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ORIGINAL CONTRIBUTION

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Expression of mortalin in patients with chronic atrial fibrillation

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Abstract *Background* In myocardium of patients with chronic atrial fibrillation (AF) the expression of the mitochondrial heat shock proteins HSP60 and HSP10 is increased. They are responsible for folding and translocation of proteins inside the mitochondria. Import of these proteins is accomplished by mortalin. The aim of our study was to investigate if the expression of the heat shock protein mortalin is also increased in patients with AF. *Methods* Right atrial samples from 18 patients undergoing elective cardiac surgery were excised and immediately frozen in liquid nitrogen: 8 patients had chronic AF (≥ 3 month) and 10 patients were in sinus rhythm (SR). Mortalin was determined by SDS-PAGE, Western blot and quantified by optical densitometry. *Results* In myocardial samples from patients with chronic AF we found a more than 2-fold increase in mortalin expression. *Conclusions* The increased expression of mortalin may represent an adaptive heat shock response to restore cellular homeostasis.

Key words Mortalin – atrial fibrillation – heat shock proteins – actin – western blot

Introduction

Atrial fibrillation (AF) is characterized by an electrical, contractile and structural remodeling [1]. Electrical remodeling [3] and structural alterations [6] seem to perpetuate AF, whereas the long-term metabolic stress leads to the upregulation of heat-shock proteins as a physiological adaptation [16].

Mortalin, also known as mitochondrial heat shock protein 70 (mthsp70), peptide binding protein 74 (PBP74), or glucose regulated protein 75 (GRP75) is a member of the Hsp70 family of proteins [2]. Mortalin was shown to have different subcellular localizations in normal and immortal cells [7].

Density gradient cell fractionation along with organelle-specific markers confirmed that mortalin is dominantly present in mitochondria [4]. It was shown to form an essential component of the mitochondrial import machinery. This includes unfolding of proteins outside mitochondria, unidirectional translocation and completion of import by acting as an ATP driven motor [10].

Inside the mitochondria, HSP60 and HSP10 are responsible for folding and translocation of nuclear-coded mitochondrial proteins. Since we found an increase in HSP60/HSP10 expression in human atrial fibrillation [17], the aim of our study was to examine the expression of the functional co-chaperonin mortalin.

Material and methods

Patients

The present study was reviewed and approved by the ethics committee of the University of Ulm. Analyses of protein levels of mortalin were performed in myocardium from 18 human hearts. Atrial myocardium was obtained after extracorporeal circulation from 10 patients in sinus rhythm and 8 patients with atrial fibrillation undergoing elective cardiac surgery for coronary revascularization and/or aortic respective mitral valve replacement. Chronic AF was considered as permanent AF for more than three months [19]. In all patients, except one, CAF was documented with serial ECG's and a history for more than 3 months. The clinical characteristics of the patients are given in Table 1: (Patient 1 through 18) 16 were male and 2 were female; their mean age was 69 ± 9 years. Anesthesia was the same for each patient. Cardiopulmonary bypass was established with a priming solution (1000 ml Ringer, 400 ml human albumin, 200 ml Trasylol, 5000 IU Heparin) at a flow rate of 2.4 l/min/m^2 body surface area. All patients were cooled to 32 °C (esophageal). In addition, there was aortic cross-clamping, with a myocardial arrest induced by antegrade infusion of cold Brettschneider cardioplegic solution at a myocardial temperature of approximately 4 °C.

Table 1 Clinical characteristics of the patients

Patient	Age	Sex	Op	Rhythm	Medication
1	65	M	ACVB	SR	BB, Dig, ACE, Diur, Nit
2	55	M	ACVB	SR	BB, Ca, CSE
3	69	M	ACVB	SR	BB, Dig, Diur,
4	78	M	ACVB	SR	BB, ACE, Nit
5	55	M	ACVB	SR	Dig, CSE
6	79	M	AVR	SR	BB
7	48	M	ACVB	SR	BB, Nit
8	60	M	MVR	SR	BB, Ca
9	75	M	ACVB	SR	BB, Diur, ACE
10	75	F	ACVB	SR	BB, ACE, Nit, CSE
1	74	M	AVR	AF> 7 months	Dig, Ca, Nit,
2	70	M	AVR	AF>11 months	Dig, ACE, Diu, Ca
3	71	M	MVR/ACVB	AF> 3 months	Dig, Amio, Diur,
4	74	F	MVR	AF> 2 months	Dig
5	69	M	MVR/ACVB	AF> 3 months	Dig, Diur
6	78	M	ACVB	AF> 3 months	Dig, Diur
7	66	M	ACVB	AF> 3 months	Dig
8	79	M	ACVB	AF>13 months	Dig, Diur, CSE, ACE

ACVB aortocoronary bypass; *MVR* mitral valve replacement/repair; *AVR* aortic valve replacement; *SR* sinus rhythm; *AF* atrial fibrillation; *BB* beta-blocker; *ACE* ACE inhibitor; *Diur* Diuretic; *Ca* Ca-antagonist; *CSE* CSE-inhibitor; *Nit* nitrates; *Dig* Digitalis; *Amio* Amiodaron

Western blot analysis

Protein preparation

Myocardium for Western blot analyses was dissected, immediately frozen in liquid nitrogen, and stored at –80 °C until use. Care was taken not to use fibrotic or adipose tissue. For protein preparation, ≈ 40 mg of frozen atrial tissue was homogenized by use of a Ultra-Turrax T8 micro dismembrator (IKA Labortechnik, Staufen) for 60 s in a nine-fold lysis buffer (mmol/L) $Na_4P_2O_7$ 100, EGTA 5, $MgCl_2$ 5, KCl 300, DTT 1, [pH 8.5] along with a protease inhibitor cocktail 0.1 mmol/L AEBSF, containing E-64, bestatin, leupeptin and aprotinin [P2714, Sigma Ltd] at 4 °C. The homogenate was centrifuged at 4000 rpm for 10 min at 4 °C in a Labofuge GL (Heraeus Sepatech). After centrifugation, the supernatant was carefully removed.

Western blot analysis

Samples of 100 µl protein of the particulate fraction were denaturated by heating to 95 °C in 900 µl 2% sodium dodecyl sulfate (SDS), 10% Glycerol, 5% 2-mercaptoethanol, 0.002% bromphenol blue, 0.0625 M Tris-HCl, [pH 6.8] and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Mortalin

SDS-PAGE was performed under reducing conditions on a 10% separation gel with 4% stacking gel. Running conditions were 200 V at 50 mA for 45 min on ice. Proteins were transferred to a Hybond-ECL nitrocellulose membrane at 5 mA/cm^2 for 30 min by use of 25 mmol/L Tris, 192 mmol/L glycine, and 20% methanol [pH 8.3] as a blotting buffer. The blot was blocked 1 hour in 10% nonfat milk solution (20 mmol/L Tris-HCl, 137 mmol/L NaCl, and 10% nonfat milk powder, pH 7.45), incubated with a 1:2000 diluted mouse anti-human mortalin monoclonal antibody solution (SPA-825, StressGen) for one hour, and washed two times for one minute and three times for five minutes in 10 ml TTBS solution. Immunodetection of the primary antibody against mortalin was carried out with a 1:5000 diluted peroxidase conjugated anti mouse secondary antibody (NA 931, Amersham Ltd) for 45 min and washed again two times for 1 min and 4 times for 15 min in TTBS. After incubation with 0.125 ml/cm^2 ECL-detection reagent (Amersham buchler Ltd) for 1 min, blots were exposed to Hyperfilm ECL (Amersham Ltd) for 1 min.

Actin

Separation was performed according to mortalin. For immunodetection, the blot was incubated with a 1:10,000

diluted mouse anti-human actin monoclonal antibody solution (Clone AC-40, Sigma) for 1 h (dilution buffer, 20 mmol/L Tris-HCl, 137 mmol/L NaCl, 0,1 % Tween 20, pH 7.45) and washed two times for 1 min and three times for 5 min in 10 ml TTBS solution. Immunodetection of the primary antibody against actin was carried out according to mortalin.

Quantification of immunoreactive bands

After development the blots were scanned with a Umax Mirage II densitometer (Umax, Freemont, CA) and a Epson Perfection 1240 Photo (Epson® Europe). Bands were quantified with an analysis software (mars 98, version 1.0.1) according to the densitometric integral derived from each sample band (see Fig. 3). The integral of the density over a measured area was taken to calculate the amount in each sample according to the known standard values. A linear relationship was found between the known protein amounts and the densitometric integrals ($r = 0.97$). On the basis of this linear relationship, mortalin in each atrial sample was calculated.

Statistical analysis

After testing for normal distribution, all data are presented as mean ± SD. To detect differences between groups, the unpaired two tailed Student t-test was performed. The $P < 0.05$ value was considered statistically significant. The correlation between standard mortalin and the densitometric integral was examined by linear regression analysis.

Results

The surgical outcome was uneventful in all patients, and there was no complication from right atrial dissection. The bypass time was approximately 114.6 ± 30 min and the ischemic time 66.7 min ± 21 min.

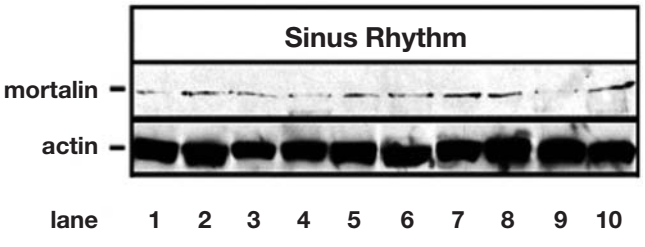

Fig. 1 A representative Western blot of SDS-PAGE showing mortalin and actin separated from human right atrial specimen in sinus rhythm

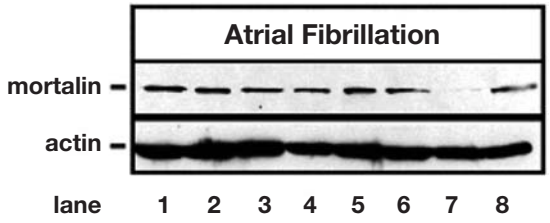

Fig. 2 Western blot of immunochemical detection of mortalin and actin from human right atrial specimen with chronic AF

Mortalin expression in myocardium of patients in sinus rhythm and atrial fibrillation

In order to evaluate whether there were any changes in mortalin expression in permanent atrial fibrillation, Western blot analysis was carried out on SDS-PAGE separations of total protein isolated from ten hearts in SR (Fig. 1) and eight hearts in AF (Fig. 2). Mortalin was present in atrial homogenates of all patients studied. Visual inspection of the actin bands demonstrates that wells in each group were loaded with equal amounts of protein. The following values refer to a concentration of nanogram protein per milligram wet heart tissue. There is a 2.19-fold increase in mortalin content (3184 ng/mg ± 1259 ng/mg) in myocardium of patients with chronic AF compared with the mean mortalin level in myocardium of patient with sinus rhythm (1457 ng/mg ± 583 ng/mg; * $p < 0.01$; Fig. 3).

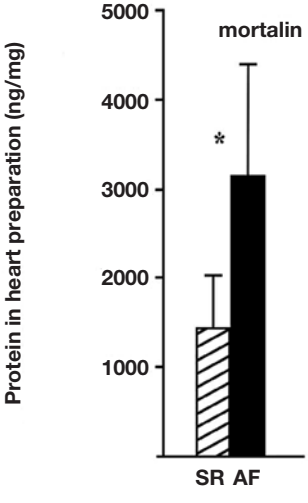

Fig. 3 Computerized densitometric mortalin levels in right atrial myocardium from patients in SR and patients with chronic AF

Discussion

The majority of mitochondrial proteins are synthesized in the cytoplasm and transported via HSP70/40 to the mitochondria [24]. Import of these proteins across the mitochondrial membranes is accomplished by mortalin [21]. It prevents premature folding, precipitation and aggregation of these mitochondrial proteins [13]. The import function is dependent on its anchoring to the inner mitochondrial membrane and interactions with an inner mitochondrial translocase TIM44 [23]. Inside the mitochondria HSP60/10 are responsible for translocation and refolding or degradation via the proteasom pathway [9].

The present study demonstrates that myocardium from chronic fibrillating right atrial appendages display a more than 2-fold increase in mortalin. Since previous studies have shown that HSP60 and HSP10 are also expressed more than 2-fold [17], AF seems to trigger a mitochondrial heat shock response. The increased atrial activity with enhanced cellular metabolism leads to an accumulation of unfolded proteins, the innate signal to restore cellular homeostasis. Subsequently a different set of proteins is synthesized by a change in the transcription program [5].

The factors that regulate the adaptive processes to cardiac muscle are not fully understood. Mortalin expression was induced by muscle activity, mitochondrial activity and biogenesis [12, 20]. Salo et al. [15] reported an acutely increased expression of skeletal muscle stress proteins immediately following treadmill running. They attributed this increase in part to the hyperthermia associated with exercise. In contrast, Skidmore et al. [18] reported an increased expression of stress proteins independent of changes in body temperature following acute treadmill running. Furthermore, Mattson et al. [11] demonstrated a chronically increased expression of HSP60 and mortalin following endurance exercise training.

Mortalin seems to play multiple functions relevant to the control of cellular proliferation and tumorigenesis. Many of the human transformed and tumor derived cells were seen to have a high level of mortalin expression. Malignant transformation, life span extension and attenuation of differentiation was explained by its p53 binding and inactivation function [22]. Thus, it seems possible that AF is associated with a change in the proliferation and differentiation program.

Other functions of mortalin, including chaperonization and mitochondrial biogenesis, may also be significantly important for control of division potential. It was shown that it binds to FGF-1 and aids in its intracellular trafficking. Also the association of mortalin with the IL-1 receptor was predicted to have a role in receptor internalization [14], and its binding to GRP94 may be involved in stress resistance and survival of cells. It has also been shown that functional inactivation causes aggregation of mitochondria, which are essential for calcium regulation, apoptotic cell death, energy supply and for cell survival [8].

In summary, our result shows an upregulation of mortalin expression in chronic AF, possibly to increase mitochondrial protein import.

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