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**GRK2 mediates arginine vasopressin-induced IL-6 production via NF- κ B signaling in neonatal rat
cardiac fibroblasts**

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Nonstandard abbreviations: AVP, arginine vasopressin; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; GRK2-ct, G-protein coupled receptor kinase C-terminal peptide; BrdU, 5-bromo-2'-deoxyuridine; DMEM, Dulbecco's Modified Eagle's Medium; β -gal, β -galactosidase; NRCFs, neonatal rat cardiac fibroblasts; ELISA, enzyme linked immunosorbent assay; $V_{1A}R$, arginine vasopressin receptor type 1A; PDTC, Pyrrolidinedithiocarbamic acid; 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; NF- κ B, Nuclear factor kappa-B; IL-6, interleukin6; IC_{50} , half maximal inhibitory concentration.

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Abstract Elevated levels of interleukin 6(IL-6) in congestive heart failure are associated with myocardial damage during acute exacerbation and with chronic inflammation. Arginine vasopressin (AVP), a hormone released in response to cardiac stress, could be a factor of inflammation and fibrosis in the pathogenesis of heart failure. Recently, we showed that AVP promotes proliferation of neonatal rat cardiac fibroblasts (NRCFs) through the V_{1A} vasopressin receptor via G protein-coupled receptor kinase2 (GRK2) signaling. The aim of the present study was to characterize the effect of AVP on IL-6 production in NRCFs and to test the possible involvement of the GRK2-dependent signaling in this effect. IL-6 mRNA and protein were measured in NRCFs by quantitative PCR and ELISA. Cellular GRK2 activities were manipulated by using a pharmacological inhibitor or by overexpressing an inhibitory peptide, GRK2-ct. Phosphorylation and activation of nuclear factor kappa-B (NF- κ B) were determined by immunoblotting and by a luciferase reporter gene assay. The results showed that: 1) AVP increased the mRNA and the protein levels of IL-6 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) pharmacological blockade of NF- κ B signaling diminished the AVP-induced IL-6 production. In summary, AVP induces IL-6 production in NRCFs by activating V_{1A} receptor signaling via a GRK2/NF- κ B pathway. These findings provide a possible molecular mechanism for inflammation occurring in heart failure and in response to other cardiac stress.

Introduction

Cardiac endocrine, paracrine, or autocrine factors are major mediators in cardiac remodeling post ischemia or pressure overload (Lionetti et al., 2010). The interleukin-6 (IL-6) family of cytokines, including IL-6, leukemia inhibitory factor and IL-11, has been implicated in these processes (Fujio et al., 2011; Taga and Kishimoto, 1997; Wang et al., 2002); the pleiotropic cytokine IL-6 could be the key factor. Recent studies demonstrated that plasma levels of IL-6 are elevated in congestive heart failure (Vasan et al., 2003), suggesting that IL-6 may serve as an inflammatory marker in acute and chronic myocardial damage (Murray and Freeman, 2003). Increased production of cytokines, especially inflammatory cytokines such as tumor necrosis factor alpha (TNF α), interleukin-1 (IL-1) or IL-6, is responsible at least in part for cardiac dysfunction in patients with heart failure and cardiac remodelling (Finkel et al., 1992; Hirota et al., 1995).

Arginine vasopressin (AVP) is secreted in response to hypovolemic or cardiac stress. It participates in various physiological processes such as the release of adrenocorticotrophic hormone, vasoconstriction, and osmotic regulation. AVP is released not only from the hypothalamus but also from peripheral immune cells in response to stress and inflammation. AVP receptors are also expressed in those immune cells, such as rat B lymphocytes and thymic epithelial cells, and mononuclear cells.(Baker et al., 2003). Thus, AVP may stimulate the production of cytokines and antibodies in an autocrine fashion during inflammation.

The physiological effects of AVP are mediated through the binding of AVP to specific membrane receptors on target cells. Three vasopressin receptor subtypes (V_{1A}R, V₂R and V_{1B}R, also termed V₃R) have been identified in humans (de Keyzer et al., 1994; Lolait et al., 1992; Morel et al., 1992). All three subtypes belong to the G protein-coupled receptor (GPCR) superfamily (Carmichael and Kumar, 1994; Thibonnier et al., 2002). Among the three subtypes, only V_{1A}R is expressed in cardiac myocytes (Hiroyama et al., 2007) and cardiac fibroblasts (Chen et al., 2016). It has been shown that administration of AVP increases cell hypertrophy in neonatal mouse cardiomyocytes (Hiroyama et al., 2007). Either constitutive or controlled overexpression of

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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; these effects are primarily mediated via Gα_q protein-dependent signaling (Li et al., 2011).

While the Gα_q-coupled V_{1A}R typically activates 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) and primarily with GRK2 (Chen et al., 2016; Zhang et al., 2016). Apart from their well-defined roles in receptor desensitization, GRKs also activate G protein-independent signaling pathways (Huang et al., 2012; Moore et al., 2007). These G protein-independent pathways have been implicated in the regulation of myocardial hypertrophy and apoptosis (Dorn, 2009; Huang et al., 2012; Metaye et al., 2005) and in the promotion of cardioprotective extracellular signal-regulated kinase 1/2 (ERK_{1/2}) signaling (Noma et al., 2007; Zhai et al., 2005). The role of GRKs in inflammation and inflammatory disease is an evolving area of research (Packiriswamy and Parameswaran, 2015). However, little is known regarding the inflammatory regulation of GRK2 in the heart. Our previous studies have shown that AVP enhances H9c2 myoblast survival (Zhu et al., 2013), A7R5 rat aortic smooth muscle cell proliferation (Zhang et al., 2016) and cardiac fibroblast proliferation (Chen et al., 2016), and that all of these effects are likely mediated by a common GRK2/β-arrestin1/ERK_{1/2}-dependent pathway. In the present study, we examined the effect of AVP on IL-6 production in neonatal rat cardiac fibroblast (NRCF) and the relative impact of GRK2-dependent signaling on this effect.

Materials and Methods

Materials

All protocols were approved by The Board of Nantong University Animal Care and Use. Neonatal Sprague Dawley rats were obtained from Animal Center of Nantong University (Nantong, China). Dulbecco's Modified Eagle's Medium (DMEM), penicillin and streptomycin were purchased from Invitrogen (Gaithersburg, MD). AVP (Sigma, V9879) were from Sigma-Aldrich (St. Louis, MO). V_{1A}R selective

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antagonist, SR49059, was from Tocris Bioscience (Cat#2310, Ellisville, MO). Paroxetine hydrochloride (Cat#1500218) was purchased from ABlcam (Cambridge MA). The adenovirus containing β -galactosidase (β -gal) was a gift from Dr. Yibin Wang (University of California, Los Angeles). The adeno-GRK2-CT virus was obtained from Dr. Walter Koch (Temple University, Philadelphia). Both NF-kB luciferase (E8491) and Renilla luciferase (E2231) were purchased from Promega (Madison, MI). Anti-P-NF-kB (S536) antibody was from Cell signaling (#3033), antibodies for GRK2 (Sc-13143), NF-kB (Sc-109) and GAPDH (Sc-25778) were from Santa Cruz (Santa Cruz, CA).

Cell culture and adenoviral infection

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 Nantong University Animal Care and Use. Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) for 4-5 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. Fibroblasts changed to serum-free medium were infected with adenoviruses containing GRK2-Ct or β -galactosidase (β -gal) at a multiplicity of infection of 100. Culture medium was added after 24 h with or without treatment reagents based on the experimental design.

Transient transfection and luciferase gene reporter assay

NRCFs (250×10^3 cells/well) were plated in 12-well plates. When the cells reached 80-90% confluency, transfection with reporter plasmids was carried out using Lipofectamine2000. Co-transfection was achieved with 0.5 μ g of NF-kB luciferase plasmids and 0.5 μ g of Renilla luciferase plasmids. For basal activity, cells were transfected with the NF-kB-luciferase plasmid (0.5 μ g) and a Renilla control vector (0.5 μ g) without AVP incubation. Transfected cells were cultured for 6 h, serum-starved overnight, and further cultured in the

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presence of AVP for 24 h. The luciferase assay was carried out according to the manufacturer's instructions using a luminometer (Thermo).

Quantitative PCR (qPCR)

Fresh tissue samples were collected from a 12-week old Sprague Dawley male rat. Messenger RNA (mRNA) levels of V_{1A}R, V_{1B}R, V₂R in NRCFs, rat brain and rat liver were assessed by reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from cultured NRCFs and fresh tissues in accordance with the protocol of the RNA extraction kit (...). Complementary DNA (cDNA) was prepared using a reverse transcription synthesis kit (TaKaRa, Dalian, China) according to the manufacturer's instructions.

NRCFs (1×10⁶ cells/well) were plated in 10-cm culture dishes. After being made quiescent at 90% confluence and stimulated with AVP, POTH and PTDC as designated for individual experiments, total RNA was extracted from cultured NRCFs with a protocol using TRIZOL Reagent (Promega, Madison, WI, USA). cDNA was amplified using a Step-one Real Time PCR System (Applied Biosystems, FosterCity, CA, USA). The double-stranded DNA-specific dye SYBR Green I was incorporated into the PCR buffer provided in the QuantiTech SYBR PCR kit (Qiagen, Valencia, CA, USA) to allow for quantitative detection of the PCR product in a 25-μl reaction volume. The primer sequences for detecting IL-6 (accession No. NM_012589) were ACTCCATCTGCCCTTCA (sense) and ACTCCATCTGCCCTTCA (antisense). The primer sequences for GAPDH (accession No. M17701) were TTCAATGGCACAGTCAAGGC (sense) and TCACCCCATTTGATGTTAGCG (antisense). The temperature profile of the reaction was as follows: 95 °C for 1 min, 40 cycles of denaturation at 95 °C for 5 s, annealing at 55 °C for 1 min, and extension at 72 °C for 30 s. GAPDH levels were employed as RNA content loading control.

Western blotting analysis

After cells were treated with AVP for 0-120 min, they were rapidly washed two times with ice-cold PBS and lysed with 250 μL of ice-cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 5

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mg/ml leupeptin, 1 mM phenylmethanesulfonyl fluoride, 20 mg/ml aprotinin, 1 mM NaF, and 1 mM Na₃VO₄).

After centrifugation at 12,000 g at 4° C for 10 min, equal amounts of total cell lysate (20 µg protein) were subjected to 4-12% SDS-PAGE, followed by immunoblotting for phosphorylated NF-κB, NF-κB, GRK2, and GAPDH. LI-COR Odyssey[®] CLx Imaging System was employed for western blot images.

Statistical analysis

A commercial software package was used for statistical analysis (Graphpad Prism Software, Inc., San Diego, CA, USA). Comparison of means ± S.E.M. was conducted with one- or two-way 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 of AVP for 0-24 h or with AVP from 0.001 to 1.0 µM for 6 h. Using q-PCR, it was 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 ELISA. As shown in Fig.1C& 1D, IL-6 levels were elevated in a dose- and time-dependent manner with AVP treatment. In contrast, low levels of IL-6 (22.4 ± 4.4 pg/ml, n=3) were detected in culture media in unstimulated cardiac fibroblasts.

Inhibition of GRK2 with paroxetine or over-expression 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 the GRK2 inhibitor paroxetine (POTH) (Schumacher et al., 2015) or by over-expression of GRK2 C-terminal peptide (GRK-Ct, inhibitory peptide of GRK2 activation). As shown in Fig. 2A, pretreatment of cells with 10 µM of POTH abolished the induction of IL-6 mRNA by AVP. Similarly, over-expression of

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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- κ B signaling mediates the AVP-induced IL-6 production

To determine whether NF- κ B signaling is necessary for AVP-induced IL-6 production, cells were pretreated with 50 μ M PDTC, an NF- κ B inhibitor (Fan et al., 2007). As shown in Fig. 3A, AVP-induced IL-6 mRNA production was diminished by PDTC pretreatment. AVP induced the phosphorylation of p65 (Fig. 3B), nuclear translocation of p65 (Supplementary data 1), and activation of NF- κ B (Fig. 3C). The activation of NF- κ B was abolished by PDTC (Fig. 3D). Together, these results suggest that AVP-induced IL-6 production is mediated through NF- κ B signaling in NRCFs.

GRK2 is necessary for AVP-evoked NF- κ B signaling

To define the causal relationship between GRK2 and NF- κ B, either pharmacological inhibition of GRK2 or over-expression of GRK2-CT was employed to abolish GRK2 signaling. As demonstrated in Fig. 4A, pretreatment of cells with POTH diminished the AVP-evoked NF- κ B luciferase activation evoked by AVP in NRCFs. As expected, GRK2-ct was dramatically expressed after cells were transduced with the adenovirus containing GRK2-ct (Fig. 4B, upper panel). Importantly, over-expression of GRK2-ct reduced AVP-induced IL-6 mRNA production (Fig. 4B, lower panel). Inconsistently, inhibition of GRK2 with POTH diminished the phosphorylation of NF- κ B p65 as shown in Fig 4C.

V_{1A}R mediates AVP-induced IL-6 production and NF- κ B activation

The only vasopressin receptor mRNA detected by RT-PCR in NRCF was that for V_{1A} (Chen et al., 2016). More importantly, V_{1A} receptor selective inhibitor, SR49059, efficiently blocked AVP-induced IL-6 expression with an IC_{50} of 2.35 ± 0.46 nM (n=3, Fig. 5A) and NF- κ B signaling with an IC_{50} of 1.25 ± 0.44 nM(n=3,

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Fig.5B). It was noted that 1 μ M of SR49059 had no effect on either basal IL-6 expression or NF-kB activation (Figure 5A & 5B).

Discussion

Interleukin-6 (IL-6) is an important cytokine participating in immune regulation and inflammation, and is associated with cardiovascular remodeling. Although the baseline mRNA expression of IL-6 is low in cardiac fibroblasts and is absent in cardiomyocytes, the level of IL-6 can be increased by β_2 AR stimulation (Yin et al., 2006), hypoxia (Wang et al., 2016), or co-culture with macrophages (Ma et al., 2012). In the present study, we 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 with a pharmacological approach or over-expression of GRK2-ct abolished AVP-induced IL-6 production and NF-kB activation; and 3) blockade of NF-kB signaling diminished the AVP-induced IL-6 production.

AVP released from the hypothalamus suppresses inflammation through its synergistic action on the pituitary. The response to corticotropin releasing hormone (CRH) via hypothalamus–pituitary–adrenal (HPA) axis response is diminished in patients with rheumatoid arthritis, as evidenced by low cortisol levels and different degrees of joint inflammation. AVP-evoked inflammation is significantly suppressed by pretreatment of a V2 receptor antagonist in murine alveolar epithelial cells (Boyd et al., 2008). While low-dose AVP does not change murine hemodynamics, the innate immune response is significantly reduced (Boyd et al., 2008). Thus, treatment of septic shock with AVP may be due to both its vasoactive and immunomodulatory properties. AVP and its receptors have been found in immune cells such as rat B lymphocytes, thymic epithelial cells and human peripheral blood mononuclear cells (PBMC). Thus, AVP could be released from those immune cells and stimulate the production of cytokines and antibodies through its receptors in response to inflammation (Baker et al., 2003). Therefore, unbalanced AVP due to an abnormal HPA axis may be one of factors contributing to the chronic inflammatory state (Chikanza and Grossman, 1998). GPCR- or stress-stimulated IL-6 secretion in cardiac fibroblasts might be responsible for cardiac inflammation, fibroblast proliferation, and cardiac

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remodeling. Data from ours and other studies have shown that AVP promotes the proliferation of cardiac fibroblasts (Chen et al., 2015; Chen et al., 2016; He et al., 2008; Yan-ping et al., 2008; Yang et al., 2003). Here, we report for the first time that AVP induces IL-6 production. Since AVP-induced transforming growth factor-beta1 (TGF-beta1) secretion is responsible for cardiac fibroblast-myofibroblast transformation (Yan-Hong et al., 2010), whether IL-6 induction plays a role on cardiac fibroblast proliferation requires further studies.

Recent studies have found that GPCR signals can be mediated by GRKs or β -arrestins in a G protein-independent manner (Tilley, 2011). Increasingly, non-traditional functions of GRKs and β -arrestins have been discovered. For example, GRK2, β -arrestin 1 and β -arrestin 2 have been shown to mediate protective β_1 -adrenergic signaling in cardiac myocytes (Noma et al., 2007). Recent studies have demonstrated a much broader function of GRK family members beyond their original function for GPCR desensitization (Ferguson, 2007). Due to their various biochemical functions in regulating the critical physiological and pathophysiological processes, GRKs have become drug targets in human diseases such as heart failure. The role of GRKs in regulating inflammation and inflammatory diseases is gradually being recognized (Packiriswamy and Parameswaran, 2015). Recently, it was reported that fibroblast-specific GRK2 knockout mice exhibit attenuated cardiac fibrosis after cardiac ischemia (Woodall et al., 2016). More importantly, this cardioprotection is closely attributed to inflammatory suppression as evidenced by reduced expression and secretion of tumor necrosis factor- α and diminished infiltration of neutrophils to the ischemic area in fibroblast-specific GRK2 knockout mice (Woodall et al., 2016), suggesting that deletion of GRK2 in cardiac fibroblasts suppresses cytokine secretion and inflammation evoked by ischemic stress. In the present study, we have shown that AVP enhances the level of IL-6 in NRCFs through a GRK2-dependent pathway. Since heart failure is marked by increased AVP in both the systemic circulation and in local cardiac tissues (Chen et al., 2015), the present findings highlight IL-6 as an effector cytokine downstream of GRK2, that may link elevated AVP levels to inflammatory cardiac damage and ultimately to cardiac remodeling.

It has recently been reported that GRKs regulates the immune response not only in immune cells but also in cardiomyocytes via the NF- κ B signaling pathway. GRK5 may directly interact with NF- κ B p105 (Patil et

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al., 2009) or may inhibit phosphorylation of p105 mediated by Toll-like receptor-4-induced I κ B kinase β (Parameswaran et al., 2006). As a functional consequence, the endotoxemia-enhanced levels of cytokines and chemokines are largely diminished in GRK5 knockout mice (Patial et al., 2011b). A follow-up study further demonstrates that GRK5 is necessary for regulating the NF- κ B pathway in cardiomyocytes (Islam et al., 2013). GRK2 was identified as a conserved regulator of NF- κ B signaling by genome-wide RNA interference in *Drosophila* cells (Valanne et al., 2010). Interaction of GRK2 with p105, suppressing NF- κ B activation, reduced the production of cytokines/chemokines (Patial et al., 2011a). On the other hand, GRK2 expression is also up-regulated by immune factors such as Toll-like receptor ligands in primary macrophages (Loniewski et al., 2008). The specific GRK isoform regulating the NF- κ B signaling could depend on the species, tissues and cells used in the experimental models. Our results corroborate these findings since AVP-induced IL-6 production in NRCFs depends on the activities of GRK2 and NF- κ B p65 signaling.

It is well-established concept that NF- κ B regulates inflammatory factors including IL-6 and is implicated in the initiation and progression of myocardial fibrosis (Kawano et al., 2006; Neves et al., 2005). NF- κ B mediates AVP-induced iNOS production and proliferation in cardiac fibroblasts (Fan et al., 2007; Fan et al., 2003; Niu et al., 2014). In the present study, AVP indeed evokes the NF- κ B signaling, evidenced by phosphorylation of the NF- κ B p65 subunit and activation of the NF- κ B luciferase reporter. Inhibition of NF- κ B signaling abolished the effects of AVP on IL-6 production, suggesting that NF- κ B signaling is responsible for AVP-induced IL-6 production.

Vasopressin receptor subtypes differ in their tissue distributions and functions. V_{1A} receptor is expressed in various tissues including vascular smooth muscle, platelets, liver and uterus. It participates in vasoconstriction, platelet aggregation, and glycogenolysis. Structurally, V_{1A} receptor 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 showed that AVP enhances cell survival via a GRK2/ β -arrestin1/ERK_{1/2}-dependent pathway (Zhu et al., 2013). The V_{1A} receptor blocker

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SR45049 abolishes AVP-induced NRCF proliferation (Chen et al., 2016) and smooth muscle cell proliferation, even though V₂ receptor is also expressed in A7R5 rat aortic smooth muscle cells (Zhang et al., 2016). Since vasopressin and oxytocin receptors 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), AVP-regulated inflammation should be distinguished between effects mediated by the V_{1A} receptor and those mediated by the oxytocin receptor. In the present study, the data further demonstrated that the V_{1A} receptor selective blocker abolished the AVP-induced IL-6 production and NF-κB signaling, suggesting that V_{1A} receptor mediates AVP-evoked inflammation in NRCFs.

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

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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.

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Footnotes

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

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2. Both Feifei Xu and Suzhen Sun are equal contributors.

Legends for Figure

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Figure 1. AVP induces IL-6 production in a time- and dose-dependent manner in neonatal rat cardiac fibroblasts. (A, B) Cells were harvested for total RNA extraction after the serum-starved cells in 6-well plates were stimulated with 1-1000 nM of AVP for 6 h or incubated with 0.1 μ M of AVP for 0-48 h. IL-6 mRNA was measured with q-PCR as described in Methods. Data are expressed as mean \pm S.E.M. of 3 separate experiments. $\$p < 0.01$ for dose-response (repeated two-way ANOVA, $n=3$), $\$p < 0.01$ for time-course (repeated two-way ANOVA, $n=3$). $*p < 0.05$, $**p < 0.01$ vs control, $n=4$; (C, D) Serum-starved cells in 12-well plates were stimulated with 0.1 μ M of AVP for 0-48 h or 0.1-1000 nM of AVP for 24 h. The supernatants were collected for measurement of IL-6 as described in Methods. $\$p < 0.01$ for time-course (repeated two-way ANOVA, $n=4$), $\$p < 0.01$ for dose-response repeated two-way ANOVA ($n=3$).

Figure 2. Inhibition of GRK2 by paroxetine or over-expression of GRK2-CT suppress the AVP-induced IL-6 mRNA production in adult rat cardiac fibroblast. (A, B) Serum-starved cells, pretreated with 10 μ M of paroxetine (POTH) for 1 h, were stimulated with 0.1 μ M of AVP for 0-24 h (A) or for 6 h in the presence of 1-100 μ M of AVP. IL-6 mRNA levels were assessed as described in Methods. Data are expressed as mean \pm S.E.M. of 4 separate experiments. $*P < 0.05$, $**P < 0.01$ vs control; $\#P < 0.05$, $\#\#\#P < 0.01$ vs AVP alone. It was noted that 10 μ M of POTH alone had no effect on IL-6 expression (0.89 ± 0.25 fold over control). (B) Upper panel: GRK2-ct was over-expressed as described in Methods. A representative blot shows expression of GRK2-ct protein in NRCFs. Lower panel: Effects of AVP on IL-6 mRNA levels were assessed as described in Methods. Average data are expressed as mean \pm S.E.M. of 3 separate experiments. $**P < 0.01$ vs control; $\#P < 0.05$ vs AVP alone.

Figure 3. NF- κ B signaling mediates the AVP-induced IL-6 production. (A) Serum-starved NCRF cells pretreated with 50 μ M of PDTC for 1 h were further incubated with 0.1 μ M of AVP for 0-24 h. IL-6 mRNA levels were measured using q-PCR as described in Methods. Data are expressed as mean \pm S.E.M. of 4 separate

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experiments. *P<0.05, **P<0.01 vs control; #P<0.05. ##P<0.01 vs AVP alone. (B) Serum-starved cells were stimulated with 0.1 μ M of AVP for 1-120 min. Phosphorylation of NF-kB was assessed in cell lysates by immunoblotting with anti-phosphorylated NF-kb antibody as described in Methods. Upper panel: representative immunoblot; Lower panel: Data are means from 3 separate experiments. #p<0.05 for time-course (repeated two-way ANOVA, n=3). *p<0.05, **p<0.01 vs control. (C) AVP activates NF-kB in a dose- and time-dependent manner. The starved cells transfected with NF-kB luciferase reporter plasmids as described in Methods were incubated with either 0.1 μ M of AVP for 0-36 h or in the presence of 1-1000 nM of AVP for 24 h. Luciferase activation was measured as described by in Methods. Data are means from 3 separate experiments. *P<0.05, **P<0.01 vs control. (D) The cells transfected with luciferase were incubated with 0.1 M of AVP in the presence or absence of PDTC for 24 h. AVP-induced NF-kB activation was assessed as described in Methods. Data are means from 3 separate experiments. **P<0.01 vs control, ##P<0.01 vs AVP alone.

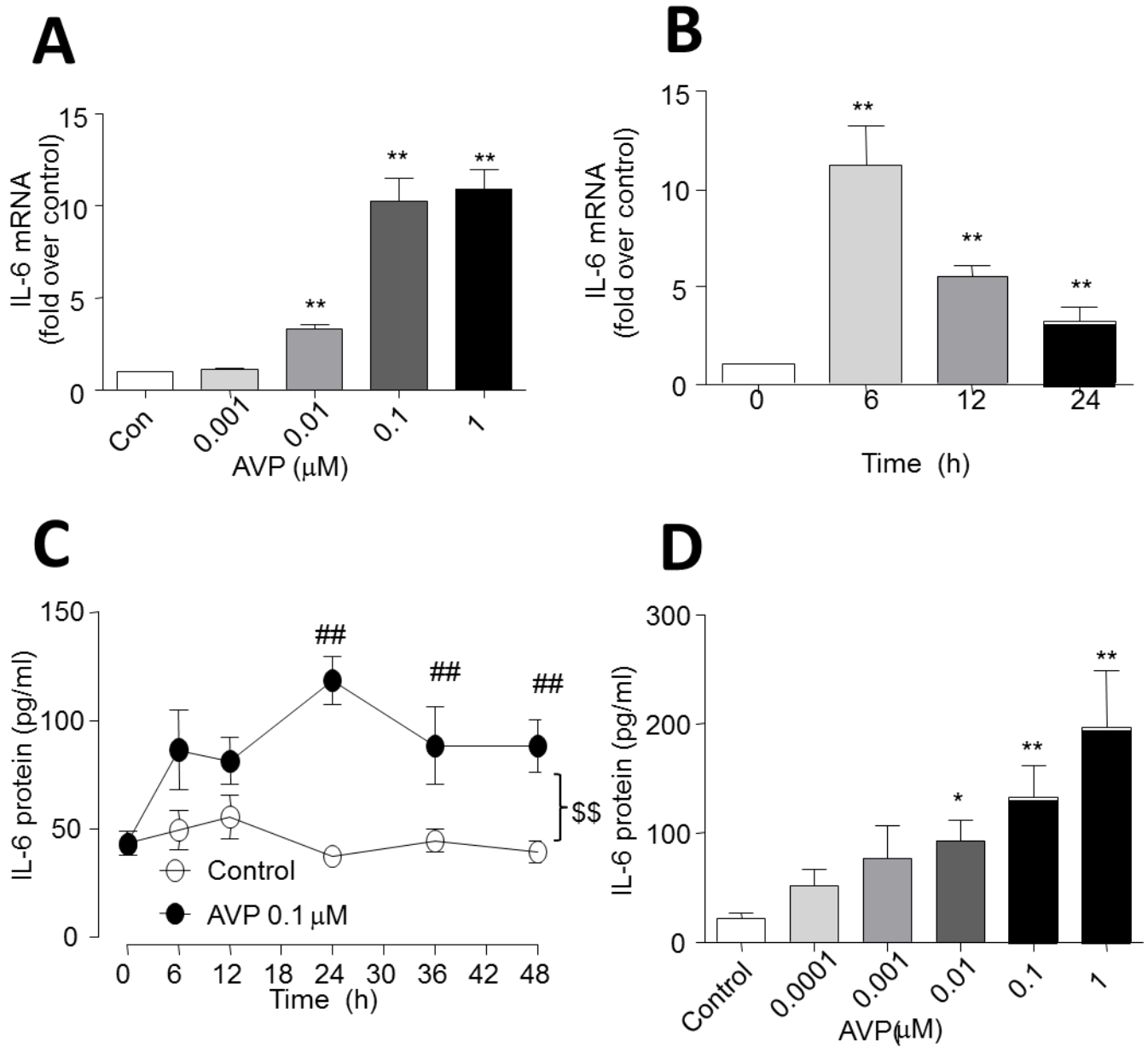
Figure 4. GRK2 is necessary for AVP-evoked NF-kB signaling. (A) Cells were transfected with plasmids containing NF-kB luciferase for 6 h, then serum-starved overnight, pre-incubated with and without 10 μ M POTH, and then stimulated with 1 μ M AVP for 24 h. Luciferase activation was assessed in cellular lysates as described in Methods. (B) AVP-induced IL-6 expression was assessed in cells with and without over-expression of GRK1-ct. Data are expressed as mean \pm S.E.M. of 4 separate experiments. **p<0.01 vs control, ##p<0.01 vs AVP alone. (C) Effects of POTH on phosphorylation of NF-kB p65 induced by AVP incubation. Upper panel: A representative immunoblot; Lower panel: Data are expressed as mean \pm S.E.M. of 3 separate experiments. **P<0.01 vs control, ##P<0.01 vs AVP alone.

Figure 5. V_{1A} receptor mediates AVP-induced NF-kB activation and IL-6 production. (A) Serum-starved NCRF cells were pretreated with 1-1000 μ M of V_{1A} receptor blocker SR45059 for 1 h, and then further stimulated with 0.1 μ M of AVP for 6 h. (A) IL-6 production was assessed as described in methods. (B) NF-kB

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activity was assessed as described in Methods. Data represent means from 3-6 separate experiments, **p<0.01 vs control, ##P<0.01 vs AVP alone. It was noted that 1 μ M of SR 49059 had no effects on the basal level of either IL-6 expression or NF- κ B luciferase activation.

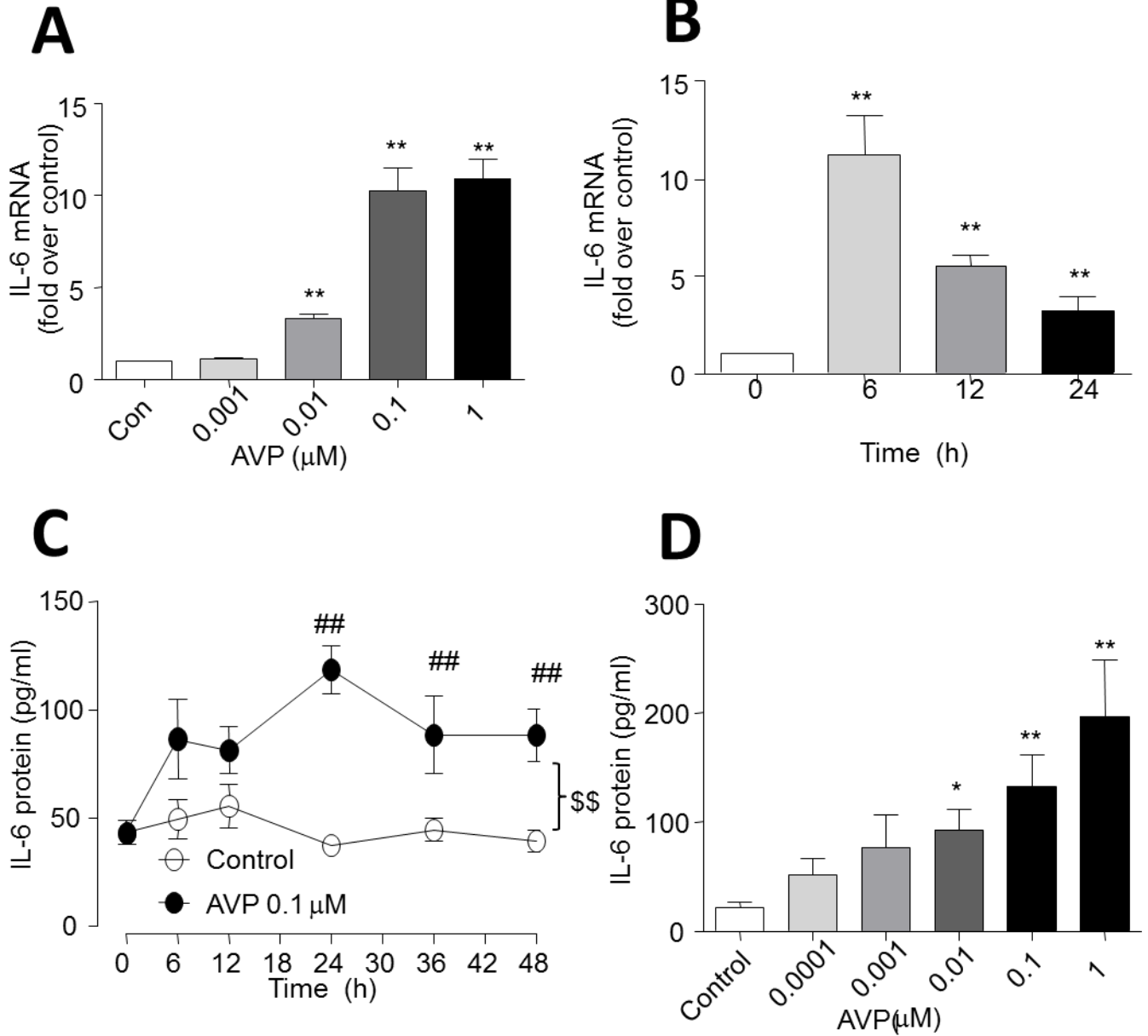
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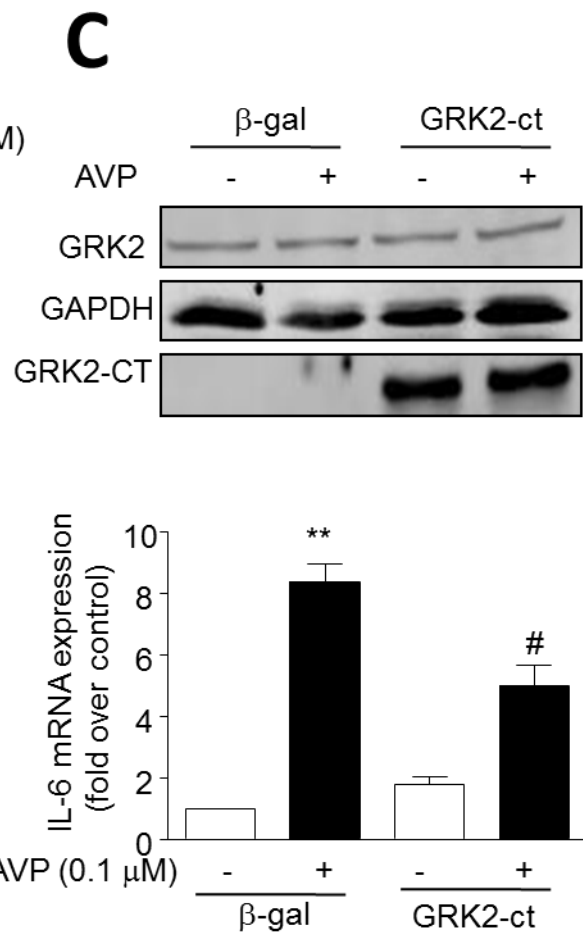
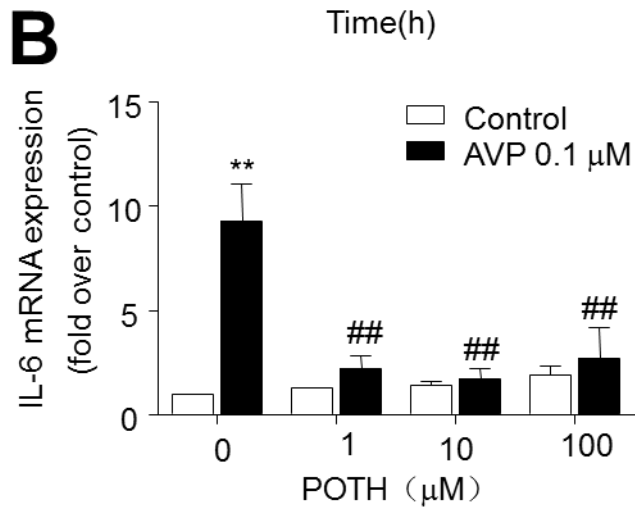
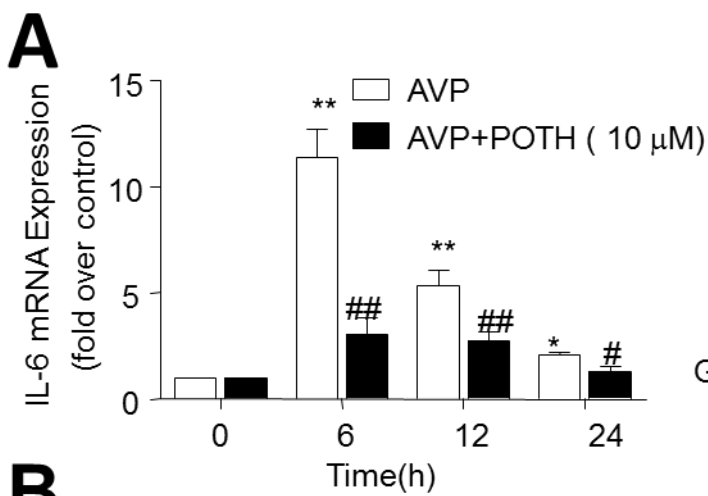
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Figure 1

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Figure 2

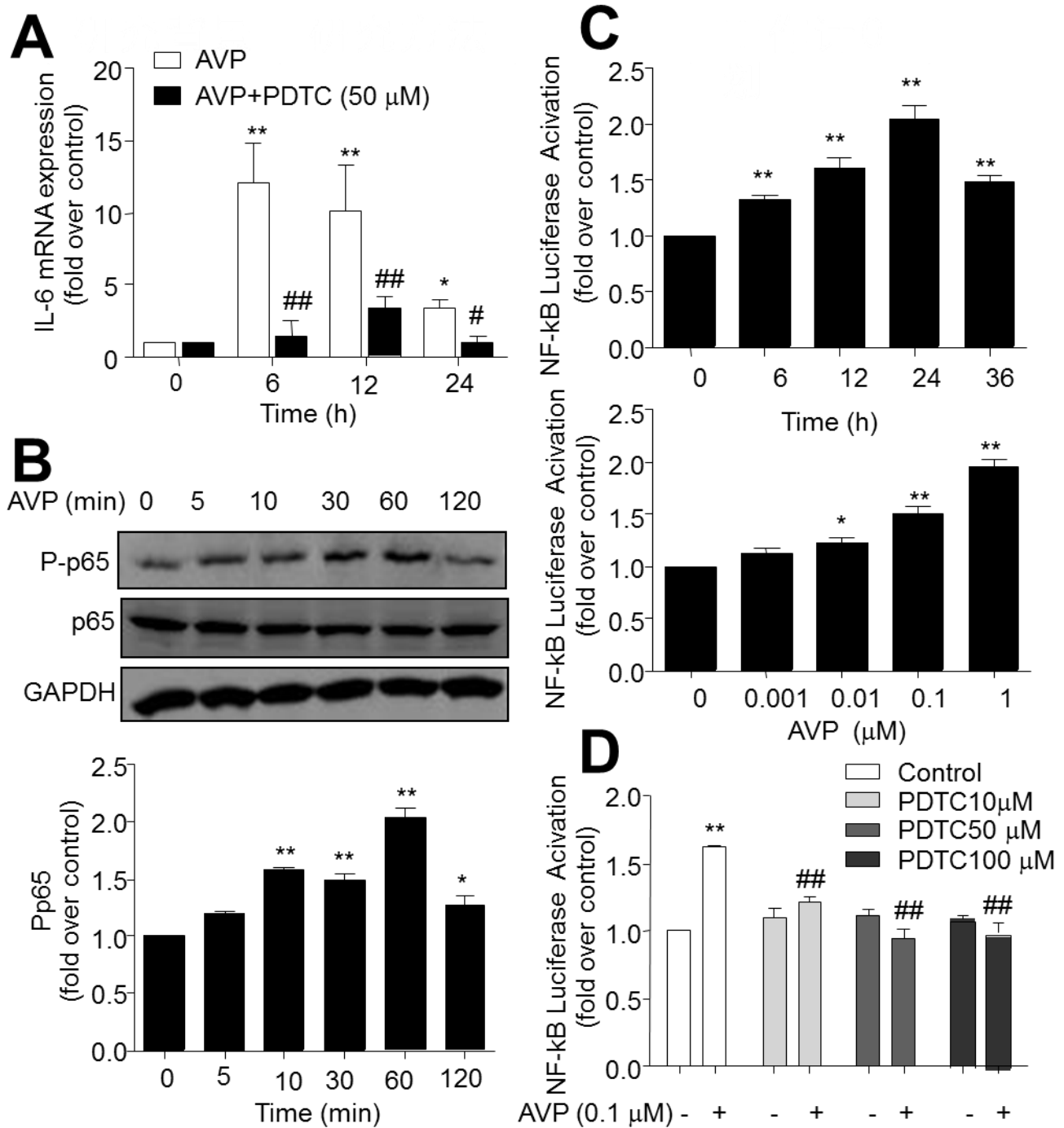


Figure 3

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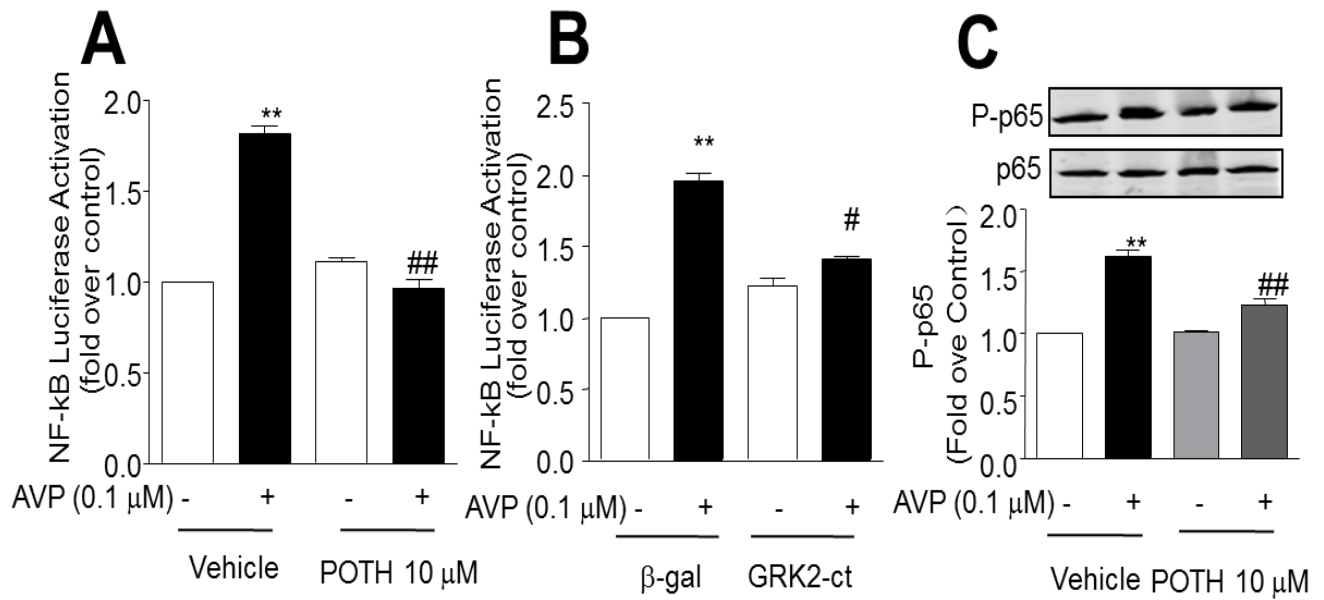


Figure 4

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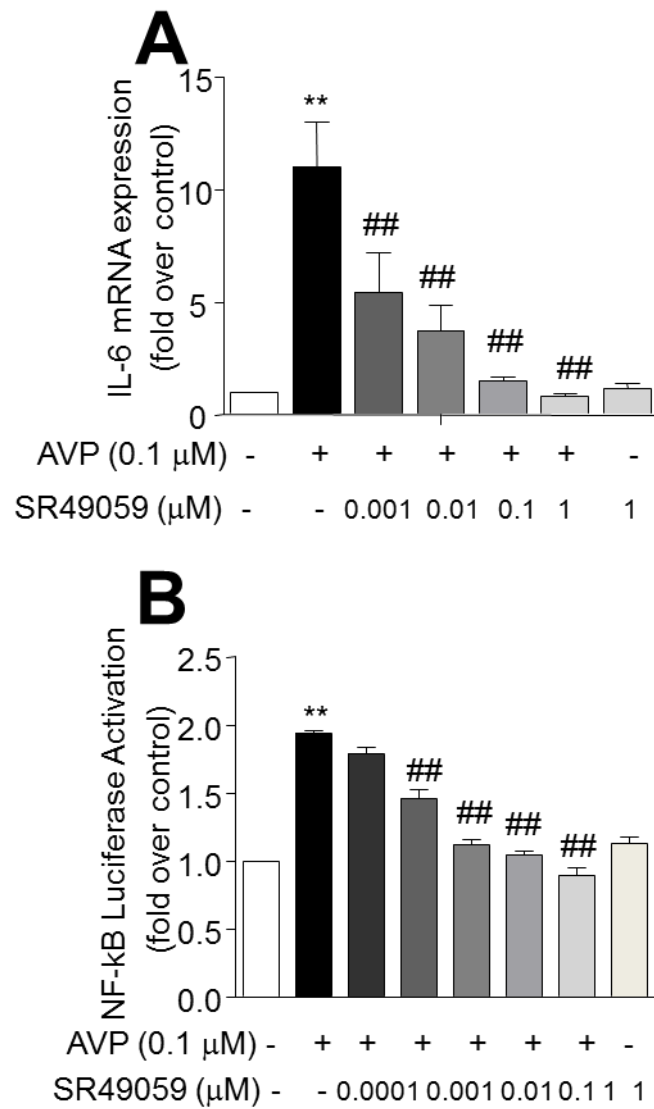


Figure 5