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

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**Running title:** GRK2 mediates IL-6 production

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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;  $\beta$ -gal,  $\beta$ -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- $\kappa$ B, Nuclear factor kappa-B; IL-6, interleukin6; IC<sub>50</sub>, 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- $\kappa$ 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- $\kappa$ B activation; and 3) pharmacological blockade of NF- $\kappa$ 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- $\kappa$ 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 $\alpha$ ), 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<sub>1A</sub>R, V<sub>2</sub>R and V<sub>1B</sub>R, also termed V<sub>3</sub>R) 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<sub>1A</sub>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<sub>1A</sub>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<sub>q</sub> protein-dependent signaling (Li et al., 2011).

While the Gα<sub>q</sub>-coupled V<sub>1A</sub>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<sub>1/2</sub>) 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<sub>1/2</sub>-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<sub>1A</sub>R selective

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antagonist, SR49059, was from Tocris Bioscience (Cat#2310, Ellisville, MO). Paroxetine hydrochloride (Cat#1500218) was purchased from ABCam (Cambridge MA). The adenovirus containing  $\beta$ -galactosidase ( $\beta$ -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  $\beta$ -galactosidase ( $\beta$ -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 ( $250 \times 10^3$  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  $\mu$ g of NF-kB luciferase plasmids and 0.5  $\mu$ g of Renilla luciferase plasmids. For basal activity, cells were transfected with the NF-kB-luciferase plasmid (0.5 $\mu$ g) and a Renilla control vector (0.5  $\mu$ 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<sub>1A</sub>R, V<sub>1B</sub>R, V<sub>2</sub>R 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 (1×10<sup>6</sup> 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 Na<sub>3</sub>VO<sub>4</sub>).

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<sup>®</sup> 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- $\kappa$ B signaling mediates the AVP-induced IL-6 production***

To determine whether NF- $\kappa$ B signaling is necessary for AVP-induced IL-6 production, cells were pretreated with 50 $\mu$ M PDTC, an NF- $\kappa$ 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- $\kappa$ B (Fig. 3C). The activation of NF- $\kappa$ B was abolished by PDTC (Fig. 3D). Together, these results suggest that AVP-induced IL-6 production is mediated through NF- $\kappa$ B signaling in NRCFs.

### ***GRK2 is necessary for AVP-evoked NF- $\kappa$ B signaling***

To define the causal relationship between GRK2 and NF- $\kappa$ 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- $\kappa$ 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- $\kappa$ B p65 as shown in Fig 4C.

### ***V<sub>1A</sub>R mediates AVP-induced IL-6 production and NF- $\kappa$ B activation***

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

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Fig.5B). It was noted that 1  $\mu$ 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  $\beta_2$ 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  $\beta$ -arrestins in a G protein-independent manner (Tilley, 2011). Increasingly, non-traditional functions of GRKs and  $\beta$ -arrestins have been discovered. For example, GRK2,  $\beta$ -arrestin 1 and  $\beta$ -arrestin 2 have been shown to mediate protective  $\beta_1$ -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- $\alpha$  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- $\kappa$ B signaling pathway. GRK5 may directly interact with NF- $\kappa$ B p105 (Patil et

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al., 2009) or may inhibit phosphorylation of p105 mediated by Toll-like receptor-4-induced I $\kappa$ B kinase $\beta$  (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- $\kappa$ B pathway in cardiomyocytes (Islam et al., 2013). GRK2 was identified as a conserved regulator of NF- $\kappa$ B signaling by genome-wide RNA interference in *Drosophila* cells (Valanne et al., 2010). Interaction of GRK2 with p105, suppressing NF- $\kappa$ 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- $\kappa$ 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- $\kappa$ B p65 signaling.

It is well-established concept that NF- $\kappa$ 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- $\kappa$ 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- $\kappa$ B signaling, evidenced by phosphorylation of the NF- $\kappa$ B p65 subunit and activation of the NF- $\kappa$ B luciferase reporter. Inhibition of NF- $\kappa$ B signaling abolished the effects of AVP on IL-6 production, suggesting that NF- $\kappa$ B signaling is responsible for AVP-induced IL-6 production.

Vasopressin receptor subtypes differ in their tissue distributions and functions. V<sub>1A</sub> receptor is expressed in various tissues including vascular smooth muscle, platelets, liver and uterus. It participates in vasoconstriction, platelet aggregation, and glycogenolysis. Structurally, V<sub>1A</sub> 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/ $\beta$ -arrestin1/ERK<sub>1/2</sub>-dependent pathway (Zhu et al., 2013). The V<sub>1A</sub> receptor blocker

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SR45049 abolishes AVP-induced NRCF proliferation (Chen et al., 2016) and smooth muscle cell proliferation, even though V<sub>2</sub> 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<sub>1A</sub> receptor and those mediated by the oxytocin receptor. In the present study, the data further demonstrated that the V<sub>1A</sub> receptor selective blocker abolished the AVP-induced IL-6 production and NF-κB signaling, suggesting that V<sub>1A</sub> 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<sub>1A</sub> receptor signaling. These findings provide a possible molecular mechanism for inflammation occurring in heart failure and other cardiac conditions.

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## 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

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- 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. *Journal of neuroendocrinology* **15**(11): 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**(2): 325-331.
- Carmichael MC and Kumar R (1994) Molecular biology of vasopressin receptors. *Semin Nephrol* **14**(4): 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 (2016) GRK2/beta-arrestin mediates arginine vasopressin-induced cardiac fibroblast proliferation. *Clinical and experimental pharmacology & physiology*.
- Chikanza IC and Grossman AS (1998) Hypothalamic-pituitary-mediated immunomodulation: arginine vasopressin is a neuroendocrine immune mediator. *British journal of rheumatology* **37**(2): 131-136.
- de Keyzer 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**(2-3): 215-220.
- Dorn GW, 2nd (2009) GRK mythology: G-protein receptor kinases in cardiovascular disease. *J Mol Med (Berl)* **87**(5): 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-kappaB activation and its relation with myocardial fibrosis. *Life sciences* **81**(4): 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 : [Acta physiologica Sinica]* **55**(4): 417-421.
- Ferguson SS (2007) Phosphorylation-independent attenuation of GPCR signalling. *Trends in pharmacological sciences* **28**(4): 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**(5068): 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. *Journal of pharmacological sciences* **117**(4): 213-222.
- Hawtin SR (2006) Pharmacological chaperone activity of SR49059 to functionally recover misfolded mutations of the vasopressin V1a receptor. *The Journal of biological chemistry* **281**(21): 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 : [Acta physiologica Sinica]* **60**(3): 333-340.
- 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. *Proceedings of the National Academy of Sciences of the United States of America* **92**(11): 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. *European journal of pharmacology* **559**(2-3): 89-97.
- Huang ZM, Gold JJ and Koch WJ (2012) G protein-coupled receptor kinases in normal and failing myocardium. *Front Biosci* **17**: 3047-3060.
- Islam KN, Bae JW, Gao E and Koch WJ (2013) Regulation of nuclear factor kappaB (NF-kappaB) in the nucleus of cardiomyocytes by G protein-coupled receptor kinase 5 (GRK5). *The Journal of biological chemistry* **288**(50): 35683-35689.
- Kawano S, Kubota T, Monden Y, Tsutsumi T, Inoue T, Kawamura N, Tsutsui H and Sunagawa K (2006) Blockade of NF-kappaB improves cardiac function and survival after myocardial infarction. *American journal of physiology Heart and circulatory physiology* **291**(3): H1337-1344.

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- Li X, Chan TO, Myers V, Chowdhury I, Zhang XQ, Song J, Zhang J, Andrel J, Funakoshi H, Robbins J, Koch WJ, Hyslop T, Cheung JY and Feldman AM (2011) Controlled and cardiac-restricted overexpression of the arginine vasopressin V1A receptor causes reversible left ventricular dysfunction through Galphaq-mediated cell signaling. *Circulation* **124**(5): 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 failure reviews* **15**(6): 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**(6376): 336-339.
- Loniewski K, Shi Y, Pestka J and Parameswaran N (2008) Toll-like receptors differentially regulate GPCR kinases and arrestins in primary macrophages. *Molecular immunology* **45**(8): 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 beta/Smad activation and cardiac fibrosis induced by angiotensin II. *PloS one* **7**(5): 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*). *General and comparative endocrinology* **226**: 88-101.
- Metaye T, Gibelin H, Perdrisot R and Kraimps JL (2005) Pathophysiological roles of G-protein-coupled receptor kinases. *Cell Signal* **17**(8): 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**(6369): 523-526.
- Murray DR and Freeman GL (2003) Proinflammatory cytokines: predictors of a failing heart? *Circulation* **107**(11): 1460-1462.
- Neves MF, Amiri F, Viridis A, Diep QN, Schiffrin EL and Hypertension CMRGo (2005) Role of aldosterone in angiotensin II-induced cardiac and aortic inflammation, fibrosis, and hypertrophy. *Canadian journal of physiology and pharmacology* **83**(11): 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-kappaB signaling pathway. *Journal of cardiovascular pharmacology* **64**(6): 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 and Rockman HA (2007) Beta-arrestin-mediated beta1-adrenergic receptor transactivation of the EGFR confers cardioprotection. *The Journal of clinical investigation* **117**(9): 2445-2458.
- Packiriswamy N and Parameswaran N (2015) G-protein-coupled receptor kinases in inflammation and disease. *Genes and immunity* **16**(6): 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 NFkappaB1 p105 and negatively regulate lipopolysaccharide-stimulated ERK1/2 activation in macrophages. *The Journal of biological chemistry* **281**(45): 34159-34170.
- Patial S, Luo J, Porter KJ, Benovic JL and Parameswaran N (2009) G-protein-coupled-receptor kinases mediate TNFalpha-induced NFkappaB signalling via direct interaction with and phosphorylation of IkappaBalpha. *The Biochemical journal* **425**(1): 169-178.
- Patial S, Saini Y, Parvataneni S, Appledorn DM, Dorn GW, 2nd, Lapres JJ, Amalfitano A, Senagore P and Parameswaran N (2011a) Myeloid-specific GPCR kinase-2 negatively regulates NF-kappaB1p105-ERK pathway and limits endotoxemic shock in mice. *Journal of cellular physiology* **226**(3): 627-637.
- Patial 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 NFkappaB

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- activation in primary macrophages and modulates inflammation in vivo in mice. *Journal of cellular physiology* **226**(5): 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. *Pharmacology, biochemistry, and behavior*.
- 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. *Science translational medicine* **7**(277): 277ra231.
- Taga T and Kishimoto T (1997) Gp130 and the interleukin-6 family of cytokines. *Annual review of immunology* **15**: 797-819.
- Thibonnier M, Coles P, Thibonnier A and Shoham M (2002) Molecular pharmacology and modeling of vasopressin receptors. *Progress in brain research* **139**: 179-196.
- Tilley DG (2011) G protein-dependent and G protein-independent signaling pathways and their impact on cardiac function. *Circulation research* **109**(2): 217-230.
- Tilley DG, Zhu W, Myers VD, Barr LA, Gao E, Li X, Song J, Carter RL, Makarewich CA, Yu D, Troupes CD, Grisanti LA, Coleman RC, Koch WJ, Houser SR, Cheung JY and Feldman AM (2014) beta-adrenergic receptor-mediated cardiac contractility is inhibited via vasopressin type 1A-receptor-dependent signaling. *Circulation* **130**(20): 1800-1811.
- Valanne S, Myllymaki H, Kallio J, Schmid MR, Kleino A, Murumagi A, Airaksinen L, Kotipelto T, Kaustio M, Ulvila J, Esfahani SS, Engstrom Y, Silvennoinen O, Hultmark D, Parikka M and Ramet M (2010) Genome-wide RNA interference in Drosophila cells identifies G protein-coupled receptor kinase 2 as a conserved regulator of NF-kappaB signaling. *Journal of immunology* **184**(11): 6188-6198.
- Vasan RS, Sullivan LM, Roubenoff R, Dinarello CA, Harris T, Benjamin EJ, Sawyer DB, Levy D, Wilson PW, D'Agostino RB and Framingham Heart S (2003) Inflammatory markers and risk of heart failure in elderly subjects without prior myocardial infarction: the Framingham Heart Study. *Circulation* **107**(11): 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. *Journal of molecular and cellular cardiology* **34**(10): 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-beta1 signaling pathway. *Laboratory investigation; a journal of technical methods and pathology* **96**(8): 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. *Circulation research* **119**(10): 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. *Journal of cardiovascular pharmacology* **55**(5): 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. *Journal of cardiovascular pharmacology* **52**(1): 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. *Journal of cardiovascular pharmacology* **42**(1): 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 beta2-adrenergic receptor-induced IL-6 production in neonatal mouse cardiac fibroblasts. *Journal of molecular and cellular cardiology* **40**(3): 384-393.
- Zhai P, Yamamoto M, Galeotti J, Liu J, Masarekar M, Thaisz J, Irie K, Holle E, Yu X, Kupersmidt S, Roden DM, Wagner T, Yatani A, Vatner DE, Vatner SF and Sadoshima J (2005) Cardiac-specific



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overexpression of AT1 receptor mutant lacking G alpha q/G alpha i coupling causes hypertrophy and bradycardia in transgenic mice. *J Clin Invest* **115**(11): 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. *European journal of pharmacology* **792**: 15-25.

Zhang W, Chen XF, Huang YJ, Chen QQ, Bao YJ and Zhu W (2012) 2,3,4',5-Tetrahydroxystilbene-2-O-beta-D-glucoside inhibits angiotensin II-induced cardiac fibroblast proliferation via suppression of the reactive oxygen species-extracellular signal-regulated kinase 1/2 pathway. *Clinical and experimental pharmacology & physiology* **39**(5): 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/beta-arrestin1/extracellular-regulated kinase 1/2-dependent pathway in H9c2 cells. *Molecular pharmacology* **84**(2): 227-235.

## 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  $\mu$ 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  $\mu$ 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  $\mu$ M of paroxetine (POTH) for 1 h, were stimulated with 0.1  $\mu$ M of AVP for 0-24 h (A) or for 6 h in the presence of 1-100  $\mu$ 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  $\mu$ M of POTH alone had no effect on IL-6 expression ( $0.89 \pm 0.25$  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- $\kappa$ B signaling mediates the AVP-induced IL-6 production.** (A) Serum-starved NCRF cells pretreated with 50  $\mu$ M of PDTC for 1 h were further incubated with 0.1  $\mu$ 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  $\mu$ 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  $\mu$ 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  $\mu$ M POTH, and then stimulated with 1  $\mu$ 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<sub>1A</sub> receptor mediates AVP-induced NF-kB activation and IL-6 production.** (A) Serum-starved NCRF cells were pretreated with 1-1000  $\mu$ M of V<sub>1A</sub> receptor blocker SR45059 for 1 h, and then further stimulated with 0.1  $\mu$ 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  $\mu$ M of SR 49059 had no effects on the basal level of either IL-6 expression or NF- $\kappa$ B luciferase activation.

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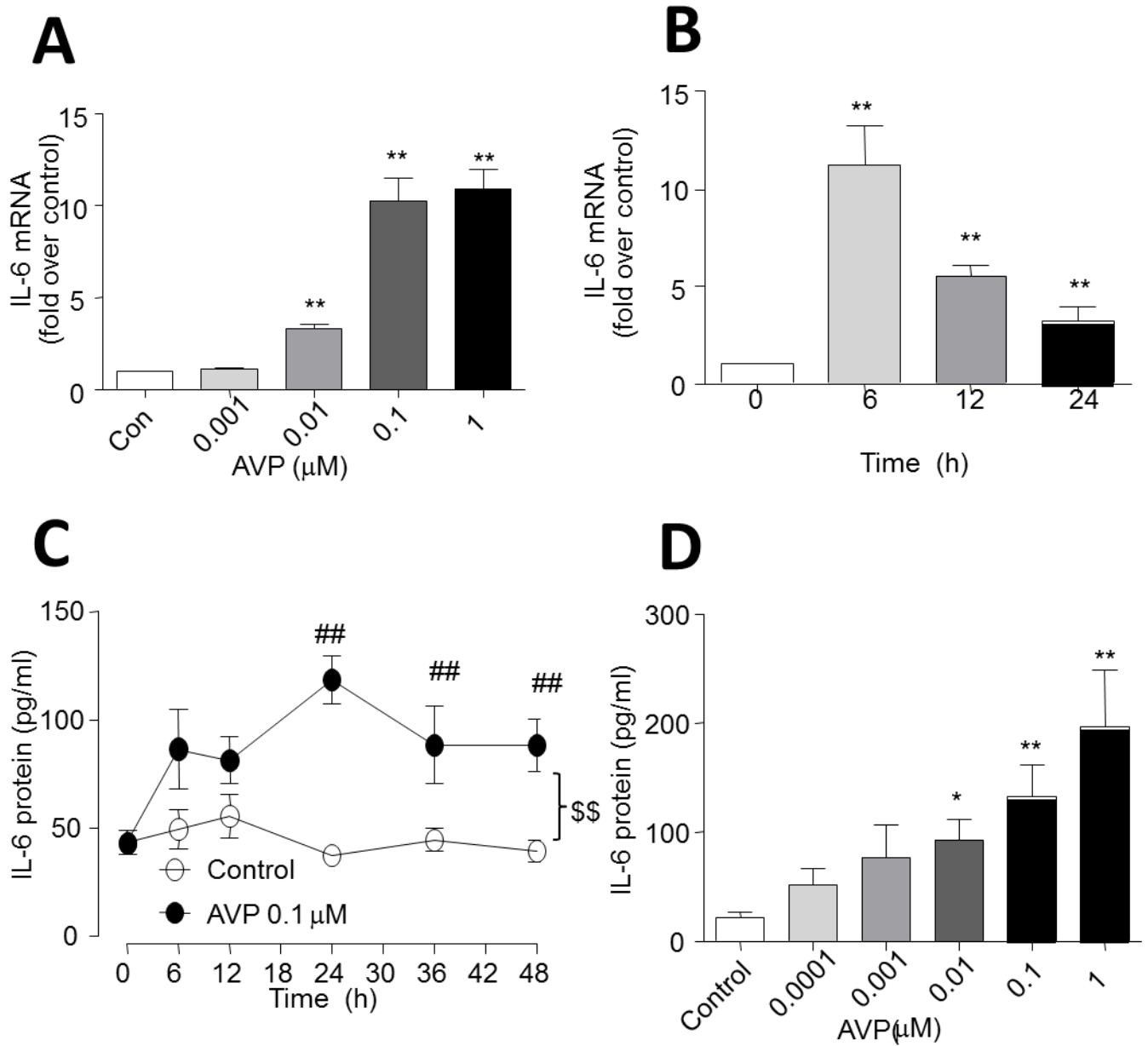
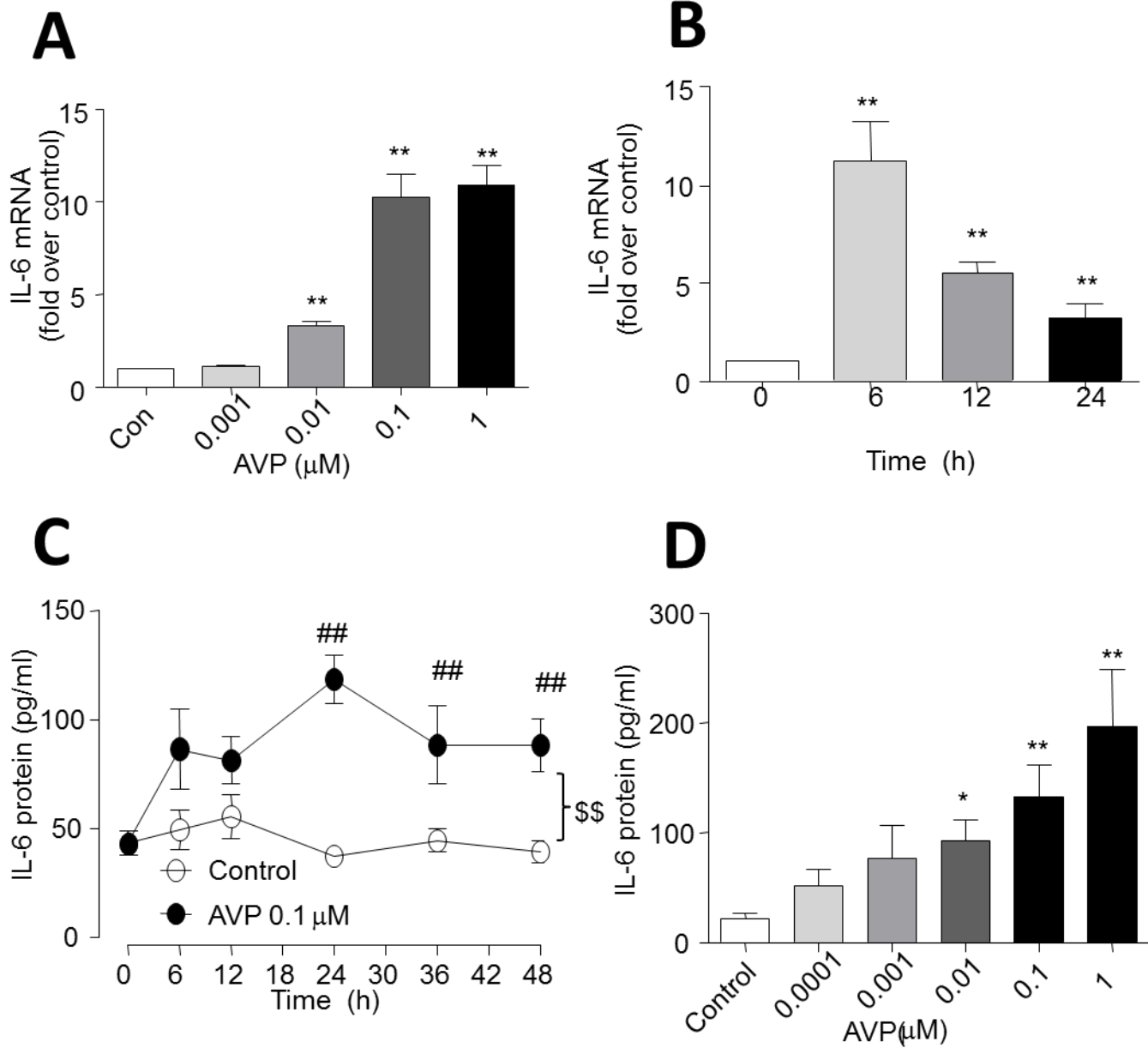
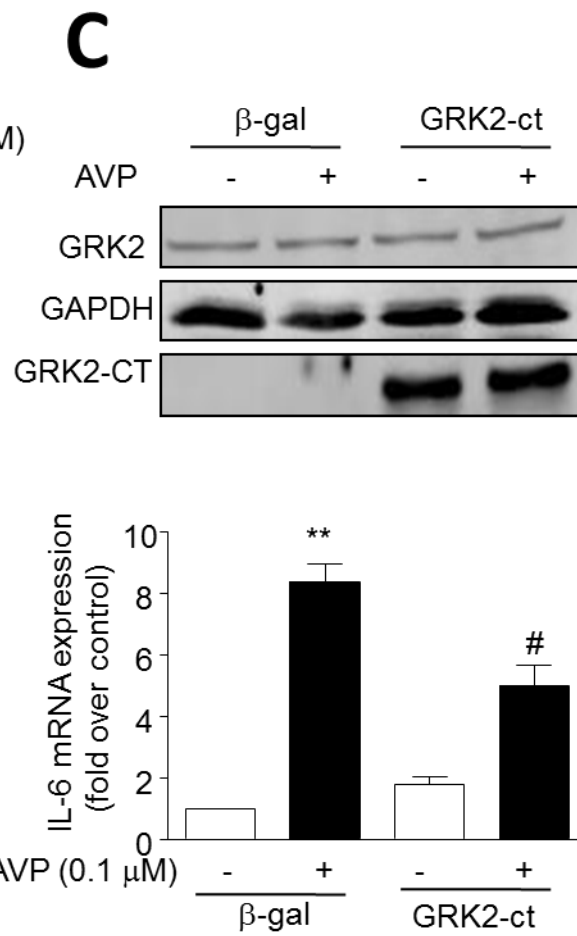
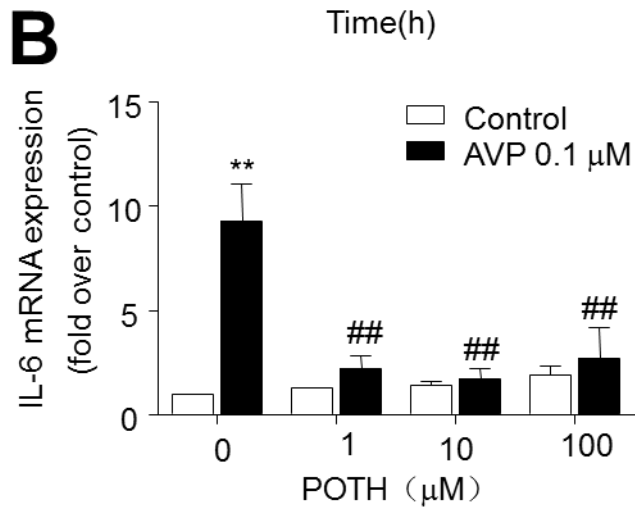
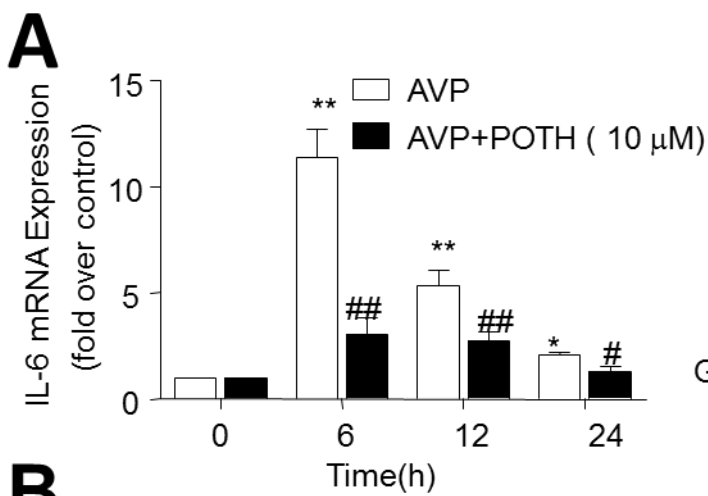


Figure 1

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Figure 2

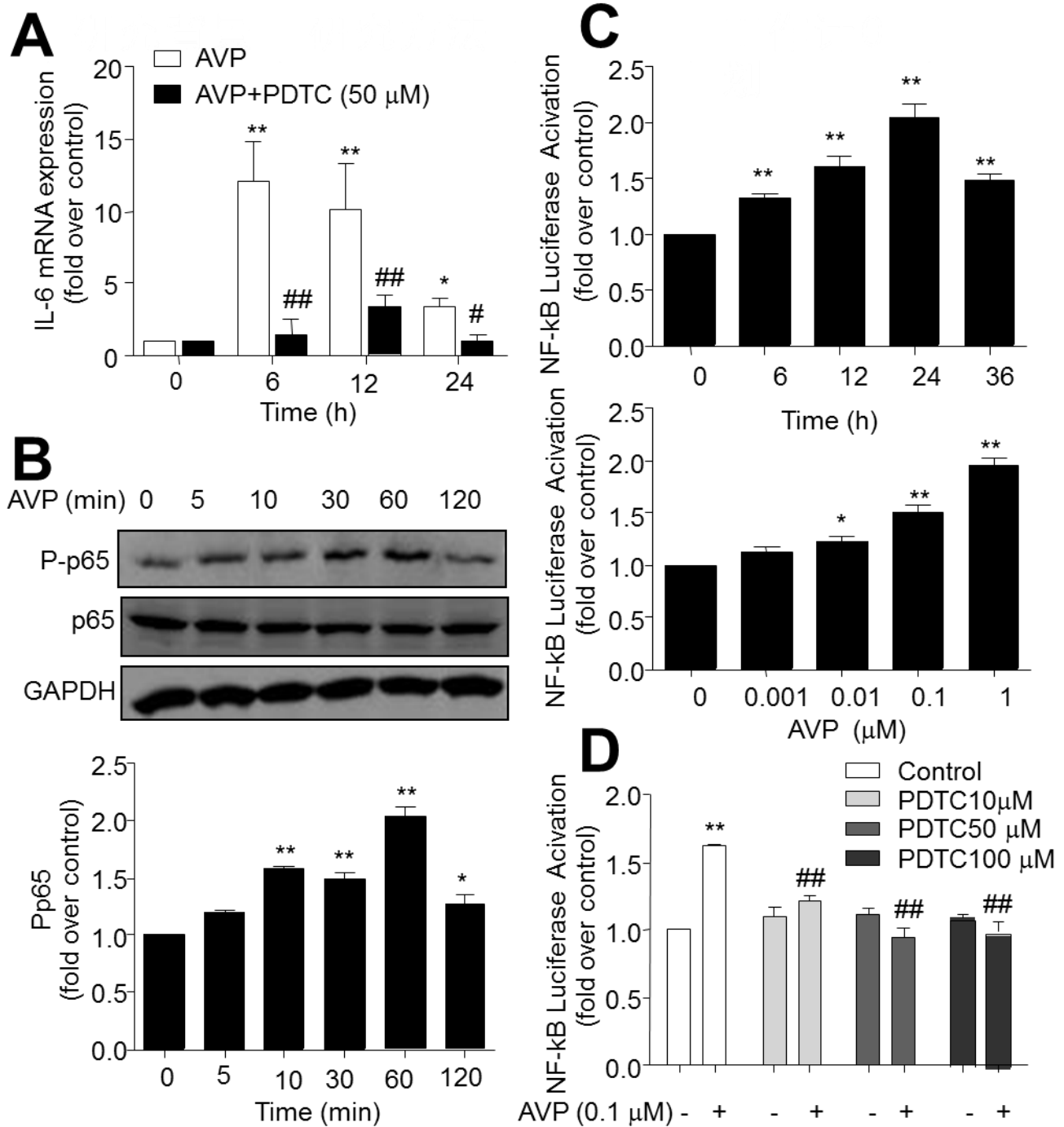


Figure 3

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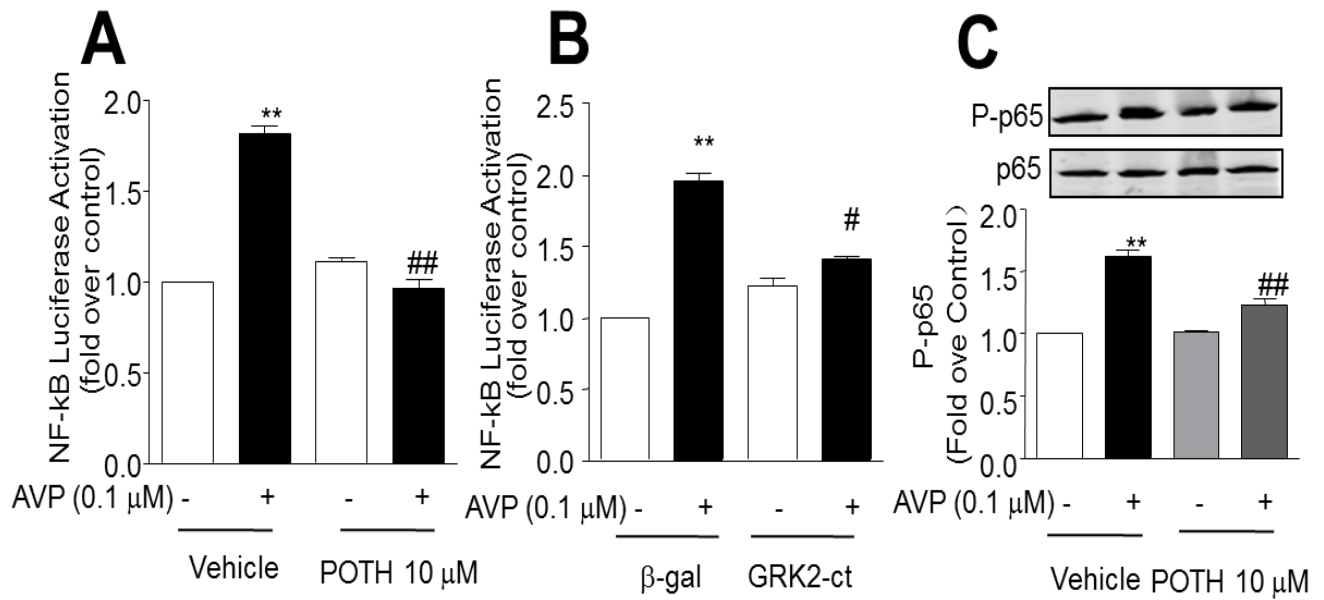


Figure 4

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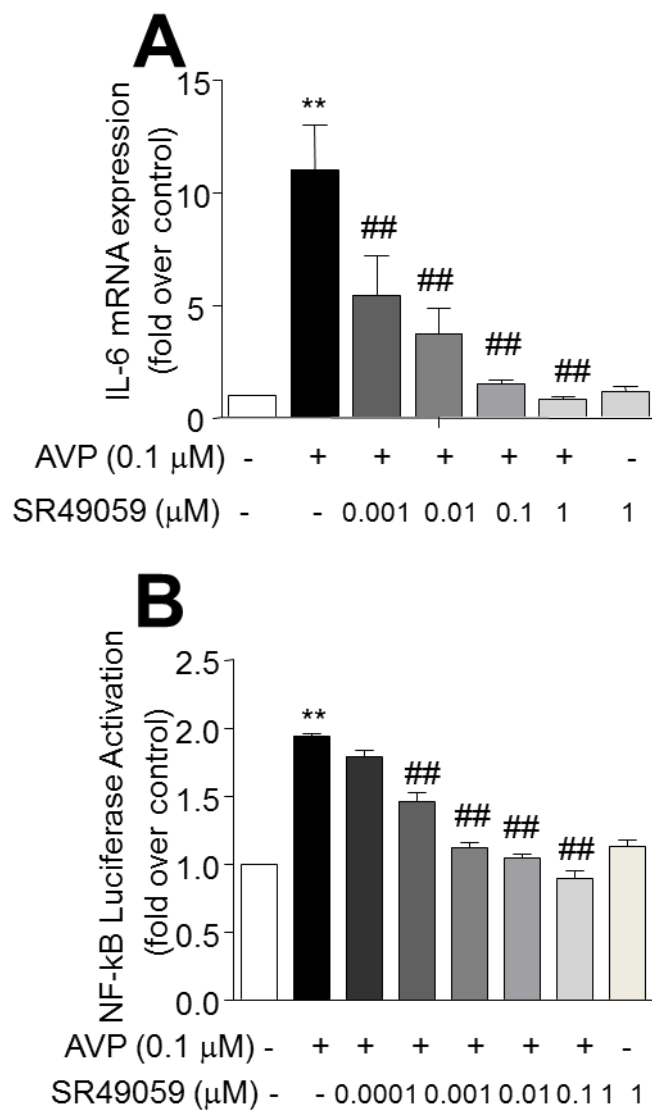


Figure 5