Kinetics of heat shock protein 70 synthesis in the human heart after cold cardioplegic arrest

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Abstract

Objective: Protection of the myocardium against ischemia/reperfusion injury is a major challenge in cardiac surgery and cardiology. A cardioprotective role of heat shock proteins (Hsp), in particular Hsp 70, against ischemia has been demonstrated. A prerequisite for clinical exploitation of high Hsp 70 levels in the heart during ischemia is the determination of the efficacy and the kinetics of cardiac Hsp synthesis in vivo. Methods: We examined Hsp 70 and other immediate early genes, that are induced by cardioplegia and reperfusion, in right atrial biopsies taken from 15 patients during coronary artery bypass grafting. Specimens were obtained before cardioplegia and after ending of reperfusion and subsequently studied by immunohistochemistry and Western blot analyses. Results: Overall Hsp 70 increased 2.0 ± 1.1-fold (P < 0.01) in the nucleus as well as in the cytosol of myocytes and endothelial cells during open-heart surgery. As determined by comparison to a dilution series of recombinant protein, Hsp 70 levels amounted up to 6‰ of total cellular protein. The increase of Hsp 70 correlated well with the duration of cardioplegia and reperfusion (P < 0.005) showing a markedly accelerated increase at periods longer than 2 h. Further, the immediate early gene c-Fos also increased 2.4 ± 2.2-fold during open-heart surgery (P < 0.05), whereas other members of the Hsp family, like Hsp 27 and Hsp 90, showed no significant changes in protein levels during cardioplegia and reperfusion. Conclusions: These findings demonstrate that protein levels of Hsp 70 in the myocardium increase to significant amounts within few hours after induction. The optimum time point for induction of Hsp 70 appears to be at least 2 h before open-heart surgery. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Ischemia/reperfusion injury; Heat shock protein; Open heart surgery; Myocardial protection; Protein synthesis

1. Introduction

Cross-clamping of the ascending aorta interrupts the physiological blood flow to the myocardium for minutes up to a few hours during open-heart surgery. Myocardial oxygen consumption is significantly reduced by cardioplegia, in addition, systemic and local cooling may be applied to protect myocytes from ischemic damage [1]. Nevertheless, in open-heart surgery as well as in heart transplantation postoperative cardiac function and patient survival show significant negative correlations with the time of cardioplegia [2,3]. A similar problem is faced during percutaneous transluminal coronary angioplasty (PTCA), that repetitively subjects parts of the working heart to ischemia for about one minute, causing measurable myocardial damage in one third of patients [4]. Thus, improvement of cardiac protection against damage due to ischemia remained a subject of intense investigations.

Heat shock proteins (Hsps) constitute an endogenous stress response that protects myocytes from damage [5,6]. As most convincingly demonstrated in transgenic mice over-expressing the inducible form of the 70 000 molecular weight heat shock protein (Hsp 70), increased Hsp 70 expression in the heart results in increased resistance against ischemia/reperfusion injury and significantly improves recovery of cardiac function [7]. These cytoprotective effects of Hsps make them tempting targets for therapeutic interventions that are related to temporary myocardial ischemia, such as open-heart surgery and PTCA. Hsp induction can be achieved by a multitude of different stimuli, among them hyperthermia, hypoxia, ischemia, oxidative stress, and various drugs as well as cardioplegia and reperfusion [5,6,8–11]. However, the time elapsing between Hsp induction and completion of protein synthesis in the heart is not

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known. Correct timing of Hsp 70 induction may be critical for successful clinical application since studies have shown a direct correlation between the amount of hsp induced and the degree of myocardial protection [8].

The purpose of the present study was to investigate the kinetics of Hsp 70 synthesis in human hearts in vivo. Therefore, Hsp 70 was analyzed in heart biopsies, taken during coronary artery bypass grafting (CABG) before and after cold cardioplegic arrest and reperfusion. We focused on the determination of (i) the relative and absolute amounts of the studied proteins before and after their induction by cardioplegia and reperfusion, (ii) the localization of Hsp 70 and (iii) the kinetics of cardiac protein synthesis. For comparison, we further analyzed the kinetics of other hsps, Hsp 27 and Hsp 90, as well as another member of the immediate early gene family, c-fos. Like hsps, the c-fos gene is activated by ischemia [12,13]. The proto-oncogene c-fos acts as a transcription factor regulating genes that may be involved in complex cellular responses such as growth, differentiation, and programmed cell death [14,15].

2. Patients

The study protocol was approved by the institutional ethics committee on human research. Informed consent was obtained from 15 patients (61.4 ± 10.4 years old, three of whom female, ejection fraction 61.0 ± 16.5%; mean ± SD) scheduled for elective CABG with multiple grafts. As in all routine cases of open-heart surgery, cardiopulmonary bypass was performed with a priming of 1400 ml Ringer solution and 250 ml 20% mannitol at a flow of 2.4–2.6 l/min m² calculated body surface area. Moderate systemic hypothermia (30–32 °C) was applied in all patients. Following aortic cross-clamping, a single dose (30 ml/kg body-weight) of cold (4 °C) Bretschneider cardioplegic solution (15 mmol/l NaCl, 9 mmol/l KCl, 1 mmol/l KH-2-ketoglutarate, 4 mmol/l MgCl₂, 180 mmol/l histidine, 18 mmol/l histidine–HCl, 2 mmol/l tryptophan, 30 mmol/l mannitol) was administered. Cold (4 °C) Ringer solution was performed for external cooling. 4.5 ± 0.8 (mean ± SD) coronary anastomoses were placed during 62.7 ± 17.4 min (mean ± SD) of cardiac arrest. Next, normothermic blood perfusion of the heart was reconstituted lasting 39.9 ± 11.9 min (mean ± SD) until termination of cardiopulmonary bypass. Using a fresh scalpel, small myocardial biopsies (about 300 mm³) from the right atrial appendage were removed in every patient before aortic cross-clamping and after weaning from the extracorporeal circulation. The area of the specimen in contact with the forceps was removed. Particular care was taken not to mechanically irritate the site of tissue harvesting during the surgical procedure or with the venous cannula of cardiopulmonary bypass. All procedures were performed by the same surgeon. There were no intraoperative complications, and no patients died perioperatively.

3. Materials and methods

3.1. Immunohistochemistry

Tissue samples were fixed in Bouin’s solution and processed in paraffin. Alternatively, they were directly immersed in OCTZ 4583 (Miles Inc.) and frozen in liquid nitrogen. Five-micrometer sections were baked overnight at 60 °C before exposure to 3% H₂O₂ for 10 min and blocking with normal sheep serum (1:20). Incubation with monoclonal antibodies and the peroxidase–antiperoxidase method were performed as described previously [10,11]. After a brief counterstain with hematoxylin for better identification of cell types, slides were coverslipped and reviewed histologically. In controls, unspecific immunoglobins replaced the primary antibodies.

3.2. Preparation of nuclear and cytosolic extracts

Myocardial specimens were snap-frozen immediately after surgical removal and stored at 80 °C until analyzed. Frozen heart muscle samples were mechanically homogenized in liquid nitrogen. Lysis was prepared in cytosolic buffer containing 10 mM HEPES (pH 7.9), 40 mM KCl, 3 mM MgCl₂, 1 mM DTT, 5% glycerol, 0.2% Nonidet P40, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM PMSF for 10 min on ice. Centrifugation at 14 000 rpm for 20 s in a microcentrifuge (Eppendorf) removed the nuclei, that were then lysed in hypertonic buffer containing 20 mM HEPES (pH 7.9), 420 mM KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.5 mM PMSF for 30 min on ice. Centrifugation for 15 min at 14 000 rpm at 4 °C removed the insoluble fractions. Cytosolic and nuclear extracts were frozen and stored at 80 °C. Protein determination was performed using a BCA kit (Sigma). Samples were normalized to equal protein concentrations.

3.3. Western blot analysis

Either nuclear extracts, cytosolic extracts or equal amounts of both fractions were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (15% gel) and then transferred to a nitrocellulose filter. Membranes were blocked using 5% dry milk. The blot was probed with monoclonal antibodies against Hsp 70, Hsp 70/Hsc 70 (detecting both the inducible and the constitutive form of the 70 kDa molecular weight protein), Hsp 27, Hsp 90 (all at 1:1000 dilution; StressGen), and cFos (1:100; Calbiochem) overnight at room temperature. The antigen–antibody complex was visualized on an X-ray film using secondary antibodies linked to horseradish peroxidase (1:1000; Amersham) and a chemiluminescence kit (ECL; Amersham). Control experiments without primary antibody were negative. Microdensitometry of the films (Molecular Dynamics) provided quantitative values. To exclude measurements obtained from saturated signals experiments
were carried out in a series of dilutions. Only densitometric data within a range of good linear correlation between signal intensity on the film and the amount of total protein loaded on the gel \((r = 0.98, P < 0.001)\) were included in the analysis. Results were normalized to recombinant standard on each autoradiograph to correct for experiment-to-experiment variation.

3.4. Statistical analysis

Data are presented as mean ± standard deviation derived from heart biopsies of 15 patients. Values before and after cardioplegic arrest and reperfusion were compared using Student’s *t*-tests. *P*-Values smaller than 0.05 were considered to be statistically significant. Linear regression analyses were used to determine the correlation between the time of cardioplegic arrest plus reperfusion and the ratio of protein levels after/before cardioplegic arrest and reperfusion.

4. Results

4.1. Localization of Hsp 70

Nuclear and cytosolic extracts of tissue samples taken after cardioplegic arrest and reperfusion were analyzed for Hsp 70 via Western blot. Hsp 70 was detected in both cellular fractions without significant differences in signal intensity (Fig. 1). For comparison with other proteins of the immediate early gene family, nuclear and cytosolic preparations were further examined for c-Fos. Western blots showed clear signals exclusively in the nuclear extracts (Fig. 1).

Immunohistochemical staining of tissue sections from heart biopsies revealed intense immunoreactivity for Hsp 70 (Fig. 2). As in the results from Western blot analyses, strong signals were obtained from the nuclei of cardiac myocytes and endothelial cells as well as from the cytoplasm. Surrounding tissue showed no labeling.

4.2. Protein levels before and after cardioplegia and reperfusion

Taken equal amounts of both nuclear and cytosolic extracts of heart biopsies, inducible Hsp 70 showed an average 2.0 ± 1.1-fold increase \((P < 0.01)\) for all 15 patients (Fig. 3). Using antibodies that detect both the inducible form (Hsp 70) and the constitutive form (Hsc 70) of Hsp 70 protein levels did not change significantly (1.3 ± 0.6-fold; \(P = 0.11\)). Hsp families other than the 70 kDa protein also exhibited non-significant changes of 1.2 ± 0.5-fold for Hsp 90 \((P = 0.20)\) and 1.0 ± 0.3-fold for Hsp 27 \((P = 0.78;\) Fig. 3). A significant increase in nuclear cFos levels of 2.4 ± 2.2-fold \((P < 0.05)\) was also observed.

4.3. Kinetics of protein synthesis

This study comprised 15 patients who underwent CABG. The time of cardioplegic arrest and reperfusion varied between 64 and 153 min \((103 ± 28 \text{ min}; \text{mean} ± \text{SD})\). Representative results from Western blot analyses of cardiac biopsies are shown in Fig. 4 for relatively short (Fig. 4a) and long (Fig. 4b) periods of cardioplegic arrest and reperfusion. Fig. 5 depicts the relationship between the individual duration of cardioplegia plus reperfusion and the relative increase of detected Hsp 70 during this period. Protein levels increased constantly with the time of cardioplegia and reperfusion \((r = 0.70; P < 0.005)\). After 1 h, almost no increase of Hsp 70 levels was observed (1.3-fold) and the increase was minor 1.5 h after aortic cross-clamping (1.4-fold). However, an accelerated more than 2-fold increase was detected after 2 h. In those patients with the longest periods of cardioplegic arrest and reperfusion...
cardiac Hsp 70 showed an average 3–4-fold (maximum 4.2-fold) increase during 2.5 h without ever reaching a peak or leveling off.

The 2.4 ± 2.2-fold increase of cFos showed no time correlation.

4.4. Absolute amount of cardiac Hsp 70

The absolute amounts of Hsp 70 in right atrial biopsies after cardioplegia and reperfusion were determined by comparison of signal intensities in autoradiographs from Western blot analyses of Hsp 70 in heart biopsies and in dilution series with recombinant Hsp 70. As indicated in Fig. 6, after an average time of cardioplegia and reperfusion, i.e. about 100 min in this study, heart biopsies contained 15–20 ng Hsp 70 per 10 μg total cellular protein, i.e. 1.5–2‰. In biopsies taken 2.5 h after aortic cross-clamping Hsp 70 amounted up to 6‰ of total cellular protein.

5. Discussion

Our previous studies have shown a significant induction of Hsp 70 RNA during cold cardioplegic arrest and reperfusion [10,11]. The patterns of Hsp protein synthesis reported here demonstrate the efficient translation of significant Hsp amounts and define a time frame for Hsp induction before an intervention involving cardiac ischemia.

As shown by immunohistochemistry (Fig. 2), Hsp 70 in the heart is exclusively expressed in myocytes and endothe-
Several hours under the conditions of cold cardioplegic arrest and reperfusion. To achieve high Hsp 70 levels with a promise of improved cardioprotection, preconditioning of the myocardium may be most efficient when performed at least 2 h before open-heart surgery. This hypothesis is underscored by previous experiments in rats where Hsp 70 levels reached about 80% of maximum not before 4 h after induction [20]. Amrani et al. [5] defined the peak of Hsp 70 levels and cardioprotective effects against ischemia even 24–30 h after heat shock.

All our data are based on findings in the right atrial appendage because appropriate amounts of heart tissue, sufficient for Western blot analysis and histochemistry but without the risk of impairing cardiac function, are most readily obtained from this site. The extrapolation of our measurements to the entire heart clearly represents a limitation of our work. However, since the stimulus for hsp synthesis, i.e. aortic cross-clamping and induction of cardioplegia through the aortic root, is imposed equally on the entire organ, we hypothesize that our findings in the right atria reflect changes in the entire heart. Nevertheless, further studies using tissue from other cardiac sites, particularly the left ventricle, are necessary to support this hypothesis.

The comparison of Hsp 70 synthesis to that of other members of the heat shock family revealed striking differences in their induction patterns. No relevant increases of Hsp 27 and Hsp 90 were observed during the surgical procedure. Either the induction of these proteins was less intense than for Hsp 70 or the translation of induced genes was highly delayed and a significant increase of Hsp 27 and Hsp 90 was not detectable within our observation period confined by the surgical procedure.

Compared to Hsp synthesis, the induction and translation of myocardial eFos was faster in our experiments. A significant increase of c-Fos was observed in heart tissue of all patients amounting to similar levels after relatively short (about 70 min) and relatively long (about 150 min) periods of cardioplegia and reperfusion. This observation supports the concept of an individual pace of induction and translation of different early immediate genes in the human heart. Further, rapid c-Fos synthesis demonstrates active translational processes in the heart during cold cardioplegic arrest and reperfusion.

Proto-oncogenes, like c-Fos, play an important role in cellular responses to stress [12,14] mediating positive, but also negative effects on cellular fate, e.g. c-Fos is also involved in apoptosis leading to myocyte death [21,22]. Since cardiomyocytes are unable to proliferate and thus cannot be replaced, loss of these cells is deleterious to the heart. Therefore, we have to aim at a principal or, if possible, exclusive induction of stress proteins like Hsp 70 to improve myocardial protection during ischemia. Among various possible routes to Hsp 70 induction, the best strategy has yet to be defined. Amrani et al. [5] suggested a critical threshold of Hsp 70 to induce cardioprotection in rat hearts. Thus, besides the accurate timing of hsp induction, the efficiency of the stimulus may be of particular importance.
Hsp 70 is induced by a multitude of stimuli, and the induction of Hsp 70 by hyperthermia has been shown to go along with improved recovery from ischemia and cardiac arrest in animal hearts [5, 8, 23]. Furthermore, acute hypertension was found to enhance the expression of Hsp 70 in the vasculature [24]. Maulik and colleagues report an improved ventricular recovery after cardiopulmonary bypass in swine after a drug-induced increase of Hsp 70 [25]. Current investigations are aiming at new strategies for Hsp 70 induction that are practicable in a clinical setting. The significant increase of Hsp 70 found in our study underscores the optimism surrounding these investigations. However, the delayed synthesis of Hsps described here recommends their early induction no later than 2 h before open-heart surgery or PTCA.

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References