CARDIOPULMONARY SUPPORT AND PHYSIOLOGY

THE EARLY RESPONSE GENES *C-JUN* AND HSP-70 ARE INDUCED IN REGIONAL CARDIAC STUNNING IN CONSCIOUS MAMMALS

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Objectives: A reversible contractile dysfunction without necrosis after transient myocardial ischemia has been termed *stunning*. The molecular mechanisms underlying this phenomenon are only now beginning to be unraveled. It is conceivable that the expression of early-response genes may play a crucial role in stunning.

Methods: The expression of HSP-70, *c-jun*, and GRP-94 was investigated in a chronically instrumented dog model (n = 9). The left anterior descending coronary artery was occluded temporarily for 10 minutes after the animals had fully recovered from instrumentation. The wall thickening fraction was measured in the left anterior descending coronary artery and the nonischemic ramus circumflex of the left coronary artery-perfused region. When the wall thickening fraction of the left anterior descending coronary artery had recovered to 50% of preocclusion values, tissue samples were obtained from the areas perfused by the left anterior descending coronary artery and the nonischemic ramus circumflex of the left coronary artery.

Results: The messenger RNA of HSP-70 was increased to $214\% \pm 26\%$ in the area perfused by the left anterior descending artery compared with that perfused by the nonischemic ramus circumflex of the left coronary artery. There was no difference in the messenger RNA of GRP-94. The HSP-70 content was elevated to $130\% \pm 14\%$ in the left anterior descending artery compared with the area perfused by the ramus circumflex of the left coronary artery, and the *c-jun* protein content was $70\% \pm 25\%$ higher in the ischemic area compared with the control area.

Conclusions: The induction of early-response genes observed here may indicate that they play an adaptive role in myocardial stunning, even in conscious mammals. (J Thorac Cardiovasc Surg 2000;119:820-5)

A brief coronary artery occlusion results in reversible contractile dysfunction of the dependent myocardium,

which is termed *stunning*.¹ Stunning may gain relevance during coronary artery bypass surgery, heart transplantation, and angina pectoris and in patients with coronary artery disease.² The underlying mechanisms are only partially known.³ In the present study we chose to measure the expression of heat shock protein (HSP) 70, *c-jun*, and glucose-related protein (GRP) 94 in myocardial stunning on the basis of prior evidence indicating their protective role in cellular function, which arose from other disease models.

After ischemia and reperfusion, some cellular proteins are damaged and misfolded. This initiates intracellular repair mechanisms, which include the induction of proteins promoting correct folding and degradation of terminally damaged proteins, which include HSP-70 and GRP-94. HSPs were first described in the *Drosophila* species salivary gland; an increase in these

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proteins is apparently a standard response to injury. HSPs, such as HSP-70, are induced in myocardial ischemia followed by reperfusion. HSPs enhance the recovery from ischemic insult and protect against subsequent ischemia.⁴ However, data on myocardial stunning in intact animals are currently not available.

A protein closely related to the canonical HSP is GRP-94. After depletion of intracellular glucose stores, increased synthesis of GRPs is induced.⁵ This protein is involved in protein folding, Ca²⁺ binding, nuclear signaling, and secretion.⁶ The glucose levels are reduced in ischemia.

Moreover, altered calcium turnover is thought to occur during myocardial stunning.^{2,3} Hence we hypothesized that these biochemical changes might increase the expression of GRP-94 on the RNA level. Previous work reported that myocardial infarction can induce c-jun in addition to HSP-70 production.^{7,8} C-jun is a transactivating transcription factor. It can activate activator protein-1-responsive elements in appropriate genes, and these genes may exert a cardioprotective role. Currently, no data are available concerning the expression of c-jun in myocardial stunning under any conditions or species.

To investigate biochemical mechanisms that accompany stunning, chronically instrumented dogs were used in the present study. This approach seems to be closer to physiologic situations. Other models, such as open-chest animals or isolated organ models, have been shown to be of limited clinical relevance and even to exhibit artifactual biochemical alterations.⁹ The aim of this study was to increase our knowledge on changes of gene expression in myocardial stunning. Clearly, new insights into protective mechanisms during myocardial ischemia may lead to new therapeutic options. It has not previously been studied whether the expression of the putative protective genes HSP-70, GRP-94, or *c-jun* is altered in myocardial stunning in conscious mammals.

Methods

Animal instrumentation and physiologic interventions.

The experimental protocol was approved by the local animal welfare board. The investigation conforms with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institute of Health. Mongrel dogs of either sex (weight, 20-26 kg) were used. Details of the instrumentation have been published previously.¹⁰

After instrumentation, the dogs were trained daily to accustom them to the experimental environment and to lie quietly in a cage when connected to the data acquisition system. Experiments were performed only after complete recovery from surgery and when normal blood gas values and hemodynamic variables were obtained.

Aortic and left atrial pressures were measured by using disposable pressure transducers. Pressure, flow velocity, and wall thickening signals were processed by using a 6-channel pulsed Doppler system (Baylor College of Medicine, Houston, Tex). The left ventricular micromanometer was calibrated to the pressures measured in the aorta and left atrium. The left ventricular pressure signal was electronically differentiated (Gould Inc, Cleveland, Ohio). All signals were recorded on an 8-channel thermal writing polygraph (Gould Inc).

After measurement of baseline values, left anterior descending coronary artery (LAD) ischemia was induced for 10 minutes. Ten minutes was chosen for comparability with our previous work and to exclude any subendocardial infarction.¹¹ During reperfusion, wall thickening fraction (WTF) was followed until 50% recovery compared with baseline values occurred. When this level of recovery was reached, the animals were anesthetized with 300 mg of propofol, 0.5 mg of fentanyl, and 15 mg of midazolam. The trachea was intubated, and the dogs' lungs were ventilated with 100% oxygen. A parasternal thoracotomy was performed, the heart was excised, and samples of the LAD-perfused and ramus circumflex of the left coronary artery (RCx)-perfused territories were frozen in liquid nitrogen. A group of 7 sham-operated animals underwent the same procedure without induction of ischemia.

Northern blotting. A modification of the method described by Chomczynski and Sacchi¹² was used to extract total RNA from the frozen samples taken from the LAD and RCx areas.

Probes of complementary DNA were constructed by reverse transcription-polymerase chain reaction (PCR). Probes for cardiac HSP-70 and GRP-94 proteins were generated by cross-species reverse transcription-PCR.^{4,13} The PCR products were visualized on 2% agarose gels, cut out, purified by dialysis,¹⁴ and used as probes in Northern blots.

For Northern blotting, total RNAs (20 µg) were separated on 1% denaturing agarose gels and transferred to nylon membranes (Amersham Buchler, Braunschweig, Germany) by capillary transfer in 20× standard saline citrate (SSC). To normalize the amount of RNA bound to membranes, all blots were also hybridized for the 18S ribosomal RNA, as previously described.¹⁴

Western blotting. Frozen cardiac myocardium from the LAD and RCx areas was homogenized in 10 mmol/L NaHCO₃ and 5% sodium dodecylsulfate. Protein concentrations were determined by using the Lowry assay.¹⁴ One hundred micrograms of homogenate sample protein was loaded per lane. These amounts were in the linear range for each protein. After gel electrophoresis, separated proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany), as previously described.¹⁵

Quantitative immunoblotting for HSP-70. Nitrocellulose sheets were incubated in Tris-buffered saline solution (TBS; 10 mmol/L Tris and 154 mmol/L NaCl) for 1 hour with TBS containing 0.1% Tween-20 (TTBS) and 2% bovine serum albumin and 10 minutes with TTBS to occupy nonspecific

Table I. Hemodynamic parameters and WTF at baseline, during ischemia, and at the time the heart was excised

	Baseline	Ischemia	Time of excision
Heart rate (beats/min)	87 ± 3.2	96 ± 6 (<i>P</i> = .02)	98.4 ± 6.8 (<i>P</i> = .001)
Mean arterial pressure (mm Hg)	106.4 ± 3	93.5 ± 3.8 (<i>P</i> = .002)	109.8 ± 4.2 (<i>P</i> = .32)
LVdp/dt _{max} (mm Hg/s)	3081 ± 201	2294 ± 166 (<i>P</i> = .0003)	2898 ± 192 (<i>P</i> = .003)
LVdp/dt _{min} (mm Hg/s)	2768 ± 144	1911 ± 180 (<i>P</i> = .0008)	2508 ± 147 (<i>P</i> = .008)
WTF (%)	100	-27 ± 6.6 (<i>P</i> = .0001)	49 ± 6 (<i>P</i> = .0001)

In chronically instrumented dogs, the LAD was occluded for 10 minutes. The stunned region was reperfused until the WTF returned to 50% of the preocclusion value. Time points given here are at preocclusion (*baseline*), 3 minutes after the beginning of the occlusion during the ischemic episode (*ischemia*), and immediately before the heart was excised (*time of excision*). *P* values are versus baseline.

LVdp/dt_{max}, Maximum rate of increase in left ventricular pressure; LVdp/dt_{min}, maximum rate of decrease in left ventricular pressure.

protein-binding sites on the nitrocellulose. The antibody against HSP-70 (StressGen, Victoria, British Columbia, Canada) at 1 µg/mL dilution in TTBS was incubated with the blot for 1 hour at room temperature. The antibody recognized a band at the expected molecular mass of 70 kDa, which was quantified. After several rinses in TTBS, the nitrocellulose was incubated with iodine-125 labeled anti-mouse immunoglobulin G (ICN Biomedicals, Eschwege, Germany) diluted 1:1000 in TBS and 2% bovine serum albumin for 1 hour at room temperature. After several washes with TBS, the nitrocellulose membranes were dried, and the radioactive bands were visualized in a PhosphorImager system (Molecular Dynamics, Division of Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom), as described above. No additional (supposedly unspecific) bands were noted.

Quantitative immunoblotting for c-jun. Nitrocellulose sheets were incubated in TBS and 5% nonfat dry milk overnight at 4°C to occupy nonspecific protein-binding sites on the nitrocellulose. The antibody against c-jun (Oncogene, Cambridge, Mass) at 2 µg/mL dilution in TBS and 1% nonfat dry milk was incubated with the blot for 2 hours at room temperature. The antibody recognized a band at the expected molecular mass of 39 kDa, which was quantified. After several rinses in TTBS, the nitrocellulose was incubated with iodine-125 labeled anti-mouse immunoglobulin G (ICN Biomedicals) diluted 1:500 in TBS and 1% nonfat dry milk for 1 hour at room temperature. After several washes with TBS, the nitrocellulose membranes were dried, and the radioactive bands were visualized in a PhosphorImager system, as described above. No additional (supposedly unspecific) bands were noted.

Statistics. Data shown are means ± SEM. Statistical analysis was performed by using the Student *t* test.

Results

Induction of ischemia by inflation of the balloon occluders led to a rapid decrease of WTF to negative values in the LAD-perfused area, whereas WTF in the RCx-perfused area remained constant (*n* = 9). Compared with preocclusion values (100%), WTF decreased to -27% ± 6.6% (*P* = .0001). The WTF

before the excision of the heart was 49.0% ± 6.0%, which was reached after 43.8 ± 6.3 minutes of reperfusion. All hemodynamic and cardiodynamic alterations are shown in Table I.

No hemodynamic alterations were noted in sham-operated dogs (*n* = 7, data not shown). The induction of ischemia resulted in an immediate decrease in the WTF to negative values, which represents a paradoxical wall motion (wall thinning), a well-described phenomenon during ischemia.¹⁶ This was followed by a gradual regression, and all criteria in the experimental design for stunning were therefore met. The occlusion time of 10 minutes was chosen to avoid any microinfarction.

mRNA levels of HSP-70 and GRP-94. Total RNA was isolated from samples of the stunned LAD-perfused myocardium and the RCx-perfused area, which served as a control. The HSP-70 mRNA content was 214% ± 25.9% in the LAD-perfused myocardium compared with that found in the RCx-perfused area (100% = 10,150 PhosphorImager units; *n* = 9; *P* = .02; Fig 1). There was no difference in the mRNA content for GRP-94 (Fig 2). In the stunned LAD-perfused territory, the content was 109% ± 10.4% compared with that found in the RCx area (100% = 185,800 PhosphorImager units), which was not statistically significant (*P* = .35). There were no differences in the HSP-70 and GRP-94 mRNA content between the RCx-perfused and LAD-perfused territories in sham-operated animals. This indicates that the alterations are genuinely the result of ischemia and not caused by regional variations in the expression of the genes of interest. Because no changes of GRP-94 RNA levels were noted, no effort was put into studying the protein level, which was not expected to be subsequently altered. Moreover, no appropriate antibody was available to us.

Quantification of HSP-70 and c-jun protein levels. Given the increase in HSP-70 RNA, it was reasonable to study the protein expression of this gene. Initial experiments showed that the measurement was linear

Fig 1. Expression of mRNA for HSP-70 in stunned canine myocardium. The LAD in chronically instrumented dogs was occluded for 10 minutes followed by reperfusion until the WTF returned to 50% of the preocclusion value. Tissue was obtained from the LAD and the RCx-perfused areas. The autoradiograph of a representative Northern blot for HSP is shown. The *left lane* contains RNA from the RCx-perfused area, and the *right lane* contains RNA from the LAD-perfused area.

between 50 and 150 μ g of protein of ventricular homogenate (data not shown). For quantitative immunoblotting, 100 μ g of protein was used. The HSP-70 content was $130\% \pm 14.3\%$ in the LAD region compared with that found in the RCx area ($100\% = 80,600$ PhosphorImager units; $P = .05$; Fig 3). The amount of c-jun was increased by $70\% \pm 25\%$ in the LAD compared with the RCx area ($100\% = 14,600$ PhosphorImager units; $P = .01$; Fig 4). Again, no difference was noted between the RCx and LAD areas in sham-operated animals (data not shown).

Discussion

The main new finding of this study was that the levels of HSP-70 and c-jun were increased during stunning in chronically instrumented conscious mammals. However, our study cannot establish a cause-and-effect relationship at this time.

Awake dogs, which were used in this study, are a well-established model for the investigation of myocardial stunning. Myocardial stunning was in fact first described in dogs.¹ Numerous studies have been published, either in awake animals or (to allow a simplified

Fig 2. Expression of mRNA coding for GRP-94 in stunned canine myocardium. The LAD in chronically instrumented dogs was occluded for 10 minutes followed by reperfusion until the WTF returned to 50% of the preocclusion value. Tissue was obtained from the LAD- and the RCx-perfused areas. The autoradiograph of a representative Northern blot for GRP-94 is shown. The *left lane* contains RNA from the RCx-perfused area, and the *right lane* contains RNA from the LAD-perfused area.

method) in anesthetized animals, with conflicting results. The biochemical and hemodynamic findings in open-chest dogs were not in agreement with those in awake dogs.⁹ The present model therefore seems to be closer to the pathophysiologic situation in patients than that found in isolated cardiac preparations.¹⁷ Although the contractile dysfunction in stunning is well characterized, the underlying and concomitant biochemical alterations are not completely understood.

HSP-70 is a protein with protective functions in the cell. It is a member of a group of proteins that are involved in the correct folding of proteins and appropriate interaction of proteins.¹⁸ Since their first description in 1962 by Ritossa,^{18a} there has been growing evidence that the induction of HSPs is a universal response to cellular stress or injury. In isolated perfused rabbit hearts, HSP-70 levels increased after brief ischemic episodes.¹⁹ The trigger for the induction of HSP-70 is not the pure ischemic insult.

Reperfusion seems to enhance the activation of HSP-70. In isolated rat hearts the time course for

Fig 3. Protein levels of HSP-70 in stunned canine myocardium. The LAD in chronically instrumented dogs was occluded for 10 minutes followed by reperfusion until the WTF returned to 50% of the preocclusion value. Tissue was obtained from the LAD- and the RCx-perfused areas. The autoradiograph of a representative Western blot is shown. The area of interest around 70 kDa is depicted. The *left lane* represents the RCx-perfused area, and the *right lane* shows the LAD-perfused area.

HSP-70 activation after a 20-minute period of ischemia peaked around 120 minutes.²⁰ Increased levels of HSP-70 may result from enhanced gene transcription or prolonged half-life of the mRNA, enhanced rates of translations, or greater protein stability.¹⁹ Our data are consistent with an important role of enhanced gene transcription in conscious mammals.

In the heart the induction of HSP-70 through preceding ischemic episodes or heat shock was accompanied by a decrease in stunning or a reduced infarct size. In transgenic mice overexpression of HSP-70 also correlated with an improved recovery of contractile force after ischemia.^{4,21} It may be asked how the time course and the magnitude of the induction observed here compares with and extends previous findings. In isolated Langendorff-perfused rabbit hearts, an occlusion of the large marginal branch of the left circumflex artery for 5 minutes, which led to stunning, doubled the content of HSP-70 mRNA, whereas 4 cycles of ischemia and reperfusion led to a 3-fold increase in HSP-70 mRNA. This increase was noted 1 hour after the start of reperfusion.¹⁹ In isolated perfused rat hearts, 20 minutes of ischemia induced an 80-fold increase in HSP-70 mRNA in ischemic versus control hearts.²⁰ Here we noted only smaller increases of RNA. The different species (dog) and a more physiologic set-up (conscious animal) may explain this; however, we noted a significant increase of HSP-70 on RNA and on the protein level. Thus HSP-70 may initiate a repair mechanism in the model we used. Another heat shock-related protein that may be altered during stunning is GRP-94. Interestingly, this protein was first identified in the dog

Fig 4. Protein levels of c-jun in stunned canine myocardium. The LAD in chronically instrumented dogs was occluded for 10 minutes followed by reperfusion until the WTF returned to 50% of the preocclusion value. Tissue was obtained from the LAD- and the RCx-perfused area. The autoradiograph of a representative Western blot is shown. The area of interest around 39 kDa is shown. The *left lane* represents the RCx-perfused area, and the *right lane* shows the LAD-perfused area.

heart in 1994.²² It was suggested to play an important role in the sarcoplasmic reticular Ca^{2+} handling, nuclear signaling, protein folding, and sorting.²³ Unlike HSP-70, which resides in the nucleus and in cytoplasmic compartments, GRP-94 is located in the cardiac sarcoplasmic reticulum.^{13,24} GRP-94 may be induced by the accumulation of unfolded proteins in the endoplasmic reticulum.⁶ Although an increase in GRP-94 has been observed during ischemic stress in the kidney,⁶ currently there are no data on the expression of GRP-94 in any model of cardiac ischemia. In the present model we noted no difference in the expression of GRP-94 after the induction of stunning, although the expression of another HSP, namely HSP-70, was increased. This argues against a function of GRP-94 in myocardial stunning.

No data have previously been available concerning c-jun expression in canine hearts (under either physiologic or pathophysiologic conditions). Together with c-fos, c-jun is a component of the transcription factor AP-1, which regulates the transcription of numerous cardiac genes, including ANF.²⁵ C-jun has been reported to be increased in the heart after infarction by using rat models but not yet in the stunned myocardium. For instance, in rats c-jun was increased in the postinfarction myocardium after ligation of the left coronary artery.²⁶ Like HSPs, c-jun was not induced by ischemia alone but required reperfusion.²⁷ Hypoxia has also been shown to induce c-jun in cardiac myocytes.²⁸ Although cyanide closely mimicked the effects of hypoxia, the time course of c-jun was different.²⁸ Therefore other factors than metabolic switching seem to be involved in the regulation of c-jun. Pretreatment of cells with protein kinase inhibitors suppressed the response.²⁹

In the present model of stunning, c-jun was increased. Increased levels³⁰ have been reported in dilated human cardiomyopathy, but data on stunned human myocardium have not been available yet.

In summary, the present study demonstrates for the first time that HSP-70 and c-jun are increased in regional stunning in conscious mammals. This induction underscores a protective role of these genes in cardiac stunning.

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