Effects of a heat shock protein inducer on the atrial fibrillation substrate caused by acute atrial ischaemia

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1. Introduction

Atrial fibrillation (AF) is a common cardiac arrhythmia associated with increased cardiovascular morbidity and mortality.1–3 Conventional anti-arrhythmic drugs often fail to provide effective sinus-rhythm maintenance in AF patients and may exert negative influences on prognosis, particularly in patients with ischaemic heart disease.4–7 There is therefore great interest in therapeutic approaches based on mechanisms of AF promotion, which seek to target the AF substrate.8

Coronary artery disease is associated with an increased risk of AF,3,9–11 which in turn is associated with an increased risk of death.12–16 Experimental manoeuvres that induce isolated atrial ischaemia (AI) produce conduction slowing in the ischaemic region, which stabilizes atrial reentry that maintains AF.17–19 Heat shock proteins (HSPs) are a group of endogenous cytoprotective factors activated by various pathological conditions. This study addressed the effects of geranylgeranylacetone (GGA), an orally active HSP inducer, on the atrial fibrillation (AF) substrate associated with acute atrial ischaemia (AI).

Methods and results

Four groups of mongrel dogs were studied: (1) a group subjected to AI without GGA (AI-CTL, n = 13 dogs); (2) dogs that underwent AI after GGA pretreatment (120 mg/kg/day; AI-GGA, n = 12); (3) dogs receiving GGA pretreatment without AI (n = 5); (4) control dogs for tissue sampling (n = 5). Isolated right AI was produced by occluding a right atrial (RA) coronary-artery branch. AI reduced ischaemic-zone conduction velocity (CV, from 94 ± 3 to 46 ± 5 cm/s; P < 0.01) and increased maximum local phase delays (P95, from 1.6 ± 0.1 to 4.6 ± 0.6 ms/mm; P < 0.01), conduction heterogeneity index (CHI, from 0.7 ± 0.1 to 2.9 ± 0.5; P < 0.01), and the mean duration of burst pacing-induced AF (DAF, from 44 ± 18 to 890 ± 323 s; P < 0.01) in AI-CTL dogs. GGA pretreatment attenuated ischaemia-induced conduction abnormalities (CV, 77 ± 8 cm/s; P95, 2.1 ± 0.4 ms/mm; CHI, 1.1 ± 0.2; all P < 0.01 vs. AI-CTL) and DAF (328 ± 249 s; P < 0.01) in AI-GGA dogs. GGA treatment alone, without ischaemia, did not alter DAF or conduction indices. AI slightly prolonged atrial refractory period, an effect also prevented by GGA. GGA significantly increased HSP70 protein expression in RA tissues of ischaemic hearts.

Conclusions

GGA prevents ischaemia-induced atrial conduction abnormalities and suppresses ischaemia-related AF. These results suggest that HSP induction might be a useful new anti-AF intervention for patients with coronary artery disease.
orally administered GGA on the atrial electrophysiological and arrhythmic consequences of acute AI in dogs.

2. Methods

2.1 Animal preparation

All animal-handling procedures were approved by the Animal Research Ethics Committee of the Montreal Heart Institute in accordance with the Canadian Council on Animal Care and followed NIH guidelines (NIH Publication No. 85-23, revised 1996). Thirty-five mongrel dogs (18–45 kg) were randomized into four groups to assess the effects of GGA on AF substrates induced by AI: (1) a group subjected to AI in the absence of GGA (AI-CTL, n = 13 dogs); (2) a group subjected to AI in the presence of GGA (120 mg/kg/day) pretreatment (AI-GGA, n = 12), beginning 1 day before electrophysiological study and continued until the morning of the electrophysiological study; (3) a non-ischaemic group (GGA-CTL, n = 5 dogs) receiving GGA therapy at the same dose as ischaemia dogs, followed for the same time with the same measurements; and (4) control (non-ischaemic, non-GGA treated) dogs for cardiac tissue sampling (n = 5 dogs).

Dogs were anesthetized with morphine (2 mg/kg s.c.) and α-chloralose (120 mg/kg i.v., followed by 29.25 mg/kg/h i.v.) and ventilated mechanically. Body temperature was maintained at 37°C with a heating blanket, and a femoral artery and both femoral veins were cannulated for blood pressure monitoring and drug administration. A median sternotomy was performed and the heart was exposed by creating a pericardial cradle. A branch of the right coronary artery (the right intermediate atrial artery) perfusing the right atrial free wall (RAFW) but not perfusing ventricular tissue was doubly ligated to cause isolated AI as previously reported. To delineate the extent of atrial hypoperfusion following coronary-artery ligation, 6% thioflavin-S dissolved in 0.9% NaCl solution was injected into a femoral vein after the conclusion of the experiment. Hypoperfused areas appeared as non-fluorescing areas under fluorescent light and were traced for subsequent quantification. Left atrial or ventricular hypoperfusion were excluded in all dogs by confirming fluorescence in these areas.

2.2 Electrophysiological measurements

Bipolar electrodes insulated except at the tips were hooked into the right and left atrial appendages for recording and stimulation. Five silicon sheets containing 240 bipolar electrodes were attached to the atria as previously described, with the results from the RAAF agar used for phase-delay analysis (see below). Electrophysiological mapping was conducted with the Cardiomap® system (Research Center, Sacré-Coeur Hospital and Biomedical Engineering Institute, École Polytechnique and Université de Montréal).

Atrial effective refractory periods (ERPs) of the right atrial appendage (RAA), RAFW, and right atrial inferior wall (RAIW) were measured at basic cycle lengths (BCLs) of 150, 200, 250, and 300 ms with 10 basic stimuli (51) followed by a premature extrastimulus (S2) with 5 ms decrements. All stimuli were twice-threshold, 2 ms pulses. The longest S1–S2 interval failing to capture the atria was defined as the ERP. ERP measurements were performed in duplicate and in cases of divergence between the two determinations, a third determination was performed, and the average of the three values retained.

To evaluate conduction properties, phase maps were constructed for the right atrial RA electrode array as previously reported. Conduction velocities (CVs) were assessed by measuring the conduction time between adjacent electrodes across the ischaemic and the non-ischaemic zones. Conduction indices based on phase-delay histograms were used to evaluate local conduction abnormalities in the RA as previously described. The local activation time differences between each electrode site and its adjacent neighbours were divided by the respective interelectrode distance and the largest activation time/distance ratio taken to indicate the phase-delay at that site, following an approach developed by Lammers et al. Histograms of all local phase-delays were then constructed at each time point in each experiment. Statistical analysis of the phase-delay histograms provides a median phase delay (P50) reflecting the average CV. The P95 (marking the phase-delay corresponding to the 5% slowest-conducting zones) reflects the values of slowest local conduction. Values of the distribution corresponding to the difference (P5-P95) between the 5% shortest (P5) and the 5% longest (P95) delays indicate the range of local phase delays in each data set, and the value normalized to the median phase-delay (P5-P95) is a conduction heterogeneity index that expresses conduction heterogeneity independently of overall mean CV changes. Results were obtained at BCLs of 150, 200, 250, and 300 ms.

To estimate mean AF duration in each dog, AF was induced by burst pacing (10 Hz, 4 times threshold, 2 ms stimuli, 1–10 s): 10 times for AF duration < 20 min and 5 times for 20–30 min AF. Prolonged AF was defined as AF > 20 min, and AF > 30 min was terminated by QRS-synchronized direct-current electrical cardioversion. A 20 min rest period was then allowed before continuing measurements. If AF > 30 min was induced twice, no further AF induction was performed. Measurements were conducted at baseline (prior to coronary-artery occlusion) and then at 0.5, 3, and 5 h post-occlusion.

2.3 Western blot analyses of HSP expression

RA tissue samples from control, AI-CTL, and Al-GGA hearts were fast-frozen in liquid nitrogen, pulverized, and stored at −80°C for Western blot analysis. To isolate proteins, powdered tissues were homogenized before electrophoresis on 10% PAA-SDS gels. Proteins were then transferred to polyvinyl-difluoride membranes (Stratagene, Amsterdam, The Netherlands), which were incubated with primary antibodies against HSP70 (SPA810) and HSP 27 (SPA801; all from StressGen Biotechnologies, Victoria, Canada). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch Labs, West Grove, PA) was used as the secondary antibody. Signals were detected by the electrochemiluminescence detection method (Amersham, Piscataway, NJ) and quantified with Quantity One software. The amount of protein chosen was in the linear immunoreactive signal range and all values are expressed relative to protein loading by Poinceau stain on the same sample. All ischaemic-region samples and all non-ischaemic region samples were loaded on single gels, along with control-dog samples from corresponding regions, to ensure equal protein loading by Poinceau stain on the same sample. Data are presented as mean ± SEM. Multiple-group comparisons were obtained with a two-factor mixed-design ANOVA with repeated measures on one factor for ERP at varying cycle lengths and conduction heterogeneity data, and a one-way ANOVA with repeated measures for time-series data. All data satisfied criteria for normality of distribution except for AF duration, for which data were analysed after normalization by log-transformation. Bonferroni-corrected t-tests were used to evaluate individual mean differences when ANOVA revealed significant group effects. Event prevalences were compared with Fisher’s exact test. A two-tailed P < 0.05 was considered statistically significant.

3. Results

3.1 Effects of GGA on the AI-related AF substrate

The hypoperfused zone size was comparable for AI-CTL and AI-GGA dogs (5.1 ± 0.4 and 5.0 ± 0.5 cm², respectively).
Al substantially and progressively increased mean AF duration in Al-CTL dogs for the full follow-up period of 5 h after coronary-artery ligation (Figure 1). GGA prevented the development of AF promotion following the induction of acute Al, with significantly shorter-lasting AF in Al-GGA than in Al-CTL dogs. Al provoked prolonged AF in six of eight (75%) of Al-CTL dogs, but in only one of seven (14%; \( P < 0.05 \) vs. Al-CTL) dogs that received GGA prior to ischemia. Dogs treated with GGA but not subjected to ischemia (GGA-CTL) had AF of only short duration that did not change over the 5 h observation period. No instances of prolonged AF could be induced in GGA-CTL dogs.

AI prolonged ERPs in the central ischaemic zone at 3 and 5 h of ischemia (RAFW, Figure 2, upper middle panel), but not in non-ischaemic tissue (RAA, upper left panel) or in the periphery of the ischaemic zone (RAIW, upper right panel). GGA prevented ischemia-induced ERP prolongation in Al-GGA dogs (center panel), but did not affect ERPs in the RAFW in the absence of Al (lower panel). The ERP did not change over time in GGA-CTL dogs.

Figure 3 illustrates representative RA activation maps of one preparation subjected to ischemia in the absence (upper row) and another preparation in the presence (lower row) of GGA at BCL of 300 ms. The ischaemic zone is delineated by shaded areas. Al caused obvious conduction delay as indicated by isochrone crowding within the ischaemic zone, and changed the pattern of RA conduction in the area of the ischaemic zone as shown by the dashed line. In the presence of GGA, ischaemia-induced conduction changes were greatly attenuated.

Mean CVs for all dogs across the ischaemic zone border and in non-ischaemic tissue at a BCL of 300 ms are shown in Figure 4A and B, respectively. Acute Al progressively slowed conduction into the ischaemic zone in the absence of GGA (white bars), decreasing CV by \( \sim 50\% \) at 5 h (from a mean pre-ischaemia value of 94 \( \pm \) 4 to 46 \( \pm \) 5 cm/s at 5 h ischaemia). In dogs pre-treated with GGA (black bars), ischaemia-related conduction slowing was greatly attenuated (Figure 4A). For example, CV averaged 77 \( \pm \) 8 cm/s in GGA-pretreated dogs, \( \sim 73\% \) greater (\( P < 0.01 \)) than the corresponding value in dogs subjected to ischemia in the absence of GGA, and not significantly altered from the pre-ischaemia baseline in GGA-treated animals. CVs in non-ischaemic tissue were not altered following Al, whether in the absence or presence of GGA, and CV did not change over time in GGA-treated dogs not subjected to ischemia (Figure 4B). The results of phase-delay histogram analyses are illustrated in Figure 4C and D. Both the P95, reflecting the regions of greatest local conduction slowing and the (P5–P95)/P50, reflecting conduction heterogeneity in the RA, were greatly increased by Al. GGA pre-treatment substantially prevented the P95 and P5–95/P50 changes induced by Al.

3.2 Effects of GGA on HSP expression

Western blots of atrial-tissue homogenates from control, Al-CTL, and Al-GGA are shown, along with corresponding mean data, for RA non-ischaemic tissue in Figure 5A and RA ischaemic tissue in Figure 5B. Bands at 70 kDa, corresponding to HSP70, were systematically more intense in GGA-pretreated dogs and were significantly increased by GGA in both ischaemic and non-ischaemic regions. Bands at 27 kDa, corresponding to HSP27, were more variable in intensity. HSP27-expression increased with ischemia, reaching statistical significance in the ischaemic zone, but was not significantly altered by GGA.

4. Discussion

We have found that oral administration of the HSP-inducer GGA prevents the atrial conduction abnormalities and AF promotion caused by acute Al. These observations suggest that orally administered agents that induce HSP expression may protect against some forms of AF in patients with coronary artery disease.

4.1 Potential importance of AF associated with myocardial ischaemia

AF occurs in up to 20% of patients with acute myocardial infarction, and is associated with longer durations of hospitalization and higher mortality rates compared with patients without AF. Besides increased atrial stretch and increased catecholamine output because of haemodynamic deterioration, acute Al is a potential contributor to the pathophysiology of AF in such patients. Postmortem studies have suggested that atrial infarction is relatively common, occurring in up to 17% of cases of myocardial infarction, and acute myocardial infarction is one of the most frequently identified causes of AF. Coronary-artery disease is a major risk factor for AF, with Al being a potential contributor to this epidemiological association. AF is a very common complication of coronary artery bypass graft surgery, and Al is implicated as a significant pathophysiological candidate mechanism. Thus, there is an evidence for a role of Al in AF over a range of important clinical contexts.

4.2 Comparison with previous literature regarding effects of HSP on ischemic substrates

The functional properties of HSPs, also known as stress proteins, have been extensively studied in various organ systems. HSPs are essential for the survival of cells confronted with environmental insults such as hyperthermia, endotoxemia, oxidative injury, hypoxia, and ischaemia,
Figure 2  Effect of ischaemia and geranylgeranylacetone on ERP in the non-ischaemic right atrial appendage (RAA) and in the centre (right atrial free wall, RAFW) and periphery (right atrial inferior wall, RAIW) of the ischaemic zone. *$P < 0.05$ vs. pre-occlusion baseline.

Figure 3  Representative RA epicardial activation maps at pre-ischaemic baseline and after 0.5, 3, and 5 h of atrial ischaemia in an atrial ischaemia without geranylgeranylacetone (AI-CTL) dog (upper panels) and an atrial-ischaemia dog pretreated with geranylgeranylacetone (AI-GGA, lower panels). Hypoperfused zones are indicated by the shaded areas. Isochronal activation times are indicated. The direction of electrical conduction from the stimulating electrode site (asterisk) through the ischaemic zone is shown by the dashed arrow. Results shown here were obtained during RAA stimulation at a cycle length of 300 ms.
Figure 4 Mean ± SEM data for conduction properties at a basic cycle length of 300 ms. Conduction velocity in ischaemic (right atrial free wall, A) and non-ischaemic zones (right atrial appendage, B), phase-delays corresponding to slowest conduction zones (P95, C) and heterogeneity index (P5–95/P50, D) are shown under baseline conditions as well as at varying times following coronary-artery occlusion. AI-CTL, dogs subjected to ischaemia without geranylgeranylacetone; AI-GGA, dogs pretreated with geranylgeranylacetone prior to ischaemia; GGA-CTL, dogs receiving GGA and monitored in the absence of ischaemia; IZ, ischaemic zone; NZ, normal zone. *P < 0.01 vs. pre-occlusion baseline, **P < 0.05, ***P < 0.01 vs. AI-CTL.

Figure 5 Effects of geranylgeranylacetone treatment on heat shock protein (HSP) expression. (A) Results for non-ischaemic RA tissue: Top: A gel showing bands for HSP70 and HSP27 from 5 dogs in each group. Bottom: Mean ± SEM values. (B) Results for ischaemic RA tissue: Same format as in A. *P < 0.05, **P < 0.01. GGA prevents ischaemia-related AF.
which result in proteins having non-native conformations. HSPs provide an organized network for quality control of protein folding, and protect the cell by stabilizing macromolecular structures and reactivating denatured proteins.

Several studies have described HSPs as mediators of myocardial protection in experimental models of ventricular ischaemia. Within the multimember family of HSPs, stress-inducible medium-sized HSP70-family proteins appear to have a particularly important role in protecting myocardial cells. Evidence for myocardial protection with HSPs has prompted several researchers to investigate the therapeutic potential of molecules that increase myocardial HSP expression, and the upregulation of HSPs has been described as a potentially valuable new therapeutic approach for cardiac pathology.

A single oral dose of GGA has been shown to improve the recovery of left ventricular function and to reduce creatine kinase release resulting from 20 and 40 min of ischaemia in isolated rat hearts. No difference was seen when ischaemia was prolonged to 60 min prior to reperfusion. This result differs from ours in that we observed protection for a full 5 h period after ischaemia onset. The discrepancy may be because of the production of global cardiac ischaemia in the isolated rat heart model, compared with limited AI in our study. In the rat heart study, GGA was found to increase the expression of HSP72 but not HSP27, similar to our result. We were unable to identify any previous studies of HSP-induction effects on AI or associated arrhythmias. In the present study, acute AI created a substrate for AF maintenance in association with strong local conduction slowing within ischaemic tissue, as previously reported. GGA suppressed both atrial conduction slowing and AF promotion caused by acute AI, whereas it had no effect on non-ischaemic region parameters. These results are similar to findings previously reported for the anti-arrhythmic agents nadolol and diltiazem, and for the gap-junction conductance enhancer rotigaptide.

### 4.3 Potential significance

As discussed in detail above, there is substantial evidence pointing to ischaemic heart disease as an important pathophysiological contributor to the occurrence of AF in a variety of clinical settings. We have recently shown that HSP induction with GGA protects against development of the AF substrate associated with atrial tachycardia remodelling. In the present study, we found that GGA prevents the negative electrophysiological and arrhythmia-promoting effects of acute AI. We have also obtained preliminary data that suggest a beneficial effect of GGA on atrial fibrosis and AF promotion resulting from congestive heart failure. Thus, HSP induction by GGA may have the ability to prevent the development of a variety of clinically relevant AF-promoting substrates. HSP induction, either by GGA or by other compounds presently in development, may therefore be an interesting new approach for ‘upstream therapy’ of AF, a strategy that holds the promise of AF prevention without the ventricular proarrhythmic risks of conventional anti-arrhythmic agents.

The molecular determinants of AF occurrence are still very poorly understood. There is evidence that HSPs may be endogenous cardioprotective molecules that are activated in a variety of cardiac stress situations. Several studies suggest that endogenous changes in HSP expression may have an important impact on the occurrence and persistence of clinical AF. The results of the present study add to the growing body of evidence that HSPs represent endogenous cardioprotective molecules that can determine the presentation of such cardiac conditions as AF. Our work also points out the need for further studies of the regulation and manipulation of HSPs in AF.

### 4.4 Potential limitations

In this study, our observations were restricted to the first 5 h after the onset of acute AI. The electrophysiological and arrhythmic consequences of chronic AI and/or a prior atrial infarction may respond differently to GGA treatment.

We sought to assess the effects of GGA on atrial electrophysiological abnormalities and arrhythmias resulting from AI per se. Accordingly, we occluded a relatively small coronary artery branch that perfuses only the RA. In clinical settings, AI or infarction is usually accompanied with similar changes at the ventricular level, which is more complex by virtue of additional potential effects on atrial pressures secondary to ventricular dysfunction, by autonomic nervous system responses and by concomitant drug therapy. Therefore, caution should be exercised in clinical extrapolation. It would be interesting to assess whether GGA therapy can also prevent AF promotion with more severe ischaemia, operating at both atrial and ventricular levels.

In the present work, we found GGA to enhance the expression of only HSP70, whereas in our previous investigation of atrial tachycardia remodelling GGA increased the expression of both HSP70 and HSP27. The discrepancy may be because of differences in the duration of GGA therapy. In the present study, only two doses of GGA were given, one the day before coronary artery occlusion and the other several hours before the intervention, whereas in our study of atrial tachycardia remodelling the drug was started 3 days before tachypacing onset and continued throughout the 7 day tachypacing period. The induction of HSP27 may require a longer period of exposure to oral GGA than the induction of HSP70. We searched the literature but were unable to obtain information about the relative time course for the induction of different HSP isoforms by GGA. In our previous study, experiments in HL-1 atrial cardiomyocytes suggested that HSP27 is required for protection against tachycardia remodelling and that HSP70 induction is ineffective. In the present study, effective protection occurred with HSP70 up-regulation only. However, the present results are consistent with a number of previous studies that showed protection against ventricular ischaemia with highly selective HSP70 expression enhancement. HSP70 promotes ATP-dependent K⁺-channel (KATP-channel) function in a newborn pig brain injury model, whereas HSP27 impairs K-ATP channel function, suggesting modulation of K-ATP channels as a potential mediator of the differential cardioprotective effect of HSP70 in ischaemia. It is possible that the specific pathophysiology of atrial tachycardia remodelling necessitates HSP27 induction for prevention.

Dose selection was a challenge because no previous data existed for cardiac HSP induction by oral GGA dosing in the dog. The dose used for cardioprotection in rat studies is...
200 mg/kg.31,54 We chose a dose of 120 mg/kg based on pilot experiments in the dog that showed this to be a well-tolerated dose that effectively induces cardiac HSP expression.32

GGA enhances the HSP response to various stressors and has a very high safety margin, to oral doses of 15 000 mg/kg,55 over 100 times the dose we used. GGA is widely used to probe selectively for HSP induction effects and we were unable to identify reports of clear actions independent of HSP induction. Nevertheless, a possible contribution of non-specific actions cannot be totally excluded, as for any pharmacological compound.

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