Ischemia of the Dog Heart Induces the Appearance of a Cardiac mRNA Coding for a Protein with Migration Characteristics Similar to Heat-Shock/Stress Protein 71

WOLFGANG H. DILLMANN, HARSHVARDHAN B. MEHTA, ALICE BARRIEUX, BRIAN D. GUTH, WILLIAM E. NEELEY, AND JOHN ROSS, JR.

SUMMARY **Recent evidence indicates that different forms of stress, including hypoxia, can induce specific proteins called heat-shock or stress proteins in various types of mammalian cells. These studies examined whether myocardial ischemia can result in increased levels of proteins with molecular weight and isoelectric point characteristics similar to those described for heat-shock or stress proteins. The left anterior descending coronary artery of the dog heart was completely occluded; normal and ischemic myocardial samples were obtained 6 hours after occlusion; and total cardiac proteins and RNA were prepared. Ribonucleic acid was translated *in vitro* in a modified rabbit reticulocyte lysate system, and [^{35}S]-methionine-labelled translational products as well as unlabelled cardiac proteins were separated by two-dimensional gel electrophoresis. Total proteins were visualized by silver staining and *in vitro* translation products quantified by fluorometry. A translatable mRNA coding for a 71,000 dalton peptide with an isoelectric point of 5.8 was markedly increased in the ischemic myocardium after 6 hours of ischemia. A protein with similar migration characteristics was detected in ischemic myocardium but not in normal myocardium. These results indicate that an mRNA coding for a translational product with similar migration characteristics of heat-shock protein 71 is induced by ischemia in the dog heart. (*Circulation Research* 1986;59:110–114)**

KEY WORDS • **myocardial infarction** • **heat-shock proteins** • **cardiac mRNAs**

ISCHEMIA-INDUCED changes in the level of specific cardiac proteins, or the mRNAs which code for them, have not been extensively investigated. This may be related in part to the view that ischemia-induced changes in cardiac proteins are a terminal event[1] occurring well after other biochemical alterations produced by ischemia that may be reversible. However, selective alterations of cardiac proteins that could result in abnormal contractile activity have been documented in ischemic myocardium by Toyo-Oko and Ross[2] who reported decreased content of the three troponin subunits in actomyosin from the endocardial half of infarcting myocardium as early as 4 hours after coronary occlusion; the myosin light chains were unchanged.

Recently accumulated evidence indicates that organs in intact animals and cells in tissue culture respond to various forms of stress by increased formation of specific proteins. These proteins, which have been called stress or heat-shock proteins,[3,4] were first described in *Drosophila* larvae exposed to elevated temperatures.[5] It has been reported that different forms of stress can induce different proteins and that their induction occurs at a pretranslational level in mammalian cells.[4] The induction of mRNAs coding for proteins with similar molecular weight and isoelectric point as heat-shock protein 71 (HSP 71) has been described in the hearts of rats submitted to banding of the ascending aorta or to hyperthermia.[6] It is typical for HSP 71 to consist of several proteins with the same molecular weight of 71,000 but different isoelectric points (pIs) ranging from 5.8 to 6.8.

In this study, we present evidence that the canine myocardium responds to ischemia by inducing proteins having similar electrophoretic properties as HSP 71. These proteins will be designated as putative cardiac stress proteins 71 (CSP 71). The appearance of the proteins is restricted to the ischemic region of the myocardium and correlates with the increased level of mRNAs coding for similar proteins. This study focuses on ischemia-induced increases in both protein and translatable RNA that occur 6 hours after the onset of ischemia. The results have been presented previously in abstract form.[7]

From the Division of Endocrinology and Metabolism (W.H.D., H.B.M., A.B.) and the Division of Cardiology (J.R., Jr, B.D.G.), Department of Medicine, and from the Department of Pathology (W.E.N.), University of California at San Diego, San Diego, California.

Address for reprints: W.H. Dillmann, MD, Department of Medicine, UCSD Medical Center, 225 Dickinson Street (H-811-C), San Diego, CA 92103.

Received October 14, 1985; accepted April 24, 1986.

Materials and Methods

Animal Preparation

Four mongrel dogs were anesthetized (pentobarbital 26 mg/kg) and ventilated with a Harvard respirator. A left lateral thoracotomy was performed in the 5th inter-

costal space, the pericardium opened, and the left anterior descending coronary artery dissected free. This vessel was then ligated distal to the first major diagonal branch. In all dogs, a cyanotic zone became apparent, and a lack of contraction of the antero-apical region distal to the ligation was observed. A map was drawn of the cyanotic region, using epicardial arteries as landmarks and to identify the site for transmural tissue samples later taken from the center of the ischemic zone.

Six hours after LAD ligation, the hearts were rapidly excised and three transmural tissue samples (2–3 g) were taken, divided into endo- and epicardial halves and immediately frozen in liquid nitrogen. The first sample was taken from the center of the ischemic region as mapped previously. The second was taken from the basal-lateral wall of the left ventricle at a site more than 4 cm from the ischemic zone. The third sample was taken from the right-ventricular free wall.

RNA Preparation, Cell-Free Translation, and Analysis of *in Vitro* Translational Products

Samples used for RNA or protein preparations were stored at −80° C for a maximum of 2 days. Cardiac RNA prepared as previously described[8] was translated in the presence of [^{35}S]-methionine in the modified rabbit reticulocyte lysate[9] prepared in our laboratory. A 5- to 10-fold increase in [^{35}S]-methionine incorporation into acid-insoluble material was observed after the addition of cardiac RNA to the reticulocyte lysate.

Samples containing equal amounts of radioactivity (250–350,000 cpm) were subjected to two-dimensional gel electrophoresis.[8] After electrophoresis, gels were prepared for fluorography and exposed to Kodak XAR-5 film at −80° C. Selection of ischemia-induced changes in spot predominance was performed by visual examination of the fluorograms before quantitation.

Quantitation of Translation Products

We quantified the selected translation products with the aid of a computerized photodiode scanning device developed by Neeley et al.[10] In order to correct for random variations between experiments, the integrated density of several spots whose predominance appeared not to be affected by ischemia was measured and compared between fluorograms derived from ischemic and control myocardium. The densities of spots 118 and 41 were not significantly altered by the ischemic state. Therefore, these two spots were selected to calculate corrected integrated densities:

$$(R/C)_N \quad \text{and} \quad (R/C)_{Exp}$$

where R = integrated density of ischemia-responsive spot, C = integrated density of spots 41 and 118 from the same fluorogram, N = normal fluorogram, and Exp = ischemic fluorogram. We measured the Mr and pI of each spot as previously described and used the spot numbering system described by Dillmann et al[8] in all fluorograms. Corrected integrated densities of identical products measured from each group were compared by the two-tailed Student's *t* test.

Preparation of Myocardial Proteins

One hundred milligrams of normal and ischemic myocardium were homogenized with a Polytron homogenizer in 0.5 mM each of tosylamide-2-phenylethylchloromethyl ketone (TPCK), tosyl-lysine chloromethyl ketone (TLCK), phenylmethylsulfonyl fluoride (PMSF) and 20 μg/ml leupeptin. After complete homogenization, an equal volume of a solution containing 40 mM Tris:HCl (pH 8), 2 mM $CaCl_2$, and 100 μg/ml micrococcal nuclease was added. The suspension was then brought to 0.1% sodium dodecylsulfate, 50 mM dithiothreitol by the addition of 0.1 volume of SDS-DTT solution, and nucleic acid digestion was completed by the addition of 0.1 volume of 100 mM Tris:HCl (pH 7.4), 50 mM $MgCl_2$, 1 mg/ml DNase I, and 0.5 mg/ml RNase A. Solid urea was then added to a final concentration of 9.5 M, Triton X-100 to 1% and ampholytes to 1%. The denatured peptides were separated by two-dimensional gel electrophoresis[8] and visualized by silver staining.[11]

Results

Proteins and RNA were prepared from the most severely ischemic endocardial region (ischemic samples) as well as from nonischemic areas of the left and right ventricles (control samples). No differences in protein pattern or predominance of translational products were obtained between the left ventricular and the right ventricular myocardium from normal regions (data not shown). When total myocardial proteins prepared from ischemic and normal myocardium were compared, the abundance of two 71,000 dalton proteins with slightly different pIs was always markedly increased in the ischemic myocardium (Figure 1).

To determine whether this increased predominance correlates with increased mRNA coding for this protein, RNA prepared from ischemic and normal myocardium was translated in a cell-free lysate. Figure 2 shows that two translation products with similar migration behavior as the myocardial polypeptides (Figure 1) were increased in fluorograms obtained from ischemic RNA when compared to normal RNA. The putative CSP 71 present in the total cardiac homogenate migrated slightly faster than the *in vitro* translation product. The increase in one of the two translation products (spot CSP 71a) having a more acidic pI was always significant in all the preparations tested. This spot was, therefore, quantitated by digital matrix photometry. It is likely that the more basic spot represents an isoform of spot CSP 71a, as noted for other heat-shock proteins[4] and for cardiac-overload-induced proteins in the rat heart.[6]

Two selected translational products of dog myocardial RNA with no apparent response to ischemia (spots 118 and 41) as well as the 71K dalton protein were quantified by photometry. The results are presented in Table 1. Only the ischemic-to-control ratio of CSP 71 was significantly different from 1, indicating that translatable RNA coding for CSP 71 was increased by a factor greater than 6 in the ischemic myocardium. Translatable RNA coding for spots 118 and 41 were

not affected by ischemia. When ischemic and control CSP 71 and spot 41 density ratios were compared (Table 1, line 2), only CSP 71 was significantly increased after ischemia. The translational activity of RNA from ischemic heart decreased slightly, indicating that the relative abundance of CSP 71 RNA appears to be specifically increased in the ischemic myocardium.

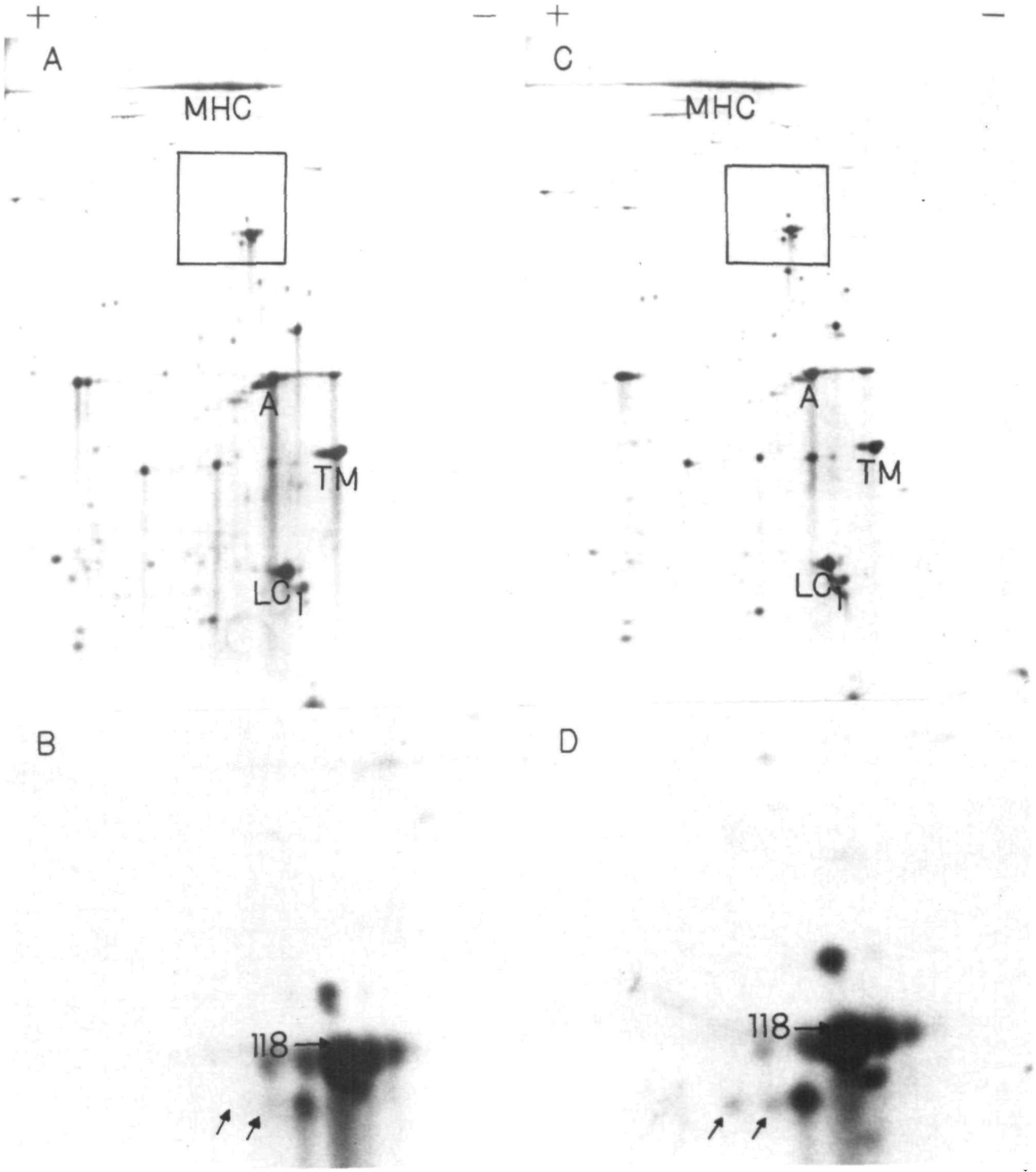

FIGURE 1. *Two-dimensional gel analysis of cardiac proteins in normal (A,B) and ischemic (C,D) myocardium of the dog heart. Crude total cardiac proteins were prepared from normal (nonischemic right ventricular myocardium) and ischemic (ischemic region of left ventricle) tissue after occluding the left anterior descending coronary artery for 6 hours. Proteins (60 μg) were separated and visualized by silver staining. B and D are magnified views of the squared areas in A and C respectively. MHC = myosin heavy chain; A = actin; TM = tropomyosin; LC_1 = light chain 1; + = basic end; − = acidic end. Horizontal arrow indicates spot 118 (the level of which does not change with ischemia). Upward pointing arrow indicates proteins (CSP 71), which increase in response to ischemic insult.*

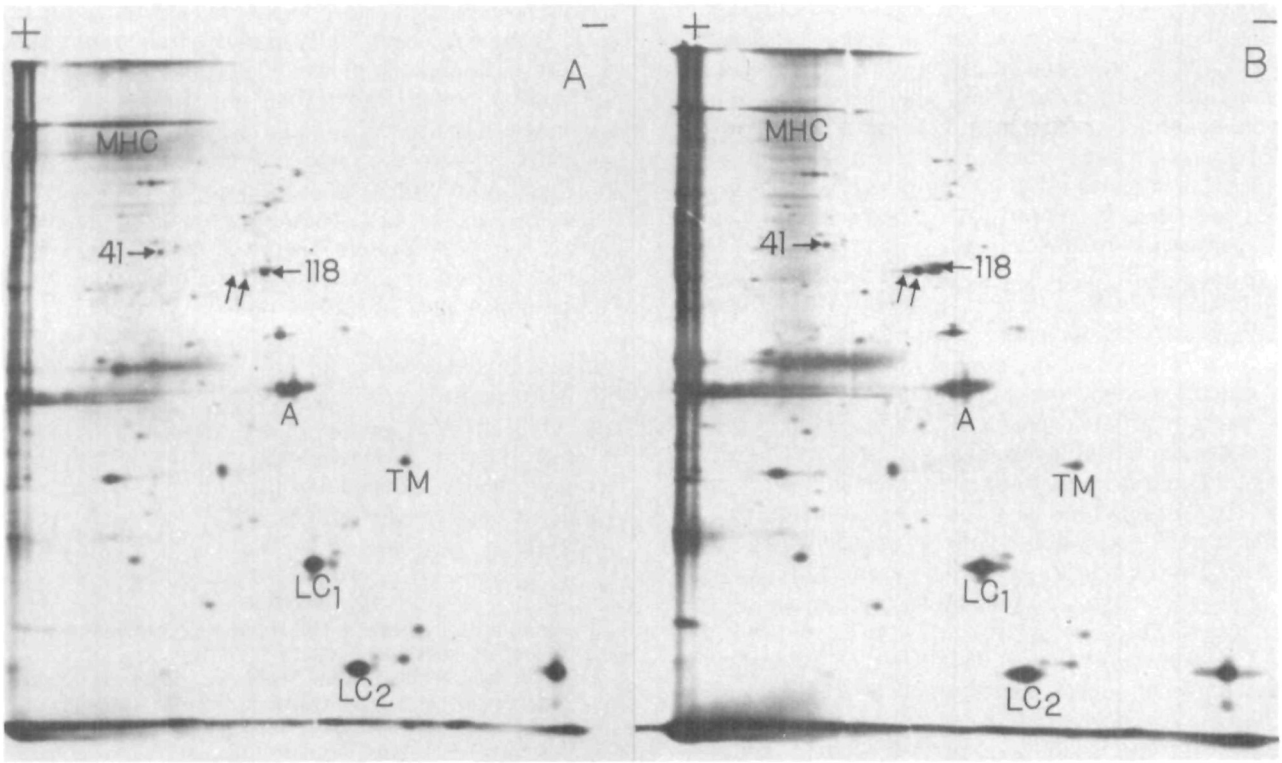

FIGURE 2. *Two-dimensional gel electrophoresis analysis of cell-free translation products from normal (A) and ischemic (B) myocardium of the dog heart. Cardiac RNA was prepared from normal (nonischemic right ventricular myocardium) and ischemic (an area of the left ventricular myocardium) tissue after 6 hours of ischemia. RNA was translated* in vitro *and [^{35}S]-methionine-labelled translation products were analyzed as described in "Materials and Methods." MHC = myosin heavy chain; A = actin; TM = tropomyosin; LC_1 = light chain; LC_2 = light chain 2; + = basic end; − = acidic end. Horizontal arrows indicate proteins (spots 41 and 118), the level of which does not change appreciably due to ischemic insult. Upward pointing arrow indicates proteins (CSP 71) which increase significantly in response to ischemia.*

In addition to the significant ischemia-induced increase in the mRNA coding for CSP 71, we noted changes in several other mRNAs isolated from ischemic myocardium, which will be characterized in future studies.

Discussion

Ischemia of 6 hours duration leads to a marked increase in specific mRNAs that code for proteins with a molecular weight of 71,000 and pIs of 5.8 and 5.9. Two authentic cardiac proteins with migration characteristics similar to those of the *in vitro* translation products are also increased in the ischemic myocardium. The slight Mr differences observed between the CSP 71 translated *in vitro* and CSP 71 present in the heart do not result from the greater protein mass present in the silver-stained gel, since the migration of the translation product CSP 71 was not altered when it was coelectrophoresed with total cardiac proteins. They may result more likely from posttranslational modifications occurring in the intact heart but not in the reticulocyte lysate. The fact that the increase in the 71,000 dalton

TABLE 1. *Integrated Densities of Control and Ischemia-Induced Translational Products*

	Spot 118		Spot 41		Spot CSP 71	
Ischemic	1.18 ± .25		1.02 ± .38		6.42 ± .93*	
Control						
	C	I	C	I	C	I
Spot 41 or CSP 71	—	—	0.24 ± .08	0.19 ± .02	0.16 ± .06	0.871 ± .27†
Spot 118						

Values are in means ± SD. RNA was prepared from ischemic and control region of the myocardium from 4 dogs and translated *in vitro*. Control samples were obtained from nonischemic portions of the left ventricle or from the right ventricle. No alterations in the mRNA predominance was found for normal left ventricular and right ventricular myocardium. The amount of translated peptides was quantified as described in "Materials and Methods." Only the more acidic spot (CSP 71a) was quantitated (see Figure 2). The ratios of ischemic ID to the normal ID for each spot are shown in the 1st row; the ratios of spot 41 and CSP 71 ID to spot 118 ID measured in any given gel are shown for control and ischemic fluorograms in the second row. C = control; I = ischemic.

*Value significantly different from 1 at $p < .01$.

†Value significantly different from control value at $p < 0.005$.

translation product and protein occurs specifically in the ischemic myocardium indicates that it is related to the ischemic state and not induced by the operative procedure. Ischemia-induced increases in CSP 71 were quantified only at the 6-hour point in this study, but additional preliminary experiments show that increases in the level of specific mRNAs coding for CSP 71 can be detected as early as 1.5 hours after the onset of ischemia (data not shown).

Translatable RNAs coding for proteins with similar migration characteristics as the proteins described in this report can be induced in the rat heart after banding of the aorta.[6] Proteins of similar molecular weight and isoelectric point become very prominent in various cell types from different species after exposure to hypoxia, heat, or inhibition of protein synthesis, among other stimuli,[4] and are commonly referred to as "heat-shock" or "stress" proteins. The mode of induction and migration characteristics of the proteins encoded by the CSP 71 mRNAs demonstrated in the present study strongly suggest that these induced cardiac proteins are related to heat-shock or stress protein 71.

The possibility that CSP 71 mRNA represents a breakdown product of cardiac mRNA appears quite unlikely. Normal and ischemic myocardial RNA preparations synthesize similar amounts of large peptides, including myosin heavy chain, and there are no appreciable differences in the predominance of low molecular weight products. Should increased RNA degradation have occurred in the ischemic heart, an increase in the frequency of low molecular weight products would be observed when RNA prepared from ischemic myocardium is translated *in vitro*. Finally, the increased cell-free synthesis of CSP 71 was observed in four different RNA preparations, indicating that the appearance of CSP 71 mRNA cannot be generated from random RNA degradation. Therefore, CSP 71 mRNA does not appear to represent a degradation product of an RNA present in a normal heart since it would require the induction of a highly selective RNase giving rise to a degraded yet functional RNA coding for a protein identical to HSP 71.

Although myocardial tissue after several hours of ischemia contains an increased number of blood-derived cells in comparison to normal myocardium,[12] it is unlikely that these cells are responsible for the increase in CSP 71 mRNA. Previous studies have shown that 1 g of hemorrhagic myocardial tissue contains at most about 80 mg of hemoglobin, which represents about 0.2 ml of blood,[13] and less than 50% of the volume of blood is constituted by red cells. White blood cells, which contain mRNA, are 1,000 times less predominant than red blood cells. White-blood-cell-derived mRNA is at least 10,000 times less abundant than the myocardial-tissue-derived mRNA, and the mRNA coding for the 71,000 dalton translational product is, therefore, most likely derived from myocardial tissue.

The precise cellular events that lead to induction of CSP 71 mRNA are currently unclear. Ischemia results in marked alterations of adenylated nucleotide levels and increase in the level of P^1; P^4-diadenosine-5′-tetraphosphate has been postulated as a stimulus for the induction of stress protein in other systems.[14] The function of the protein is also unknown. Evidence accumulated in other cell systems has indicated that heat-shock or stress proteins serve a protective effect against subsequent insult.[4]

The appearance of CSP 71 mRNA and the corresponding proteins during the first several hours of ischemia may allow use of these proteins as a marker for ischemic damage. It is uncertain whether CSP 71 appear in the blood and, if so, whether the amount liberated into the bloodstream correlates with the extent of ischemia-induced myocardial damage. Investigations to answer some of these questions are currently in progress.

References

1. Jennings RB, Reimer KA: Lethal myocardial ischemic injury. *Am J Pathol* 1981;102:241–255
2. Toyo-Oka T, Ross J Jr: Ca^{2+} sensitivity change and troponin loss in cardiac natural actomyosin after coronary occlusion. *Am Phys Soc* 1981;240:H704–H708
3. Hightower LE, White FP: Cellular responses to stress: Comparison of a family of 71-73 kilodalton proteins rapidly synthesized in rat tissue slices and canavanine-treated cells in culture. *J Cell Physiol* 1981;108:261–275
4. Schlesinger MJ, Ashburner M, Tissieres A (eds): *Heat Shock from Bacteria to Man*. Cold Spring Harbor, NY, Cold Spring Harbor, Laboratory, 1982
5. Tissieres A, Mitchell HK, Tracy U: Protein synthesis in salivary glands of Drosophila melanogaster. Relation to chromosome puffs. *J Mol Biol* 1974;84:389–398
6. Hammond GL, Lai Y-K, Markert CL: Diverse forms of stress lead to new patterns of gene expression through a common and essential metabolic pathway. *Proc Natl Acad Sci USA* 1982; 71:3485–3488
7. Dillmann WH, Guth BD, Barrieux A, et al: Ischemia of the dog heart induces the appearance of a cardiac mRNA coding for a protein with migration characteristics identical to stress protein 71. *Circulation* 1984;70:84(II)
8. Dillmann WH, Barrieux A, Neeley WE, et al: Influence of thyroid hormone on the *in vitro* translational activity of specific mRNAs in the rat heart. *J Biol Chem* 1983;258:7738–7745
9. Pelham HRB, Jackson RJ: An efficient mRNA dependent translational system from reticulocyte lysates. *Eur J Biochem* 1976;67:247–256
10. Neeley WE, Epstein D, Zettner A: An instrument for digital matrix photometry. *Clin Chem* 1981;27:1665–1668
11. Morrissey JH: Silver stain for proteins in polyacrylamide gels: A modified procedure with enhanced uniform sensitivity. *Anal Biochem* 1981;117:307–310
12. Engler RL, Schmid-Schonbein GW, Pavelec RS: Leukocyte capillary plugging in myocardial ischemia and reperfusion in the dog. *Am J Pathol* 1983;111:98–111
13. Higginson LAJ, White F, Heggtveit HA, et al: Determinants of myocardial hemorrhage after coronary reperfusion in the anesthetized dog. *Circulation* 1982;65:62–69
14. Lee PC, Bochner BR, Ames BN: AppppA, heat-shock stress and cell oxidation. *Proc Natl Acad Sci USA* 1983;80:7496–7500