**Valsartan Reduced Atrial Fibrillation Susceptibility by Inhibiting Atrial Parasympathetic Remodeling through MAPKs/Neurturin Pathway**

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**Key Words**
Parasympathetic remodeling • Neurturin • Atrial fibrillation • Angiotensin II • Valsartan • MAPKs

**Abstract**

**Background/Aims:** Angiotensin II receptor blockers (ARBs) have been proved to be effective in preventing atrial structural and electrical remodeling in atrial fibrillation (AF). Previous studies have shown that parasympathetic remodeling plays an important role in AF. However, the effects of ARBs on atrial parasympathetic remodeling in AF and the underlying mechanisms are still unknown. **Methods:** Canines were divided into sham-operated, pacing and valsartan + pacing groups. Rats and HL-1 cardiomyocytes were divided into control, angiotensin II (Ang II) and Ang II + valsartan groups, respectively. Atrial parasympathetic remodeling was quantified by immunocytochemical staining with anti-choline acetyltransferase (ChAT) antibody. Western blot was used to analysis the protein expression of neurturin. **Results:** Both inducibility and duration were increased in chronic atrial rapid-pacing canine model, which was significantly inhibited by the treatment with valsartan. The density of ChAT-positive nerves and the protein level of neurturin in the atria of pacing canines were both increased than those in sham-operated canines. Ang II treatment not only induced atrial parasympathetic remodeling in rats, but also up-regulated the protein expression of neurturin. Valsartan significantly prevented atrial parasympathetic remodeling, and suppressed the protein expression of neurturin. Meanwhile, valsartan inhibited Ang II-induced up-regulation of neurturin and MAPKs in cultured cardiac myocytes. Inhibition of MAPKs dramatically attenuated neurturin up-regulation induced by Ang II. **Conclusion:** Parasympathetic remodeling was present in animals subjected to rapid...
pacing or Ang II infusion, which was mediated by MAPKs/neurturin pathway. Valsartan is able to prevent atrial parasympathetic remodeling and the occurrence of AF via inhibiting MAPKs/neurturin pathway.

Introduction

Atrial fibrillation (AF) is the most common and challenging arrhythmia with many etiologies and multiple treatment options in clinical practice [1]. Angiotensin II (Ang II), a main bioactive component of the renin-angiotensin system (RAS), plays a crucial role in AF [2]. Ang II receptor blockers (ARBs) have been proven to have preventive effect on AF by ameliorating atrial structural and electrical remodeling in addition to their anti-hypertension activities [3]. However, the detailed mechanisms of preventive and therapeutic effects of ARBs on AF are not fully understood.

Accumulative evidence suggests that atrial autonomic nervous system (ANS) remodeling is involved in the pathogenesis of AF, which is manifested by increased sympathetic and parasympathetic activity [4-7]. Heightened atrial sympathetic innervation has been found in persistent AF patients and sustained AF canine model [8, 9], and angiotensin II (Ang II) plays an important role in atrial sympathetic remodeling [9-11]. A growing body of clinical and experimental studies has showed that ARBs could down regulate sympathetic adrenergic activity by blocking the effects of Ang II on sympathetic nerve release and reuptake of norepinephrine [12, 13]. Recently, evidence indicates that atrial parasympathetic hyperinnervation contributed to AF maintenance in congestive heart failure dogs [14]. But whether atrial parasympathetic remodeling is present in AF and the effects of ARBs on atrial parasympathetic remodeling are still unknown.

Neurturin (NRTN), a member of the glial cell line-derived neurotrophic factor (GDNF) family, has been proved to be essential for the development and survival of parasympathetic neurons [15, 16]. Gene deletion of NRTN or either component of NRTN receptor could disrupt the development and function of parasympathetic nerve in the rodent heart [17-19]. However, it is unclear whether the change of NRTN expression exists in atria during AF.

Mitogen-activated protein kinases (MAPKs) are important mediators in Ang II-induced cardiac structural remodeling in AF [20]. The three best-characterized MAPKs superfamily members are p38 MAPK, extracellular signal-regulated kinase 1/2 (ERK1/2) and c-Jun N-terminal kinases (JNK). MAPKs could regulate the expression of GDNF while the regulatory effect on NRTN is still unknown [21].

In the present study, we aim to use immunohistochemical techniques to analyze atrial parasympathetic nerve density in different AF animal models and determine the effects of valsartan, one of the mostly used ARBs on atrial parasympathetic remodeling. We also attempt to elucidate the underlying molecular mechanisms by evaluation the expression of NRTN and MAPKs.

Materials and Methods

Preparation of animal models susceptible to AF

All animal procedures were approved by the Institutional Animal Care Committee of the First Affiliated Hospital of Harbin Medical University. The entire experiments performed in the present study were designed following the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Twenty-one mongrel canines of either sex (Experimental Animal Center of the First Affiliated Hospital, Harbin Medical University, Harbin, China), weighting 15 to 25 kg, were randomly divided into sham-operated (SO), pacing and pacing + valsartan (valsartan) groups. As described previously, canines in the pacing and valsartan groups were paced at 400 beats per minutes for 6 weeks [22]. Valsartan was administered orally
(30 mg/kg/day, Diovan, Novartis Pharmaceuticals Corp., Basel, Switzerland) one week before pacing and continued until completion of pacing procedure.

Fifteen male Wistar rats (weight 200-250g) (Experimental Animal Center of the First Affiliated Hospital, Harbin Medical University, Harbin, China) were randomly divided into control, Ang II and Ang II + valsartan (valsartan) groups. Ang II (2 mg/kg/day, Sigma-Aldrich, St. Louis, MO, USA) was infused into the rats with a subcutaneously implanted osmotic minipump (Alzet, Cupertino, CA, USA) for 14 days. In addition, the rats in the valsartan group were orally administered with valsartan (40mg/kg/day) during the Ang II infusion period.

**Cell culture and treatment**

HL-1 cardiomyocytes (ATCC, Manassas, VA, USA) were cultured in Claycomb medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 100 μM norepinephrin, 10% fetal bovine serum (PAA Laboratories GmbH, Linz, Austria), 4 mM L-glutamine (Gibco, Grand Island, NY, USA) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin, Hyclone, Logan, UT, USA), then incubated in humidified 5% CO₂ atmosphere at 37°C, and the medium was changed every 2-3 day.

To investigate the role of valsartan in the regulation of NRTN expression induced by Ang II *in vitro*, cells were randomly divided into control group (maintained in Claycomb medium), Ang II group (treated with 1μM Ang II for 48 hours) and Ang II + valsartan (valsartan) group (treated with 1μM Ang II and 10μM valsartan, Sigma-Aldrich, St. Louis, MO, USA for 48 hours).

The effects of MAPKs on the expression of NRTN induced by Ang II were also evaluated. Cells were randomly divided into control group (maintained in Claycomb medium), Ang II group (treated with 1μM Ang II for 48 hours), Ang II plus MAPKs inhibitors group (treated with 1μM Ang II and inhibitors of each MAPK for 48 hours) and MAPKs inhibitors group (treated with individual MAPKs inhibitors only). MAPKs inhibitors included: SB203580 (inhibitor of p38 MAPK, 10μM, Sigma-Aldrich, St. Louis, MO, USA), PD98059 (inhibitor of ERK1/2, 10μM, Sigma-Aldrich, St. Louis, MO, USA) and SP600125 (inhibitor of JNK, 30μM, Sigma-Aldrich, St. Louis, MO, USA).

**Electrophysiological measurements of AF**

In canine model, AF inducibility and duration were measured 6 weeks after continuous rapid atrial pacing before and after pharmacological blockade with atropine in all groups. Atrial burst pacing lasting for 10 seconds at a pacing cycle length of 100 milliseconds was used for 10 times to assess the inducibility and duration of AF [22]. To examine the effect of parasympathetic nerve on AF vulnerability, AF inducibility and duration were examined before and after blockade of parasympathetic nerve with intravenous injection of atropine (0.04 mg/kg, Tianjin Pharmaceutical Group Co. Ltd., China).

In rat model, short episodes of AF were induced every 2 minutes by open-chest burst pacing (83Hz) of RA for 30 seconds followed by 90 seconds of intrinsic heart rhythm. Inducibility of AF was defined as the induction rate of 10 times’ burst pacing [23, 24].

**Immunohistochemical studies**

PowerVision™ two-step immunohistochemical staining method was performed on sections taken from the right atrial posterior wall (RAPW), left atrial posteriormedial wall (LAPW), right atrial anterior wall (RAAW) and left atrial anterior wall (LAAW). Cardiac ganglia were defined as nerve bundles containing 1 or more neuronal cell bodies [25, 26]. Briefly, the deparaffinized and hydrated sections were incubated with primary anti-choline acetyltransferase (ChAT, MILLIPORE, Boston, MA, USA) antibody overnight at 4°C, and then with secondary antibody (IgG-HRP conjugates, Zhongshan, Beijing, China) at 37°C for 20 minutes. The tissue sections were subsequently visualized with substrate DAB (Diaminobenzidine) and counterstained with haematoxylin. The slides were coded so that the investigator who counted the nerves was blinded to the canine identification at the time of nerve count. Quantitation of the immunohistochemical staining of atrial parasympathetic nerve was analyzed by using Image-Pro Plus version 6.0 (Media Cybernetics, Rockville, MD, USA). Mean density of cardiac ganglia was calculated by the ChAT-positive nerve area divided by the cardiac ganglia area examined [14]. We analyzed 15 to 30 fields to cover the entire region of canine tissue section. Mean nerve density was calculated by the average of all fields. The 3 highest and the 3 lowest nerve density fields in the tissue section were chosen, and the difference of these averages was also calculated and defined as regional heterogeneity of the slide [27, 28].
Western blot analysis

Proteins were extracted from tissues or cells by using RIPA lysis buffer (Higene, Shanghai, China). Total protein (50 μg) was fractionated by SDS-PAGE and transferred onto nitrocellulose filter membranes. The membranes were incubated overnight at 4°C with different primary antibodies to the following target proteins: NRTN (1:500, abcam, Cambridge, MA, USA), muscarinic acetylcholine receptor M2 (M2ACh-R, 1:200, Bioss, Beijing, China), p38 MAPK (1:1000, abcam, Cambridge, MA, USA) phospho-p38 MAPK (P-p38 MAPK, 1:1000, abcam, Cambridge, MA, USA), ERK1/2 (1:500, abcam, Cambridge, MA, USA), phospho-ERK1/2 (P-ERK1/2, 1:500, abcam, Cambridge, MA, USA), JNK1:200, Bioss, Beijing, China), phospho-JNK (p-JNK, 1:200, Bioss, Beijing, China), GAPDH (1:2000, Santa Cruz, Dallas, Texas, USA) and β-actin (1:2000, Santa Cruz, Dallas, Texas, USA). Subsequently, the membranes were rinsed and incubated with horseradish peroxidase-conjugated secondary antibodies (Higene, Shanghai, China) for 2 hours at 37°C. Band intensity was quantified by Quantity One Software (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

Statistical analysis was performed by using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). All data were expressed as mean ± SEM. Two-group comparisons were performed by Student’s t-test. Multiple-group comparisons were carried out using one-way ANOVA followed by Dunnett post-hoc test. The Pearson’s product-moment correlation analysis was used to determine the relationship between the levels of NRTN protein and the mean density of parasympathetic nerves. A value of P < 0.05 was considered statistically significant.

Results

Inducibility of AF in canines and rats subjected to rapid pacing and Ang II infusion

In atrial rapid pacing canine model, the inducibility and duration of AF were both dramatically increased in the pacing group compared with the SO group, which were inhibited by the treatment with valsartan (Fig. 1A, B). We also found that the increased AF inducibility and AF duration could be significantly reduced by atropine treatment (Fig. 1A, B).

In Ang II infused rat model, AF inducibility was much higher in the Ang II group than that in the control group, which was mitigated by valsartan (Fig. 1C).

Parasympathetic remodeling in rapid pacing induced canine AF model

As shown in Fig. 2A, B, the mean density of ChAT-positive cardiac ganglia in RAPW and LAPW and nerve fibers in RAAW and LAAW were both higher in the pacing group than those in the SO group, which were alleviated by the treatment with valsartan. The regional heterogeneity of ChAT-positive nerve in the pacing group was also higher than that in the SO group (Fig. 2C). Valsartan treatment also significantly diminished the regional heterogeneity of ChAT-positive nerve in both RA and LA (Fig. 2C).

Expression of M2ACh-R and NRTN in rapid pacing induced canine AF model

Compared with the SO group, the protein level of M2ACh-R was increased in RA and LA in the pacing group, and valsartan treatment dramatically decreased the expression of M2ACh-R in both RA and LA (Fig. 3A, B).

After pacing, the protein level of NRTN was increased, and valsartan treatment significantly decreased the expression of NRTN in both RA and LA (Fig. 3C, D). Moreover, the protein level of NRTN was positively correlated with the mean density of parasympathetic nerve fibers in all groups (r = 0.7351, P < 0.01 for RA and r = 0.7075, P < 0.01 for LA).

Parasympathetic remodeling and NRTN expression in Ang II infused rat model

The mean densities of ChAT-positive nerves in atria were markedly higher in the Ang II group than that in the control group (Fig. 4). The Ang II induced atrial parasympathetic hyperinnervation was significantly reduced after treatment with valsartan (Fig. 4).
Consistently, the level of NRTN protein expression was also higher in Ang II group than that in the control group, which was dramatically suppressed by valsartan treatment (Fig. 5).

**MAPKs mediated the over-expression of NRTN in Ang II incubated cells**

Consistent with animal experiment data, the expression of NRTN protein in HL-1 cardiomyocytes was obviously increased in the Ang II-treated group, which was attenuated by valsartan treatment (Fig. 6A). In addition, valsartan inhibited the over-expression of P-p38 MAPK, P-ERK1/2 and P-JNK that were induced by Ang II treatment (Fig. 6B, C, D). Inhibitors of p38 MAPK, ERK1/2 and JNK significantly suppressed the up-regulation of NRTN induced by Ang II, respectively (Fig. 7A, B, C).

**Discussion**

**Major findings**

The present study showed that significant parasympathetic nerve sprouting and heterogeneous hyperinnervation were present both in prolonged atrial rapid pacing canine model and Ang II infusion rat model. Atrial parasympathetic remodeling was accompanied by the up-regulation of M2ACh-R and NRTN, and a positive correlation was revealed between atrial parasympathetic nerve density and the level of NRTN protein in canine models. Valsartan treatment prevented atrial parasympathetic remodeling by attenuating the up-regulation of NRTN.

It is interesting to notice that valsartan also inhibited the up-regulation of NRTN and MAPKs induced by Ang II in HL-1 cardiomyocytes. Inhibitors of MAPKs suppressed the up-regulation of NRTN induced by Ang II, which suggested that MAPKs/NRTN signaling...
pathway may contribute to the processes of parasympathetic remodeling of the atria. To the best of our knowledge, this is the first study to show the inhibiting effect of valsartan on atrial parasympathetic nerve sprouting and heterogeneous hyperinnervation, and reveal the molecular mechanism responsible for the development of atrial parasympathetic remodeling in AF animal models.

**Atrial parasympathetic remodeling and AF**

Previous studies suggested that the parasympathetic nerve plays a critical role in the dynamics of AF initiation and maintenance [11]. Parasympathetic stimulation has been demonstrated to be proarrhythmic in the atrium through refractory period shortening and increased heterogeneity of repolarization [29]. Hyperactivity of the parasympathetic system could cause the release of excessive amount of acetylcholine from presynaptic nerve terminals in the atrium [30]. Acetylcholine induces activation of the inhibitory G proteins by combining with M2ACh-R and subsequently results in dramatic decrease in the action potential duration (APD) and refractoriness of the atria [31]. M2ACh-R, which was considered as the most important muscarinic acetylcholine receptor in the heart, could induce the G protein coupled inward rectifier K+ channel (I K_Shr) [31]. Activation of atrial I K_ACh leads to a potent atrial effective refractory period (AERP) shortening and promotes atrial arrhythmias [32].
Previous findings hint that atrial parasympathetic nerve distribution pattern could alter atrial electrophysiology [26]. The regional differences in the distribution of the parasympathetic innervation could result in the nonuniformity of acetylcholine concentration and shortening of AERP when parasympathetic nerve was stimulated [33]. Lomax et al. reported that the heterogeneous distribution of parasympathetic ganglia when combined with the heterogeneity of \(I_{K_{ACH}}\) current density in atria may augment the dispersion of atrial refactoriness [34].

In the present study, we found chronic rapid atrial pacing resulted in a significant increase in the density of nerve fibrils, cardiac ganglías containing parasympathetic neuronal cell bodies, and the heterogeneity of atrial parasympathetic innervation in canine atria. Furthermore, the level of M2ACHE-R in canine atria was significantly higher in pacing group than that in SO group. These findings indicate that parasympathetic remodeling changed the parasympathetic distribution patterns and increased the vulnerability of AF. To explore the effect of parasympathetic on AF electrophysiological parameters, we measured AF inducibility and duration before and after blocking parasympathetic nervous system with atropine. We found that, atropine pretreatment markedly decreased AF susceptibility of canines subjected to long-term rapid pacing. These data suggested that parasympathetic hyperinnervation enhanced the susceptibility to AF.

MAPKs/NRTN pathway participates in atrial parasympathetic remodeling in AF

Neurotrophic factors were required for the maintenance of cardiac innervation. It has been illustrated that NGF plays a crucial role in regulating the sympathetic innervation of the heart and the excessive NGF production leads to sympathetic hyperinnervation [35].
Fig. 4. Effect of valsartan on the atrial parasympathetic remodeling by immunohistochemical staining of ChAT in Ang II infusion rat. (A), (B), (C) Atrial parasympathetic nerve in control, Ang II and Ang II +valsartan groups. (D) Statistical analysis of atrial parasympathetic nerve. Data are expressed as mean ± SEM (n=5). * P < 0.05 vs. control group, # P < 0.05 vs. Ang II group. The arrows indicate the ChAT-positive staining nerve fibers.

Fig. 5. Changes of NRTN expression in Ang II infused rat model. Data are expressed as mean ± SEM (n=5). * P<0.05 vs. control group, # P < 0.05 vs. Ang II group.

In contrast, much less is known about neurotrophic factor requirements of cardiac parasympathetic innervation. Recent findings suggest that NRTN plays a critical role in the development and survival of cardiac parasympathetic neurons. NRTN acts preferentially through GDNF family receptor α2 (GFRα2) and the transmembrane Ret tyrosine kinase. Both components of the NRTN receptor are expressed in adult cardiac parasympathetic neurons, and deletion of either components (i.e. GFRα2 or Ret) will destroy the development of cholinergic parasympathetic innervation of the heart [18, 19]. Studies in animal models revealed that hearts from NRTN knockout mice contained only 35% the normal number of cholinergic neurons, and the residual cholinergic neurons were 15% smaller than that in wild type mice [18]. These experiments provide evidence that NRTN has an essential role in normal cholinergic innervation of the heart. Our findings indicated that chronic atrial rapid pacing resulted in significant up-regulation of atrial NRTN expression, which is positively correlated with the density of parasympathetic nerve in AF canines. We also discovered that
Fig. 6. Effects of Ang II and valsartan on the expression of NRTN (A), P-p38 MAPK (B), P-ERK1/2 (C) and P-JNK (D) in HL-1 cardiomyocytes. Data are expressed as mean ± SEM. * P < 0.05 vs. control group, * P < 0.05 vs. Ang II group.

Fig. 7. Effects of inhibitors of p38 MAPK (A), ERK (B) and JNK (C) on the expression of NRTN in HL-1 cardiomyocytes. Data are expressed as mean ± SEM. ** P < 0.01 vs. control group, * P < 0.05 vs. control group, ** P < 0.01 vs. Ang II group, * P < 0.05 vs. Ang II group.

Ang II could up-regulate the expression of NRTN in both an in vivo rat model and cultured HL-1 cardiomyocytes.

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MAPKs, which participate in atrial structural remodeling in AF, have been demonstrated to regulate GDNF expression [20]. In this study we found that, the expressions of p38 MAPK, ERK1/2 and JNK were up-regulated accompanied with NRTN in HL-1 cardiomyocytes treated with Ang II. When the MAPKs were inhibited, the up-regulation of NRTN was canceled, suggesting that MAPKs act as the upstream regulators of NRTN expression in Ang II-treated atrial myocytes and activation of the MAPKs/NRTN pathway plays an important role in atrial parasympathetic remodeling.

Effects and underlying mechanism of valsartan on atrial parasympathetic remodeling in AF

In light of the important role of the ANS in the creation of AF substrate, a variety of strategies by targeting at autonomic denervation have been developed in recent years [36-38], while specific and accurate surgical methods also had the added disadvantage resulting from transmural atrial tissue damage [39]. The ideal therapeutic technique should be more precise and safe in targeting the nerves involved in the genesis of AF.

There has been compelling evidence supporting the important role of RAS in AF through atrial remodeling. Inhibition of the RAS with ARBs has been shown to be beneficial in preventing AF by attenuating atrial structural remodeling mediated by MAPKs [40]. Our previous studies showed that valsartan, one of the most commonly used ARBs, could suppress atrial structural remodeling by inhibiting the up-regulation of calpain I and prevent the induction and promotion of AF in chronic atrial rapid-pacing dogs [22]. Besides, valsartan could down-regulate cardiac sympathetic nerve activity and left ventricular performance in patients with CHF [13].

The present study provides the first insight into the effects of valsartan on atrial parasympathetic remodeling in AF. We found that valsartan could prevent parasympathetic remodeling by down-regulating NRTN expression both in long-term atrial rapid pacing canine model and Ang II infused rat model. Meanwhile, we demonstrated that valsartan could significantly suppress the over-expression of NRTN induced by Ang II via MAPKs-dependent pathway.

Limitation

Further studies such as electrophysiological measurements under parasympathetic stimulation are required to identify the role of parasympathetic remodeling in contributing to electrical remodeling in AF and the direct effect of valsartan on MAPKs/NRTN pathway in vivo were also need to be clarified.

Conclusions

The present study demonstrated that chronic atrial tachypacing and Ang II infusion could induce atrial parasympathetic remodeling. The up-regulation of MAPKs/NRTN pathway participated in the atrial parasympathetic remodeling process. Valsartan could inhibit parasympathetic remodeling via down-regulating MAPKs/NRTN pathway, which may represent a novel approach to modify autonomic influences on AF.

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Disclosures Statement

The authors declare no conflicts of interest.
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