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Cardioprotective effects of 70-kDa heat shock protein in transgenic mice

NINA B. RADFORD*†, MAGGY FINA*, IVOR J. BENJAMIN*, RANDALL W. MOREADITH*, KATHY H. GRAVES*, PIYU ZHAO‡, SANDHYA GAVVA‡, ANDREA WIETHOFF‡, A. DEAN SHERRY†‡, CRAIG R. MALLOY*†, AND R. SANDERS WILLIAMS*§¶

Departments of *Internal Medicine, §Biochemistry, and †Radiology, University of Texas Southwestern Medical Center, Dallas, TX 75235; and ‡Department of Chemistry, University of Texas at Dallas, Richardson, TX 75080

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ABSTRACT **Heat shock proteins are proposed to limit injury resulting from diverse environmental stresses, but direct metabolic evidence for such a cytoprotective function in vertebrates has been largely limited to studies of cultured cells. We generated lines of transgenic mice to express human 70-kDa heat shock protein constitutively in the myocardium. Hearts isolated from these animals demonstrated enhanced recovery of high energy phosphate stores and correction of metabolic acidosis following brief periods of global ischemia sufficient to induce sustained abnormalities of these variables in hearts from nontransgenic littermates. These data demonstrate a direct cardioprotective effect of 70-kDa heat shock protein to enhance postischemic recovery of the intact heart.**

Previous studies have demonstrated a cytoprotective function for heat shock proteins during thermal stress or energy deprivation in cultured cells (1–6). Expression of endogenous 70-kDa heat shock protein (hsp70) is induced by ischemia in intact hearts (6, 7), and this response correlates with a reduced susceptibility to ischemic injury (8–11). Preconditioning stimuli that induce hsp70, however, also invoke other responses that may mitigate myocardial dysfunction resulting from ischemia (12–14). The proposition that stress proteins exert direct cardioprotective effects remained conjectural until the development of transgenic mouse models, which provide an opportunity for rigorous tests of this hypothesis by examination of relationships between heat shock protein expression and various descriptors of ischemic injury. The goal of this work was to assess the effects of forced constitutive expression of hsp70 on recovery of high-energy phosphate metabolism (assessed by ^{31}P NMR spectroscopy) following periods of global ischemia in isolated perfused mouse hearts.

MATERIALS AND METHODS

Transgenic Mouse Lines. Transgenic mice (FVB/N strain) were generated by standard methods using a linearized DNA fragment containing full-length human inducible hsp70 cDNA (15) inserted downstream of the human β-actin promoter (16) and upstream of the polyadenylylation site and small t antigen intron from the simian virus 40 early promoter. Animals were screened by Southern analysis of tail DNA after digestion with *Sca* I, which cuts once within the transgene. Of five original founder animals, two animals representing different chromosomal insertion sites were bred to establish lines used in these experiments (transgenic lines 813 and 820). Genotypes were confirmed by repeat Southern analysis of DNA extracted from hearts at the termination of the perfusion experiments.

Analysis of Transgene Expression. Expression of human hsp70 mRNA was assessed by a reverse transcriptase–PCR assay. RNA was extracted from myocardial tissue samples by standard methods, and cDNA was synthesized with reverse transcriptase by using an oligo(dT) primer. Primer pairs were specific either for murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (loading control) or human hsp70 cDNA and generated amplification products of 400 or 496 bp, respectively. RNA isolated from heat shocked human HeLa cells served as a positive control. The identity of the PCR product generated from the transgenic hearts when using human hsp70 primer pairs was confirmed by cleavage with *Cla* I, the restriction site for which is absent from the murine hsp70 sequence, to produce a 453-bp fragment. Proteins extracted from whole hearts were separated by isoelectric focusing and SDS/PAGE, and two-dimensional immunoblot assays (17) were conducted by using an exquisitely sensitive rabbit anti-human inducible hsp70 antibody.

Langendorff Perfusion. The care and treatment of all animals in this study were in accord with the recommendations of the National Institutes of Health and the U. S. Department of Health and Human Services. The protocol was approved by the Institutional Review Board for Animal Research. Hearts were isolated from anesthetized transgenic mice (25–30 g) and perfused in the standard Langendorff mode with a perfusion pressure of 90 mmHg with a phosphate-free Krebs–Henseleit solution containing 118 mM NaCl, 4.7 mM KCl, 2.5 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 5.5 mM glucose, 3.0 mM $CaCl_2$, and 0.5 mM Na_2 EDTA (18). This bicarbonate-buffered medium was bubbled continuously with 95% O_2/5% CO_2; the pH was 7.4. Heart rate and left ventricular developed pressure were recorded on a Colbourne (Lehigh Valley, PA) model R14-18 strip-chart recorder by using an open-ended, fluid-filled catheter placed into the left ventricle through the left atrium and connected in turn to a Gould (Cleveland) model P23 ID pressure transducer. These conditions were established as optimal on the basis of preliminary studies of over 50 wild-type animals.

^{31}P NMR Spectroscopic Methods. A General Electric model GN 500 (11.75 T) spectrometer operating at 202 MHz for ^{31}P was equipped with a 10-mm probe which could be tuned to either ^{23}Na or ^{31}P. Magnetic field homogeneity was adjusted by using the ^{23}Na free-induction decay; a linewidth of approximately 12 Hz was usually obtained. Fully relaxed ^{31}P spectra were acquired with a 11.75-sec interpulse delay, using a 90° pulse for a total of 360 acquisitions over 1.2 h. Subsequent ^{31}P spectra were acquired with a 250-msec interpulse delay for a total of 360, 600, or 1200 acquisitions over 3, 5, or 10 min, respectively. ^{31}P spectra were obtained during three intervals: at baseline after equilibration, during global ischemia for 6, 10, 20, or 25 min, and during 30 min of recovery.

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Abbreviations: hsp70, 70-kDa heat shock protein; TG, transgenic; WT, wild type.

¶To whom reprint requests should be addressed.

Data Analysis. Investigators performing the perfusion studies were blinded to the genotype of the initial experimental animals until after analysis of spectroscopic data was complete. A complete set of spectroscopic data was obtained from 32 heterozygote transgenic animals (TG) and 19 wild-type nontransgenic littermates (WT). Initial studies of hearts subjected to 6 or 10 min of ischemia (n = 19 TG and 10 WT) were conducted by using animals from both of the two independent transgenic lines representing different chromosomal insertion sites (lines 813 and 820), and no differences were apparent between the two transgenic lines. Consequently, in subsequent studies in which hearts were subjected to 20 or 25 min of ischemia (n = 13 TG and 9 WT), a single line of transgenic animals was examined (TG line 813).

Three ^{31}P spectra were summed at baseline and at the end of recovery to enhance signal to noise for the measurement of the resonance areas for phosphocreatine, inorganic phosphate, and β-ATP, which were measured in each spectrum by using an automated curve fitting routine. pH was calculated from the chemical-shift difference between the inorganic phosphate and the phosphocreatine peaks (19). Differences among the pooled data from the three groups subjected to 6 or 10 min of ischemia (TG line 813, TG line 820, and WT) were compared by using repeated measures analysis of variance with the Student–Newman–Keuls test. Differences between the two groups subjected to 20 or 25 min of ischemia (TG line 813 and WT) were compared by using Student's t test.

RESULTS

The human hsp70 transgene was transcribed in hearts isolated from both of the two transgenic mouse lines studied in this analysis (Fig. 1*A*). Expression of human inducible hsp70 protein in these hearts was detected by two-dimensional immunoblot analysis (Fig. 1*B*). No differences in transgene expression were evident between the two independent transgenic lines. Of note, we did not detect the mouse inducible hsp70 in wild-type hearts as others have shown when using this antibody (20). This may be related to the smaller sample of protein used in our assay (600 μg of total protein from each heart was used for immunoblots, except for no. 6, which contained 1.2 mg; Fig. 1*B*). Overexpression of human hsp70 expression did not appear to interfere with normal development: body weights, gross appearance of internal organs at necropsy, and histological appearance of the hearts from transgenic animals were indistinguishable from those of nontransgenic littermates. A clear phenotypic difference resulting from expression of the transgene was revealed, however, by examination of isolated hearts subjected to ischemic stress.

Representative ^{31}P NMR spectra from wild-type and transgenic hearts acquired at baseline, after 6 min of global ischemia, or following 30 min of reperfusion are shown in Fig. 2. In the absence of ischemia, all metabolic variables were stable for up to 120 min of perfusion (data not shown). The 6-min duration of ischemia was sufficient to deplete high-energy phosphate stores and induce acidosis in both transgenic and wild-type hearts. In addition, developed pressure fell to zero in all hearts during the ischemic period.

During recovery, however, significant differences were observed in the transgenic hearts compared with the wild-type controls. Representative spectra are shown in Fig. 2 and demonstrate that β-ATP and phosphocreatine levels were increased and that inorganic phosphate levels declined toward baseline values in the transgenic heart, while severe abnormalities of these variables persisted in the wild-type control heart.

This improved recovery of high-energy phosphate stores in both the 813 and 820 lines of transgenic hearts was significant in hearts subjected to 6 or 10 min of global ischemia and 30 min of reperfusion. This effect of transgene expression was lost, however, after longer durations of global ischemia (20 or 25 min). Data illustrating recovery of ATP concentrations are summarized in Fig. 3. While there was no difference in pH among the two transgenic lines and the wild-type hearts at baseline, ischemia-induced intracellular acidosis was corrected during recovery from ischemia in transgenic hearts following 6 min of ischemia (pH 7.06 ± 0.07 vs. pH 6.90 ± 0.10; $P < 0.05$). These differences were not observed in hearts experiencing more prolonged ischemia.

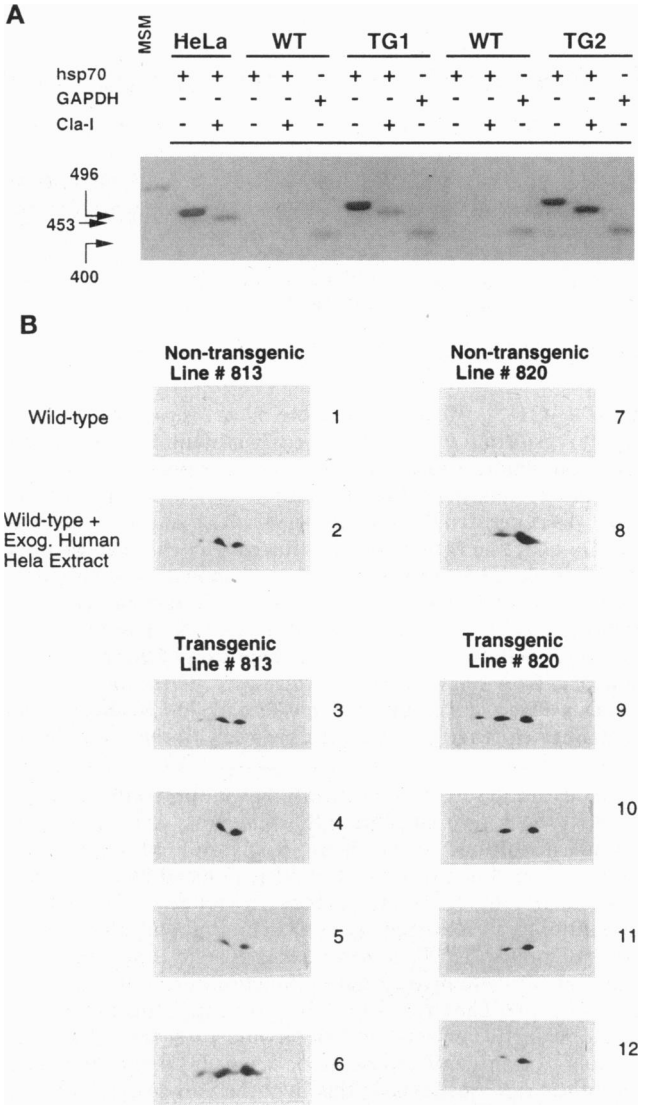

FIG. 1. (*A*) Reverse transcriptase–PCR assay of hsp70 mRNA from each of two independent hsp70 transgenic lines (TG1 and TG2). The 496- and 453-bp fragments represent intact and *Cla* I-digested human hsp70 cDNA, respectively. The 400-bp fragment represents glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, reverse transcribed from the endogenous mouse transcript, and serves as a control for the integrity of RNA samples from each heart. MSM, molecular size marker. (*B*) Two-dimensional immunoblot analysis of hsp70 protein expression in transgenic and wild-type hearts. Protein extracted from heat-shocked HeLa cells was mixed with protein extracted from a wild-type mouse heart to provide an unambiguous source of human hsp70 as a positive control. The rabbit anti-human polyclonal antibody crossreacts with human inducible hsp70 which is present in transgenic hearts but not in wild-type hearts (600 μg of total protein from each heart was used for immunoblots, except for no. 6, which contained 1.2 mg). A similar pattern was observed in four representative animals in both of the two independent transgenic lines.

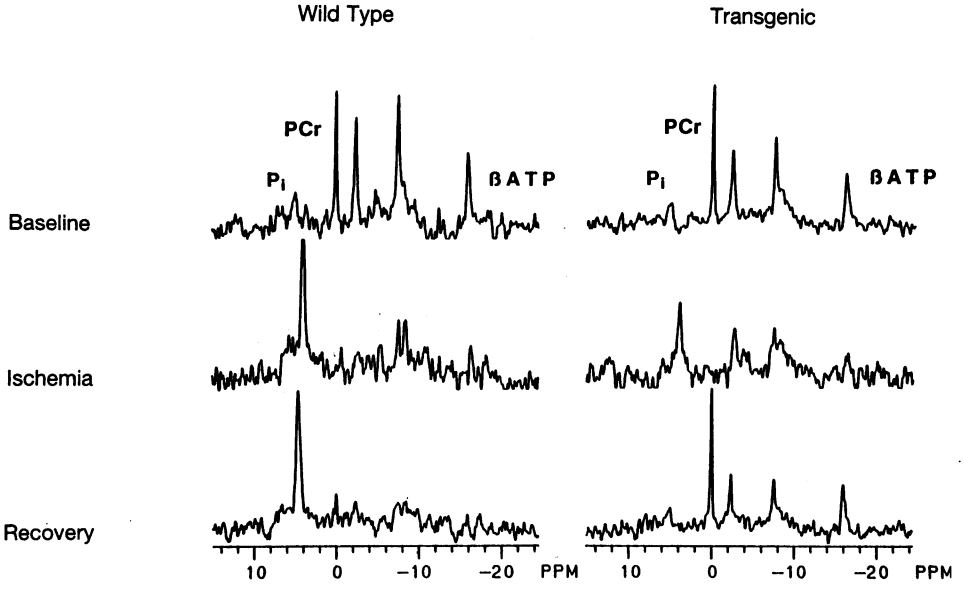

FIG. 2. ^{31}P NMR spectra from a wild-type and a transgenic perfused mouse heart at baseline, following 6 min of global ischemia, and following 30 min of reperfusion. The positions of peaks representing inorganic phosphate (P_i), phosphocreatine (PCr), and β-adenosine triphosphate (βATP) are indicated.

The salutary effects of hsp70 transgene expression on metabolic recovery were accompanied by enhanced recovery of contractile function. After 6 min of global ischemia, the recovery of developed pressure (systolic pressure minus diastolic pressure) at 30 min was essentially complete in both transgenic lines while wild-type hearts recovered to only 40% of baseline (data not shown). Following longer time periods of ischemia, however, the differences in recovery of mechanical function were not significantly different among all hearts.

DISCUSSION

Major stress proteins, including but not limited to hsp70, are clearly involved in promoting thermotolerance in prokaryotes, unicellular eukaryotes, insects, and in mammalian cell lines (21). In addition, we (1) and others (2) have previously demonstrated salutary effects of hsp70 on cell survival and metabolic recovery in cultured cells subjected to conditions simulating ischemia. Our present findings demonstrate that metabolic recovery from *bona fide* ischemia is enhanced by forced expression of hsp70 in the more complex environment of the intact heart and provide direct evidence for a cytoprotective function for stress proteins in tissues, as opposed to isolated cells, of vertebrate organisms. These findings compliment studies done concurrently in two separate laboratories with transgenic mouse lines which showed enhanced mechanical recovery in isolated perfused mouse hearts which overexpressed hsp70 (20, 22).

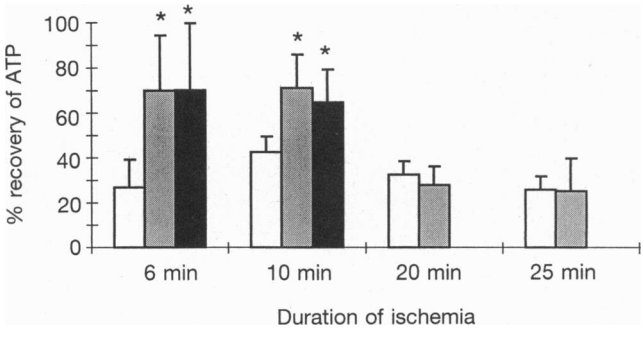

FIG. 3. Summary of changes in ATP following ischemia and reperfusion in WT (open bars) and hsp70 TG (813 line, gray bars; 820 line, black bars) hearts. Histograms represent mean ± SD within groups after 30 min of reperfusion following the indicated periods of global ischemia, expressed relative to baseline values in each heart (*, $P < 0.05$ compared with WT).

The molecular basis for the cardioprotective activity of hsp70 remains to be established, but presumably is related to enhanced refolding to functional conformations of critical cellular proteins that become partially denatured during the ischemic stress. Studies in cell-free systems have demonstrated an enhancing effect of major stress proteins on recovery of enzymatic function of purified proteins following thermal denaturation (23, 24). In addition, based on the chaperone function of hsp70 with respect to nascent polypeptides (25–27), a greater abundance of hsp70 in the recovery period may promote more rapid replacement of irreversibly damaged proteins with newly synthesized polypeptides.

Our study design did not permit systematic assessment of a dose–response relationship between concentrations of hsp70 and indices of myocardial recovery following ischemia. However, the magnitude of overexpression of hsp70 is sufficient to evoke favorable effects. Thus, the constitutive overexpression of human hsp70 transgene that appears not to alter the induction of endogenous hsp70 gene expression (20, 22) is directly responsible for the observed phenotype in postischemic metabolic recovery. This result is also consistent with previous studies in rat or rabbit hearts preconditioned with heat shock 24 h before coronary occlusion in which cardioprotective effects were associated with a similarly induction of expression of endogenous hsp70 (8–10).

Enhanced metabolic recovery resulting from expression of the hsp70 transgene in the intact heart was not observed in hearts subjected to longer durations of ischemia (≥20 min). This result is similar to our previous findings in cultured cells grown under conditions of simulated ischemia (1) and indicates, not surprisingly, that the cytoprotective function of hsp70 can be overwhelmed if the ischemic insult is sufficiently severe. While higher levels of hsp70 expression may extend the time period in which cytoprotective effects are observed, there is evidence that massive overexpression of hsp70 in unstressed cells may have deleterious effects. Clones of mammalian or insect cells stably transformed in culture with a foreign hsp70 gene exhibit abnormal growth properties, suggesting negative selection against sustained, high-level expression of this pro-

tein (1, 2, 4). Future studies of relationships between expression of hsp70 and cellular recovery from ischemia or other stresses should consider the potentially narrow concentration range of hsp70 that may evoke favorable, as opposed to deleterious, effects.

The observation that forced expression of hsp70 enhances recovery from ischemic stress in the intact heart has potential clinical implications (11, 28). Preconditioning maneuvers or gene transfer strategies to augment expression of heat shock proteins could provide clinical benefits to patients undergoing surgical or catheter-based procedures that involve a high risk of myocardial ischemia.

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