MOLECULAR PHARMACOLOGY IN CHINA

GRK2 Mediates Arginine Vasopressin-Induced Interleukin-6 Production via Nuclear Factor- κ B Signaling Neonatal Rat Cardiac Fibroblast^S

Feifei Xu, Shuzhen Sun, Xiaojun Wang, Eran Ni, Lingling Zhao, and Weizhong Zhu

Laboratory of Cardiovascular Science, Department of Pharmacology, Nantong University School of Pharmacy, Nantong, China

Received November 30, 2016; accepted January 20, 2017

ABSTRACT

Interleukin 6 (IL-6), which is elevated in patients with congestive heart failure and acts as both a chronic marker of inflammation and an acute-phase reactant, is associated with myocardial damage. Circulating levels of arginine vasopressin (AVP) are elevated during cardiac stress and could be a factor for cardiac inflammation and fibrosis. Our previous study has shown that AVP promotes the proliferation of neonatal rat cardiac fibroblasts (NRCFs) through V_{1A} vasopressin receptor-mediated G protein-coupled receptor kinase 2 (GRK2) signaling. In the present study, we investigated the impact of the GRK2-dependent signaling. Using quantitative polymerase chain reaction and enzyme-linked immunosorbent assay, we measured the levels of interleukin-6 (IL-6) mRNA and protein in NRCFs, respectively. Manipulation of GRK2 activation either pharmacologically or through overexpression of GRK2-ct was used to determine

the role of GRK2 in regulating the effects of AVP on IL-6 production. Phosphorylation and activation of nuclear factor κ -B (NF- κ B) evoked by AVP stimulation were measured by immunoblot and NF- κ B luciferase reporter gene transfected in NRCFs, respectively. Present studies have found that: 1) AVP increased the level of IL-6 protein and mRNA in a dose- and time-dependent manner in NRCFs; 2) inhibition of GRK2 abolished the AVP-induced IL-6 production and NF- κ B activation; and 3) blocking NF- κ B signaling using the pharmacologic approach diminished AVP-induced IL-6 production. In summary, AVP induces IL-6 production of NRCFs by activating V_{1A} receptor signaling via a GRK2/NF- κ B pathway. These findings provide a possible molecular mechanism for inflammation that occurs in heart failure and other types of cardiac stress.

Introduction

Cardiac remodeling is initiated by endocrine, paracrine, or autocrine factors that evoke multiple intracellular signaling pathways and ultimately regulate transcription factors and related gene expression (Lionetti et al., 2010). The interleukin-6 (IL-6) family of cytokines, including IL-6, leukemia inhibitory factor, and interleukin-11, may play an important role in these processes (Taga and Kishimoto, 1997; Wang et al., 2002; Fujio et al., 2011). IL-6, a pleiotropic cytokine, is highlighted in the developmental process of myocardium

remodeling because of its clinical importance. An increasing number of investigations have demonstrated that the plasma levels of IL-6 were elevated in patients with congestive heart failure (Vasan et al., 2003) and that IL-6 could act as both an acute-phase reactant and a chronic marker of inflammation associated with myocardial damage (Murray and Freeman, 2003). Increased production of cytokines, especially inflammatory cytokines such as tumor necrosis factor α , interleukin-1, or IL-6, is responsible, at least in part, for cardiac dysfunction in patients with heart failure (Finkel et al., 1992; Hirota et al., 1995).

Arginine vasopressin (AVP) is secreted in response to hypovolemic or cardiac stress. It participates in physiologic processes such as osmotic regulation, vasoconstriction, and the release of adrenocorticotrophic hormone. AVP has also been found in rat B lymphocytes, thymic epithelial cells, and human peripheral blood mononuclear cells, in which AVP

This project was supported by the National Natural Science Foundation of China (No. 81370345, No. 81541008) and by funding from the Nantong University Co-Innovation Program (NTU 2016-1).

F.X. and S.S. contributed equally to this work.

<https://doi.org/10.1124/mol.116.107698>.

^S This article has supplemental material available at molpharm.aspetjournals.org.

ABBREVIATIONS: ANOVA, analysis of variance; AVP, arginine vasopressin; ERK1/2, extracellular signal-regulated kinase 1/2; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; GRK2-ct, G protein-coupled receptor kinase C-terminal peptide; IL-6, interleukin 6; NF- κ B, nuclear factor- κ B; NRCF, neonatal rat cardiac fibroblast; PCR, polymerase chain reaction; PDTC, pyrrolidinedithiocarbamic acid; POTH, paroxetine hydrochloride; SR49059, (2S)-1-[(2R,3S)-5-Chloro-3-(2-chlorophenyl)-1-[(3,4-dimethoxyphenyl)sulfonyl]-2,3-dihydro-3-hydroxy-1H-indol-2-yl]carbonyl]-2-pyrrolidinecarboxamide; V_{1A}R, arginine vasopressin receptor type 1A.

receptors exist as well. Thus, AVP stimulates the production of cytokines and antibodies through these receptors in response to inflammation. AVP is released not only from the hypothalamus but also from peripheral immune cells in response to stress and inflammation (Baker et al., 2003). These physiologic effects of AVP are mediated through the binding of AVP to specific membrane receptors on target cells. To date, three vasopressin receptor subtypes ($V_{1A}R$, $V_{2}R$, and $V_{1B}R$, also termed V_3R) have been found in humans (Lolait et al., 1992; Morel et al., 1992; de Keyser et al., 1994). All three subtypes belong to the G protein-coupled receptor (GPCR) superfamily (Carmichael and Kumar, 1994; Thibonnier et al., 2002). Among the three subtypes of the vasopressin receptors, only $V_{1A}R$ is expressed in cardiac myocytes (Hiroyama et al., 2007) and in cardiac fibroblasts (Chen et al., 2017). Administration of AVP increases cell hypertrophy in neonatal mouse cardiomyocytes (Hiroyama et al., 2007), and either constitutive or controlled overexpression of $V_{1A}R$ in cardiac myocytes induces hypertrophy and dilation of the left ventricles, diminishes contractile performance of the myocardium, and reprograms the heart failure gene profile in transgenic mice; its effects are mediated primarily via $G\alpha_q$ protein-dependent signaling (Li et al., 2011).

Although $G\alpha_q$ -coupled $V_{1A}R$ typically induces protein kinase C, an activator of the myocardial hypertrophic gene programs (Li et al., 2011), it also interacts with GPCR kinase (GRK) isoforms (Tilley et al., 2014), primarily GRK2 (Chen et al., 2015, 2017; Zhang et al., 2016). Besides their well defined roles in receptor desensitization, GRKs also activate the G protein-independent signaling pathways (Moore et al., 2007; Huang et al., 2011). These G protein-independent pathways have been attributed to the regulation of myocardial hypertrophy and apoptosis (Métayé et al., 2005; Dorn, 2009; Huang et al., 2011) and to the promotion of a cardioprotective extracellular signal-regulated kinase 1/2 ($ERK_{1/2}$) signaling (Zhai et al., 2005; Noma et al., 2007). GRK in inflammation and inflammatory disease is an evolving area of research (Packiriswamy and Parameswaran, 2015), but there is little information on the inflammatory regulation of GRK2 in hearts. Our previous study has shown that AVP enhances the survival of H9c2 myoblasts (Zhu et al., 2013), proliferation of rat smooth muscle (Zhang et al., 2016), and cardiac fibroblasts (Chen et al., 2015, 2017) via a $GRK2/\beta$ -arrestin1/ $ERK_{1/2}$ -dependent pathway. The present study investigated the effect of AVP on IL-6 production in neonatal rat cardiac fibroblasts (NRCFs) and the relative impact of the GRK2-dependent signaling on this effect.

Materials and Methods

Materials. Neonatal Sprague-Dawley rats were obtained from the Animal Center of Nantong University (Nantong, China). All protocols were approved by the board of Nantong University Animal Care and Use committee. Dulbecco's modified Eagle's medium, penicillin, and streptomycin were purchased from Invitrogen (Gaithersburg, MD). AVP (Sigma, V9879) was from Sigma-Aldrich. $V_{1A}R$ -selective antagonist (SR49059) was from Tocris Bioscience (Minneapolis, MN), paroxetine hydrochloride (cat no. 1500218) was purchased from ABCam, the adenovirus containing β -galactosidase was a gift from Dr. Yibin Wang (University of California, Los Angeles), and the adeno-GRK2-Ctvirus was obtained from Dr. Walter Koch (Temple University, Philadelphia, PA). Both NF- κ B luciferase (E8491) and Renilla (E2231) were purchased from Promega (Madison, WI). Anti-

P-NF- κ B(S536) antibody was from Cell Signaling Technology (no. 3033; Danvers, MA); antibody for GRK2 (Sc-13143), NF- κ B(Sc-109), and GAPDH (Sc-25778) were from Santa Cruz Biotechnology, Dallas, TX).

Cell Culture and Adenoviral Infection. The NRCFs were prepared from the hearts of 1- to 3- day-old Sprague-Dawley pups as previously described (Zhang et al., 2012). Procedures for the handling and use of animals were approved by the board of the Nantong University Animal Care and Use committee. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum for 4 to 5 days before passaging. Cells were grown to confluence and subsequently passaged 1:3 using 0.25% trypsin. Experiments were conducted on cells from passages 3–5. After replacing with serum-free medium, fibroblasts were infected with adenoviruses containing GRK2-Ct or β -galactosidase at a multiplicity of infection of 100. Culture medium was added after 24 hours with or without treatment reagents based on the experimental design.

Transient Transfection of NF- κ B Promoter Luciferase Reporter Gene and Measurement of NF- κ B-Luciferase Activation. The NRCFs were plated in 12-well plates (250×10^3). When confluence of growing cells reached 80%–90%, transfection of reporter plasmid was carried out using Lipofectin 2000(Invitrogen) (Zhang et al., 2016). Cotransfection assay was achieved with 1 μ g of NF- κ B luciferase plasmid and 0.5 μ g of Renilla plasmids. For basal activity, cells were transfected with the NF- κ B-luciferase plasmid (0.5 μ g) and a Renilla control vector (0.5 μ g) without AVP incubation. Transfected cells were cultured for 6 hours and further cultured in the presence of AVP for 24 hours. The luciferase assay was carried out according to the manufacturer's instructions on the luminometer (Thermo Fisher Scientific, Somerset, NJ).

Quantitative PCR for Measurement of IL-6. NRCFs with a density of 1×10^6 cells/well were plated in 10-cm culture dishes. After being made quiescent at 90% confluence and stimulated with AVP, paroxetine hydrochloride (POTH), and pyrrolidinedithiocarbamic acid (PDTC), as designed in individual experiments, total RNA was isolated using TRIZOL reagent (Promega, Madison, WI). First-strand cDNA synthesis was performed using 1 μ g of total RNA in a 20- μ l reaction mixture according to the recommended conditions in the Moloney murine leukemia virus reverse transcription kit (Promega). For real-time PCR, the cDNA was amplified using a Stepone Real Time PCR System (Applied Biosystems, Foster City, CA). The double-stranded DNA-specific dye SYBRGreen I was incorporated into the PCR buffer provided in the QuantiTech SYBR PCR kit (Qiagen, Valencia, CA) to allow for quantitative detection of the PCR product in a 25- μ l reaction volume. The primer sequences for IL-6 were 5'-ACT-CCA-TCT-GCC-CTT-CA-3' (sense) and 5'-ACT-CCA-TCT-GCC-CTT-CA -3' (antisense); IL-6 accession no. NM_012589). The primer sequences for GAPDH were 5'-TTC-AAT-GGC-ACA-GTC-AAG-GC-3' (sense) and 5'-TCA-CCC-CAT-TTG-ATG-TTA-GCG-3'(antisense; GAPDH accession No. M17701). The temperature profile of the reaction was 95°C for 1 minute, 40 cycles of denaturation at 95°C for 5 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 30 seconds. GAPDH was used to normalize differences in RNA isolation, RNA degradation, and the efficiencies of the real-time PCR.

Western Blotting Analysis. After the cells were treated with AVP for 0–120 minutes, they were rapidly washed twice with ice-cold PBS and lysed with 250 μ l of ice-cold lysis buffer (50 mM Tris-HCl, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, 5 mg/ml leupeptin, 20 mg/ml aprotinin, 1 mM NaF, and 1 mM Na_3VO_4). After centrifugation at 12,000g for 10 minutes, equal amounts of total cell lysate (20 μ g protein) were subjected to 4%–12% SDS-PAGE, followed by immunoblotting for phosphorylated NF- κ B, NF- κ B, GRK2, and GAPDH. The images of immunoblots were detected with LI-COR Odyssey (Lincoln, NE).

Statistical Analysis. A commercial software package was used for statistical analysis (Graph Pad Software, Inc., San Diego, CA). Comparison of means $\pm$ S.E.M. was conducted with one- or two-way

Fig. 1. AVP induces IL-6 production in a time- and dose-dependent manner in neonatal rat cardiac fibroblasts. (A, B) AVP enhanced the level of IL-6 mRNA in a dose- (A) and time- (B) dependent manner in NRCFs. Cells were harvested for total RNA after the starved cells in six-well plates were stimulated with 1–1000 nM AVP for 6 hours or incubated with 0.1 μM AVP for 0–48 hours. IL-6 mRNA was measured with q-PCR described in *Materials and Methods*. Data are expressed as mean ± S.E.M. of three separate experiments. * $P < 0.05$, ** $P < 0.01$ versus control, $n = 4$; (C, D) AVP increased the level of IL-6 protein in time- and dose-dependent manner. The starved cells in 12-well plates were stimulated with 0.1 μM AVP for 0–48 hours or 0.1–1000 nM AVP for 24 hours. The supernatants were collected for measurement of IL-6 as described in *Materials and Methods*. ** $P < 0.01$ for time-course (repeated two-way ANOVA, $n = 4$); *** $P < 0.01$ for dose-response repeated two-way ANOVA ($n = 3$).

analysis of variance (ANOVA) followed by Bonferroni's test. A value of $P < 0.05$ was considered statistically significant.

Results

Arginine Vasopressin Induces IL-6 Production in Neonatal Rat Cardiac Fibroblasts. To determine the effects of AVP on IL-6 production, NRCFs were starved overnight and then incubated with or without 0.1 μM AVP for 0–24 hours or with AVP in the range of 0.001–1.0 μM for 6 hours. Using quantitative PCR, we found that incubation of the cells with AVP increased IL-6 mRNA production in a concentration-dependent (Fig. 1A) and time-dependent manner (Fig. 1B). In addition, the supernatants were then collected at different time points post-AVP stimulation for the measurement of IL-6 protein by enzyme-linked immunosorbent assay. As shown in Fig. 1, C and D, in unstimulated cardiac fibroblasts, low levels of IL-6 were detected in culture medium (22.4 ± 4.4 pg/ml, $n = 3$). IL-6 levels, however, were elevated in dose- and time-dependent manner.

Inhibition of GRK2 with Paroxetine Overexpression of GRK2-CT Suppresses the AVP-Induced IL-6 in Neonatal Rat Cardiac Fibroblasts. To determine whether GRK2 signaling is responsible for AVP-induced IL-6 production, GRK2 activity was disrupted by GRK2 inhibitor paroxetine (POTH) (Schumacher et al., 2015) or overexpression of GRK2 C-terminal peptide (GRK-ct, inhibitory peptide of GRK2 activation). As shown in Fig. 2A, pretreatment of cells with 10 μM POTH significantly abolished the level of IL-6

mRNA induced by AVP stimulation. Similarly, overexpression of GRK2-ct (Fig. 2B, upper panel) efficiently suppressed the AVP-induced IL-6 mRNA production (Fig. 2B). These results suggest that GRK2 is involved in the AVP-induced IL-6 production.

NF-κB Signaling Mediates the AVP-Induced IL-6 Production. To determine whether NF-κB signaling is necessary for AVP-induced IL-6 production, cells were pretreated with 50 μM NF-κB inhibitor PDTC (Fan et al., 2007). As shown in Fig. 3A, the AVP-induced IL-6 mRNA production was diminished by PDTC pretreatment. AVP indeed induced the NF-κB nuclear translocation (Supplemental Material), phosphorylation (Fig. 3B), and activation (Fig. 3C). Activation of NF-κB was abolished by PDTC (Fig. 3D). Together, our results have shown that AVP-induced IL-6 production is through NF-κB signaling in NRCFs.

GRK2 Is Necessary for AVP-Evoked NF-κB Signaling. To define the causal effects between GRK2 and NF-κB, either pharmacologic inhibitor of GRK2 or overexpression of GRK2-CT was used to abolish GRK2 signaling. As demonstrated in Fig. 4A, pretreatment of cells with POTH diminished the AVP-evoked NF-κB luciferase activation evoked by AVP incubation in NRCFs. As expected, GRK2-ct was dramatically expressed after cells were transduced with the adenovirus containing GRK2-ct (Fig. 4B, upper panel). Overexpression of GRK2-ct reduced the AVP-induced IL-6 mRNA production (Fig. 4B, lower panel).

V_{1A}R Mediates AVP-Induced Inflammation. To determine which subtypes functionally mediate AVP-induced IL-6 production, mRNAs for V_{1A} receptor were detected only by reverse transcription-PCR in NRCFs (Chen et al., 2017). More importantly, the V_{1A} receptor selective inhibitor SR49059 efficiently blocked the AVP-induced IL-6 expression with 2.35 ± 0.46 nM, IC_{50} ($n = 3$, Fig. 5A), and NF-κB signaling with 1.25 ± 0.44 nM, IC_{50} ($n = 3$, Fig. 5B); 1 μM SR49059 had no effect on either basal IL-6 expression or NF-κB activation (Fig. 5, A and B).

Discussion

IL-6 is an important cytokine that participates in multiple biologic activities in immune regulation and inflammation. IL-6 has been associated with cardiovascular remodeling. Although the baseline mRNA expression of IL-6 in cardiac fibroblasts was low and absent in cardiomyocytes, the level of IL-6 will be increased upon β₂AR stimulation (Yin et al., 2006), hypoxia (Wang et al., 2016), or coculture with macrophages (Ma et al., 2012). In the present study, we have found that 1) AVP increased the level of IL-6 protein and mRNA in dose- and time-dependent manner in NRCFs; 2) inhibition of GRK2 via a pharmacologic approach or overexpression of GRK2-ct abolished the AVP-induced IL-6 production and NF-κB activation; and 3) blocking NF-κB signaling diminished AVP-induced IL-6 production.

Both AVP and corticotrophic releasing hormone act synergistically on the pituitary to release adrenocorticotrophic hormone, stimulating the adrenal cortex to release corticosteroid, which in turn suppresses inflammation. The hypothalamic-pituitary-adrenal axis response is diminished in patients with rheumatoid arthritis, manifested by a low cortisol level for the degree of joint inflammation. AVP promotes inflammation

Fig. 2. Inhibition of GRK2 by paroxetine or overexpression of GRK2-CT suppresses the AVP-induced IL-6 mRNA production in adult rat cardiac fibroblast. (A,B) Pretreatment of cells with paroxetine (POTH) suppressed the AVP-induced IL-6 mRNA expression. The starved cells pretreated with 10 μM POTH were stimulated with 0.1 μM AVP for 0–24 hours (A) or for 6 hours in the presence of 1–100 μM. Data are expressed as mean ± S.E.M. of four separate experiments. * $P < 0.05$; ** $P < 0.01$ versus control; ### $P < 0.01$ versus AVP alone; 10 μM POTH alone has no effects on IL-6 expression (0.89 ± 0.25 -fold over control). (C) Overexpression of GRK2-CT diminished the AVP-induced IL-6 expression. Upper panel: A representative blot to show the expression of GRK2-ct protein in NRCFs. Lower panel: Average data were expressed as mean ± S.E.M. of three separate experiments. ** $P < 0.01$ versus control; # $P < 0.05$ versus AVP alone.

in human alveolar epithelial cells, which was significantly suppressed in mice pretreated with a V2 receptor antagonist (Baker et al., 2003; Boyd et al., 2008). Low-dose AVP injected in mice did not affect hemodynamics but significantly reduced the innate immune response (Boyd et al., 2008). Thus, the efficacy of AVP in septic shock may be due to both its vasoactive and immunomodulatory properties. AVP has been found in rat B lymphocytes and thymic epithelial cells and in human peripheral blood mononuclear cells, in which AVP receptors also exist. Thus, in response to inflammation, AVP can stimulate the production of cytokines and antibodies through these receptors in immune cells (Baker et al., 2003). AVP is also a proinflammatory peptide that can stimulate the release of prolactin, a proinflammatory peptide, which can also exacerbate inflammation. AVP stimulates the release of cytokines, increases T helper-1 cell actions, and augments mixed lymphocytic response, further worsening inflammation. Immunoneutralization of serum AVP in rats has been shown to diminish inflammation. Thus, excessive production of AVP from an abnormal hypothalamic-pituitary-adrenal axis may contribute to the chronic inflammatory state (Chikanza and Grossman, 1998). In cardiac fibroblasts, secretion of IL-6 by GPCR stimulation or cardiac stress such as ischemic might be responsible for the cardiac inflammation, fibroblast proliferation, and cardiac remodeling. Our and other studies have shown that AVP promotes the proliferation of cardiac fibroblasts (Yang et al., 2003; He et al., 2008; Yan-ping et al., 2008; Chen et al., 2015; 2017); insofar as we know, this is the first finding that AVP induces IL-6 production. Whether IL-6 is the mediator for cardiac fibroblast proliferation should be further studied (Yan-Hong et al., 2010).

Fig. 3. NF-κB signaling mediates the AVP-induced IL-6 production. (A) Inhibition of NF-κB with PDTC abolished the AVP-induced IL-6 mRNA expression. The starved cells pretreated with 50 μM PDTC for 1 hour were further incubated with 0.1 μM of AVP for 0–24 hours. IL-6 mRNA was measured by q-PCR as described in *Materials and Methods*. Data are expressed as mean ± S.E.M. of four separate experiments. * $P < 0.05$; ** $P < 0.01$ versus control; # $P < 0.05$; ### $P < 0.01$ versus AVP alone. (B) AVP induced the phosphorylation of the NF-κB in NRCFs. The starved cells were stimulated with 0.1 μM of AVP for 1–120 minutes. The cellular lysate was performed for phosphorylation of NF-κB with anti-phosphorylated NF-κB antibody as described in *Materials and Methods*. Upper panel: A representative blot. Lower panel: Average data from three separate experiments. * $P < 0.05$ for time course (repeated two-way ANOVA, $n = 3$). * $P < 0.05$; ** $P < 0.01$ versus control. (C) AVP activates NF-κB in dose- and time-dependent manner. The starved cells transfected with NF-κB luciferase reporter plasmid as described in *Materials and Methods* were incubated with either 0.1 μM AVP for 0–36 hours or in the presence of 1–1000 nM of AV for 24 hours. Luciferase activation was measured according to the kit instructions. Average data were from three separate experiments. * $P < 0.05$; ** $P < 0.01$ versus control. (D) Inhibition of NF-κB with PDTC abolished the AVP-evoked NF-κB activation. The cells transfected with luciferase were incubated with 0.1 μM AVP in the presence or absence of PDTC for 24 hours. Average data from three separate experiments. ** $P < 0.01$ versus control; ## $P < 0.01$ versus AVP alone.

Recent studies have found that GPCR signals could be mediated by GRKs or β -arrestins in a G protein-independent manner (Tilley, 2011). Increasing nontraditional functions of GRKs and β -arrestins have been discovered. For example, GRK2, β -arrestin 1, and β -arrestin 2, mediate protective β_1 -adrenergic signaling in cardiac myocytes (Noma et al., 2007). G protein-coupled receptor kinases (GRKs) are serine/threonine protein kinases originally discovered for their role in G protein-coupled receptor (GPCR) phosphorylation. Recent studies have demonstrated a much broader function for this kinase family, including phosphorylation of cytosolic substrates involved in cell-signaling pathways stimulated by GPCRs, as well as by non-GPCRs. In addition, GRKs modulate signaling via phosphorylation-independent functions (Ferguson, 2007). Because of these various biochemical functions, GRKs affect critical physiologic and pathophysiologic processes and thus are considered as drug targets in

Fig. 4. GRK2 is necessary for AVP-evoked NF- κ B signaling. (A) Pretreatment of POTH abolished the AVP-evoked NF- κ B activation. Cells were transfected with plasmid containing NF- κ B luciferase for 6 hours; cells were starved overnight and further stimulated with 1 μ M AVP for 24 hours. The cellular lysates were done for activation of luciferase activation. ** P < 0.01 versus control; ## P < 0.01 versus AVP alone (B) Inhibition of GRK2 with overexpression of GRK2-CT abolished the AVP-induced IL-6 expression. Data were expressed as mean $\pm$ S.E.M. of four separate experiments. ** P < 0.01 versus control; # P < 0.05; ## P < 0.01 versus AVP alone. (C) GRK2 inhibition with POTH abolished the phosphorylation of NF- κ B p65 induced by AVP incubation. Upper panel: A representative image. Lower panel: Data were expressed as mean $\pm$ S.E.M. of three separate experiments. ** P < 0.01 versus control; ## P < 0.01 versus AVP alone.

diseases such as heart failure. The role of GRKs in inflammation and inflammatory diseases is an evolving area of research seen in review (Packiriswamy and Parameswaran, 2015). GRK2 fibroblast knockout mice also had decreased fibrosis and fibrotic gene expression (Woodall et al., 2016). These protective effects correlated with decreased infiltration of neutrophils to the ischemia site and decreased levels of tumor necrosis factor- α expression and secretion in GRK2 fibroblast knockout mice (Woodall et al., 2016), suggesting that GRK2 deletion suppresses the cytokine secretion and inflammation evoked by ischemia stress in cardiac fibroblasts. In the present study, an increased local cardiac and circulating AVP in heart failure (Chen et al., 2015) enhanced the level of IL-6 in NRCFs, suggesting that IL-6 mediates the development of AVP-increased heart failure.

GRKs, in particular GRK2 and GRK5, have been reported to modulate the NF- κ B signaling pathway in immune and nonimmune cells. It has been shown that GRK5 directly interacts with NF- κ B p105 (one of the I κ B members) (Patil et al., 2009) and inhibits Toll-like receptor-4-induced I κ B kinase β -mediated phosphorylation of p105 (Parameswaran et al., 2006). Consistent with these biochemical findings, the levels of cytokines and chemokines were largely attenuated in GRK5 knockout mice compared with the wild-type mice in an endotoxemia model (Patil et al., 2011b). Using GRK5 knockout mice, we found that GRK5 indeed positively regulates the NF- κ B pathway in cardiomyocytes (Islam et al., 2013). Compared with GRK5, GRK2 has also been shown to interact with I κ B α and p105. Genome-wide RNA interference in *Drosophila* cells identifies GRK2 as a conserved regulator of NF- κ B signaling (Valanne et al., 2010). GRK2 negatively regulates p105 signaling in primary peritoneal (Patil et al., 2011a) macrophages via interaction with p105. Toll-like receptor ligands enhance GRK2 expression in primary macrophages (Loniewski et al., 2008). These specific GRK isoforms regulating the NF- κ B signaling could be dependent on the species, tissues, and cells used in the experiment models. Our results corroborate these findings since AVP-induced IL-6 production of NRCFs depends on the activities of GRK2 and NF- κ B signaling.

Fig. 5. V_{1A} receptor mediates AVP-induced NF- κ B activation and IL-6 production. (A) V_{1A} receptor subtype-selective blockers efficiently inhibited AVP-induced IL-6 production in NRCFs. After the starved cells were pretreated with 1–1000 μ M of V_{1A} receptor blocker SR49059 for 1 hour, cells were further stimulated with 0.1 μ M of AVP for 6 hours. (B) SR49059 blocked the AVP-induced NF- κ B activity in dose-dependent manner. Average data were from three to six separate experiments. ** P < 0.01 versus control; ## P < 0.01 versus AVP alone. It was noted that 1 μ M of SR 49059 has no effects on the basal level of either IL-6 expression or NF- κ B luciferase activation.

It is a well established concept that NF- κ B, a redox-sensitive transcription factor, is a key transcription factor that regulates inflammatory factor, including IL-6 and other cytokines. NF- κ B has been implicated in the initiation and progression of pathogenesis in myocardium fibrosis (Neves et al., 2005; Kawano et al., 2006). NF- κ B mediates the AVP-induced iNOS production and proliferation in cardiac fibroblasts (Fan et al., 2003, 2007; Niu et al., 2014). In the present study, AVP indeed evoked NF- κ B signaling, evidenced by NF- κ B p65 phosphorylation and activation as shown with the NF- κ B luciferase reporter as being enhanced by AVP stimulation; in addition, inhibition of NF- κ B signaling with the pharmacologic approach abolished the effects of AVP on the production of IL-6, suggesting that NF- κ B signaling is responsible for the AVP-induced IL-6 production. Further study is required to determine whether IL-6 production promotes the proliferation of cardiac fibroblasts.

Vasopressin receptor subtypes differ in their tissue distribution and function. $V_{1A}R$ is expressed in various tissues, including vascular smooth muscle, platelets, liver, and uterus. It participates in vasoconstriction, platelet aggregation, and glycogenolysis. It has been shown that immunomodulatory action of AVP was significantly suppressed when mice were pretreated with a $V_{2}R$ antagonist in human alveolar epithelial

cells. Structurally, the V_{1A}R has a conserved DRY motif in its second intracellular loop, and its C-terminus contains multiple serine or threonine residues that can be phosphorylated by GRKs (Hawtin, 2006). Our previous study in H9c2 cells has shown that AVP enhances cell survival via a GRK2/ β -arrestin1/ERK1/2-dependent pathway (Zhu et al., 2013) and that V_{1A}R blocker SR45049 abolished AVP-induced NRCF proliferation (Chen et al., 2017) and smooth muscle cell proliferation, although the V₂R also exists in A7R5 rat aorta smooth muscle (Zhang et al., 2016). Vasopressin receptor and oxytocin receptor belong to the same subfamily of the GPCR superfamily (Mayasich and Clarke, 2016), and AVP is a partial agonist of the oxytocin receptor (Ramos et al., 2016). Thus, AVP-regulated inflammation should distinguish V_{1A}R from oxytocin receptor. In the present study, the data further demonstrated that V_{1A}R-selective blocker abolished AVP-induced IL-6 production and NF- κ B signaling, suggesting that V_{1A}R mediates AVP-evoked inflammation in NRCFs.

In summary, AVP induces IL-6 production of NRCFs by activating V_{1A}R signaling via a GRK2/NF- κ B pathway. These findings provide a possible molecular mechanism for inflammation that occurs in heart failure and other cardiac conditions.

Acknowledgments

The authors thank Drs. Walter Koch (Temple University School of Medicine, Philadelphia) and Yibin Wang (University of California, Los Angeles) for providing the GRK2-ct and β -gal adenovirus, respectively.

Author Contributions

Participated in research design: Xu, Sun, Zhu.

Conducted experiments: Xu, Sun, Wang, Ni, Zhao.

Performed data analysis: Sun, Xu, Zhu.

Wrote or contributed to the writing of the manuscript: Xu, Sun, Zhu.

References

- Baker C, Richards LJ, Dayan CM, and Jessop DS (2003) Corticotropin-releasing hormone immunoreactivity in human T and B cells and macrophages: colocalization with arginine vasopressin. *J Neuroendocrinol* **15**:1070–1074.
- Boyd JH, Holmes CL, Wang Y, Roberts H, and Walley KR (2008) Vasopressin decreases sepsis-induced pulmonary inflammation through the V2R. *Resuscitation* **79**:325–331.
- Carmichael MC and Kumar R (1994) Molecular biology of vasopressin receptors. *Semin Nephrol* **14**:341–348.
- Chen X, Lu G, Tang K, Li Q, and Gao X (2015) The secretion patterns and roles of cardiac and circulating arginine vasopressin during the development of heart failure. *Neuropeptides* **51**:63–73.
- Chen Y, Xu F, Zhang L, Wang X, Wang Y, Woo AH and Zhu W (2017) GRK2/ β -arrestin mediates arginine vasopressin-induced cardiac fibroblast proliferation. *Clin Exp Pharmacol Physiol* **44**:285–293.
- Chikanza IC and Grossman AS (1998) Hypothalamic-pituitary-mediated immunomodulation: arginine vasopressin is a neuroendocrine immune mediator. *Br J Rheumatol* **37**:131–136.
- de Keyser Y, Auzan C, Lenne F, Beldjord C, Thibonnier M, Bertagna X, and Clauser E (1994) Cloning and characterization of the human V3 pituitary vasopressin receptor. *FEBS Lett* **356**:215–220.
- Dorn, 2nd GW (2009) GRK mythology: G-protein receptor kinases in cardiovascular disease. *J Mol Med (Berl)* **87**:455–463.
- Fan YH, Zhao LY, Zheng QS, Dong H, Wang HC, and Yang XD (2007) Arginine vasopressin increases iNOS-NO system activity in cardiac fibroblasts through NF- κ B activation and its relation with myocardial fibrosis. *Life Sci* **81**:327–335.
- Fan YH, Zhao LY, Zheng QS, Xue YS, Yang XD, Tian JW and Xu L (2003) [Arginine vasopressin-induced nitric oxide content changes in cultured cardiac fibroblasts and its relation to nuclear factor-kappaB]. *Sheng Li Xue Bao* **55**(4):417–421 [article in Chinese].
- Ferguson SS (2007) Phosphorylation-independent attenuation of GPCR signalling. *Trends Pharmacol Sci* **28**:173–179.
- Finkel MS, Oddis CV, Jacob TD, Watkins SC, Hattler BG, and Simmons RL (1992) Negative inotropic effects of cytokines on the heart mediated by nitric oxide. *Science* **257**:387–389.
- Fujio Y, Maeda M, Mohri T, Obana M, Iwakura T, Hayama A, Yamashita T, Nakayama H, and Azuma J (2011) Glycoprotein 130 cytokine signal as a therapeutic target against cardiovascular diseases. *J Pharmacol Sci* **117**:213–222.
- Hawtin SR (2006) Pharmacological chaperone activity of SR49059 to functionally recover misfolded mutations of the vasopressin V1a receptor. *J Biol Chem* **281**:14604–14614.
- He YP, Zhao LY, Zheng QS, Liu SW, Zhao XY, Lu XL and Niu XL (2008) Arginine vasopressin stimulates proliferation of adult rat cardiac fibroblasts via protein kinase C-extracellular signal-regulated kinase 1/2 pathway. *Sheng Li Xue Bao* **60**(3):333–340 [article in Chinese].
- Hirota H, Yoshida K, Kishimoto T, and Taga T (1995) Continuous activation of gp130, a signal-transducing receptor component for interleukin 6-related cytokines, causes myocardial hypertrophy in mice. *Proc Natl Acad Sci USA* **92**:4862–4866.
- Hiroyama M, Wang S, Aoyagi T, Oikawa R, Sanbe A, Takeo S, and Tanoue A (2007) Vasopressin promotes cardiomyocyte hypertrophy via the vasopressin V1A receptor in neonatal mice. *Eur J Pharmacol* **559**:89–97.
- Huang ZM, Gold JL, and Koch WJ (2011) G protein-coupled receptor kinases in normal and failing myocardium. *Front Biosci (Landmark Ed)* **16**:3047–3060.
- Islam KN, Bae JW, Gao E, and Koch WJ (2013) Regulation of nuclear factor κ B (NF- κ B) in the nucleus of cardiomyocytes by G protein-coupled receptor kinase 5 (GRK5). *J Biol Chem* **288**:35683–35689.
- Kawano S, Kubota T, Monden Y, Tsutsumi T, Inoue T, Kawamura N, Tsutsui H, and Sunagawa K (2006) Blockade of NF- κ B improves cardiac function and survival after myocardial infarction. *Am J Physiol Heart Circ Physiol* **291**:H1337–H1344.
- Li X, Chan TO, Myers V, Chowdhury I, Zhang XQ, Song J, Zhang J, Andrel J, Funakoshi H, Robbins J, et al. (2011) Controlled and cardiac-restricted overexpression of the arginine vasopressin V1A receptor causes reversible left ventricular dysfunction through G α q-mediated cell signaling. *Circulation* **124**:572–581.
- Lionetti V, Bianchi G, Recchia FA, and Ventura C (2010) Control of autocrine and paracrine myocardial signals: an emerging therapeutic strategy in heart failure. *Heart Fail Rev* **15**:531–542.
- Lolait SJ, O'Carroll AM, McBride OW, Konig M, Morel A, and Brownstein MJ (1992) Cloning and characterization of a vasopressin V2 receptor and possible link to nephrogenic diabetes insipidus. *Nature* **357**:336–339.
- Loniewski K, Shi Y, Pestka J, and Parameswaran N (2008) Toll-like receptors differentially regulate GPCR kinases and arrestins in primary macrophages. *Mol Immunol* **45**:2312–2322.
- Ma F, Li Y, Jia L, Han Y, Cheng J, Li H, Qi Y, and Du J (2012) Macrophage-stimulated cardiac fibroblast production of IL-6 is essential for TGF β /Smad activation and cardiac fibrosis induced by angiotensin II. *PLoS One* **7**:e35144.
- Mayasich SA and Clarke BL (2016) The emergence of the vasopressin and oxytocin hormone receptor gene family lineage: Clues from the characterization of vasotocin receptors in the sea lamprey (*Petromyzon marinus*). *Gen Comp Endocrinol* **226**:88–101.
- Métayé T, Gibelin H, Perdrisot R, and Kraimps JL (2005) Pathophysiological roles of G-protein-coupled receptor kinases. *Cell Signal* **17**:917–928.
- Moore CA, Milano SK, and Benovic JL (2007) Regulation of receptor trafficking by GRKs and arrestins. *Annu Rev Physiol* **69**:451–482.
- Morel A, O'Carroll AM, Brownstein MJ, and Lolait SJ (1992) Molecular cloning and expression of a rat V1a arginine vasopressin receptor. *Nature* **356**:523–526.
- Murray DR and Freeman GL (2003) Proinflammatory cytokines: predictors of a failing heart? *Circulation* **107**:1460–1462.
- Neves MF, Amiri F, Virdis A, Diep QN, and Schiffrin EL; CIHR Multidisciplinary Research Group on Hypertension (2005) Role of aldosterone in angiotensin II-induced cardiac and aortic inflammation, fibrosis, and hypertrophy. *Can J Physiol Pharmacol* **83**:999–1006.
- Niu X, Xue Y, Li X, He Y, Zhao X, Xu M, and Zhao L (2014) Effects of angiotensin-(1-7) on the proliferation and collagen synthesis of arginine vasopressin-stimulated rat cardiac fibroblasts: role of mas receptor-calcineurin-NF- κ B signaling pathway. *J Cardiovasc Pharmacol* **64**:536–542.
- Noma T, Lemaire A, Naga Prasad SV, Barki-Harrington L, Tilley DG, Chen J, Le Corvoisier P, Violin JD, Wei H, Lefkowitz RJ, et al. (2007) Beta-arrestin-mediated beta1-adrenergic receptor transactivation of the EGFR confers cardioprotection. *J Clin Invest* **117**:2445–2458.
- Packiriswamy N and Parameswaran N (2015) G-protein-coupled receptor kinases in inflammation and disease. *Genes Immun* **16**:367–377.
- Parameswaran N, Pao CS, Leonhard KS, Kang DS, Kratz M, Ley SC, and Benovic JL (2006) Arrestin-2 and G protein-coupled receptor kinase 5 interact with NF- κ B p105 and negatively regulate lipopolysaccharide-stimulated ERK1/2 activation in macrophages. *J Biol Chem* **281**:34159–34170.
- Patil S, Luo J, Porter KJ, Benovic JL, and Parameswaran N (2009) G-protein-coupled-receptor kinases mediate TNF α -induced NF- κ B signalling via direct interaction with and phosphorylation of I κ B α . *Biochem J* **425**:169–178.
- Patil S, Saini Y, Parvataneni S, Appledorn DM, Dorn, 2nd GW, Lapres JJ, Amalfitano A, Senagore P, and Parameswaran N (2011a) Myeloid-specific GPCR kinase-2 negatively regulates NF- κ B p105-ERK pathway and limits endotoxemic shock in mice. *J Cell Physiol* **226**:627–637.
- Patil S, Shahi S, Saini Y, Lee T, Packiriswamy N, Appledorn DM, Lapres JJ, Amalfitano A, and Parameswaran N (2011b) G-protein coupled receptor kinase 5 mediates lipopolysaccharide-induced NF- κ B activation in primary macrophages and modulates inflammation in vivo in mice. *J Cell Physiol* **226**:1323–1333.
- Ramos L, Hicks C, Caminer A, Couto K, Narlawar R, Kassiou M, and McGregor IS (2016) MDMA ('ecstasy'), oxytocin and vasopressin modulate social preference in rats: a role for handling and oxytocin receptors. *Pharmacol Biochem Behav* **150**:115–123.
- Schumacher SM, Gao E, Zhu W, Chen X, Chuprun JK, Feldman AM, Tesmer JJ, and Koch WJ (2015) Paroxetine-mediated GRK2 inhibition reverses cardiac dysfunction and remodeling after myocardial infarction. *Sci Transl Med* **7**:277ra31.
- Taga T and Kishimoto T (1997) Gp130 and the interleukin-6 family of cytokines. *Annu Rev Immunol* **15**:797–819.
- Thibonnier M, Coles P, Thibonnier A, and Shoham M (2002) Molecular pharmacology and modeling of vasopressin receptors. *Prog Brain Res* **139**:179–196.

- Tilley DG (2011) G protein-dependent and G protein-independent signaling pathways and their impact on cardiac function. *Circ Res* **109**:217–230.
- Tilley DG, Zhu W, Myers VD, Barr LA, Gao E, Li X, Song J, Carter RL, Makarewich CA, Yu D, et al. (2014) β -adrenergic receptor-mediated cardiac contractility is inhibited via vasopressin type 1A-receptor-dependent signaling. *Circulation* **130**:1800–1811.
- Valanne S, Myllymäki H, Kallio J, Schmid MR, Kleino A, Murumägi A, Airaksinen L, Kotipelto T, Kaustio M, Ulvila J, et al. (2010) Genome-wide RNA interference in *Drosophila* cells identifies G protein-coupled receptor kinase 2 as a conserved regulator of NF- κ B signaling. *J Immunol* **184**:6188–6198.
- Vasan RS, Sullivan LM, Roubenoff R, Dinarello CA, Harris T, Benjamin EJ, Sawyer DB, Levy D, Wilson PW, and D'Agostino RB; Framingham Heart Study (2003) Inflammatory markers and risk of heart failure in elderly subjects without prior myocardial infarction: the Framingham Heart Study. *Circulation* **107**:1486–1491.
- Wang F, Trial J, Diwan A, Gao F, Birdsall H, Entman M, Hornsby P, Sivasubramanian N, and Mann D (2002) Regulation of cardiac fibroblast cellular function by leukemia inhibitory factor. *J Mol Cell Cardiol* **34**:1309–1316.
- Wang JH, Zhao L, Pan X, Chen NN, Chen J, Gong QL, Su F, Yan J, Zhang Y, and Zhang SH (2016) Hypoxia-stimulated cardiac fibroblast production of IL-6 promotes myocardial fibrosis via the TGF- β 1 signaling pathway. *Lab Invest* **96**:839–852.
- Woodall MC, Woodall BP, Gao E, Yuan A, and Koch WJ (2016) Cardiac fibroblast GRK2 deletion enhances contractility and remodeling following ischemia/reperfusion injury. *Circ Res* **119**:1116–1127.
- Yan-Hong F, Hui D, Qing P, Lei S, Hai-Chang W, Wei Z, and Yan-Jie C (2010) Effects of arginine vasopressin on differentiation of cardiac fibroblasts into myofibroblasts. *J Cardiovasc Pharmacol* **55**:489–495.
- Yan-ping H, Lian-you Z, Qiang-sun Z, Shao-wei L, Xiao-yan Z, Xiao-long L, Xiao-lin N, and Xia L (2008) Mitogenic effect of arginine vasopressin on adult rat cardiac fibroblast: involvement of PKC-erk1/2 pathway. *J Cardiovasc Pharmacol* **52**:72–81.
- Yang XD, Zhao LY, Zheng QS, and Li X (2003) Effects of arginine vasopressin on growth of rat cardiac fibroblasts: role of V1 receptor. *J Cardiovasc Pharmacol* **42**:132–135.
- Yin F, Wang YY, Du JH, Li C, Lu ZZ, Han C, and Zhang YY (2006) Noncanonical cAMP pathway and p38 MAPK mediate β 2-adrenergic receptor-induced IL-6 production in neonatal mouse cardiac fibroblasts. *J Mol Cell Cardiol* **40**:384–393.
- Zhai P, Yamamoto M, Galeotti J, Liu J, Masurekar M, Thaisz J, Irie K, Holle E, Yu X, Kupersmidt S, et al. (2005) Cardiac-specific overexpression of AT1 receptor mutant lacking G α_q /G α_i coupling causes hypertrophy and bradycardia in transgenic mice. *J Clin Invest* **115**:3045–3056.
- Zhang L, Wang X, Cao H, Chen Y, Chen X, Zhao X, Xu F, Wang Y, Woo AY, and Zhu W (2016) Vasopressin V1A receptor mediates cell proliferation through GRK2-EGFR-ERK1/2 pathway in A7r5 cells. *Eur J Pharmacol* **792**:15–25.
- Zhang W, Chen XF, Huang YJ, Chen QQ, Bao YJ, and Zhu W (2012) 2,3,4',5-Tetrahydroxystilbene-2-O- β -D-glucoside inhibits angiotensin II-induced cardiac fibroblast proliferation via suppression of the reactive oxygen species-extracellular signal-regulated kinase 1/2 pathway. *Clin Exp Pharmacol Physiol* **39**:429–437.
- Zhu W, Tilley DG, Myers VD, Coleman RC, and Feldman AM (2013) Arginine vasopressin enhances cell survival via a G protein-coupled receptor kinase 2/ β -arrestin1/extracellular-regulated kinase 1/2-dependent pathway in H9c2 cells. *Mol Pharmacol* **84**:227–235.

Address correspondence to: Dr. Weizhong Zhu, Laboratory of Cardiovascular Science, Department of Pharmacology, Nantong University School of Pharmacy, 19 Qixiu road, Nantong City, 226001, P.R. China. E-mail: Zhuwz@ntu.edu.cn