Induction of Heat Shock Response Protects the Heart Against Atrial Fibrillation

Bianca J.J.M. Brundel,* Akiko Shirotshita-Takeshita,* XiaoYan Qi, Yung-Hsin Yeh, Denis Chartier, Isabelle C. van Gelder, Robert H. Henning, Harm H. Kampina, Stanley Nattel

Abstract—There is evidence suggesting that heat shock proteins (HSPs) may protect against clinical atrial fibrillation (AF). We evaluated the effect of HSP induction in an in vitro atrial cell line (HL-1) model of tachycardia remodeling and in tachypaced isolated canine atrial cardiomyocytes. We also evaluated the effect of HSP induction on in vivo AF promotion by atrial tachycardia–induced remodeling in dogs. Tachypacing (3 Hz) significantly and progressively reduced ~Ca~ in transients and cell shortening of HL-1 myocytes over 4 hours. These reductions were prevented by HSP-inducing pretreatments: mild heat shock, geranylgeranylacetone (GGA), and transfection with human HSP27 or the phosphorylation-mimicking HSP27-DDD. However, treatment with HSP70 or the phosphorylation-deficient mutant HSP27-AAA failed to alter tachycardia-induced ~Ca~ transient and cell-shortening reductions, and downregulation (short interfering RNA) of HSP27 prevented GGA-mediated protection. Tachypacing (3 Hz) for 24 hours in vitro significantly reduced L-type ~Ca~ current and action potential duration in canine atrial cardiomyocytes; these effects were prevented when tachypacing was performed in cells exposed to GGA. In vivo treatment with GGA increased HSP expression and suppressed refractoriness abbreviation and AF promotion in dogs subjected to 1-week atrial tachycardia–induced remodeling. In conclusion, our findings indicate that (1) HSP induction protects against atrial tachycardia–induced remodeling, (2) the protective effect in HL-1 myocytes requires HSP27 induction and phosphorylation, and (3) the orally administered HSP inducer GGA protects against AF in a clinically relevant animal model. These findings advance our understanding of the biochemical determinants of AF and suggest the possibility that HSP induction may be an interesting novel approach to preventing clinical AF. (Circ Res. 2006;99:1394-1402.)

Key Words: atrial fibrillation  ■  heat shock protein  ■  remodeling

The most common sustained clinical tachyarrhythmia, atrial fibrillation (AF), is characterized in part by its self-perpetuating nature.1 AF self-perpetuation is caused by complex changes in cardiomyocyte electrical and contractile function resulting from atrial activation-rate increases.3 AF-treatment approaches that focus on cardiac electrical properties have limited effectiveness and significant potential complications.2 There is, therefore, increased interest in therapeutic approaches that target mechanisms, such as electrical remodeling, that contribute to the AF substrate.1

Induction of the heat-shock response provides cytoprotective effects that may be beneficial for a variety of acute diseases.3 Because such action depends on the timely induction of heat-shock proteins (HSPs), drugs that boost endogenous heat-shock responses may be of particular interest.4,5 Atrial HSPs are increased in clinical AF,6,9 and this response correlates with reduced AF perpetuation.9 Here, we assess the role of HSP induction in preventing the effects of AF-related atrial tachycardia remodeling in an in vitro HL-1 myocyte model system that is appropriate for genetic manipulation (transient transfection) and in tachypaced isolated canine atrial cardiomyocytes. Because HSP induction prevented electrical and contractile remodeling in vitro, we extended our study to a clinically relevant in vivo model to determine whether HSP induction by an orally administered (co)inducer, geranylgeranylacetone (GGA), protects against AF.

Materials and Methods

HL-1 Cell Culture Conditions, Transfections, and Constructs

HL-1 cells10 were obtained from William Claycomb (Louisiana State University, New Orleans) and cultured as previously described.11 Transient transfection was performed with Lipofectamine (Life Technologies). pHSP70-wt encodes human HSP70 and pHSP27-wt encodes human HSP27, both under control of cytomegalovirus

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From the Department of Medicine and Research Center (B.J.J.M.B., A.S.-T., X.Y.Q., Y.-H.Y., D.C., S.N.), Montreal Heart Institute and Université de Montréal, Canada; Departments of Radiation and Stress Cell Biology (B.J.J.M.B., H.H.K.), Cardiology (I.C.v.G.), and Clinical Pharmacology (R.H.H.), University Medical Center, University of Groningen, The Netherlands; and First Cardiovascular Division (Y.-H.Y.), Chang Gung Memorial Hospital, Chang Gung University, Tao-Yuan, Taiwan.

*Both authors contributed equally to this work.

Correspondence to Stanley Nattel, Montreal Heart Institute, 5000 Belanger St E, Montreal, Quebec H1T 1C8, Canada. E-mail stanley.nattel@icm-mhi.org

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promoter (Clontech). For phosphorylation studies, we used the phosphorylation-deficient mutant HSP27-AAA, in which the 3 known phosphorylation sites (Ser15, Ser78, and Ser82) in HSP27-wt are mutated to alanine, or a phosphorylation-mimicking mutant HSP27-DDD, with these serine residues replaced by negatively charged aspartates.13 Empty pSP64 vector was used as a control. Myocytes were cotransfected with CD-8 cDNA and successfully transfected myocytes selected with anti-CD8 Dynabeads (Dynal).

**Pacing and Induction of HSP Expression**

HL-1 myocytes cultured on coverslips showed spontaneous contraction at ~0.5 Hz. The cells were tachypaced in C-Dish100 culture dishes with a C-Pace100 pacer (IonOptix). HL-1 myocytes were stimulated at 3 Hz with square-wave 5-ms pulses. Results in paced cells were compared with nonpaced cells studied in parallel. We required capture efficiency of >90% cells (microscopic examination of cell shortening [CS]) throughout stimulation. HSP expression was induced: (1) by subjecting cells to modest heat shock (43°C for 15 minutes) followed by overnight incubation at 37°C; (2) by incubation with GGA 4 hours before and during pacing; or (3) by transfection of pHSP70-wt, pHSP27-wt, or pHSP27-AA/DDD 24 hours before in vitro study.

**Short Interfering RNA**

The pSUPER-RNAi system13 was used to develop mouse HSP27 short interfering RNA (siRNA) (all from 5′ to 3′): forward, GATCCCC GACCAAGATGCGTGGTT TCTAGA GACCCAGC- CATCTTGTTG CTG (HSP27 siRNAII); reverse, AGCTTAAAAA GCTGCAATTCGA CATCGGATTT TGCTCTCAGG CAATCCAC (HSP27 siRNAIII). HEK293 cells were transfected with mouse HSP27-GFP construct and siRNAII, siRNAIII, or mock siRNA.

**Calcium Transient and CS Measurements**

These measurements were performed as described previously.14,15 The CaT amplitude (ΔR_{CaL}) was the difference between diastolic and systolic values. Mean amplitude for each experimental condition was measured as densities (pA/pF). Junction potentials averaged 15.9 mV and were corrected for APs only. Contaminating effects of I_{CaL} rundown were minimized by beginning all studies 5 minutes after membrane rupture and bracketing protocols by I_{CaL} measurements, with experiments rejected if I_{CaL} varied by >5% over the protocol. Tyrode’s solution contained (in mmol/L) NaCl 126, CaCl2 2, KCl 5.4, MgCl2 0.8, NaH2PO4 0.33, dextrose 10, and HEPES 10, pH 7.4 (NaOH). The pipette solution for AF recording contained (in mmol/L) GTP 0.1, K-aspartate 110, KCl 20, MgCl2 0.1, and TFP-Mg 5, HEPES 10, Na-phosphocreatine 5, and EGTA 0.05, pH 7.4 (KOH). The extracellular solution for I_{CaL} measurement contained (in mmol/L) tetraethylammonium chloride 136, CsCl 5.4, MgCl2 0.8, CaCl2 2, NaH2PO4 0.33, dextrose 10, and HEPES 10, pH 7.4 (CsOH). Niflumic acid (50 μmol/L) was added to inhibit Ca2+-dependent Cl− current, and 4-aminoypyridine (2 mmol/L) was added to suppress I_{CaL}. The pipette solution for I_{CaL} recorded containing (in mmol/L) CsCl 120, tetraethylammonium chloride 20, MgCl2 1, EGTA 20, ATP-Mg 5, HEPES 10, and GTP (lithium salt) 0.1, pH 7.4 (CsOH).

**In Vivo Model**

Animal-handling procedures followed guidelines of the National Institutes of Health and were approved by the Animal Research Ethics Committee of the Montreal Heart Institute. Fifteen mongrel dogs (28 to 38 kg) were anesthetized with ketamine (5.3 mg/kg IV), diazepam (0.25 mg/kg IV), and halothane (1.5%). Unipolar pacing leads were inserted into the right ventricular apex and right atrial (RA) appendage under fluoroscopic guidance and were connected to pacemakers (Vitatron) in subcutaneous pockets in the neck. Antiro-ventricular block was created by radiofrequency catheter ablation to avoid excessively rapid ventricular responses during atrial tachypacing. The right ventricular demand pacemaker was programmed to 80 bpm. After 24-hour recovery, 7-day atrial tachypacing at 400 bpm was instituted.

Results in 5 atrial tachypaced dogs with GGA treatment were compared with 5 tachypaced dogs without GGA treatment and 5 nonpaced control dogs. GGA was given orally (120 mg/kg per day), starting 3 days before and continuing throughout atrial tachypacing. At the end of the preparation period, dogs were anesthetized with morphine (2 mg/kg SC) and ace-chloralose (120 mg/kg IV, followed by 20.25 mg/kg per hour) and ventilated mechanically. A median sternotomy was performed, and bipolar electrodes were hooked into the RA and LA appendages. Sheaths containing 240 bipolar electrodes were attached to the atria.17,18 The effective refractory period (ERP) was measured with 10 basic stimuli (S1) followed by premature extrastimuli (S2s) with 5-ms decrements. All stimuli were twice-threshold current, 2-ms square-wave pulses. The longest S1 to S2 interval failing to capture defined the ERP. AF was induced with 1- to 10-second burst pacing (10 Hz, 4×threshold current). To estimate mean AF duration in each dog, AF was induced 10 times for AF duration <20 minutes and 5 times for 20- to 30-minute AF. AF >30
minutes was terminated by direct-current electrical cardioversion. A 20-minute rest period was allowed before continuing measurements. If prolonged AF was induced twice, no further AF induction was performed. Atrial ERP's were measured at multiple basic cycle lengths in the RA appendage and at a basic cycle length of 300 ms at 7 additional sites: LA appendage, RA and LA posterior wall, RA and LA inferior wall, RA and LA Bachmann's bundle. AF vulnerability was the percentage of atrial sites at which AF was induced by single extrastimuli. Hearts were preserved in formalin for analysis of cell death (hematoxylin/phloxine/saffron stain) and fibrosis (Masson's Trichrome).

Western Blot Analysis
Frozen RAs and LAs were used for protein isolation.19 For protein isolation from HL-1 myocytes, cells were lysed by adding SDS-PAGE sample buffer followed by sonication before separation on 10% polyacrylamide–sodium dodecyl sulfate gels (105 cells/slot). After transfer to nitrocellulose membranes (Stratagene), membranes were incubated with primary antibodies against GAPDH (Affinity Reagents), rodent HSP27 (SPA801), human HSP27 (SPA800), or HSP70 (SPA810; all from StressGen). Horseradish peroxidase–conjugated anti-mouse or anti-rabbit IgG (Santa Cruz Biotechnology) was used as secondary antibody. Signals were detected by ECL detection (Amersham) and quantified by densitometry.

Data Analysis
Data are presented as mean±SEM. Multiple-group comparisons were obtained by ANOVA with Bonferroni corrected post hoc t tests. All data fulfilled criteria for parametric analysis, except AF duration, which was normalized by logarithmic transformation. A 2-tailed P<0.05 was considered statistically significant.

Results
Effect of HSP Induction on Tachypaced HL-1 Myocytes
We first examined the effect of HSP induction in cultured HL-1 cells, an in vitro model of atrial tachycardia remodeling.9,11,20 HSP expression was increased by preexposure to GGA or heat shock (Figure 1). Cell tachypacing reduced CaT and contractile function, effects prevented by HSP induction (Figure 2A and 2B). To assess the efficacy of individual HSPs, HL-1 myocytes were transiently transfected with human wild-type (wt) HSP70 or HSP27 before pacing. Transfection with HSP27-wt prevented tachycardia-induced CaT (Figure 2A and 2C) and CS (Figure 2A and 2C) depression, whereas HSP70-wt was ineffective (Figure 2C). In addition, we synthesized short hairpin RNAs that act as siRNA-like molecules13 to specifically knock down HSP27 expression in GGA-treated myocytes and compared their response with cells transfected with mock siRNA (containing multiple mismatches to murine HSP27 sequence). Two HSP27 siRNA molecules (directed at different parts of the HSP27 sequence) were used: either prevented GGA-mediated protection against CaT and CS reduction (Figure 3). The results in Figures 2C and 3 indicate that HSP27 is sufficient and required for GGA-induced protection. Recent studies in smooth muscle cells demonstrated that protective effects of HSP27 on contractile function depend on its phosphorylation status.21,22 Therefore, HL-1 myocytes were transfected with either phosphorylation-deficient HSP27 (HSP-AAA) or a phosphorylation-mimicking mutant (HSP27-DDD).12 Only the phosphorylation-mimicking mutant prevented reductions in CaT and CS (Figure 2C), showing that the protective actions of HSP27 require its phosphorylation.

In Vitro Effect of GGA Treatment on Electrical Remodeling in Dog Atrial Myocytes
Figure 4A shows typical Ical recordings on 200-ms depolarizing pulses from −50 mV to +10 mV. Mean data at all test potentials for each group are provided in Figure 4B. In the absence of GGA, tachypacing reduced Ical amplitude (Figure 4, left panels). For example, Ical density at +10 mV averaged −1.9±0.4 pA/pF in 3-Hz paced (P3) cells (n=13), 40% of the value of −4.8±1.6 pA/pF in 1-Hz paced (P1) cells (n=9, P<0.001). There were no appreciable differences between P1 and nonpaced (P0) cells. GGA prevented tachypacing-induced reductions in Ical, with changes being greatly attenuated at 10 μmol/L and virtually abolished at 100 μmol/L.
APs were recorded at multiple frequencies after 24-hour pacing at 0, 1, or 3 Hz in P0, P1, and P3 cells. Resting membrane potential was not altered by rapid pacing, averaging -71.4±1.5 mV (n=11) in P0 cells compared with -73.8±1.8 mV (n=16) in P1 cells and -73.8±1.1 mV (n=19) in P3 cells (P=NS). APs recorded during 1-Hz stimulation from P1 and P3 atrial cardiomyocytes are illustrated in Figure 5 (left panels). Results were not significantly different in P0 versus P1 cells; therefore, for simplicity, only the P1 and P3 data are shown. Mean AP duration (APD) data at 90% repolarization (APD90) are shown as a function of recording frequency in the right panels. Tachypacing reduced APD and attenuated APD rate dependence, changes characteristic of in vivo atrial tachycardia remodeling.1,16,17 GGA treatment prevented tachypacing-induced APD changes.

To assess possible direct electrophysiological effects of GGA, we recorded I_{CaL} and AP properties before and after drug superfusion. As shown in Figures I and II in the online data supplement, available at http://circres.ahajournals.org, GGA had no statistically significant direct effects at concentrations that prevented tachypacing-induced APD changes.

**In Vivo Effect of HSP Induction**

Having demonstrated that HSP induction in an in vitro atrial-derived cell model protects against tachycardia-induced remodeling and GGA administration in isolated dog atrial myocytes prevents electrical remodeling, we studied in vivo applicability. Tachypacing alone did not affect HSP expression, but GGA treatment significantly increased HSP expression in both RA and LA (Figure 6). There were no significant differences among hemodynamic variables, but GGA-treated dogs were slightly larger than the other groups (Table). Results of electrophysiological studies after 7 days of atrial tachypacing in GGA-treated and nontreated dogs are shown in Figure 7, along with results in nonpaced control dogs. Atrial tachypacing in the absence of GGA produced the changes typical of atrial tachycardia remodeling, reducing atrial ERP and ERP rate adaptation (Figure 7A). The atrial tachypacing–induced ERP decreases were attenuated by GGA therapy. Atrial tachypacing without GGA reduced ERP in a statistically significant fashion at most atrial sites (Figure 7B). Atrial tachypacing–induced ERP decreases were regionally variable, as previously described,23 with the largest changes occurring in the RA inferior wall, posterior wall, and appendage, as well as the LA appendage. GGA significantly attenuated atrial tachypacing effects on ERP in the RA appendage, atria, posterior wall, inferior wall, and Bachmann’s bundle. The mean duration of induced AF was increased by tachycardia remodeling from 30 seconds to 15 minutes (Figure 7C), and atrial vulnerability to AF induction by premature extrastimuli increased from 10% to
50\% (Figure 7D). These AF-promoting changes were suppressed by GGA treatment.

We considered the possibility that the prevention of tachypacing-induced I_{CaL} downregulation and APD abbreviation might come at the expense of impaired cellular viability. Therefore, we compared atrial cell death and fibrous tissue content in atrial tissue samples taken after euthanasia of control, atrial tachypacing nontreated, and atrial tachypacing GGA-treated dogs. The results (supplemental Figure III) show no negative impact of GGA therapy. We also analyzed cell-death rate in 24 hours in vitro tachypaced cardiomyocytes. Tachypacing in the absence of GGA reduced cell viability, whereas GGA eliminated this effect (supplemental Table I), suggesting that HSP induction has, if anything, favorable effects on tachypaced cardiomyocyte stability.

**Discussion**

It is well known that AF promotes its own maintenance by causing tachycardia-induced remodeling. We evaluated the effect of HSP induction in an in vitro tachypaced atrial cell line (HL-1) model of AF-related tachycardia remodeling and in tachypaced isolated atrial myocytes from dogs, as well as in vivo on AF promotion by atrial tachycardia–induced remodeling in dogs.

HSP induction by heat shock or GGA protected HL-1 myocytes against suppression of cellular Ca\(^{2+}\) release and contractility resulting from tachypacing. Protective effects were also seen on transfection with HSP27 and a phosphorylation-mimicking HSP27 mutant, but not by HSP70 or a nonphosphorylatable HSP27 mutant construct. Knockdown of HSP27 with short-hairpin forming siRNA prevented GGA-mediated protection. These results indicate that HSP induction protects against tachypacing effects on HL-1 cells, that HSP27 (but not HSP70) is sufficient to reproduce this protective effect, that knockdown of HSP27 prevents protection because of GGA-induced HSP induction, and that HSP27 must be in a phosphorylatable form for protection to occur.

To translate our results to more physiologically relevant systems, we developed an isolated atrial cardiomyocyte model and found both that it reproduced in vivo consequences of atrial tachycardia remodeling and that it demonstrated protective effects with GGA. Finally, we found that protective effects with GGA were also manifest in vivo.

**GGA Induces HSP Expression**

GGA is a nontoxic 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.6,7,24 GGA induces HSP expression through activation of the heat shock transcription factor HSF1.24,25 Oral administration of GGA rapidly upregulates HSP expression in response to a variety of stresses, although this effect is weaker under nonstress conditions.26 The protective effect of GGA-induced HSP expression on atrial remodeling that we observed in in vitro and in vivo models of atrial tachycardia–induced AF promotion suggests that HSP induction might have potential value for clinical AF.

**Relationship to Previous Observations Regarding Drug Effects on Atrial Tachycardia–Induced Remodeling**

Pharmacological approaches to prevent atrial remodeling are being studied, with the hope that they might be useful in treating AF. L-type Ca\(^{2+}\) channel blockers, a Na\(^+/H^+\) exchange inhibitor and an angiotensin-converting enzyme inhibitor, are ineffective in preventing remodeling caused by >24 hours of atrial tachycardia.27 Drugs with T-type Ca\(^{2+}\) channel blocking action, such as mibebradil28 and amiodarone,29 prevent atrial tachycardia remodeling, although their precise mechanism of action is unclear. Interventions with antiinflammatory and/or antioxidant actions, such as glu-
corticoids and statins, prevent atrial remodeling and may have some efficacy in clinical AF. Our results suggest that HSP induction is a novel antiremodeling intervention.

HSPs, Cardioprotection, and Arrhythmias

HSPs, also known as “stress proteins,” are induced by a variety of stressors and show significant cardioprotective actions. HSP27 (which in various species has molecular
HSP expression is increased in both experimental AF.34 and clinical8 AF. Higher levels of HSP may contribute to preventing atrial tachycardia remodeling. The qualitative similarity of the in vitro paced canine cardiomyocyte model permitted molecular manipulation that demonstrated the importance of HSP-mediated protection against atrial remodeling14,15 and can be monitored in intact cells, because these parameters are affected by atrial tachycardia remodeling14,15 and can be monitored in intact cells, and should be interpreted in this light.

**Potential Limitations**

We studied CaTs and CS as indices of remodeling in HL-1 cells, because these parameters are affected by atrial tachycardia remodeling14,15 and can be monitored in intact cells, avoiding the effects of dialysis with tight-seal patch clamp on cellular function. CaT downregulation, APD abbreviation and loss of APD rate adaptation) make the in vitro model potentially interesting for further studies of the pathophysiology of atrial remodeling.

**Novelty and Potential Significance**

HSPs have been shown to be cardioprotective in a variety of paradigms.32 Our study is the first to show that HSP induction protects against AF in an in vivo model. HSPs have potentially significant antioxidant properties,32 and there is evidence that oxidant stress contributes to the pathophysiology of AF40–42 and that compounds with antioxidant properties protect against atrial remodeling.17,18 Thus, prevention of oxidant stress–induced injury is another potential contributor to HSP-mediated protection against atrial remodeling and associated AF promotion.

**General Properties at Open-Chest Study**

<table>
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<th>Property</th>
<th>NP</th>
<th>ATP</th>
<th>ATP + GGA</th>
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<tr>
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<td>30±1</td>
<td>36±1*</td>
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<td>124±16</td>
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<td>LVEDP, mm Hg</td>
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<tr>
<td>LAP, mm Hg</td>
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<td>6±2</td>
<td>5±1</td>
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</tbody>
</table>

NP indicates nonpaced control; ATP, atrial tachypacing only; ATP + GGA, atrial tachypacing with GGA treatment; BP, blood pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end diastolic pressure; LAP, LA pressure. *P<0.05 vs atrial tachypacing only.
rectifier K+ currents) that participate in atrial tachycardia–induced AF promotion. However, the extensive additional experiments needed to address these issues go beyond the context of the present study.

GGA-treated dogs were slightly larger than the other groups (Table). This difference should, if anything, have favored AF maintenance in atrial tachypacing GGA-treated dogs and should have decreased our chances of showing GGA-induced protection.

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Nathalie L’Heureux, Chantal Maltais, and Chantal St-Cyr provided expert technical assistance; France Thériault helped greatly with manuscript preparation and submission; Jurre Hageman helped to prepare the siRNA constructs; and Eisai Pharmaceuticals, Japan, generously provided GGA.

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Disclosures
None.

References


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Online Table 1

Percentage of live versus dead canine atrial cardiomyocytes after 24 hours of in vitro pacing at the frequencies shown

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<th>Live cells</th>
<th>Dead cells</th>
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*P<0.05 vs P 0 and P 1 Hz cells.
Online Figure Legends

Online Figure 1. Direct effects of GGA (100 µmol/L, left; 1 mmol/L, right) on $I_{CaL}$ in canine atrial cardiomyocytes. Top: Typical recordings before and after GGA in individual cells; Bottom: mean data. Neither concentration significantly reduced $I_{CaL}$.

Online Figure 2. Direct effects of GGA on AP properties at a concentration (100 µmol/L) that fully prevented tachypacing-induced APD alterations. Top: Recordings before and after GGA in one cell. Bottom: Mean AP properties in absence and presence of GGA.

Online Figure 3. Analyses of atrial cell-death and fibrous tissue content. Left atrial samples were removed at the end of in vivo studies and immersed in 10%-neutral buffered formalin for >24 hours. Microscopic images of Masson’s trichrome-stained sections at 400× magnification were digitized (Scion Image Software) and analyzed with Sigmascan 4.0 (Jandel Scientific). Connective tissue content was quantified as a percentage of surface area, excluding blood vessels. To analyze cell-death, sections were stained with hematoxylin-phloxin-safran (HPS). Dead (acidophilic) and viable cells were counted in 5-10 transverse-section fields at 400×. A. Typical HPS sections from each group. B. Typical Masson’s Trichrome sections from each group. C. Mean±SEM dead-cell counts. D. Mean±SEM fibrous tissue contents.
Online Figure 1
Online Figure 2

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<th>APA (mV)</th>
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<th>APD&lt;sub&gt;90&lt;/sub&gt; (ms)</th>
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<tr>
<td>GGA 100 µM (n=8)</td>
<td>-77 ± 2</td>
<td>118 ± 4</td>
<td>80 ± 15</td>
<td>144 ± 14</td>
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</table>
Online Figure 3

A

HPS stain

NP  ATP  ATP+GGA

B

Masson’s Trichrome stain

NP  ATP  ATP+GGA

C

Acidophilic cells (%)

D

Fibrous Tissue (%)

NP  ATP  ATP+GGA