

RESEARCH ARTICLE

Norepinephrine-Induced Adrenergic Activation Strikingly Increased the Atrial Fibrillation Duration through β 1- and α 1-Adrenergic Receptor-Mediated Signaling in Mice

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Abstract

Background

Atrial fibrillation (AF) is the most common arrhythmias among old people. It causes serious long-term health problems affecting the quality of life. It has been suggested that the autonomic nervous system is involved in the onset and maintenance of AF in human. However, investigation of its pathogenesis and potential treatment has been hampered by the lack of suitable AF models in experimental animals.

Objectives

Our aim was to establish a long-lasting AF model in mice. We also investigated the role of adrenergic receptor (AR) subtypes, which may be involved in the onset and duration of AF.

Methods and Results

Trans-esophageal atrial burst pacing in mice could induce AF, as previously shown, but with only a short duration (29.0±8.1 sec). We found that adrenergic activation by intraperitoneal norepinephrine (NE) injection strikingly increased the AF duration. It increased the duration to more than 10 minutes, i.e., by more than 20-fold (656.2±104.8 sec; P<0.001). In this model, a prior injection of a specific β 1-AR blocker metoprolol and an α 1-AR blocker prazosin both significantly attenuated NE-induced elongation of AF. To further explore the mechanisms underlying these receptors' effects on AF, we assessed the SR Ca²⁺ leak, a major trigger of AF, and consequent spontaneous SR Ca²⁺ release (SCR) in atrial myocytes. Consistent with the results of our in-vivo experiments, both metoprolol and prazosin

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significantly inhibited the NE-induced SR Ca^{2+} leak and SCR. These findings suggest that both β 1-AR and α 1-AR may play important roles in the development of AF.

Conclusions

We have established a long-lasting AF model in mice induced by adrenergic activation, which will be valuable in future AF study using experimental animals, such as transgenic mice. We also revealed the important role of β 1- and α 1-AR-mediated signaling in the development of AF through in-vivo and in-vitro experiments.

Introduction

Atrial fibrillation (AF) is the most common arrhythmias, especially among elderly people, and causes harmful effects to the patients [1,2]. The lifetime risk of AF for middle-aged people has been estimated to be greater than 20% [3,4], and the prevalence of AF is reported to be increasing in developed countries [5]. In addition to uncomfortable chest symptoms and reduction of cardiac function, AF patients may face a strikingly increased risk of stroke, due to thrombus formation in left atrium. It is thus recommended that such patients should take anti-coagulant medication. However, the adverse complication of the anticoagulant therapy, such as bleeding, is a major clinical problem. All of these issues significantly affect patients' quality of life. There have been many animal and human studies aimed at reducing the risk of AF to minimize its harmful consequences as suggested in clinical guide lines [6]. However, animal studies of AF, in particular, have been hampered by the lack of suitable AF model because the known AF model has only a short AF duration of seconds [7], not of minutes. Thus it is difficult to evaluate the effect of drug in detail with such a short duration.

Numerous studies have demonstrated that activity of the autonomic nervous system is closely involved in the onset and maintenance of AF [8]. However, the molecular mechanism of autonomic activation-induced AF has not yet been fully elucidated, as the autonomic nervous system regulates the function of cardiomyocytes in a highly complex manner. In addition, research has been hindered by the absence of useful animal models for autonomic activation-induced AF. In this study, we have established a long-lasting AF in mice by the use of transesophageal pacing. We will demonstrate that intraperitoneal NE injection strikingly and reliably elongated the duration of atrial burst pacing-induced AF in mice. We have also determined the role of AR subtypes involved in adrenergic activation-induced AF in our model. Because NE activates both α - and β -adrenergic receptors, either or both may play an important role. To further explore the mechanisms by which abovementioned receptors-mediated signaling affect the duration of AF, we assessed the sarcoplasmic reticulum (SR) Ca^{2+} leak, which is known to be a major trigger for AF, and the consequent spontaneous SR Ca^{2+} release (SCR) in mouse atrial myocytes. Our results have indicated that not only β 1-AR but also α 1-AR-mediated signaling are involved in the NE-induced SR Ca^{2+} leak and SCR as well as maintenance of AF.

Materials and Methods

Animals

Male C57BL/6 mice aged 11–12 weeks were purchased from Japan SLC (Shizuoka, Japan). Standard food and water were provided ad libitum to mice. Twelve- to 14-week-old male mice

were used for the experiments performed in this study. All animal experiments were approved by the Animal Care and Use Committee of Yokohama City University School of Medicine.

Induction of atrial fibrillation

Simple and minimally invasive AF models have been established in small animals such as rats and mice. We induced AF using rapid transesophageal atrial pacing according to previously reported methods with some modifications [7]. Briefly, mice were anesthetized by means of isoflurane inhalation (1.5–2.0% for maintenance). A 1.1 French octapolar catheter with eight 0.5-mm circular electrodes and an interelectrode distance of 1 mm (EPR800; Millar Instruments, Houston, TX, USA) was carefully advanced through the esophagus of each mouse. The catheter was placed at the site with the lowest threshold for atrial capture [9]. To ensure the correct position of the pacing catheter, atrial capture with 1:1 atrioventricular conduction was documented prior to the burst pacing period [10]. Transesophageal atrial burst pacing was then conducted for 10 seconds at a stimulation amplitude of 1.5 mA with 10 msec cycle lengths and a pulse width of 3 mA.

Drug treatment

All the reagents used in this study were purchased from Sigma Aldrich (St. Louis, MO, USA) unless described otherwise. For sympathetic activation, norepinephrine (NE) bitartrate dissolved in natural saline (Otsuka Pharmaceutical, Tokyo, Japan) was intraperitoneally injected 10 minutes before the induction of AF. For the selective blockade of adrenergic receptor (AR), mice were intraperitoneally injected with either 2 mg/kg of metoprolol (a β 1-AR-selective antagonist), 1 mg/kg of prazosin (an α 1-AR selective antagonist), or natural saline 45 minutes before NE administration.

Isolation of atrial myocytes

Atrial myocytes were prepared from adult mice as previously described with some modifications [11,12]. Briefly, C57BL/6 mice at 12–14 weeks of age were anesthetized by intraperitoneal injection of pentobarbital (2.3 mg per mouse) with heparin (150 units per mouse). The heart was excised, and the aorta was cannulated and perfused with 2 mL of modified Joklik's minimal essential medium (JMEM) (Life Technologies, Carlsbad, CA, USA) consisting of 113 mM NaCl, 4.7 mM KCl, 0.6 mM KH_2PO_4 , 0.6 mM Na_2HPO_4 , 1.2 mM MgSO_4 , 12 mM NaHCO_3 , 20 mM D-glucose, 10 mM HEPES, 30 mM taurine, 2 mM creatinine, 2 mM carnitine and 5 mM butanedione monoxime (pH 7.4). The atria were cut into several small pieces and incubated in JMEM containing 0.02 mg/mL Liberase TH (Roche, Indianapolis, IN, USA) with occasional agitation for 60 minutes followed by gentle trituration for 5 minutes at 37°C. The same volume of JMEM with 1% (w/v) BSA was added and the suspension was filtered through a 100 μm mesh (BD, Franklin Lakes, NJ, USA). Cells were precipitated by centrifugation for 2 minutes at 40 x g and the pellet was gently resuspended in JMEM with 1% (w/v) BSA. Ca^{2+} reproduction was gradually performed to a concentration of 1.25 mM. Myocytes were precipitated again and resuspended in attaching media consisting of Medium 199 (Life Technologies) with 4% (v/v) FBS and 1% (v/v) penicillin/streptomycin (Wako). The cells were then plated onto laminin-coated glass cover-slips and incubated at 37°C in humidified air with 5% CO_2 for 1 hour. Finally, attaching media was exchanged for maintaining media (Medium 199 containing 1% BSA and 1% penicillin/streptomycin). The prepared myocytes were bathed in maintaining media at 37°C before Ca^{2+} transient was measured.

Measurement of Ca^{2+} transient

The measurement of Ca^{2+} transient was performed according to previously reported protocols with several modifications [11,13]. All experiments were performed at room temperature. Myocytes were loaded with 5 μM fluo-4 AM (Dojindo, Kumamoto, Japan) in normal Tyrode solution (140 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 10 mM glucose and 10 mM HEPES, pH 7.4 adjusted with NaOH) containing 1.8 mM Ca^{2+} for 15 minutes. Cells were washed twice with normal Tyrode solution and transferred to a chamber equipped with platinum electrodes. The chamber was placed on a Ti2000 confocal microscope system (Nikon, Tokyo, Japan) in a dark room. To detect SCRs, the external solution was rapidly switched from normal Tyrode to $0\text{Na}^+/0\text{Ca}^{2+}$ Tyrode (140 mM LiCl, 5 mM KCl, 1 mM MgCl_2 , 10 mM glucose, 1 mM EGTA, and 10 mM HEPES adjusted pH to 7.4 with LiOH). Myocytes were bathed for 30 seconds in $0\text{Na}^+/0\text{Ca}^{2+}$ Tyrode solution, and SCRs were counted during this period. After SCR measurement, diastolic Ca^{2+} leak from the SR was estimated by a quick treatment with tetracaine. The ryanodine receptor (RYR) was rapidly and reversibly blocked by 1 mM tetracaine, causing Ca^{2+} uptake from the cytosol into the SR. The tetracaine-dependent shift of Ca^{2+} from the cytosol to the SR was considered to be proportional to the SR Ca^{2+} leak [14]. Finally, 10 mM caffeine was rapidly applied for the estimation of SR Ca^{2+} content.

For drug-treated samples, myocytes were pre-incubated with 1 μM of AR antagonist (prazosin or metoprolol) and 1 μM of NE for 5 minutes prior to the measurement of Ca^{2+} transient. The protocol described above for the measurement of SCR and Ca^{2+} leak was repeated using normal Tyrode or $0\text{Na}^+/0\text{Ca}^{2+}$ Tyrode solution supplemented with AR antagonist (1 μM) and NE (1 μM).

Statistical analysis

All values were represented as mean \pm SEM. All statistical analyses were performed by Student's *t* test (two-tailed) or one-way ANOVA followed by the Tukey-Kramer post-hoc study for multiple comparisons. P value <0.05 was considered to indicate statistical significance in this study.

Results

Norepinephrine strikingly elongates the duration of AF

AF was inducible by trans-esophageal pacing, as previously reported [10]. AF was defined as an irregular heart rhythm with loss of P-waves lasting at least 2 sec before spontaneous conversion into normal sinus rhythm (Fig 1A and 1B) [15,16]. During pacing-induced arrhythmic events, however, there occurred not only AF, but also intermittent regular atrial activities (Fig 1C and 1D), which is most likely atrial flutter (Afl). We thus measured the time from the end of burst pacing to spontaneous conversion into normal sinus rhythm (NSR), which indeed included both AF and Afl. Further, such duration was not always identical among different pacings. We thus performed three series of burst pacing over a 3-minute interval for each mouse, and the duration of the longest AF was used as index in this study (Fig 2A).

With the above-mentioned method, AF was reliably induced, but with a short duration, as pointed out in previous studies [7,17]. The duration was only a few to tens of seconds (Figs 1 and 2B). With such a short duration, we thought that it will be difficult to convincingly examine the effect of pharmacological stimulation.

Because it has been suggested, in human, that autonomic imbalance may trigger the onset and duration of AF, we investigated the effect of adrenergic activation by intra-peritoneal NE injection. We found that NE administration strikingly and stably elongated the duration of AF

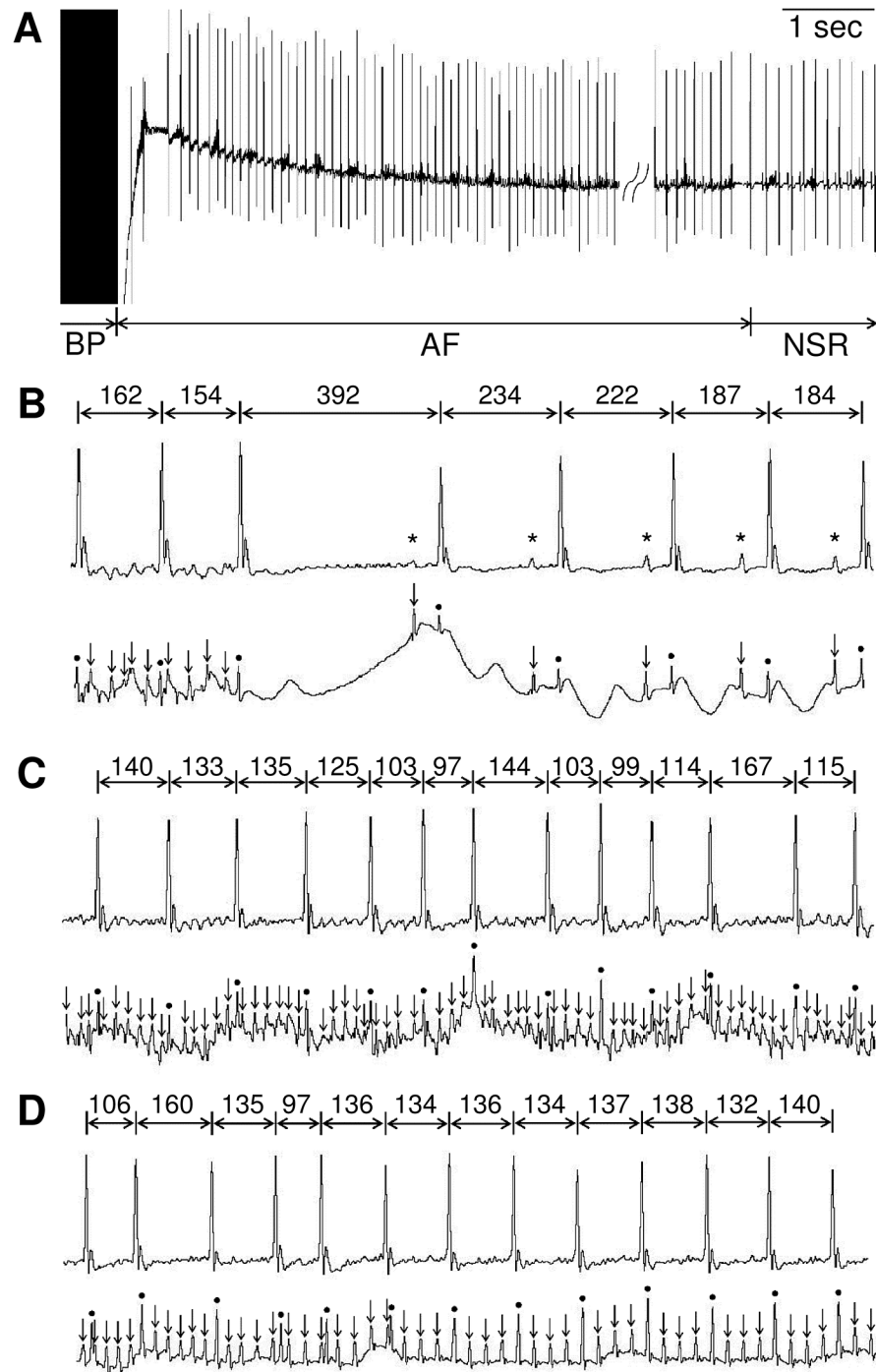
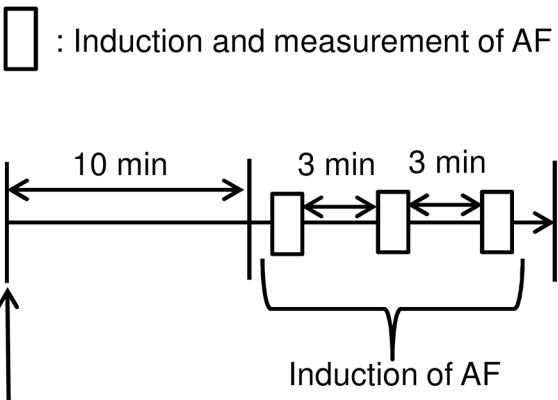


Fig 1. Induction of AF by transesophageal atrial burst pacing in mice. Representative lead II body surface electrocardiogram (ECG) recordings (A). Simultaneous recordings of Lead II body surface ECG (upper) and esophageal ECG (lower) (B-D). (A) AF was induced by transesophageal atrial burst pacing (BP). An AF lasted about 32 seconds before spontaneous conversion into normal sinus rhythm (NSR). (B) Spontaneous conversion from AF to NSR. (C) Representative example of AF episode with disorganized fibrillatory atrial activities and irregular ventricular responses. (D) Conversion from AF to Afl with 4:1 atrioventricular-nodal conduction. Asterisks, arrows and circles indicate P-waves, atrial- and ventricular-electrograms, respectively. All R-R intervals are expressed in milliseconds.

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A  : Induction and measurement of AF duration

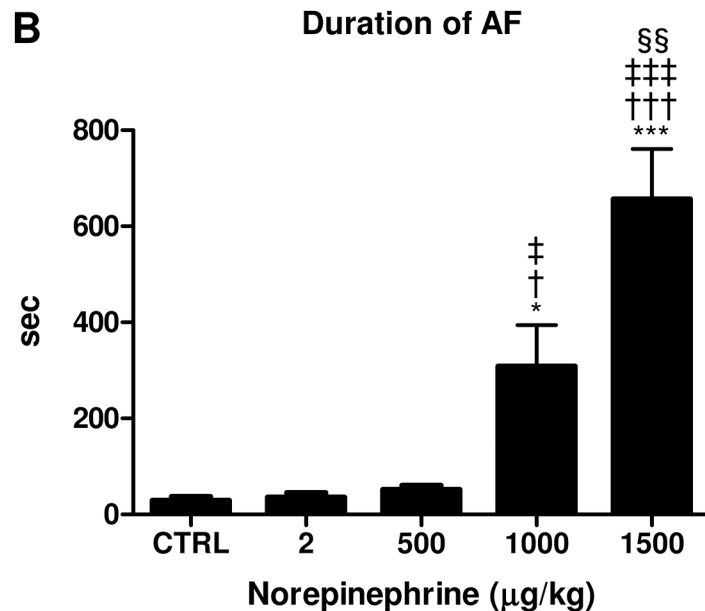
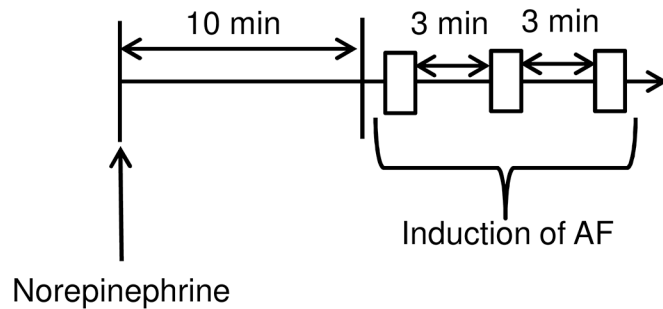


Fig 2. Norepinephrine strikingly elongates the duration of AF. (A) Schematic diagrams of experimental protocol to induce AF after sympathetic activation in mice. Mice were treated with 1.5 mg/kg of norepinephrine (NE) by intraperitoneal injection followed by transesophageal atrial burst pacing to induce AF. The rectangle represents the period from the start of burst pacing to the termination of AF. Note that, for each individual animal, the longest duration among 10 trials was taken to be the duration of AF after NE administration. (B) The duration of AF was strikingly increased after NE administration in a dose-dependent manner. NE was intraperitoneally injected into each mouse at one of several doses as indicated below, and was followed by transesophageal atrial burst pacing (n = 6–8, *P<0.05 vs CTRL, †P<0.05 vs 2 µg/kg, ‡P<0.05 vs 500 µg/kg) (n = 6–8, ***P<0.001 vs CTRL, †††P<0.001 vs 2 µg/kg, ‡‡‡P<0.001 vs 500 µg/kg, §§P<0.01 vs 1000 µg/kg).

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in a dose-dependent manner (Fig 2B). The AF duration was less than 30 seconds (29.0±8.1 sec) in the absence of NE, but increased to 35.7±9.4 sec with NE (2 µg/kg), 51.8±8.3 sec (500 µg/kg), 308.3±86.2 sec (1 mg/kg), and 656.2±104.8 sec (1.5 mg/kg). Thus, the duration was increased by more than 20-fold, to more than 10 minutes.

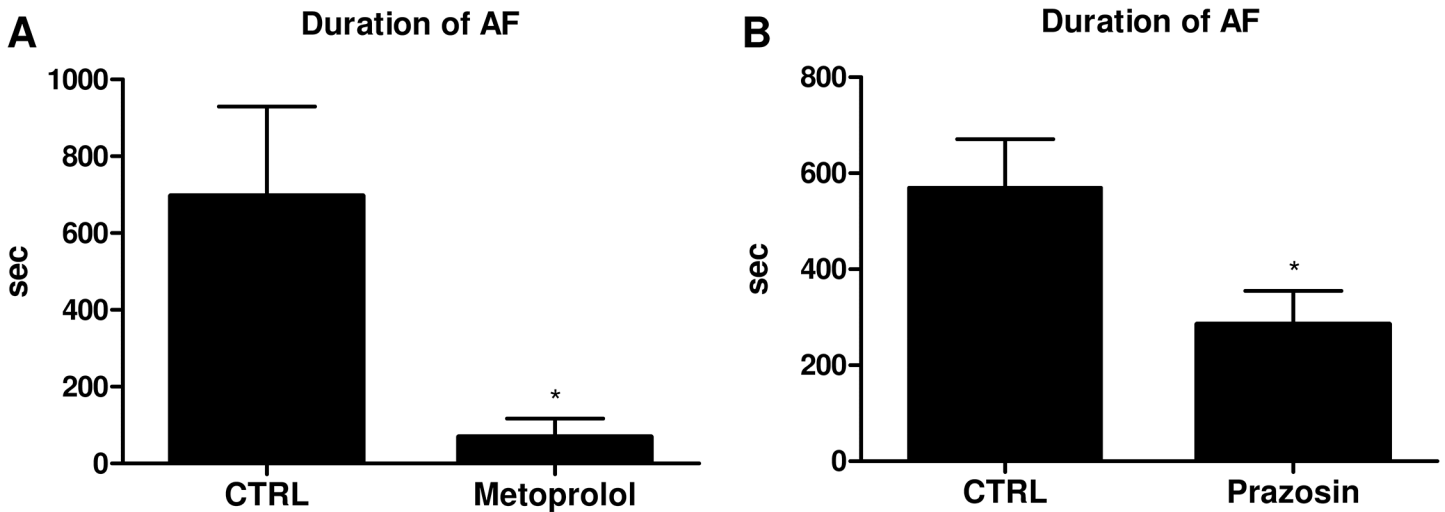


Fig 3. Norepinephrine elongates AF duration through β 1- and α 1-adrenergic receptor-mediated signaling. (A) 2 mg/kg of metoprolol, (B) 1 mg/kg of prazosin or natural saline (CTRL) was intraperitoneally injected into conscious mice 45 min before the administration of NE (1.5 mg/kg). Both metoprolol and prazosin treatment significantly shortened the NE-elongated AF. (n = 10–13, *P<0.05, #P<0.1 vs CTRL) (n = 10–15, *P<0.05).

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Norepinephrine elongates AF duration through β 1- and α 1- adrenergic receptor-mediated signaling

To determine which types of AR-mediated signaling play important roles in the NE-induced elongation of AF, we examined the effects of prazosin and metoprolol on the duration of AF in our model. The doses of metoprolol and prazosin were determined based on previous reports [18,19]. The heart rate (HR) just before the AF induction by burst pacing was significantly lower in metoprolol treated group compared with the control group (control 490.6±11.0 sec vs metoprolol 421.4±30.4 sec, P<0.05). On the other hand, no significant difference was observed in the HR between prazosin treated group and control group (control 504.4±11.3 sec vs prazosin 481.9±8.7 sec, not significant). The duration of the AF was significantly shortened by both metoprolol (control 696.6±232.8 sec vs metoprolol 69.1±47.6 sec, P<0.05) (Fig 3A) and prazosin (control 569.7±101.1 sec vs prazosin 285.2±69.6 sec, P<0.05) (Fig 3B), indicating that both β 1-AR and α 1-AR signaling pathways play important roles in the NE-induced elongation of AF.

Norepinephrine induces SR Ca²⁺ leak and spontaneous Ca²⁺ releases via β 1- and α 1-AR-mediated signaling in atrial myocytes

To elucidate the mechanism underlying the adrenergic activation-induced elongation of AF, we next examined the effect of NE on the SR Ca²⁺ leak and SCR in isolated atrial myocytes. The magnitude of the diastolic Ca²⁺ leak from SR was expressed as a value relative to the caffeine-releasable SR Ca²⁺ content [20]. The adrenergic activation by NE increased the SR Ca²⁺ leak (~1.4-fold compared to control, P<0.05) and the rate of SCR (~2.1-fold, P<0.01) (Fig 4A and 4B). Consistent with the findings of our in-vivo study (Fig 3), treatment with 1 μ M metoprolol significantly suppressed the NE-induced increase in the SR Ca²⁺ leak (~31% lower than that seen in untreated myocytes, P<0.05) and SCR (~54% lower, P<0.01) in a dose-dependent manner. In addition, prazosin treatment also significantly attenuated the SR Ca²⁺ leak (~30% lower, P<0.05) and SCR (~55% lower, P<0.001) (Fig 5A and 5B). These results suggest that

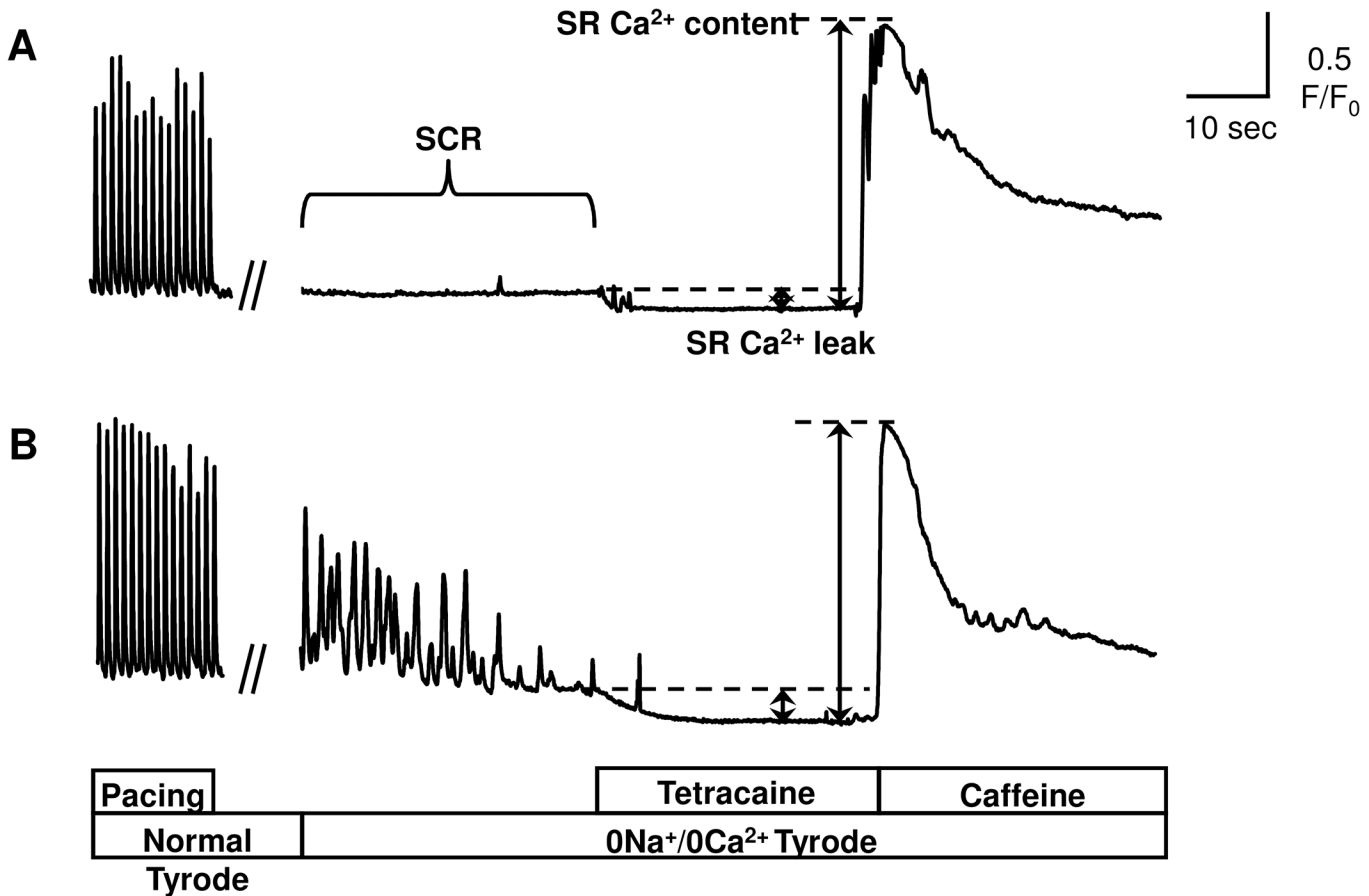


Fig 4. Measurement of Ca²⁺ transient in atrial myocytes. Representative Ca²⁺ traces of atrial myocytes in the absence (A) or presence (B) of 1 μ M NE. Fluo-4 loaded myocytes were electrically paced at 1 Hz for 15 seconds followed by a rapid switch of the extracellular solution from normal Tyrode to 0Na⁺/0Ca²⁺ Tyrode. The spontaneous Ca²⁺ release (SCR) was counted for 30 seconds. The diastolic Ca²⁺ leak from sarcoplasmic reticulum (SR), SR Ca²⁺ leak, was estimated by measuring the downward shift in fluorescence after 1 mM tetracaine treatment. Finally, 10 mM caffeine was administered rapidly to estimate the SR Ca²⁺ content.

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not only β 1-AR but also α 1-AR-mediated signaling are involved in the NE-induced SR Ca²⁺ leak and SCR in atrial myocytes.

Discussion

In this study, we established an adrenergic activation-induced long-lasting AF model in mice. NE injection significantly elongated transesophageal burst pacing-induced AF from around 30 seconds [7] to 10 minutes. By this method we were able to stably induce, what is to our knowledge, the longest AF ever reported in genetically-unmodified mice. The development of AF has been demonstrated by genetic overexpression of several molecules that are involved in β -AR or α -AR mediated signaling [21], such as cAMP-response element modulator [22], G α q [23], or Rho A [24]. However, it has been desired to induce AF in genetically-unmodified normal mice. In this regard, the short duration of AF in genetically-unmodified animal models has been a major problem in investigating the mechanisms of AF. Long-term observation of AF episodes in our model will enable us to examine in greater detail the mechanisms involved in AF maintenance, such as AF-induced atrial remodeling [25]. In addition, it will provide researchers with time to inject potentially useful drugs after the onset of AF, in order to evaluate the

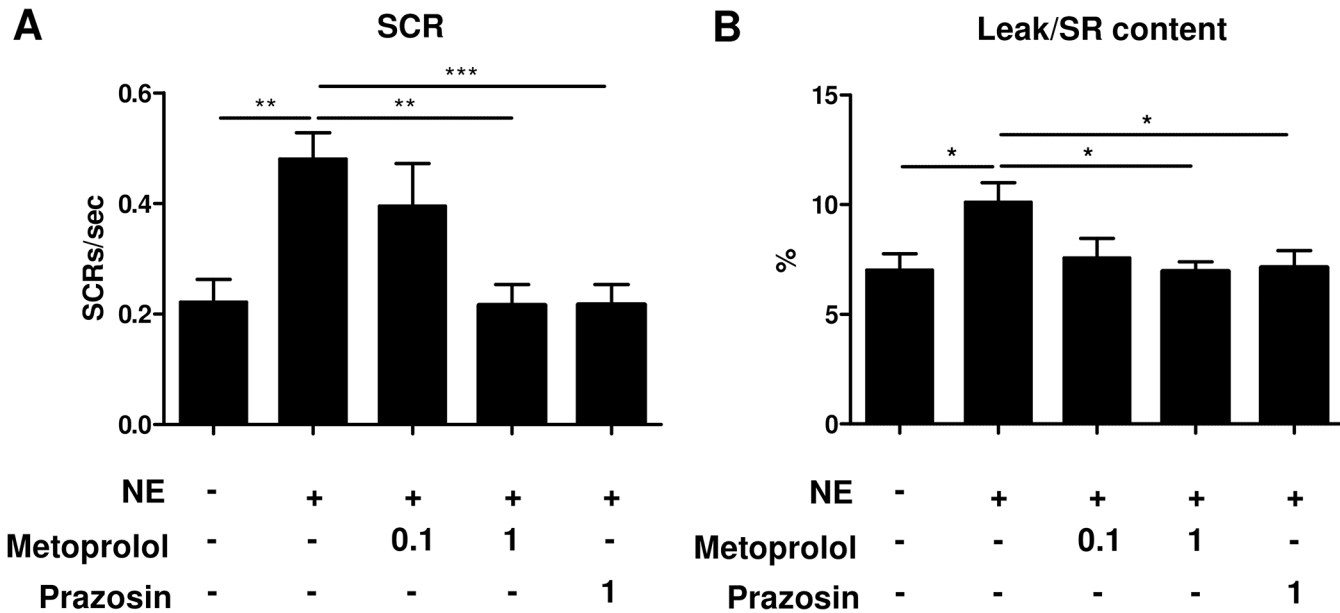


Fig 5. Norepinephrine induces SR Ca²⁺ leak and spontaneous Ca²⁺ releases via β 1- and α 1-AR mediated signaling. Quantification of spontaneous Ca²⁺ release (A) and SR Ca²⁺ leak (B). Atrial myocytes were incubated with metoprolol or prazosin in the absence (-) or presence (+) of 1 μ M NE. Metoprolol and prazosin significantly reversed the NE-enhanced SCR (n = 14–22, **P<0.01, ***P<0.001) and SR Ca²⁺ leak (n = 14–22, *P<0.05). The magnitude of SR Ca²⁺ leak is expressed as a relative value to SR Ca²⁺ content. Values under the graph represent the concentrations of adrenergic receptor antagonists (μ M).

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efficiency of such a drug for defibrillation of AF. Furthermore, this is a minimally invasive model, requiring no surgical procedure e.g. intravascular catheterization [26]. Thus, we can repetitively examine susceptibility to AF over a long observation period. Additionally, the applicability of this model to genetically-modified mice will enable us to obtain more solid evidence of the importance of specific molecules in AF development.

Using the model, we next investigated which AR subtype was more prominently involved in AF maintenance. The sympathetic and parasympathetic nervous systems play pivotal roles in the development of AF [8]. Consistently, activation of the sympathetic and parasympathetic systems is observed before the onset of AF [27]. In addition, autonomic nervous system function is thought to be involved in the arrhythmogenic mechanisms of several risk factors underlying AF including hyperthyroidism, exercise and ischemic heart disease [28–30]. Moreover, atrial tachyarrhythmias can be induced by activating the mediastinal nerves, which causes activation of the sympathetic and parasympathetic systems in the heart [31]. Based on these findings, the inhibition of inappropriate autonomic nervous system activation has been adopted to prevent development of AF. As expected, the usefulness of β 1-AR blockers [32,33] and autonomic denervation [34] have been demonstrated in several human studies. Yet the efficacy of those treatments is limited, at least in part because of our incomplete understanding of the mechanisms underlying autonomic activation-induced atrial arrhythmogenesis. Thus the identification of the signaling pathway that is predominantly involved in catecholamine-induced arrhythmogenesis is an important step toward developing more effective strategies for AF prevention. Consistent with previous reports [35], a β 1-AR-specific blocker was effective at preventing NE-induced elongation of AF in our mouse model. In addition, an α 1-AR-specific blocker also shortened the NE-induced elongation of AF. These findings support the recently proposed concept that both α -AR and β -AR play important roles in the development of AF.

To further investigate the mechanism by which both types of receptor-mediated signaling contribute to maintaining AF, we assessed the SR Ca^{2+} leak and the consequent spontaneous SR Ca^{2+} release in cultured atrial cardiomyocytes. Catecholamine-induced phosphorylation of RYR by protein kinase A or Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) is reported to cause diastolic SR Ca^{2+} leak, leading to delayed afterdepolarization (DAD), which is recognized as a major source of ectopic activity [8,36]. Ectopic activity is accepted as one of the major mechanisms responsible for the onset and maintenance of AF [8]. β -AR activation has been reported to induce SR Ca^{2+} leak and spontaneous SR Ca^{2+} release [37]. Consistently, the β 1-AR-specific blocker metoprolol attenuated the NE-induced SR Ca^{2+} leak and the spontaneous SR Ca^{2+} release. In keeping with the results of our in-vivo study, the α 1-AR-specific blocker prazosin had a similar effect.

Recent reports have documented an important role of α 1-AR-mediated signaling including Gq, phospholipase C, inositol triphosphate receptor (IP3R), protein kinase C, and CaMKII in the regulation of Ca^{2+} transient in cardiomyocytes [38–40]. In addition, endothelin, which also elicits IP3R, Ca^{2+} , and CaMKII-mediated signaling, has been reported to induce elevation of intracellular Ca^{2+} concentration through SR Ca^{2+} release from IP3R, leading to spontaneous Ca^{2+} release from RYR in atrial myocytes [41]. Thus the α 1-AR activation-induced SR Ca^{2+} leak that was observed in the present study may have been caused by a similar mechanism.

On the other hand, it has been reported that α -AR signaling induces the activation of cardiac neurons [42,43]. In addition, α -adrenergic activation can inhibit inwardly rectifying K^+ current, thereby enhancing automaticity [44]. These factors can be also considered among the candidate mechanisms that may be responsible for α 1 AR activation-induced AF.

These findings imply that consideration of α 1-AR-mediated signaling may also be important in the management of AF. Along the same lines, a recent report showed that the α , β -blocker carvedilol is more useful than the β 1 selective blocker metoprolol in preventing AF after cardiac surgery [45].

A possible limitation of this study is that we measured Ca^{2+} transient at room temperature following the method of previous reports [13,14]. The temperature might have affected the response of cardiomyocytes.

In conclusion, we established an adrenergic activation-induced long-lasting AF model in mice. Using the model, we demonstrated the important role of β 1- and α 1-AR-mediated signaling in the maintenance of AF. In addition, we showed that not only β 1-AR but also α 1-AR activation are involved in the SR Ca^{2+} leak in atrial cardiomyocytes. This model and the knowledge we have obtained through its use will be useful in establishing novel therapeutic targets and agents for the treatment of AF.

Author Contributions

Conceived and designed the experiments: TF SO YI. Performed the experiments: KS NH WC HJ YH RP. Analyzed the data: KS TF SO YI. Contributed reagents/materials/analysis tools: KS TF MU UY MS SO YI. Wrote the paper: KS TF YI.

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