MOLECULAR PHARMACOLOGY IN CHINA

GRK2 Mediates Arginine Vasopressin-Induced Interleukin-6 Production via Nuclear Factor-κB Signaling Neonatal Rat Cardiac Fibroblast

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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 V1A 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-κB (NF-κB) evoked by AVP stimulation were measured by immunoblot and NF-κB 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-κB activation; and 3) blocking NF-κB signaling using the pharmacologic approach diminished AVP-induced IL-6 production. In summary, AVP induces IL-6 production of NRCFs by activating V1A receptor signaling via a GRK2/NF-κB 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, vasconstriction, and the release of adrenocorticotropic hormone. AVP has also been found in rat B lymphocytes, thymic epithelial cells, and human peripheral blood mononuclear cells, in which AVP

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-κB, nuclear factor-κB; NRCF, neonatal rat cardiac fibroblast; PCR, polymerase chain reaction; PDTC, pyrrolidinedithiocarbamic acid; POTH, paroxetine hydrochloride; SR49059, (S)-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; V1aR, 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 (V1aR, V1bR, and V2R) have been found in humans (Lolait et al., 1992; Morel et al., 1992; de Keyzer 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 V1aR 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 cardiac fibroblasts (Chen et al., 2017) via AVP enhances the survival of H9c2 myoblasts (Zhu et al., 2009; Huang et al., 2011). These G protein–dependent pathways have been attributed to the regulation of myocardial hypertrophy and apoptosis (Métyá et al., 2005; Dorn, 2009; Huang et al., 2011) and to the promotion of a cardiotrophic extracellular signal–regulated kinase 1/2 (ERK1/2) 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/β-arrestin1/ERK1/2-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, V85789) was from Sigma-Aldrich. V1aR-selective antagonist (SR49059) was from Tocris Bioscience (Minneapolis, MN), paroxetine hydrochloride (cat no. 1500218) was purchased from ABcam, the adenovirus 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-kB Promoter Luciferase Reporter Gene and Measurement of NFkB-Luciferase Activation. The NRCFs were plated in 12-well plates (250 × 104). 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 μg of NF-kB luciferase plasmid and 0.5 μg of Renilla plasmids. For basal activity, cells were transfected with the NF-kB-luciferase plasmid (0.5μg) and a Renilla control vector (0.5 μg) 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, Somers, NJ).

Quantitative PCR for Measurement of IL-6. NRCFs with a density of 1 × 105 cells/well were plated in 10-cm culture dishes. After being made quiescent at 90% confluence and stimulated with AVP, paroxetine hydrochloride (POTH), and pyrrolidine dihydrocarboxamide (PDTC), as designed in individual experiments, total RNA was isolated using TRIzol reagent (Promega, Madison, WI). First-strand cDNA synthesis was performed using 1 μg of total RNA in a 20-μl 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 QuantTech SYBR PCR kit (Qiagen, Valencia, CA) to allow for quantitative detection of the PCR product in a 25-μl reaction volume. The primer sequences for IL-6 were 5′-ACT-CCA-TCT-GCC-CPT-CA-3′ (sense) and 5′-ACT-CCA-TCT-GCC-CPT-CA-3′ (antisense); IL-6 accession no. NM_012589). The primer sequences for GAPDH were 5′-TTC-AAT-GGC-ACA-GTC-AAG-GC-3′ (antisense) and 5′-TCA-CCC-CAT-TTG-ATG-TTA-GCG-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 μl of ice-cold lysis buffer (50 mM Tris-HCl, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 mg/ml leupeptin, 20 μg/ml aprotinin, 1 mM NaF, and 1 mM Na3VO4). After centrifugation at 12,000g for 10 minutes, equal amounts of total cell lysate (20 μg protein) were subjected to 4%–12% SDS-PAGE, followed by immunoblotting for phosphorylated NF-kB, NF-kB, 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 ± S.E.M. was conducted with one- or two-way
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 manner (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 Inhibits the AVP-Induced IL-6 Production 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 pre-treated 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 NK-kB 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 NK-kB 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).

V1AR Mediates AVP-Induced Inflammation. To determine which subtypes functionally mediate AVP-induced IL-6 production, mRNAs for V1A receptor were detected only by reverse transcription-PCR in NRCFS (Chen et al., 2017). More importantly, the V1A receptor selective inhibitor SR49059 efficiently blocked the AVP-induced IL-6 expression with 2.35 ± 0.46 nM, IC50 (n = 3, Fig. 5A), and NF-κB signaling with 1.25 ± 0.44 nM, IC50 (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).
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 lymphocyte 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 signaling 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).

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 β1-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
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-induced heart failure.

GRKs, in particular GRK2 and GRK5, have been reported to modulate the NF-kB signaling pathway in immune and nonimmune cells. It is has been shown that GRK5 directly interacts with NF-kB p105 (one of the IκB members) (Patial et al., 2009) and inhibits Toll-like receptor-4–induced IkB 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 (Patial et al., 2011b). Using GRK5 knockout mice, we found that GRK5 indeed positively regulates the NF-kB pathway in cardiomyocytes (Islam et al., 2013). Compared with GRK5, GRK2 has also been shown to interact with IκBα and p105. Genome-wide RNA interference in Drosophila cells identifies GRK2 as a conserved regulator of NF-kB signaling (Valanne et al., 2010). GRK2 negatively regulates p105 signaling in primary peritoneal (Patial 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-kB 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-kB signaling.

It is a well established concept that NF-kB, a redox-sensitive transcription factor, is a key transcription factor that regulates inflammatory factor, including IL-6 and other cytokines. NF-kB has been implicated in the initiation and progression of pathogenesis in myocardium fibrosis (Neves et al., 2005; Kawano et al., 2006). NF-kB 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-kB signaling, evidenced by NF-kB p65 phosphorylation and activation as shown with the NF-kB luciferase reporter as being enhanced by AVP stimulation; in addition, inhibition of NF-kB signaling with the pharmacologic approach abolished the effects of AVP on the production of IL-6, suggesting that NF-kB 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. V1aR 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 V1aR antagonist in human alveolar epithelial
cells. Structurally, the V2R 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-arrestin-1/ERK1/2-dependent pathway (Zhu et al., 2013) and that V1AR 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 V1AR partial agonist of the oxytocin receptor (Ramos et al., 2016).

In summary, AVP induces IL-6 production of NRCFs by activating V1AR signaling via a GRK2/NF-kB pathway. These findings provide a possible molecular mechanism for inflammation that occurs in heart failure and other cardiac conditions.

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GRK2 mediates arginine vasopressin-induced IL-6 production via NF-kB signaling in neonatal rat cardiac fibroblast

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Molecular Pharmacology

Supplemental Figure 1 AVP induced the nuclear translocation of NF-kB. (A) AVP evoked the nuclear translocation of NK-kB. The starved cells were stimulated with 0.1 μM of AVP for 30 min. The cells were washed 3 times with 4°C of PBS and fixed with 4% of formaldehyde for 10 min and then washed 2 times with PBS. The fixed cells were blocking and permeabilizing with 3% BSA, 1% Triton in 1X PBS for 20 min. The cells were incubating in anti-NF-kB (rabbit, 1:500) overnight. After washing 3 times, the cells were incubated with second antibody for anti-rabbit for 1 h. The cells are count-staining with DAPI (10 μg/ml) for 15 min. The images were taken with 436 and 588 nm wavelength. (B) AVP induced the NF-KB nuclear translocation The starved cells were stimulated with 0.1 μM of AVP for 1-120 min. The cytosol and nuclear protein were separated with cellular sub-organ separation kit ( P0023, Biyuntian, Nantong).
A

Vehicle

AVP 1 \mu M (30 min)

B

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NF--kB

GAPDH

H2