Ischemia Induces Changes in the Level of mRNAs Coding for Stress Protein 71 and Creatine Kinase M

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Hyperthermia, hypoxia, and other conditions induce the appearance of heat shock or stress proteins in cells. We have previously shown that in the ischemic dog myocardium the level of a messenger RNA (mRNA) coding for a protein with migration characteristics similar to heat shock/stress protein 71 increases. Using a human heat-shock protein (hHSP) 70 genomic clone and anti-HSP70 antibodies as probes, we demonstrate in this report that heart stress protein (SP) 71 mRNA and its translational products (71 kDa polypeptides) are members of the stress protein family. In rabbit hearts, the ischemia-induced mRNAs translate into three isoforms with different isoelectric points (6.0, 6.1, and 6.15), in contrast to dog heart mRNA that translates into a protein with a pI of 5.8. The levels of SP71 mRNA in the dog and rabbit ischemic myocardium increased by sixfold and 18-fold, respectively. In the same samples, the levels of creatine kinase M mRNA decreased by about 40%, whereas those of myosin heavy chain mRNA remain unaltered. Our comparative analysis of three different mRNAs indicates that ischemia manifests its effects by differentially changing the levels of specific mRNAs coding for proteins with separate and distinct roles in the cell. (Circulation Research 1988; 63:512-517)

Myocardial infarction is one of the major causes of death in Western society. Despite the clinical importance of ischemia-induced cardiac changes, little is known about the regulation of gene expression and alterations in the level of specific messenger RNAs (mRNAs) that occur in the ischemic myocardium. Since the energy required for cardiac function is primarily derived from aerobic metabolism, decreased perfusion of the heart during ischemia adversely affects cardiac cells. It is now recognized that living organisms respond to specific stresses such as hyperthermia and hypoxia by synthesizing a set of proteins called stress proteins. Although the precise function of these proteins is not known, indirect evidence suggests that they afford protection to cells against subsequent insults. It is believed that stress creates metabolic disturbances leading to the accumulation of putative intracellular stressors or alarms, which in turn activate the genes for stress proteins. We previously indicated that in dog hearts, 6 hours of ischemia increased the level of an mRNA coding for a putative stress protein (SP) 71 using an in vitro translation assay. In this study, we compared the time course of appearance of stress-induced mRNA after coronary ligation in the dog and rabbit hearts and also analyzed the levels of mRNAs coding for creatine kinase M (CK-M) and myosin heavy chain (MHC) using specific DNA probes.

Materials and Methods

Animal Preparation

Ischemia was induced as previously described. Briefly, mongrel dogs were anesthetized with pentobarbital (26 mg/kg body wt) while rabbits received a combination of ketamine (25 mg/kg) and rompun (25 mg/kg). Animals were ventilated with a respirator and were kept on a 37°C bed. A left lateral thoracotomy was performed, the pericardium was opened, and the left anterior descending (LAD) coronary artery was ligated. A cyanotic zone became apparent within a few minutes of ligation. Control and ischemic myocardial tissues of the same heart were excised after ligating the LAD for various lengths of time. Alternatively, animals were anesthetized and either kept immersed in a water bath at 43.5°C for 45 minutes (heat-stressed) or kept at...
room temperature. Myocardial tissue was frozen in liquid N₂ quickly after removal from the animals.

RNA Preparation, Cell-Free Translation, and Analysis of In Vitro Translation Products

Total RNA was prepared by the Proteinase K method and translated in rabbit reticulocyte lysate containing [³⁵S]methionine. Translation products (2 × 10⁵ cpm) were separated by two-dimensional gel electrophoresis and gels were enhanced and exposed to Kodak XAR-5 films at -70°C for 6-7 days. All chemicals used were of reagent grade and were purchased from Bio Rad, Richmond, California, or Sigma, St. Louis, Missouri, except for enzymes used for RNA and lysate preparations, which were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Indiana, and [³⁵S]methionine, which was purchased from Amersham, Arlington Heights, Illinois.

Antibody Production and Immunoprecipitation

Antibodies used in this experiment were raised against a synthetic peptide. The amino acid sequence of human heat stress protein (HSP) 70 was used to synthesize a peptide containing amino acids Tyr-Gly-Ser-Gly-Pro-Thr-Ile-Glu-Glu-Val-Asp, C terminal. Lysine was added as the N-terminal residue to allow chemical coupling to a carrier protein (Keyhole limpet hemocyanin) by the glutaraldehyde method. Protein conjugated peptide was emulsified in Freund's complete adjuvant and injected intramuscularly into rabbits. Booster doses were injected in Freund's incomplete adjuvant 4 and 6 weeks later. The blood was withdrawn 8 weeks after primary injection, and serum was prepared. For immunoprecipitation, the method described earlier was used with some modifications. Briefly, sulfuryl-35 labeled translation products were heat denatured (90°C for 3 minutes) in buffer A (25 mM Tris-Cl, 50 mM β-mercaptoethanol, 1% sodium dodecyl sulfate [SDS], and 10% glycerol at pH 7.4) and diluted 10-fold (final volume, 500 μl) in RIPA buffer (phosphate-buffered saline, pH 7.2, containing 1.0% Triton X-100, 1.0% sodium deoxycholate, and 0.5 mM each of tosylamide-2-phenylethylchloromethylketone [TPCK], tosylsinechloromethylketone [TLCK], and phenylmethyl sulfonfluoride [PMSF], and 20 μg/ml leupeptin). A preincubation with a 50-μl sample of a 10% solution of formalin-treated Staphylococcus protein A was carried out at room temperature for 30 minutes, and the mixture was microfuged at 4°C for 10 minutes. The supernate was reacted at room temperature for 60 minutes, first with 5 μl of either anti-human heat shock protein (hHSP) 70 (C-terminal decapeptide) antiserum or preimmune serum and then with 50 μl of 10% Staphylococcus protein A for 30 minutes. The reaction mixture was spun at 4°C in the microfuge, and the pellet was washed six times with 750 μl RIPA containing 0.1% SDS. The pellet was resuspended in 30 μl of gel loading buffer, vortexed, boiled for 3-5 minutes, and pelleted in the microfuge at room temperature for 5 minutes. The supernate was analyzed by SDS-polyacrylamide gel electrophoresis (10%), the gel enhanced and exposed to Kodak XAR-5 film at -70°C. The reactivity of the antipeptide antibodies to 72 kDa (inducible) and 73 kDa (constitutive) heat stress proteins of HeLa cells was assayed by Western blotting using biotinylated second antibodies and horseradish peroxidase. The reagents and the protocol were purchased from Vectastain, Burlingame, California.

Northern and Dot-Blot Analysis

Total RNA (10-15 μg) was denatured in the presence of 2.2 M formaldehyde and 50% formamide (deionized) at 65°C for 6-8 minutes and was size-separated by electrophoresis through 0.8% agarose gel made in 40 mM MOPS (pH 7.0), 10 mM sodium acetate, 1 mM EDTA, and 2.2 M formaldehyde. The positions of 28S and 18S mRNAs were

Figure 1. Time course of appearance of ischemia-induced cardiac stress protein messenger RNA (mRNA) in the heart of dogs and rabbits. Cell free translation products of RNA made from nonischemic (a–c) vs. corresponding ischemic (h–m) tissue of the dog heart in which the left anterior descending coronary artery was ligated for 0.3 (a and h), 0.75 (b and k), and 3.0 (c and m) hours. Similarly, in vitro synthesized proteins from RNA preparations of nonischemic (d–f) versus respective ischemic (n–p) rabbit hearts in which the left anterior descending coronary artery was ligated for 0.3 (d and n), 0.75 (e and o) and 3.0 (f and p) hours. The heat shock-induced mRNA translation product of the rabbit heart (r) is compared with the control (g).
marked by staining with acridine orange in 10 mM sodium phosphate buffer (pH 7.2) and 1.1 M formaldehyde. The RNA was transferred onto Nytran membrane (Schleicher and Schuell, Keene, New Hampshire) in the presence of 10 x standard saline citrate (SSC) (1 x SSC is 150 mM sodium chloride and 15 mM sodium citrate, pH 7.2). Alternatively, denatured RNA was spotted at different concentrations on Nytran strips. The membrane was air dried, baked at 80° C in a vacuum oven, and prehybridized at 42° C for 10-12 hours in solution containing 5 x SSPE (1 x SSPE is 0.15 M sodium chloride, 0.01 M sodium dihydrogen phosphate, 0.001 M EDTA), 2.5 x Denhardt (1 x Denhardt is 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, and 0.02% bovine serum albumin), 25 μg of sheared herring sperm DNA per milliliter, 0.1% SDS, and deionized formamide at a final concentration of 50%. The radiolabeled DNAs were denatured by boiling for 10 minutes, chilled on ice, and added to the prehybridization solution, and hybridization was performed. The filter was washed sequentially twice with 2 x SSC and 0.1% SDS at room temperature for 20 minutes, twice with 1 x SSC and 0.1% SDS at 50° C for 30 minutes, and once with 0.1 x SSC and 0.1% SDS at 50° C for 30 minutes, and autoradiograms were obtained after exposure to Kodak XAR films at -70° C.

**Labeling of Probes**

All the probes were labeled using the nick translation protocol of Maniatis et al. The concentration of cold and [32P]deoxy nucleotide triphosphate (dNTPs) were adjusted to obtain specific activities between 1 x 10^7-1 x 10^8 cpm/μg DNA. [32P]dNTPs (3,000 Ci/mmol) were obtained from New England Nuclear, Boston, Massachusetts. An HSP70 genomic clone (pH 2.3) was a gift from R. Morimoto; a rat cardiac CK-M cDNA clone (pCKM7) was isolated in our laboratory using a dog heart CK-M complementary DNA clone (a gift from A. Strauss); and MHC complementary DNA clone (p86C) was a gift from A. Barrieux, UCSD Medical Center, San Diego, California. All the probes were purified on Bio-Gel A-5m gel A5m columns (Bio Rad).

**Results**

RNA was prepared from ischemic and nonischemic myocardium of dog and rabbit hearts after LAD occlusion for varying lengths of time and translated in vitro. Two-dimensional gel analysis of labeled in vitro translational products show that although both dog and rabbit hearts respond to ischemic stress, the nature of the translational products of stress-induced mRNAs and the time course of appearance of the messages is different for dogs and rabbits. Ischemic stress in the dog heart induces an mRNA coding for a major protein with a molecular mass of 71 kDa and an isoelectric point (pI) of 5.8 (Figure 1, k and m), whereas in the rabbit heart mRNAs are induced which code for isoforms with different pIs at 6.0, 6.1, and 6.15 (Figure 1, n-p). The rabbit heart responds to ischemic insult within a short period (0.3 hours of ligation of LAD) as judged by the appearance of translational products (Figure 1, n), which further increased as the LAD was occluded for longer periods of time (Figure 1, o and p). In dogs, stress protein coding mRNA was not detected at the 0.3-hour point (Figure 1, h) but began to appear at 0.75 hours (Figure 1, k) of LAD occlusion. It may be noted that at all the time points, both in dog and rabbit hearts, the nonischemic myocardium of the same heart showed minimal or no synthesis of stress protein mRNA (compare Figure 1, a-f with h-p). The induction was, therefore, not due to the stress of surgery (Figure 1, g and a-f).

To show that 71 kDa polypeptides of ischemia-induced mRNAs from dogs and rabbits (Figure 1) are heat-shock related SPs, we raised polyclonal antibodies to a C-terminal peptide (deduced from nucleotide sequence of human HSP70 gene). Figure 2 shows results of immunoprecipitation of in vitro translational products of RNA from ischemic and nonischemic dog and rabbit hearts. We noticed that the antibodies reacted to two products: a
FIGURE 3. Northern analysis of RNA from control and ischemic dog and rabbit hearts. Appropriate nick translated radioactive probes (about 1–1.5 x 10^6 cpm/ml of hybridization mix) were used. Hybridization to a labeled human stress protein 70 probe (clone pH 2.3) with total RNA from the dog heart (control, lane A; and ischemic, lane B), ischemic for 6 hours; rabbit heart (control, lane C; and ischemic, lane D), ischemic for 3 hours; and normal (lane E) and heat-shocked (lane F) rabbit heart. Lanes G to J were hybridized to a labeled rat cardiac creatine kinase M (CK-M) cDNA (clone pCKM7) and lanes K to N were hybridized to a labeled myosin heavy chain complementary DNA (clone pMyH7, a gift from A. Barrieux). The dog heart RNAs from control (lanes G and K) and ischemic (lanes H and L) tissue were from 1.5 hours postligation. The rabbit heart RNAs from control (lanes I and J) and ischemic (lanes M and N) myocardium were from 0.75 hours postligation. The position of the 28S and 18S ribosomal RNA bands after staining with acridine orange is indicated.

Northern analysis (Figure 3) provided further evidence for the relatedness of ischemia and heat shock-induced mRNAs. RNAs from ischemic and normal myocardium of dogs and rabbits were size separated on denaturing agarose gels. The labeled hHSP70 genomic probe showed specific crossohybridization to 2.6 kilobase transcripts present at elevated levels in the ischemic dog (Figure 3B) and rabbit (Figure 3D) hearts. The heat shock-induced mRNA in the rabbit hearts was of the same size as ischemia-induced mRNAs (Figure 3F). It may be noted that hHSP70 genomic sequence does not cross hybridize to mRNA coding for constitutively expressed protein (Figure 3A, C, and E; Wu et al18). In response to ischemic stress, SP71 mRNA gradually increased (Figure 4A). In the dog heart ischemic for 6 hours, the mRNA levels were sixfold higher, whereas about 18-fold increase was noted at 3 hours postischemia in the rabbit hearts.

In addition to SP71 mRNA, the level of CK-M mRNA and of MHC mRNA were determined in normal and ischemic myocardium. CK is a key enzyme in the phosphocreatine shuttle that transfers high-energy phosphates to the myofibrils; CK-M (as an MM dimer) is the major form of CK in the mammalian heart. While the total enzyme activity of CK has been shown to decrease 15–20% in ischemic rabbit hearts and in human hearts with coronary artery disease,22 mRNA levels for CK have not been quantified. A labeled rat CK-M cDNA hybridized to a 1.6 kilobase transcript in both dogs and rabbits (Figure 3G, H, I, and J). CK-M mRNA levels were up to 40% lower in ischemic RNA preparations and varied with the duration of ischemia (Figure 4B). In contrast, MHC cDNA hybridizable transcripts showed no ischemia-induced changes (Figure 3K, L, M, N; and Figure 4B). The quantitation of MHC mRNA was undertaken because myosin is an important component of the ATP-utilizing contractile unit. We have seen that MHC (200 kDa) and its mRNA are not degraded during ischemia of at least 6 hours’ duration.10

Discussion

We show that ischemia of the dog and rabbit hearts results in increased levels of mRNAs coding for proteins that are members of the stress protein family (Figure 1). The dog heart mRNA coded for a major protein of 71 kDa and a pI of 5.8, whereas the rabbit cardiac mRNAs translated into three isoforms with pIs of 6.0, 6.1, and 6.15 (Figure 1). We do not know, at present, if these three isoforms are made from a single mRNA or from three different but related mRNAs. In mouse L cells, heat stress induced three mRNAs with an almost identical coding region (C-terminal 250 amino acid stretch) but with a less conserved 3' untranslated region.24
Microheterogeneity of the amino acid sequence in protein coding areas may result in πl shifts. Our data suggest species specific differences in the sequence of messages synthesized in response to myocardial ischemia. In contrast, in the rabbit heart two different stimuli, ischemia and heat, appear to induce messages that code for similar proteins as judged by molecular mass and isoelectric point (Figure 1, p and r). Similar results have been obtained by Hammond et al,25 showing that heat shock and aortic banding induce similar stress proteins in the rat heart. We also noted that the appearance of cardiac stress protein mRNAs in response to LAD ligation was faster in rabbits than in the dog hearts. One explanation for the later onset of induction of mRNAs in the dog may be that the canine heart is supplied by more extensive collateral vessels than the rabbit heart.26 During short ligation periods, the levels of putative intracellular stressor(s) responsible for activating stress protein gene may, therefore, be lower in dog hearts. Experimental results using antibodies raised against human HSP70 sequence (C-terminal 10 amino acid peptide) suggest that products of ischemia-induced mRNAs are members of the heat shock/stress protein gene family, and that the C-terminal portion is conserved in the constitutive and inducible isoforms in different mammalian systems. This may imply a common function for the C-terminal portion in spite of slight differences in molecular masses and πls. Using deletion mutants, Lewis and Pelham27 made Drosophila HSP70 truncated at the C end. Their results indicated that C-terminal deletion resulted in less efficient interaction with ATP and reduced entry into nucleoli.

Our results show that myocardial ischemia increases the level of the mRNA coding for SP71 in dog and rabbit hearts for up to 6 hours and 3 hours of ischemia, respectively, indicating that the myocardium is not inactive but possesses synthetic activity as late as 6 hours into ischemia. Also, at these time points, we failed to see a general degradation of either proteins or RNAs.10 Allen et al28 have reported functional recovery after 6 hours of regional ischemia by careful control of conditions of reperfusion and composition of reperfusate. It has been proposed that the function of stress proteins is protection against stress-induced intracellular damage.6-7 For example: 1) Chinese hamster ovary cells survived and recovered better from acute heat shock when preexposed to sublethal heat stress and allowed to synthesize HSP29,2) HSPs bind and hydrolyze ATP and associate promiscuously with a variety of intracellular macromolecules. It is suggested by Pelham that this association either refolds denatured proteins or prevents stress denaturation of these molecules by stabilizing them in an active configuration.6,27 One could, therefore, speculate that the appearance of SP71 mRNA in the hearts subjected to ischemic stress results in increased formation of a protein that exerts a protective function. Ischemia-stressed myocardium is an energetically compromised tissue. It is possible that decreased high energy phosphate utilization by contractile proteins may prevent further damage. This may occur through reduction in CK-M mRNA and its product, which might further limit energy delivery to the contractile protein system and thereby exert a protective function. The mechanism by which CK-M mRNA decreases is not clear and also it is not understood as to why CK-M mRNA levels are restored at 6 hours after coronary ligation.
In summary, our comparative analysis of three different mRNAs indicate that ischemia manifests its effects by differentially changing the levels of specific mRNAs coding for proteins with separate and distinct roles in the cell.

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