

# Electrical and histological remodeling of the pulmonary vein in 2K1C hypertensive rats: Indication of initiation and maintenance of atrial fibrillation

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## ABSTRACT

**Objective:** Hypertension is a significant risk factor for atrial fibrillation (AF). The role of pulmonary vein (PV) remodeling in the mechanistic association between hypertension and AF is not definitive. In this study, we aimed to identify changes in the electrophysiology and histology in PVs in two-kidney, one-clip (2K1C) hypertensive rats.

**Methods:** Fifty male Sprague-Dawley rats were classified into the 2K1C and sham-operated groups. The systolic blood pressure was measured every 2 weeks. The left atrial diameter was measured by transthoracic echocardiography. Left superior PV (LSPV) and left atrial (LA) fibrosis was evaluated by Masson's trichrome staining. The expression of fibrosis markers [angiotensin II (Ang II), transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), matrix metalloproteinase-2 (MMP-2), and collagen I (Col I)] and ion channels [Kir2.1, Kir2.3, Ca<sub>v</sub>1.2, and Na<sub>v</sub>1.5] in LSPV was quantified by western blot. Conventional microelectrodes were used to record the action potential duration at 90% repolarization (APD90) and effective refractory period (ERP) in isolated LA.

**Results:** At 4 months, the 2K1C hypertensive rats developed LA dilation. Col deposition in LSPV and left atrium and expression of TGF- $\beta$ 1, MMP-2, and Col I in LSPV were significantly increased in 2K1C hypertensive rats. In addition, hypertension reduced the expression of Na<sub>v</sub>1.5 and Kir2.1, although there were no significant differences in APD90; ERP; and expression of Ang II, Kir2.3, and Ca<sub>v</sub>1.2 between the two groups.

**Conclusion:** Hypertension may lead to changes in the electrophysiology and histology of rats PVs, which is characterized by significant reduction in the expression of Na<sub>v</sub>1.5 and Kir2.1 and increase in interstitial fibrosis. These observations may clarify the role of PVs in the mechanistic association between hypertension and AF. (*Anatol J Cardiol* 2018; 19: 169-75)

**Keywords:** atrial fibrillation, fibrosis, hypertension, pulmonary vein, remodeling

## Introduction

The relationship between atrial fibrillation (AF) and hypertension has been established in epidemiological as well as histological studies (1-5). Clinical and animal studies have shown that hypertension can lead to histological and electrophysiological remodeling of the atrial tissue, which results in easier maintenance of AF (1, 5). Previous studies have demonstrated that spontaneous ectopic activity in the pulmonary veins (PVs) plays a critical role in the initiation of AF (6-8). However, most previous studies on associations between hypertension and AF have specifically focused on the atria. There is currently a lack of data on the arrhythmogenic role of PVs in the direct link between hypertension and AF.

Histological and electrophysiological remodeling of PV has some links with AF, including PV interstitial fibrosis and altered expression or function of cardiac ion channels (9). Some cardiac diseases modify the operation of ion channels in a way that promotes the occurrence of cardiac rhythm disturbances; the process is termed as "arrhythmogenic remodeling" (10). Therefore, changes in the ion channels may play an important role during the arrhythmogenic process.

We hypothesized that hypertension leads to AF not only through atrial remodeling but also through PV arrhythmogenesis. We investigated the effects of hypertension on histological and electrical remodeling of PV in a rat model. It has been reported that expression of collagen I (Col I), matrix metalloproteinase-2

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(MMP-2), and transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), which imply fibrosis of the tissue, and expression of Na<sub>v</sub>1.5 and Kir2.1 channel protein are indicators of electrical remodeling. In the present study, interstitial fibrosis, indicated by the expression of Col I, MMP-2, and TGF- $\beta$ 1, and the cardiac ion channels level at PV were evaluated.

## Methods

### Animal model of hypertension

All experiments 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 1996). Male Sprague-Dawley rats (180–200 g, n=50) were maintained in a 12-h light/dark cycle at 22°C with free access to rat chow and water.

Two-kidney, one-clip (2K1C) hypertension was induced in rats, as described previously (11). The rats were randomly assigned to 2K1C (n=30) and sham-operated (n=20) groups, and anesthetized using chloral hydrate (300 mg/kg, intraperitoneally). After a mid-line laparotomy, a silver clip with an internal diameter of 0.20 mm was placed around the left renal artery close to the aorta. Sham-operated rats underwent the same surgical procedure, except for the placement of the silver clip. Tail systolic blood pressure (SBP) was measured by tail-cuff plethysmography (BP-2000, Visitech Systems, USA) every 2 weeks. The mean of three or four measurements was used as the blood pressure estimate, and the rats were considered to be hypertensive if their SBP exceeded 160 mm Hg after 2 weeks of ligation (11). Approximately 20% of the operated rats failed to achieve this level of SBP.

### Doppler echo study

Transthoracic echocardiography was performed by independent, blinded to physicians. The rats were anesthetized with isoflurane, and transthoracic echocardiography (70 Mhz, Visual-Sonics company, Canada, Toronto) was conducted at 2, 3, and 4 months postoperatively. Left atrial diameter (LAD), interventricular septal thickness (IVS; d), and left ventricular ejection fraction (EF, %) were measured.

### Tissue preparation

At the end of the experimental period, the animals (n=12/group) were anesthetized by intraperitoneal injection of sodium pentobarbital (80 mg/kg). After confirming suppression of the nociceptive reflex, the chest cavity of the rats was opened. The heart and lungs were excised in a block and repeatedly rinsed in ice cold 0.9% saline solution. For each rat, the intact heart was weighed. The left PV (LPV, 5–6 mm long, zone 1 as reported by Bronquard et al.) (12) and the left atrium were then excised from the block. Portions of LPV and LA tissues were immediately fixed in 4% polyformaldehyde for histological studies; the remaining tissues were snap frozen in liquid nitrogen and then stored at –80°C for Western blot analysis.

### Histological study

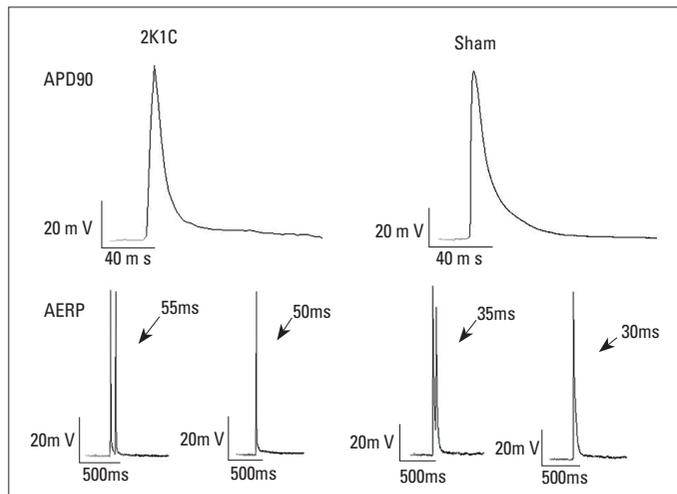
LSPV tissue samples (n=6/group) were fixed in 4% polyformaldehyde for 36 h at room temperature and embedded in paraffin. Deparaffinized tissues were cut into sections (4  $\mu$ m thick), which were mounted on glass slides and stained with Masson's trichrome stain (Nanjing Jiancheng Technology Company, China) to evaluate the area occupied by Col fibers. With this technique, cardiomyocytes are stained bright red, whereas Col fibers are stained blue. Images were captured under a microscope, and quantitative analysis of interstitial fibrosis was performed using Image-Pro Plus 6.0 software (USA).

### Western blotting

Immunoblotting was performed, as described previously (13). The LSPV tissue (approximately 40 mg) (n=6/group) was homogenized and cleared by ultracentrifugation (14,000 $\times$ g) at 4°C for 15 min. Homogenates (containing 40  $\mu$ g of protein) were subjected to 8% SDS-polyacrylamide gel electrophoresis (Beyotime, China) and transferred to PVDF membranes. Membranes were blocked in TBST containing 5% bovine serum albumin at room temperature for 1.5 h before incubation with corresponding primary antibodies: goat polyclonal anti-Col I and rabbit polyclonal anti-MMP-2 (1:200, Santa Cruz, Japan); rabbit monoclonal anti-Ang II (1:2,000, Abcam, UK), rabbit polyclonal anti-TGF- $\beta$ 1 (1:500, Abcam, UK); and rabbit polyclonal anti-Kir2.1, rabbit monoclonal anti-Kir2.3, rabbit monoclonal anti-Cav1.2 and rabbit monoclonal anti-Nav1.5 (1:200, Alomone, Israel). Rabbit monoclonal anti-glyceraldehyde phosphate dehydrogenase (GAPDH; 1:1,000, CST, America) antibody was used as a protein loading control. After overnight incubation with primary antibodies at 4°C, the membranes were washed and incubated with anti-rabbit or anti-goat secondary antibodies (1:5,000, Immunology Consultants Laboratory, USA) for 2 h at room temperature. Membranes were washed, and immunodetection was visualized using an enhanced chemiluminescence method (Thermo, USA) and then developed using X-ray film. Resulting images were analyzed using a densitometer to quantify the protein expression after normalization to GAPDH (Image Lab, BIO-RAD, USA). Mean values of the 2K1C group were compared with the corresponding mean values of the sham-operated group.

### Electrophysiological studies on LA

Electrophysiological studies on the LA tissue were performed, as previously described (14). Four months postoperatively, the rats (500–600 g; n=4 for the 2K1C group and n=3 for the sham-operated group) were anesthetized with sodium pentobarbital (45 mg/kg, intraperitoneally) and given heparin. Left atrium was rapidly removed and the endocardial surface was pinned open in a tissue bath. The atria were superfused with Tyrode's solution containing KCl, 4.0; NaCl, 131; CaCl<sub>2</sub>, 1.8; NaHCO<sub>3</sub>, 24.9; MgSO<sub>4</sub>, 1.2; KH<sub>2</sub>PO<sub>4</sub>, 1.2; and dextrose, 11.1 mmol/L (T=36.5°C $\pm$ 0.5°C, pH 7.4). Bipolar stimulating electrodes were placed on the free wall of left atrium. Conventional microelectrode techniques were used to record action potential duration at 90% repolarization (APD90). Recording sites were located 3–4 mm from the stimulating electrodes. Af-



**Figure 1.** Representative action potential duration at 90% repolarization (APD90) and effective refractory period (ERP) of the left atrium in the 2K1C and sham-operated groups  
 APD90, action potential duration at 90% repolarization; ERP, effective refractory period

ter 20-min equilibration in Tyrode’s solution with pacing from the left atrium at cycle length (CL)=0.4 s, an intermittent burst pacing protocol was applied. This was developed to mimic an intermittently bursting focus in the left atrium. The period of intermittence started at 150 ms, until the atrial effective refractory period (AERP) was recorded and then decreased by 5 ms each time.

**Statistical analysis**

All data are presented as mean±standard deviation (SD) and analyzed using unpaired Student’s t-test in GraphPad Prism 5.0 (GraphPad Software, USA). The Kolmogorov–Smirnov test was used to identify whether data were normally distributed. P<0.05 was considered to indicate statistical significance.

**Results**

**Systolic blood pressure in 2K1C hypertensive rats**

Clipping the left renal artery (2K1C) induced a marked rise in SBP (mm Hg), which was clearly observed, and reached a plateau at 2 weeks postoperatively. Table 1 showed SBP measured using the tail-cuff method at 2 weeks and 1, 2, 3, and 4 months postoperatively.

**LA remodeling during the development of 2K1C hypertension**

Hypertension-induced morphological alterations in the left atrium of 2K1C hypertensive rats. Increased LAD was observed 3 months postoperatively in the 2K1C groups compared with that in the sham-operated groups (Table 2; p=0.002). However, at 4 months postoperatively, there were no significant differences in heart weight/body weight ratio, (IVS; d) and EF% between the groups (Table 2).

APD90 and ERP were recorded in the excised, perfused LA tissue from the rats in the 2K1C and sham-operated groups. There were no significant differences between 2K1C hypertensive and

**Table 1. Comparison of SBP in different time periods between two groups**

	2K1C	Sham	P
Two weeks	168.60±21.28	121.20±11.12	<0.001
One month	170.10±15.11	121.20±12.23	<0.001
Two months	170.20±24.42	125.40±13.59	<0.001
Three months	177.70±21.62	130.70±9.94	<0.001
Four months	176.60±22.20	127.30±17.59	<0.001

Data are expressed as mean±SD (n=15). SBP - systolic blood pressure

**Table 2. Effects of hypertension on cardiac remodeling at four months after surgery**

	2K1C	Sham	P
2-month LAD (mm)	5.13±0.51	4.48±0.36	0.099
3-month LAD (mm)	5.63±0.29	4.32±0.41	0.002
4-month LAD (mm)	6.01±0.29	4.78±0.51	0.002
Heart/body weight (%)	0.33±0.07	0.27±0.02	0.132
IVS; d (mm)	1.83±0.09	1.76±0.13	0.589
EF (%)	62.83±4.53	63.98±8.75	0.818
APD90 (ms)	29.53±9.84	43.5±14.24	0.399
ERP (mm)	56±24	38±14	0.499

N=6 for LAD, Heart weight/body weight ratio, (IVS; d) and EF%. N=4 for 2K1C group and N=3 for sham group for APD90, ERP of LA. Data are expressed as mean±SD. APD90 - action potential duration at 90% repolarization; (EF, %) - left ventricular ejection fraction; ERP - effective refractory period; (IVS; d) - interventricular septal thickness; LAD - left atrial diameter

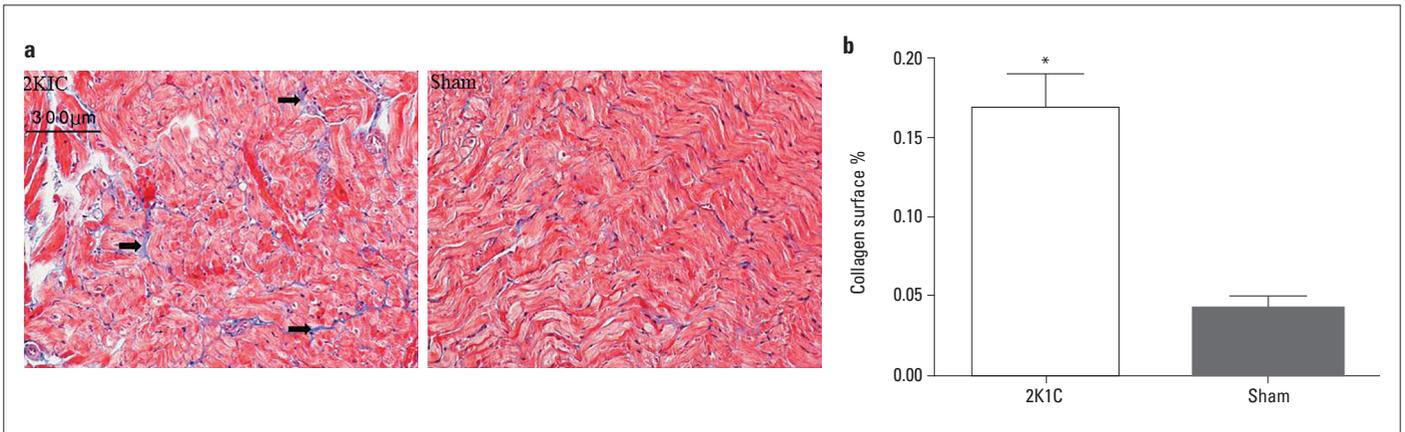
**Table 3. Comparison of protein expression between two groups**

	2K1C	Sham	P
Coll	0.37±0.11	0.19±0.06	<0.001
TGF-β1	0.25±0.08	0.09±0.03	0.029
MMP-2	0.31±0.17	0.12±0.03	0.029
AngII	0.99±0.27	1.11±0.47	0.574
Na <sub>v</sub> 1.5	0.40±0.20	1.08±0.52	0.032
Kir2.1	0.78±0.15	1.11±0.27	0.041
Ca <sub>v</sub> 1.2	0.25±0.05	0.30±0.02	0.4
Kir2.3	2.78±0.47	2.65±0.81	0.691

N=6 for each group. AngII - Angiotensin II; Ca<sub>v</sub>1.2 - L-type Ca<sup>2+</sup>-subunit Ca<sub>v</sub>1.2; Coll - collagen; Kir - Inward rectifying potassium channel; MMP-2 - matrix metalloproteinase-2; Na<sub>v</sub>1.5 - Voltage-gated sodium channel subunit Na<sub>v</sub>1.5; TGF-β1 - transforming growth factor-β1

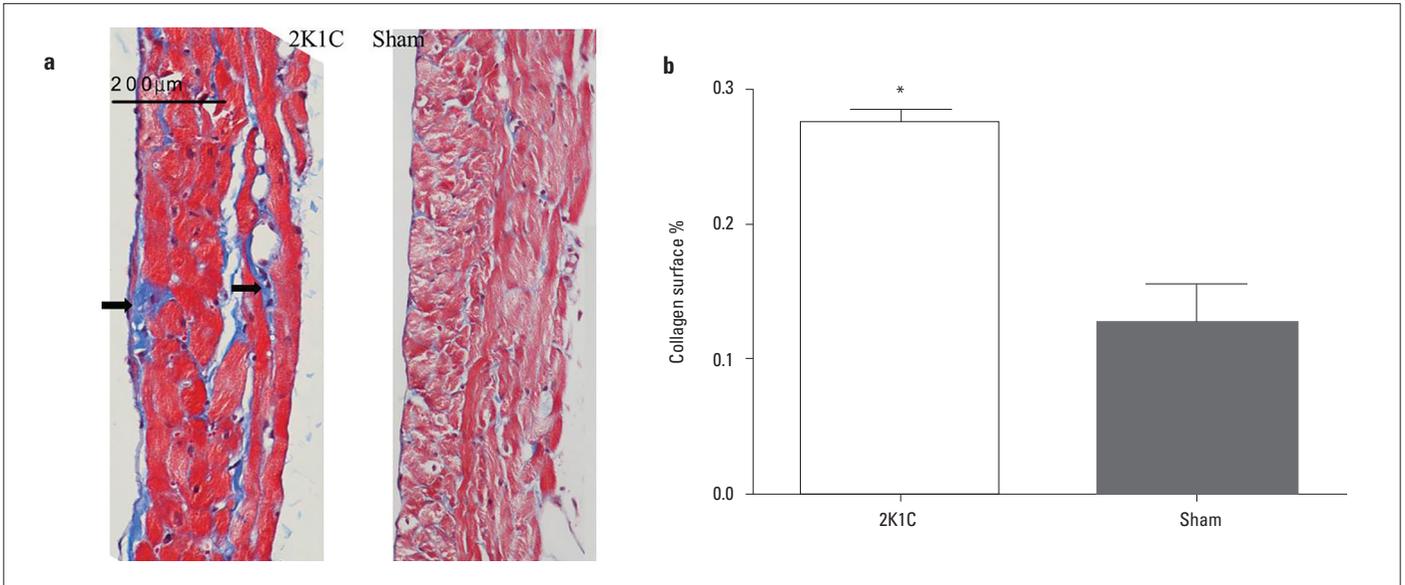
sham-operated rats in terms of APD90 and ERP (Table 2 and Fig. 1).

LA fibrosis was examined by Masson’s trichrome staining of tissue sections. At 4 months, the degree of LA fibrosis was markedly greater in the 2K1C group than in the sham-operated group (Fig. 2a). The mean percentage of fibrosis in sections from the 2K1C group was also greater than that observed in the sham-operated group (Fig. 2b; 16.96%±3.57% vs. 4.32%±1.98%; p=0.012).



**Figure 2.** Atrial fibrosis in 2K1C hypertensive and sham-operated rats (a) Representative examples of Masson's trichrome-stained left atrial sections from 2K1C hypertensive and sham-operated rats (original magnification 200x; arrows indicate areas of fibrosis). (b) Percentage fibrosis measured as the area that was stained blue as a percentage of the total area using Image-Pro Plus 6.0

Data are expressed as mean±SD. \*p=0.012 vs. sham-operated group (n=6)



**Figure 3.** Left superior pulmonary vein (LSPV) fibrosis in 2K1C hypertensive and sham-operated rats (a) Representative examples of Masson's trichrome-stained LSPV sections from 2K1C hypertensive and sham-operated rats (original magnification 200x; arrows indicate areas of fibrosis). (b) Percentage fibrosis measured as the area that was stained blue as a percentage of the total area using Image-Pro Plus 6.0

Data are expressed as mean±SD. \*p=0.016 vs. sham-operated group (n=6)

### Histological and electrophysiological remodeling in LSPV

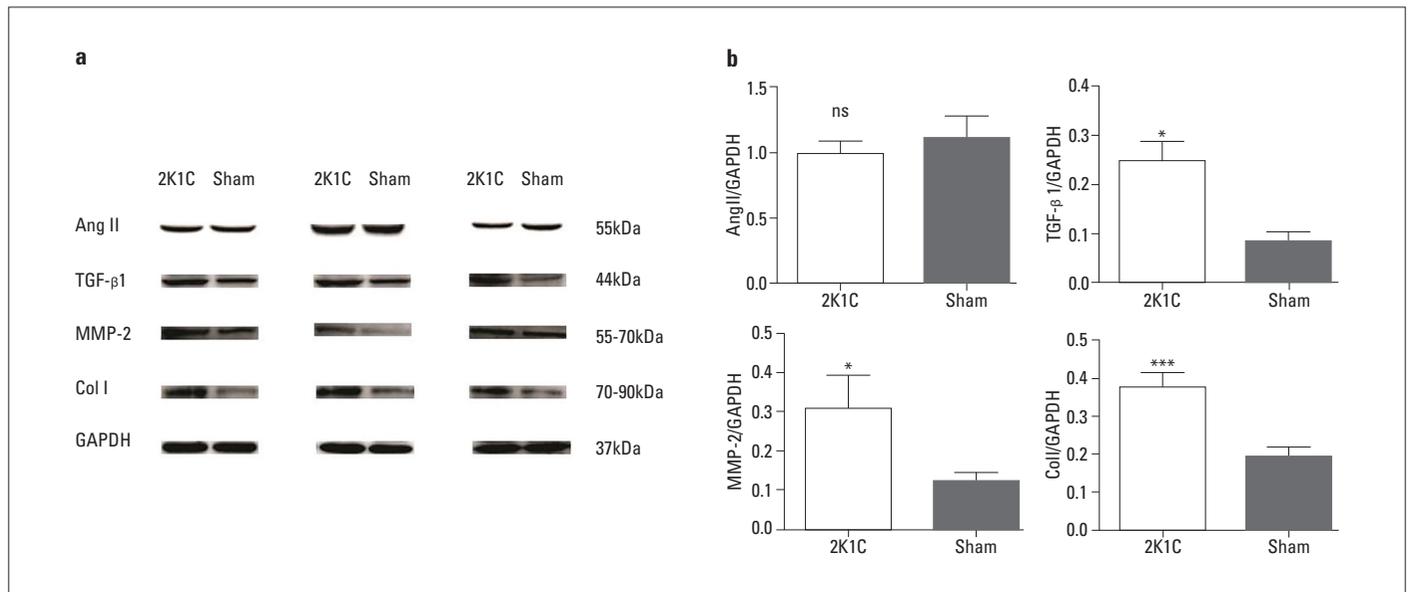
Increased interstitial fibrosis in LSPV was detected by Masson's staining in 2K1C hypertensive rats compared with that in sham-operated rats (Fig. 3; 27.55%±2.20% vs. 12.71%±9.09%; p=0.016). Expression of Col I (0.37±0.11 vs. 0.19±0.06; p<0.001), TGF-β1 (0.25±0.08 vs. 0.09±0.03; p=0.029), and MMP-2 (0.31±0.17 vs. 0.12±0.03; p=0.029) were increased, while expression of Ang II was unchanged in 2K1C hypertensive rats compared with that in sham-operated rats (p=0.574) (Table 3; Fig. 4). Changes in ion channel protein were detected as decreased expression of Na<sub>v</sub>1.5 (0.40±0.20 vs. 1.08±0.52; p=0.032) and Kir2.1 (0.78±0.15 vs. 1.11±0.27; p=0.041), although no major changes in the expression of Ca<sub>v</sub>1.2 and Kir2.3 were observed (p=0.4, p=0.691) (Table 3; Fig. 5).

### Discussion

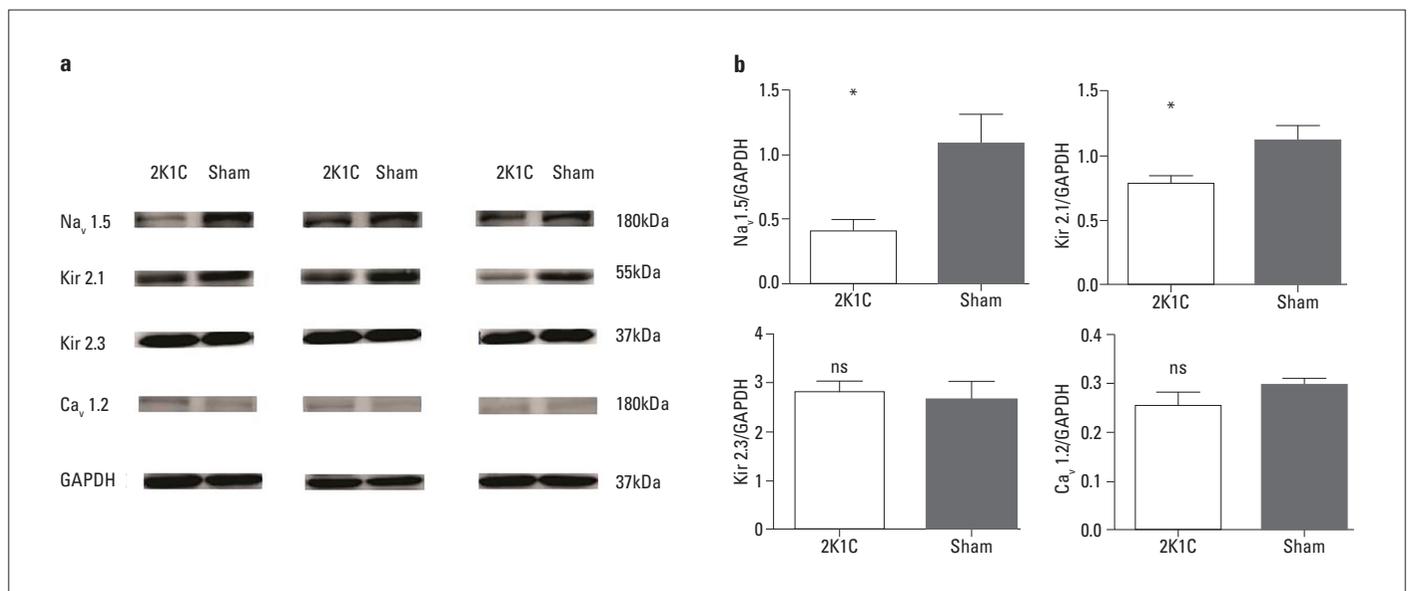
In this study, we demonstrated structural and electrophysiological remodeling in PVs in a rat model of hypertension. This shows a newly discovered mechanistic association between hypertension and AF beyond atrial remodeling, which has been established as an arrhythmogenic substrate for AF (15).

#### Mechanism of PV arrhythmogenicity

It has been nearly 20 years since Haissaguerre found that ectopic activity originated from PVs accounts for initiation of paroxysmal AF (6). Since then, numerous studies have focused on electro-anatomic features of PVs. Triggered automaticity has



**Figure 4.** Protein levels of the fibrotic markers Ang II, TGF-β1, MMP-2, and Col I, analyzed by Western blot and normalized to GAPDH. Data are expressed as mean±SD. \*p<0.05 vs. sham-operated group. \*\*\*p<0.01 vs. sham-operated group (n=6). Ang II, angiotensin II; Col I, collagen I; GAPDH, glyceraldehyde phosphate dehydrogenase; MMP-2, matrix metalloproteinase-2; ns, not significant; TGF-β1, transforming growth factor-β1



**Figure 5.** Protein levels of the ion channel components Na<sub>v</sub>1.5, Kir2.1, Kir2.3, and Ca<sub>v</sub>1.2, analyzed by western blot and normalized to GAPDH. Data are expressed as mean±SD. \*p<0.05 vs. sham-operated group (n=6). Ca<sub>v</sub>1.2, L-type Ca<sup>2+</sup>-subunit Ca<sub>v</sub>1.2; GAPDH, glyceraldehyde phosphate dehydrogenase; Kir, inward rectifying potassium channel; Na<sub>v</sub>1.5, voltage-gated sodium channel subunit Na<sub>v</sub>1.5; ns, not significant

been implicated as possible mechanisms of PV firing (16-18). In addition, PV fibrosis may maintain a possible re-entry in the development of AF through impaired anisotropic conduction (9, 19).

#### Structural and electrical remodeling of the atrium and PV in 2K1C hypertensive rats

In our study, we attempted to elucidate changes in fibrosis and expression of ion channel proteins within PV in 2K1C hypertensive rats. Experimental 2K1C hypertension led to marked histological and electrophysiological remodeling of LSPV; the former refers to

changes in fibrosis associated with increased expression of Col I, TGF-β1, and MMP-2, whereas the latter is involved in downregulation of the expression of Nav1.5 and Kir2.1 channel proteins. To date, these hypertension-related changes in PVs remain unclear.

The precise mechanism leading to the onset and persistence of AF has not been completely elucidated. Structural remodeling, particularly atrial fibrosis, is regarded as the major mechanism accounting for AF persistence (20). In addition to fibrosis, atrial dilatation has also been associated with heterogeneity of conduction (21). Previous studies have also indicated that structural remod-

eling is the main contributor to the persistence of AF and might be present before the development of AF caused by associated diseases (22). Our histological study confirmed the presence of a higher degree of fibrosis in the left atrium of 2K1C hypertensive rats compared with that observed in sham-operated rats. Furthermore, transthoracic echocardiography showed LA dilatation in 2K1C hypertensive rats. These structural changes in the atria may contribute to increased susceptibility to AF in patients with hypertension.

LA dilatation and increased fibrosis were observed in 2K1C hypertensive rats. These characteristics are considered the hallmark of structural remodeling in AF and the substrate for AF perpetuation (15). However, there were no significant differences in APD90 and AERP between the groups. Previous studies have demonstrated numerous changes in APD and ERP in the atria of hypertensive animals, such as shortened APD and ERP (1, 23) and prolonged (24, 25) or unchanged (26, 27) APD and ERP. This variability could be due to species differences, blood pressure level, or even measurement techniques. Inconsistency in APD and ERP also illustrated diverse mechanisms of AF among different disease models.

As important profibrotic factors, the expression of Ang II, TGF- $\beta$ 1, and MMP-2 were investigated to elucidate the mechanisms by which fibrosis develops within PV. However, the difference in the expression of the Ang II protein between the two groups did not reach the level of statistical significance in our study. This result may be due to two factors. First, angiotensin I-converting enzyme activity increased in 2K1C hypertensive rats, but decreased to normal levels after 10 weeks. This finding suggests that the renin-angiotensin-aldosterone system activation plays an important role in the initial phase of 2K1C hypertension, but not in the late phase (28). Second, 2K1C results in a sustained increase in blood pressure because of increased renin activity, which in turn, increases circulating Ang II levels. In the early stages, hypertension is renin-angiotensin dependent because of the absence of salt and water retention as the second, unclipped kidney is intact. However, after approximately 6 weeks, the increased Ang II releases aldosterone leading to a gradual retention of salt and water, which in return decreases renin production (29). The critical role of TGF- $\beta$ 1 in the development of AF has been confirmed in transgenic mice overexpressing TGF- $\beta$ 1, which led to selective atrial fibrosis, enhanced AF susceptibility, and increased conduction heterogeneity, although atrial action potential duration and ventricular structure and function were normal (30). Researchers have hypothesized that TGF- $\beta$ 1 levels in PVs may be related to the arrhythmogenicity of these vessels (31); however, expression of TGF- $\beta$ 1 in PVs has not been investigated widely. Also, accumulating evidence obtained in animal studies has implicated imbalanced MMP activity, particularly of MMP-2, in hypertension-induced vascular remodeling (32, 33). In the present study, we observed a significant increase in the expression of Col I protein and increased fibrosis in LSPV in 2K1C hypertensive rats. These effects may be, at least in part, caused by TGF- $\beta$ 1 and MMP-2.

In our study, we observed decreased expression of the ion channel proteins Na<sub>v</sub>1.5 and Kir2.1 in LSPV in 2K1C hypertensive rats compared with that in sham-operated rats. It is reported that reduction in the expression of I<sub>Na</sub> not only contributes to conduction retardation, which favors re-entry (34), but also promotes early afterdepolarization (35). The 2K1C hypertensive rats in the present study exhibited reduced expression of the Kir2.1 protein compared with that in sham-operated rats, while no significant differences in the expression of Kir2.3 protein were observed. Kir2.1 and Kir2.3 encode the principal cardiac inward-rectifier I<sub>k1</sub> subunit. Previous studies have shown that reduction in I<sub>k1</sub> causes increased membrane resistance, leading to a larger voltage deflection for a given quantity of depolarizing membrane current; this may be an important mechanism underlying the promotion of afterdepolarization-induced arrhythmias (26, 36). Decreased expression of the protein may implicate a decrease in the relevant channel function. However, the direct functional link between expression of the protein and ion channels needs to be further studied.

#### Study limitations

The study was performed at 4 months after 2K1C surgery. However, more obvious changes in the structure and electrophysiology may have been observed if the observation time was extended. Moreover, electrophysiological LPV data, such as APD, ERP, and ion current, was not calculated.

#### Conclusion

Hypertension is associated with histological and electrophysiological PV remodeling characterized by fibrosis and some changes in the expression of the ion channel protein. These observations provide new mechanistic association between hypertension and AF beyond the previously well-established role of atrial remodeling.

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**Peer-review:** Externally peer-reviewed.

**Authorship contributions:** Concept – W.Z.J.; Design – W.Z.J.; Supervision – W.Z.J.; Materials – P.P.X., L.J.L.; Data collection &/or processing – R.D.Q., J.J.S.; Analysis &/or interpretation – P.P.X., R.D.Q.; Literature search – M.L.C.; Writing – P.P.X., L.J.L.; Critical review – W.Z.J.

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