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Pinocembrin attenuates autonomic dysfunction and atrial fibrillation susceptibility via inhibition of the NF-κB/TNF-α pathway in a rat model of myocardial infarction

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ABSTRACT

Previous studies indicate that myocardial infarction (MI) may contribute to atrial fibrillation (AF). Emerging evidence has shown that pinocembrin protects myocardial ischemic injury (I/R)-induced cardiac fibrosis and arrhythmias in animals via its anti-inflammatory or antioxidant activities. However, the effects of pinocembrin on MI-induced atrial arrhythmias remain unknown. Thus, this study aimed to investigate the effects of pinocembrin on autonomic dysfunction and AF susceptibility in MI rats and the possible mechanism. In a standard experimental MI model, Sprague-Dawley rats received permanent ligation of the left anterior descending (LAD) coronary artery and were treated with pinocembrin or saline for 6 days. Our results demonstrated that pinocembrin treatment significantly decreased sympathetic activity, augmented parasympathetic activity, improved heart rate variability (HRV), prolonged the atrial effective refractory period (ERP) and action potential duration (APD), shortened activation latency (AL), lowered the indicibility rate of AF, attenuated atrial fibrosis, and decreased concentrations of norepinephrine (NE), tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 in the serum and the left atrial (LA). Furthermore, pinocembrin treatment significantly increased the expression levels of Cx43 and Cav1.2 and suppressed the phosphorylation of inhibitor-κBα (IκBα) and the activation of nuclear factor-kappa B (NF-κB) subunit p65. In conclusion, the findings indicate that pinocembrin treatment decreases autonomic remodeling, lowers atrial fibrosis, ameliorates atrial electrical remodeling, and suppresses MI-induced inflammatory responses, which suggests a potential novel strategy for atrial arrhythmias.

1. Introduction

Atrial fibrillation (AF) is the most prevalent tachyarrhythmia, with a 0.9% overall prevalence rate in the US and Europe [1]. AF is often associated with increased mortality, morbidity, and a series of deleterious comorbidities, such as stroke and heart failure [2]. Previous studies have demonstrated that AF and myocardial infarction (MI) often coexist [3,4]. In addition, MI is recognized as a traditional risk factor for AF [5]; however, little is known about the signaling basis. Autonomic remodeling, atrial electrical remodeling, and atrial structural remodeling are considered to be the predominant mechanisms of AF [6].

Inflammatory responses can be activated after MI, not only at the local sites of injury, but also at the remote sites through the circulating blood. At the local sites of injury, MI accounts for cardiomyocyte injury and even death if prolonged, followed by elevated inflammatory cytokines [e.g., tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6] in the myocardium [7]. Systemic inflammation may also be activated after MI with increased levels of inflammatory cytokines in plasma [8]. Clinical studies show that post-MI patients with arrhythmias have higher circulating levels of inflammatory cytokines compared with patients with sinus rhythm, which implies that inflammation may promote the occurrence of arrhythmia [9,10]. Furthermore, inflammation is also related to the initiation and maintenance of AF, providing a new strategy for upstream therapies of AF via the suppression of inflammatory responses [11,12].

The transcription factor nuclear factor-kappa B (NF-κB) is a pivot inflammatory signaling molecule that regulates inflammatory responses

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and other important biological processes upstream [13]. NF-κB is inactive when bound to inhibitor-κB (IκB) proteins. Faced with stimuli, NF-κB dissociates from IκB and translocates into the nucleus to regulate its target genes, including inflammatory cytokines such as TNF-α, IL-1β, and IL-6, thus resulting in adverse consequences [14,15].

Pinocembrin (5,7-dihydroxyflavonone), an abundant flavonoid isolated from propolis and some plants, shows various biological effects, such as anti-inflammatory, antioxidant, and antimicrobial activities [16]. Studies have showed that pinocembrin has protective effects against cerebral ischemic injury (I/R) [17–19]. Recent studies have also attempted to determine whether pinocembrin has a beneficial effect on the heart. For instance, pinocembrin was found to improve cardiac function, reduce ventricular arrhythmias, and decrease the myocardial infarct area in myocardial I/R rats [20,21]. However, it remains unknown whether pinocembrin has beneficial effects on atrial arrhythmias, especially in a MI model. In certain studies, pinocembrin suppresses inflammatory responses via the inhibition of the NF-κB pathway [22–24]. TNF-α, an important inflammatory marker, is closely related to an increased risk of atrial arrhythmias and is primarily regulated by NF-κB [15]. In the present study, we implemented a rat model of MI to investigate the incidence and the underlying mechanisms of AF in MI. We hypothesized that pinocembrin could attenuate autonomic dysfunction and AF susceptibility, which is possibly associated with the suppression of the NF-κB/TNF-α signaling pathway.

2. Materials and methods

2.1. Animals

A total of 106 male Sprague-Dawley (SD) (200 ± 20 g) rats were randomly assigned into five treatment groups: (i) Sham group (n = 24): Sham + saline; (ii) MI group (n = 24): MI + saline; (iii) MI + P group (n = 24): MI + pinocembrin (5 mg/kg); (iv) MI + L group (n = 17): MI + saline + lipopolysaccharide (LPS, 4 mg/kg); and (v) MI + P + L group (n = 17): MI + pinocembrin + LPS. Pinocembrin (5 mg/kg; purity ≥ 98%, Sigma–Aldrich) or saline was injected intravenously (IV) via the tail vein from day 1 to day 6. LPS (4 mg/kg; Escherichia coli 055:B5) was injected intraperitoneally 1 h after administration of saline or pinocembrin on day 6. All rats were housed under standard conditions with a 12/12-hour light/dark cycle and food and water ad libitum. Animal use protocols were followed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and were approved by the animal experimental administration of Wuhan University, China.

2.2. Myocardial infarction model establishments

Rats were anesthetized with sodium pentobarbital (3%, Sigma) before surgery and received continuous electrocardiogram (ECG; lead II) monitoring (PowerLab 4/35, AD Instruments, Australia). The MI model in rats was created by permanent ligation of the left anterior descending (LAD) coronary artery [25]. The left ventricle, especially the apex, became pale immediately post-ligation and the ST segment increased. Rats in the Sham group underwent a similar procedure without LAD ligation. Rats in the MI group and the MI + L group were administrated IV saline 30 min before ischemia on the first day and rats in the MI + P group and the MI + P + L group were administrated IV pinocembrin. Then, rats continued to get injections once a day at the same time for 5 days until they were euthanized on day 7. The rats in the Sham group were treated with IV saline at the same time each day for 6 days.

2.3. Heart rate variability analysis

The ECG was recorded using the PowerLab system (4/35, AD Instruments, Australia), and the data were analyzed using LabChart 8.0 software [26]. RR variability measurement was conducted on segments of 250 to 300 cycles [27]. Heart rate variability (HRV) was measured both in the time and the frequency domain. In the time domain, the mean heart rate (HR), the mean of all normal RR-intervals (mean RR), the standard deviation of normal RR intervals (SDNN) and the square root of the mean squared differences of successive RR intervals (RMSSD) were calculated. In the frequency domain, we evaluated the low frequency (LF) at 0.25 to 0.75 Hz and the high frequency (HF) at 0.75 to 2.50 Hz [27]. The LF/HF ratio was also counted to assess the autonomic balance.

2.4. Electrophysiological study

2.4.1. Isolated heart preparation

The atrial electrophysiological parameters were detected (n = 10 per group). The rats were anesthetized (3% sodium pentobarbital; Sigma) and heparinized (400 U, heparin sodium; Sigma). Subsequently, the hearts were captured and instantly perfused in accordance with the Langerdorff technique (AD Instruments, Dunedin, New Zealand) at constant pressure (70–90 cm H_2O) and temperature (37.0 °C ± 0.5 °C). The isolated heart was perfused with HEPES-buffered Tyrode solution of several components [28]. Hearts with irreversibly irregular spontaneous rhythms were discarded [29].

2.4.2. Monophasic action potential analysis

The experimental protocol was conducted according to our previous study [28,29]. Briefly, the paired platinum-stimulating electrodes delivered programmed stimulation, and the two custom-made Ag–AgCl electrodes measured monophasic action potentials (MAPs) from the left atrial appendage (LAA). The distance between the two electrodes was strictly 1 cm in all groups. The atrial activation latency (AL) and effective refractory period (ERP) were detected via programmed stimulation, which consisted of eight successive fundamental stimuli (S1) [cycle length (CL): 250 ms] along with a preceding stimulus (S2), the CL of which was progressively decreased from 100 ms to 1 ms. The AL referred to the time difference between S2 and the action potential duration (APD) peak. The myocardium is captured in the last S2, in which the maximum AL was detected and compared. The ERP was defined as the longest S2 interval that failed to catch the myocardium. The PowerLab system (AD Instruments) and Chart 8.0 software were used to measure and record all the signals. The S1S1 stimulation procedure was conducted to construct APD curves at orderly pacing cycle lengths (PCLs) of 250 ms, 200 ms, 150 ms, and 100 ms with 10 stimuli. The APD at LAA was measured at 90% repolarization (APD_{90}), 50% repolarization (APD_{50}), and 20% repolarization (APD_{20}). The PCL was gradually decreased by 10 ms until APD alternans was detected. 50 Hz burst pacing was used within six times to assess AF susceptibility. AF was determined as rapid irregular atrial rhythm lasting for at least 2 s.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Rats were anaesthetized with sodium pentobarbital (3%, Sigma) intraperitoneally to collect blood and hearts for ELISA or western blot assays (n = 7 per group). Blood samples were extracted from the opened abdomen via the inferior vena cava in an ethylenediamine tetraacetic acid (EDTA)-containing vacutainer and separated into serum aliquots by centrifuging (Beckman Coulter, USA) at 3000 g for 10 min at 4 °C. The separated serum was stored at − 80 °C until assayed. Tissues were obtained from the LA. Norepinephrine (NE), TNF-α, IL-1β, and IL-6 concentrations in the serum and LA were measured using ELISA according to the manufacturers' specification.

2.6. Masson staining

The tissues dissected from the LA after the HRV analysis were fixed in 4% paraformaldehyde, then embedded in paraffin (n = 7 per group).

We cut the dissected tissues in 5-μm sections for histological examination. The degree of fibrosis was measured using the Masson trichrome method to stain heart sections for collagen. The fibrotic area was examined under a microscope with magnification of 200 × using Image-Pro-Plus 6.0 software (Media Cybernetics, Inc, Rockville, MD).

2.7. Western blot analysis

Rats were euthanized in the state of anesthesia (3%, sodium pentobarbital; Sigma), and tissues obtained from the LA were dissected rapidly (n = 7 per group). The protocol was conducted according to our previous study [30]. In brief, after extraction of the LA tissues, the equivalent amount of protein was separated by 10% to 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to the polyvinylidene difluoride (PVDF) membrane. Cx43 (1:1000; Abcam), Cav1.2 (1:200; Abcam), IκBα (1:1000; CST), p-IκBα (1:500; CST), p65 (1:2000; CST), and p-p65 (1:1000; CST) proteins were measured with primary antibodies, respectively, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH 1:10000; Abcam) served as a housekeeping reference protein. Image J software was used to visualize and analyze the image densities.

2.8. Statistical analysis

The continuous variables were described as mean ± standard deviation. Proportions were presented as percentages. The differences between two groups were assessed by the Student's *t*-test or the Pearson chi-squared test when appropriate, whereas those among multiple groups were assessed by one-way analysis of variance (ANOVA) followed by the Tukey multiple comparisons test. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Pinocembrin improved HRV in MI rats.

After 1 week of intervention, a decrease in the mean RR was shown in the MI group. However, the alteration was significantly attenuated after administration of pinocembrin in the MI + P group (Fig. 1A). In the time-domain analysis, SDNN and RMSSD showed a significant decrease in the MI group compared with the Sham group. In the frequency-domain analysis, the LF and LF/HF ratio were significantly increased whereas the HF was significantly decreased in the MI group compared with the Sham group. However, compared with the MI group, pinocembrin contributed to a significant increase in SDNN, RMSSD, and HF and a decrease in LF and LF/HF ratio in the MI + P group (Fig. 1B–E).

3.2. Pinocembrin ameliorated atrial electrical remodeling in MI rats.

Fig. 2A showed the representative atrial electrogram in the S1S2 pacing protocol. The maximum AL was significantly longer in the MI group than in the Sham group (30.60 ± 5.34 vs. 15.30 ± 2.31 ms, $P < 0.01$; Fig. 2B). The mean ERP was significantly decreased in the MI group compared with the Sham group (20.40 ± 4.79 vs. 47.20 ± 6.41 ms, $P < 0.01$; Fig. 2C). However, pinocembrin administration markedly shortened the maximum AL and increased the mean ERP (all $P < 0.01$; Fig. 2A, B). Fig. 3A showed representative APDs recorded from the LAA in the three groups. The APD_{90} was markedly shorter in the four PCLs in the MI group than in the Sham group, but pinocembrin significantly restored the decreased APD_{90} in the MI + P group (all $P < 0.01$; Fig. 3B). APD_{20} and APD_{50} showed similar results to those of the APD_{90} (all $P < 0.01$; Fig. 3C). Moreover, the maximum PCL of the APD alternans CL in the MI group was significantly increased compared with that of the Sham group ($P < 0.01$), which was markedly decreased by pinocembrin in the MI + P group ($P < 0.01$; Fig. 4A, B). A burst pacing protocol was applied to assess AF inducibility. AF was inducible in the MI and MI + P group, but not in the Sham group (Fig. 5A, B). In addition, the incidence of AF was 30% in the MI + P group and 80% in the MI group (Fig. 5B). However, no significant difference was found in the average duration of AF between the MI group and the MI + P group (35.60 ± 17.01 vs. 13.00 ± 8.16, $P > 0.05$; Fig. 5C).

3.3. Pinocembrin decreased concentrations of NE, TNF-α, IL-1β, and IL-6 in the serum and LA in MI rats.

The NE concentration in the serum and LA was significantly higher

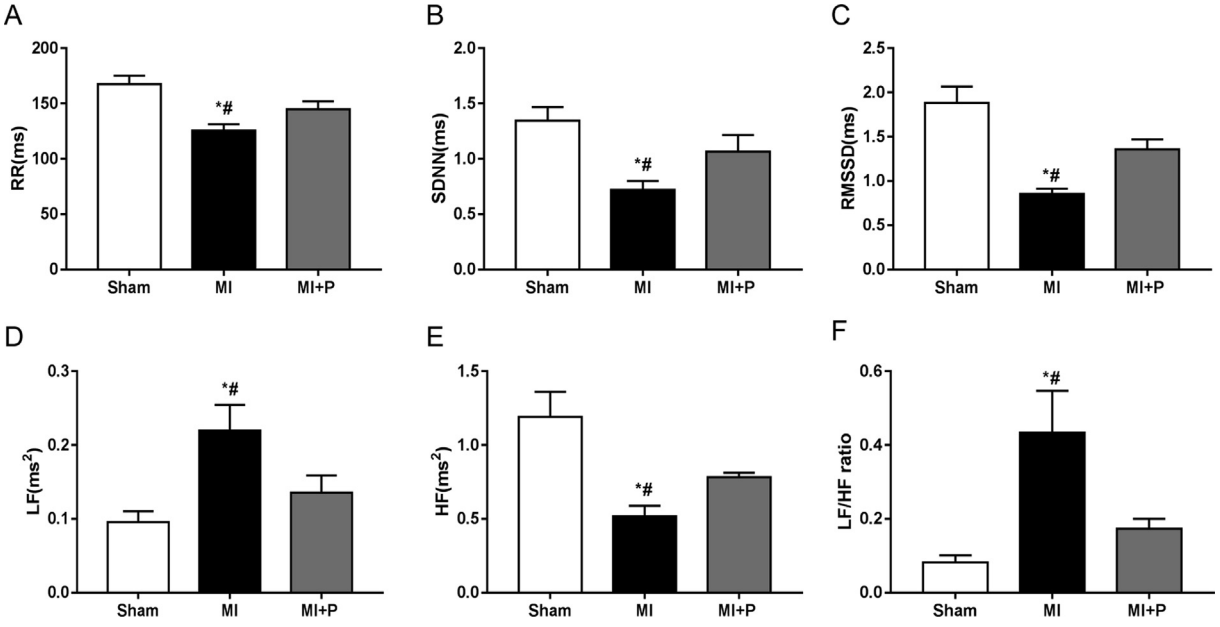

Fig. 1. HRV. n = 7 per group. (A–F) Statistical analysis of the mean RR, SDNN, RMSSD, LF, HF, and LF/HF ratio of the three groups, respectively. *P < 0.01 vs. Sham; #P < 0.01 vs. MI + P. HRV, heart rate variability; mean RR, the mean of all normal RR-intervals; SDNN, the standard deviation of normal-to-normal intervals; RMSSD, the square root of the mean squared differences of successive RR intervals; LF, low-frequency; HF, high-frequency; Hz, hertz.

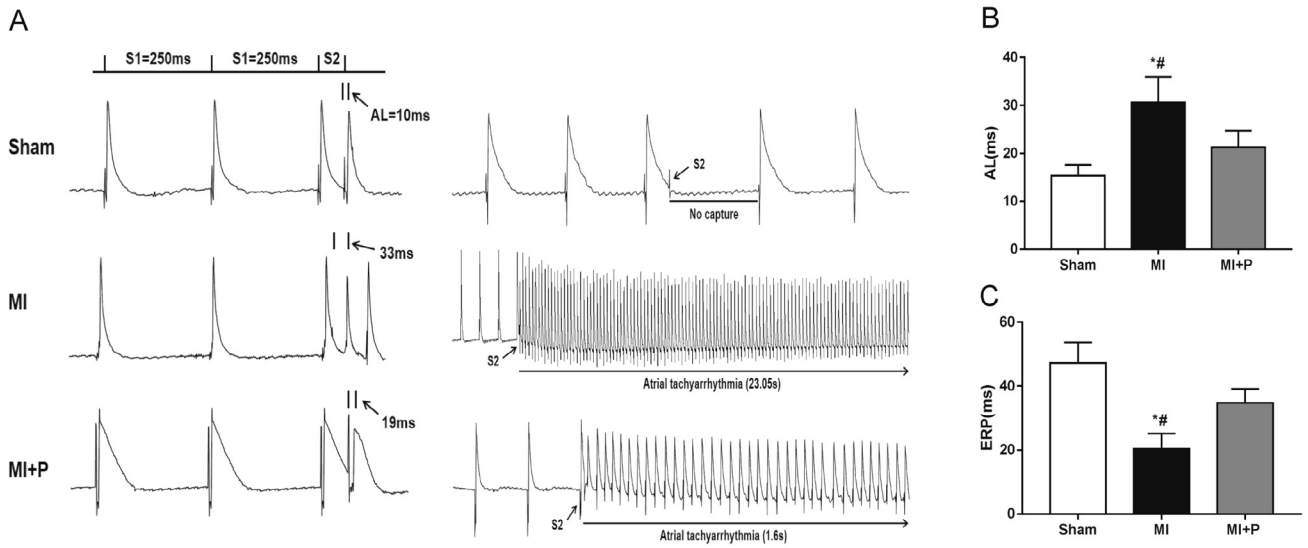

Fig. 2. Monophasic action potentials. (A) Representative recordings of atrial electrogram in the three groups. (B,C) AL and ERP in the three groups, respectively. n = 10 per group. *P < 0.01 vs. Sham; #P < 0.01 vs. MI + P. AL, activation latency; ERP, effective refractory period.

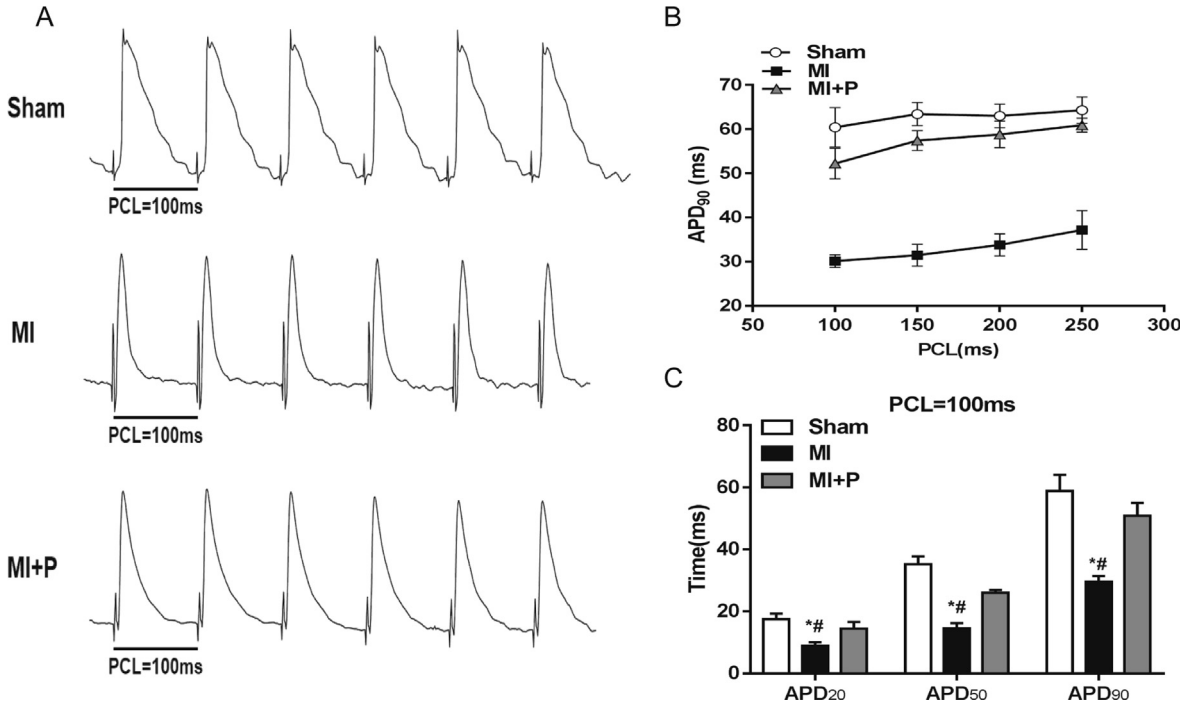

Fig. 3. Atrial repolarization. (A) Representative recordings of APD at PCL of 100, 150, 200, and 250 ms. (B) The spatial dispersions of APD90 in the three groups, respectively. n = 10 per group. (C) APD20, APD50, and APD90 at PCL of 100 ms in the three groups, respectively. n = 10 per group. *P < 0.01 vs. Sham; #P < 0.01 vs. MI + P. PCL, pacing cycle length; APD_{90}, APD_{50}, and APD_{20}, action potential duration at 90% repolarization, 50% repolarization, and 20% repolarization, respectively.

in the MI group than in the Sham group (P < 0.01), demonstrating a hyperactive sympathetic activity in MI rats. However, it was markedly decreased in the MI + P group compared with the MI group (P < 0.01), indicating that the hyperactive sympathetic activity is partially inhibited after administration of pinocembrin (Fig. 6A). Furthermore, the concentrations of TNF-α, IL-1β, and IL-6 were significantly increased in the MI group compared with the Sham group (all P < 0.01). However, the aforementioned inflammatory markers were significantly lower in the MI + P group than in the MI group (all P < 0.01; Fig. 6B, C), demonstrating that pinocembrin treatment inhibits MI-induced inflammatory responses.

3.4. Pinocembrin decreased atrial fibrosis and increased Cx43 and Cav1.2 expression in MI rats.

Fig. 7A showed the representative images of Masson staining, indicating a significant increase of the LA myocardial interstitial collagen deposition in the MI group compared with the Sham group. However, a significant decrease of atrial fibrosis was shown in the MI + P group compared with the MI group (all P < 0.01; Fig. 7B). Western blot assays showed that the expression levels of Cx43 and Cav1.2 were significantly decreased in the MI group compared with the Sham group. However, those were significantly higher in the MI + P group than in the MI group (all P < 0.01; Fig. 7C-E).

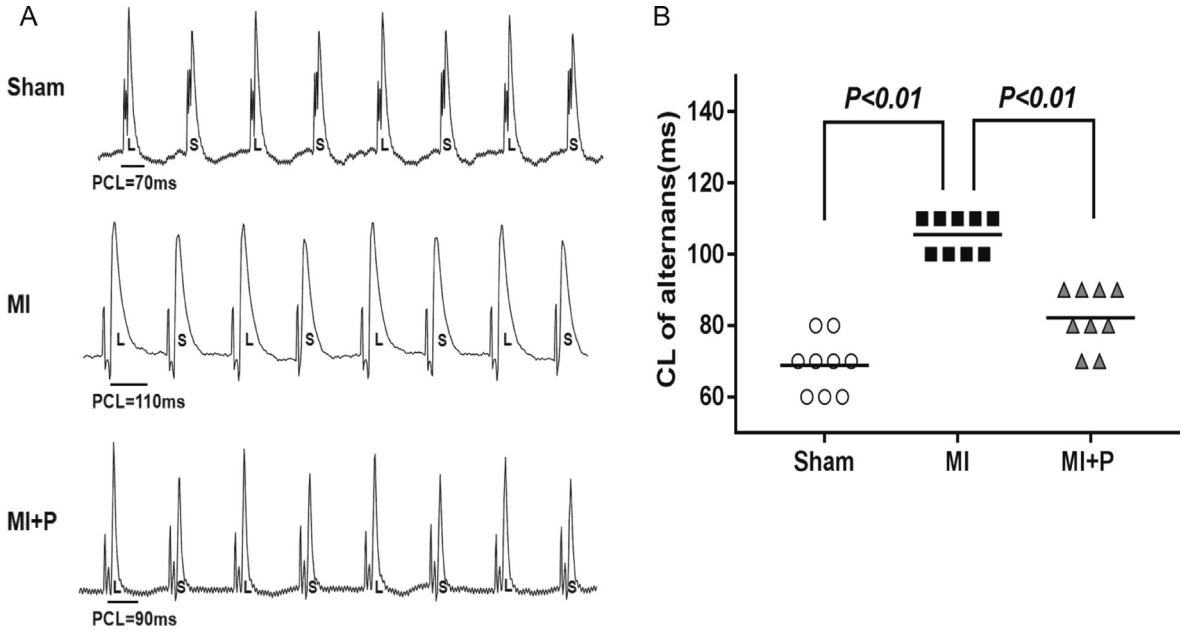

Fig. 4. APD alternans. (A) Representative recordings of APD alternans in the three groups. (B) Threshold cycle length of APD alternans in the three groups, respectively. n = 10 per group. APD, action potential duration.

3.5. Pinocembrin lowered the protein expression levels of p-p65 and p-IκBα in MI rats.

The expression of p-p65 was significantly increased in the MI group compared with the Sham group (0.52 ± 0.04 vs. 0.09 ± 0.01, $P < 0.01$), but it was significantly reduced in the MI + P group compared with the MI group ($P < 0.01$; Fig. 8A, B), indicating that administration of pinocembrin inhibited MI-induced NF-κB activation. The expression of p-IκBα was higer in the MI group than in the Sham groups (0.30 ± 0.02 vs. 0.03 ± 0.01, $P < 0.01$), but it was significantly lower in the MI + P group than in the MI group ($P < 0.01$; Fig. 8A, B), demonstrating that pinocembrin treatment inhibited MI-induced IκBα activation. However, no significant differences were found in the expression of p65 and IκBα among the three groups.

3.6. Pinocembrin and the NF-κB/TNF-α pathway in MI rats

To further explore the mechanism of pinocembrin in regulating the autonomic nervous system (ANS) and AF susceptibility, the MI + L group and the MI + P + L group were conducted. As Fig. 9A showed, although no significant difference was found between the MI group and the MI + L group in the mean ERP, pinocembrin treatment significantly

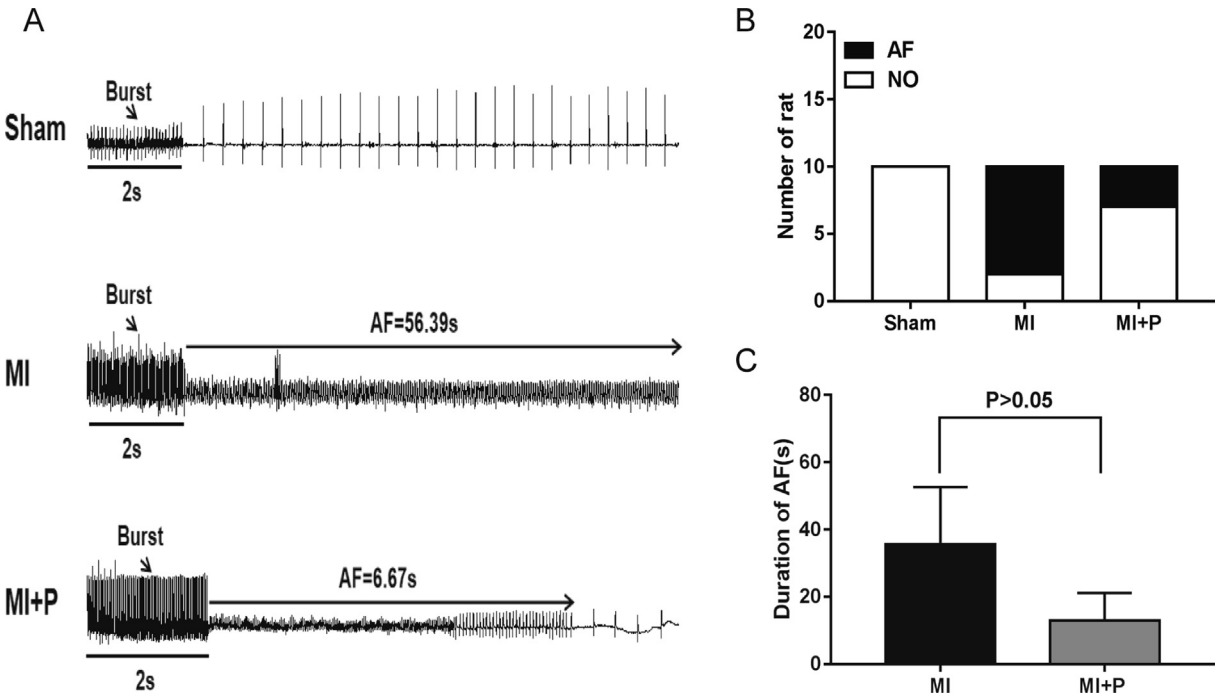

Fig. 5. AF vulnerability. (A) Representative recordings of burst pacing in the three groups. (B) Inducibility of AF in the three groups, respectively. n = 10 per group. (C) Duration of AF in the MI and MI + P group. AF, atrial fibrillation.

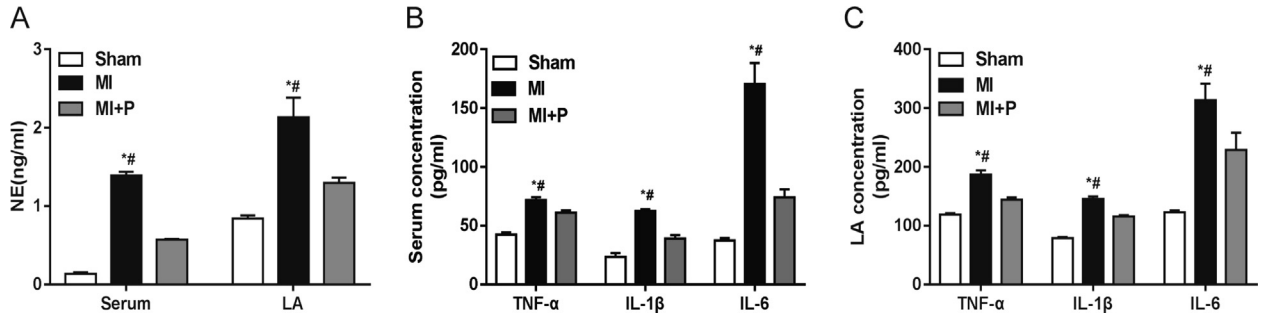

Fig. 6. (A) NE, (B, C) TNF-α, IL-1β, and IL-6 concentrations in the serum and LA. n = 7 per group. *P < 0.01 vs. Sham; #P < 0.01 vs. MI + P.

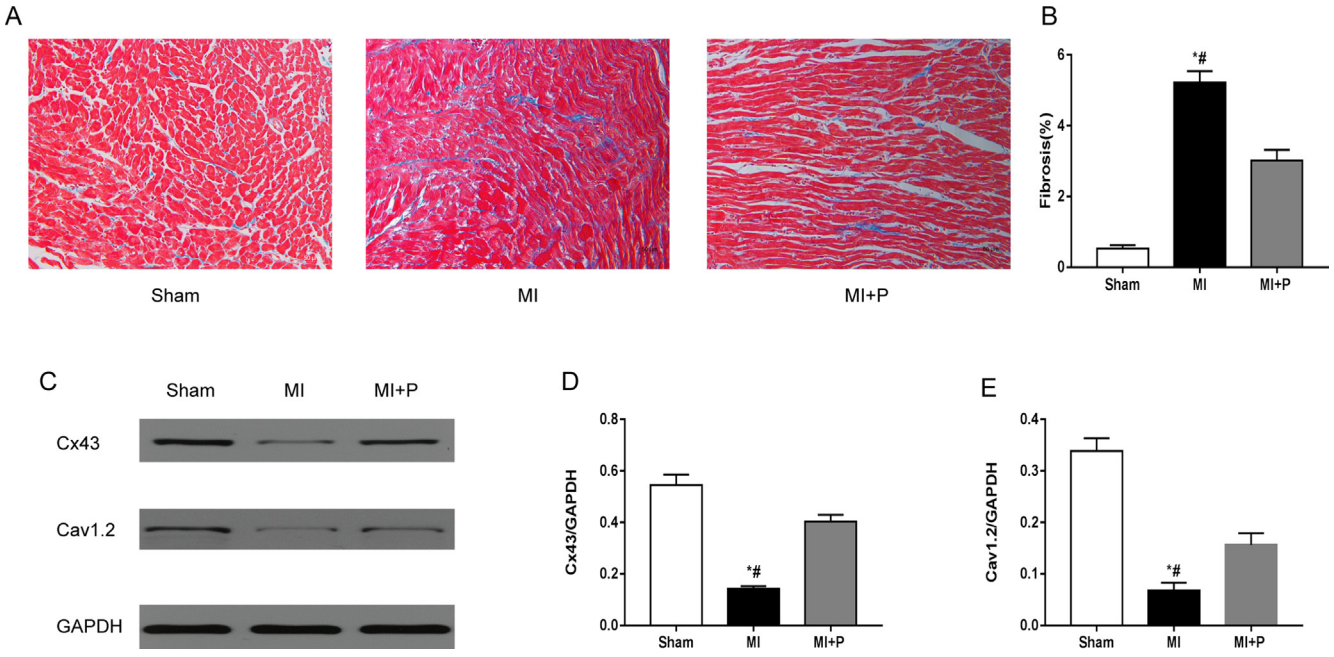

Fig. 7. Atrial myocardial fibrosis and protein expression of Cx43 and Cav1.2. (A) Representative images of Masson staining of atrial (original magnification: ×200). (B) Quantification of the fibrotic area per group. n = 7 per group. (C) Immunoblotting of Cx43 and Cav1.2. n = 7 per group. (D, E) The expression ratio of Cx43 and Cav1.2. *P < 0.01 vs. Sham; #P < 0.01 vs. MI + P.

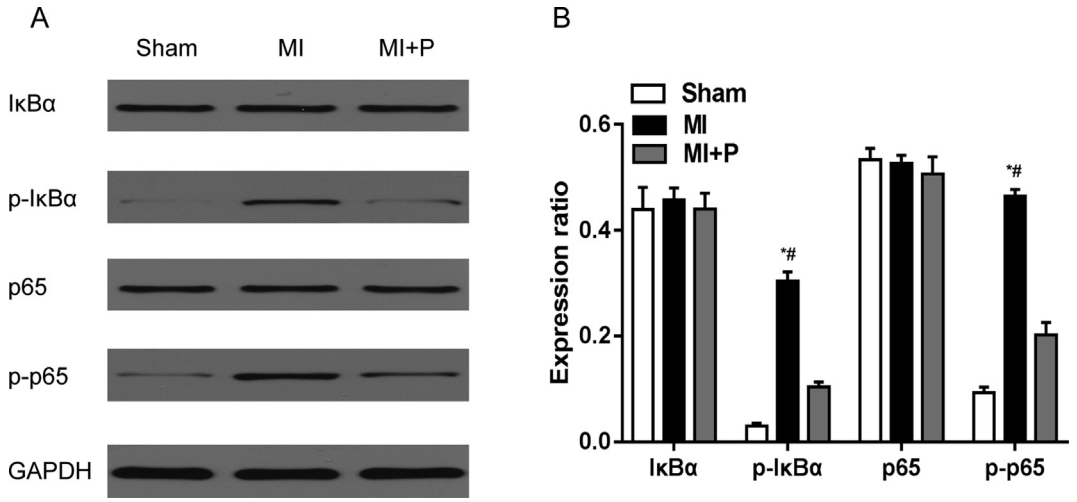

Fig. 8. Protein expression of IκBα, p-IκBα, p65, and p-p65. n = 7 per group. (A) Immunoblotting of IκBα, p-IκBα, p65, and p-p65 per group, respectively. (B) The expression ratio of IκBα, p-IκBα, p65, and p-P65 per group, respectively. *P < 0.01 vs. Sham;#P < 0.01 vs. MI + P.

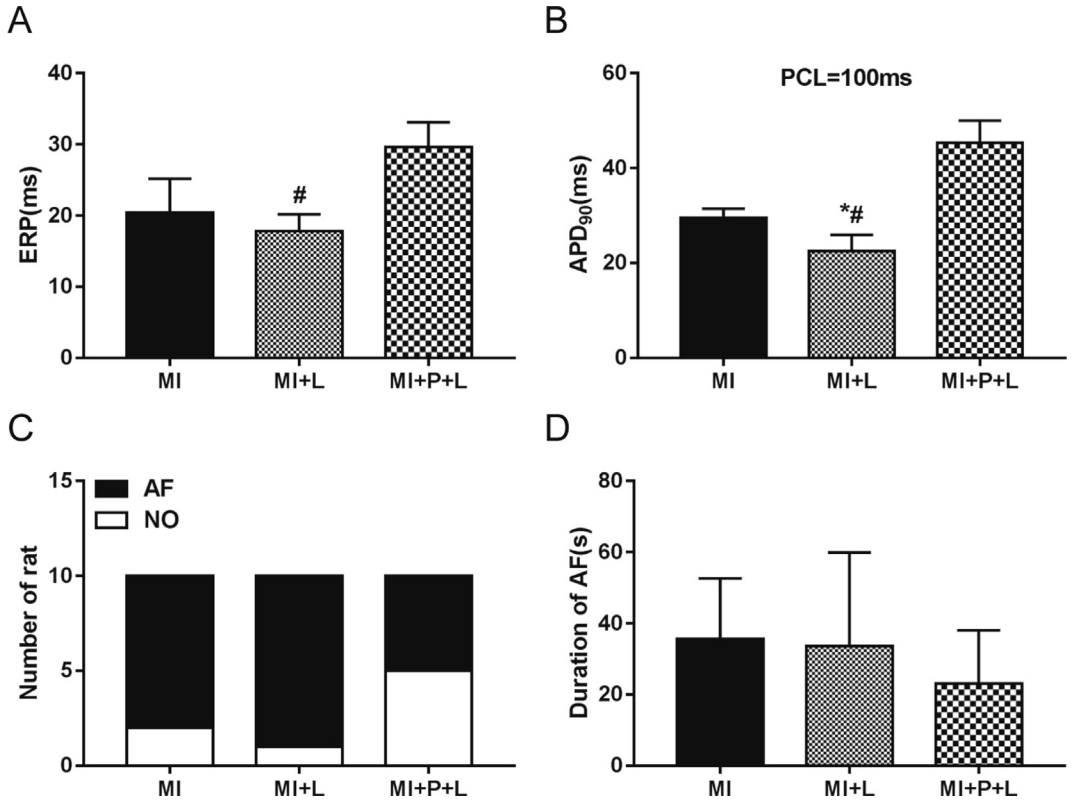

Fig. 9. (A–D) ERP, APD90 at PCL of 100 ms, inducibility of AF and duration of AF, respectively. n = 10 per group. *P < 0.01 vs. MI; #P < 0.01 vs. MI + P + L. ERP, effective refractory period; APD90, action potential duration at 90% repolarization; PCL, pacing cycle length; AF, atrial fibrillation.

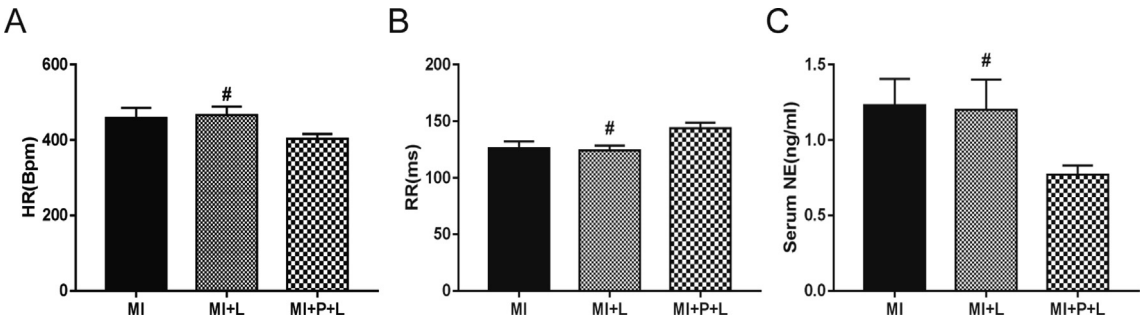

Fig. 10. (A–C) The mean HR, mean RR and serum NE concentration, respectively. n = 7 per group. #P < 0.01 vs. MI + P + L. HR, heart rate; mean RR, the mean of all normal RR-intervals.

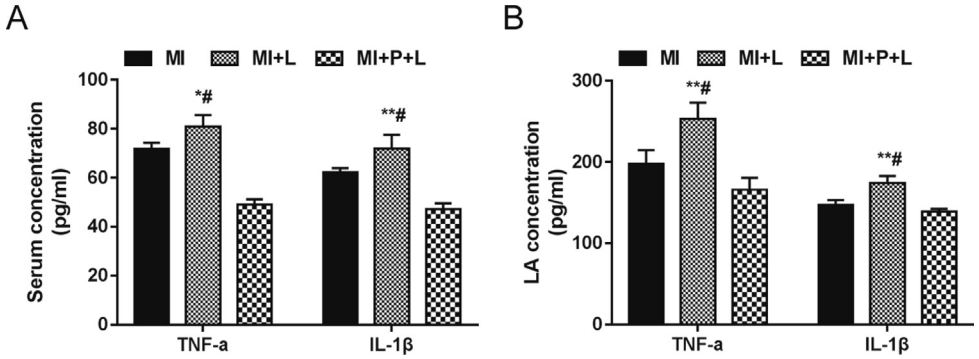

Fig. 11. (A, B) The concentrations of TNF-α and IL-1β in the serum and LA. n = 7 per group. *P < 0.01 vs. MI; **P < 0.05 vs. MI; #P < 0.01 vs. MI + P + L.

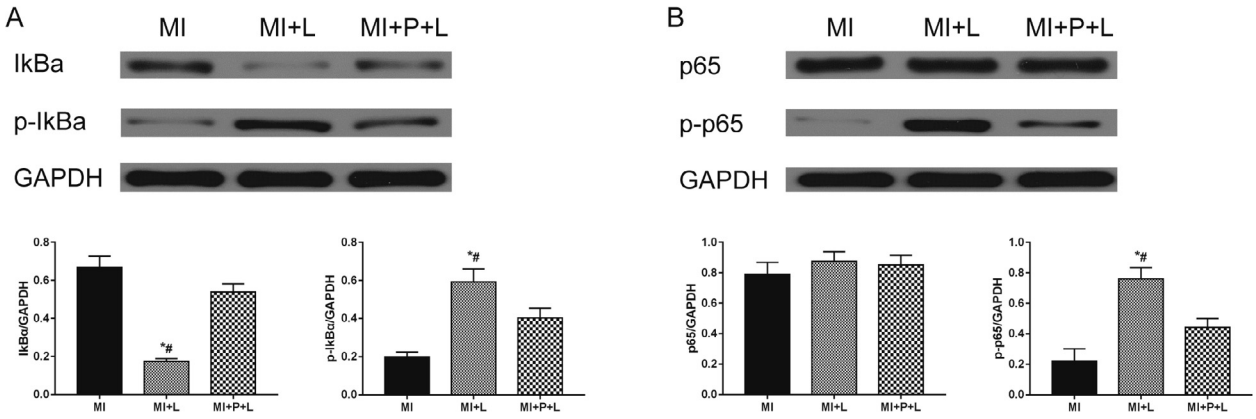

Fig. 12. (A, B) Protein expression of IκBα, p-IκBα, p65, and p-p65. n = 7 per group. *P < 0.01 vs. MI; #P < 0.01 vs. MI + P + L.

increased the mean ERP in the MI + P + L group. Compared with the MI group, the APD_{90} was significantly shortened in the MI + L group; however, it was significantly increased in the MI + P + L group (Fig. 9B). As Fig. 9C showed, AF was inducible in the three groups in the burst pacing protocol, and the incidence of AF was 80% in the MI group, 90% in the MI + L group, and 50% in the MI + P + L group; however, no significant difference was found in the average duration of AF in the three groups (Fig. 9D). Fig. 10A, B showed no significant difference between the MI group and the MI + L group in the mean HR and mean RR, respectively, but pinocembrin administration significantly reduced the mean HR and increased the mean RR in the MI + P + L group. Although no significant difference was found between the MI group and the MI + L group in the serum NE concentration, the MI + P + L group exhibited a significant decrease in the concentration of NE compared with the MI + L group (Fig. 10C). Furthermore, the concentrations of TNF-α and IL-1β in the serum and LA were significantly increased in the MI + L group compared with the MI group, but those were significantly decreased in the MI + P + L group (Fig. 11A, B). In addition, Fig. 12A showed that pinocembrin treatment significantly increased the expression of IκBa in the MI + P + L group compared with the MI + L group; however, the expression levels of p-IκBa and p-p65 were significantly reduced by pinocembrin (Fig. 12A, B), illustrating that the NF-κB pathway was inhibited after administration of pinocembrin.

4. Discussion

The present study evaluates the effects of pinocembrin on autonomic dysfunction and AF susceptibility in rat models of MI. Our results demonstrate the following findings: (i) a higher incidence of atrial arrhythmias in the MI group compared with the Sham group; (ii) pinocembrin decreased cardiac autonomic remodeling and improved HRV in MI rats; (iii) pinocembrin ameliorated atrial electrical remodeling in MI rats; (iv) pinocembrin decreased atrial fibrosis and increased expression of Cx43 in MI rats; (v) pinocembrin increased the expression of Cav1.2 and thus upregulated L-type calcium currents in MI rats; and (vi) pinocembrin decreased concentrations of TNF-α, IL-1β, IL-6, and expression of p-p65 and p-IκBα in MI rats, indicating that pinocembrin administration suppresses inflammatory responses via inhibition of the NF-κB/TNF-a pathway in a rat model of MI.

4.1. Pinocembrin and HRV

The ANS, including the sympathetic and parasympathetic system, plays an important role in cardiac electrophysiology. Autonomic remodeling promotes the persistence and recurrence of AF [31]. Our results showed the decreased HF, mean RR, SDNN, and RMSSD and increased LF and LF/HF in the MI group. HF reflects the parasympathetic activity, whereas LF indicates a combination of both sympathetic and parasympathetic activities. The LF/HF ratio is mainly used as an effective indicator of sympathetic activity. Moreover, the SDNN measures the total RR variations, and the RMSSD shows the short-term and high-frequency variations of RR intervals, both of which reflect the parasympathetic activity. Decreased HF, mean RR, SDNN, and RMSSD are associated with the attenuated parasympathetic activity, whereas increased LF and LF/HF ratio are related to the augmented sympathetic activity [29]. All these factors contribute to a higher risk of cardiovascular diseases, especially the arrhythmias [31,32]. These findings, along with the high concentration of NE in the serum and LA, demonstrated that MI enhanced sympathetic activity, decreased parasympathetic activity, and contributed to the sympathovagal imbalance. However, pinocembrin treatment increased the mean RR, SDNN, RMSSD, and HF and decreased the LF and LF/HF ratio in the MI + P group compared with the MI group, illustrating that pinocembrin could decrease MI-induced cardiac autonomic remodeling. However, additional studies are still needed to investigate the accurate mechanisms of these results.

4.2. Pinocembrin and atrial fibrillation

Atrial electrophysiological parameters were measured to confirm whether pinocembrin is beneficial to atrial electrical remodeling. Previous studies have demonstrated that AF is often associated with decreased atrial ERP and APD [33,34,35]. AL curbs the atrial response interval resulted from the delayed activation and slow conduction velocity. Moreover, the increased AL may steepen APD restitution, which is related to the initiation of AF [36]. APD alternans is widely considered as a predictor and risk factor of arrhythmia including AF, in which L-type Ca^{2+} channels play an important role [37]. Our work showed similar results. Compared with the Sham group, a significant decrease in the mean ERP and APD and increase in the AL, APD alternans CL, and inducibility rate of AF were observed in the MI group. However, pinocembrin significantly increased the mean ERP and APD and decreased the AL, APD alternans CL, and inducibility rate of AF in the MI + P group compared with the MI group, demonstrating that pinocembrin decreased MI-induced AF susceptibility by ameliorating atrial electrical remodeling.

Various ion channels are associated with electrical remodeling, of which the calcium channel is the most important. In our study, the expression of Cav.1.2 was significantly decreased in the MI group compared with the Sham group, but it was markedly increased in the MI + P group compared with the MI group. Calcium release promoting triggered activity is a crucial mechanism of AF initiation. Decreased L-type Ca^{2+} current density was found in AF patients with decreased APD [38]. L-type Ca^{2+} channel blocks were also observed to ameliorate atrial electrical remodeling by prolonging ERP [39]. Furthermore,

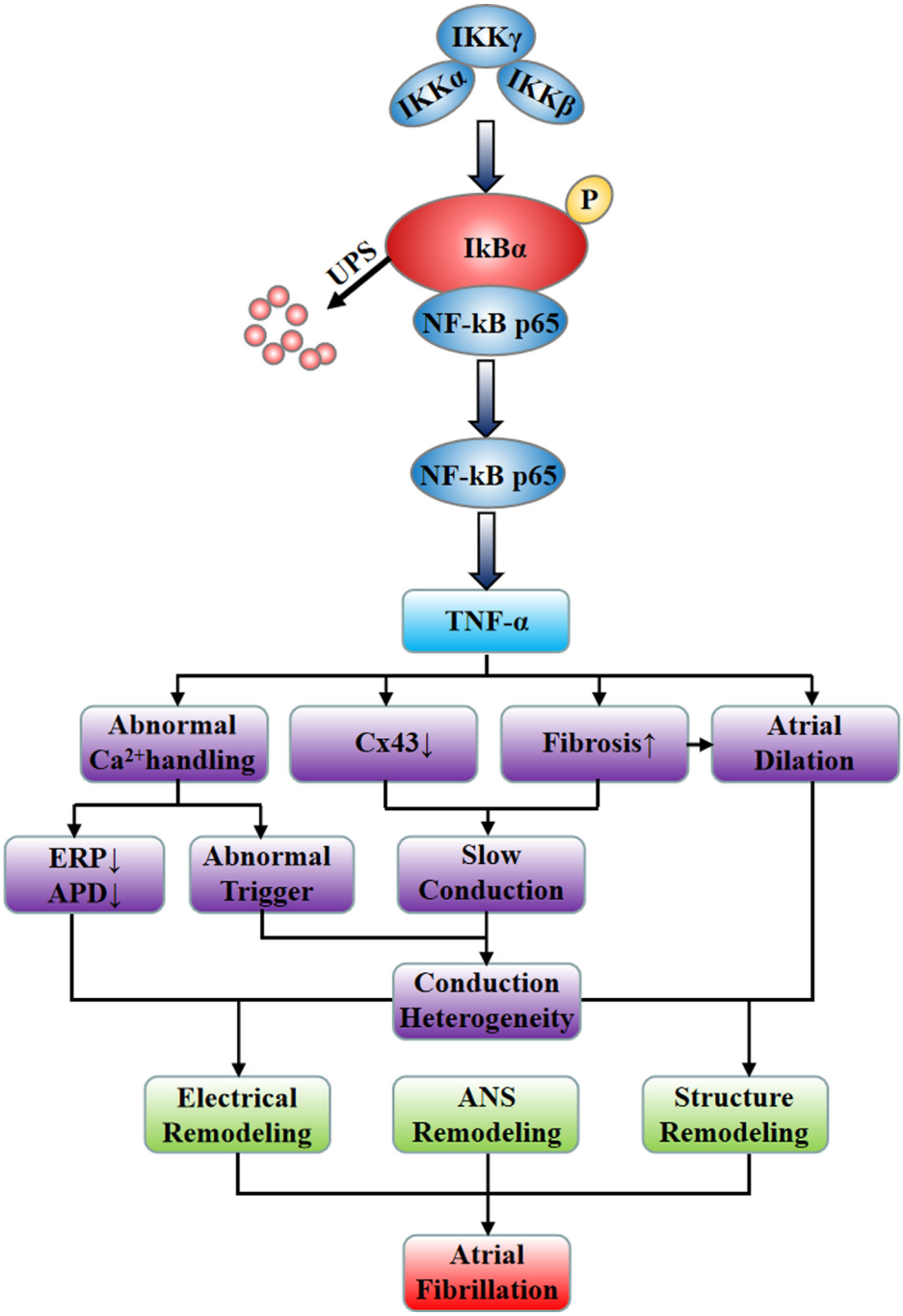

Fig. 13. NF-κB regulates TNF-α, which is closely associated with the development and maintenance of AF. ERP, effective refractory period; APD, action potential duration; ANS, autonomic nervous system; AF, atrial fibrillation.

dysfunction of Ca^{2+} signaling was proved to generate APD alternans [37]. All the aforementioned studies indicate that the calcium channel may be a vital factor in atrial electrical remodeling and the occurrence and maintenance of AF [40].

The present study showed a significant increase in atrial fibrosis and decrease in Cx43 expression in the MI group compared with the Sham group, but pinocembrin significantly decreased atrial fibrosis and increased the expression of Cx43 in the MI + P group compared with the MI group. Atrial fibrosis, an important form of atrial structural remodeling, is closely associated with AF. Fibrosis interferes with electric continuity and slows down the conduction [41]. Moreover, AF itself may accelerate atrial structural remodeling, which helps to generate a permanent form of AF [42]. Gap junctions, mainly composed of connexins, connect myocardial cells to each other with a low resistance. Cx43 is a crucial kind of connexin expressed in atrial. The loss of Cx43 results in atrial heterogeneity and slow conduction. These factors provide an abnormal substrate for the initiation and maintenance of AF [43,44].

4.3. Pinocembrin and the NF-κB/TNF-α signaling pathway

Previous studies have indicated that the Toll-like receptor (TLR) family plays an important role both in innate and adaptive immune responses [45,46]. TLRs can recognize specific molecular patterns, subsequently triggering various activities, in particular the inflammatory responses [46]. Numerous studies have demonstrated that

LPS, the cell-wall of gram-negative bacteria, can be recognized by TLR4, thus contributing to the activation of the NF-kB pathway both in animals and humans [47].

A previous study has shown that LPS treatment induces inflammatory responses in vivo with the RAW macrophage cells and in invitro experiments with the murine model, along with activated NF-κB and increased inflammatory cytokines. However, pinocembrin administration markedly suppresses inflammatory responses through the inhibition of the NF-κB pathway, indicating the function of pinocembrin in regulating the NF-κB pathway [24]. The present study showed similar results. Inflammatory cytokines, including TNF-α, IL-1β, and IL-6 in the serum and LA, were significantly higher in the MI group than in the Sham group. Furthermore, the expression of p-p65 and p-IκBα were significantly increased in the MI group compared with the Sham group. However, those were dramatically decreased in the MI + P group compared with the MI group, illustrating that pinocembrin administration inhibited MI-induced NF-κB and IκBα activation to suppress inflammatory responses.

Excessive activation of inflammatory responses often leads to adverse consequences, in which inflammatory cytokines play a key role [9]. As described previously, inflammatory cytokines (e.g., TNF-α, IL-1β) are found in MI both at the local injured sites and at the remote sites throughout the circulating blood [7,8]. As a vital upstream regulator of the inflammatory responses, NF-κB remains a low basal transcriptional activation in normal conditions and it is activated with its inhibitory protein IκBα degraded when faced with stress. Activated NF-κB gets into the nucleus and upregulates the transcription of target genes, including TNF-α, IL-1β, and IL-6, which then results in problematic outcomes including arrhythmias [13,14,15]. Increased TNF-α, IL-1β, and IL-6 levels are associated with the occurrence and progression of AF and the prognosis after AF ablation [48,49]. As an inflammatory biomarker, TNF-α is closely related to the development and maintenance of AF, primarily in the following way (Fig. 13): (i) TNF-α promotes atrial fibrosis and atrial dilatation and alters the expression of connexins such as Cx43, causing a heterogeneous conduction; (ii) TNF-α may also directly change Ca^{2+} handling in cardiomyocytes, providing a substrate for the initiation of AF. Similar to TNF-α, IL-1β interferes with electrophysiology, primarily due to its changes in Ca^{2+} handling and cell-cell coupling [50]. Higher serum IL-6 is also associated with a higher risk of the occurrence and recurrence of AF by influencing the cardiomyocyte Ca^{2+} handling [49,51]. Our findings indicate that pinocembrin inhibits the NF-κB/TNF-α signaling pathway and lowers pro-inflammatory cytokines, which may help to ameliorate the susceptibility of AF.

In fact, to further investigate the effects and mechanism of pinocembrin on regulating the ANS and AF susceptibility, we used the LPS to activate the NF-κB/TNF-α signaling pathway in MI rats. As Fig. 9C showed, AF inducibility was 90% in the MI + L group, which was reduced to 50% in the MI + P + L group. Along with the longer ERP and APD in the MI + P + L group than in the MI + L group, the results demonstrated that AF susceptibility was attenuated after administration of pinocembrin in MI rats. The mean HR and mean RR are considered to be the common measure of the ANS, particularly the parasympathetic system. The increased HR is often associated with the reduced parasympathetic activity [52]. As the inverse of the HR, the decreased mean RR also reflects the decreased parasympathetic activity [53]. In our present study, although no significant differences were found between the MI group and the MI + L group in the mean HR and mean RR, pinocembrin treatment significantly decreased the mean HR and increased the mean RR in the MI + P + L group. Along with the reduced NE concentration in the serum, the findings indicate that pinocembrin treatment augments the parasympathetic activity and depresses the sympathetic activity, contributing to a sympathovagal balance in MI rats.

In addition, the concentrations of TNF-α and IL-1β in the serum and LA were significantly higher in the MI + L group than in the MI group, indicating that LPS promoted the inflammatory responses. However, pinocembrin treatment significantly decreased the inflammatory markers, demonstrating that the inflammatory response was effectively suppressed by pinocembrin. Moreover, the expression of p-p65 and p-IκBα was significantly increased in the MI + L group compared with the MI group, showing that the NF-κB pathway was further activated by LPS in a rat model of MI. However, compared with the MI + L group, pinocembrin treatment significantly reduced the expression of p-p65 and p-IκBα. The results confirm that pinocembrin suppresses inflammatory responses by inhibiting the NF-κB/TNF-α signaling pathway in MI rats. The aforementioned findings indicate that pinocembrin attenuates autonomic dysfunction and AF susceptibility probably resulted from the inhibition of the NF-κB/TNF-α pathway in a rat model of MI.

5. Limitations

The present study has several limitations. First, although much attention was given to avoid unnecessary disturbances, anesthetized animals still have some limitations in the assessment of autonomic activity. However, animals were given similar anesthesia, which could counteract the differences among groups. Second, MI may lead to not only the inflammatory responses but also to some other stress responses (e.g., atrial ischemia, oxidative stress) that lead to adverse cardiac consequences, which needs to be investigated in the subsequent studies. Finally, inflammatory responses often reach a peak 3 to 5 days after stress, whereas the present study detected inflammatory markers only on day 7; our subsequent study should investigate these findings.

6. Conclusions

In summary, the present study showed that pinocembrin ameliorated autonomic remodeling, atrial electrical remodeling, atrial fibrosis, and AF susceptibility and decreased the inflammatory cytokine production in MI rats. In addition, pinocembrin inhibited NF-κB and IκBα activation to suppress MI-induced inflammatory responses. These findings provide new insights into the relationship between pinocembrin and AF.

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Declaration of Competing Interest

None.

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