β-Arrestin 2 mediates arginine vasopressin-induced IL-6 induction via the ERK_{1/2}-NF-κB signal pathway in murine hearts

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Evidence to date suggests that β-arrestins act beyond their role as adapter proteins. Arginine vasopressin (AVP) may be a factor in inflammation and fibrosis in the pathogenesis of heart failure. In the present study we investigated the effect of AVP on inflammatory cytokine IL-6 production in murine hearts and the impact of β-arrestin 2-dependent signaling on AVP-induced IL-6 production. We found that administration of AVP (0.5 U/kg, iv) markedly increased the levels of IL-6 mRNA in rat hearts with the maximum level occurred at 6 h. In β-arrestin 2 KO mouse hearts, deletion of β-arrestin 2 decreased AVP-induced IL-6 mRNA expression. We then performed in vitro experiments in adult rat cardiac fibroblasts (ARCFs). We found that AVP (10^{-9}–10^{-6} M) dose-dependently increased the expression of IL-6 mRNA and protein, activation of NF-κB signaling and ERK_{1/2} phosphorylation, whereas knockdown of β-arrestin 2 blocked AVP-induced IL-6 increase, NF-κB activation and ERK_{1/2} phosphorylation. Pharmacological blockade of ERK_{1/2} using PD98059 diminished AVP-induced NF-κB activation and IL-6 production. The selective V₁A receptor antagonist SR49059 effectively blocked AVP-induced NF-κB phosphorylation and activation as well as IL-6 expression in ARCFs. In AVP-treated mice, pre-injection of SR49059 (2 mg/kg, iv) abolished AVP-induced NF-κB activation and IL-6 production in hearts. The above results suggest that AVP induces IL-6 induction in murine hearts via the V₁A receptor-mediated β-arrestin 2/ERK_{1/2}/NF-κB pathway, thus reveal a novel mechanism of myocardial inflammation in heart failure involving the V₁A/β-arrestin 2/ERK_{1/2}/NF-κB signaling pathway.

Keywords: myocardial inflammation; β-Arrestin 2; arginine vasopressin; IL-6; adult rat cardiac fibroblasts; ERK_{1/2}

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INTRODUCTION

GPCRs transduce various environmental signals and coordinate cellular responses to environmental stimuli. β-Arrestin 1 and 2 are adapter proteins that primarily terminate GPCR signaling via receptor desensitization and internalization [1, 2]. Recent evidence has demonstrated that these proteins also act as scaffold proteins for multiple signaling molecules. For example, these proteins link GPCR signaling to ERK_{1/2}, Akt, and Src kinases [3] and interact with the transcriptional factor NF-κB [4, 5]. Therefore, β-arrestins have emerged as key regulators of cellular functions [6], including controlling GPCR-dependent chemotactic signals in immune cells during inflammatory reactions.

The IL-6 family of cytokines, including IL-6, has been implicated in cardiac pressure overload and postischemic remodeling [7–9]. Recent studies have demonstrated that IL-6 plasma levels are elevated during congestive heart failure [10], which suggests that IL-6 can be used as a marker of inflammation in acute and chronic myocardial injury [11]. The increased production of cytokines, particularly inflammatory cytokines, such as tumor necrosis factor alpha (TNFα), interleukin-1 (IL-1) and IL-6, is at least partially responsible for heart dysfunction in patients with heart failure and cardiac remodeling [12, 13].

Arginine vasopressin (AVP) is secreted in response to hypovolemic or cardiac stress. Accordingly, AVP receptors are also found in some immune cells, such as rat B lymphocytes and thymic epithelial cells [14]. Therefore, AVP may stimulate the production of cytokines and antibodies in an autocrine manner during inflammation. The physiological effects of AVP are mediated via binding to specific membrane receptors on target cells. Three vasopressin receptor subtypes (V₁AR, V₂R, and V₃R [also termed V₃R]) have been identified in humans [15–17]. All of these receptors belong to the GPCR superfamily [18, 19]. However, only V₁AR is found in cardiac myocytes [20] and cardiac fibroblasts [21, 22]. G_{q/11}-coupled V₁AR typically induces protein kinase C, which is an activator of gene programming for cardiac hypertrophy [23], but it also interacts with GPCR kinase (GRK) isoforms [24], primarily GRK2 [21, 25]. GRKs activate G protein-independent signaling pathways in addition to having a clearly defined role in receptor desensitization [26, 27]. These pathways are related to the regulation of cardiac hypertrophy and apoptosis [28] and the promotion of cardioprotective extracellular signal-regulated kinase 1/2 (ERK_{1/2}) signal transduction via β-arrestin 2 [29]. However, little is known about the inflammatory regulation of β-arrestin 2 in the heart. The present study investigated the effect of AVP on IL-6.
production in murine hearts and the impact of β-arrestin 2-dependent signaling on AVP-mediated IL-6 production.

MATERIALS AND METHODS

Drug and reagents

The Animal Care and Use Committee of Nantong University approved all of the procedures. Adult Sprague-Dawley rats were obtained from the Animal Center of Nantong University (Nantong, China). β-Arrestin 2 KO mice were gifted by the laboratory of Dr. Lefkowitz (Duke University, Durham, NC, USA). Dulbecco’s modified Eagle’s medium (DMEM), penicillin and streptomycin were purchased from Invitrogen (Gaithersburg, MD, USA). Arginine vasopressin (AVP; V9879) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The V1aR selective antagonist SR49059 was purchased from Tocris Bioscience (Minneapolis, MN, USA). A rat IL-6 ELISA kit (#BMS625) was purchased from ThermoFisher Scientific (Waltham, MA, USA). The adenovirus containing β-arrestin 2 or a lentivirus containing shRNA targeting β-arrestin 2 were purchased from Genechem (Shanghai, China). NF-κB luciferase (E8491) and renilla luciferase (E2231) were purchased from Promega (Madison, WI, USA). Anti-P-NF-κB (sc-3033) and anti-β-arrestin 2 (A6745) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against NF-κB and GAPDH were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Animals

Male rats and mice (8–12 weeks old) were administered 0.5 U/kg AVP via the tail vein. Murine heart tissues were harvested for RNA and protein isolation at designated timepoints after AVP administration. The Animal Care and Use Committee of Nantong University approved all of the procedures.

Cell culture and adenoviral or lentiviral infection

ARCFs were prepared from the hearts of adult (8- to 12-week-old) Sprague-Dawley rats as previously described [30]. The cells were cultured in DMEM containing 10% FBS and 100 U/mL penicillin-streptomycin for 4–5 days before being passaged. When the cells reached 90% confluence, they were passaged at a ratio of 1:3 with 0.25% trypsin. Experiments were consistently performed on cells from passages 3–5. The serum was replaced with serum-free medium, and the ARCFs were transfected with an adenovirus containing β-arrestin 2 or a lentivirus containing shRNA targeting β-arrestin 2 at an MOI of 100. Medium with or without treatment agent was added after 24 h according to the experimental design.

Table 1. The primers for murine V1aR, V1bR, IL-6, GAPDH, and β-actin

| Gene     | Primers                                                                 | Sizes (bp) |
|----------|-------------------------------------------------------------------------|------------|
| V1aR     | F:5'-GTGGCTGGCTTCCCTTGAACA-3' R:5'-TGAGTTGAACGGTGTCGG-3'                | 154        |
| V1bR     | F:5'-CTGGACACCTGGGATCACA-3' R:5'-CTGGACACCTGGGATCACA-3'                | 190        |
| II   | F:5'-GGCGAGGATGAGCTTGGTTAAATG-3' R:5'-GGCGAGGATGAGCTTGGTTAAATG-3'      | 222        |
| II   | F:5'-ATGGCTCTCTCTGCTTGACTGTA-3' R:5'-ATGGCTCTCTCTGCTTGACTGTA-3'        | 217        |
| β-actin  | F:5'-AAACCCCCACACGCTATCCC-3' R:5'-AAACCCCCACACGCTATCCC-3'              | 191        |
| GAPDH    | F:5'-ATGGCTCTCTCTGCTTGACTGTA-3' R:5'-ATGGCTCTCTCTGCTTGACTGTA-3'        | 101        |
| IL-6     | F:5'-AGCCGAGGATGAGCTTGGTTAAATG-3' R:5'-AGCCGAGGATGAGCTTGGTTAAATG-3'    | 222        |

Western blotting

Cells were treated with AVP for 0–120 min, washed twice with ice-cold PBS and lysed with 250 μL of ice-cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 50 mg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mg/mL aprotinin, 1 mM NaF, and 1 mM Na3VO4). After centrifugation at 12 000 × g for 15 min at 4 °C, equal amounts of total cell lysates (20 μg of protein) were loaded onto 4–12% SDS-PAGE gels, and immunoblotting for phosphorylated NF-κB, NF-κB, β-arrestin 2, phosphorylated ERK1/2, and GAPDH was performed.

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ELISA for IL-6
Heart homogenates or cell culture supernatant samples (50 μL) were used to measure IL-6 using an ELISA kit, as described in the kit manual. The analytical sensitivity of the kit was 12.0 pg/mL and 31.3–2000 pg/mL, respectively.

Statistical analysis
The data and statistical analyses complied with the recommendations on experimental design and analysis in pharmacology [32]. An experimenter who was blinded to the experimental protocol randomized the animals into groups in the animal experiments on AVP-induced IL-6 production. The number of rats in each group in the in vivo study was less than needed to reduce the number of euthanized animals because the difference between the control and treatment groups was significant in a preliminary study. Randomization was not applicable in any of the other experiments. Comparisons were performed using one- or two-way ANOVA followed by Bonferroni’s test. All values are presented as the means ± SEM. P < 0.05 was considered statistically significant.

RESULTS
β-Arrestin 2 mediates AVP-induced IL-6 expression in murine hearts
To determine whether AVP evokes IL-6 induction in the murine myocardium, AVP (0.5 U·kg⁻¹) was administered via the tail veins of the rats. The level of troponin I in the serum was not increased 24 h after AVP administration (data not shown). AVP-evoked IL-6 expression in the heart, and the maximum expression of IL-6 mRNA and protein occurred 6 h (Fig. 1a, b) after AVP administration. Notably, the deletion of β-arrestin 2 reduced AVP-induced IL-6 mRNA expression (Fig. 1c) in β-arrestin 2 KO mouse hearts (Supporting Information Data S1).

To further investigate the mechanistic role of β-arrestin 2 in AVP-mediated IL-6 induction, we performed in vitro experiments using cultured adult rat cardiac fibroblasts (ARCFs). AVP-induced IL-6 expression in a dose- and time-dependent manner in cultured ARCFs (Fig. 2). The silencing of β-arrestin 2 with shRNA (Fig. 3a and Supporting Information Data 3) or the overexpression of β-arrestin 2 with a lentiviral vector (Fig. 3b and Supporting Information Data 2)
were used to manipulate the levels of β-arrestin 2. The silencing of β-arrestin 2 suppressed the AVP-induced mRNA and protein levels of IL-6 in ARCFs (Fig. 3c, d). Notably, the overexpression of β-arrestin 2 did not alter the induction of IL-6 expression. These results suggest that β-arrestin 2 is necessary, but not sufficient, for the AVP-induced induction of IL-6 in murine hearts.

β-Arrestin 2 is required for AVP-induced NF-κB activation

Our previous study demonstrated that AVP induces IL-6 production via NF-κB signaling in neonatal rat cardiac fibroblasts [33] and cultured ARCFs (Fig. 4). To determine whether β-arrestin 2 is involved in the AVP-mediated induction of NF-κB activation, we measured the levels of phosphorylated NF-κB and the luciferase activity of NF-κB.
under different conditions. We found that the silencing of β-arrestin 2 by shRNA efficiently suppressed AVP-induced NF-κB phosphorylation (Fig. 5a, b) and NF-κB luciferase activity (Fig. 5c). In contrast, the overexpression of β-arrestin 2 did not alter the activation or phosphorylation levels of NF-κB (Fig. 5b, c). Notably, AVP-evoked NF-κB activity in a PKC-independent manner, as a PKC inhibitor did not block AVP-induced NF-κB activity in ARCFs (Supporting information Data 3). These results suggest that β-arrestin 2 is necessary, but not sufficient, for AVP-evoked NF-κB signaling, which is consistent with its effect on AVP-induced IL-6 production.

β-Arrestin 2-dependent ERK1/2 phosphorylation is essential for AVP-induced NF-κB and IL-6 production

β-Arrestin 2 is a critical component in the mediation of the β2-adrenergic stimulation of ERK1/2 signaling [34]. We next tested our hypothesis that β-arrestin 2-dependent ERK1/2 signaling is responsible for AVP activity in the heart. AVP induced the phosphorylation of ERK1/2 in a PD98059-sensitive manner in cultured ARCFs and intact rat hearts (Fig. 6). The deletion of β-arrestin 2 by shRNA or β-arrestin 2 adenovirus reduced the AVP-evoked phosphorylation of ERK1/2, and the overexpression of β-arrestin 2 did not further enhance AVP-induced ERK1/2 activation. These results suggest that β-arrestin 2 is required for the AVP-induced phosphorylation of ERK1/2. Accordingly, β-arrestin 2 deficiency and ERK1/2 inhibition with PD98059 abolished the AVP-induced activation of NF-κB (Fig. 7a) and induction of IL-6 expression (Fig. 7b) in mouse hearts. A PD98059-sensitive pathway mediated AVP-induced IL-6 expression and NF-κB phosphorylation in mouse hearts, which was evidenced by the inhibition of NF-κB
phosphorylation and the induction of IL-6 by PD98059 (Fig. 8). Taken together, these data support the hypothesis that β-arrestin 2-mediated ERK1/2 signaling contributes to the AVP-induced NF-κB/IL-6 inflammatory response in the heart.

We used RT-PCR and demonstrated that the V1A receptor is the only vasopressin receptor subtype in ARCFs (Fig. 9a). Notably, the V1A receptor-selective inhibitor SR49059 efficiently blocked AVP-induced NF-κB phosphorylation with an IC50 of 3.20 ± 0.13 nM (Fig. 9b), NF-κB phosphorylation and the induction of IL-6 by PD98059 (Fig. 8).
activation with an IC50 of 1.25 ± 0.44 nM (Fig. 9c), and IL-6 expression with an IC50 of 0.55 ± 0.16 nM in ARCFs (Fig. 9d). Notably, 1 µM of SR49059 had no effect on basal IL-6 expression or NF-κB activation in ARCFs. Similarly, pretreating mice with 2 mg/kg SR49059 prior to AVP administration (0.5 U/kg) diminished NF-κB activation (Fig. 9f) and IL-6 production (Fig. 9e, g).
DISCUSSION

The cytokine IL-6 is important for immune system regulation, inflammatory responses and cardiovascular remodeling. IL-6 has a low baseline mRNA expression level in cardiac fibroblasts and is absent in cardiomyocytes, but IL-6 levels are stimulated by β2AR [35], hypoxia [36] or coculture with macrophages [37]. The following results were found in the present study: (1) AVP increased the mRNA and protein levels of IL-6 in murine hearts; (2) the silencing or deletion of β-arrestin 2 reduced AVP-induced IL-6 production, NF-κB activation and ERK 1/2 phosphorylation; (3) the pharmacological inhibition of ERK1/2 signaling diminished AVP-induced NF-κB activation and IL-6 production; and (4) the blockade of the V1A receptor by the selective antagonist SR49059 abolished AVP-evoked NF-κB phosphorylation and IL-6 induction in intact hearts and ARCFs.

AVP is an antidiuretic hormone that is secreted by the hypothalamus-pituitary-adrenal axis. AVP significantly affects vaso-constriction, immune regulation and body temperature regulation. Ventricular remodeling occurs when end-stage heart failure develops, and inflammation is an important mechanism for the development of ventricular remodeling. When heart failure occurs, the concentration of catecholamines increases, which induces cardiac fibroblasts to secrete the pro-inflammatory factor IL-6 via β2 adrenergic receptors [38]. IL-6 is involved in the remodeling of the extracellular matrix of cardiomyocytes, which induces or aggravates the development of ventricular remodeling, which promotes chronic heart failure [39]. The level of AVP may be used as an early warning sign of the disease, and it may provide new ideas for the treatment of cardiovascular diseases in the future.

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GPCR- or stress-stimulated IL-6 secretion from cardiac fibroblasts may lead to cardiac inflammation, fibroblast proliferation and cardiac remodeling. Data from our and other studies have demonstrated that AVP promotes the proliferation of cardiac fibroblasts [21, 40–43]. We report for the first time that AVP induces IL-6 production in the murine myocardium. AVP-induced transforming growth factor-beta 1 (TGF-beta 1) secretion is...
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responsible for cardiac fibroblast-myofibroblast transformation [44], but whether the induction of IL-6 plays a role in cardiac fibroblast proliferation requires further study.

A large number of studies have confirmed that β-arrestin is primarily involved in negative feedback regulation, such as the desensitization and internalization of GPCRs and seven transmembrane receptors (7TMRs), and recent studies have demonstrated that β-arrestin also participates in G protein-independent signaling pathways, which also mediate the physiological and pathological functions of cells. For example, β-arrestin 1 and β-arrestin 2 mediate protective β1-adrenergic signaling in cardiac myocytes [29]. Due to their various biochemical functions in the regulation of critical physiological and pathophysiological processes, including inflammation, β-arrestins have become a drug target in human diseases, such as heart failure. Heart failure is marked by increased AVP levels in the systemic circulation and local cardiac tissues [43]. Therefore, the present findings highlight IL-6 as the effector cytokine downstream of β-arrestin 2 that may link elevated AVP levels to inflammatory cell models and pathological conditions. The cardioprotective effect of β-arrestin 2 may occur via GPCR-dependent [45] and GPCR-independent [46, 47] signaling pathways. It was recently reported that β-arrestin regulates the immune response in immune cells via the NF-κB signaling pathway. β-Arrestin 2 directly interacts with IkBα (an NF-κB inhibitor and the key molecule in innate and adaptive immunity) [5] and prevents the phosphorylation and degradation of IkBα [4, 5]. Consequently, β-arrestin 2 effectively modulates NF-κB activation and the expression of NF-κB target genes [4, 5]. Moreover, the stimulation of β2-adrenergic receptors significantly enhances the interaction between β-arrestin 2 and IkBα and greatly promotes the stabilization of IkBα by β-arrestin 2, which indicates that β-arrestin 2 mediates crosstalk between β2-adrenergic receptors and NF-κB signaling pathways [4]. This interaction suggests a novel mechanism for the regulation of the immune system by the sympathetic nervous system. In contrast, Withrow et al. found that the overexpression of β-arrestin 1 or β-arrestin 2 leads to a marked inhibition of NF-κB activity. Conversely, the suppression of the expression of β-arrestin 1, but not of β-arrestin 2, using RNA interference leads to a threefold increase in TNF-stimulated NF-κB activity [5]. The direct interaction between β-arrestin and NF-κB regulates NF-κB signaling, and the present study demonstrates that the phosphorylation of NF-κB is mediated by β-arrestin 2 signaling via ERK1/2 activation in cardiac cells.

It is well established that NF-κB regulates the expression of inflammatory factors (including IL-6) and the initiation and progression of myocardial fibrosis [48, 49]. The present study demonstrated that AVP, as an endogenous pro-inflammatory factor, activates NF-κB in fibroblasts, as evidenced by NF-κB phosphorylation, nuclear translocation and luciferase activity. PDTC is an inhibitor of NF-κB that inhibits the inflammatory effect of AVP to a certain extent via the inhibition of the activation of NF-κB p65 and alleviates AVP-induced inflammation and organ damage. Therefore, the proinflammatory effect of AVP depends on the regulation of NF-κB to some extent. By targeting the mechanisms of NF-κB regulation, we hope to develop new ideas for the treatment of inflammation and myocardial fibrosis in cardiovascular diseases.

There are three vasopressin receptor subtypes, namely, V1A, V1B, and V2. V1A is widely distributed in the central system, and V2 is concentrated in the renal collecting duct. Early studies by our laboratory revealed that only V1A receptors are present in neonatal rat cardiac fibroblasts. The distribution and function of the three receptors are different. The primary roles of the V1A receptor are vasocostriction, platelet aggregation, blood pressure regulation, and hepatic glycogen metabolism [50]. The V1A receptor mediated the AVP-induced inflammatory response in this experiment, and its selective antagonist SR49059 significantly inhibited this response. Our data further demonstrate that a selective V1A receptor blocker efficiently abolishes AVP-induced NF-κB signaling and IL-6 production in intact hearts, which suggests that the V1A subtype mediates AVP-evoked inflammation in the murine myocardium.

β-Arrestin 2/ERK1/2/NF-κB signaling may not be specific to the V1A receptor because cardiomyocytes also contain other Gq-coupled receptors, which could induce the activation of such a pathway and participate in the inflammatory response in the heart. Notably, the Gq-PLC pathway was not involved in the V1A/AVP-mediated activation of NF-κB in our study (as shown in Supplementary Information Data 3) or in cellular protection via a PKC-independent pathway in our previous study [9]. However, the AVP-mediated activation of ERK1/2 has been previously demonstrated in H9c2 cells [9, 51] and cardiomyocytes [52]. Therefore, further study on whether different signaling mechanisms between Gq-PLC and β-arrestin mediate the diverse biological functions evoked by AVP is valuable because AVP is increased during the development of heart failure.

CONCLUSION

In summary, our data reveal an important role of β-arrestin 2 in the AVP-mediated inflammatory response, which highlights a novel inflammatory mechanism whereby AVP induces IL-6 production in intact murine hearts via a β-arrestin 2/ERK1/2/NF-κB pathway. These findings shed new light on our molecular understanding of inflammation in heart failure and possibly other cardiac stress conditions.

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AUTHOR CONTRIBUTIONS

SZS and WZZ contributed to the conception of the work, the interpretation of the data and the critical revision of the paper. SZS, NY, XFZ, LLZ, QZ, LLZ, EAN and HC contributed to data acquisition. SZS and WZZ drafted and finalized the paper. All authors contributed to the final approval of the manuscript.

ADDITIONAL INFORMATION

The online version of this article (https://doi.org/10.1038/s41410-019-0292-y) contains supplementary material, which is available to authorized users.

Conflict of interest: The authors declare that they have no conflict of interest.

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