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Original Research

β-arrestin2 Mediates the Arginine Vasopressin-Induced Expression of IL-1β in Murine Hearts

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Abstract

Background: Circulating levels of arginine vasopressin (AVP) are elevated during cardiac stress and this could be a factor in cardiac inflammation and fibrosis. Herein, we studied the effects of AVP on interleukin-1β (IL-1β) production and the role(s) of β-arrestin2-dependent signaling in murine heart. **Methods**: The levels of IL-1β mRNA and protein in adult rat cardiofibroblasts (ARCFs) was measured using quantitative PCR and ELISA, respectively. The activity of β-arrestin2 was manipulated using either pharmacologic inhibitors or through recombinant β-arrestin2 over-expression. These experiments were conducted to determine the roles of β-arrestin2 in the regulation of AVP-induced IL-1β and NLRP3 inflammasome production. The phosphorylation and activation of NF-κB induced by AVP was measured by immunoblotting. β-arrestin2 knockout (KO) mice were used to investigate whether β-arrestin2 mediated the AVP-induced production of IL-1β and NLRP3, as well as the phosphorylation of the NF-κB p65 subunitin mouse myocardium. Prism GraphPad software(version 8.0), was used for all statistical analyses. **Results**: AVP induced the expression of IL-1β in a time-dependent manner in ARCFs but not in cultured adult rat cardiomyocytes (ARCMs). The inhibition of NF-κB with pyrrolidinedithiocarbamic acid (PDTC) prevented the AVP-induced phosphorylation of NF-κB and production of IL-1β and NLRP3 in ARCFs. The deletion of β-arrestin2 blocked the phosphorylation of p65 and the expression of NLRP3 and IL-1β induced by AVP in both mouse hearts and in ARCFs. **Conclusions**: AVP promotes IL-1β expression through β-arrestin2-mediated NF-κB signaling in murine heart.

Keywords: arginine vasopressin; β-arrestin 2; NF-κB p65; NLRP3 inflammasome; interlukin-1β; inflammation; murine

1. Introduction

Inflammatory response is a key mechanism driving cardiovascular disease, and a critical risk factor for the development of cardiovascular conditions. In response to heart injury, necrotic cardiac cells release a variety of damage-associated molecular patterns (DAMPs) that activate NLR family pyrin domain containing 3 (NLRP3) inflammasomes in cells of the innate immune system [1]. The NLRP3 inflammasome is a key multiprotein signaling platform for activation of inflammatory responses, and plays an important role in heart injury by activating caspase-1 for the subsequent maturation of pro-inflammatory cytokines, interleukin-1β (IL-1β) and interleukin-18 (IL-18) [2]. Both IL-1β and IL-18 are members of the pro-inflammatory cytokine IL-1 superfamily and are often found in the serum during inflammatory stimulation. Cardiac-derived IL-1β can impair cardiac contractility by inducing a calcium leak from the sarcoplasmic reticulum, ultimately promoting cell death and tissue remodeling [3].

Arginine vasopressin (AVP) is a vasopressive peptide composed of nine amino acids in mammals. As a neurohormone and hemodynamics factor AVP maintains fluid balance homeostasis, vascular tonus, and the regulation of the endocrine stress system [4]. These physiological effects are mediated in humans via binding to at least three G-protein-coupled receptors (GPCRs) subtypes, which have been identified as $V_{1A}R$, V_2R and $V_{1B}R$ (also termed V3R) [5]. However, only $V_{1A}R$ is found in cardiac myocytes and cardiac fibroblasts [6]. AVP can be synthesized in the heart and elicits local and potentially systemic effects via cardiac paracrine [7] signaling. Of note, AVP levels are elevated relative to the severity of heart failure or left ventricular dysfunction [8]. In our earlier studies, AVP induced the expression of IL-6 in rat cardiac fibroblasts [9]. Both IL-6 and IL-1β belong to the interleukin family, so it is possible that AVP could induce the expression of IL-1β in heart cells and whether this process was related to the NLRP3 inflammasome.

β-arrestins are commonly expressed cytoplasmic adapter proteins, which function, along with heterotrimeric G proteins, as critical regulators and signal transducers for GPCRs [10]. β-arrestins mediate neuropeptide-driven inflammatory disease and GPCR-mediated inflammation [10,11]. Specifically, β-arrestins regulate toll-like receptor-interleukin 1 receptor (TLR-IL-1R)-induced signal transduction in macrophages which promotes inflam-

mation [12]. Moreover, TLRs upregulate the NLRP3 inflammasome by inducing the activation of NF-κB. In addition, β-arrestin1, which is 78% identical to β-arrestin2 at the amino acid level, is essential for the full activation of NLRP3 [13]. Earlier studies from our group have shown that β-arrestin2 regulates inflammation by activating the AVP-mediated $ERK_{1/2}$-NF-κB (ERK: extracellular regulated protein kinases) signaling pathway which induces expression of IL-6 [6]. In this study, the role of AVP in regulating the expression of IL-1β, as well as the roles of β-arrestin2 and the NLRP3 inflammasome in regulating this process, were further explored both *in vivo* and *in vitro* and this work may provide a new therapeutic approach for the treatment of cardiovascular diseases.

2. Materials and Methods

2.1 Animals

The adult Sprague-Dawley rats were obtained from the Animal Center of Nantong University (Nantong, China). The β-arrestin2 knockout mice were obtained from the laboratory of Dr. Lefkowitz (Duke University, Durham, USA). Male rats and mice (8–12 weeks old) were administered with 2 μg/g of AVP via tail vein injection. Heart tissues were harvested and RNA and protein obtained at designated timepoints after AVP administration. The study was approved by the Board of Nantong University Animal Care and Use.

2.2 Materials

NF-κB p65 antibody (D14E12), p-NF-κB antibody (93H1), anti-rabbit IgG, anti-mouse IgG, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody, and β-arrestin1/2 antibody (D24H9) were purchased from Beijing Green Herbs Biological Co. LTD (Beijing, China). NLRP3 antibody (ab214185) and IL-1β antibody (ab205924) were purchased from Abcam (Cambridge, UK). The primers for murine IL-1β and GAPDH were purchased from Shanghai Sangon Biotech (Shanghai, China). Total RNA Extraction Kit and Total DNA Extraction Kit were from Zymo Research (Beijing, China). Rat IL-1β ELISA Kit (E-EL-R0017c) and mouse IL-1β ELISA Kit (E-EL-M0079c) were obtained from Elabscience (Wuhan, China). PDTC (S1808) was obtained from Shanghai Beyotime Biotechnology (Shanghai, China). ShRNA that target β-arrestin2 (shRNA898, shRNA899, shRNA900) was purchased from Shanghai Genechem Co.Ltd (Shanghai, China).

2.3 Methods

2.3.1 Isolation and Culture of Cardiomyocyte and Cardiac Fibroblasts

Adult rat cardiac fibroblasts (ARCFs) were obtained from the hearts of adult (250–300 g) male Sprague Dawley rats after anesthesia with 10% chloralhydrate (0.5 mL/kg body weight) as previously described [9]. Experiments were consistently performed on passage 3–8 cells. Adult rat cardiac myocytes (ARCMs) were isolated and cultured in serum-free Medium199 with 100 U/mL penicillin-streptomycin solution for 2–4 h before passaging as previously described [14].

2.3.2 Lentiviral Infection

ARCFs were washed twice with sterile PBS (phosphate buffer solution) and infected with lentivirus encoding shRNA targeting β-arrestin2 (shRNA898, shRNA899, shRNA900) at a MOI = 50. Cells were subsequently incubated in fresh medium for 12 h, and the transfection efficiency was observed under a fluorescence microscope after 72 h.

2.3.3 Propidium Iodide Assay

A propidium iodide (PI) assay was used to quantify cardiomyocyte death (ST512, Shanghai, China). Cardiomyocytes were rinsed with PBS after AVP treatment and incubated with Hoechst 33258 (C1018, Beyotime, China) for 15–30 min at 37 °C for cell counting. The cells were then rinsed three times with PBS and incubated with PI for 20–30 min at 37 °C to count dead cells. A Leica fluorescence microscope was used to capture images.

2.3.5 Immunofluorescence

Mouse hearts were fixed in 4% paraformaldehyde and embedded at 4 °C overnight. The hearts were cut into 5μm of tissue slices with a tissue slicer and were fixed in the cover slips. After wax removal and rehydration of tissue slices, the myocardium samples were incubated with primary antibody to IL-1β or NLRP3 diluted in 0.4% Triton X-100 for permeabilization. Following incubation with primary antibody, slides were washed 3 times with permeabilizing buffer, and the slides were then labeled with RITC (rhodamine isothiocyanate)—labeled goat anti-mouse IgG secondary antibody. Fluorescence density was assayed using Image J software version 1.8.0.112 (NIH, Bethesda, MD, USA).

2.3.6 Quantitative PCR (qPCR) for Analyzing IL-1β mRNA Levels

The level of the IL-1β and GAPDH RNA in ARCMs was quantified by qPCR. Total RNA from cultured cardiomyocytes was extracted using Trizol reagent (Promega, Madison, WI, USA) according to the manufacturer's protocol. The Step-one Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used to amplify the cDNA. Primer sequences used to detect murine IL-1β and GAPDH are shown in Table 1. Thermocycling conditions were as follows: 95 °C for 1 min, 35 cycles at 95 °C, denaturation for 5 s, then annealing at 60 °C for 1 min. The level of GAPDH was employed as RNA content loading control. Data were analyzed using the ΔCT-method ($2^{-\Delta\Delta CT}$).

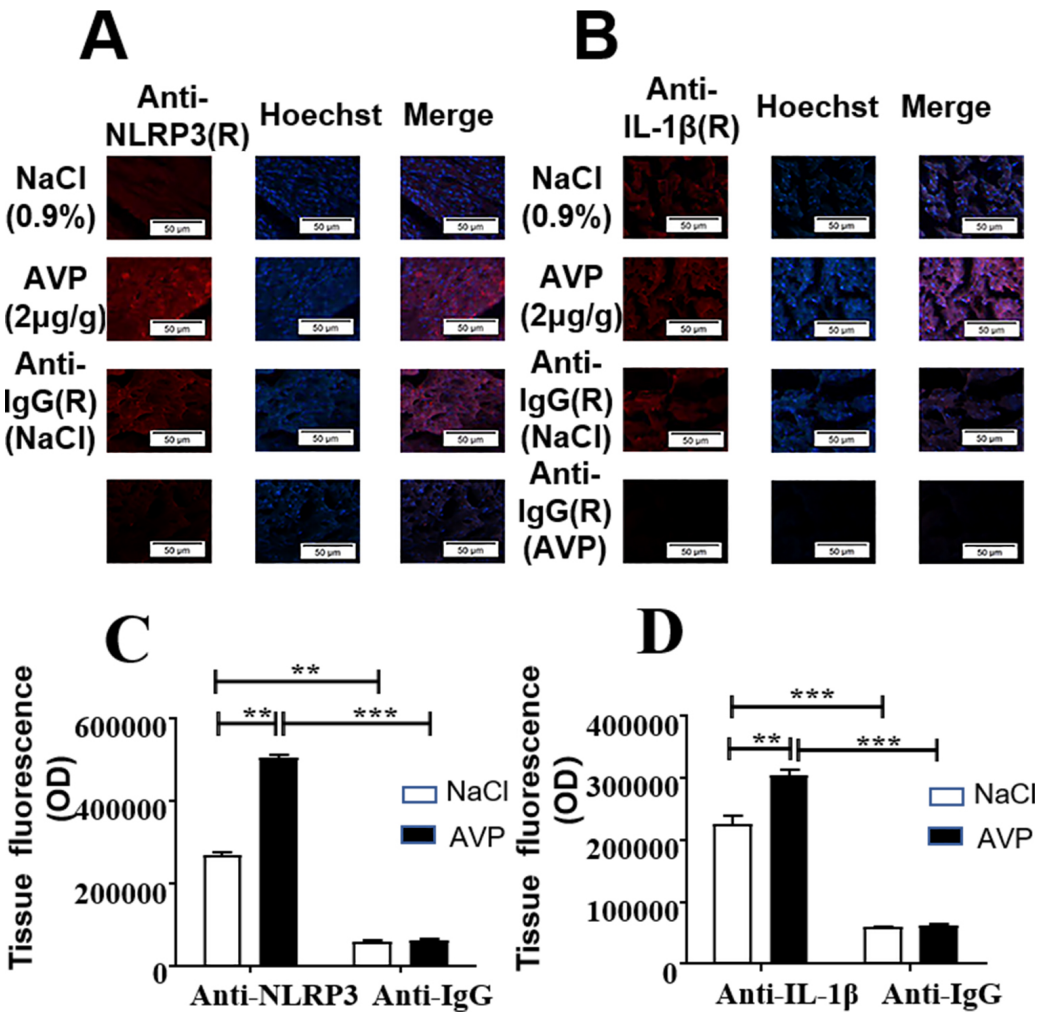

Fig. 1. AVP induced expression of the NLRP3 inflammasome and IL-1β in mouse myocardium following AVP administration. (A,B) Representative images of wild type mice injected with 2 μg/g body weight of AVP or 0.9% of NaCl via the tail vein. Frozen sections were prepared from dissected heart tissue to determine the protein level of the NLRP3 inflammasome and IL-1β 6 h after dosing with NaCl or AVP. (C,D) Fluorescent optical density was determined and data expressed as mean $\pm$ S.E.M. of 3 separate animals with triplicate tissue sections, $^{**}p < 0.01$, $^{***}p < 0.001$. one-way ANOVA test, followed by Bonferroni's post hoc test. Note: Non-specific binding staining was examined using mouse IgG. Scale bar = 50 μm.

Table 1. The primers for rat IL-1β and GAPDH.

Gene	Primers	Sequences (5′-3′)
IL-1β	Forward	TTCTTGGGACTGATGT
	Reverse	GAATGACTCTGGCTTTG
GAPDH	Forward	TTCAATGGCACAGTCAAGGC
	Reverse	TCACCCCATTTGATGTTAGCG

2.3.7 ELISA for IL-1β

Mouse serum, heart homogenates, or cell culture supernatant samples (100 μL) were used to measure IL-1β using an ELISA kit. The analytical sensitivity of the kit was 12.0 pg/mL and has a range of 31.3–2000 pg/mL, respectively.

2.3.8 Western Blots

Tissue and cell samples were ground with RIPA buffer, and the mixture was centrifuged at 12,000 g for 15 min at 4 °C. Supernatants were stored at –20 °C, and the protein concentration was measured using a bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology). In total, 100 μg of proteins were separated using 5%–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to PVDF membranes. Following transfer, the membranes were treated with blocking buffer (5% BSA) for 2 h and subsequently incubated with primary antibodies against the NF-κBp65s subunit (1:1000), phospho-NF-κB (1:1000), β-arrestin1/2 (1:1000), NLRP3 (1:1000), IL-1β (1:1000), or GAPDH (1:5000) at 4 °C overnight. After washing three times for 10 min with washing buffer, the

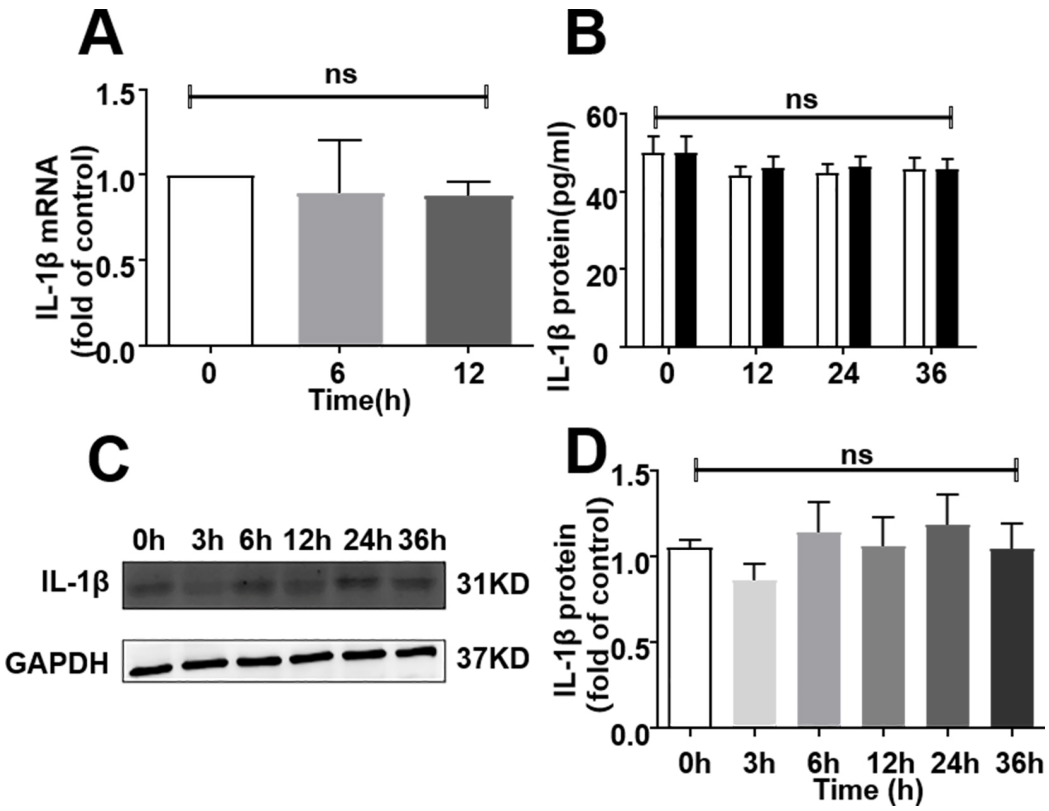

Fig. 2. AVP did not induce IL-1β expression in adult rat cardiomyocytes (ARCMs). (A) AVP treatment did not alter the level of IL-1β mRNA in ARCMs. Cells were treated with 10^{-7} M AVP for 0–36 h and subsequently harvested for total RNA. IL-1β mRNA was subsequently determined by q-PCR. Data were expressed as mean $\pm$ S.E.M. of 4 separate experiments. (B,C,D)AVP stimulation did not increase the level of IL-1β protein in ARCMs. The cells in 6-well plates were stimulated with 10^{-7}M AVP for 0–36 h. The supernatant was collected and ELISA assays conducted to measure IL-1β. Data are expressed as mean $\pm$ S.E.M. of 6 separate experiments. (B) Supernatant in cultured ARCMs. (C,D) A represent blots and average data in ARCMs. One-way ANOVA test, ns: no significance.

membranes were incubated with anti-rabbit IgG (1:1000) at room temperature for 2 h. After another three washes for 10 min with washing buffer, the protein bands were scanned and quantified using Image J software.

2.3.9 Statistical Analysis

Prism GraphPad software (version 8.0) (San Diego, USA) was used for all statistical analyses. In each biological experiment, the data for each individual western blot was normalized to a value of density in control (setting all control values to 1). For control group, the data was normalized to the average value (setting average values to 1) of biological replicates in order to perform the statistical analysis. Data are presented as mean $\pm$ S.E.M. Comparisons were conducted using one or two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. $p < 0.05$ was considered statistically significant.

3. Results

3.1 AVP Induces Expression of the NLRP3 Inflammasome and IL-1β in Mouse Myocardium

To determine if AVP perfusion induced expression of the NLRP3 inflammasome and IL-1β in mouse myocardium, mice were injected with 2 μg/g body weight of AVP or 0.9% of NaCl, as a control, via the tail vein. Mouse hearts were cut for frozen sections 6 h post AVP dosing, and we subsequently performed immunohistochemistry as shown in Fig. 1A–B. Fluorescent optical density was determined using Image J. As shown in Fig. 1C,D, the expression of the NLRP3 inflammasome and IL-1β protein in heart tissue were significantly increased following AVP injection when compared with controls.

3.2 AVP Specifically Induced Expression of IL-1β in ARCFs but not in ARCMs

To investigate whether AVP affected the viability of ARCMs, *in vitro* experiments using ARCMs were performed. The cells were incubated with 10^{-7} M AVP for 0 h, 12 h, 24 h, and 36 h followed by Hoechst/PI staining, and the number of stained cells was counted under fluores-

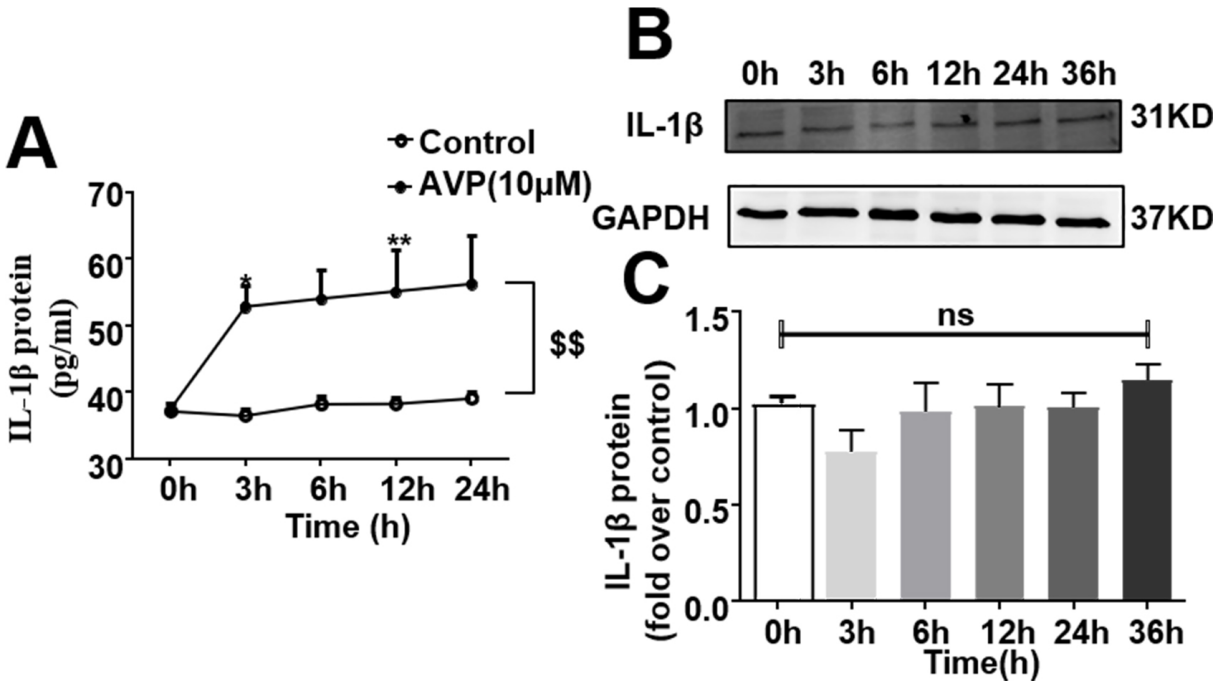

Fig. 3. AVP induced the expression of IL-1β protein in adult rat cardiac fibroblasts (ARFCs) in a time-dependent manner. Starved cells in 6-well plates were stimulated with 10^{-7} M AVP for 0–24 h. (A) AVP increased the level of IL-1β protein in a time-dependent manner in supernatant of cultured cells. IL-1β was measured in culture media using ELISA. Data were expressed as mean $\pm$ S.E.M. of 6 separate experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{\$\$}p < 0.01$ for time-course (repeated two-way ANOVA). (B,C) AVP did not enhance the IL-1β expression in the cellular lysates of ARCFs by either western blotting or ELISA. Data were expressed as mean $\pm$ S.E.M. of 3 separate experiments. One-way ANOVA test, ns, no significance.

cence microscopy. The viability of cells was below 10% within 36 h of culture, indicating that AVP did not induce the cell death (data not shown). To further investigate the effect of AVP on IL-1β expression in ARCMs, primary rat cardiomyocytes were stimulated with 10^{-7} M AVP for 0 h, 3 h, 6 h, 12 h, 24 h, or 36 h, and total RNA and protein were isolated from ARCMs. The levels of IL-1β mRNA measured by qPCR did not change significantly in ARCMs (Fig. 2A). In addition, the results of ELISA (Fig. 2B–D) and western blotting (Fig. 2C) revealed that AVP did not alter the expression of either IL-1β mRNA or protein in ARCMs during these time periods.

The same procedure was conducted with adult rat cardio fibroblasts (ARCFs) to explore the effect of AVP on IL-1β induction. Starved ARCFs were incubated with or without 10^{-7} M AVP for 0–24 h and cellular lysates were harvested. The level of IL-1β in the supernatant of cultured medium was detected by ELISA. Fig. 3A indicated that the incubation of ACRFs with AVP increased the level of IL-1β protein in the supernatant of the cultured ARCFs in a time-dependent manner compared with the vehicle control group. However, the level of IL-1β protein in the cellular lysates determined by western blot was not changed following stimulation, implying that mature IL-1β were secreted from ARCFs, and the extra cellular IL-1β protein did not degrade within 24 h as shown in Fig. 3B,C.

3.3 AVP Induced the Expression of NLRP3 Inflammasome and IL-1β through NF-κB Signaling Pathway

To determine whether AVP induced NF-κB p65 phosphorylation and whether this process can be specifically blocked by PDTC, an inhibitor of NF-κB, starved ARCFs were pretreated with 50 μM of PDTC followed by 10^{-7} M AVP. The results showed that AVP induced NF-κB p65 phosphorylation, while PDTC specifically blocked AVP-induced this phosphorylation event (Fig. 4A,B). Moreover, PDTC itself had no effect on NF-κB p65 phosphorylation.

Accordingly, as displayed in Fig. 4C–E, the inhibition of NF-κB with PDTC6, significantly attenuated the effects of AVP in the induction of NLRP3 in ARCFS, as well as enhancing the level of IL-1β in the supernatant of cultured ARCFs compared to controls. Additionally, PDTC itself had no effect on the expression of the NLRP3 inflammasome and IL-1β.

3.4 The Silencing of β-arrestin2 Prevented NLRP3 Expression Induced by AVP Stimulation in ARCFs

β-arrestin2 was knocked down in ARCFs using shRNA-encoding lentivirus. 48 h post infection, more than 90% of the cells were infected as evidenced by cells displaying virally-encoded fluorescent reporter (data not shown). Subsequently, western blotting was used to determine that either shRNA898 or shRNA900 significantly down regu-

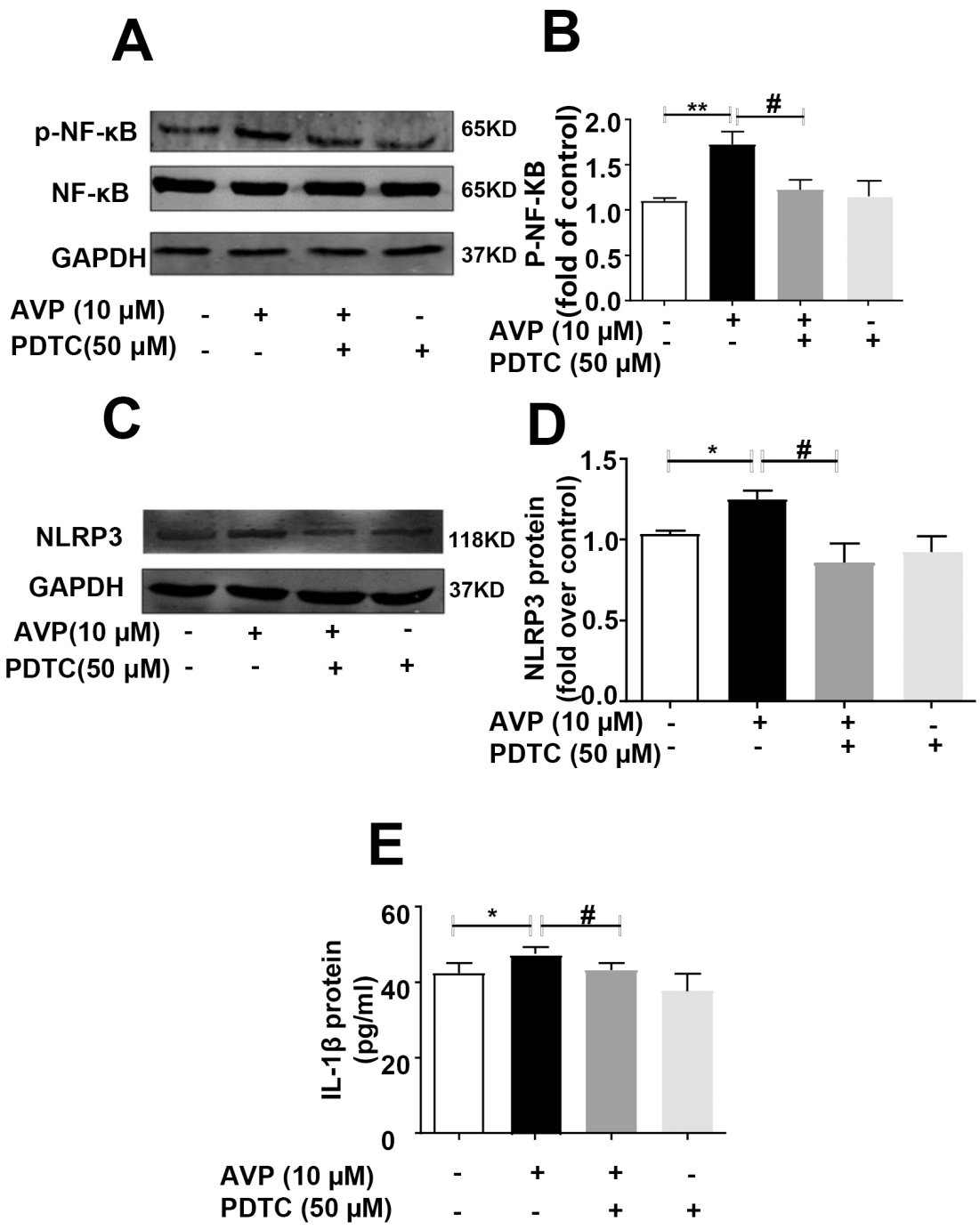

Fig. 4. PDTC abolished AVP-induced the phosphorylation of NF-κB p65, expression of NLRP3 inflammation, and IL-1β in ARCFs. Starved cells were cultured with 50μM PDTC for 1 h, followed by stimulation with 10^{-7} M of AVP for 1 h. Cellular lysates were subsequently used in western blotting experiments. (A) Representative blot. (B) Average data. Data were expressed as mean $\pm$ S.E.M. of 6 separate experiments. $^{**}p < 0.01$, $^{\#}p < 0.05$, one-way ANOVA test, followed by Bonferroni's post hoc test. (C) PDTC abolished the AVP-induced production of IL-1β protein in supernatant of cultured ARFCs. (D,E) PDTC abolished the AVP-induced expression of NLRP3 inflammasome in ARFCs. (D) A representative blot, (E): average data from 6 separate experiments. $^{*}p < 0.05$, $\#p < 0.05$, one-way ANOVA test, followed by Bonferroni's post hoc test.

lated the expression of β-arrestin2 compared to a scrambled shRNA control, as shown in Fig. 5A,B. In ARCFs with and without knocked down β-arrestin2 expression, western blot analysis indicated that AVP induced the expression of the NLRP3 inflammasome in ARCFs, but not in ACRFs with knocked down β-arrestin2 expression (Fig. 5C,D).

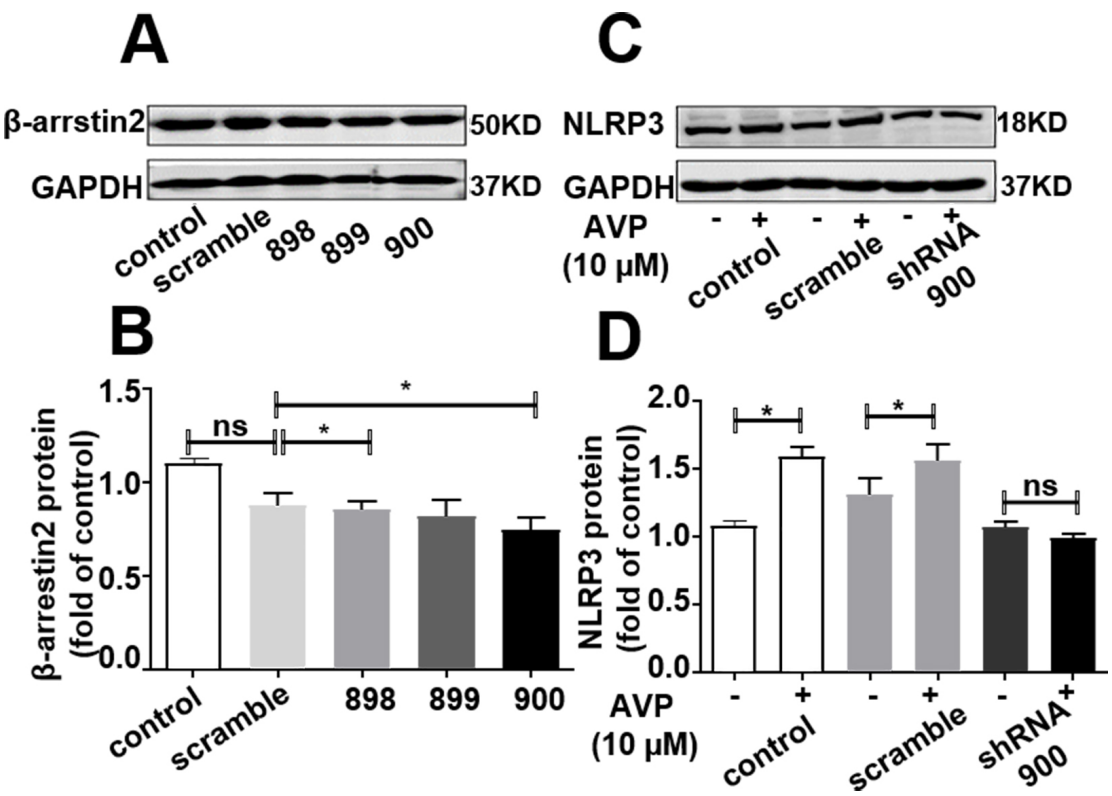

Fig. 5. Silencing of β-arrestin2 abolished NLRP3 expression induced by AVP stimulation in ARCFs. (A,B) Silencing of β-arrestin2 protein expression by different β-arrestin2 shRNA in ARCFs. Western blotting confirmed decreased the expression of β-arrestin2 in ARCFs. (A) A representative blot showing that shRNA-encoding lentivirus efficiently knocked down expression of β-arrestin2. (B) Average data. Data were presented as the mean $\pm$ S.E.M. from 6 independent experiments. $^{*}p < 0.05$ vs scramble, one-way ANOVA test, followed by Bonferroni's post hoc test. (C,D) Knockdown of β-arrestin2 abolished the AVP-induced NLRP3 inflammation expression in ARCFs. (C) A representative blot. (D) Average data. Data were presented as the mean $\pm$ S.E.M. from 5 independent experiments. $^{*}p < 0.05$, one-way ANOVA test, followed by Bonferroni's post hoc test. ns, no significance.

This suggested that silencing β-arrestin2 abolished AVP-induced expression of NLRP3 in ARCFs.

3.5 β-arrestin2 was Necessary for AVP-Induced IL-1β and NLRP3 Inflammasome Expression in Mouse Myocardium

To investigate the specific role of β-arrestin2 in AVP-induced inflammatory response, β-arrestin2 knockout mice and wild-type mice were injected with AVP (2 μg/g body weight) through the tail vein. An equal volume of normal saline with 0.9% NaCl was injected into the vehicle group. The protein level of IL-1β in blood serum, as measured by ELISA, indicated that dosing of AVP enhanced the level of IL-1β in the serum of wild type mice, but not in β-arrestin2 knockout mice (Fig. 6A). Further, IL-β protein expression of in the left ventricle of mouse heart measured by ELISA (Fig. 6B) and western blot (Fig. 6C,D) demonstrated that AVP induced the expression of IL-1β in myocardium of wild type mice, but not in β-arrestin2 knockout mice. These results suggest that AVP-induced IL-1β expression in cardiac tissue is dependent on β-arrestin2. Similarly, as shown in Figs. 6E,6F, dosing of AVP induced the expression of the NLRP3 inflammasome in the heart tissue of normal mice, but not in β-arrestin2 knockout mice. Therefore, AVP-induced NLRP3 inflammasome expression dependent on β-arrestin2 signaling.

3.6 β-arrestin2 is Involved in AVP-Induced Activation of the NF-κB Signaling Pathway in Mouse Myocardium

To identify the mechanism mediating the effect of β-arrestin2 on AVP-induced expression of IL-1β and the NLRP3 inflammasome, mice were treated as mentioned in 2.5. Western blot analysis showed that AVP induced NF-κB p65 phosphorylation in the heart tissue of wild-type mice compared to controls. However, AVP had no effect on NF-κB p65 phosphorylation in β-arrestin2 knockout mice (Fig.7A,B). In addition, the deletion of β-arrestin2 inhibited the basal level of NF-κB p65 phosphorylation.

4. Discussion

Arginine vasopressin (AVP) is a mammalian vasopressin peptide synthesized mainly in neuroendocrine neurons in the supraoptic, paraventricular, and suprachiasmatic nuclei of the hypothalamus [15]. A number of experimental and clinical studies have shown that the concentration

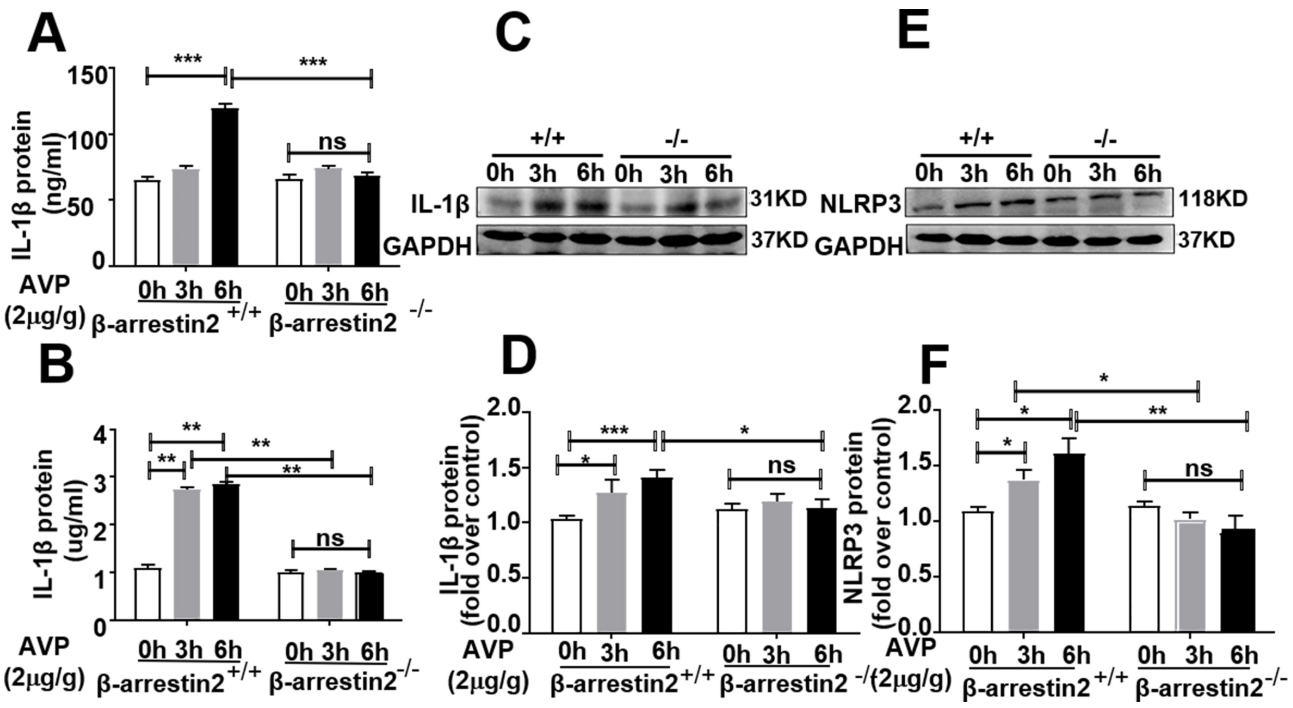

Fig. 6. β-arrestin2 was necessary for AVP-induced IL-1β expression in mouse myocardium. (A) AVP induced the expression of IL-1β protein in serum, but not in β-arrestin2 knockout mice. Lysates from the left ventricle and serum were harvested 0–6 h post injection with 2 μg/g of AVP or 0.9% of NaCl into tail veins. The serum was collected for measurement of IL-1β as described in Methods. Data were expressed as mean $\pm$ S.E.M. of 5 separate experiments. $^{***}p < 0.001$ vs control, one-way ANOVA test, followed by Bonferroni's post hoc test. (B,C,D) Deletion of β-arrestin2 abolished AVP-induced IL-1β expression in mouse myocardium. (B) Content of IL-1β in mouse myocardium. (C) A representative blot. (D) Average data. Data were expressed as mean $\pm$ S.E.M. of 4 separate animals. $^{*}p < 0.05$, $^{***}p < 0.001$, one-way ANOVA test, followed by Bonferroni's post hoc test. (E,F) Knockout of β-arrestin2 abolished AVP-induced NLRP3 expression. (E) A representative blot. (F) Average data. Data were expressed as mean $\pm$ S.E.M. of 6 separate animals. $^{*}p < 0.05$, $^{**}p < 0.01$. One-way ANOVA test, followed by Bonferroni's post hoc test. ns, no significance.

of AVP in the blood of patients with hypertension and post-infarct heart failure is significantly increased. Further, increased levels of inflammatory cytokines (TNF-α, IL-1β, IL-6) [16], and high levels of AVP can lead to heart failure [17]. Early studies have reported that AVP induces the expression of IL-6 in cardiac fibroblasts, promotes the proliferation of neonatal rat AVP in cardiac fibroblasts [6], and this leads to myocardial fibrosis [18]. The present study found that: (1) AVP induces expression of the NLRP3 inflammasome and IL-1β in mouse heart and cultured rat cardio fibroblasts; (2) AVP-induced expression of IL-1β in adult cardiac fibroblasts but not in adult rat cardiomyocytes; (3) AVP induces the expression of NLRP3 inflammasome and IL-1β through the NF-κB signaling pathway; (4) AVP-induced expression of IL-1β and activation of NF-κB signaling is dependent on β-arrestin2. Therefore, AVP promotes the expression of IL-1β through the β-arrestin2-mediated NF-κB signaling pathway, and participates in the regulation of inflammation.

Fibroblasts are traditionally considered extracellular matrix-producing cells that become activated following injury and participate in scar formation [19]. Nevertheless, role of cardiac fibroblasts in promoting inflammation have attracted more attention recently. Cardiomyocytes [20], cardiac endothelial cells [21], cardiac fibroblasts [6,22,23], and cardiac infiltrates [24,25] were all reported to produce inflammatory cytokines that cause cardiac remodeling and dysfunction. Among them, IL-1β mainly comes from cardiac endothelial cells and cardiac fibroblasts and is strictly regulated by inflammasomes [26,27]. Cardiac fibroblasts are particularly sensitive to the pro-inflammatory cytokine IL-1 in post-myocardial infarction remodeling [28]. IL-1 is involved in the pathogenesis of a variety of inflammatory diseases, in which both IL-1β and IL-18 are generally not expressed in healthy cells, but their inactive precursors can be synthesized after cell injury and both molecules are secreted as active molecules. The present study used ARCMs and ARCFs as the experimental subjects. Interestingly, the expression of IL-1β could not be detected in ARCMs, but the levels of IL-1β and NLRP3 inflammasome expression were increased in ARCFs. This was confirmed in another study that showed that isoproterenol induced the expression of IL-18, but not IL-1β, in cardiomyocytes [29]. The difference of reactivity between the cardiac cell types might

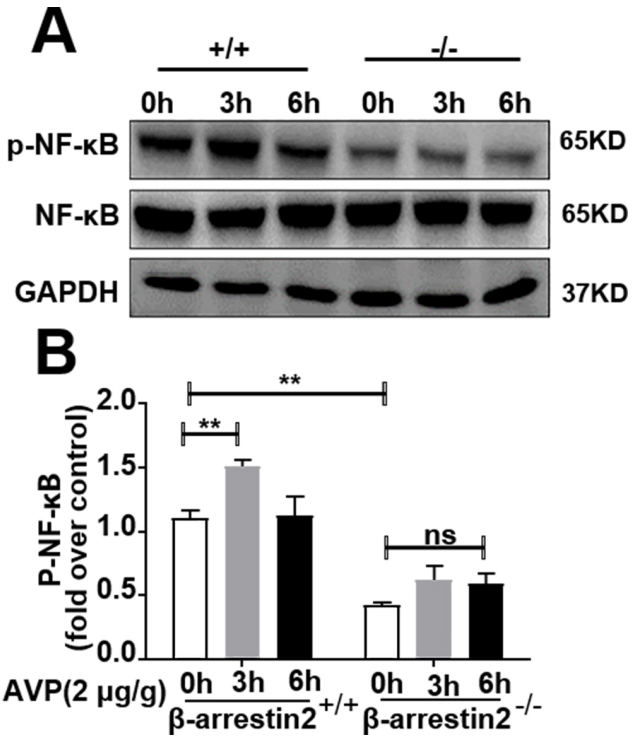

Fig. 7. Deletion of β-arrestin2 diminished phosphorylation of NF-κB p65 induced by AVP in mouse myocardium. Left ventricle were harvested 0–6 h post injecting 2 μg/g body weight of AVP or 0.9% of NaCl into veins of the tails. The myocardium lysate was subsequently assayed for phosphorylation of NF-κB p65 with anti-phospho NF-κB p65 antibody by western blotting. (A) A representative blot. (B) Average data were from 3 individual animals, $^{*}p < 0.05$, $^{**}p < 0.01$, one-way ANOVA test, followed by Bonferroni's post hoc test. Note: β-arrestin2 KO reduced the basal level of NF-κB p65 phosphorylation.

provide experimental basis for drug selectivity in clinical practice.

It is well known that β-arrestins are scaffold proteins involved in GPCR desensitization and down-regulation, and can transduce receptor signals independently of G-proteins. A recent study reported that cardiac β-arrestin2 expression is increased in mice with myocardial infarction and modulates the inflammatory response [30]. Additionally, earlier work in our laboratory revealed that the β-arrestin1 and β-arrestin2 pathways are involved in AVP-induced cardiac fibroblast proliferation and extracellular matrix synthesis, and that β-arrestin2 is also involved in AVP-induced fibroblast IL-6 expression. This study used β-arrestin2 knockout mice and β-arrestin2 knockdown ARCFs as experimental models. The effects of β-arrestin2 on AVP-induced expression of the NLRP3 inflammasome and IL-1β were investigated in both animal and cellular models. A highlight of this study is the discovery of the relationship between β-arrestin2 and the NLRP3 inflammasome during cardiac inflammation. Specifically, we document that β-arrestin2 is a necessary molecule for the AVP-induced expression of the NLRP3 inflammasome.

An involvement of the NLRP3 inflammasome has been shown in cardiovascular diseases. A few pharmacological agents that selectively target the NLRP3 inflammasome signaling have been tested in animals, and early phase human studies show promising results [31,32]. It is well known that there are different inflammasomes that are comprised of a complex of proteins. They activate caspase-1 which, in turn, promote maturation of pro-IL-1 and IL-18 into their active forms. Among the different types of inflammasomes such as NLRP1, NLRP2, NLRP3 and AIM, the role and regulation of Nodlike receptor family, pyrin domain containing 3 (NLRP3) inflammasome is well studied [33]. Such as in the seminal plasma of varicocele patients, NLRP3 inflammasome components include interleukin-1b (IL-1b), IL-18, caspase-1, and apoptosis associated speck-like protein (ASC) [34]. Non-NLRP3 inflammasomes may be significant in cardiovascular diseases as well [35,36]. It is worthwhile to further study if other components are involved in AVP-induced NLRP3 inflammasome and non-NLRP3 inflammasome signaling beyond the IL-1β, although our lab reported that IL-6 mediates AVP-evoked the cardiac inflammation [6].

It is well known that NF-κB is an important nuclear transcription factor that induces inflammatory signaling and the expression of inflammatory factors. Further, NF-κB has been proved to be involved in the occurrence and development of various immune and inflammatory responses, apoptosis, and proliferation-related heart diseases [37]. It has been shown that the TLR4/MyD88/NF-κB signaling pathway is involved in myocardial inflammation after coronary microembolization, evidenced by increased serum levels of TNF-α, IL-1β, and increased expression levels of TNF-α, IL-1β and IL-18 in myocardium. Among these, TLR4 was described as the first member of the TLR family, while MyD88 is a key adapter molecule in the Toll-like receptor signaling pathway, connecting the activation of NF-κB and the NLRP3 inflammasome [38]. The present study indicated that the NF-κB signaling pathway was involved in the arginine vasopressin-induced expression of the NLRP3 inflammasome and IL-1β *in vivo* and *in vitro*. We further showed that PDTC, an inhibitor of NF-κB, inhibited the inflammatory response to some extent by inhibiting the phosphorylation of NF-κB p65. In addition, knockout of β-arrestin2 proved to inhibit NF-κB 65 phosphorylation as well. The regulatory effect of NF-κB suggests that it may provide insights into inflammation in cardiovascular disease.

5. Conclusions

In conclusion, this study shows that AVP induces the expression of IL-1β specifically in ARCFs, and β-arestin2 is necessary for the activation of the arginine vasopressin-induced NF-κB signaling pathway. Therefore, silencing of

β-arrestin2, abolishing NF-κB signaling, and inhibiting the NLRP3 inflammasome all provide a novel approach for the clinical treatment of inflammatory cardiovascular diseases.

Abbreviations

AVP, Vasopressin; ARCFs, adult rat cardiac fibroblasts; ARCMs, adult rat cardiomyocyte; NF-κB, Nuclear factor-Kappa B; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; IL, Interleukin; TNF, Tumor Necrosis Factor; ELISA, Enzyme-linked immunosorbent assay.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

NY, BG, and WZ designed the research study. NY, BG, YW, YH, XZ, JC, YL, YQ, and HS performed the research. NY, BG, and WZ analyzed the dat. NY, BG, and WZ wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

The study was approved by the Board of Nantong University Animal Care and Use (Ethical approval number: NTU220191418).

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Conflict of Interest

WZ is serving as one of the Guest Editors of this journal. We declare that WZ had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to SI and MMA.

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