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Oral geranylgeranylacetone treatment increases heat shock protein expression in human atrial tissue

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BACKGROUND Heat shock proteins (HSPs) are important chaperones that regulate the maintenance of healthy protein quality control in the cell. Impairment of HSPs is associated with aging-related neurodegenerative and cardiac diseases. Geranylgeranylacetone (GGA) is a compound well known to increase HSPs through activation of heat shock factor-1 (HSF1). GGA increases HSPs in various tissues, but whether GGA can increase HSP expression in human heart tissue is unknown.

OBJECTIVE The purpose of this study was to test whether oral GGA treatment increases HSP expression in the atrial appendages of patients undergoing cardiac surgery.

METHODS HSPB1, HSPA1, HSPD1, HSPA5, HSF1, and phosphorylated HSF1 levels were measured by western blot analysis in right and left atrial appendages (RAAs and LAAs, respectively) collected from patients undergoing coronary artery bypass grafting (CABG) who were treated with placebo ($n = 13$) or GGA 400 mg/da($n = 13$) 3 days before surgery. Myofilament fractions were isolated from LAAs to determine the levels of HSPB1 and HSPA1 present in these fractions.

RESULTS GGA treatment significantly increased HSPB1 and HSPA1 expression levels in RAA and LAA compared to the placebo group, whereas HSF1, phosphorylated HSF1, HSPD1, and HSPA5 were unchanged. In addition, GGA treatment significantly enhanced HSPB1 levels at the myofilaments compared to placebo.

CONCLUSION Three days of GGA treatment is associated with higher HSPB1 and HSPA1 expression levels in RAA and LAA of patients undergoing CABG surgery and higher HSPB1 levels at the myofilaments. These findings pave the way to study the role of GGA as a protective compound against other cardiac diseases, including postoperative atrial fibrillation.

KEYWORDS Atrial appendage; Coronary artery bypass grafting; Geranylgeranylacetone; Heat shock protein; Proteostasis

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Introduction

Proteostasis, which is the balance in protein synthesis, folding, assembly, trafficking, and clearance by protein degradation systems, is controlled by the protein quality control (PQC) system.¹ The PQC is an exquisitely regulated network in which the most important chaperones, heat shock proteins (HSPs), play a crucial role. In response to changes in the intra- and extracellular environment, cells upregulate HSPs to maintain proteostasis. HSPs guide protein folding

of nascent proteins, stabilize misfolded proteins, prevent protein aggregation, guide protein refolding, and facilitate degradation of unwanted and damaged proteins.¹

HSPs consist of 5 HSP families: HSPA (HSP70), HSPB (small HSPs), HSPC (HSP90), HSPD (HSP60), and DnaJB (HSP40), each with several family members and (specific) cofactors in various cellular localizations, with distinct and overlapping functions.¹ Previous studies revealed that HSPBs are especially abundant in cardiomyocytes and that they safeguard a balanced proteostasis in cardiomyocytes.² HSPBs interact with contractile and microtubule proteins, thereby stabilizing the cardiomyocyte structure and conserving the contractile and electrophysiological function of the cardiomyocytes.^{3–5} Upon cellular stress, such as heat shock, hypoxia, oxidative stress, inflammation, and ischemia, the heat shock response is stimulated by activation of heat shock transcription factors (HSF). HSF1

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is the major regulator of HSP transcription in eukaryotes.⁶ Upon phosphorylation, the activated HSF1 translocates to the nucleus and binds to the heat shock elements (HSEs) in *hsp* genes, thereby resulting in the transcription and expression of HSPs to maintain proteostasis.⁷ However, HSP levels decline with age and long-term stress, leading to a derailment in proteostasis by altered stability of proteins and accumulation of protein damage, unfolding, and breakdown.^{1,8} Therefore, impairment of tissue HSP levels is associated with aging-related misfolded protein diseases, such as Parkinson, Huntington, and Alzheimer disease, as well as cardiac diseases, including atrial fibrillation (AF) and myocardial infarction.¹ It is because of the central role of HSPs in PQC that pharmacologic modulation of HSP levels represents an interesting target for treatment of aging-related diseases.

Geranylgeranylacetone (GGA) is compound well known to induce HSP levels via activation of heat shock transcription factor-1 (HSF1).⁹ Since 1984 this nontoxic acyclic isoprenoid compound has been clinically applied as an anti-ulcer drug in Asian countries.^{10,11} GGA also has been examined in numerous disease models in animals, in which it has been found to induce HSPs in various tissues, including gastric mucosa, intestine, liver, peritoneum, kidney, retina, central nervous system, skeletal muscle, and myocardium.^{9,12–14} Moreover, pharmacologic induction of HSPs by GGA revealed pleiotropic protective effects by attenuating electrical as well as structural changes in experimental models of heart failure, myocardial infarction, desmin-related cardiomyopathy, and AF.^{4,8,15–19} Therefore, GGA may also represent a protective agent in human heart diseases, but whether GGA can increase HSP expression in human heart tissue is unknown. In the current study, we tested whether oral GGA treatment can increase HSF1-mediated HSP expression in atrial appendages of patients undergoing cardiac surgery.

Materials and methods

Study population

Patients scheduled for coronary artery bypass grafting (CABG) surgery received GGA (Selbex; Teprenone, Eisai, Tokyo, Japan) ($n = 13$) or placebo ($n = 13$) for 3 days before and 3 days after on-pump CABG at a dosage of 400 mg/d. Patients selected for this study did not have a history of AF, which was confirmed by Holter monitoring and high-resolution ECG measurement of signal-averaged P-wave duration (cutoff point >150 ms for AF indication). Both patients and physicians were blinded as to treatment. Patients were recruited at the A.N. Bakulev National Medical Research Center of Cardiovascular Surgery (Moscow, Russia). The study was approved by the Institutional Committee on Human Research (MEC Bakulev 12-029/12323). Before study enrollment, each patient was provided an oral and written explanation of the study procedure, and written informed consent was obtained from all patients. The study was performed according to the principles of the Declaration

of Helsinki. Clinical data from the patients were obtained from electronic patient files.

Sampling

Tissue samples of right atrial appendage (RAA) and left atrial appendage (LAA) were collected just before aortic cross-clamping, snap frozen in liquid nitrogen, and stored at -80°C .

Whole protein isolation

Part of the frozen tissue was homogenized on ice with SDS sample buffer (10% SDS, 50% glycerol, 0.33M Tris-HCl pH 6.8, 10% β -mercaptoethanol, 0.05% bromophenol blue, and protease and phosphatase inhibitors) using an Ultra-Turrax (IKA, the Netherlands). The lysates were centrifuged and the supernatant was collected, passed through an insulin syringe, and boiled for 6 minutes.

Myofilament fractionation

Part of the frozen tissue was dissected in F60 buffer (0.447% KCl, 0.204% imidazole, and 0.041% $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) containing protease and phosphatase inhibitors. Supernatant containing cell debris was discarded. A precooled metal homogenizer bead was placed in the sample tubes, and 2 cycles of (1) adding cold F60 buffer including 1% triton, (2) homogenization in the Qiagen TissueLyser II, the Netherlands, for 3 minutes at maximum speed, (3) centrifugation at 14,000g (10 minutes at 4°C), and (d) removal of the supernatant (soluble fraction) were performed. Subsequently the pellet was homogenized in RIPA buffer (R0278; Merck, the Netherlands). Supernatant was collected by obtaining the myofilament fraction, which was mixed with $4\times$ NuPage buffer, the Netherlands, (6.68% Tris-HCl, 6.84% Tris base, 8% lithium dodecyl sulfate, 40% glycerol, 0.060% EDTA, 0.075% Serva Blue G250, 0.025% phenol red) and 10% dithiothreitol.

Western blot analysis

Equal amounts of protein were analyzed by western blot analysis as detailed in the [Supplementary Materials and Methods](#) and [Supplementary Table S1](#).

Statistical analysis

A detailed description of the statistical analysis can be found in the [Supplementary Materials and Methods](#).

Results

Study population

The study population consisted of 26 CABG patients, 13 were treated with placebo and 13 with GGA. All patients received beta-blockers before surgery. All patients had normal resting heart rate between 65 and 70 bpm and normal cardiac output, with normal left ventricular ejection fraction (LVEF) between 59% and 60%. Baseline characteristics of the study population are summarized in [Table 1](#). There was no difference with regard to age (placebo 53 [47–59] vs

Table 1 Clinical characteristics

Treatment	Patient no.	Age (y)	Gender	Multifocal atherosclerosis	LVEF (%)	Anterior–posterior LA size (cm)	NYHA or CCS class	No. of CABG conduits	poAF
Placebo	1	58	Male	Yes	60	4.3	II	3	No
	2	46	Male	No	58	4.3	II	3	No
	3	54	Male	Yes	59	5.5	II	2	Yes*
	4	62	Male	Yes	65	3.9	II	1	No
	5	53	Female	Yes	60	4.1	II	2	No
	6	46	Male	No	58	4.0	II	3	No
	7	48	Male	No	59	3.7	II	2	No
	8	59	Male	Yes	56	4.2	II	2	No
	9	57	Male	No	60	4.9	II	3	No
	10	48	Male	Yes	60	4.4	II	3	No
	11	41	Female	No	63	4.7	II	2	No
	12	59	Male	Yes	60	4.7	II	1	No
	13	50	Male	No	58	4.5	II	2	No
GGA	1	61	Male	Yes	56	4.5	II	3	No
	2	60	Male	Yes	60	5.3	III	2	Yes*
	3	50	Male	No	59	4.2	II	3	No
	4	45	Male	Yes	56	4.0	III	2	No
	5	50	Male	Yes	60	4.2	II	3	No
	6	56	Male	No	60	4.0	II	3	No
	7	53	Male	Yes	62	4.0	II	2	No
	8	42	Male	Yes	56	4.4	III	2	No
	9	55	Male	Yes	57	4.3	III	3	No
	10	51	Male	No	58	4.4	III	3	No
	11	63	Male	Yes	60	4.9	II	3	No
	12	50	Male	Yes	60	4.2	II	3	No
	13	50	Male	Yes	55	4.5	III	2	No

CABG = coronary artery bypass grafting; CCS = Canadian Cardiovascular Society grading scale for angina; GGA = geranylgeranylacetone; LA = left atrium; LVEF = left ventricular ejection fraction; NYHA = New York Heart Association; poAF = postoperative atrial fibrillation.

*Tachy-form of atrial fibrillation with heart rate 180–190 bpm developed on second day after surgery, lasted for 8–12 hours, and was eliminated after amiodarone infusion.

GGA 51 [50–58] years, $P = .797$); gender (placebo 11 [84.6%] male vs GGA 13 [100%] male, $P = .480$); multifocal atherosclerosis (placebo 7 [53.8%] vs GGA 10 [76.9%], $P = .411$); LVEF (placebo $59.7\% \pm 2.29\%$ vs GGA $58.4\% \pm 2.18\%$, $P = .149$); and left atrial (LA) size (placebo 4.40 ± 0.474 cm vs GGA 4.38 ± 0.375 cm, $P = .892$). However, LA enlargement was correlated with increased risk of postoperative atrial fibrillation (poAF) (cutoff value >5.0 cm; $P < .001$). All patients in the placebo group were in New York Heart Association (NYHA) functional class II, whereas in the GGA group 7 patients (53.8%) were in NYHA class II and 6 (46.2%) were in NYHA class III ($P = .015$). In total, 2 patients (1 in the placebo group and 1 in the GGA group) developed poAF. Both patients had enlarged LA (cutoff value >5.0 cm), which significantly correlates with poAF ($P < .001$).

GGA induces HSPB1 and HSPA1 expression in atrial tissue

To study whether GGA increases the expression of HSF1-related HSPs, HSPA1, HSPB1, HSPD1, HSF1, and phosphorylated HSF1 levels were measured in the atrial appendages. Expression of HSPA5, which is not HSF1 related, was determined as a negative control. GGA

significantly induced HSPB1 and HSPA1 levels compared to placebo ($P = .019$ and $P = .021$, respectively) (Figures 1A and 1B). Similar levels for HSPD1, a mitochondrial HSP, and HSPA5 were observed in both the placebo and GGA groups (Figures 1C and 1D). HSF1 and phosphorylated HSF1 levels were measured to test whether the HSP-inducing effect was via HSF1 activation. GGA did not increase protein expression levels of HSF1 and pHSF1 (Figures 1E and 1F). Lower HSPD1 levels correlated with larger LA size ($P < .01$); higher HSF1 levels correlated with larger LA size ($P < .05$); and higher HSPA1 levels correlated with lower LVEF ($P < .05$) (Supplementary Table S2).

GGA enhances HSPB1 levels at the myofilaments

Whereas HSPA1 exerts its protective actions mainly in the cytosol,⁸ HSPB1 (co)localizes with myofilament proteins, including α -actinin, actin, and myosin, to stabilize the myofilaments and/or shield the contractile proteins from cleavage by cysteine proteases, such as calpain.^{8,19,20} Both HSPB1 and HSPA1 levels were higher in RAA and LAA of patients treated with GGA in the RAA and LAA, with the effect more clearly observed for HSPB1 in LAA ($P < .05$) (Figure 2). To investigate whether the GGA-induced increase in HSPB1 and HSPA1 resulted in

Figure 1 Geranylgeranylacetone (GGA) increases heat shock factor-1 (HSF1)-mediated heat shock protein (HSP) expression in atrial appendages of patients undergoing coronary artery bypass grafting surgery. Representative western blots (left 3 lanes placebo; right 3 lanes GGA treated) and quantified western blot results for HSPB1 (A), HSPA1 (B), HSPD1 (C), HSPA5 (D), HSF1 (E), and phosphorylated HSF1 (pHSF1) (F) relative to GAPDH in left atrial appendage (LAA) and right atrial appendage (RAA) of patients treated with placebo or GGA. * $P < .05$ vs placebo. GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

enhanced colocalization at the myofilaments in human heart tissue, myofilament fractions were isolated from LAAs and utilized for western blot analysis. Of interest, GGA significantly enhanced HSPB1 levels ($P = .042$) but not HSPA1 levels ($P = .851$) at the myofilaments compared to placebo (Figure 3).

Discussion

Preclinical studies showed HSF1-mediated HSP induction by GGA treatment and subsequent protection against various cardiac diseases.^{4,8,15–19} In this study we aimed to obtain proof of concept for oral GGA treatment to increase HSP levels in human heart tissue. HSPB1,

HSPA1, HSPD1, HSPA5, HSF1, and phosphorylated HSF1 levels were measured by western blot analysis in RAAs and LAAs collected from patients undergoing CABG surgery who were treated with placebo or GGA (400 mg/d for 3 days). GGA treatment was associated with higher HSPB1 and HSPA1 levels, which are both HSF1 regulated, whereas it did not have an effect on mitochondrial HSPD1 and endoplasmic reticulum HSPA5, the latter is not HSF1 regulated. HSF1 and the activated form of HSF1 (phosphorylated HSF1) were both unchanged. Previous studies showed HSPB1 protected against cardiac diseases by (co)-localization to the myofilaments, thereby safeguarding cardiomyocytes from protein damage and derailment of proteostasis by

Figure 2 GGA enhances HSPB1 more in LAA than RAA. Quantified western blot results for HSPB1 in RAA and LAA of patients treated with placebo and GGA. * $P < .05$ vs placebo. Abbreviations as in Figure 1.

stabilizing the contractile apparatus.^{8,19,20} By utilizing isolated myofilaments, we were able to show that HSPB1 but not HSPA1 levels were enhanced at the myofilaments by GGA treatment, in contrast to the placebo-treated atrial tissue samples. Taken together, these data indicate for the first time that 3 days of GGA treatment (400 mg/d) was associated with higher HSPB1 and HSPA1 expression levels in atrial tissue samples of patients undergoing CABG surgery and increased localization of HSPB1 levels, specifically at the myofilaments. These findings pave the way to study the role of GGA as a protective compound in cardiac diseases, including (po)AF.

Increased HSPA1 and HSPB1 levels in heart tissue by GGA

The observed increase in HSPB1 and HSPA1 expression levels in human atrial tissue is in line with the effect of GGA reported in preclinical studies. In rabbits, oral GGA treatment increased HSPA1 expression in the heart (LA), which was associated with protection against heart failure-induced electrical and structural remodeling.¹⁵ Rats showed enhanced

recovery after ischemia–reperfusion injury when treated with GGA (oral 200 mg/kg). In the GGA-treated rats, HSPA1 expression was increased and thereby preserved the structure of the cardiomyocytes compared to controls.¹⁶ In a myocardial infarction model in rats, oral GGA treatment increased HSPB1 and HSPB8 levels, resulting in preserved mitochondrial and cardiac pump function.¹⁷ Also, oral GGA treatment in HSPB5 R120G transgenic mice, a model for desmin-related cardiomyopathy, improved cardiac function and increased survival via HSPB1 and HSPB8 upregulation.¹⁸ Furthermore, the protective effects of GGA have been extensively studied in various experimental models of AF, including tachypaced HL-1 cardiomyocytes, *Drosophila melanogaster*, and dogs, in which GGA treatment increased HSPB1 levels, protected against structural and electrical remodeling, and conserved contractile function upon tachypacing.^{4,8,19,21} The observed increase in HSPB1 and HSPA1 expression levels in human atrial tissue revealed a more pronounced effect in LAA vs RAA. This is in line with previous studies showing enhanced structural remodeling in LAA compared to RAA and suggests an increased stress response in LAA.²²

Although no dose–response and pharmacokinetic studies were conducted in the dog model for AF, an oral dosage of 4 g/d was used to reveal a protective effect against AF progression.^{4,21} The registered clinical dose for treating gastric ulcers in Japan is GGA 150 mg/d, revealing a maximal concentration of 2.1 $\mu\text{g/mL}$ GGA in serum at 5 hours after administration. In the current study, 3 days of oral treatment with 400 mg GGA was sufficient to increase HSPB1 and HSPA1 levels in human atria. To date, whether this clinical dose will suffice in protecting the heart from developing poAF is unknown. On patient each in both the placebo and GGA-treated groups developed poAF, and these patients revealed LA size >5 cm and average HSPA1 and HSPB1 levels, suggesting atrial dilation was the cause of poAF onset. Future studies in larger patient groups should elucidate whether 3 days of treatment

Figure 3 GGA enhances HSPB1 and not HSPA1 levels at the myofilaments compared to placebo. Quantified western blot results for HSPB1 (A) and HSPA1 (B) relative to α -actinin in LAA of patients treated with placebo (P) and GGA (G). * $P < .05$ vs placebo. Abbreviations as in Figure 1.

with 400 mg GGA is sufficient for protection against development of poAF.

Enhanced HSPB1 levels at the myofilaments upon GGA treatment

Preclinical studies previously showed that the protective action of GGA against contractile dysfunction is critically dependent on the induction of HSPB1, as knockdown of HSPB1 abrogated the protective effect.^{4,9} The current study revealed that GGA treatment was associated with higher HSPB1 levels at the myofilaments in human atrial tissue. This suggests that HSPB1 (re)localizes to the myofilaments, thereby shielding the contractile machinery during stress.^{8,19,20} This is in line with experimental studies in rats, in which HSPB1 binds to cytoskeletal, structural, and contractile proteins to conserve proteostasis and thereby contractile function.^{23–25} We recently observed that GGA-induced HSPB1 expression also restores contractile function, an experimental model for AF recovery,⁹ indicating that, next to prevention of damage, HSPB1 also aids in recovery from contractile protein damage. With this proof of concept for GGA to increase HSPs in human atrial tissue, the next logical step is to investigate in larger study populations whether GGA protects against cardiac diseases, including AF, heart failure, myocardial infarction, and cardiomyopathy. Monitoring HSP levels before and, periodically, after GGA treatment in serum/plasma could facilitate prediction of treatment outcome and possible progression of the disease.

Possible mechanisms of GGA to increase HSP expression

The exact mode of action of HSP induction by GGA is unknown. GGA is thought to act via HSF1 activation. Although we did not observe an increase in (phosphorylated)HSF1 by GGA treatment in the current study, this can be attributed to (phosphorylated)HSF1's early and temporary activation upon stress.⁹ Recently, we observed that a GGA derivative enhances hyperphosphorylation of HSF1, resulting in prolonged binding of HSF1 to the HSE in the promoter regions of *hsp* genes, thereby prolonging *hsp* gene transcription and HSP protein expression.⁹ GGA is expected to work by a similar mechanism.

Next to enhanced phosphorylation of HSF1 by GGA, GGA is described to bind the C-terminus of HSPA1 *in vitro*, thereby dissociating HSPA1 from HSF1, which in nonstressed situations are bound in the cytosol. Free HSF1 can become phosphorylated, resulting in translocation to the nucleus and binding to the HSE within the promoter regions of *hsp* genes.²⁶ As an alternative mechanism, we previously observed that active RhoA abrogates HSF1 transcriptional activity by suppressing HSF1 binding to the HSE of *hsp* genes.²⁷ Activation of RhoA is regulated by naturally occurring prenylation. Prenylation is the addition of C15 (farnesyl) or C20 (geranylgeranyl) isoprenoids to proteins, which act as posttranslational modifiers of proteins and

thereby regulate protein function.²⁸ GGA may compete with endogenous geranyl groups, thereby resulting in inhibition of RhoA activation and consequently enhancing binding of HSF1 to the HSE region.²⁹ As such, GGA may enhance HSF1-mediated HSP induction via inhibition of RhoA activation. However, this mode of action of GGA should be further explored by genetic ablation and competition and enhanced binding experiments to elucidate the mechanism of action.

Potential protective role of HSP induction in AF

Various studies in human atrial tissue revealed that induction of HSPA1 and HSPB1 expression may have cardioprotective effects against AF. Higher HSPA1 expression levels in atrial tissue are related to a lower incidence of poAF.^{30,31} In the current study, we showed that GGA treatment was associated with higher expression levels of HSPA1 and HSPB1 in human atria; therefore, it is of interest to further explore the effect of GGA in preventing poAF.

A possible protective role of HSPB1 in patients was previously indicated by 2 independent studies that reported elevated HSPB1 expression levels in atrial tissue from patients with short duration of AF and low levels of atrial structural changes, including degradation of sarcomeres.^{8,32} In addition, elevated HSPB1 levels were found in patients with paroxysmal AF, whereas patients with persistent AF had lower levels of HSPB1, suggesting that the HSP response is activated upon short duration of AF, whereas it becomes exhausted with longer duration of AF. Lower levels of HSPB1 possibly may indicate derailment of proteostasis, which in turn leads to progression of structural changes and persistence of AF. Thus, securing adequate HSP(B) levels with GGA treatment may limit the induction and progression of AF.

Clinical perspectives

Several experimental studies have already indicated a potential beneficial role of HSP induction via GGA in preventing cardiac diseases, including AF, heart failure, ischemia-reperfusion injury, myocardial infarction, and desmin-related cardiomyopathy.^{4,8,15–19} GGA has been marketed in various Asian countries for decades, is well tolerated, and has a solid safety profile,³³ so GGA is a viable approach to treatment of various cardiac diseases. Future studies including larger study populations may reveal possible associations between HSP(B1) levels and demographic and clinical parameters, including NYHA functional class, atrial pressure, cardiac output, and atrial interstitial fibrosis levels. In the current study, lower HSPB1 levels correlated with larger LA size, higher HSF1 levels correlated with larger LA size, and higher HSPA1 levels correlated with lower LVEF, which can be explained by a larger stress response and thus increased HSF1 and HSPA1 levels in these patients. Because the size of the current patient groups was small,

further studies are warranted to study the relationship between HSPs and LA size and LVEF.

It would be of particular interest to study the effect of GGA in reducing the incidence of poAF because poAF affects 20%–40% of patients undergoing CABG surgery,³⁴ with an even higher prevalence among patients undergoing valve surgery, either alone or in combination with CABG.³⁵ In addition, GGA may enhance successful recovery of heart function after elective electrical cardioversion of patients with paroxysmal and/or persistent AF. If successful, this approach may ultimately result in reduced hospital time, increased quality of life for the patient, and lower health care costs for the society.

Because most patients with cardiac disease reveal cardiomyocyte remodeling at the time of diagnosis, compounds that reverse cardiomyocyte remodeling are clinically relevant. In a recent study, GGA and GGA derivatives both were shown to prevent and also reverse contractile dysfunction in experimental models of AF.⁹ As such, this study substantiates previous findings that GGA treatment has high potential as a novel approach to prevent and reverse cardiac diseases, including AF.

Conclusion

Three days of GGA treatment was associated with higher HSPB1 and HSPA1 expression levels in RAA and LAA of patients undergoing CABG surgery and enhances HSPB1 levels at the myofilaments. These findings pave the way to further study of the protective effects of GGA against cardiac diseases in larger study populations.

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Appendix

Supplementary data

Supplementary data associated with this article can be found in the online version at <https://doi.org/10.1016/j.hrthm.2019.07.010>.

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