. 7. CK efflux was significantly reduced in transgene positive hearts reflecting the greater contractile recovery seen in this group. The CK activities at each time point had considerable variability, hence standard errors are large and individual time points fail to reach statistical significance.

Myocardial infarct size. Tetrazolium stains viable myocardium deep red. Figs. 8 and 9 show the staining pattern of slices from a transgene positive and transgene negative heart, respectively. In this example there is a small discrete, predominantly subendocardial area of infarction in the transgene positive heart. The area of infarction in the transgene negative heart is less contiguous and much more extensive. Like the heart in Fig. 8, a number of hearts showed epicardial necrosis that was geographically separate from the larger area of endocardial necrosis (see Discussion). When the slices from all the hearts were analyzed, the total volume of myocardial infarction was significantly greater in the transgene negative hearts. Moreover, these differences in infarct size became even more significant when volumes of infarction for each heart were normalized by total volume at risk of infarction (see Fig. 10).

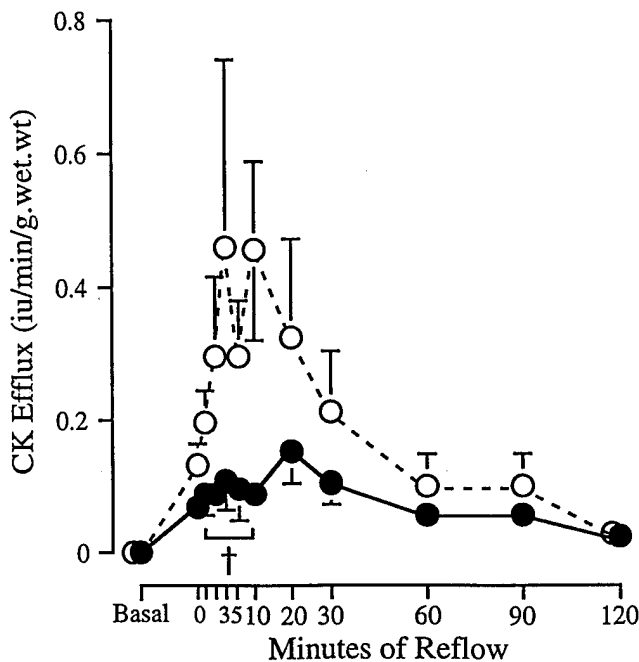


Figure 7. CK efflux during reflow after 20 min of global ischemia. There was no detectable CK activity in the coronary effluent at baseline. During reflow, the CK contents of the coronary effluents varied widely, but at each time point activity was less in transgene positive hearts. \circ , transgene negative hearts; \bullet , transgene positive hearts. Bars represent one standard error of mean. There was no significant difference between groups at each time point, two way analysis of variance $P = 0.04$ for the effect of group. Between 1 and 10 min of reflow the differences between the groups were marginally significant $0.09 > P > 0.05$. Basal to 30 min reflow, $n = 15$ for each group; 60–90 min of reflow, $n = 8$ for each group.

After ischemia the percentage of contractile recovery (see Fig. 4) was less than the percentage of viable myocardium (see Fig. 10). The implication was that in both groups some portion of the heart, though viable, was not contributing to contraction. Since perfusion had been restored, the most likely explanation for this discrepancy was that there was some degree of contractile stunning (30). Nonetheless, in individual hearts, normalized infarct size correlated significantly with contractile performance (see Fig. 11). This observation suggests that infarction also contributed significantly to the contractile deficit.

Discussion

Previous studies have shown that myocardial protection follows whole body heat stress. The cause for this protection is uncertain, although increases in myocardial hsp70i and possibly myocardial catalase have been considered (8, 9). In this study we have shown that myocardial protection occurs in transgenic mice overexpressing hsp70i in their hearts, without alteration in myocardial catalase. This observation strongly supports the hypothesis that hsp70i is a cytoprotective protein within the heart and is at least partially responsible for the myocardial protection that follows whole body heat stress. Our findings are in keeping with previous observations that overexpression of hsp70 confers protection against simulated ischemia and thermal stress in isolated heart or muscle-derived cells (12, 13, 31, 32).

The mechanisms by which hsp70 may result in myocardial protection. hsp70i is a member of the family of proteins known as chaperones (33). These proteins function in a variety of well described circumstances to promote correct protein folding and prevent inappropriate protein interactions (34, 35). The mechanisms whereby such functions can protect the myocardium from ischemic damage are necessarily speculative since the precise cause for cell death during ischemia is unknown.

During ischemia the cellular internal milieu changes profoundly with the intracellular accumulation of protons and sodium ions (36). These changes are compounded by the free-radical stress and the marked increase in intracellular calcium associated with reperfusion (37). Under these circumstances, the tertiary structure of proteins may change sufficiently to alter function. Such ischemia-induced changes in protein conformation and function have been described for the key metabolic enzyme carnitine palmitoyltransferase (38). In our experiments, the presence of an excess of hsp70 may prevent these adverse conformational changes or promote the correct refolding of denatured proteins once the cell reenergizes during reperfusion. Further evidence in support of this hypothesis is the fact that myocardial ischemia is a potent stimulus for the induction of hsp70i (2, 17, 39). This suggests that some component(s) of the ischemic injury is able to activate hsp70 gene expression, a process known to be triggered by the presence of denatured proteins (40, 41) and ATP depletion (32, 41). The intronless gene arrangement and the preferential translation of hsp70 in such circumstances intimate its special role in the ischemic/reperfused heart (42). It is therefore possible that an overabundance of hsp70 before ischemia, as occurs in the transgenic heart, is able to attenuate the consequences of ischemia at the protein level. A similar explanation is thought to underly the resistance to thermal injury that accompanies overexpression of hsp70 (31, 43) and the sensitization to thermal injury that accompanies diminished expression of hsp70 (44). Thus, thermal and ischemic injuries may be prevented by overexpression of hsp70 because they have protein denaturation as a common pathology.

During ischemia, ATP levels fall, preventing protein translation (45). In this circumstance, nascent polypeptides, representing incompletely translated mRNA, are exposed to the ionic perturbations described above. Under normal conditions, during the course of translation, these immature proteins associate with a series of chaperones and chaperonins including hsp70 (46). These associations are thought necessary to suppress and reverse polypeptide chain interactions that would otherwise result in a nonfunctional, incorrectly folded protein (46, 47). Consequently, chaperones may also play a role in the recovery of translation with the restoration of useful protein synthesis on reperfusion. The availability of these newly synthesized proteins may be crucial to the recovery of the ischemically injured cell.

Implications and future directions. Our findings show that it is probably possible to overexpress hsp70i within the heart without any apparent detrimental effect. This finding is surprising since in cell culture overexpression of the human inducible hsp70 gene slows cell growth (12). However, this gene is able to confer protection independent of its effect on cell growth (12). In addition, it is thought that hsp70 regulates its own transcription by interacting with the heat shock transcription factor to prevent binding to the heat shock element (48). One might expect, therefore, that overexpression of the rhsp70i may reduce the expression of the endogenous mouse hsp70s. How-

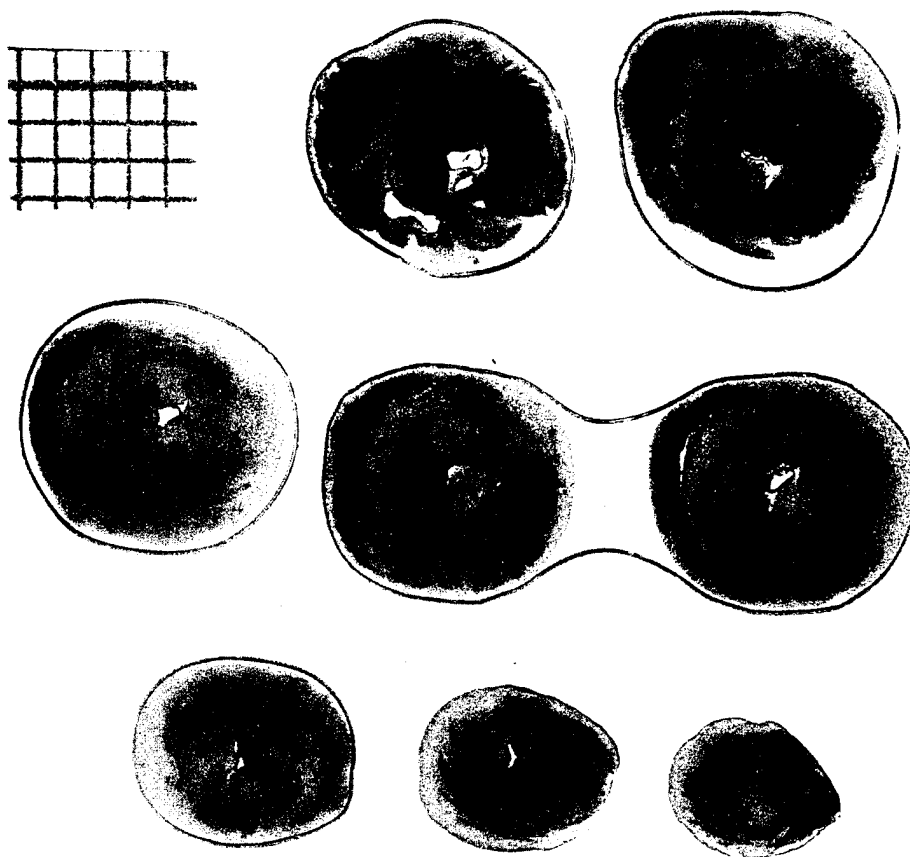


Figure 8. Myocardial infarction caused by 20 min of global ischemia delineated by tetrazolium staining in a heart from a transgene positive mouse. The heart has been sliced transversely from base to apex. Tetrazolium fails to stain nonviable myocardium, which remains pale. The pale area of necrosis is predominantly subendocardial with a separate, almost circumferential, area of epicardial necrosis closer to the apex of the heart. The calibration squares are 1 mm.

ever, no reduction was seen in the mouse hsp70c mRNA of protein. This finding reflects our previous observation in a myogenic cell line where overexpression of human hsp70i did not alter the expression of the rat hsp70c (12). We conclude, therefore, that it is possible to overexpress hsp70i without disturbing the expression of the endogenous constitutive hsp70 genes. However, we have not excluded an effect of the transgene on expression of endogenous hsp70i and other hsp genes at the protein level.

In a previous study, constitutive overexpression of hsp70 in *Drosophila* cells led to the sequestration of hsp70 into granules, where it was inactivated and unable to confer thermoresistance (49). However, similar sequestration was not seen in rodent cells overexpressing hsp70 (50) which, consistent with our findings, had a protected phenotype.

Our ability to overexpress hsp70i within the heart and protect the myocardium without any apparent detrimental effects introduces the possibility of future therapeutic opportunities.

In the past decade, the treatment of acute myocardial infarction has been revolutionized by interventions which achieve early reperfusion, such as thrombolytic therapy and aspirin (51). Unfortunately, the mortality benefit of these interventions diminish if treatment is delayed (51). The finding that mortality can be reduced just by increasing the rate of infusion of a thrombolytic agent further underlines the importance of early reperfusion (52). Therefore, the ability of hsp70i to slow the

progress of myocardial necrosis would act to increase the time window for myocardial reperfusion and thereby could further decrease mortality. Similar considerations are likely to apply in patients with unstable angina and in those undergoing cardiopulmonary bypass or high risk coronary angioplasty. In addition, the preservation of explanted hearts before transplantation may be improved. In all these situations the ability of hsp70i to delay the progression of ischemic myocardial damage could favorably influence the outcome.

If hsp70i was to be used as an adjunct to thrombolytic therapy, it would be necessary to increase the expression of this protein within the myocardium before infarction. Our results suggest that in patients at risk of myocardial infarction it may be possible to produce long-term overexpression of hsp70i without causing harm.

Study limitations. The use of tetrazolium to delineate regional infarction is a recognized technique (26). However, we are not aware that it has been used to demarcate infarction resulting from global ischemia in the mouse, although a similar technique has been used in other species (53, 54). In light of this, our results with respect to infarct size should be treated with caution.

A further difficulty we encountered with the assessment of infarction were areas of apical subepicardial loss of tetrazolium staining (see Fig. 8). These areas were probably related to the apical suture causing local myocardial distortion which impeded

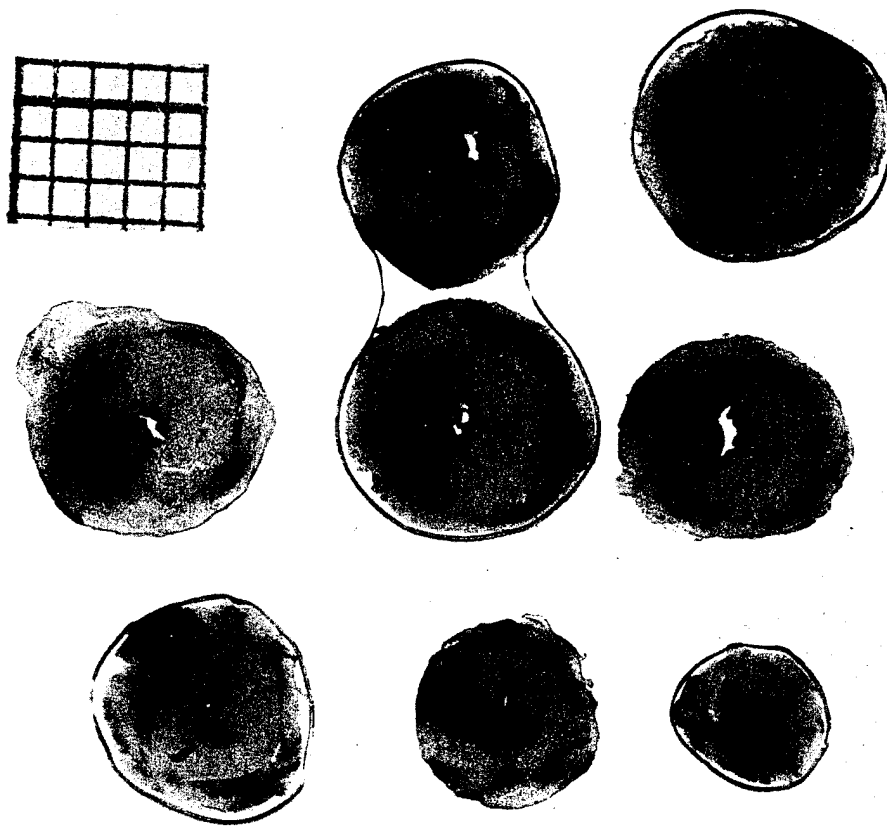


Figure 9. Myocardial infarction caused by 20 min of global ischemia delineated by tetrazolium staining in a heart from a transgene negative mouse. See legend to Fig. 8. Compared with the heart in Fig. 8, the area of necrosis is more extensive and less well circumscribed.

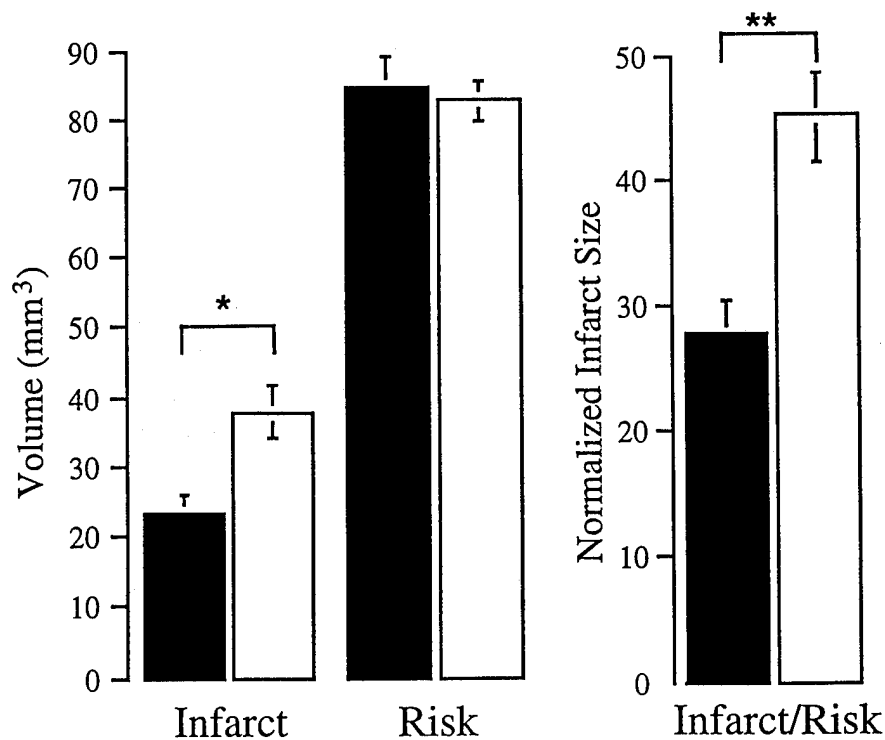


Figure 10. Myocardial, infarct, and risk volume and normalized infarct size after 20 min of global ischemia. Hearts were reperused for 30 or 120 min. □, transgene negative, $n = 12$; ■, transgene positive, $n = 15$. Bars represent one standard error of mean * $P \leq 0.05$, ** $P \leq 0.01$.

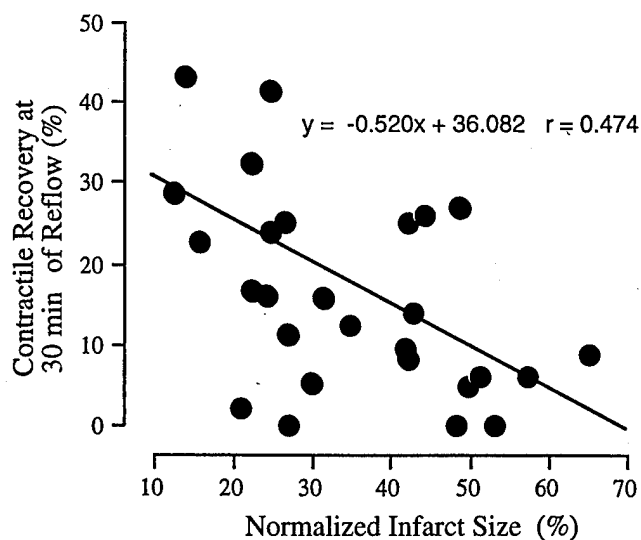


Figure 11. The relationship between normalized infarct size and contractile recovery at 30 min of reflow after 20 min of global ischemia. The data from transgene positive and negative experiments were both used for this analysis. There is a significant negative correlation between infarct size and contractile function $P = 0.01$. The ordinate intercept is 36.1%. This implies that even without infarction contractile recovery would have been incomplete and that therefore an element of contractile stunning is probably present (see Results).

capillary perfusion during early reperfusion when ischemic contracture was greatest. In a preliminary experiment performed without an apical suture, this artifact was absent. However, since the volumes of apical subepicardial infarction were small, they only had a significant effect on overall infarct size in those hearts with small volumes of subendocardial infarction. Thus, infarct size would be overestimated in those hearts with small infarcts and this would have mitigated against the separation we observed in infarct size between groups.

By using a chimeric promoter for the rhsp70i gene, we achieved high levels of transcription and translation within the myocardium. At a cellular level this high level of expression may have been at the expense of a lower level of expression of other genes which had to compete for the same transcriptional and translational machinery. It is therefore possible, but unlikely, that the protective benefits that we observed were not due to overexpression of hsp70i.

Conclusions. Hearts harvested from transgenic mice overexpressing myocardial hsp70i were resistant to ischemia. In these mice, postischemic contractile function was enhanced, intracellular enzyme efflux was reduced, and infarct size was diminished. In contrast to the changes occurring within the heart after whole body heat stress, an increase in myocardial catalase activity did not accompany the expression of the transgene. We conclude that hsp70i is able to protect the heart from ischemic injury and that it is probably responsible for the myocardial protection that follows whole body heat stress.

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Review

Heat Shock Proteins and Protection Against Myocardial Ischemia

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Introduction

The incidence of myocardial infarcts has significantly decreased in the past decade. Research aimed at identifying the cause of atherosclerosis and efforts to prevent it have contributed to this decrease in myocardial infarction. However in spite of early reperfusion, loss of functional myocardium leading to subsequent severe cardiac failure still remains a significant medical problem (American Heart Association, 1990). The salvage of additional myocardium is, therefore, a highly desirable aim. Recent evidence indicates that endogenous protective mechanisms are activated in the ischemic cardiomyocyte. A better understanding of these endogenous protective mechanisms will most likely lead to additional myocardial salvage in the reperfused myocardium.

Ischemia is known to produce a number of intracellular changes within the cardiomyocyte. These changes include among others increased cellular calcium levels, altered osmotic control, membrane damage, free radical production, decreased intracellular pH, depressed ATP levels, oxygen depletion, decreased intracellular glucose levels, etc. (Bonventre, 1988). These events represent a form of metabolic or hypoxic stress which is known to produce protein denaturation within the cell. Interestingly, an increase in denatured proteins in the cell has been reported to result in the onset of the heat shock response which increased the synthesis of the so-called heat shock proteins (HSPs) (Ananthan *et al.*, 1986). The heat shock response has been shown to occur in all organisms examined to date. This heat shock response consists of a transient rearrangement of cellular activities to

cope with the stress period by protecting essential components within the cell so as to permit it to resume normal activity during recovery from the stress (Lindquist, 1986). This ability of the cell for self-preservation has attracted the attention of several investigators in the field. Several studies have shown that a hyperthermic treatment of experimental animals can result in a significantly improved myocardial salvage following coronary occlusion and reperfusion *in vivo* (Donnelly *et al.*, 1992; Currie *et al.*, 1993) as well as in an isolated perfused heart model (Walker *et al.*, 1993). Interestingly, recent studies have now demonstrated a direct correlation between the amount of one of these heat shock proteins (HSP70) and the degree of myocardial protection following a hyperthermic treatment in experimental animals (Hutter *et al.*, 1994; Marber *et al.*, 1994). A closer examination of this group of proteins (HSPs) and their involvement in cardioprotection during myocardial infarction is then extremely important as a means to understand the cardiac cell's ability to protect itself against ischemic injury. The present review will attempt to cover what is known about these proteins and the more recent studies related to their expression and myocardial protection.

The heat shock proteins

The heat shock phenomenon was first observed in *Drosophila* by Ritossa in 1962. Subsequently, Tissieres *et al.* (1974) discovered the major heat shock proteins by analyzing newly synthesized proteins in *Drosophila melanogaster* larvae that had been incubated at 37.5 C for 20 min. Electrophoretic

analysis of the salivary gland proteins showed the induction of several new polypeptides as well as the reduction in the expression of preexisting mRNAs. The newly induced proteins were not synthesized when low levels of actinomycin D or α -amanitin had been administered prior to heat shock. These results imply that the induced synthesis of these proteins is dependent on *de novo* RNA transcription.

A heat shock response similar to that found in *Drosophila* has been reported in a wide range of organisms from bacteria to man (Schlesinger *et al.*, 1982). This suggests that the heat shock phenomenon is universal. In virtually all organisms studied, a similar number of heat shock proteins are synthesized following an increase in temperature. This high conservation of the heat shock response through the evolutionary ladder suggests that these proteins must serve a vital function within the cell. In addition to an increase in temperature, a diversity of other agents induce the heat shock response. The HSPs are mainly expressed following a noxious stress such as: heat shock (42 °C in mammals), hypoxia, hydrogen peroxide, changes in pH levels, amino acid analogues, heavy metals, viral infections, arsenite, ethanol and ischemia/reperfusion.

The heat shock proteins are members of the family of stress proteins which also includes the glucose regulated proteins (GRPs), ubiquitin, α B-crystallin and heme oxygenase, among others (Table 1). The glucose regulated proteins are involved in the stabilization and formation of intracellular protein complexes. One example is the GRP78 or BIP (immunoglobulin heavy chain binding protein) which stabilizes the immunoglobulin heavy chain before its assembly with the immunoglobulin light chains in the lumen of the endoplasmic reticulum. Contrary to the other stress proteins mentioned, the GRPs are induced by a diversity of stresses to the cell (e.g. glucose deprivation, calcium influx, prolonged hypoxia and so forth) but not by a heat shock (for review on GRPs, see Lee, 1987). Presently, no strong link has been found between the expression of the GRPs and myocardial infarction.

Mammalian heat shock proteins as well as ubiquitin, α B-crystallin and heme oxygenase which are also induced by an increase in temperature and thus can also be considered heat shock proteins and can be grouped in three subgroups according to their molecular mass (Table 1). The high molecular mass HSPs includes three major members, namely HSP110, HSP90- α and HSP90- β . The last two HSPs have attracted much interest due to their ability

to bind to steroid receptors and are presumably involved in the regulation of these molecules with their ligands (Pratt, 1993). The HSP70 family is the most abundant of the HSPs. This subgroup includes HSP70 and HSP60 proteins. The HSP70 family is made up of at least 3 to 4 members in mammalian cells (Lowe and Moran, 1986; Harrison *et al.*, 1987). One of the HSP70s is expressed constitutively in all cells and is slightly increased in expression by a heat shock or other oxidative stresses. The remaining three HSP70 members are inducible isoforms which are expressed exclusively when the cell is under stress with the exception of primate cells which constitutively express a certain amount of these inducible forms of HSP70 even under normal conditions (Welch *et al.*, 1983). HSP60 is nuclearly encoded but resides in the mitochondria where it is believed to be involved in the assembly of macromolecular complexes (Ostermann *et al.*, 1989).

The small molecular mass HSPs are composed of HSP47, HSP27, α B-crystallin (20 kD), heme oxygenase (32 kD) and ubiquitin (8 kD). The HSP47 is an endoplasmic reticulum resident protein which has a high binding affinity for collagen (Nagata *et al.*, 1991) and recently has been postulated to serve as a molecular chaperone (Sauk *et al.*, 1994). The HSP27 protein is encoded by four distinct genes in mammalian cells (McGuire *et al.*, 1989) and its main feature is being the target of phosphorylation in response to mitogens and tumour promoters (Welch, 1985). The α B-crystallin is highly homologous to small heat shock proteins (Ingolia and Craig, 1982; Southgate *et al.*, 1983) and has recently been reported to be induced by a heat shock (Klemenz *et al.*, 1991). In addition, α B-crystallin has been found to be an abundant protein in cardiac muscle cells (Bennardini *et al.*, 1992). The heme oxygenase besides being induced by several of the common stressors (heat shock in rodents, hypoxia, hydrogen peroxide, cadmium) as other HSPs is also induced by heme as are other stress proteins (Sistonen *et al.*, 1992). Ubiquitin, the smallest member in this subgroup, is induced by similar stresses as the major HSPs (heat shock, amino acid analogues, denatured proteins) and plays a vital role in the process of protein degradation (Mayer *et al.*, 1991).

As a consequence of the rapidity and intensity of the heat shock response, the heat shock genes have been an ideal system for molecular biologists in the study of the regulation of transcription. Therefore, the promoter of HSP genes has received much attention and scrutiny. The region containing the TATA box of the heat shock gene was found to be protected from exonuclease digestion both before

Table 1 Mammalian stress proteins*

		Heat Shock Proteins	
Protein Type	Features	Stimulus	Function
High molecular mass HSPs			
HSP 110	Nucleolar location	Heat	Yeast homolog (HSP 104) is involved in thermotolerance
HSP 90	Two isoforms (alpha and beta) Binds to actin and steroid receptors	Heat	Regulates activity of the steroid receptors
HSP 70 Family			
HSP 70	Most abundant of the HSPs Several inducible isoforms One constitutively expressed form	Heat, heavy metals, arsenite, ethanol, hypoxia, amino acid analogs, ischemia/reperfusion, etc	Binds to nascent and denatured proteins Serves as molecular chaperone, imports proteins into mitochondria and endoplasmic reticulum (ER)
HSP 60	Nuclear encoded and located in mitochondria	Heat, hypoxia, anti-cancer drugs	Assembly of macromolecular complexes in the mitochondria
Small molecular mass HSPs			
HSP 47	Binds to collagen Located in endoplasmic reticulum	Heat, cell differentiation	Involved in translocation of collagen into ER
HSP 27	Encoded by four separate genes Highly phosphorylated	Heat, estrogen, mitogens, cytokines, inducers of differentiation	Involved in regulation of actin microfilament dynamics
Alpha B-crystallin	Found in lens and very abundant in cardiac tissue Homologous to HSP 27 20 kD	Heat, low pH	Closely associated to cytoskeletal proteins (e.g. actin and desmin)
Heme oxygenase	Two isoforms, 32 kD	Heat (in rodents), heavy metals, hydrogen peroxide, hemin, UV radiation, hypoxia	An essential enzyme in heme catabolism
Ubiquitin	8 kD	Heat, denatured proteins, amino acid analogs	Involved in selective degradation of short-lived and abnormal proteins
		Glucose Regulated Proteins	
Protein Type	Features	Stimulus	Function
GRP 78	Identical to BIP (binding immunoglobulin protein) Closely related to HSP 70	Glucose starvation Malfolding of proteins	Proteins export out of ER Binds ATP
GRP 94	Closely related to HSP 90	Calcium ionophores Same as GRP 78	Function similar to GRP 78 in ER and Golgi

*For references see text.

and during heat shock. This implies a constant state of readiness of heat shock genes which are activated extremely rapidly as soon as the cell is stressed. A region upstream from the TATA box is only protected during heat shock and this suggests that this region contains binding sites for a heat shock factor (HSF). This heat shock factor is reported to bind to heat shock elements (HSE) present in HSP promoters. The HSF exists in a monomer form when inactive and as a trimer when activated by a stress, at which point, it is able to bind to HSE (Clos *et al.*, 1990). It is this binding of HSF to HSE of the HSP promoters that is believed to stimulate

their transcription (Bienz and Pelham, 1987). Recent studies have postulated that the trigger for activation of HSFs is ATP depletion caused by stress, especially in the case of metabolic stress (Beckmann *et al.*, 1992). According to this model, HSP70 is believed to bind HSF and therefore maintains the HSF as a monomer and, therefore, inactive. The HSP70-HSF complex can then be dissociated by an increase in denatured or nascent proteins that will require binding of HSP70 to preserve their unfolded state within the cell (Beckmann *et al.*, 1990). The dissociation of HSP70 from denatured proteins is known to be an ATP-dependent process; therefore,

a decrease in the amount of intracellular ATP caused by stress (e.g. metabolic stress) would reduce the pool of free HSP70 by diminishing the recycling of HSP70 already bound to denatured proteins. Consequently, this will cause an increase in the dissociation of the HSF-HSP70 complex leaving HSF free to adopt its timer structure which then binds to HSEs inducing the synthesis of additional HSP70 (Beckmann *et al.*, 1992; Baler *et al.*, 1992; Morimoto, 1993). Although this might well be the mechanism of activation of HSFs during metabolic stress and heat shock, our recent results show that this is not the case during hypoxia.

When cardiomyocytes are placed under hypoxic conditions, the inducible HSP70s are expressed prior to any detectable decline in total cellular ATP levels (Iwaki *et al.*, 1993), although it cannot be excluded that particular intracellular subpools of ATP might decline in the process. This difference in the mechanism of activation between heat, metabolic and hypoxic stress could be explained by the use of different HSFs since recent studies have shown that there are at least two distinct HSFs in eukaryotic cells (Sarge *et al.*, 1993).

For example, a recent study has shown that during hemin induction it is actually the binding of HSF2 to HSE that activates the synthesis of the human HSP70 (Sistonon *et al.*, 1992). We have also examined this possibility and found that the identical HSF form (HSF1) binds to HSEs during heat shock as well as during hypoxia (Mestril *et al.*, 1994a). In conclusion, these results indicate that this particular HSF form (HSF1) must be activated by at least two distinct mechanisms (Fig. 1). One of these activation pathways would then be ATP-dependent (heat and metabolic stress) while a second pathway would be ATP-independent (hypoxia).

The function of heat shock proteins

The cellular location of HSPs has been extensively studied in the search for a possible function of these proteins within the cell. The most abundant among the HSPs are the HSP70 members. The constitutively expressed HSP70 (apparent molecular weight 73 kd) is found in the cytoplasm under non-stress conditions, while the inducible HSP70, (apparent molecular weight 72 kd), is found in the nucleus and more precisely in the nucleolus during a heat shock. The heat shock proteins besides having the common feature of being induced by stress have been found to bind denatured or nascent polypeptides in the different compartments of the cell. The characteristic of heat shock proteins has

made them a good candidate to be involved in a cellular defense mechanism. The present level of knowledge points to heat shock proteins being involved in protecting the cell during metabolic or oxidative stress (Schlesinger, 1990).

The protective nature of heat shock proteins is documented by the observation that a mild heat shock (42 °C) confers resistance to the cell against a subsequent lethal heat shock (45 °C) (Li and Werb, 1982). This phenomenon is known as thermotolerance which is a term used to refer to a transient resistance to cytotoxic effects from subsequent lethal hyperthermic treatments induced by short exposure to a non-lethal heat treatment. The synthesis and degradation of heat shock proteins precedes the development and decay of thermotolerance (Li and Mak, 1985). This fact has been taken as evidence that these proteins are involved in the acquisition, maintenance and decay of thermotolerance.

It has been reported that the constitutively expressed HSP70 in yeast is an "unfoldase" which functions to facilitate the transport of proteins through the membranes of the endoplasmic reticulum (ER) and mitochondria (Deshaies *et al.*, 1988; Chirico *et al.*, 1988). Due to this property of facilitating the translocation of other proteins through the membranes of the different intracellular compartments; the HSPs have been classified as molecular chaperones. Still other investigators have found that the mammalian constitutive HSP70 (73 kd) is involved in a mechanism that targets intracellular proteins for lysosomal degradation during periods of serum withdrawal. According to their findings, HSP70 recognizes and binds a short peptide sequence found in certain intracellular proteins which are preferentially degraded when cells are deprived of serum (Chiang *et al.*, 1989). Interestingly, the intracellular concentration of HSP70 increases in response to serum withdrawal. These findings suggest that HSPs have a vital function within the cell even under non-stress conditions.

The increased synthesis of these HSPs may reflect the need for these proteins to protect different protein structures within the diverse cellular compartments. Evidence that seems to support this hypothesis comes from two reports that show when HSP70 is depleted in cells either by microinjection of antibodies specific to the HSP70 (Riabowal *et al.*, 1988) or by reducing the expression of HSP70 by genetic means (promoter competition) (Johnston and Kucey, 1988) cells are rendered sensitive to a subsequent heat shock. Another report has shown that purified HSP70 when added to a rabbit reticulocyte *in vitro* translation system at a tem-

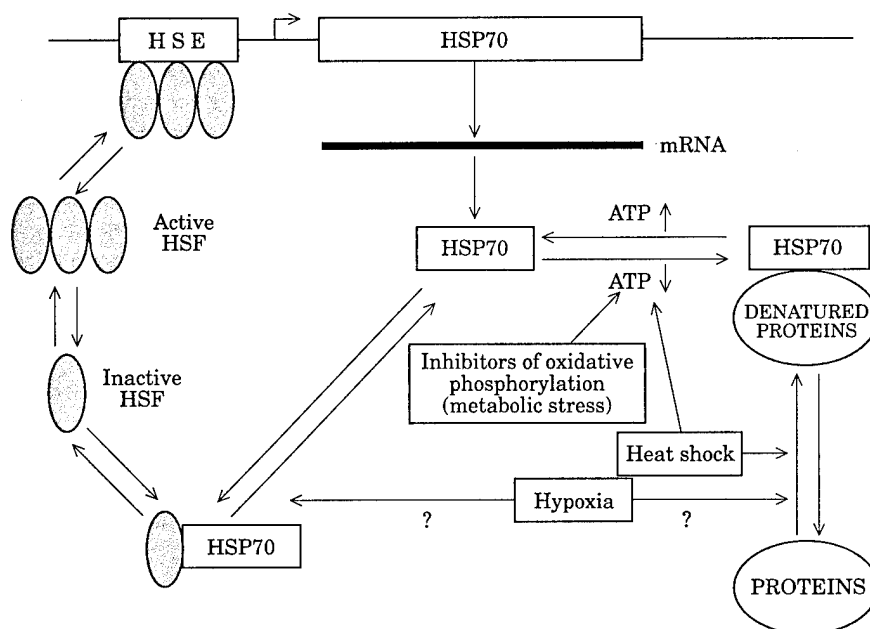


Figure 1 Model of HSP70 Autoregulation and Postulated Mechanism of Activation of Selected Inducers. Heat shock increases the amount of denatured proteins and simultaneously decreases intracellular ATP. Both events augment the quantity of HSP70-denatured protein complexes reducing the free pool of HSP70 and consequently producing the dissociation of the HSF-HSP70 complex. Once free, the HSF adopts the trimer configuration, binds to HSEs on the promoters of heat shock genes and activates their expression. Subsequently, when sufficient HSP70 has been produced to replenish the free pool of HSP70, HSP70 will then bind to HSF and revert it to its inactive monomeric form. Metabolic stress produced by inhibitors of oxidative phosphorylation causes a decrease in the amount of intracellular ATP and in this manner triggers the heat shock response. Meanwhile, hypoxia induces the heat shock response prior to any detectable decrease in ATP levels and, therefore, must trigger the expression of the HSPs by either protein denaturation of an oxygen binding protein or at some other ATP-independent step.

perature that inhibits protein synthesis (42 C) partially restores activity to the translation machinery to levels found at control temperatures (30 C) (Mivechi and Ogilvic, 1989). Still another group of investigators has reported that DNA K, the *E.coli* HSP70 homologue, is capable of protecting purified *E.coli* RNA polymerase from heat inactivation *in vitro*. They found that during heat inactivation (10 min at 45 C), RNA polymerase forms aggregates which exhibit no transcriptional activity, but if DNA K (HSP70) is present during the heat inactivation, the RNA polymerase will be protected from aggregation and will preserve its enzymatic activity. Furthermore, they find that heat inactivated RNA polymerase aggregates can be rescued by incubation with DNA K and hydrolyzable ATP (Skowyra *et al.*, 1990).

The most convincing evidence for the protective role of HSP70 against heat stress has been recently reported in two parallel studies. In these studies, the constitutive expression of a stably transfected HSP70 gene either in a rat fibroblast cell line (Li *et al.*, 1991) or in simian CV cells (Angelidis *et al.*, 1991) resulted in a higher resistance to thermal stress. In summary, it would seem that HSP70s are involved in vital functions within the cell and that

its presence is of crucial importance for cell survival during a heat shock. In addition, numerous studies have shown that increased levels of HSPs by a heat shock will also protect against other stresses and vice versa (Mizzen and Welch, 1988; Hahn and Li, 1990; Polla *et al.*, 1991). This phenomenon of cross-protection or cross-tolerance has attracted the attention of many investigators, especially those attempting to find new means of protecting cardiac myocytes against ischemia-induced injury.

Heat shock proteins and the cardiac cell

In the past decade, several studies have shown that heat shock proteins are readily synthesized in cardiac cells during tissue trauma (Currie and White, 1981), aortic banding and hyperthermia (Hammond *et al.*, 1982). Further studies have shown that HSP70 is induced to high levels of expression following conditions similar to those encountered during myocardial ischemia. Ligation of the left anterior descending coronary artery in the heart of experimental animals for several hours produces acute myocardial ischemia which was found to increase expression of the HSP70 inducible

gene (Dillmann *et al.*, 1986; Mehta *et al.*, 1988). Isolated perfused rat hearts that were either treated to hyperthermia *in vivo* or *in vitro* and to *in vitro* ischemia were found to accumulate high levels of HSP70 protein (Currie, 1988a). Hypoxia decompression which produces hypoxia, a form of oxygen depletion, was found to also increase the synthesis of HSP70 *in vivo* in rodent cardiac tissue (Howard and Geoghegan, 1986).

Recently, we have found that the expression of HSP70 is also induced in cultured rat neonatal myocytes during stresses similar to those encountered in ischemia. Neonatal rat myocytes exposed to ATP depletion using metabolic inhibitors or oxygen depletion exhibit high levels of HSP70 mRNA and protein (Iwaki *et al.*, 1993). The rapid induction of HSP70 in cardiac tissue during oxidative stress has prompted interest in investigating the possible protective role that it may play in the heart during myocardial ischemia. It has shown that a whole animal pre-heat shock treatment of rats confers enhanced post-ischemic recovery in an isolated reperfused rat heart model system (Currie *et al.*, 1988b).

Currie and co-workers found that isolated perfused hearts from rats which had received a 15 min heat treatment at 42 C, 24 h previously, exhibited an improved contractile recovery after a 30 min period of low-flow ischemia followed by reperfusion as compared to hearts from non-heat treated animals. In addition, these investigators found that the pre-heat treatment of animals produced less ultrastructure disruption of the mitochondria and a decrease in creatine kinase release in rat heart tissue following ischemia/reperfusion injury. Upon examination of the changes in the rat heart after the heat treatment, they found increased levels of HSP70 protein and an increase in enzymatic activity for the anti-oxidative enzyme: catalase. The increase in catalase activity following whole-body heat stress remains unclear and subsequent studies have shown that it does not involve any changes in transcription activation of the gene coding for catalase (Currie and Tanguay, 1991). In addition, it has recently been suggested that the heat-induced increase in catalase activity may be secondary to a direct heat shock protein interaction which modulates the activity of the enzyme (Kukreja and Hess, 1992).

Obviously, a whole-body heat stress results in many cellular changes in an organism besides an increase in the expression of heat shock proteins that could be responsible for the observed protection against myocardial ischemia. Nonetheless, recent studies have shown that HSPs and, in particular, the amount of HSP70 present following a whole-body heat shock is directly related to the degree of myocardial protection

obtained (Hutter *et al.*, 1994; Marber *et al.*, 1994). Further direct evidence that HSP70 is able to cross-protect against ischemic injury has recently been obtained using myogenic cell lines. It was found that when myogenic cells that had previously received a mild heat shock were submitted to conditions mimicking ischemia *in vitro* (hypoxia, glucose deprivation, hypotonicity, restricted intercellular volume) or simulated ischemia, these cells were then able to survive significantly better than cells that had not been pre-heat shocked (Mestril *et al.*, 1994b). Similar results were obtained when a stably transfected HSP70 was over-expressed in myogenic cells. Overexpression of human HSP70 in rodent myogenic cell lines either by transient or stable transfection has shown to confer a protective effect against metabolic stress (Williams *et al.*, 1993) and simulated ischemia (Mestril *et al.*, 1994b).

In summary, these results indicate that HSP70, if not solely responsible, must play an important role in the myocardial protection obtained following a whole body heat stress. Conclusive evidence that HSP70 plays this protective role *in vivo* as well as *in vitro* is within our present day experimental possibilities. Delivery of exogenous copies of HSP70 using viral vectors (e.g. adenovirus) to heart tissue of experimental animals or stable integration of exogenous copies of the HSP70 gene in the germ line of transgenic mice should elicit in the near future if the exclusive increase of HSP70 is capable of conferring protection against myocardial ischemia.

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