Beta1-adrenergic receptor autoantibodies contributes to atrial remodeling by PTEN-mediated repression of cardiomyocyte autophagy and aggravation of cardiomyocyte apoptosis

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Research Article

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Abstract

Purpose β1 adrenergic receptor autoantibodies (β1-AAbs) can promote atrial electrical remodelling and structural remodelling, ultimately leading to the development of atrial fibrillation (AF). Phosphatase and tensin homologue (PTEN) has been confirmed to be involved in AF, but its role in β1-AAb-induced AF is unclear. This study aimed to investigate the involvement of PTEN in the occurrence and development of β1-AAb-induced AF and explore the potential mechanism underlying its effect.

Methods A β1-AAb-induced AF rat model was established by active immunization. The first section was divided into 3 groups: the control group, β1-AAb group, and β1-AAb+bisoprolol group. The second section was divided into 3 groups: the control group, β1-AAb group, and β1-AAb+Oroxin B group. Serum levels of β1-AAb, atrial tissue levels of cyclic monophosphate (cAMP), atrial electrophysiological parameters, cardiac structure and function, mitochondrial structure, autophagy levels, cardiomyocyte apoptosis, and myocardial fibrosis were examined.

Results The results showed that β1-AAb induced electro-anatomical remodelling of the atrium, inhibited autophagy and increased apoptosis in atrial tissue. Blocking β1-AR could partially offset these effects. β1-AAb decreased PTEN expression in the atrium. In addition, activating PTEN with a specific agonist (Oroxin B) could inhibit the AKT/mTOR and NF-κB signalling pathways, increase autophagy, reduce apoptosis, and significantly improve atrial remodelling.

Conclusion β1-AAbs inhibit PTEN protein expression and activate downstream signalling mediators (AKT/mTOR and NF-κB), thereby inhibiting autophagy and increasing apoptosis, which are involved in atrial remodelling. Bisoprolol and PTEN agonists ameliorate these effects.

Introduction

Atrial fibrillation (AF) is the most prevalent persistent arrhythmia and a major contributor to stroke, heart failure, sudden death, and cardiovascular disease. These detrimental events can result in substantial medical expenses and impose significant burdens on public health[1]. The pathophysiological mechanism of AF is complex. In the conceptual framework of AF pathogenesis, the substrate plays a crucial role in AF occurrence. It is currently believed that various pathophysiological changes contribute to the development of atrial substrates[2].

Self-targeting antibodies (including anti-β antibodies, anti-M2 antibodies, and anti-HSP antibodies) are involved in the occurrence and development of AF[3]. A sympathomimetic β1 adrenergic receptor (β1-AR) antibody was produced in active immunization models by using β1-AR second extracellular cyclic peptide, which could shorten the atrial effective refractory period (AERP) and increase AF inducibility[4]. Similarly, our previous studies demonstrated that β1 adrenergic receptor autoantibodies (β1-AAbs) were involved in atrial remodelling[5, 6], but the exact molecular mechanisms are unknown.
Autophagy maintains cellular homeostasis, and apoptosis is a programmed form of cell death regulated by proapoptotic factors and antiapoptotic signals[7]. Myocardial autophagy exerts a counterbalanced effect on cardiomyocyte apoptosis, which is specifically characterized by reduced autophagy-mediated apoptosis[8]. Furthermore, an increase in cardiomyocyte apoptosis promoted cardiac self-repair by inducing myocardial fibrosis[9]. These pathophysiological changes in autophagy, apoptosis, and fibrosis jointly participate in the occurrence and maintenance of AF[10, 11]. However, there have been no relevant reports on whether cross-talk between atrial autophagy and cardiac apoptosis occurs in β1-AAb-induced atrial remodelling, and the underlying mechanism remains unclear.

Phosphatase and tensin homologue (PTEN) is a protein and lipid phosphatase that is highly expressed in the heart[12]. PTEN plays a role in a variety of cardiovascular diseases, such as coronary heart disease and heart failure, by regulating autophagy and apoptosis[13, 14]. However, whether PTEN mediates cardiomyocyte autophagy and apoptosis in β1-AAB-induced AF and the specific mechanisms remain unclear.

We designed in vivo experiments to determine whether PTEN plays an important role in atrial remodelling induced by β1-AAbs, the potential mechanism, and whether pharmacological intervention of PTEN could exert a protective effect similar to the common β1-receptor blocker bisoprolol and prevent β1-AAb-induced AF.

### Materials and Methods

#### Animal model and experimental protocol

All animal experiments were performed with the approval of the Ethics Committee of Xinjiang Medical University (No. IACUC20170420-03). The rats were housed in pathogen-free conditions at a temperature of 20°C and were exposed to 12 hours of light and 12 hours of darkness each day. Before the experiment, the rats were given a regular diet for 1 week to acclimatize to the environment.

The animal experiments were divided into two parts. The first part aimed to observe any changes in atrial autophagy, apoptosis, and PTEN expression in the β1AAAb-induced AF susceptibility model. Male Sprague–Dawley (SD) rats, aged 6 to 8 weeks and weighing 180–220 g, were obtained from the Animal Center of Xinjiang Medical University. A total of thirty SD rats were randomly assigned to three groups (ten rats in each group): the control group, β1-AAb group, and β1-AAb + bisoprolol group (bisoprolol group). The experimental design is shown in Fig. 1A. We used the method described in our previous study to establish an active immune AF model by injecting the second extracellular loop (ECL2) peptide of β1-AR (Biotechnology Inc., Beijing, China)[5, 6]. A solution of 0.32 mg of β1-AR-ECL2 dissolved in 0.16 ml of saline was fully emulsified using an equal volume of complete Freund's adjuvant (Sigma–Aldrich, St. Louis, MO, United States). At 0 weeks, the solution was injected at multiple points on the back of each rat, and 2 weeks later, booster immunizations of emulsified β1-AR-ECL2 solution with incomplete Freund's adjuvant were performed three times at an interval of 2 weeks. The control group received injections of
the same amount of vehicle without β1-AR-ECL2. Bisoprolol, which is a selective β1-receptor blocker, was orally administered to the rats in the bisoprolol group at a dose of 1 mg/kg/d (Merck Inc., Germany) for 2 weeks after successful establishment of the AF model.

The second part aimed to determine the role of PTEN in β1-AAb-induced atrial remodelling and the regulation of cardiomyocyte autophagy and apoptosis. A total of 30 rats were randomly divided into 3 groups (10 rats in each group): the control group, β1AAb group, and β1AAb + Oroxin B group (Oroxin B group). The experimental design is shown in Fig. 5A. Oroxin B is a PTEN agonist that enhances PTEN activity and inhibits Akt phosphorylation[15]. After the active immunization model was established, rats in the Oroxin B group were intraperitoneally injected with Oroxin B (18.56 mg/kg/2 d, purity ≥ 98%, Must Biotechnology, Chengdu, China) for 6 weeks[16].

**Enzyme-linked immunosorbent assay (ELISA)**

Serum and atrial tissue homogenate samples were collected, and the concentrations of β1-AAb and cyclic monophosphate (cAMP) were measured, respectively. Prior to each immunization and at the 8-week mark, 3 ml of venous blood was extracted from the medial canthus vein of rats. The blood was then centrifuged at 3470 rpm for 20 min to obtain serum, which was stored at -80°C until further processing. Serum β1AAb levels were measured using a β1AAb ELISA kit (MBB-20171029, Biosynthesis Biotechnology Inc., Beijing, China) according to the instructions provided by the manufacturer.

cAMP concentrations in atrial tissue homogenates were measured using a rat cAMP ELISA kit (ml1002907-2, Mibio, China). Atrial tissue and PBS were thoroughly ground in a tube at a weight-to-volume ratio of 1:9, and the supernatant was collected. Standard wells contained 50 µl of various concentrations of standards, and sample wells contained 50 µl of the test sample. Subsequently, 100 µl of horseradish peroxidase (HRP)-labelled detection antibody was added and incubated for 1 h at 37°C. After the liquid was discarded, the cells were rinsed thoroughly with 5 times with wash solution. Substrates A and B (50 ml each) were added to each well, 50 µl of termination solution was added after the plate was incubated at 37°C for 15 min in the dark, and the OD value of each well was measured at a wavelength of 450 nm. The sample concentration was calculated based on the OD value.

**Evaluation of ECG parameters**

Electrocardiograms were performed at 0 and 8 weeks in each group. The rats were anaesthetized with isoflurane and placed on a small animal temperature-controlled heating pad. The rats were fixed in a supine position and connected to electrocardiogram leads. Heart electrical signals were recorded in real time using the PowerLab physiological recorder and Bio Amp. Each electrocardiogram was continuously recorded for 30 seconds before the recording was stopped, and there were at least 3 recordings made for quantitative calculations. Electrocardiographic parameters such as average heart rate, P duration, P amplitude, PR interval, and QTC were measured. The data were analysed using LabChart (ADInstruments, Australia).

**Echocardiographic imaging**
Echocardiography was performed at 0, 8, 10 and 14 weeks in each group. The rats were anaesthetized with isoflurane, and the hair on the chest area was shaved. The rats were then placed in the left lateral decubitus position for measurement. Cardiac structure and function were examined using a Doppler ultrasound machine (Philips Inc., Bothell, WA, USA) on a small animal temperature-controlled heating pad (RWD Life Sciences, Shenzhen, China). Measurements were taken for left atrial diameter (LAD), ventricular end-diastolic dimensions (LVEDd), left ventricular end-systolic dimensions (LVESd), left ventricular ejection fraction (LVEF), and left ventricular fractional shortening (LVFS). The sampling time for these measurements was at least 3 cardiac cycles[5].

**MEA assay**

To assess the conduction and conduction heterogeneity of the left atrial (LA)-attached epicardial surface in vivo, MEA measurements were conducted under sinus rhythm. The rats were weighed and anaesthetized with 1% pentobarbital sodium (30 mg/kg) via intraperitoneal injection, and their body temperature was maintained using a small animal temperature-controlled heating pad. The heart was fully exposed, and a flexible MEA chip with 36 electrodes (arranged in a 6 × 6 grid with a distance of 300 µm between electrodes and an electrode diameter of 30 µm) was placed against the surface of the left atrial appendage. Once the monopolar electrogram recorded by the MEA became stable, a conductivity map was generated, and the conduction velocity (CV) was calculated. The nonuniformity index was then determined as the coefficient of variation of CV (P95 − P5/P50)\[17\]. Data were collected at a sampling rate of 10 kHz per channel and analysed using Cardio2D + software (Multi channel Systems, Reulingen, Germany) [5].

**Electrophysiological measurement**

Electrophysiological tests were performed after the MEA test. Body surface ECG leads were placed on the limbs of the animals, and open-chest electrophysiological stimulation was performed using a Lead-7000 equipment (Jinjiang Electronic Science and Technology Inc., Chengdu, China). After full exposure of the chest, a 4-French 10-pole electrode was placed on the surface of the atrium[18]. The AERP was measured using the S1-S2 protocol. In this protocol, 8 basic S1 stimuli were applied with an S1S1 interval of 150 ms and a pulse width of 0.5 ms. These were followed by an advanced S2 stimulus with an initial pacing length of 100 ms, which was decreased by 5 ms until S2 could no longer capture depolarization. AERP is defined as the longest S1-S2 interval that failed to be captured. The AERP measurement was performed three times, and the average value was calculated. AF inducibility was detected using the S1-S1 mode with a 4v 10 s stimulation and an S1S1 interval of 50 ms[6]. AF is characterized by irregular atrial electrograms and irregular ventricular responses lasting more than 1 second[18]. The atrial arrhythmia induction rate was calculated for each rat in which atrial arrhythmia occurred at least once. The duration of AF was determined by counting the cumulative number of AF after 10 burst pacings for each animal. The rate of AF induction was calculated as the ratio of the number of AF occurrences to the total number of AF inductions[6]. At the end of the experiment, rats were euthanized by intraperitoneal injection of 50 mg/kg sodium pentobarbital.

**Histopathological staining**
After each experiment, the rat hearts were collected and rinsed with physiological saline. The atria were then rapidly removed, fixed in 4% paraformaldehyde, and embedded in paraffin. The tissue was cut into 5 mm-thick sections. Atrial morphology was observed using haematoxylin-eosin (HE) staining. Atrial fibrosis was evaluated using Masson's trichrome staining and Sirius red staining. Five sections from each group were randomly selected for quantification, and each field of view was magnified 20 times. Fibrosis was quantified using ImageJ software (version 1.8.0). The collagen volume fraction (CVF) was calculated as the collagen area divided by the total area, multiplied by 100%[18].

**Transmission electron microscopy (TEM)**

Atrial tissue was isolated (3 rats in each group), and the tissue size was approximately 1 mm³. The tissue was fixed using electron microscopy fixative (Servicebio, Wuhan, China) and 1% osmic acid (Ted Pella Inc., CA, USA) in the dark at room temperature. The sample was then dehydrated using a graded ethanol series, embedded with 812 embedding medium, and subjected to ultrathin sectioning (60–80 nm-thick). To prevent light staining, a 2% uranyl acetate saturated alcohol solution was used, and to avoid carbon dioxide staining, a 2.6% lead citrate solution was applied. Finally, a transmission electron microscope (HT7800/HT7700, HITACHI, Tokyo, Japan) was used for observation, and image analysis was performed.

**Detection of apoptosis in the atrium**

Myocardial cell apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assays. Atrial tissues were fixed with 4% paraformaldehyde, dehydrated with different concentrations of sucrose (15%, 20%, 30%), frozen and embedded with Tissue-Tek® OCT Compound (Zorakura, USA). Then, the tissue was cut into 6 mm-thick sections. The TUNEL assay kit (Elabscience, Wuhan, China) was used to detect apoptotic cells in frozen tissue sections according to the manufacturer's instructions. Then, 100 µl of 1× proteinase K working solution was added to the glass slides and incubated at 37°C for 10 min. A mixture of TdT enzyme, labelling solution, and TdT calibration buffer was added and incubated at 37°C for 1.5 h in the dark, and then the samples were incubated with DAPI working solution for 5 min at room temperature in the dark. The nuclei were counterstained, and the samples were mounted with fluorescence quenched mounting solution (Solarbio, Beijing, China). Images were acquired using a Nikon confocal microscope. Three different fields were randomly selected, and TUNEL-positive cells and the total number of cells in each field were counted. The apoptosis rate is expressed as the apoptosis index (number of apoptotic cells/total number of cells).

**Immunohistochemistry**

The paraffin tissue sections were dewaxed and hydrated. They were then blocked with 3% hydrogen peroxide for 10 min. Afterwards, the sections were heated and repaired using Tris-EDTA antigen retrieval solution (Proteintech, Wuhan, China) for 15 min. Goat serum was used for blocking for 20 min. The sections were incubated overnight with a primary antibody against PTEN (diluted 1:100). A universal secondary antibody kit (ZSGB-BIO, Wuhan, China) was used, and the samples were incubated at 37°C for 1 hour. After DAB colour development (Bosterbio, Wuhan, China), haematoxylin was used to counterstain the nuclei. The slides were then differentiated, dehydrated, cleared, and mounted. Each field of view was
magnified 20 times, and the images were quantified using ImageJ software (version 1.8.0). The average optical density (AOD) was used to calculate the areas of immunohistochemically positive cells[19].

**Western blot analysis**

The left atrial tissue was homogenized in RIPA lysis buffer (Solarbio, R0010, Beijing, China). After centrifugation at 12,000 × g for 15 min at 4°C, the supernatant was collected. The protein concentration was determined using a BCA protein assay kit (Solarbio, PC0020, Beijing, China). The protein samples were subjected to electrophoresis to separate proteins of different sizes and electrotransferred, and the membranes were incubated at 4°C with the corresponding primary antibodies (PTEN (1:1000, Abcam, ab267787), Beclin1 (1:2000, Abcam, ab207612), Bax (1:1000, Abcam, ab32503), Bcl-2 (1:1000, Abcam, ab196495), AKT (1:1000, CST, 9272S), p-AKT (1:1000, CST, 4060s), mTOR (1:1000, CST, 2983T), p-mTOR (1:1000, CST, 2971S), p62 (1:1000, MBL, PM045), LC3 (1:1000, MBL, PM036), NF-κB p65 (1:1000, Affinity Biosciences, AF5006), p-NF-κB p65 (1:1000, Affinity Biosciences, AF2006), and GAPDH (1:5000, Proteintech, 60004-1-Ig)) and appropriate secondary antibodies (1:5000; Proteintech, Wuhan, China) for 1.5 h at room temperature. Protein levels were normalized against GAPDH.

**Statistical analysis**

The data were statistically analysed using SPSS 26.0 software. Measurement data are expressed as the mean ± standard deviation, and component ratios are expressed as percentages. Comparisons between 3 groups were made using univariate analysis of variance (ANOVA) with post hoc comparisons by Bonferroni's (chi-square) or Tamhane's T2 correction (chi-square). Pearson's chi-square test was used to analyse the component ratios of the three groups. Repeated-measures data were examined by one-way repeated-measures ANOVA and Bonferroni post hoc comparisons. Two-tailed P values < 0.05 were considered statistically significant, and all graphics were exported from GraphPad Prism 9.

**Results**

**β1-AAbs alter electrocardiogram parameters**

In the first stage, we used ELISA to detect serum β1AAb levels in the three groups from 0–8 weeks. Consistent with our previous results[6], after the first vaccination with β1-AR-ECL2, β1-AAb concentrations in the β1-AAb group and the bisoprolol group were higher than those in the control group from week 2 to week 8 (P < 0.05, Fig. 1B). Moreover, cAMP levels in the atrial tissue of rats in the immunization group and the bisoprolol group were increased (P < 0.05, Fig. 1C), suggesting that β1-AR activation was induced by β1-AAbs. We used the PowerLab multichannel physiological recorder to detect ECG parameters in the 3 groups. Compared with those in the control group, heart rate (HR), P wave duration, P wave amplitude, and PR interval in the β1-AAb group and bisoprolol group were significantly increased (P < 0.05, Fig. 1D-H). Compared with that in the control group, the QTC in the β1-AAb group was significantly shortened (P < 0.05, Fig. 1I).
Blocking β1-AR improves the electro-anatomical substrate induced by β1-AAbs

Similar to our previous findings[6], our results showed that β1-AAbs shortened the AERP and increased the AF induction rate and AF duration in rats with an active immune response, and blocking β1-AR attenuated AF susceptibility induced by β1-AAbs (P < 0.05, Table S1). In addition, the echocardiography results showed that the LAD of rats in the β1-AAb group was significantly higher than that in the control group, and bisoprolol could alleviate atrial structural changes after 2 weeks of treatment (P < 0.05, Table S2). The LVEDD and LVESD in the β1-AAb group were significantly higher than those in the control group, the LVEF and LVFS were significantly lower than those in the control group, and blocking β1-AR significantly restored these changes (P < 0.05, Table S2).

Compared with those in the normal group, tissue slices in the β1-AAb group showed significant atrial morphological disorders, myocardial disarrangement and a decrease in sarcomeres, as shown by H&E staining, and these abnormal myocardial morphologies were partially ameliorated by bisoprolol (Fig. 2A). Quantitative analysis of the CVF by Masson's trichrome staining and Sirius red staining showed significant collagen deposition and extracellular matrix assembly in the myocardium of rats in the β1-AAb group compared with the control group, and this effect was significantly alleviated by bisoprolol treatment (P < 0.05, Fig. 2A-C). In addition, we recorded the conductance of LA. The heatmap shows that the conductance of LA in the control group was uniform, orderly and directed towards the surrounding tissues. In the β1-AAb group, LA conduction was disordered, and wave conduction had abnormal conduction locations, but in the bisoprolol group, these effects were restored (Fig. 2D). The left atrial CV of the β1-AAb group was significantly lower than that of the control group, and this effect was significantly improved in the bisoprolol group (P < 0.05, Fig. 3E). The LA conduction heterogeneity index of the β1-AAb group was higher than that of the control group and was decreased by bisoprolol treatment (P < 0.05, Fig. 2F).

An increase in β1-AAbs reduces autophagy and increases apoptosis in atrial tissue

TEM showed that rats in the β1-AAb group had reduced numbers of autophagosomes per field and significantly increased numbers of vacuolated mitochondria per field compared with those in the control group. In contrast, these ultrastructural changes were reversed in the bisoprolol treatment group (P < 0.05, Fig. 3A, B). Then, the autophagy markers LC3, Beclin 1 and p62 in the atrial tissue were examined. Compared with that in the normal group, the protein expression of LC3-II and Beclin1 was decreased, while the protein expression of p62 was increased, and these changes were reversed by bisoprolol treatment (P < 0.05, Fig. 3C,D).

The level of apoptosis in atrial tissue was detected by TUNEL staining. TUNEL-positive signals were increased in rats with high β1-AAb titres compared to those in the control group, and this abnormality was
effectively normalized by bisoprolol treatment (P < 0.05, Fig. 3E). Bax and Bcl-2 are considered markers of apoptosis. In our experiment, western blot analysis of atrial tissue showed that compared with that in rats in the normal group, Bax protein expression as increased in the β1-AAb group, and Bcl-2 protein expression was reduced; these effects were normalized by blocking β1-AR (P < 0.05, Fig. 3F-G).

An increase in β1-AAbs disturbs the balance between myocardial autophagy and apoptosis by downregulating PTEN/AKT/mTOR signalling

Previous studies have reported that the AKT/mTOR and NF-κB pathways are key regulators of autophagy in cardiovascular diseases[20]. To determine the molecular signalling pathways of β1-AAb-induced autophagy, we focused on the PTEN, AKT/mTOR and NF-κB pathways. First, we evaluated the protein expression level of PTEN in atrial tissue in the β1-AAb group. In the IHC experiments, positive PTEN expression was indicated by brown–yellow particles in the atrium, and the number and shadings of these particles corresponded to the target protein level. Compared with that in the control group, the number of PTEN-positive granules was reduced in the β1-AAb group, and the number of PTEN-positive granules was enhanced by bisoprolol treatment (P < 0.05, Fig. 4A, B). In addition, western blotting showed that PTEN protein expression was reduced in the β1-AAb group, while the protein levels of p-AKT, p-mTOR and p-NF-κB p65 were significantly enhanced compared to those in normal rats. These changes were reversed by bisoprolol treatment (P < 0.05, Fig. 4C-G).

Activating PTEN prevents autophagy and apoptosis induced by β1-AAbs

The PTEN agonist Oroxin B was used to upregulate PTEN protein expression. The IHC results showed that the expression of PTEN-positive granules was reduced in the β1-AAb group, and overexpression of PTEN rescued the decrease in PTEN expression induced by β1-AAb (P < 0.05, Fig. 5B). Compared with those in the β1-AAb group, rats in the Oroxin B group showed increased numbers of autophagosomes per field and a significant decrease in the numbers of vacuolated mitochondria per field, as shown by TEM (Fig. 5C). In addition, compared with the β1-AAb group, orexin B increased the expression of PTEN and downregulated the protein expression of p-akt, p-mtor and p-NF-kb p65 (P < 0.05, Fig. 5D,E).

To examine autophagy progression, we measured the protein levels of LC3, Beclin1, p62, Bax and Bcl-2 after PTEN overexpression. The β1-AAb group showed decreased protein expression of LC3-II and Beclin1 but increased protein expression of p62, and this effect could be ameliorated by overexpression of PTEN (P < 0.05, Fig. 5F, G). The protein expression of Bax was increased, and the protein expression of Bcl-2 was decreased in the β1-AAb group; however, the increase in apoptosis induced by β1-AAb was alleviated by the overexpression of PTEN (P < 0.05, Fig. 6F,G).

Activating PTEN ameliorates β1-AAb-induced atrial remodelling
The AF induction rate was significantly lower and the duration of AF was significantly shorter in response to oroxin B than β1-AAb under short-pulse pacing conditions (P < 0.05, Fig. 6A,B). Further atrial electroconductivity showed that atrial conduction in the β1-AAb group was disordered and there were abnormal conduction positions of the wave front, and oroxin B treatment could alleviate these atrial conduction abnormalities (P < 0.05, Fig. 6C,D). Compared to rats in the β1-AAb group, the echocardiographic results demonstrated that oroxin B treatment significantly decreased the LAD (P < 0.05, Fig. 6E,F). Additionally, the Sirius red staining results indicated that oroxin B treatment significantly reduced the extent of atrial fibrosis compared to that in rats in the β1-AAb group (P < 0.05, Fig. 6E-H).

Discussion

There are several important findings in this study. First, this study demonstrated for the first time that β1-AAbs inhibited autophagy and induced apoptosis in atria, which may involve inhibiting PTEN expression and activating the downstream AKT/mTOR and NF-κB signalling pathways, leading to AF susceptibility and anabatic electro-anatomical remodelling. Second, the therapeutic effects of bisoprolol on atrial remodelling, autophagy, apoptosis, and fibrosis caused by β1-AAbs may be partly attributed to PTEN activation. Third, the PTEN activator oroxin B, which is a natural flavonoid glycoside with multiple biological activities, has a similar antiarrhythmic effect as bisoprolol on β1-AAb-induced AF.

The P wave index (duration or dispersion) and PR interval can indirectly reflect electro-anatomical remodelling of the atrium, which is specifically characterised by an increase in P wave duration and a prolongation of the PR interval[21]. Our previous results [6] showed that an increase in P wave dispersion occurred in the β1-AAb-induced rabbit AF model. In this study, we used a novel electrocardiogram assay to better detect rat electrocardiograms, and we provided new evidence of changes in the P-wave index, including the P wave duration and P wave amplitude. LA diameter is a marker of electro-anatomical remodelling[22]. AF and pathological alterations are the anatomical basis for atrial electrical heterogeneities associated with AF maintenance. Our echocardiographic results demonstrated that β1-AAbs increased the LA internal diameter, and histopathological results showed that β1-AAbs significantly exacerbated atrial tissue disorganization and fibrosis. Uneven shortening of the AERP, ion channel remodelling, and changes in CV create a vulnerable substrate[23]. Our electrophysiological results showed that β1-AAbs reduced the AERP and CV in the atria. Our ECG, electrophysiological, cardiac ultrasound and histopathological results suggest that an increase in β1-AAbs mediates the electro-anatomical substrate of the atria.

Autophagy is an evolutionarily conserved catabolic pathway that physiologically maintains cellular dynamic balance and remodelling during the self-degradation of intracellular substances[24]. Autophagy plays an important role in maintaining cardiac function and cardiac cellular homeostasis. However, only physiological autophagy has a protective effect and maintains cardiac function. Overexpression or inhibition of autophagy can promote the development of cardiovascular diseases[20]. Inhibiting autophagy in the ischaemic heart aggravates cardiomyocyte apoptosis, thereby inducing the progression of myocardial infarction towards cardiac dysfunction[25]. In addition, autophagy can improve myocardial
function by reducing apoptosis and thereby inhibiting fibrosis[26]. Early studies have shown that autophagy is significantly impaired in patients with AF[27], which can increase and participate in the process of AF[28]. In terms of signal transduction, β1-AAbs can shift β1-AR into an agonist-coupled high-affinity state and activate the typical cAMP-dependent protein kinase A signalling pathway in cardiomyocytes. Continued activation by β1-AAbs induces caspase-3 activation and cardiomyocyte apoptosis[29]. β1-AAbs have been shown to decrease cardiomyocyte autophagy and thereby promote an increase in cardiomyocyte apoptosis, and reducing cardiomyocyte autophagy is the key to the occurrence of cardiomyocyte apoptosis[30, 8]. In β1-AR knockout mice, it was verified that β1-AAbs inhibit cardiomyocyte autophagy in the ventricle via β1-AR[31]. Previous studies of β1-AAb-induced changes in autophagy and apoptosis have mostly focused on ventricles, and no study has reported whether β1-AAbs play the same role in atria. In contrast, our study showed that β1-AAb-induced changes in autophagy reductions and apoptosis increases were also evident in the atria, and treatment with the β1-AR blocker bisoprolol reversed these changes. We therefore suggest that β1-AAb-induced autophagy inhibition and increase in apoptosis occur in the whole heart and exert detrimental effects on the whole heart.

PTEN is a potent tumour suppressor that regulates cell growth, cell proliferation, vesicle trafficking, angiogenesis, anabolism and cancer by negatively regulating the PI3K/AKT signalling pathway[32]. PTEN is closely associated with the dynamic regulation of cellular autophagy and apoptosis[13]. For example, PTEN deficiency inhibited phagosome closure and autolysosome formation, thereby aggravating apoptosis[33]. In hypoxia-reoxygenated human cardiomyocytes (HCMs), a reduction in PTEN expression accelerates cardiomyocyte apoptosis induced by myocardial ischaemia–reperfusion injury[34]. PTEN can activate the AKT/GSK3 and AKT/mTOR signalling pathways and play a key role in isoproterenol (ISO)-induced cardiac hypertrophy and fibrosis[35, 36]. Ang II increases the activity and expression of immunoproteasome subunits (β2i and β5i), increases PTEN degradation and activates downstream signalling (AKT/mTOR, TGF-β1-Smad2/3, NF-κB, NOX and Cx43), leading to AF and atrial fibrosis. Inhibiting immunoproteasome activity, attenuating PTEN degradation and inhibiting downstream signalling can exert cardioprotective effects, and these protective effects can be reversed by blocking PTEN activity with the PTEN-specific inhibitor VO-Ohpic[37, 38]. Our results suggest that β1-AAbs decrease PTEN expression and activate the AKT/mTOR and NF-κB signalling pathways, thereby inhibiting autophagy, increasing apoptosis, and ultimately inducing atrial fibrosis and participating in atrial electro-anatomical remodelling. Therefore, we speculate that β1-AAb activates the AKT/mTOR and NF-κB signalling pathways by stimulating β1-AR or inhibiting PTEN expression.

Oroxin B is a flavonoid and the main bioactive component of *Oroxylum indicum* (L.) Vent[39]. Li et al. showed for the first time that oroxin B had a therapeutic effect on liver cancer by upregulating PTEN expression and downregulating PI3K and p-AKT expression[40]. Oroxin B can play a protective role in osteoarthritis by inhibiting the PI3K/AKT/mTOR signalling pathway and enhancing autophagy[41]. In addition, oroxin B can attenuate liver inflammation and the progression of metabolism-related fatty liver disease by enhancing the intestinal barrier by upregulating the NF-κB signalling pathway[42]. Our data show that after oroxin B treatment, PTEN expression in rat atrial tissue was increased, AKT/mTOR and NF-κB signal transduction pathways were inhibited, autophagy was increased, and apoptosis was
decreased, which ultimately improved β1-AAb-induced atrial remodelling. This study provides new ideas and therapeutic targets for the prevention and treatment of β1-AAb-induced AF.

However, our study also has limitations. First, our study showed that β1-AAb could affect autophagy and subsequent changes by activating the AKT/mTOR and NF-κB signalling pathways, but we did not use pathway inhibitors to further verify the necessity of these pathways. Second, recovery experiments are needed to further verify the upstream and downstream relationships between autophagy and apoptosis. Finally, our research mainly focused on in vivo animal experiments without further verification in cells. Subsequent drug experiments on cell models are needed to verify the deeper mechanisms.

In summary, we demonstrated that β1-AAb reduced PTEN expression in atrial tissues, and the use of a β1 receptor blocker (bisoprolol) significantly attenuated the β1-AAb-induced decrease in PTEN expression, increase in myocardial autophagy and apoptosis, and increase in fibrosis, thereby alleviating the electrical and structural remodelling associated with AF. Furthermore, activation of PTEN using a specific agonist (Oroxin B) significantly abolished the effects of the increase in β1-AAbs on structural and electrical remodelling in rat AF. Thus, our results suggest that activating PTEN expression to inhibit downstream mediators prevents β1-AAb-induced atrial remodelling.

Declarations

Competing Interests

The authors declared that there is no conflict of interest associated with this study.

Authors’s Contributions

Xianhui Zhou, Luxiang Shang, Huaxin Sun and Na Yang conceptualized the project. Na Yang, Linqiang Xi, Qianhui Wang, Jiaru Cao, Jie Song performed experiments. Na Yang, Huaxin Sun and Linqiang Xi analyzed and interpreted data. Baopeng Tang, Ling Zhang and Yanmei Lu provided technical guidance. Na Yang, Huaxin Sun and Luxiang Shang wrote the manuscript. Xianhui Zhou, Luxiang Shang and Baopeng Tang supplied funds to support. All reviewed and approved the manuscript.

Ethics approval

All studies conformed to the Basel Declaration and the principle of the Association for Assessment and Accreditation of Laboratory Care (AAALAC). All experiments were performed with the approval of the Ethics Committee of Xinjiang Medical University (No. IACUC20170420-03).

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References


Figures
Figure 1

β1-AAbs alter electrocardiogram parameters

A Animal grouping and timeline of the first part of the in vivo experiment. ECL2 was injected at four time points and per stage persisted 2 weeks (control group, n=10; β1-AAb group, n=10; bisoprolol group, n=10). Bisoprolol was administered after 8-week active immunization for 2 weeks. B Plasma β1-AAb
concentrations of 3 groups at five time points, (n=6) per group, *P<0.05 vs. baseline of each group at different time points; 6P<0.05 vs. control group at same time points among different groups. C Detection of atrial cAMP contents of 3 groups after extracting atrial tissues, (n=6) per group. D Schematic of ECG measurements using Powerlab and ECGs of the control and immunised groups. E-I repeating heart rate, P duration, P amplitude, PR interval, and QTC measurements per animal for 3 times of 3 groups, (n=6) per group. *P<0.05 as indicated.
Figure 2

Blocking β1-AR improves the electro-anatomical substrate induced by β1-AAbs

ARepresentative images of H&E staining, Masson's trichrome staining and Sirius red staining of the atrium in 3 groups of rat. B,C Quantification of CVF of the atrium in 3 groups of rat. D Representative isochronous maps in the left atrium as obtained by multielectrode array (MEA) recording. E,F Analysis of CV and LA conduction heterogeneity index of 3 groups, (n=6) per group. CVF: Collagenvolume fraction; CV: conduction Velocity; *P<0.05 indicates statistical significance.
Figure 3

An increase in β1-AAbs reduces autophagy and increases apoptosis in atrial tissue

**A,B** Representative images of TEM of the atrium in 3 groups of rat, and quantification of autophagosomes and vacuolated mitochondria per field in 3 groups of rat. Red arrows and blue arrows represent autophagosomes and mitochondrial phagosomes, respectively. Green arrows represent mitochondria.
with vacuolization. Yellow arrows represent lysosomes. C-D Representative Western blot images and quantification of Beclin1, LC3, and p62 expressions in the atrium of 3 groups of rat. E Representative immunohistochemical staining of TUNEL in the atrium of 5 groups of mice. TUNEL-positive cells appear red, DAPI-counterstained nuclei are blue. And Area occupied by TUNEL-positive cells in atrium, as determined by image analysis. F-G Representative Western blot images and quantification of Bcl-2 and Bax expressions in the atrium of 3 groups of rat. *P<0.05 indicates statistical significance.

**Figure 4**

An increase in β1-AAbs disturbs the balance between myocardial autophagy and apoptosis by downregulating PTEN/AKT/mTOR signalling.

A-B Representative immunohistochemical staining and quantification of PTEN in the atrium of 3 groups of rat. C Representative Western blot images of PTEN, phosphor-Akt (p-Akt), total-Akt (Akt), phosphor-mTOR (p-mTOR), total-mTOR (mTOR), phosphor-NF-κB p65 (p-p65) and total-NF-κB p65 (p65) expressions in the atrium of 3 groups of rat. D-G Quantification of PTEN, p-mTOR/mTOR, p-Akt/AKT, p-p65/p65 expressions in the atrium of 3 groups of rat.
Figure 5

Activating PTEN prevents autophagy and apoptosis induced by β1-AAbs

A Animal grouping and timeline of the second part of the in vivo experiment. ECL2 was injected at four time points and per stage persisted 2 weeks (control group, n=10; β1-AAb group, n=10; Oroxin B group, n=10). Oroxin B was administered after 8-week active immunization for 6 weeks. Six animal models per
group were chosen for the next experiments. B Representative immunohistochemical staining images and quantification of immunohistochemical staining of PTEN in the atrium of 3 groups of rat. C Representative images of TEM of the atrium in 3 groups of rat, and quantification of autophagosomes per field in 3 groups of rat. Red arrows and blue arrows represent autophagosomes and mitochondrial phagosomes, respectively. Green arrows represent mitochondria with vacuolization. Yellow arrows represent lysosomes. D Representative Western blot images of PTEN, phosphor-Akt (p-Akt), total-Akt (Akt), phosphor-mTOR (p-mTOR), total-mTOR (mTOR), phosphor-NF-κB p65 (p-p65) and total-NF-κB p65 (p65) expressions in the atrium of 3 groups of rat. E Quantification of PTEN, p-mTOR/mTOR, p-Akt/AKT, p-p65/p65 expressions in the atrium of 3 groups of rat. F Representative Western blot images of Beclin1, LC3, p62, Bcl-2 and Bax expressions in the atrium of 3 groups of rat. G Quantification of Beclin1, LC3, p62, Bcl-2 and Bax expressions in the atrium of 3 groups of rat. *P<0.05 indicates statistical significance.
Figure 6

Activating PTEN ameliorates β1-AAb-induced atrial remodelling

A Typical images of AF induction with burst pacing at 50 ms S1-S1 interval among three groups.

B Repeating AF inducibility per animal 10 times and accumulated durations of AF episodes during AF induction of 3 groups of rats, (n=6) per group.

C Representative isochronous maps in the left atrium as
obtained by multielectrode array (MEA) recording. **D** LA conduction heterogeneity index of 3 groups, (n=6) per group. **E** Typical M-mode echocardiographic images of the atria of 3 groups of rats. The red line represents the left atrial diameter size. **F** Analysis of LAD of 3 groups, (n=6) per group. **G,H** Representative images of Sirius red staining and Quantification of CVF.*P<0.05 indicates statistical significance.

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