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### **Research Article**

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# A Single Myocardial Stretch or Decreased Systolic Fiber Shortening Stimulates the Expression of Heat Shock Protein 70 in the Isolated, Erythrocyte-Perfused Rabbit Heart

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## Abstract

The regulation of heat shock protein 70 (HSP 70) expression was examined in the isolated, red blood cell-perfused rabbit heart by Northern and Western blot analysis. In the isovolumic (balloon in left ventricle), isolated perfused heart, HSP 70 mRNA was increased threefold after 30 min and sevenfold at 2 and 4 h compared to normal, nonperfused hearts. To further elucidate the etiology of the increase in HSP 70 mRNA, the effects of decreased systolic shortening (isovolumic heart) and of a single ventricular stretch were examined. Perfusion without the application of a stretch or the presence of a balloon resulted in no increase in HSP 70 mRNA; while a single stretch resulted in a threefold increase in HSP 70 mRNA. These changes were accompanied by an increase in HSP 70 protein by Western blot analysis. To elucidate the signalling mechanism mediating the increase in HSP 70, hearts were perfused with H7, a protein kinase C inhibitor. H7 did not prevent the induction of HSP 70. These results indicate that initiation of expression of myocardial HSP 70 can be stimulated by a single myocardial stretch or by prevention of systolic shortening. These mechanisms may contribute to the rapid expression of HSP 70 after coronary occlusion when dyskinesia, reduced systolic shortening, and increased diastolic segment length all occur. (*J. Clin. Invest.* 1991. 88:2018–2025.) Key words: mechanical transduction • protein kinase C • hypertrophy

## Introduction

Mechanical overload of the heart has been shown to induce expression of heat shock protein (HSP)<sup>1</sup> 70 (1, 2) and several protooncogenes (1). Such mechanical overload has been postulated to induce gene expression by imposing a stretch (i.e., by causing elongation of the myocytes, activating “stretch recep-

tors,” which in turn “signal” the activation of gene expression (3, 4). We recently reported an increase in HSP 70 expression in both the ischemic and nonischemic regions of a rabbit heart subjected to coronary occlusion (5). In addition to the ischemia, which occurred in the ischemic regions after coronary occlusion, both the ischemic and nonischemic regions had an altered level of wall stress and an altered pattern of systolic shortening. The nonischemic region experienced an increased load as the result of the coronary occlusion markedly decreasing contractile function of the ischemic region, and the ischemic region underwent a mild degree of increase in segment length in both systole and diastole (5). Accordingly, we hypothesized that such an increase in segment length or fiber stretch, could induce expression of HSP 70 as has been shown for several protooncogenes. However, in our previous studies of coronary occlusion in the open-chested rabbit we could not exclude neural or humoral factors (e.g., increased circulating catecholamine levels) as factors responsible for the increased expression of HSP 70.

The purpose of this study was to use an isolated perfused rabbit heart, free of circulating neural and humoral factors, to study the effect of myocardial fiber stretch on the expression of HSP 70. During these experiments, we also observed that reduced systolic fiber shortening, independent of stretch, was a potent stimulus to HSP 70 expression and we compared the effect of stretch and reduced systolic fiber shortening to ischemia, to determine their relative efficacy in inducing HSP 70 expression. Because other workers have suggested that protein kinase C activation may be part of the pathway, which signals the gene that a mechanical overload has occurred, we assessed the effect of 1-(5-isoquinolyn-sulfonyl)-2-methyl piperazine (H7), a potent inhibitor of protein kinase C, on the induction of HSP 70 expression.

## Methods

To accomplish our experimental goals, we used an isolated blood-perfused rabbit heart preparation (6, 7). The extent of systolic fiber shortening was varied by the absence or presence of a fluid-filled intraventricular balloon, the presence of the balloon preventing systolic fiber shortening. Myocardial fiber stretch was caused by transiently elongating the isolated heart along the long axis of the left ventricle by inserting a cannula through the mitral valve orifice and pressing it against the endocardial surface of the left ventricle until visible elongation of the heart was apparent. In other experiments a brief myocardial stretch was imposed by transiently cross-clamping the ascending aorta of the anesthetized open chest rabbit for several seconds until visible distension of the heart occurred. These impositions of stretch were compared to the imposition of global ischemia.

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1. Abbreviations used in this paper: GAPD, glyceraldehyde-3-phosphate dehydrogenase; HSP, heat shock protein; LVEDP, left ventricular end diastolic pressure.

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### Experimental preparation

Male New Zealand white rabbits weighing ~ 2.0 kg were anesthetized with 75 mg/kg of intravenous sodium pentobarbital and the thorax was rapidly opened. We used an isolated, isovolumic, working red cell-perfused heart preparation that was developed in our laboratory based on the work of Marshall (8) and has been described previously in detail (6, 7, 9). A short perfusion cannula was inserted into the aortic root and the coronary arteries were perfused via the aortic root with a perfusate containing red blood cells. Less than 20 s elapsed between opening the thorax and the initiation of coronary perfusion in all experiments.

Perfusion pressure was monitored via a sidearm of the aortic cannula connected to a pressure transducer (Gould-Statham P-23 dB; Gould Inc., Oxnard, CA). An apical drain was inserted through the left ventricle in selected experiments to drain any Thebesian circulation. Because insertion of the apical drain caused a transient stretch of the heart, in selected experiments this step was omitted (see below). The pulmonary artery was cannulated, and a second drain was inserted through the pulmonary artery into the right ventricular apical region to completely collect coronary venous effluent and to empty the right ventricle. A pacing wire (model 59; Grass Instrument Co., Quincy, MA) and a thermistor (model 400; Yellow Springs Instrument Co., Yellow Springs, OH) were inserted into the right ventricle via the superior vena cava and right atrium. A collapsed thin-walled latex balloon was placed in the left ventricle via the left atrium and secured in place. The balloon was connected to a pressure transducer (Gould-Statham P23 dB) to measure left ventricular pressure and its first derivative. The fidelity of our pressure recording system satisfies the criteria required for accurate measurement of left ventricular pressure and its first derivative (10) and has been previously reported (11). The heart was then placed in a water-jacketed constant temperature chamber and submerged in saline.

The perfusion system consisted of a "venous" reservoir, a variable flow pump, an oxygenator, a water jacket arterial reservoir, and a filter of 20- $\mu$ m pore size. In this system the perfusion pressure was controlled by a valve that adjusted the pressure of the arterial reservoir. Coronary blood flow was allowed to vary dependent on coronary vasomotor autoregulation.

A red cell perfusate as previously described (7, 8) was used, consisting of bovine red blood cells at a final hematocrit of 40% in Krebs-Henseleit buffer. Fresh whole cow blood containing 15,000 U of sodium heparin per liter was spun in a refrigerated centrifuge at 5°C with a rotor speed of 3,000 rpm for 15 min. The supernatant was discarded and the resulting packed red cells were mixed 1:1 (by volume) with Krebs-Henseleit buffer. The cells were washed three times with Krebs-Henseleit buffer and centrifuged at 3,000 rpm for 15 min at 5°C. The resulting packed cells were essentially white cell and platelet free. For future use the packed cells were stored at 4°C and washed daily.

Red blood cells were suspended in a Krebs-Henseleit buffer that contained: 118 mM NaCl, 4.7 mM KCl, 2.0 mM CaCl<sub>2</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 1.2 mM NaHCO<sub>3</sub>, 5.5 mM glucose, 1.0 mM lactate, 0.4 mM palmitic acid (as a source of fatty acids), 4 g% essentially fatty acid-free BSA (Sigma Chemical Co., St. Louis, MO). The buffer was prepared fresh daily. Gentamicin (2 mg/liter) was added to the red blood cell perfusate to retard bacterial growth. The perfusate was equilibrated with 20% O<sub>2</sub>/3% CO<sub>2</sub>/77% N<sub>2</sub> to achieve a PO<sub>2</sub> higher than 120 mmHg and a pH of 7.40.

### Data collection and analytic procedures

Coronary perfusion pressure, left ventricular pressure, and its first derivative ( $dP/dt$ ) were recorded continuously on a multichannel physiologic recorder (Gould Inc., Cleveland, OH). Coronary blood flow was measured by timed collection of the coronary venous effluent. Arterial and venous blood gas analysis (blood gas analyzers; Allied Instruments Laboratory, Dakota, GA) was done intermittently on arterial and venous samples. Myocardial oxygen consumption was calculated from the arterial venous oxygen content differences. Oxygen content of arte-

rial and venous samples were derived from oxygen saturation for the resuspended Krebs's buffer over the experimental range of pH and PO<sub>2</sub> values.

**RNA isolation and Northern blot analysis.** At the completion of nonischemic perfusion protocols the left ventricle was flushed with saline to remove the blood, dissected from the right ventricle, and then frozen in liquid nitrogen. In the ischemia protocol the hearts were snap frozen on the perfusion apparatus, and the frozen apex was subsequently used for RNA isolation. After homogenization of the tissue in guanidinium thiocyanate, RNA was isolated by the method of Chirgwin as previously described (5, 12). RNA samples were analyzed by electrophoresis on 0.9% agarose gels, and equivalence of RNA was verified by visual inspection of the ribosomal 28s and 18s RNA. Samples were then transferred to a nylon membrane (Genescreen; New England Nuclear, Boston, MA) by either capillary action or by vacuum transfer (Stratavac; Stratagene Inc., La Jolla, CA). Membranes were crosslinked in a Stratalinker apparatus (Stratagene). Prehybridization and hybridization were carried out at 65°C as described previously. cDNA probes were synthesized by random hexamer priming with dCTP[<sup>32</sup>P] using a commercial kit (Amersham Corp., Arlington Heights, IL). The Northern blots were exposed to preflashed X-Omat film (Kodak) for 4–18 h at –70°C. Laser densitometry was performed when appropriate (Molecular Dynamics, Sunnyvale, CA).

**Dot blot analysis.** Dot blot analysis was done to allow comparison of relative amounts of HSP 70 mRNA in multiple samples. 2, 4, and 10  $\mu$ g of RNA were applied to a nylon membrane using a dot blot apparatus (Bio-Rad Laboratories, Richmond, CA). Hybridizations and quantitation were carried out as described previously (5).

**cDNA's.** The 2.3-kb Bam HI/Hind III fragment of pH 2.3 (American Type Culture Collection [ATCC], Rockville, MD) was used to probe for HSP 70. This cDNA is for the human HSP 70 and has been previously described (13, 14). For glyceraldehyde-3-phosphate dehydrogenase (GAPD) the 500-bp Hind III/Xba I fragment of pUC 13 was used (ATCC, 15).

**Western blot analysis.** Western blot analysis was performed as previously described (16). Ventricles were thoroughly flushed with saline via the aortic cuff at the completion of the protocols to remove albumin present in the perfusate. As albumin has a similar molecular weight to HSP 70 and is present in large amounts in the perfusate, it can block the interaction of HSP 70 with antibody on the Western blot. Samples were prepared as described previously (16), and after measurement of protein levels by BCA reagent (Pierce Chemical Co., Rockford, IL), aliquots containing 20–30  $\mu$ g protein were separated by SDS-PAGE using a 12% gel. For Western blot analysis samples were run on 8% gels to maximize the separation of HSP 70 and albumin. After transfer to nitrocellulose, the membranes were treated with 5% blotto (5% milk powder in Tris-buffered saline with 0.05% Tween 20), and incubated with monoclonal antibody to HSP 70 (marketed as anti-HSP 72, the antibody reacts with both the inducible and the constitutive forms of the protein, Amersham, 5). An anti-mouse-IgG-horseradish peroxidase second antibody (Sigma) was used at a 1:1,000 dilution, and membranes were then incubated with luminol using a commercial kit (ECL; Amersham). Membranes were exposed to X-Omat film from 1 to 2 min to achieve a satisfactory signal.

**Statistical analysis.** Data were compared using an analysis of variance to determine if there were differences between groups. Significance was then determined using the Student *t* test. A *P* < 0.05 was considered significant. A linear regression was performed to determine if there was an association between two variables.

### Experimental protocols

#### (a) Oxygenated, isovolumic perfusion for 30, 120, or 210 min

In these experiments the isolated hearts were paced at 3 Hz (180 beats/min), a left ventricular apical drain was inserted, and the intraleft ven-

tricular balloon was inserted and balloon volume adjusted to produce a left ventricular end diastolic pressure (LVEDP) of 10 mmHg. Coronary perfusion pressure was maintained at 100 mmHg. This protocol was intended to be our "control protocol," but surprisingly, hearts perfused in this manner exhibited a rapid induction of HSP 70 expression (see results below). Accordingly, subsequent protocols were performed to delineate the specific mechanical "signal" responsible for inducing HSP 70 expression.

#### (b) Variation in intraventricular balloon volume

In this protocol, the intra-LV balloon volume was varied over a volume range from an empty LV balloon to a balloon volume that resulted in an LVEDP of 20 mmHg.

#### (c) Effect of a single myocardial stretch

This protocol was performed to determine whether placement of the apical drainage cannula in our standard perfusion technique could stimulate induction of HSP 70. In the standard perfusion technique a stiff polyethylene cannula was passed through the apex of the left ventricle to drain the cavity of any Thebesian drainage. Placement of the cannula causes a single transient myocardial stretch as the cannula is passed across the mitral valve orifice and pushed against the LV apex until the apex is punctured. To assess the effect of a single ventricular stretch in this protocol we studied hearts perfused for two hours without a balloon or apical drain; in those hearts a catheter was placed across the mitral valve orifice to empty the ventricle of any Thebesian drainage.

#### (d) Aortic cross-clamping

In two hearts, myocardial stretch was avoided during the isolated heart perfusion by means of the transmitral catheter, but a ventricular stretch was deliberately imposed by cross-clamping the aorta for 7 s before removing the heart from the chest of the anesthetized rabbit. Such cross-clamping produced visible dilation of the heart, which was then perfused for 2 h with neither an apical drain nor a ventricular balloon.

#### (e) Effect of ischemia

Three different ischemia protocols were performed after a 30-min warmup period, using the isovolumic preparation as in protocol *a* above with the intra-left ventricular balloon volume adjusted to produce an LVEDP of 10 mmHg before onset of ischemia. In one protocol hearts were subjected to 1 h of low flow ischemia with coronary perfusion pressure reduced to decrease coronary flow by 85 to 90%, followed

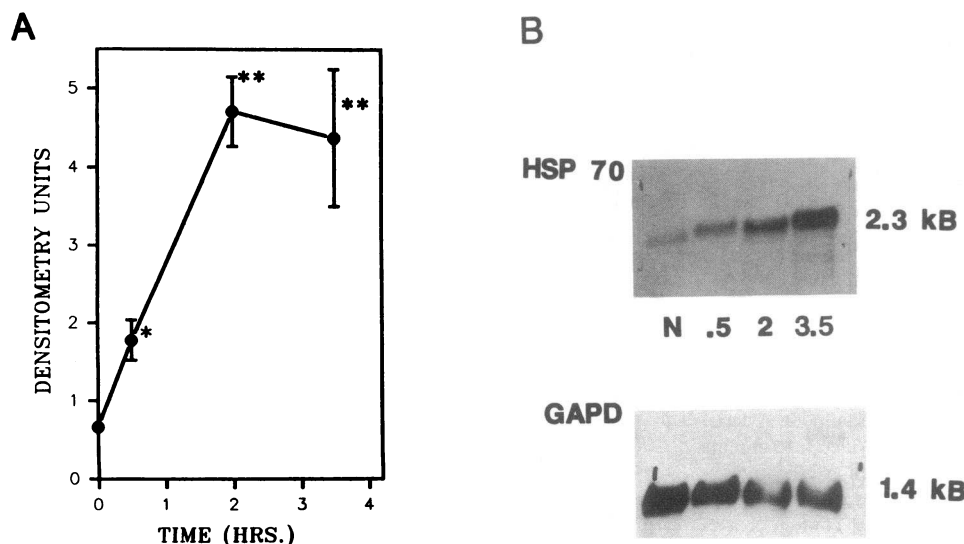
by 30 min of reperfusion. In a second protocol hearts were subjected to 2.5 h of low flow ischemia with coronary perfusion pressure reduced as above and 30 min of reperfusion. In these protocols LVEDP was observed to increase to  $38.5 \pm 5.9$  mmHg at the end of ischemia (ischemic contracture); to assess the influence of an increase in LVEDP per se on HSP 70 gene expression, a third ischemia protocol was performed where hearts were subjected to 2.5 h of low flow global ischemia as above and progressive reduction of intra-LV balloon volume during the ischemia period to maintain LVEDP < 10 mmHg. Hearts subjected to these ischemia/reperfusion protocols were compared to hearts perfused with well oxygenated perfusate at a coronary perfusion pressure of 100 mmHg with an isovolumic intra-LV balloon and LV apical cannula for comparable periods of times for comparison of steady-state levels of HSP 70 mRNA.

#### (f) Protein kinase C inhibition

To test the hypothesis that protein kinase C plays a role in the activation of HSP 70 gene expression we added to the perfusate an inhibitor of protein kinase C, H7, (1-(5-isoquinolyn-sulfonyl)-2-methyl piperazine, Sigma). During a 10-min "baseline" perfusion period, isolated hearts were perfused with careful attention to avoid any myocardial stretch, i.e., no intra-LV balloon or apical drain was placed and a catheter was placed across the mitral valve to keep the LV decompressed. During this zero stretch baseline perfusion period the hearts were perfused with perfusate containing H7 at either 10 or 50  $\mu$ M dissolved in water or ethanol, respectively. A 2-ml aliquot was added to 250–300 ml of perfusate. After 10 min of exposure to H7 the intra-left ventricular balloon and LV apical drain were placed and sufficient volume added to intra-LV balloon to generate an LVEDP of 10 mmHg. Perfusion at a coronary perfusion pressure was then continued for a total perfusion time of 1 h in the presence of H7. Control hearts were perfused in a similar fashion with the addition of vehicle but no H7 to the perfusate.

## Results

*Oxygenated, isovolumic studies; decreased systolic shortening.* When hearts were perfused under standard conditions (intra-left ventricular balloon and apical drain) with an LVEDP of 10 mmHg, there was a rapid induction of HSP 70 mRNA within the first 30 min of perfusion (Fig. 1). Fig. 1 *A* summarizes densitometric analysis of dot blots using total RNA obtained from hearts that were either not perfused at all, or perfused for



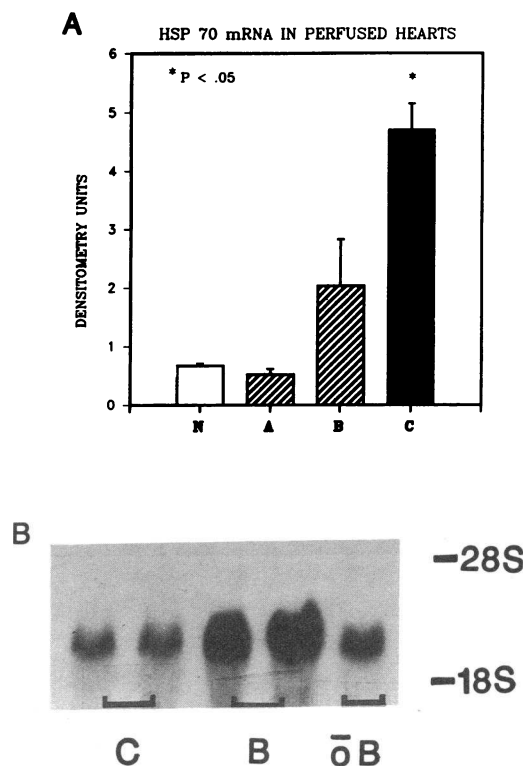
**Figure 1.** (A) The results of densitometric analysis of the time course of the increase in HSP 70 mRNA levels in the normoxic, isolated perfused heart. Results are  $\pm$ SEM. Time 0 represents normal, nonperfused left ventricle.  $n = 6, 7, 7$ , and 6 for the respective time points. \* $P < 0.001$  vs. 0 (normal ventricle); \*\* $P < 0.01$  vs. 0 and 0.5 h. (B) A representative Northern blot demonstrating the time course of the increase in steady-state levels HSP 70 mRNA with perfusion. The lower panel shows the results of hybridizing the same blot with GAPD. N, normal, nonperfused left ventricle; numbers refer to 0.5, 2, and 3.5 h of perfusion.

30, 120, or 210 min. After 2 h of perfusion HSP 70 mRNA levels were sevenfold greater than that seen in normal hearts, and the elevation persisted at 3.5 h. At all time points HSP 70 mRNA levels were significantly increased over baseline ( $P < 0.001$ ). Fig. 1 *B* shows a representative Northern blot analysis of samples from different perfusion times, illustrating the observed increase in HSP 70. To determine if this increase in steady-state levels of HSP 70 mRNA was specific, the same samples were examined for the expression of GAPD. As shown in the lower panel of Fig. 1 *B*, while HSP 70 levels increased with time, GAPD mRNA levels declined slightly during the perfusion.

**Effect of mechanical stretch.** The possibility that mechanical stretch might have a role in induction of HSP 70 mRNA was tested in a series of experiments where hearts were perfused for 2 h under either standard conditions, which involves insertion of an apical drain and a balloon in the left ventricle, or using conditions where the apical drain or the balloon were omitted. All comparisons were made using RNA obtained from freshly removed hearts and the densitometric analysis of HSP 70 expression is shown in Fig. 2 *A*. Perfusion for 2 h without inserting an apical drain and without placing the balloon in the ventricle (*A*) resulted in no increase in HSP 70 mRNA over baseline levels found in the normal ventricle (*N*). Furthermore, placement of the apical drain, with the single stretch during placement, and an empty balloon in the ventricle (*B*) resulted in a threefold increase in HSP 70 mRNA levels compared to the sevenfold increase in hearts perfused with an apical drain and an LVEDP of 10 mmHg (*C*;  $P < 0.05$  vs. normal, and  $< 0.01$  vs. 2 h). In Fig. 2 *B* a representative Northern blot shows the difference observed between normal ventricles (*C*) and hearts perfused for 2 h with an apical drain and a fluid-filled balloon (LVEDP 10 mmHg) present (*B*). In contrast, the final lane of the Northern blot shown in Fig. 2 *B*, illustrates the similarity to normal left ventricular mRNA in the amount of HSP 70 mRNA present in a heart perfused for 2 h without a balloon or an apical drain ( $\bar{O}B$ ).

In two hearts mechanical stretch was achieved by cross-clamping the aorta as described in Methods. Both hearts showed induction of HSP 70 as illustrated in Fig. 3 (*A*). Furthermore, if neither a balloon nor an apical drain was placed in the ventricle, but the ventricle became distended by Thebesian drainage, a mild to moderate increase can be seen in HSP 70, as shown in letter *B* of Fig. 3. For comparison two normal, non-perfused ventricles (*N*) and a ventricle perfused for 2 h without an apical drain or a balloon (*C*) are shown. The lower panel of Fig. 3 shows the results of hybridization of the same blot for GAPD. As seen in Fig. 1, levels of GAPD declined slightly compared to normal.

**Ischemia.** The expression of HSP 70 was measured in a series of isovolumic hearts subjected to ischemia with an initial LVEDP of 10 mmHg. During ischemia all hearts showed a marked reduction of developed pressure and a rise in LVEDP (Table I). As illustrated in Fig. 4, there was no significant effect of ischemia to increase HSP 70 mRNA levels after 2 or 3.5 h. There was no significant difference among hearts perfused for 1 h of ischemia and 30 min of reperfusion (*B*), 2.5 h of ischemia and 30 min of reperfusion (*D*), 2.5 h of ischemia with the balloon volume reduced to lower contracture pressure (LVEDP) below 10 mmHg followed by 30 min of reperfusion (*E*), and hearts perfused for 2 or 3.5 h under normoxic, isovolumic con-

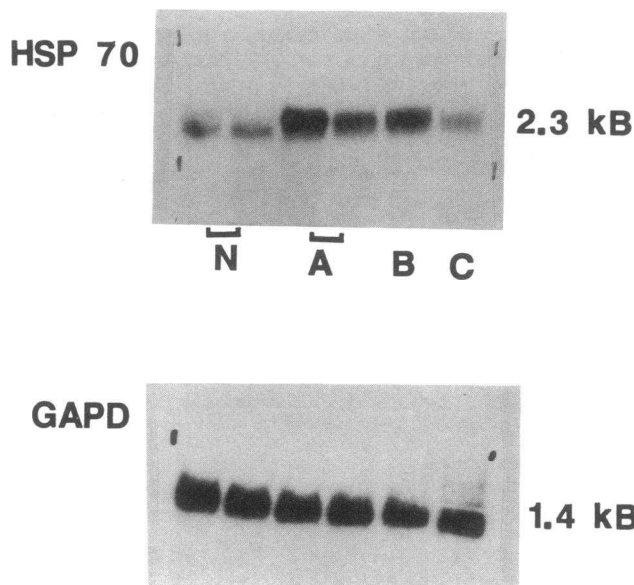


**Figure 2.** (*A*) Densitometric analysis of the effect of a single stretch and isovolumic contraction on steady-state mRNA levels for HSP 70. N, normal, nonperfused hearts ( $n = 6$ ); (*A*) perfusion for 2 h without balloon or apical drain ( $n = 3$ ); (*B*) perfusion for 2 h with an apical drain and an empty balloon (LVEDP =  $-4$  mmHg;  $n = 5$ ); (*C*) perfusion for 2 h with apical drain and a balloon with sufficient volume for an LVEDP of 10 mmHg. Results are  $\pm$ SEM.  $*P < 0.05$  compared to all other groups. B vs. N,  $P < 0.05$ . (*B*) A representative Northern blot illustrating the amount of HSP 70 mRNA present with and without balloon and apical drain. C, normal left ventricle without perfusion; B, 2-h perfusion with apical drain and isovolumic balloon present with LVEDP 10 mmHg.  $\bar{O}B$ , 2-h perfusion without either apical drain or ventricular balloon.

ditions (*A* and *C* in Fig. 4, respectively). In all hearts levels of HSP 70 were significantly increased compared to normal steady-state levels of HSP 70 mRNA in hearts frozen immediately after removal from the thorax ( $P < 0.005$ ).

**Hemodynamics.** The hemodynamic findings in the normoxic and ischemic hearts are summarized in Table I. All groups had similar developed pressure after 30 min of preischemia perfusion. During ischemia developed pressure decreased to  $14.3 \pm 2.6$  mmHg in the 1-h ischemic group, and to  $4.2 \pm 1.0$  mmHg in the 2.5-h ischemic group. In the group where the LVEDP was maintained at  $< 10$ , the developed pressure was  $16.6 \pm 2.1$  mmHg at the end of 2.5 h of ischemia. As expected, the LVEDP increased with ischemia, except in the case of those hearts where balloon volume was adjusted to maintain LVEDP  $< 10$  mmHg.

**Role of LVEDP (altered balloon volume).** LVEDP and developed pressure were analyzed to determine if there were a correlation with a change in the steady-state levels of mRNA for HSP 70 (protocol 2). When the LVEDP was varied from  $-4$  to 20, no association was seen between HSP 70 mRNA levels



**Figure 3.** Northern blot analysis demonstrating effect of stretch on HSP 70 mRNA levels. N, normal, nonperfused hearts; A, hearts perfused for 2 h after cross-clamping the aorta for 7 s to cause passive stretch of the ventricle; B, a 2-h perfusion without apical drain or ventricular balloon in which Thebesian drainage briefly accumulated in the ventricle resulting in distension and stretch of the ventricle; C, a 2-h perfusion with no apical drain, no ventricular balloon, and venting of Thebesian drainage via a small catheter across the mitral valve. The lower panel shows the same blot hybridized with GAPD as a control.

and LVEDP ( $n = 15$ ,  $P = 0.306$ ,  $r = 0.284$ ). Likewise, there was no correlation between developed pressure and HSP 70 mRNA levels ( $n = 11$ ,  $P = 0.514$ ,  $r = 0.199$ ).

**Western blot analysis of HSP 70.** To determine if the increase in HSP 70 steady-state levels of mRNA was accompanied by an increase in HSP 70 protein, Western blot analysis was done on tissue from hearts perfused for 2 h with an LVEDP of 10 mmHg (apical drain and balloon present). As shown in Fig. 5, these hearts all showed a marked increase in HSP 70 compared with normal ventricles. Laser densitometry showed the mean level of HSP 70 in these perfused hearts to be  $1,312 \pm 552$  vs.  $276 \pm 34$  (densitometry units) in the two normal hearts shown in this blot. A single heart perfused with an LVEDP of  $-4$  (apical drain, empty balloon) is shown on the far right of Fig. 5. This heart had a fourfold increase in the amount of HSP 70 protein compared with the two normal hearts. In four of the hearts with an LVEDP of 10 mmHg, both mRNA and protein levels for HSP 70 were determined. These hearts had a 7.3-fold increase in HSP 70 levels compared to normal, nonperfused left ventricles.

**Protein kinase C inhibition.** The effect of H7, a potent inhibitor of protein kinase C, on the induction of HSP 70 was examined using standard 1-h perfusions. Vehicle alone was added to the perfusate for controls. Neither 10 nor 50  $\mu$ M H7 blocked the increase of HSP 70 steady-state mRNA observed with a 1-h perfusion with an apical drain and a ventricular balloon (LVEDP 10 mmHg) as illustrated in Fig. 6 A. The second nor-

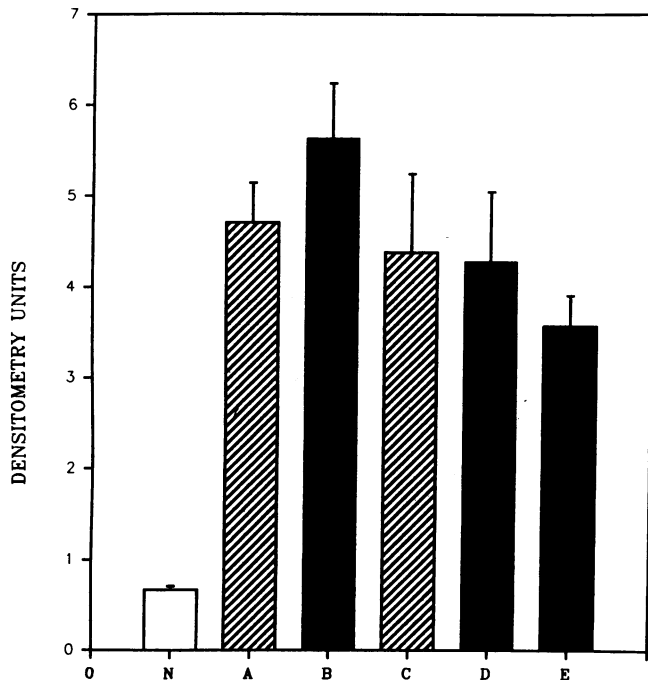
**Table I.** Hemodynamics in Normoxic and Ischemic Hearts

Group	Developed pressure	
	30 min preischemia	End ischemia
mmHg		
2-h perfusions		
Control	$89.0 \pm 10.5$	$74.7 \pm 7.8$
1-h ischemia	$96.8 \pm 4.6$	$14.3 \pm 2.6$
3.5-h perfusions		
Control	$92.0 \pm 7.2$	$78.5 \pm 6.2$
2.5-h ischemia	$95.5 \pm 7.3$	$4.2 \pm 1.0$
2.5-h ischemia, LVEDP < 10	$85.0 \pm 4.4$	$16.6 \pm 2.1$
LVEDP		
2-h perfusions		
Control	$9.9 \pm 0.4$	$7.5 \pm 0.3$
1-h ischemia	$8.8 \pm 0.3$	$29.7 \pm 5.2$
3.5-h persusions		
Control	$9.0 \pm 0.6$	$6.2 \pm 0.6$
2.5-h ischemia	$12.8 \pm 2.3$	$38.5 \pm 5.9$
2.5-h ischemia, LVEDP < 10	$9.9 \pm 0.1$	$9.1 \pm 0.3$

mal heart used as a control in these experiments had slightly higher levels of HSP 70 mRNA than we usually observe. Samples of the same hearts (used in A) were used to determine changes in HSP 70 at the protein level. As illustrated in Fig. 6 B, H7 had no affect on HSP 70 levels as determined by Western blot analysis. The same moderate increase was seen in both the control hearts and the H7-treated hearts. A single control heart showed no increase in HSP 70 protein despite the loading of equivalent amounts of protein on the gel. This may reflect both the early time point examined (60 min) as well as biological variation in response. The one sample that did not show a marked increase in HSP 70 had a larger amount of albumin present on SDS-PAGE than the other samples, and this may have interfered with the Western blot analysis as discussed in Methods.

Although H7 did not inhibit the increase in HSP 70 levels, it did increase coronary flow. Coronary flow at 30 min was 7 ml/min for the 10- $\mu$ M dose group compared to 3.4 ml/min in the control hearts. In the 50- $\mu$ M dose group coronary flow was  $13.4 \pm 0.9$  ml/min vs.  $3.9 \pm 0.1$  ml/min in the controls (ethanol, the vehicle, added to perfusate). When the flow was expressed as a function of wet weight only the 50- $\mu$ M group had a significantly increased flow,  $2.3 \pm 0.2$  ml/min per g vs. a control of  $0.9 \pm 0.1$  ml/min per g ( $P < 0.001$ ) as shown in Table II. The 10- $\mu$ M H7 group had a flow of  $1.1 \pm 0.1$  ml/min per g at 30 min. Coronary flow remained elevated throughout the 60-min perfusion period. In pilot studies the increase in coronary flow was observed to decrease after 60 min; therefore, as this was considered an indirect measure of protein kinase C inhibition, subsequent perfusions to examine the effect of H7 on HSP 70 levels were done only for 1 h. Both H7 groups initially had lower developed pressures than control hearts, but this difference was not statistically significant.

# EFFECT OF ISCHEMIA ON HSP 70 EXPRESSION IN THE ISOLATED PERFUSED HEART

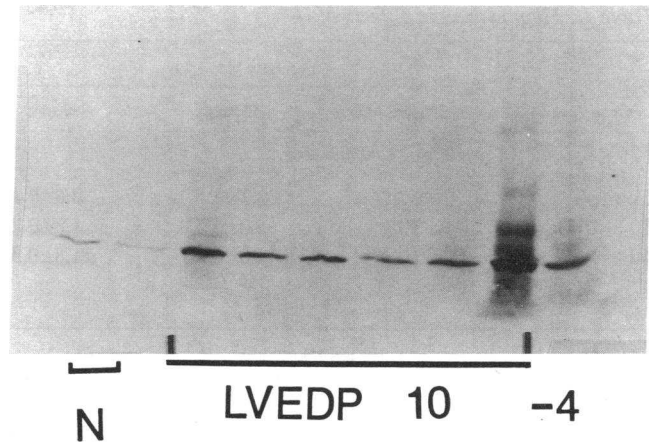


**Figure 4.** Densitometric analysis of steady-state HSP 70 mRNA levels in normoxic and globally ischemic perfused hearts. N, normal, non-perfused left ventricle ( $n = 6$ ). All perfusions were done in the standard manner with an apical drain and an isovolumic intraventricular balloon with an LVEDP of 10 mmHg. A, 2 h of nonischemic perfusion ( $n = 7$ ); B, 30 min of nonischemic perfusion followed by 1 h of ischemia and 30 min of reperfusion ( $n = 6$ ); C, 3.5 h of nonischemic perfusion ( $n = 6$ ); D, 0.5 h of nonischemic perfusion followed by 2.5 h of ischemia followed by 30 min of reperfusion ( $n = 4$ ); E, same protocol as D, but balloon volume reduced to keep LVEDP < 10 mmHg during ischemia ( $n = 7$ ).

## Discussion

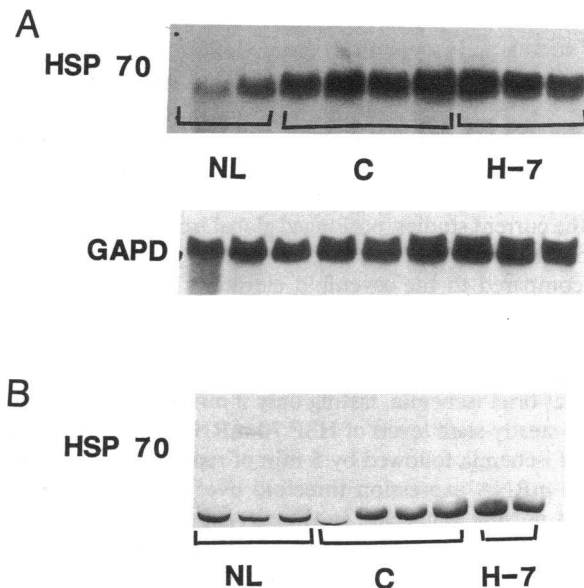
These studies demonstrated that normal, isolated, isovolumic blood-perfused heart preparations exhibit induction of HSP 70 despite the absence of ischemia, or other factors, such as heat stress, which are known to induce HSP 70. Two factors that caused an increase in steady-state levels of HSP 70 mRNA were identified: a single myocardial stretch and isovolumic contraction.

A single stretch, produced either by placement of the apical drain or by cross-clamping of the aorta, increased the steady-state levels of HSP 70 mRNA. The role of stretch-activated channels in organisms is increasingly recognized (17, 18). Several groups have reported stretch-related changes mediated by the endothelium (19, 20), and stretch-activated channels have been described in neonatal ventricular myocytes (21). Stretch as a mechanism of induction for cardiac genes has been described for atrial natriuretic factor (22, 23), as well as for *c-fos* and *c-jun* induction in a cultured cardiac myocyte model (24, 25). Stretch may also be a mechanism by which acute volume overload and acute pressure overload stimulate an increase in



**Figure 5.** Western blot analysis of HSP 70 levels in: N, normal, non-perfused left ventricle; LVEDP 10, hearts perfused for 2 h with an apical drain and a ventricular balloon with an LVEDP of 10 mmHg; -4, apical drain and an empty balloon and an LVEDP of -4 mmHg.

HSP 70 mRNA. Since stretch produced in the present studies may simulate in part the dysfunctional states produced by congestive heart failure, hypertensive crisis, and myocardial infarction, it is a plausible activator for HSP 70 in the heart.



**Figure 6.** Effect of H7 on HSP 70 expression. (A) Northern blot analysis demonstrating steady-state mRNA levels of HSP 70 after 1 h of isolated perfusion. NL, normal, nonperfused left ventricle; C, hearts perfused for 1 h with an apical drain and a balloon inserted after 10 min of perfusion as described in text, LVEDP = 10 mmHg; H7, hearts perfused in the presence of 50  $\mu$ M H7 with an apical drain and a balloon placed after 10 min of perfusion, LVEDP = 10 mmHg. The lower panel shows the results of hybridization of the same blot with GAPD as a control. (B) Western blot analysis of homogenates prepared from the same hearts (for C and H7) as shown in A. NL, normal, nonperfused left ventricle; C, 1 h of perfusion as described above; and H7, perfusion in the presence of 50  $\mu$ M H7 as described above.



Table II. Hemodynamic Effects of H7

Group	Coronary blood flow		
	30 min	45 min	60 min
	<i>ml/min per g</i>		
Controls	0.8±0.1	0.7±0.1	0.6±0.1
10 $\mu$ M H7	1.1±0.1	1.0±0.2	1.0±0.2
50 $\mu$ M H7	*2.3±0.2	*2.0±0.2	*1.8±0.3
	Developed pressure		
	<i>mmHg</i>		
Controls	83.0±11.7	71.8±11.6	57.0±7.8
10 $\mu$ M H7	62.5±7.5	80.2±1.8	78.0±3.0
50 $\mu$ M H7	63.5±2.5	71.0±4.6	65.6±4.9
	LVEDP		
	<i>mmHg</i>		
Controls	8.9±1.2	5.5±1.0	5.2±1.2
10 $\mu$ M H7	9.6±1.9	7.1±0.9	6.3±2.1
50 $\mu$ M H7	10.3±0.3	7.9±1.3	9.2±2.1

\*  $P < 0.01$  vs. controls.

Stretch alone produced a threefold increase in HSP 70 steady-state mRNA levels. As shown in Fig. 2A, the combination of stretch and isovolumic contraction produced a sevenfold increase in HSP 70 mRNA levels. Thus isovolumic contraction was a further stimulus to HSP 70 induction. Like stretch, reduced shortening is observed in disease states such as congestive heart failure, hypertensive crisis, and myocardial ischemia.

In the current studies, prolonged global ischemia of 1–2.5 h duration resulted in no further increase in HSP 70 mRNA levels compared to the sevenfold elevation seen in the well oxygenated control hearts, which had been perfused in the standard manner with an apical drain and an isovolumic balloon present in the left ventricle. In contrast we observed previously that brief ischemia, lasting only 5 min, was sufficient to increase steady-state levels of HSP 70 mRNA (5). Four 5-min cycles of ischemia followed by 5 min of reperfusion increased HSP 70 mRNA expression threefold over the baseline level found in normal ventricles. In the current study we were unable to detect an increase in HSP 70 mRNA above that produced by the isovolumic state in the perfused hearts despite 1–2.5 h of ischemia followed by reperfusion. The isovolumic perfused heart with an apical drain has a sevenfold increase in HSP 70 mRNA. This was similar to the largest increase that we have previously observed, which occurred after 2.5 h of ischemia in the *in vivo* rabbit heart (5). The failure of ischemia to further increase the steady-state levels of HSP 70 mRNA in the current study may reflect the fact that HSP 70 was already maximally induced by the isovolumic nature of the contraction and by the myocardial stretch applied by placement of the apical drain.

Variation in LVEDP, which was achieved by altering balloon volume, had no correlation with HSP 70 mRNA levels.

This lack of increase in HSP 70 mRNA may reflect maximal induction of HSP 70 by the perfusion method used which prevented normal systolic shortening in all hearts.

A parallel increase in HSP 70 protein accompanied the increase in mRNA. Some variability was seen for HSP 70 protein among the six samples from isovolumic perfusion experiments with an LVEDP of 10 mmHg examined by Western blotting. However, all samples showed a clear increase. Although the four hearts from which both RNA and protein samples were derived had similar increases in both HSP 70 mRNA and protein, this similarity may only be coincidental and does not preclude regulation at the translational level as well as the transcriptional level.

Protein kinase C was selected as a likely mediator of HSP 70 induction by stretch and/or decreased systolic shortening. Studies in neonatal rat cardiac myocytes have provided evidence that cardiac hypertrophy is at least in part mediated through  $\alpha$ 1-adrenergic receptors via the phosphoinositide/protein kinase C pathway (26–29). Both pressure and volume overload in the heart induce HSP 70 as well as the two protooncogenes, *c-myc* and *c-fos* (1, 2). Furthermore,  $\alpha$ -adrenergic stimulation has been observed to induce HSP 70 as well as *c-myc* and *c-fos* in one study (30). In view of the coexpression of HSP 70 with *c-myc* and *c-fos* during cardiac hypertrophy and the indirect evidence linking the expression of these genes to protein kinase C, we postulated that protein kinase C was a link between the mechanical signals of stretch and decreased shortening with changes in the expression of HSP 70. To explore this question, we used the protein kinase C inhibitor, H7, which readily crosses cell membranes. Other investigators have achieved inhibition of protein kinase C with concentrations of H7 similar to those we used (31–33). Although we did not measure protein kinase C activity, we did observe a doubling of coronary blood flow per gram of wet tissue weight with the 50- $\mu$ M dose of H7 compared with control perfusions to which vehicle alone was added indicating that an effective concentration of H7 was used. This effect of protein kinase C inhibition on coronary blood flow has been more fully described elsewhere (Knowlton, A. A., W. M. Vogel, and C. S. Apstein. Manuscript in preparation.) No inhibition of the increase in steady-state HSP 70 mRNA levels occurred with either 10 or 50  $\mu$ M H7 suggesting that protein kinase C does not play an important role in initiating HSP 70 expression. Further studies are needed to determine the mediators of HSP 70 induction in response to myocardial stretch and/or reduced systolic fiber shortening.

Our results have significant clinical implications as well as relevance for commonly used experimental models of myocardial ischemia and infarction. The rapid expression of HSP 70 after a single myocardial stretch or with reduced systolic fiber shortening suggests that it may be induced if the heart is over-distended and the fibers stretched with acute pulmonary edema, malignant hypertension, or after cardiac surgery. Similarly, many experimental studies of isolated heart muscle employ isometric or isovolumic preparations. Even experimental preparations in which ventricular ejection or fiber shortening occurs may nonetheless be subjected to some stretching during their surgical preparation. Our results indicated that such stretching rapidly induces expression of HSP 70, causing a subsequent increase in HSP 70 protein that is detectable within 60 min.



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