Heat shock protein upregulation protects against pacing-induced myolysis in HL-1 atrial myocytes and in human atrial fibrillation

Bianca J.J.M. Brundel a,⁎, Robert H. Henning b, Lei Ke a, Isabelle C. van Gelder c, Harry J.G.M. Crijns d, Harm H. Kampinga a

a Department of Radiation and Stress Cell Biology, Groningen University Institute for Drug Exploration (GUIDE), University Medical Center of Groningen, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands
b Department of Clinical Pharmacology, Groningen University Institute for Drug Exploration (GUIDE), University Medical Center of Groningen, The Netherlands
c Department of Cardiology, Groningen University Institute for Drug Exploration (GUIDE), University Medical Center of Groningen, The Netherlands
d Department of Cardiology, University of Maastricht, The Netherlands

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Abstract

Atrial fibrillation (AF) causes myocyte stress by inducing structural changes, predominantly myolysis, which is related to the progression of AF. As heat shock proteins (Hsp) protect against cellular stress, their efficacy in preventing myolysis was investigated in a tachy-paced cell model for AF and in patients with AF. HL-1 atrial myocytes were subjected to tachy-pacing, which induced myolysis. Hsp overexpression was accomplished by a mild heat shock or by the drug geranylgeranylacetone (GGA). Hsp-gene-transfection studies were carried out to investigate roles of individual Hsp. In left and/or right atrial appendages from patients with paroxysmal (n = 14), persistent (n = 17) AF and controls (n = 13) in sinus rhythm (SR), Hsp levels (Westerns) and localization (confocal microscopy) were determined. Heat shock and GGA administered prior to tachy-pacing resulted in almost complete protection against tachy-pacing-induced myolysis. Overexpression of Hsp27, but not of Hsp70, also provided complete protection against pacing-induced myolysis. In patients with paroxysmal AF, Hsp27 expression was significantly increased compared to SR and persistent AF. No changes in Hsp40, Hsc70, Hsp70 and Hsp90 expression levels were observed. Hsp27 levels correlated inversely with the duration of paroxysmal and persistent AF and the extent of myolysis. Furthermore, Hsp27 was localized on myofibrils in tachy-paced HL-1 myocytes and in human cardiomycocytes. These data demonstrate that upregulation of Hsp, especially Hsp27, protects tachy-paced atrial myocytes from myolysis. Therefore, the observed elevated Hsp27 expression in patients with paroxysmal AF might serve to protect myocytes from myolysis and limit the progression to persistent AF. Pharmacological induction of Hsp, with drugs such as GGA, may represent a novel therapeutic approach in AF.

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1. Introduction

Atrial fibrillation (AF) is the most common cardiac arrhythmia which has the tendency to become more persistent over time [1]. Research exploring the underlying mechanisms of the self-perpetuation of AF has demonstrated that AF leads to shortening of atrial effective refractory periods (AERP), heterogeneity of the electrical activation pattern and loss of contractile function [2]. When the arrhythmia continues, AF induces changes at the structural level, predominantly myolysis, which are associated with the progression of AF [3–8]. Myolysis is characterized by disruption of the myofibril structure [3,9,10] and observed after various forms of cell stress such as ischemic stress [11] and hypoxia [12]. Furthermore, it is well known that some Hsps, especially Hsp70 and Hsp27, protect against the degradation of myofibrils. Hsp70 is able to bind and protect microtubule network and therefore limit myofibril disruption after ischemic stress in myocardium [13]. Also, in cells overexpressing Hsp27, F-actin and myofibril structure are protected against heat- or ischemia-induced disruption [14–18] and recovery after disruption is accelerated.
Because of these protective roles of Hsp, we studied whether upregulation of Hsps prior to and during pacing can protect against tachy-pacing-induced myolysis in an established cell model for AF [20,21]. In this model, induction of Hsp synthesis was performed by a mild heat shock or by the drug geranylgeranylacetone (GGA). GGA, clinically employed as an antiulcer agent, is a non-toxic acyclic isoprenoid compound with a retinoid skeleton that induces Hsp synthesis in various tissues including gastric mucosa, intestine, liver, myocardium, retina and central nervous system under stress [22–24]. In the present study, we found that Hsp upregulation protects against pacing-induced myolysis. Notably, the sole overexpression of one member of Hsp, Hsp27, was sufficient for this protection.

2. Methods and materials

2.1. HL-1 cell culture conditions, transfections and constructs

The HL-1 atrial myocytes, developed from adult mouse atria [25], were obtained from Dr. William Claycomb (Louisiana State University, New Orleans, LA, USA) and cultured as described before [20].

Lipofectamine (Life technologies, The Netherlands) was used for transient transfections according to instructions of the manufacturer. pHsp70-YFP encodes a functional human Hsp70 fused to YFP under control of a CMV promoter. pHsp27 encodes human Hsp27 under control of CMV promoter (Clontech, The Netherlands). Hsp27 transfected myocytes were detected by immunofluorescent staining with the human specific Hsp27 antibody (SPA800, StressGen Biotechnologies, Victoria, Canada) and myocytes overexpressing human Hsp70-YFP were distinguished from non-transfected myocytes on the basis of the YFP signal.

2.2. Pacing and induction of Hsp expression

HL-1 myocytes (≥1×10⁶ myocytes) were cultured on coverslips and subjected to a 10-fold rate increase (tachy-pacing) by the use of a C-Pace100™-culture pacer and C-Dish100™-culture dishes (IonOptix Corporation, The Netherlands). Myocytes were stimulated at 5 Hz with square pulses of 5 ms duration and a pulse voltage set to 45 V. Evidence of capture was examined by microscopic examination of cell shortening at the beginning and before termination (after 24 h pacing) of stimulation. Capture efficiency at these time points was >90% for myocytes attached on gelatin/fibronectin-coated coverslips. Furthermore, the role of non-specific factors, such as electrolysis at the electrodes, was excluded by placing control myocytes outside the electrical field by the use of a specially developed pacing system. In this system, coverslip-cultured myocytes can be placed either within the electric field or outside the electric field. Control myocytes placed outside the electric field showed no signs of myocyte remodeling, whereas myocytes placed in the electric field revealed myocyte remodeling [20].

Elevation of Hsp expression in cultured myocytes was accomplished in 3 ways: (I) by subjection to a modest heat shock at 43 °C for 30 min followed by overnight incubation at 37 °C, (II) by incubation with 0.1 μM geranylgeranylacetone (GGA, gift from Eisai Co. Japan) 2 h prior to and during pacing and (III) by transfection of pHsp70-YFP or pHsp27 24 h prior to pacing. The concentration-dependent increase in Hsp expression by GGA was tested at 0.1, 1 and 10 μM. Since 90% of the maximum effect on induction of Hsp expression was already reached at 0.1 μM GGA, all experiments were performed at this concentration.

2.3. Patients

Prior to surgery, one investigator assessed the clinical characteristics of the patients (Table 1). The patient’s arrhythmia history was classified according to Gallagher [26].

| Table 1 Baseline characteristics of patients with paroxysmal AF (PAF), persistent AF (PeAF) and control patients in sinus rhythm |
|-----------------|-----------------|-----------------|
|                | SR              | PAF             | PeAF            |
| n               | 13              | 14              | 17              |
| RAA (n)         | 11              | 12              | 16              |
| LAA (n)         | 5               | 12              | 16              |
| Age             | 61±4            | 50±3            | 53±3            |
| Duration of AF  | (median, range (months)) | 11.6 (0.1–56) |
| Duration SR before surgery (median, range (days)) | 10 (0.5–210) |
| Duration of last episode AF (median, range (h)) | 12 (0.2–24) |
| AF/day (median, range (%)) | 2 (0.2–70) |
| Underlying heart disease (n) and/surgical procedure |     |
| Coronary artery disease/CABG | 8* | 0 | 0 |
| Lone AF/Maze | 0 | 8 | 9 |
| MVD/MV replacement/repair | 5 | 6 | 8 |
| New York Heart Association for exercise tolerance |     |
| Class I | 10* | 6 | 5 |
| Class II | 3 | 5 | 8 |
| Class III | 0 | 3 | 4 |
| Systolic blood pressure | 147±4 | 141±9 | 135±7 |
| Diastolic blood pressure | 84±2 | 81±3 | 86±2 |
| Echocardiography | | | |
| Left atrial diameter (parasternal) | 42±3 | 42±4 | 48±4 |
| Left ventricular end-diastolic diameter (mm) | 50±4 | 52±3 | 52±3 |
| Left ventricular end-systolic diameter (mm) | 34±4 | 38±3 | 34±5 |
| Medication (n) | | | |
| Ace-inhibitors | 4 | 5 | 7 |
| Digitalis | 0 | 1 | 7* |
| Verapamil | 6 | 3 | 7 |
| Beta-blocker | 4 | 3 | 4 |

Values are presented as mean value±SEM or number of patients. CABG: Coronary Artery Bypass Grafting; Maze: atrial arrhythmia surgery; MVD: mitral valve disease. *p<0.05.
The persistent \((n=17)\) and paroxysmal \((n=14)\) group contained patients with lone AF or AF with underlying mitral valve disease (MVD). All patients were euthyroid and had normal left ventricular function. Coumarin therapy was interrupted 3 days before surgery and class I and III antiarrhythmic drugs were discontinued for at least 5 half-times. Right and/or left atrial appendages (RAAs and LAAs, respectively) were obtained from all patients, except for the control patients undergoing CABG from whom only the RAA was gathered prior to cardiopulmonary bypass (Table 1). After excision, the atrial appendages were immediately snap-frozen in liquid nitrogen and stored at \(-85^\circ C\). The Institutional

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**Fig. 1.** The effect of induction of Hsp levels on pacing-induced myolysis. (A) Western blots show that a heat shock (HS) or GGA treatment (GGA) induces the expression of endogenous Hsp27 and Hsp70 in time, but do not change GAPDH levels, compared to non-treated myocytes (control). Increased levels are maintained during pacing. (B) Immunofluorescent staining of myosin (green) in non-paced myocytes (Con), heat-shocked control myocytes (Con HS) and GGA-treated control myocytes (Con GGA) compared to 16 h paced myocytes (Paced), paced HS myocytes and paced GGA-treated myocytes. Paced myocytes reveal disruption of myosin (myolysis), whereas myosin staining remains diffusely distributed in the cytoplasm of paced myocytes treated with either HS or GGA. (C) Quantification of percentage myocytes showing myolysis as a function of time of pacing in control and heat-shocked myocytes (non-paced myocytes ○, paced HS myocytes ●, paced GGA myocytes ▪). (D) Quantification of percentage myolysis in GGA-treated myocytes (non-paced control myocytes ○, non-paced GGA myocytes □, paced myocytes ● and paced GGA myocytes ■). * = significant increase compared to non-paced myocytes \((p<0.01)\); # = significant reduction compared to paced myocytes \((p \leq 0.05)\).
Review Board approved the study and patients gave written informed consent.

2.4. Protein extraction and Western blot analysis

For Western blot analysis, frozen RAAs and LAAs were used for protein isolation as described previously [27]. For the isolation of proteins from HL-1 myocytes, the cells were lysed by the addition of SDS-PAGE sample buffer followed by sonication before separation on 10% PAA-SDS gels (1.10⁵ cells/slot). After transfer to nitrocellulose membranes (Stratagene, The Netherlands), membranes were incubated with primary antibodies against GAPDH (Affinity Reagents, USA), rodent Hsp25 (SPA801), human Hsp27 (SPA800), Hsp40 (SPA400), Hsc70 (SPA815), Hsp70 (SPA810) or Hsp90 (SPA835) (all StressGen Biotechnologies, Victoria, Canada). Horseradish peroxidase-conjugated anti-mouse, anti-rat or anti-rabbit IgG (Santa-Cruz Biotechnology, The Netherlands) was used as secondary antibody. Signals were detected by the ECL-detection method (Amersham, The Netherlands) and quantified by densitometry. The amount of protein chosen was in the linear immunoreactive signal range and expressed relative to GAPDH.

2.5. Immunofluorescent staining, quantification and confocal analysis

After subjecting HL-1 myocytes to tachy-pacing, the cells were fixed for 10 min in 100% methanol (−20 °C), dried and blocked in 5% BSA (20 min room temperature). Antibodies against myosin heavy chain (MF-20, Developmental Studies Hyridoma Bank, Baltimore, MD, USA) or Hsp27 (StressGen Biotechnologies, Vicotria, Canada) were used as primary antibody. Fluorescein labeled isothiocyanate (FITC) anti-mouse and anti-rabbit (Jackson ImmunoResearch, The Netherlands) or N,N′-(dipropyl)-tetramethyl-indocarbocyanine Cy3 anti-mouse (Amersham, The Netherlands) was used as secondary antibody. Nuclei were visualized by 4′,6-diamidino-2-phenylindole (DAPI) staining. Images of FITC, YFP or CY3 and DAPI fluorescence were obtained by using a Leica confocal laser-scanning microscope (Leica TCS SP2).

For the quantification of the amount of myolysis, 5 fields or more were examined with a total amount of 250–500 myocytes, and myosin disruption (characteristic for myolysis [3]) was scored by three independent observers blinded for the experimental groups. An atrial myocyte was defined as myolytic when >10% of the myocyte surface was free from myosin staining [3,28]. Mean scores of the observers were used.

2.6. Definitions

2.6.1. Persistent AF

Continuous presence of AF until the moment of cardiac surgery, i.e. at least two consecutive electrocardiograms of AF more than 1 week apart, without intercurrent sinus rhythm. Persistent AF has a non-spontaneously converting character. Previously, this type of AF was classified as chronic AF [29].

2.6.2. Paroxysmal AF

AF typically occurring in episodes with a duration shorter than 24 h (but longer lasting paroxysms are not unusual) with intermittent sinus rhythm. Paroxysmal AF either converts spontaneously or is terminated by intravenously administered antiarrhythmic drug. It is non-controlled whether paroxysmal AF is present at the moment of cardiac surgery [29].

2.7. Statistical analysis

Results are expressed as mean±SEM. All Western blot procedures were performed in duplicate series and morphological quantifications were performed in duplo series of at least n=6 wells per series. Mean values were used for statistical analysis. ANOVA was used for multiple group comparisons. Correlation was determined using the Spearman correlation test. All p values were two-sided, a p value of <0.05 was considered statistically significant. SPSS version 8.0 was used for all statistical evaluations.

3. Results

3.1. Hsp protect HL-1 myocytes from myolysis

To address whether Hsp can protect from myolysis induced by AF, we applied a tachy-paced cell model for AF which reveals characteristic features of AF [20], including the induction of myolysis (Fig. 1B). To test whether increased Hsp expression is capable of protecting against tachy-pacing-induced myolysis, myocytes were pretreated with a mild non-lethal heat shock (30 min at 43 °C) and tachy-paced from 16 h afterwards. This mild heat shock induced all major heat shock proteins, including Hsp27 (in rodents often referred to as Hsp25) and Hsp70 prior to and during pacing (Fig. 1A). Heat-treated myocytes showed less pacing-induced myolysis than non-pretreated paced myocytes (Figs. 1B, C).

To test whether pharmacological induction of Hsp with the non-toxic drug GGA was able to protect from myolysis, GGA was applied 2 h prior to and during pacing. Pacing in combination with GGA treatment led to significant elevations in Hsp27 and Hsp70 expression (Fig. 1A), which coincided with a significant reduction in pacing-induced myolysis compared to normal paced myocytes (Figs. 1B, D).

3.2. Hsp27 overexpression is sufficient for protection from tachy-pacing-induced myolysis

To conclusively establish whether or not Hsp upregulation directly protects from pacing-induced myolysis and to study which Hsp conveys this protection, HL-1 atrial myocytes were transiently transfected with plasmids either encoding human Hsp27 or human Hsp70. In myocytes transiently overexpressing human Hsp27, as detected by immunofluorescent staining with a human specific Hsp27...
antibody (Fig. 2A, left panel), a significant reduction of pacing-induced myolysis compared to normal paced myocytes was found (Fig. 2B). The protection mediated by overexpression of Hsp27 alone was as efficient as seen after heat-shock or GGA treatment. Furthermore, partial co-localization of Hsp27 with myosin was observed (Fig. 2A), suggesting an interaction between Hsp27 and relevant target proteins. In contrast, myocytes transiently overexpressing human Hsp70-YFP, distinguished from non-transfected myocytes on the basis of the YFP signal (Fig. 2A, right panel), were not protected against pacing-induced myolysis (Fig. 2B). Together, the results demonstrate that a general induction of Hsp expression protects against pacing-induced myolysis and that the sole overexpression of Hsp27, but not Hsp70, is sufficient for this protection.

3.3. Hsp expression correlates with duration of persistent AF and structural changes

In extension to the findings in the tachy-paced cell model for AF, Hsp levels in atrial tissue from patients with AF were determined. Protein isolated from atrial appendages was used for immunological detection of Hsp27, Hsp40, Hsc70, Hsp70 and Hsp90. Changes in protein expression were studied in relation to protein levels of GAPDH, which did not differ between the groups (data not shown). The protein expression of Hsp27 (Fig. 3A) was significantly increased in RAAs and LAAs from patients with paroxysmal AF compared to samples from control patients ($p<0.0001$) and patients with persistent AF ($p<0.0001$). A trend for increased Hsp70 expression ($p=0.05$) was observed in patients with paroxysmal AF compared to control patients (Fig. 3B, Table 1). No significant differences in the expression levels of Hsp40, Hsc70 and Hsp90 (Fig. 3B, Table 1) were found between the groups and no significant differences between RAA and LAA within the groups were observed. Also, no significant differences in Hsp27 levels were observed between tissue from lone AF patients and AF patients with underlying valve disease. Since increased Hsp27 levels were only found in tissue from paroxysmal AF patients, this may reflect that Hsps are merely activated in acute stress conditions and become exhausted during prolonged stress [30,31]. To test this, a correlation was made between the duration of persistent AF and Hsp27 levels. A significant inverse correlation was observed between the duration of persistent AF and Hsp27 expression (Fig. 3C). Patients with a shorter duration of AF revealed higher levels of Hsp27 expression. Also a significant inverse correlation was observed between the duration of the last episode of AF and the Hsp27 levels in patients with paroxysmal AF (Fig. 3D). Paroxysmal AF patients with short period of AF display higher levels of Hsp27 expression. No significant correlation between Hsp27 expression and age, or medication was observed (data not shown).

Previously, we reported on (ultra)structural changes in atrial tissue of a part of this patient population [28]. In brief, in 7 patients with lone persistent AF, a substantial fraction of cells was myolytic (30.0±14.5%), whereas the percentage of myocytes with myolysis in tissue of 6 patients with lone paroxysmal AF was low (6.9±6.1%) and similar to that in control patients (5.5±3.6%). In this sub-group of AF patients,
we found an inverse correlation between the amount of myolysis and Hsp27 expression (Fig. 4A), i.e. high Hsp27 levels in tissue of patients were associated with low amounts of myolysis. Furthermore, confocal microscopy revealed that, like in the tachy-paced cell model for AF, Hsp27 was preferably localized on myofibrils in cardiomyocytes (both of SR and AF patients), whereas Hsp70 showed diffuse cytosolic and extracellular matrix staining (Fig. 4B).

4. Discussion

The present study identifies a protective role for Hsp in AF. Using the HL-1 cell model for AF [20], we provided evidence that upregulation of Hsp by a mild heat shock and pharmacologically by the drug GGA, attenuates myolysis. Furthermore, transfection experiments directly demonstrated that elevated expression of Hsp27 alone was sufficient for this protection. The results were extended to human AF, where a highly significant increase of Hsp27 expression was observed only in atrial appendages of patients with paroxysmal AF. In addition, the expression levels of Hsp27 correlated inversely with the duration of the arrhythmia and with the amount of myolysis in paroxysmal and persistent AF. Finally, like in the tachy-paced cell model for AF, Hsp27 was found to be localized at the myofilaments in human atrial myocytes. Our data imply that Hsp upregulation protects against tachy-pacing-induced myolysis.

4.1. Mechanism of Hsp protection

Several mechanisms may explain how Hsp27 protects myocytes from tachy-pacing-induced myolysis. Pacing directly,
or via increases of intracellular free calcium and calpain activation [20,28,32], results in disruption of myofibril structures [3]. A first possibility is that Hsp27 binds to the myofibril structure, as suggestive from the (co)localization of Hsp27 with myosin in HL-1 myocytes and at myofilaments in human atrial myocytes. Such binding would be in line with previous studies in human and rat heart [14,17] and suggest that by binding to contractile proteins, Hsp27 stabilizes myofibrils during AF similar to the observed Hsp27-mediated protection of cytoskeletal and contractile elements after heat or ischemic stress in myocardium and smooth muscle cells [15,19,33]. Alternatively, binding of Hsp27 to contractile proteins may shield them from cleavage by cystein proteases. Cystein proteases are known to become activated during AF and result in cleavage of myofilamental proteins [20,28,34,35]. Finally, Hsp27 may accelerate the recovery of myofilamental proteins after disruption, similar to its action on accelerating actin-repolymerization [19].

4.2. Hsp27 expression in paroxysmal and persistent AF

In atrial tissue of AF patients, Hsp27 expression was only significantly elevated in atrial tissue from paroxysmal AF patients and not in persistent AF patients. Moreover, in paroxysmal and persistent AF, the variation in Hsp27 levels was large and found to be inversely correlated with duration of AF and with the amount of myolysis. Generally speaking, upregulation of heat shock proteins in response to a stressful event depends both on the intensity and duration of the stress. While it is known that the magnitude of the response is dependent on the degree of stress [36], inevitably, the response gets exhausted in time if the stress continues [37]. Given these features of the heat shock response and the antmyolytic effect of Hsp27 in tachy-paced myocytes, the patient data may be interpreted as follows. For intermittent periods of stress, such as paroxysmal AF in which arrhythmias are separated by non-stressful intervals of normal sinus rhythms, myocytes are capable of increasing Hsp levels, which might prevent myofibril disruption. As a result, patients with paroxysmal AF may be able to overcome AF paroxysms without the induction of structural changes such as myolysis. The observed inverse correlation between Hsp27 levels and duration of persistent AF and paroxysmal AF suggests that the heat shock response gets exhausted in time, leading to a loss of its protective effects, thereby promoting the progression to persistent AF. Indeed, it is shown that the heat shock response gets temporarily activated in acute but not in chronic diseases [30], during cardiac differentiation [38], and it attenuates with age [37].

In our study, patients in sinus rhythm with underlying coronary artery disease or mitral valve disease were used. Previously, it was described that Hsp27 levels in failing hearts were significantly increased compared to normal hearts [39]. This would suggest that the, in the present study, observed increase in Hsp27 levels in patients with paroxysmal AF compared to control patients in sinus rhythm but without underlying heart diseases would be even more significant. Furthermore, the NYHA class I was significantly different in both patients with paroxysmal and persistent AF compared to SR patients, and also more patients with persistent AF used digitalis compared to paroxysmal AF and SR patients. These differences could potentially have influenced the results on myolysis and Hsp levels.

In summary, the results from both the tachy-paced cell model for AF and human AF provide evidence that elevated expression of Hsp27 protects myocytes from tachy-pacing-induced myolysis. The Hsp response that gets temporarily activated in patients with AF, seems to exhaust in time, thereby losing the ability to prevent structural changes like myolysis, thus leading to the progression of AF. Future experiments are therefore warranted to identify the therapeutic usefulness of drugs that boost the Hsp response, such as GGA, in AF.

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Fig. 4. An inverse correlation was found between the extent of myolysis and protein expression levels of Hsp27 (A) in RAAs of patients with paroxysmal AF (○) and persistent AF (●). (B) Typical example of an immunofluorescent staining of Hsp27 (green), Hsp70 (red) and nuclei (DAPI, blue) in atrial tissue of a patient in sinus rhythm (SR) and with paroxysmal AF (PAF). Hsp27 is localized on the myofibrils in cardiomyocytes (arrowhead), whereas Hsp70 is located in the cytosol and extracellular matrix.
References


[8] H1322


