Basic Res Cardiol 91: 283 – 288 (1996)

ORIGINAL CONTRIBUTION

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Regional distribution of HSP70 proteins after myocardial infarction

Received: 6 November 1995
Returned for revision: 21 December 1995
Revision received: 10 January 1996
Accepted: 15 January 1996

This study was supported in part by a grant from the American Heart Association – Kansas Affiliate (KS-94-GS-30)

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Abstract Hypoxia and altered hemodynamic status, both components of myocardial infarction, have been shown to be potent inducers of the 70 kD family of heat shock proteins (HSP70). We hypothesized that after infarction, the surviving myocardium would synthesize HSP70 proteins in a temporally and regionally distinct pattern. We believed that there would be a lack of an HSP70 response in the infarcted area (I), reflecting the loss of viable cells. We further postulated that tissues bordering infarctions (M) would have a compromised HSP70 response. Conversely, we proposed that HSP70 would be induced in septal tissues (S) of the infarcted heart, as a hypertrophic adaptation. A rat model of myocardial infarction was used to examine the changes in relative concentration and distribution of three major HSP70 family proteins; cytoplasmic HSP72, mitochondrial HSP75, and endoplasmic reticular GRP78 (glucose regulated protein) during 21 days of recovery. While all three HSP70 family proteins investigated were detected in all hearts from all groups at all time periods, experimental treatment (infarction) induced changes in relative protein concentrations that varied with time and sample site location. Relative concentrations of HSP72 and GRP78 were unchanged in the 24 h following infarction while relative HSP75 concentrations were halved in M tissues during the same time period. Between days 5 and 7, several changes were noted. M samples displayed nearly twice the relative concentrations of HSP75 and GRP78 after infarction, but showed no change in HSP72. S tissues showed two-fold or larger increases in all three HSP70 family proteins. I samples showed unanticipated increases in HSP75 and GRP78 during this time period. After 14 to 21 days of recovery, HSP70 family protein concentration levels in M, S, and I tissues from infarcted hearts had returned to levels similar to those seen in control animals. We conclude that the myocardium is unable to, or does not, mount an immediate HSP70 response after infarction but does recover such activity by 5 – 7 days after infarction.

Key words Infarction – HSP72 – HSC73 – HSP75 – GRP78

BRC 982

Basic Research in Cardiology, Vol. 91, No. 4 (1996)

Introduction

Studies demonstrating the sparing effect of heat shock protein 70 (HSP70) in the heart prior to ischemia and infarction (preconditioning) have generated a widespread effort to understand the underlying protective mechanisms (5, 8, 19). As most studies have focused on ischemia/reperfusion models, it is appropriate to investigate changes in HSP70 levels response to permanent coronary artery occlusion. The first step, a description of infarction-induced changes that occur in non-preconditioned hearts is presented.

Hypoxia and altered hemodynamic pressure have been shown to be potent inducers of HSP70 (4, 14). Since both of these stresses are consequences of myocardial infarction, we hypothesized that the surviving myocardium would synthesize HSP70 proteins in the period following infarction. While previous research has demonstrated a direct correlation between induced HSP70 and reduced infarct size, there is a lack of information regarding the specific areas of the heart where increases occur. Data suggests that there may be regionally dependent HSP70 response. Salvage of the pre-conditioned myocardium is enhanced by rescue of tissues at the margins of the infarct and, while no data exists clearly demonstrating such, it is possible that the HSP70 response is important in sparing tissues bordering infarctions in the normal heart (not preconditioned). We proposed that the HSP70 response would be minimal in the infarcted area, reflecting the loss of metabolically competent cells (1).

We also proposed that HSP70 would be induced in septal tissues of the infarcted heart. Delcayre (4) demonstrated that an increased HSP70 content follows an increase in hemodynamic load in 2 – 4 days. It is probable that this increase is linked to an adaptive increase in protein synthesis in the overloaded myocardium. Most septal myocardium is not ischemically compromised with permanent coronary artery occlusion in the rat, but is subject to altered mechanical forces and, as a result, the septum actively remodels and enlarges in response to freewall infarction (1). If cardiac muscle acts in a manner similar to skeletal muscle during hypertrophy (10, 12), it would be expected to have a hypertrophy-related, long-term increase in HSP70 concentrations in this region of the post-infarction myocardium. To test these hypotheses, we used a rat model of myocardial infarction to examine the changes in concentration and distribution of three major HSP70 family proteins during 21 days of recovery. The three HSP chosen for study, HSP72, HSP75, and GRP78 are found in specific cellular compartments within the cell (cytosol/nucleus, mitochondria, and endoplasmic reticulum, respectively) enabling examination of the HSP70 response at both tissue and subcellular levels.

Methods

Rat model of myocardial infarction

Thirty female Sprague-Dawley rats (mean bodyweight, 258 ± 44 g) were used as subjects, 15 randomly assigned to the experimental treatment group and 15 assigned to a control group. Infarctions were induced by the method described by Musch et al. (16). Animals were anesthetized with a combination of 60 mg/kg ketamine HCI (Ketaset, Fort Dodge) and 12 mg/kg xylazine (Rompun, Miles) injected IP. Subjects were then intubated, placed on a rodent respirator (Harvard model 680) and maintained on room air. Following left thoracotomy and dissection of the pericardium, the left main coronary was ligated at a point approximately 2 mm distal to the edge of the atrium with 6-0 TI-CRON suture (Davis and Geck). Muscle, fascia, and skin were then closed, animals extubated and allowed to recover under constant supervision. Control (sham-operated) animals underwent an identical procedure except the coronary artery suture was not tied off.

Intact hearts were harvested at several time points after coronary artery ligation or sham-operation; 1 day, 5 – 7 days (mean = 5.8 days), and 14 – 21 days (mean = 16.8 days). Subjects were anesthetized with 100 mg/kg of pentobarbital sodium (Fort Dodge), the thoracic cavity opened, and intact hearts were rapidly excised, trimmed of vessels, fat, and connective tissue. Infarcted tissue, margin tissue, and uncompromised septal tissues were dissected from infarcted hearts (Fig. 1), or from corresponding locations in control animals, and immediately snap-frozen in liquid N_2. Infarctions were confirmed by ECG and by visual observation of left ventricular wall thinning prior to inclusion in the experiment.

Laboratory animals were given appropriate care consistent with the guidelines of the American Physiological

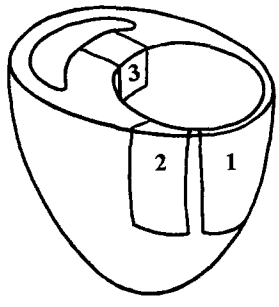

Fig. 1 Sampling sites. Samples were obtained from three sites after experimental infarction or from equivalent locations following sham-operation: 1) infarcted, 2) margin, and 3) septal tissues.

Society and housed in a facility accredited by the American Association for Accreditation of Laboratory Animal Care.

Tissue preparation

Frozen tissues were rapidly thawed and proteins extracted by homogenization in 5 volumes of 10 mM TRIS-acetate, 10 mM NaCl, 0.1 mM EDTA, and 15 mM Mercaptoethanol (pH 7.6). Following homogenization, samples were centrifuged at 12000 × G for 20 min at 4 °C. Protein concentrations were determined using the Bio-Rad™ Protein Assay. Supernatants were prepared for slot-blot analysis by adjusting protein concentrations to 20 μg/100 μl with homogenization buffer.

Slot Blot and Western Blot Analysis

Prepared samples (20 μg total protein) were applied to PVDF membranes (Micron Separations Inc.) using a Bio-Dot SF apparatus (BioRad). Following slot blot, membranes were probed overnight with monoclonal antibodies specific for the HSP of interest; anti-HSP72 mouse monoclonal (SPA-810, StressGen), anti-HSP75 mouse monoclonal (SPA-825, StressGen), anti-GRP78 mouse monoclonal (SPA-827, StressGen). Primary antibodies were diluted 1:10000 in TROPIX I-Block. One-hour incubations with the appropriate biotinylated anti-mouse IgG secondary antibody (B764, Sigma Immunochemicals) and an alkaline phosphatase conjugate followed (Ultra-Avidin-Alkaline Phosphatase, A108, Leinco). Both the secondary antibody and the alkaline-phosphatase conjugate were diluted 1:5000 in TROPIX I-Block. Membranes were prepared for autofluorography with TROPIX AMPPD chemiluminescent substrate and visualized on Kodak XAR film. Films were scanned on a Hoefer GS300 densitometer to obtain integrated peak areas to quantify relative amounts of membrane bound HSP70. Negative (10 μg bovine serum albumin) and positive HSP72 (0.1 μg purified HSP72, StressGen) controls were included on each blot to demonstrate specificity of primary antibodies. Purified HSP72 was a positive control on HSP72 assays only, it served as an additional negative control on HSP75 and GRP78 assays.

Statistical analysis

Densitometric results were analyzed using an ANOVA procedure with Bonferroni post-hoc tests. A p-value of less than 0.05 was considered significant.

Results

Each HSP70 family protein investigated was detected in all hearts from all groups at all time periods. Experimental treatment (infarction) altered relative protein concentrations to various extents over time and by sample site location. Concentrations of HSP72 and GRP78 did not change, regardless of sample site, in the 24 h following infarction (Table 1A and 1C; Fig. 2A and 2C). A 57 % reduction in relative HSP75 concentrations was noted in margin tissues during the same time period (Table 1B; Fig. 2B).

At the Day 5 – 7 time point, several changes were noted. Samples from infarct margins displayed a 1.9-fold increase in relative concentrations of HSP75, a 1.8-fold increase in GRP78, but lacked a HSP72 response. Septal tissues showed a 2.2-fold increase in relative HSP72 concentration, a 2.8-fold increase in HSP75, and a 2.0-fold increase in GRP78. Infarcted ventricular freewall samples showed increases in HSP75 (2.8-fold increase) and GRP78 (1.8-fold increase) during this time period. By 14 to 21 days HSP70 family protein concentration levels in margin, septum, and infarct tissues from experimentally treated hearts had returned to levels similar to those seen in control animals.

Discussion

This study demonstrates several points. First, the myocardium does not mount a rapid HSP70 response in and around infarcted tissues after permanent coronary artery occlusion. While it is an attractive hypothesis to believe that damage to the myocardium can be controlled or reduced by increasing concentrations of HSP70 family proteins, such a response is not rapid enough after naive infarction to produce a sparing effect. The absence of HSP72 and GRP78 elevations, coupled with the depression of HSP75 responses in margin tissues 24 h after infarction, points out the potential importance of treatments, compounds, or activities that could induce HSP70 family proteins prior to insult.

Several studies have demonstrated the protective effect of preconditioning with brief (5 – 15 min) ischemia or heat shock (3, 5, 21). The current study suggests a compromised HSP70 response after infarction in naive hearts and provides further evidence of the potential therapeutic value of HSP70 induction prior to infarction. Although the overwhelming majority of research in this area focuses on a single HSP70 family protein, HSP72, it is probable that other HSP70 family proteins may also be involved in the adaptations or protection afforded by preconditioning.

Table 1 Immunoreactivity of margin, septum, and infarct tissues from sham operated and infarcted rat hearts. Regional comparisons were made between infarcted tissues and the corresponding sham-operated tissue sample. Values are means ± SEM of densitometric results, * = < 0.05 compared to sham-operated values. Abbreviations: SO = Sham-operated, INF = experimentally infarcted.

A. HSP72 Immunoreactivity

Day	MARGIN		SEPTUM		INFARCT	
	SO	INF	SO	INF	SO	INF
1	1863 ± 182	1700 ± 236	2272 ± 298	2162 ± 461	1553 ± 200	1700 ± 320
5–7	1511 ± 279	1857 ± 290	1225 ± 116	2636 ± 246*	1374 ± 123	1331 ± 122
14–21	1971 ± 127	2552 ± 325	2000 ± 289	2186 ± 228	1579 ± 261	1706 ± 271

B. HSP75 Immunoreactivity

Day	MARGIN		SEPTUM		INFARCT	
	SO	INF	SO	INF	SO	INF
1	1655 ± 249	728 ± 236*	963 ± 115	796 ± 171	1447 ± 263	720 ± 146
5–7	828 ± 90	1590 ± 85*	747 ± 47	2110 ± 458*	705 ± 34	1976 ± 303*
14–21	994 ± 70	985 ± 45	717 ± 50	774 ± 32	1180 ± 362	740 ± 83

C. GRP78 Immunoreactivity

Day	MARGIN		SEPTUM		INFARCT	
	SO	INF	SO	INF	SO	INF
1	1391 ± 179	1083 ± 153	875 ± 98	1077 ± 157	1044 ± 217	1200 ± 321
5–7	1381 ± 144	2493 ± 124*	1444 ± 202	2904 ± 440*	1219 ± 57	2147 ± 384*
14–21	933 ± 288	1188 ± 213	832 ± 113	899 ± 167	778 ± 192	941 ± 158

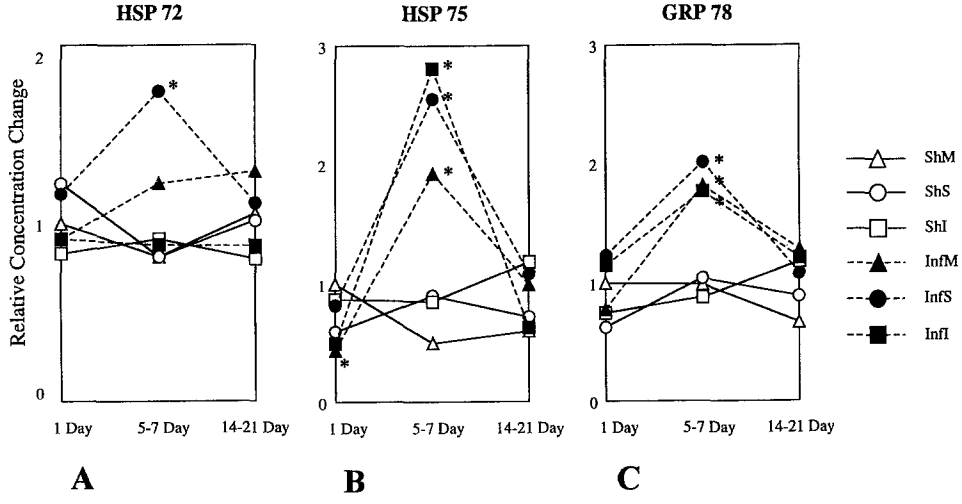

Fig. 2 Time-course and distribution of HSP72 after coronary artery ligation. "Margin" and "infarct" samples of sham-operated animals were obtained at equivalent sites, and all values shown were normalized to margin tissues of sham-operated animals. Statistical analysis was performed on raw densitometric data. Abbreviations: ShM = Sham-operated margin, ShS = Sham-operated septum, ShI = Sham-operated infarct area, InfM = Infarcted margin, InfS = Infarcted septum, InfI = Infarct area, *= $p < 0.05$ compared to sham samples.

With the exception of an HSP75 decrease in the margin, each of the HSP70 family proteins investigated here responded to infarction in a similar manner. The significance of the lower relative concentrations of mitochondrial HSP75 in the infarct margin 24 h after coronary artery ligation is not known but, it may be indicative of the loss of functional mitochondria.

A second point demonstrated by this investigation is that all regions of the infarcted heart retain, or recover, the ability to synthesize HSP70 family proteins. Margin tissues showed an enhanced synthesis of mitochondrial HSP75 and endoplasmic reticular GRP78 between days 5 – 7. These changes may be related to increases in protein traffic or recovery of enzyme function occurring during

this time. Similarly, in the septum there was an increase in all three HSP70 family proteins with experimental infarction. Because this area of the heart hypertrophies to compensate for lost myocardial mass after infarction, the changes noted most likely reflect adaptations accompanying increased protein synthetic activity after pressure load changes (15). Previous workers have also demonstrated that hemodynamic overload elicits increases in HSP72 by 4 days which remains elevated at least 7 days after load imposition (4). Alternatively, these elevations may result from relative ischemia in the septal myocardium due to mismatched energy supply and demand.

The increase in HSP75 and GRP78 seen in infarction zones was unexpected as this area loses a majority of its viable myocytes after infarction. It is therefore probable that infiltrating endothelial cells, leukocytes, and fibroblasts are responsible for HSP70 production in this region (7, 17, 20). Since this region is exposed to low oxygen tensions during this period, hypoxic induction of HSP70 in surviving cells is also possible.

The present study also shows that chronic HSP70 adaptations during compensatory hypertrophy of the infarcted heart differs from HSP70 changes seen in long-term skeletal muscle hypertrophy (10, 12). By days 14 – 21, myocardial HSP70 concentrations had returned to control levels, a finding fundamentally different from the elevated levels seen at 28 days of skeletal muscle hypertrophy. While we expected to see similar elevations in the hypertrophying myocardial septum, Ianuzzo and coworkers (9) showed an absence of a whole-heart HSP72 response with hypertrophy caused by aortic banding. In that study, aortic banding led to a 78 % increase in ventricular mass in 30 days but did not result in an elevation of HSP72. While Ianuzzo's findings corroborate long-term data from other studies, their short-term data are inconsistent with ours and others findings showing HSP70 induction at earlier time points (4, 11).

The lack of a sustained HSP70 elevation in the septum through 21 days might indicate that the majority of remodeling has been completed by that time. However, studies do not support this conclusion, since the majority of myocyte hypertrophy after infarction occurs between 2 and 4 weeks post-infarction (18). Similarly, infiltration of the infarcted region by fibroblasts and the related deposition of extracellular matrix components is also at its peak by 19 days after infarction (6), therefore an increased HSP70 content would be expected at this time point if HSP70 is required in new protein synthesis and cardiac remodeling. Since long-term elevations in myocardial HSP70 were not seen, but their role in protein synthesis (2) and links to remodeling have been well documented (10, 12), further mechanistic work is necessary to assign other potential functions to HSP70 in this context.

As the current study shows an immediate lack of response in naive, non-preconditioned hearts after infarction, future studies addressing utilizing appropriate forcings for *in vivo* HSP70 induction and maintenance are necessary to test the hypothesis that HSP70 induction might contribute to myocardial sparing after infarction. Yellon and coworkers found that while prior thermal induction of HSP72 reduced infarct size in isolated rabbit hearts (21), it was ineffective in a similar *in vivo* model (22) and concluded that the physiological perturbations in other systems induced by hyperthermia may have negated the beneficial effects of HSP70 induction. Although several other groups have shown a reduction in infarct size using various *in vivo* models (3, 5, 8), it remains that the methods of induction can often result in subject morbidity (5).This demonstrates a need for the development of additional methods of *in vivo* HSP70 induction which can be exploited clinically, such as exercise (13, 17).

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