Original Paper

The β_3 Adrenergic Receptor Agonist BRL37344 Exacerbates Atrial Structural Remodeling Through iNOS Uncoupling in Canine Models of Atrial Fibrillation

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Key Words

Atrial fibrillation • β_3 -adrenergic receptor • Atrial structural remodeling • Oxidative stress • Nitric oxide synthase • Tetrahydrobiopterin

Abstract

Background/Aims: The role of the β_3 -adrenergic receptor (β_3 -AR) agonist BRL37344 in atrial fibrillation (AF) structural remodeling and the underlying mechanisms as a therapeutic target were investigated. **Methods:** Four groups of dogs were evaluated: sham, pacing, β_3 -AR agonist BRL37344 (β_3 -AGO), and β_3 -AR antagonist L748337 (β_3 -ANT) groups. Dogs in the pacing, β_3 -AGO and β_3 -ANT groups were subjected to rapid atrial pacing for four weeks. Atrial structure and function, AF inducibility and duration, atrial myocyte apoptosis and interstitial fibrosis were assessed. Atrial superoxide anions were evaluated by fluorescence microscopy and colorimetric assays. Cardiac nitrate+nitrite levels were used to assess nitric oxide (NO) production. Protein and mRNA expression of β_3 -AR, neuronal NO synthase (nNOS), inducible NO synthase (iNOS), endothelial NO synthase (eNOS) and guanosine triphosphate cyclohydrolase-1 (GCH-1) as well as tetrahydrobiopterin (BH4) levels were measured. **Results:** β_3 -AR was up-regulated in AF. Stimulation of β_3 -AR significantly increased atrial myocyte apoptosis, fibrosis and atrial dilatation, resulting in increased AF induction and prolonged duration. These effects were attenuated by β_3 -ANT. Moreover, β_3 -AGO reduced BH4 and NO production and increased superoxide production, which was inhibited by the specific iNOS inhibitor, 1400w. β_3 -AGO also increased iNOS but decreased eNOS and had no effect on nNOS expression in AF. **Conclusions:** β_3 -AR stimulation resulted in atrial structural remodeling by increasing iNOS uncoupling and related oxidative stress. β_3 -AR up-regulation and iNOS uncoupling might be underlying AF therapeutic targets.

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Introduction

Atrial fibrillation (AF) is the most common self-sustained cardiac arrhythmia closely associated with stroke and heart failure, and its prevalence is rapidly increasing in the aging population [1]. AF is self-perpetuating because AF stimulates atrial electrical, structural and metabolic remodeling [2-4]. Atrial remodeling, in turn contributes to AF development and maintenance, creating a vicious cycle [5, 6]. After AF cardioversion to sinus rhythm, atrial electrical changes can be completely recovered within a few days, but structural damages may not be fully reversed. Moreover, structural remodeling breaks myocardial continuity, slows electrical conduction, and damages myocardial energy metabolism [4, 7]. Therefore, atrial structural remodeling is vital in AF development as an important substrate. However, the pathophysiology underlying atrial structural remodeling remains poorly understood.

β -adrenoceptors (β -ARs), including β_1 , β_2 and β_3 subtypes, are important modulators in cardiovascular function. β_1 - and β_2 -ARs classically mediate the effects of catecholamines on cardiac contraction and vascular smooth muscle relaxation [8]. However, β_3 -AR differs from the β_1 - and β_2 subtypes in its molecular structure and pharmacological profile [9]. Although many studies have demonstrated that β_3 -AR upregulation contributes to dysfunction in heart failure [10, 11], few reports have described the potential role of β_3 -AR in AF. A recent study revealed that β_3 -AR activation induced atrial electrical remodeling in rapid paced atrial myocytes [12], suggesting that β_3 -AR promotes AF development. However, the role of β_3 -AR in atrial structural remodeling associated with AF remains unclear and might present a new therapeutic target.

Oxidative stress is paramount when promoting AF pathogenesis by affecting atrial structural remodeling [13]. Reactive oxygen species (ROS) generated from nitric oxide synthase (NOS) uncoupling may be an underlying contributor [14]. NO is synthesized from L-arginine and oxygen by NOS using tetrahydrobiopterin (BH4) as a cofactor. Under conditions of low BH4 bioavailability relative to NOS, NOS is uncoupled to produce superoxide anion (O_2^-) instead of NO (Fig. 1) [14]. There are three isoforms of NOS: neuronal (nNOS), inducible (iNOS), and endothelial (eNOS) [15]. It is well established that negative inotropy associated with β_3 -AR results from the eNOS-NO pathway. However, it remains unclear whether the effect of β_3 -AR on atrial structural remodeling in AF is related to NOS uncoupling.

Here, we investigated the potential role of β_3 -AR in AF canine models induced by rapid atrial pacing and tested the hypothesis that the preferential β_3 -AR agonist BRL37344 exacerbates atrial structural remodeling in AF by increasing oxidative stress from iNOS uncoupling.

Materials and Methods

Animal Welfare and Ethical Statement

All animal procedures were approved by the Experimental Animal Ethics Committee at the Harbin Medical University. All procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised in 1996).

Fig. 1. Left: In the process of nitric oxide (NO) production, nitric oxide synthase (NOS) enzymes require the substrates L-arginine and molecular oxygen (O_2) and the cofactor tetrahydrobiopterin (BH4). Right: Reduced BH4 bioavailability relative to NOS protein results in “uncoupled” NOS characterized by the production of superoxide (O_2^-).

Animals

A total of 24 male and female mongrel dogs (2 - 3 years of age, 15 - 25 kg) were randomized into four groups: sham group (sham surgery) (n = 6); pacing group (no drug) (n = 6); β_3 -AGO group (β_3 -AR agonist, BRL37344) (n = 6); and β_3 -ANT group (β_3 -AR antagonist, L748337) (n = 6). After adequate anesthesia with sodium pentobarbital (25 mg/kg i.v.), the dogs were rapidly intubated and mechanically ventilated. Thoracotomy was performed from the right fourth intercostal space. A thin silicon lead was implanted into the right atrial free wall and affixed to a pacemaker (Harbin Science and Technology University, China). One week post-surgery, the dogs in the pacing, β_3 -AGO and β_3 -ANT groups were paced at 600 beats per minute (bpm) for four weeks according to a previous study [16]. In the β_3 -AGO and β_3 -ANT groups, the dogs were continuously given an intraperitoneal injection of BRL37344 (1.5 μ g/kg/h, Sigma-Aldrich B169) and L748337 (2 μ g/kg/h, Sigma-Aldrich L7045), respectively, by an osmotic pump for four weeks during the rapid atrial pacing phase. All experiments were performed in the presence of nadolol, an antagonist for β_1 - and β_2 -ARs (1 mg/kg, i.p. injection; cat. no. N1892; Sigma-Aldrich). A surface electrocardiogram was recorded daily to ensure sustained atrial pacing at 600 bpm.

Echocardiography

Left atrium (LA) and left atrial appendage (LAA) structure and function were serially assessed by transthoracic and transesophageal echocardiography (Philips 7500, Washington, USA) prior to surgery and heart removal. Parameters related to the LA maximal volume (LAVmax), LAA maximal volume (LAAVmax), LA minimal volume (LAVmin), and LAA minimal volume (LAAVmin) were recorded. The LA ejection fraction was determined as $(LAV_{max} - LAV_{min}) / LAV_{max} \times 100\%$, and the LAA ejection fraction was determined as $(LAAV_{max} - LAAV_{min}) / LAAV_{max} \times 100\%$.

Electrophysiological assessment of AF

AF inducibility and duration were measured at baseline and at the end of the four-week protocol. AF inducibility and duration were assessed using 10 Hz stimuli (burst pacing at 10 Hz for 10 s) at five-minute intervals with a pacing cycle length of 100 ms. AF was defined as a rapid and irregular atrial rhythm. AF lasting more than 30 minutes was terminated by direct current electrical cardioversion, and the AF duration was recorded for 30 minutes.

Histological analysis of cellular morphometry

Dogs were anesthetized, and hearts were rapidly excised. Current evidence suggests that the left atrium dominates the occurrence of AF [17, 18]. Therefore, left atrial samples adjacent to the left atrium appendage were collected, snap-frozen in liquid nitrogen and then divided into four portions: frozen sections for determination of superoxide (O_2^-) immediately after excision; tissue fixed with 10% paraformaldehyde and embedded in paraffin for morphological evaluation; tissue fixed in glutaraldehyde at 4°C for electron microscopy; and samples frozen in liquid nitrogen for subsequent assays. Sections (5 μ m) of the atrium were stained using a Masson trichrome and imaged using light microscopy. The interstitial collagen volume fraction (CVF) was used to evaluate fibrosis. After staining, collagen fibers and cardiomyocytes were stained blue and red, respectively. One section was selected from each sample, and five randomly selected fields from each section were evaluated. The mean area percentage of the collagen fibers was recorded as the collagen volume fraction (CVF%). Perivascular collagen was not included in the quantification. Sections were evaluated by two professionals who were blinded to the treatment. Digital images were analyzed using Image-Pro Plus 6.0 software.

Ultrathin sections (50 - 100 nm) pretreated with glutaraldehyde were prepared for electron microscopy (HITACHI, H7650, Japan) by two observers blinded to the treatments. Sections were prepared according to the method of Zhao et al. [19].

Terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) analysis

Tissues embedded in paraffin were sectioned to 7- μ m thickness, dewaxed and rehydrated prior to TUNEL staining. TUNEL staining was performed using an In Situ Cell Death Detection kit (Roche Applied Science) according to the manufacturer's instructions. Tissue sections were incubated in 0.1 M citrate buffer and 0.1% Triton, pH 6.0, and subsequently exposed to 350 W of microwave irradiation for 5 minutes. The sections were then rapidly cooled using distilled water (20 - 25°C) and rinsed with phosphate buffered saline (PBS) at 15 - 25°C. Subsequently, 50 μ l of TUNEL reaction mixture was added to the sections and

incubated for 60 minutes at 37°C. Finally, slides were rinsed three times with PBS. Negative control sections were incubated with 50 μ l of labeling solution. Positive control sections were incubated with DNase I (Sigma-Aldrich). Sections were subsequently imaged and microscopically analyzed for TUNEL-positive cells. The percentage of TUNEL-positive nuclei was calculated and recorded as the apoptotic index (AI%). Five randomly selected fields per section corresponding to approximately 300 cells were examined per treatment condition at high magnification (200 x).

Western blot (WB) analysis of β -3AR, nNOS, iNOS, eNOS and guanosine triphosphate cyclohydrolase-1 (GCH-1) protein expression

Protein samples were extracted from tissues, and a BCA protein assay kit was used to calculate protein concentrations. A total of 50 μ g of protein for β -3AR, iNOS, eNOS and GCH-1 and 150 μ g protein for nNOS was loaded and separated on SDS gels (8-12%) followed by blotting onto polyvinylidene difluoride (PVDF) membranes. The membranes were subsequently blocked with 5% nonfat dry milk in Tris-buffered saline with Tween (TBST) (0.05% Tween 20, pH 7.4). Membranes were incubated overnight at 4°C with primary antibodies in TBST and subsequently washed in TBST; the membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 hours at 37°C. The primary antibodies included rabbit anti- β 3-AR (1:500, Abcam, ab76249), nNOS (1:100, Santa Cruz, sc648), iNOS (1:150, Abcam, ab3523), synthetic peptide corresponding to aa598-612 (P^{598} YNSSPRPEQHKSYK 612 C) of bovine eNOS (1:1000, Biomol, ALX-210-505) and mouse anti-GCH-1 (1:400, Santa Cruz, sc134574). Glyceraldehyde phosphate dehydrogenase (GAPDH) levels were measured as an internal control with anti-GAPDH (1:1000) antibodies (Abcam, Cambridge, MA, USA). Immunoreactive bands were detected, and the signal intensities were quantified using a chemiluminescent protein detection system (Bio-Rad Laboratories).

Real time polymerase chain reaction (RT-PCR) analysis to detect β 3-AR, nNOS, iNOS, eNOS and GCH-1 mRNA expression

Total RNA was isolated using Trizol according to the manufacturer's instructions. Reverse transcription was performed in a 20 μ l reaction mixture with SYBR[®]Green qPCR reagents, TaKaRa PrimeScript TM RT reagent Kit and gDNA Eraser (Code No. RR047A) according to the manufacturer's recommendations. Quantitative RT-PCR was performed using an ABI 7500 Real Time PCR system (Applied Biosystems). Canine β 3-AR, nNOS, iNOS, eNOS, and GCH-1 steady state mRNA levels were measured. Specific primers used to amplify these genes are listed in Table 1. GAPDH was used as an internal control.

Fluorescence measurement of superoxide anion ($O_2^{\cdot-}$)

The cell-permeable dihydroethidium (DHE) is oxidized to fluorescent hydroethidium (HE) by $O_2^{\cdot-}$, which is then intercalated into DNA. The generation of $O_2^{\cdot-}$ in atrial tissue was determined based on DHE fluorescence, as previously described [20] and according to the manufacturer's instructions (GENMED SCIENTIFICS INC. USA, 10111.2 v. A).

Table 1. Primers for real-time PCR

beta3-AR	Forward primer	AGGAGAGGCTTCCAATGTCACCA
	Reverse primer	GTCATGTCCAGCACACACCACG
nNOS	Forward primer	CTGACGAAGTGGTCCCATGTTT
	Reverse primer	GAGTCTCCTCCCTGTAGATGTGGTC
iNOS	Forward primer	GGCTCTACACTTCCAATGTGACCTG
	Reverse primer	TTGGACTCTGTAGATTCCGTTGTGA
eNOS	Forward primer	CTATCAGACAGACCCGTGGAAGG
	Reverse primer	GGCAGAGATCTTCACCGCATTG
GCH-1	Forward primer	TCTTCACCAAGGGCTACCAGGA
	Reverse primer	GTCCCTTCACAATCACCATCTCATCA
GAPDH	Forward primer	GTGTCCCCACCCCAATGTATCAGT
	Reverse primer	CAGTGTAGCCCAGGATGCCTTTGAG

Fresh left atrial samples collected within 1 hour after surgery were placed in ice-cold saline and then embedded for cyrosectioning at the optimal cutting temperature (-20°C). Each sample was cut into five 10- μm -thick sections and then placed on glass slides. Sections were washed with 0.5 ml of Reagent A followed by topical application of a DHE stain working solution (10 μM , 0.2 ml). Sections were then incubated for 20 minutes at 37°C in a light-protected chamber and subsequently washed with 0.5 ml of reagent A to remove any unbound DHE. Sections were then fixed, secured with a coverslip, and imaged using a laser scanning confocal microscope (ZEISS, LSM510.META). Fluorescence intensity was quantified using Image-Pro Plus 6.0 software.

Colorimetric assay of superoxide anions ($\text{O}_2^{\cdot-}$)

To further measure $\text{O}_2^{\cdot-}$ levels and evaluate the effect of the iNOS inhibitor, 1400w, a colorimetric assay was used to measure $\text{O}_2^{\cdot-}$ in atrial tissues. The assay was performed on fresh left atrial tissues, which were collected within 1 hour after surgery. Canine atrial tissue (50 mg) that was directly adjacent to the LA appendage was used for the assay. Tissues were subsequently homogenized, and proteins were extracted. The extracts were divided into two aliquots (50 μl each), and the concentrations were measured. Nicotinamide adenine dinucleotide phosphate (NADPH) solution (1X) was added to tissue homogenates (one of the 50 μl aliquots) after 30 minutes at 4°C to a total volume of 1 ml, which resulted in a final NADPH concentration of 100 μM . The other tissue homogenate (the other 50 μl aliquot) was pre-incubated with 100 μM iNOS inhibitor, 1400w in a total volume of 1 ml for 30 minutes at 4°C . Staining was performed according to the GENMED Colorimetric assay kit instructions (GENMED SCIENTIFICS INC. U.S.A, 10096.2v.A). The optical density (OD) was determined at a wavelength of 580 nm using a microplate reader. The results were subtracted from the total OD to determine iNOS-dependent $\text{O}_2^{\cdot-}$ generation.

Measurement of cardiac NO production

Cardiac NO levels were determined using a two-step measurement of total nitrate+nitrite concentrations and the Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI, Item No.780001). Atrial tissues (60 mg) were homogenized in PBS at pH 7.4 and subsequently centrifuged at 10,000 $\times$ g for 20 minutes. Supernatants were diluted to 6 ml and ultracentrifuged at 100,000 $\times$ g for 30 minutes (BECKMAN COULTER, OptimaTML-100XP Ultracentrifuge). Tissue homogenates (80 μl) were generated and used in triplicate. The absorbance of samples was between 0.05 - 1.2 absorbance units. A total of 80 μl of the tissue homogenate was added to the sample wells, and 200 μl of assay buffer was added to blank wells as a negative control. Subsequently, the enzyme cofactor mixture (10 μl) and nitrate reductase mixture (10 μl) were added to each well (standards and experimental samples). The plate was then incubated at room temperature for 1 hour. Subsequently, Griess Reagent R1 (50 μl) and Griess Reagent R2 (50 μl) were added to each of the wells. The color was then allowed to develop for 10 minutes at room temperature. The absorbance was read at 540 nm using a Bio-Rad iMarkTM microplate reader.

High-performance liquid chromatography (HPLC) for the assessment of BH4

Atrial tissue (100 mg) was analyzed using HPLC. Tissue was homogenized and diluted to 1000 μl prior to processing (500 μl each). Protein concentrations were assessed using the BCA method. Samples (450 μl) were incubated with a 50 μl acid solution (1.5 M HClO_4 and 2 M H_3PO_4 mixture of equal proportions) at 4°C and subsequently centrifuged at 13,000 g/min for 10 minutes. The supernatant (450 μl) was transferred to a new Eppendorf tube and incubated with 50 μl of acidic iodine solution (1% iodine + 2% KI dissolved in 1 M HCl) at room temperature in the dark for 30 minutes. Subsequently, 25 μl of freshly prepared ascorbic acid (20 mg/ml) was added at 4°C and centrifuged at 13,000 g/min for 10 minutes. A portion of the supernatant (40 μl) was used to determine the total acid oxidation levels of biopterins (BH_4 + BH_2 + biopterin). Simultaneously, 400 μl of the same protein extract was incubated with a mixture of 50 μl of 1 M NaOH and 50 μl of alkaline iodine (1% iodine + 2% KI dissolved in 1 M NaOH) in the dark at room temperature for 60 minutes. Subsequently, 100 μl of 1 M H_3PO_4 and 25 μl of a freshly prepared ascorbic acid solution (20 mg/ml) were added to the protein extracts at 4°C and centrifuged at 13,000 g/min for 10 minutes. A portion of the supernatant (40 μl) was then used to measure biopterins that excluded BH_4 (BH_2 + biopterin). After oxidation (either in acidic or alkaline solution), the sample was immediately used for HPLC analysis. The mobile phase of the HPLC consisted of methanol/water (10/90) at a flow rate of 10 ml/min. Results were obtained by comparison to a standard curve and the results from analytical standards, which were obtained

from Sigma-Aldrich (B-1527 and 37272) and diluted to various concentrations (1, 10, 20, 50 and 100 nM). BH4 levels were calculated as the difference between the two measurements (acid and alkaline oxidation) as described by Takeshi and colleagues [21]. Detection of BH4 was assessed by fluorescence detection at excitation and emission wavelengths of 350 nm and 440 nm, respectively, using a Waters 515 HPLC pump, 717 plus autosamplers, and a 2475 multi λ fluorescence instrument.

Statistics

All data are expressed as the mean $\pm$ standard deviation. Echocardiographic, electrophysiological data and iNOS-dependent $O_2^{\cdot-}$ generation were compared using repeated measures analysis of variance (RM-ANOVA). Group data were compared using a one-way ANOVA with a Tukey's post-hoc test for multiple comparisons. A P value of less than 0.05 was considered statistically significant. GraphPad Prism 5.0 and 6.0 (La Jolla, CA) were used for the statistical analysis.

Results

Upregulation of β 3-AR in canine AF models was induced by rapid atrial pacing

RT-PCR analysis and WB results revealed that β 3-AR mRNA and protein levels were significantly higher in the pacing group compared to the sham group ($P < 0.01$). Furthermore, the expression of β 3-AR mRNA and protein was significantly increased in the β 3-AR agonist (BRL37344) β 3-AGO group ($P < 0.01$) and decreased in the β 3-AR antagonist (L748337) β 3-ANT group ($P < 0.05$) compared to the pacing control group (Fig. 2A, B, C).

Fig. 2. β 3-AR mRNA and protein levels in the left atrium. (A) β 3-AR mRNA expression in the sham, pacing, β 3-AGO and β 3-ANT groups. (B) Western bolt analysis of β 3-AR protein levels using specific β 3-AR antibodies. GAPDH was used as a loading control. (C) Quantitative analysis of β 3-AR protein levels relative to GAPDH levels. ** $P < 0.01$ vs. sham group, ## $P < 0.01$, # $P < 0.05$ vs. pacing group. β 3-AGO refers to the β 3-AR agonist BRL37344 group, and β 3-ANT refers to the β 3-AR antagonist L748337 group.

Table 2. Electrophysiological evaluation of canine atria before and after rapid atrial pacing. AF cases refer to the number of dogs successfully induced (10 inductions per dog) to develop AF during electrophysiological examination in each group. AF frequency refers to the total number of AF episodes that were induced during electrophysiological examination in all dogs within a group (with the understanding that AF may have been repeatedly induced in the same dog within the scope of its 10 inductions). AF inducibility is calculated as AF frequency/total stimulations, eg. 60. * $P < 0.05$, ** $P < 0.01$ vs. baseline; # $P < 0.05$, ## $P < 0.01$ vs. sham group; † $P < 0.05$, †† $P < 0.01$ vs. pacing group

Group	AF cases	AF frequency	AF inducibility(%)	AF duration (s)
sham group				
baseline	2	8	13.33	57.83±15.30
5 weeks post-operation	2	11	18.33	59.83±14.63
pacing group				
baseline	2	8	13.33	59.50±17.16
4 week pacing	6	50	83.33**	934.17±191.71***
$\beta 3$ -AGO group				
baseline	1	7	11.67	56.50±28.60
4 week pacing	6	59	98.33**	1276.00±126.54***
$\beta 3$ -ANT group				
baseline	2	9	15.00	54.50±18.99
4 week pacing	6	28	46.67**	622.00±135.41***

$\beta 3$ -AR stimulation induced and maintained AF in canines

In the pacing groups, AF inducibility ($P < 0.01$) and duration ($P < 0.01$) were markedly increased compared to the sham group. Moreover, the $\beta 3$ -AR agonist (BRL37344; $\beta 3$ -AGO) further increased AF inducibility ($P < 0.01$) and prolonged AF duration ($P < 0.01$) compared to the pacing alone group. Interestingly, the $\beta 3$ -AR antagonist (L748337) significantly inhibited ($P < 0.01$) AF induction and maintenance (Table 2).

$\beta 3$ -AR stimulation resulted in atrial dilation and exacerbated atrial dysfunction in the rapid pacing canine model of AF

Compared to the sham groups, LAVmax, LAVmin, LAAVmax, and LAAVmin were significantly increased in the pacing group ($P < 0.01$), while LAEF and LAAEF were decreased ($P < 0.01$). Compared to the pacing group, the LAVmax, LAVmin, LAAVmax and LAAVmin were significantly increased in the $\beta 3$ -AGO group ($P < 0.01$), while LAEF and LAAEF were decreased ($P < 0.01$). Interestingly, the $\beta 3$ -AR antagonist L748337 abrogated the effects of atrial dilation and dysfunction in the canine pacing model ($P < 0.05$) (Table 3).

$\beta 3$ -AR stimulation exacerbated atrial myocyte apoptosis and myocardial interstitial fibrosis in the rapid pacing canine model of AF

Representative histological staining results are shown in Fig. 3. Compared to the sham group, the percentage of TUNEL-positive cells (apoptosis index, AI) (5.72 ± 4.41 vs. $21.15 \pm 12.3\%$, $P < 0.01$) and the CVF ($3.00 \pm 1.18\%$ vs. $10.29 \pm 1.84\%$, $P < 0.01$) were markedly increased in the pacing group. $\beta 3$ -AR stimulation increased atrial myocyte apoptosis ($29.71 \pm 10.38\%$ vs. $21.15 \pm 12.31\%$, $P < 0.01$) and myocardial interstitial fibrosis ($12.88 \pm 3.77\%$ vs. $10.29 \pm 1.84\%$, $P < 0.01$) compared to the pacing group. In contrast, $\beta 3$ -AR inhibition reversed the damage to atrial myocytes ($13.83 \pm 4.61\%$ vs. $21.15 \pm 12.31\%$, $P < 0.01$) as well as the effects on interstitial fibrosis ($8.01 \pm 2.07\%$ vs. $10.29 \pm 1.84\%$, $P < 0.01$).

$\beta 3$ -AR stimulation deteriorated atrial myocardial ultrastructure in the rapid pacing canine model of AF

Representative electron micrographs highlighting the ultrastructural changes in the atria are shown in Fig. 4. Atrial myocytes in the sham group presented with regular

Table 3. Atrial dimensions and function in canines before and after a four-week rapid pacing protocol. *P < 0.05, **P < 0.01 vs. baseline, ##P < 0.01 vs. sham group, †P < 0.05, ‡P < 0.01 vs. pacing group. LAV max, LA maximal volume; LAVmin, LA minimal volume; LAEF, LA ejection fraction; LAAVmax, LAA maximal volume; LAAVmin, LAA minimal volume; LAAEF, LAA ejection fraction. 5 weeks refers to 5 weeks post-operation

Group	LAVmax (ml)	LAVmin (ml)	LAEF (%)	LAAVmax (ml)	LAAVmin (ml)	LAAEF (%)
sham group						
baseline	12.73±1.26	6.25±0.84	51.88±5.55	1.87±0.21	0.83±0.14	52.27±4.84
5 weeks	12.88±1.19	6.33±0.86	52.42±4.50	1.92±0.33	0.91±0.19	53.10±4.71
pacing group						
baseline	13.05±1.11	6.37±1.12	52.55±5.19	1.93±0.31	0.87±0.14	53.10±5.03
4 week	24.47±1.76***	15.57±1.42***	37.27±5.51***	3.10±0.37***	2.15±0.27***	35.77±4.14***
β_3 -AGO group						
baseline	12.77±1.13	6.37±0.94	52.68±5.13	1.97±0.21	0.90±0.19	52.35±6.22
4 week	29.37±1.41**	19.32±1.28**	27.58±4.86**	3.85±0.46**	2.83±0.30**	28.22±1.87**
β_3 -ANT group						
baseline	13.05±1.17	6.48±0.97	51.93±5.12	1.83±0.26	0.85±0.15	53.17±4.91
4 week	18.94±1.50**	10.08±1.32**	45.41±5.06†	2.48±0.32†	1.28±0.23**	44.81±2.81*

Fig. 3. (A) Assay of apoptosis in the atrial myocardium. TUNEL-positive (apoptotic) nuclei are stained dark brown, while healthy nuclei are stained blue. The magnification is 200 x. (B) Apoptotic index (AI%) in the atrial myocardium of the sham, pacing, β_3 -AGO and β_3 -ANT groups. (C) Masson's trichrome stain in the atrial myocardium of the sham, pacing, β_3 -AGO and β_3 -ANT groups. Collagen fibers are stained blue, and cardiomyocytes are stained red. The magnification is 200 x. (D)

Collagen volume fraction (CVF%) in atrial myocardium of the sham, pacing, β_3 -AGO and β_3 -ANT groups. Note the increased atrial AI and CVF in the pacing group compared to the sham group. An increase in the AI and CVF was observed in the β_3 -AGO group, whereas a decrease in the AI and CVF was observed in the β_3 -ANT group compared to the pacing group. **P < 0.01 vs. sham group; ##P < 0.01 vs. pacing group. β_3 -AGO refers to the β_3 -AR agonist BRL37344 group, and β_3 -ANT refers to the β_3 -AR antagonist L748337 group.

sarcomere organization and nuclear morphology as well as abundant mitochondria with normal morphology and clear cristae (Fig. 4A). Following the four-week atrial pacing

Fig. 4. Electron micrographs of the atrial ultrastructure in the sham, pacing, β_3 -AGO and β_3 -ANT groups. (A) Sham group: Note the regular sarcomere organization, normal nuclear morphology, abundant mitochondria with clear cristae and distinct intercalated discs. (B) Pacing group: Note the disorganized and sparsely arranged myofilaments, mitochondria swelling with decreased cristae and increased interstitial connective tissue. (C) β_3 -AGO group: Note the severe disarray and dissected myofilaments with evident mitochondria swelling and vacuolation as well as increased interstitial connective tissue. (D) β_3 -ANT group: Note the significantly improved ultrastructural changes in atria with limited disorganized myofilaments, mitochondria swelling and fibrous tissue infiltration. The magnification is 8,000x (A,C) and 10,000x (B, D). Mit, mitochondria; Myo, myofilaments; Nuc, nucleus; Dis, intercalated discs. β_3 -AGO refers to the β_3 -AR agonist BRL37344 group, and β_3 -ANT refers to the β_3 -AR antagonist L748337 group.

protocol, ultrastructural damage could be readily observed in the atria and was characterized by sparsely arranged myofilaments that appeared in disarray as well as mitochondria swelling with decreased cristae (Fig. 4B). Moreover, the β_3 -AR agonist BRL37344 worsened the chronic tachypacing-induced ultrastructural defects (Fig. 4C). In contrast, the β_3 -AR antagonist L748337 suppressed the ultrastructural damage (Fig. 4D).

β_3 -AR stimulation increased superoxide anion production and reduced cardiac NO production in the rapid pacing canine model of AF

Compared to the sham group, the level of atrial superoxide anion increased in the rapid pacing canine model (1721.0 ± 483.5 a.u. vs. 1161.0 ± 370.3 a.u., $P < 0.01$) (Fig. 5A and B), suggesting a reaction to oxidative stress. Atrial NO production was decreased in the pacing group (204.4 ± 34.36 $\mu\text{mol/g}$ protein vs. 279.2 ± 14.09 $\mu\text{mol/g}$ protein, $P < 0.01$) compared to the sham group, as measured by the sum of the concentrations of the NO metabolites (nitrate and nitrite) (Fig. 5C). In contrast, the β_3 -AR agonist BRL37344 aggravated the atrial oxidative stress, as evidenced by the increase in the superoxide anion level (2470 ± 392.5 a.u. vs. 1721.0 ± 483.5 a.u., $P < 0.01$), as the NO generation was decreased (147.7 ± 16.61 $\mu\text{mol/g}$ protein vs. 204.4 ± 34.36 $\mu\text{mol/g}$ protein, $P < 0.01$) in the pacing canines compared to the pacing group with no drug. Again, the β_3 -AR antagonist L748337 suppressed these biochemical alterations (superoxide anion, 938.5 ± 232.4 a.u. vs. 1721.0 ± 483.5 a.u., $P < 0.01$; NO, 248.8 ± 20.20 $\mu\text{mol/g}$ protein vs. 204.4 ± 34.36 $\mu\text{mol/g}$ protein, $P < 0.05$).

β_3 -AR stimulation decreased BH4 levels, increased iNOS expression and modulation of nNOS and eNOS in the rapid pacing canine model of AF

We showed that atrial BH4 generation (0.218 ± 0.030 pmol/mg protein vs. 0.118 ± 0.021 pmol/mg protein, $P < 0.01$) (Fig. 5D) and GCH-1 expression (both mRNA and protein) ($P < 0.05$) (Fig. 6) were decreased in the pacing groups compared to the sham control group. Furthermore, β_3 -AR stimulation reduced atrial BH4 production (0.050 ± 0.007 pmol/mg protein vs. 0.118 ± 0.021 pmol/mg protein, $P < 0.01$) (Fig. 5D) and GCH-1 expression ($P < 0.01$) (Fig. 6) in the pacing β_3 -AGO group, whereas the β_3 -antagonist L748337 attenuated

Fig. 5. (A) Representative images of superoxide anion production in the left atrium as assayed based on DHE fluorescence in the sham, pacing, β_3 -AGO and β_3 -ANT groups. Red fluorescence indicates superoxide anion production. (B) Quantitative analysis of superoxide production in the sham, pacing, β_3 -AGO and β_3 -ANT groups. ** $P < 0.01$ vs. Sham group, ## $P < 0.01$ vs. Pacing group. (C) Nitrate/nitrite represents NO production and was measured by the Griess assay in the sham, pacing, β_3 -AGO and β_3 -ANT groups. ** $P < 0.01$ vs. sham group, # $P < 0.05$, ## $P < 0.01$ vs. pacing group. (D) BH4 levels in the left atria of the sham, pacing, β_3 -AGO and β_3 -ANT groups. ** $P < 0.01$ vs. sham group, ## $P < 0.01$ vs. pacing group. BH4: tetrahydrobiopterin. (E) nNOS, iNOS and eNOS mRNA expression levels in the sham, pacing, β_3 -AGO and β_3 -ANT groups. * $P < 0.05$ vs. sham group, ## $P < 0.01$, # $P < 0.05$ vs. pacing group. (F) Western blot analysis of nNOS, iNOS and eNOS expression using specific nNOS, iNOS and eNOS antibodies. (G) nNOS, iNOS and eNOS protein expression in the sham, pacing, β_3 -AGO and β_3 -ANT groups. * $P < 0.05$ vs. sham group, # $P < 0.05$ vs. pacing group. β_3 -AGO refers to the β_3 -AR agonist BRL37344 group, and β_3 -ANT refers to the β_3 -AR antagonist L748337 group.

these effects (BH4, 0.173 ± 0.029 pmol/mg protein vs. 0.118 ± 0.021 pmol/mg protein, $P < 0.01$; Fig. 5D) (GCH-1 upregulation by L748337 is shown in Fig. 6; $P < 0.01$).

Fig. 6. GCH-1 mRNA and protein levels in atria of the sham, pacing, β_3 -AGO and β_3 -ANT groups. (A) GCH-1 mRNA levels in atria of the sham, pacing, β_3 -AGO and β_3 -ANT groups. * $P < 0.05$ vs. sham group, ## $P < 0.01$ vs. pacing group. (B) Western blot analysis of GCH-1 protein expression in the sham, pacing, β_3 -AGO and β_3 -ANT groups using specific GCH-1 antibodies. (C) Quantitative analysis of GCH-1 protein in all groups. ** $P < 0.01$ vs. sham group, ## $P < 0.01$ vs. pacing group.

Both iNOS mRNA and protein expression were increased in the pacing group compared to the sham group ($P < 0.05$) (Fig. 5E, F and G). iNOS expression was also increased by the β_3 -AR agonist and suppressed by the β_3 -AR antagonist in the pacing groups compared to pacing alone ($P < 0.05$).

We further demonstrated that nNOS expression (mRNA and protein) was increased ($P < 0.05$) and eNOS (mRNA and protein) expression was decreased ($P < 0.05$) in the pacing groups compared to the sham group (Fig. 5E, F, and G). Parallel to other findings, the β_3 -AR agonist BRL37344 markedly reduced eNOS expression ($P < 0.05$), whereas the β_3 -AR antagonist L748337 reversed these effects ($P < 0.05$) (Fig. 5E, F, and G). Interestingly, nNOS levels did not vary among the pacing groups in the presence of the β_3 -AR agonist and antagonist ($P > 0.05$) (Fig. 5E, F, and G).

β_3 -AR stimulation resulted in superoxide anion generation, which could be decreased by the specific iNOS inhibitor

Using a superoxide colorimetric assay, we demonstrated that atrial homogenates had increased superoxide production in the β_3 -AR agonist group (0.064 ± 0.008 $\mu\text{mol}/\text{mg}$ protein vs. 0.099 ± 0.024 $\mu\text{mol}/\text{mg}$ protein, $P < 0.01$) and that these effects were inhibited in the β_3 -

Fig. 7. Quantitative analysis of superoxide anion production in the sham, pacing, β_3 -AGO and β_3 -ANT groups using a colorimetric assay. (A) Comparisons of superoxide levels were directly assayed after tissue collection in the sham, pacing, β_3 -AGO and β_3 -ANT groups and after incubation with the iNOS inhibitor 1400w. **P < 0.01. (B) Comparisons of total superoxide levels amongst all groups. The results were subtracted from the total levels to obtain the iNOS-dependent superoxide levels. **P < 0.01 vs. sham group, ##P < 0.01 vs. pacing group.

AR antagonist group (0.046 ± 0.006 $\mu\text{mol/mg protein}$ vs. 0.064 ± 0.008 $\mu\text{mol/mg protein}$, $P < 0.01$) (Fig. 7), which was consistent with the DHE fluorescence results (Fig. 5B). Interestingly, pretreatment of the atrial homogenates with 100 μM of the specific iNOS inhibitor 1400W abolished the increase in superoxide production observed with β_3 -AR agonist stimulation (0.062 ± 0.025 $\mu\text{mol/mg protein}$ vs. 0.099 ± 0.024 $\mu\text{mol/mg protein}$, $P < 0.01$) (Fig. 7).

Discussion and Conclusions

This present study revealed for the first time that β_3 -AR activation exacerbates atrial structural remodeling by activating iNOS uncoupling/oxidative stress, which could be considered a novel therapeutic target for AF.

β_3 -AR activation promoted AF by aggravating atrial structural remodeling and dysfunction

β_3 -AR belongs to a group of G protein-coupled receptors characterized by seven transmembrane domains having features that determine their resistance to long-term downregulation and significance in regulating cardiac performance following acute and chronic stress. β_3 -AR levels were upregulated, which aggravated cardiac dysfunction during heart failure [10, 11]. Additionally, in humans and dogs, β_3 -AR is detected in lung and peripheral arteries and causes vasodilation [22, 23]. Therefore, β_3 -AR had beneficial effects in perinephritic hypertension [24]. Meanwhile, β_3 -AR stimulation has antiarrhythmic effect in canine ventricular tachycardia [25]. Different levels of β_3 -AR in the cardiac chambers at different stages of the pathologies and various downstream pathways may determine the functional characterization of β_3 -AR in different cardiovascular diseases.

In our study, we observed an upregulation of β_3 -AR in AF canines induced by rapid atrial pacing. β_3 -AR stimulation further increased atrial apoptosis and interstitial fibrosis, indicative of an exacerbation of atrial structural remodeling. Atrial structural remodeling in turn promotes AF development and self-persistence. Apoptosis and interstitial fibrosis during atrial structural remodeling are both considered potential therapeutic targets for AF [26-31]. Subsequent apoptosis and reparative fibrosis replaces dead cardiomyocytes, which

interrupts fiber bundle continuity, thereby slowing conduction [29, 32, 33]. Therefore, atrial structural remodeling has a strong impact on electrical remodeling. Our study also indicated that β_3 -AR activation increased AF inducibility and prolonged AF duration, which might be associated with the exacerbating effect of β_3 -AR on atrial structural remodeling. Yu and colleagues recently highlighted that β_3 -AR activation promotes atrial electrical remodeling by decreasing the abundance of L-type calcium channels as well as increasing the inward rectifier potassium current (I_{K1}) and transient outward potassium current (I_{to}) in acute AF models of rabbit and atrial myocytes [12]. Furthermore, Liu et al. recently demonstrated that β_3 -AR activation disturbed cardiac energy metabolism and aggravated AF in a rabbit AF model [34]. Moreover, in this present study, therapies using the β_3 -AR antagonist L748337 attenuated atrial apoptosis and interstitial fibrosis in AF canines, which prevented AF development.

Generally, atrial dilatation enhances cardiac vulnerability to AF, which in turn results in progressive atrial dilatation. Therefore, AF and atrial dilatation are mutually promoting and constitute a vicious circle. Atrial dilatation results in contractile dysfunction associated with typical features of AF [35]. Atrial size is a key determinant of AF reentry [36]. Therefore, therapies targeted at reducing atrial size may prevent AF. Treatment with the β_3 -AR antagonist L748337 in our AF model resulted in decreased atrial volume and attenuation of impaired atrial function, which was effective in preventing AF.

β_3 -AR activation increased iNOS uncoupling and oxidative stress

Oxidative stress is a major underlying pathology that promotes AF [13]. Excess ROS can result in a loss of enzyme function, mitochondrial dysfunction and cellular death, all of which strongly correlate with atrial structural remodeling [13]. Thus, inhibition of cardiac ROS may be effective in circumventing AF. Of the numerous cellular sources of ROS, uncoupled NOS is the major source in the human heart [13, 37].

NOS uncoupling is involved in many pathologies, such as diabetes mellitus, hypertension, ageing, and atherosclerosis [38], all of which were associated with AF. In the process of NO production, the L-arginine concentration does not affect the reaction [39-41], although low BH4 bioavailability relative to NOS results in uncoupled NOS and excessive superoxide production [14]. In this present study, we observed a decrease in BH4 levels, an increase in atrial myocardial superoxide anion levels and a decrease in NO production by β_3 -AR stimulation in the AF canine model. This indicates that β_3 -AR activation induces NOS uncoupling and oxidative stress.

We measured endogenous changes in the three isoforms of NOS following β_3 -AR stimulation in AF canines to investigate which isoform dominated the effects of β_3 -AR on NOS uncoupling. The heart normally expresses the constitutive enzymes nNOS and eNOS, whereas iNOS is inducibly expressed in pathological states [15, 42]. Additionally, iNOS is closely associated with inflammation [15] and oxidative stress [37], both of which are involved in the atrial structural remodeling in AF [43]. Han and colleagues also reported that iNOS protein levels are increased in the atria of patients with permanent AF [44].

β_3 -AR mediates negative inotropic effects on the ventricular myocardium by eNOS activation [45, 46]. In contrast, β_3 -AR activation increases the contractility of the human right atrium [45]. Moreover, we revealed that BRL37344 inhibited eNOS expression in the atrium of AF canines. Different effects of β_3 -AR on the atria and ventricle occur potentially due to lower β_3 -AR expression in the atria [47] and different activation mechanisms of β_3 -AR on eNOS [48]. Additionally, β_3 -AR activation mediated anti-hypertrophic and antioxidant effects on hypertrophic ventricular cardiomyocytes via a nNOS-dependent mechanism [49]. In contrast, our data revealed that BRL37344 activated iNOS but did not influence nNOS levels in the AF canine model. Moreover, the specific iNOS inhibitor 1400W abolished the induction of superoxide production by the agonist of β_3 -AR, which validated our previous hypothesis. Consequently, mediation of different signaling pathways may determine the diverse performance of β_3 -AR in atria and ventricles. Therefore, β_3 -AR stimulation aggravated atrial oxidative stress, which likely resulted from iNOS activation. The importance of iNOS was

Fig. 8. Schemata of the mechanisms underlying the role of β_3 -AR stimulation in atrial structural remodeling associated with AF.

further validated by our β_3 -AR antagonist, L748337, which reversed the aberrant expression of NOS isoforms.

BH4 is synthesized from guanosine triphosphate (GTP) by GTP cyclohydrolase, with GCH-1 acting as the rate-limiting enzyme [14]. Therefore, BH4 concentrations strongly correlate with GCH-1 expression. This present study showed that BH4 down-regulation by BRL37344 may occur due to reduced levels of GCH-1. Animal studies have confirmed that BH4 supplementation can reduce oxidative stress [50] and attenuate cardiac remodeling post-infarction [51]. However, due to the high cost and poor feasibility of BH4 in clinical practice, it is not utilized here in this study. β_3 -AR inhibition with L748337 in this study increased GCH-1 and BH4 levels, indicating a more feasible therapy for reducing atrial oxidative stress and structural remodeling in AF.

In conclusion, our study demonstrates that β_3 -AR activation triggers a cascade of events resulting in atrial structural remodeling (Fig. 8), and these effects might be partially attributed to iNOS uncoupling and oxidative stress in AF.

Clinical implications

The β_3 -AR antagonist L748337 was clearly effective in suppressing the progression of atrial structural remodeling, and the specific iNOS inhibitor attenuated atrial oxidative stress. Thus, both mediators have an important effect on the arrhythmogenic substrate for AF and could have therapeutic roles in clinical practice. Therefore, this present study revealed two targets for AF treatment.

Abbreviations

AF (Atrial fibrillation); AI (Apoptotic index); B (Biopterin); BH4 (Tetrahydrobiopterin); BH2 (Dihydrobiopterin); DHE (Cell-permeable dihydroethidium); β_3 -AR (β_3 adrenergic receptor); β_3 -AGO (β_3 adrenergic receptor agonist); β_3 -ANT (β_3 adrenergic receptor antagonist); CVF (Collagen volume fraction); eNOS (Endothelial NOS); GCH-1 (Guanosine triphosphate cyclohydrolase-1); HPLC (High-performance liquid chromatography); HE (Hydroethidium); HRP (Horseradish peroxidase); iNOS (Inducible NOS); LA (Left atrium); LAA (Left atrial appendage); LAVmax (LA maximal volume); LAAVmax (LAA maximal volume); LAVmin (LA minimal volume); LAAVmin (LAA minimal volume); LAEF (LA ejection fraction); LAAEF (LAA ejection fraction); NO (Nitric oxide); nNOS (Neuronal NOS); NOS

(Nitric oxide synthase); NADPH (Nicotinamide adenine dinucleotide phosphate); ROS (Reactive oxygen species); TBST (Tris-buffered saline and tween 20); WB (Western blot); PVDF (Polyvinylidenedifluoride); O_2^- (Superoxide anion).

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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