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Expression of Heat Shock Proteins in Chronic Atrial Fibrillation

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The role of heat shock proteins in AF pathophysiology was investigated in this study. We analysed mRNA and protein expression of heat shock protein (Hsp) 70, Hsp 60 and Hsp 27 in two groups of patients undergoing cardiac surgery. The first group (NAF) included six patients with coronary heart disease and no AF, the second group (CAF) included six patients with coronary heart disease and chronic atrial fibrillation. There were no significant changes in Hsp 70 protein or mRNA expression. In CAF group Hsp 60 protein and mRNA expression was also unaltered compared to NAF. Hsp 27 mRNA expression showed a significant decrease of 16±5% (NAF vs. CAF) yet accompanied with no statistically significant change of protein expression. The protein expression of Hsp 27, Hsp 60 and Hsp 70 was not altered significantly in patients with chronic AF. In contrast to heart failure, heat shock proteins seem to play a minor role in AF pathophysiology.

Key words: Atrial fibrillation, heat shock protein, western blot, RT-PCR, cellular stress, Hsp 70, Hsp 60, Hsp 27, atrial remodeling

INTRODUCTION

Atrial Fibrillation (AF) is the most common sustained arrhythmia in man characterized by a variety of electrophysiological, mechanical and structural alterations caused by the arrhythmia itself. This process is termed atrial remodeling in AF as described by Wijffels *et al.* (1995). Electrophysiological alterations of ionic currents were extensively studied (Dobrev and Ravens, 2003), but further mechanisms of underlying regulatory processes are poorly understood.

In response to adverse environmental changes, cells from many organisms increase the expression of a large class of proteins called heat shock or stress proteins to cope with the stress period by protecting essential components within the cell (Lindquist, 1986; Morimoto *et al.*, 1996). Furthermore heat shock proteins have a wide spectrum of action in the cell even in absence of stress factors.

Because pathophysiological processes-on electrical as well as on haemodynamic level-are a kind of cellular stress, a potential alteration in heat shock protein (Hsp) expression in AF is likely. The changes in protein and mRNA expression levels of heat shock proteins in patients with or without chronic atrial fibrillation were studied.

MATERIALS AND METHODS

Patients: All patients underwent open heart surgery using cardiopulmonary bypass. Chronic AF was considered as permanent AF for more than three months duration. Characteristics of included patients are shown in Table 1.

Atrial tissue collection: Cardiac specimen (100-200 mg) were immediately frozen in liquid nitrogen and stored at -70°C until further analysis. All procedures were in accordance with the institutional guidelines of the University of Tübingen.

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR): Total RNA was extracted using Trizol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol and quantified by spectrophotometry. First-strand cDNA was synthesized by reverse transcription of 5 µg total RNA with random hexamer primers (Promega, Mannheim, Germany) and a modified MMLV reverse transcriptase (SuperScript II, Invitrogen, Karlsruhe, Germany). Specific oligo-nucleotide

primers were designed using the following GenBank accession numbers: human Hsp27 (NM_001540), human Hsp 60 (NM_002156), human Hsp 70B (NM_002155) and human housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as standard (NM_002046). Primer pairs and PCR product lengths are shown in Table 2. All of the PCR reactions involved initial denaturation at 94°C for 2 min followed by 35 cycles (Hsp) of 94°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec or 30 cycles (GAPDH) of 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min. After the last cycle, 72°C elongation step was extended to 10 min. Electrophoresis of the amplicons was performed on 2% agarose gels containing ethidium bromide. Gels were photographed with a MP-4 gel documentation system (Polaroid, Offenbach, Germany) and density of each band was analysed with a GS-800 densitometer (Bio-Rad Laboratories, Munich, Germany).

Western blotting: Protein extraction was performed as previously described (Bosch *et al.*, 2003). Protein content was determined by a modified Lowry assay. Seventy micrograms of protein were fractionated on 8% SDS-polyacrylamid gels and transferred to nitrocellulose membranes (Amersham Biosciences, Freiburg, Germany) according to standard protocols. Specific antibodies were used to detect Hsp 70, Hsp 60 and Hsp 27 (Stressgene, Canada) and G3PDH (Biotrend, Köln, Germany). The protein bands were visualized by HRP conjugated anti-mouse IgG (Sigma, Taufkirchen, Germany) by ECL reagents and Hyper film ECL (Amersham Biosciences, Freiburg, Germany). Films were densitometrically evaluated using Quantity One Software (Bio-Rad, München, Germany). Expression of respective heat shock proteins were normalized using G3PDH as standard.

Data analysis: Data were expressed as mean±SEM. A two-tailed p-value <0.05 was considered statistically significant. Analysis was performed by Sigmaplot, (Jandel Scientific) and Excel (Microsoft-Corp).

Table 1: Patients characteristics

Characteristics	NAF	CAF
Age	69±4	73±7
Male/female	4/2	5/1
EF (%)	52±29	63±11
Atrium dimension [mm]	38±5	48±5
Beta-blocker	6	6
Other Antiarrhythmics	2	1
Calciumchannel-blockers	0	1

NAF = No (chronic) atrial fibrillation. CAF = Chronic atrial fibrillation. EF = Ejection fraction

Table 2: PCR primers and optimized PCR conditions for the amplification of human heat shock proteins

Genes	PO	Primer sequences	AT	PL
hsp27	Sense	5'-CACGAGGAGCGGCAGGACGAG-3'	58°C	285 bp
	Antisense	5'-CAGTGGCGGCAGCAGGGGTGG-3'		
hsp60	Sense	5'-TGTTTTGGGAGGGGTTGTGC-3'	58°C	309 bp
	Antisense	5'-AACAGAGAGGCCACACCAGCA-3'		
hsp70	Sense	5'-CTCCAGCATCCGACAAGAAGC-3'	58°C	234 bp
	Antisense	5'-ACGGTGTGTGGGGGTTTCAGG-3'		
GAPDH	Sense	5'-GCTCCTGGAAGATGGTGATGG-3'	60°C	230 bp
	Antisense	5'-AGGTGAAGTCCGGAGTCAACG-3'		

PO = Primer Orientation. AT = Annealing Temperature. PL = RTPCR product length

RESULTS AND DISCUSSION

In this study, we evaluated mRNA and protein expression levels of heat shock proteins 70, 60 and 27 by semiquantitative RT-PCR and western blot analysis in atrial tissue specimens from patients with (CAF) or without (NAF) chronic atrial fibrillation.

Comparing CAF group with NAF group, no statistical significant difference of mRNA and protein expression of Hsp 70 could be observed (Fig. 1, upper row).

In CAF group, Hsp 60 protein and mRNA expression was also unaltered compared to NAF (Fig. 1, middle row).

Finally, Hsp 27 mRNA expression showed a significant decrease of 16±5% (NAF vs. CAF) yet accompanied with on statistically significant change of protein expression (Fig. 1, lower row).

In this study, we investigated changes in atrial heat shock proteins expression levels of Hsp 70, Hsp 60 and Hsp 27 at patients with chronic atrial fibrillation. Our data suggests a minor role of heat shock proteins in chronic atrial fibrillation since there was no significant change in protein expression level.

Among all heat shock proteins, the 70 kDa family (Hsp 70) is the most frequently studied in eukaryotic cells. Like other stress proteins, gene expression and/or tissue levels of Hsp70 family members, which are located in the cytosol and nucleus of cells (Srivastava, 2002), are increased by many stress stimuli, e.g., ischemia (Yellon and Marber, 1994), suggesting their biological action might be to maintain cellular integrity and viability, as an endogenous defense mechanism (Iwaki *et al.*, 1993; Knowlton *et al.*, 1998; Suzuki *et al.*, 2000; Tanonaka *et al.*, 2001; Tanonaka *et al.*, 2004).

Indeed, Hsp 70 is a molecular chaperone assisting in protein assembly, folding, transport and translocation as well as preventing denaturation of intramyocardial enzymes (Mayer and Bukau, 2005). After cell stress or death, Hsp peptide complexes may be presented on the cell surface or released to the surrounding, thereby activating cells of the adaptive immune system (Srivastava, 2002).

As already mentioned, the main role of Hsp70 is to correct protein folding. In chronic AF, the structural changes of cardiomyocytes are in a final state and there

might be no extended protein biosynthesis and turnover. The complex composed of Hsp 60 and Hsp 10 is the major site of protein folding of multimeric enzymatic complexes in mitochondria and is therefore extremely important for the viability of the cell (Lin *et al.*, 2001). It has been demonstrated that increased Hsp 60 and/or Hsp 10 expression protects mitochondrial function and prevents apoptotic cell death induced by simulated ischemia-reoxygenation (Lin *et al.*, 2001; Shan *et al.*, 2003).

In this study, Hsp 60 protein and mRNA expression was not altered significantly. Apoptosis seems to play some role in AF (Aime-Sempe *et al.*, 1999), but it seems that other protection mechanisms are activated in CAF. Hsp 27 is part of a family of proteins that bind denatured proteins in times of stress to prevent aggregation (Fink, 1999) and therefore protects cardiomyocytes against ischemia/reperfusion injury (Martin *et al.*, 1997; Hollander *et al.*, 2004; Efthymiou *et al.*, 2004). Furthermore, Hsp27 plays an important role in dynamic regulation of cytoskeleton processes, especially actin regulation is an important factor (Lavoie *et al.*, 1995). Actin is involved in locating the L-Typ Calcium channel which seems to be very important in electrical remodeling in AF (Dobrev and Ravens, 2003).

Significant changes in mRNA expression might point to a possible contribution of Hsp 27 to atrial remodeling, but this has to be evaluated in further studies.

Present data showed that in chronic AF Hsp 27, Hsp 60 and Hsp 70 mRNA and protein expression is not altered significantly compared to control values. But one cannot exclude that heat shock protein expression is different at earlier stages after onset of atrial fibrillation and levels off when AF becomes chronic. Such a progression was described in a chronic heart failure model, where e.g., heat shock protein 72 expression increased right after onset of cardiac ischemia and then almost returned to control values after a certain time (Tanonaka *et al.*, 2001). Such a possible time course can only be evaluated in animal models with different durations of AF.

Few studies are available defining the role of heat shock protein expression in AF. Lower preoperative intracellular, but not serum levels of Hsp 70 (Mandal *et al.*, 2005) and higher blood levels of Hsp 65

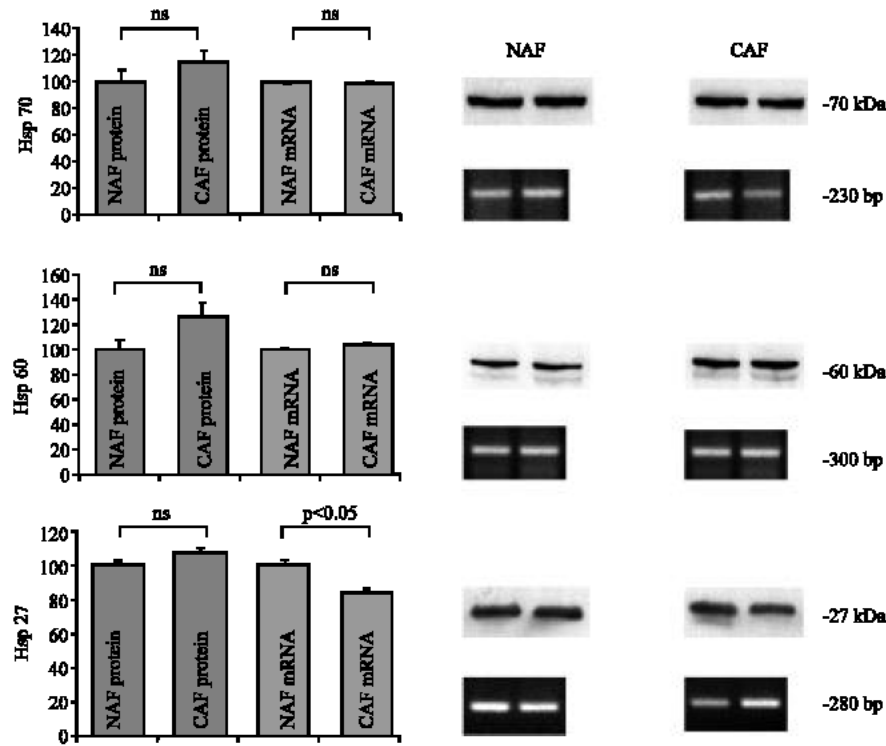


Fig. 1: Expression of Hsp 70 (upper row), Hsp 60 (middle row) and Hsp 27 (lower row) protein and mRNA of patients with (CAF, 6 patients) and without (NAF, 6 patients) chronic atrial fibrillation. Protein and mRNA expression of NAF group was normalized as 100%. At each Hsp row, original western blot (upper bands) and representative gel electrophoresis (lower band) of the respective group (NAF/CAF) are shown. ns = not significant (paired t-test)

(Mandal *et al.*, 2004) seem to be a risk factor for postoperative AF. The pathophysiological role of these findings is not clear at the moment.

Schäfler *et al.* (2002a) investigated Hsp 60 expression, Hsp 60/10 coexpression (Schäfler *et al.*, 2002b) and Mortalin (mitochondrial heat shock protein 70) expression (Kirmanoglou *et al.*, 2004) and found a more than 2 fold overexpression in each case in chronic AF patients undergoing open heart surgery for several reasons. In this study, actin was used as housekeeping gene, but cytoskeletal changes are very important in chronic AF and actin mRNA levels might be affected in chronic AF. For this reason, we used G3PDH, a protein of the cytosolic primary pathways as housekeeping gene. Furthermore all our patients underwent aorticoronary bypass surgery and no valve replacement was performed. Changes in heat shock protein expression by other processes like atrial dilatation or atrial stress through an increase of atrial pressure might have contributed to increased Hsp expression. Our study gives a detailed insight in heat shock protein expression patterns in chronic AF. We found effects of AF on heat shock

expression levels implying a minor role of heat shock proteins as protective mediators in AF pathophysiology at least in the chronic state.

But one cannot exclude an important role of heat shock proteins in the early phases of manifestation of AF. Further studies are needed to answer these important questions.

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