MOLECULAR PHARMACOLOGY IN CHINA

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

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

Laboratory of Cardiovascular Science, Department of Pharmacology, Nantong University School of Pharmacy, Nantong, China
Received November 30, 2016; accepted January 20, 2017

ABSTRACT
Interleukin 6 (IL-6), which is elevated in patients with congestive heart failure and acts as both a chronic marker of inflammation and an acute-phase reactant, is associated with myocardial damage. Circulating levels of arginine vasopressin (AVP) are elevated during cardiac stress and could be a factor for cardiac inflammation and fibrosis. Our previous study has shown that AVP promotes the proliferation of neonatal rat cardiac fibroblasts (NRCFs) through 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; Hirotu et al., 1995).

Arginine vasopressin (AVP) is secreted in response to hypovolemic or cardiac stress. It participates in physiologic processes such as osmotic regulation, vasoconstriction, and the release of 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, (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; 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 cardiac myocytes (Hiroyama et al., 2007), and either constitutive or controlled overexpression of V1aR in cardiac myocytes induces hypertrophy and dilation of the left ventricle, diminishes contractile performance of the myocardium, and reprograms the heart failure gene profile in transgenic mice; its effects are mediated primarily via Goq protein–dependent signaling (Li et al., 2011).

Although Goq-coupled V1aR typically induces protein kinase C, an activator of the myocardial hypertrophic gene programs (Li et al., 2011), it also interacts with GPCR kinase (GRK) isoforms (Tilley et al., 2014), primarily GRK2 (Chen et al., 2015, 2017; Zhang et al., 2016). Besides their well-defined roles in receptor desensitization, GRKs also activate the G protein–independent signaling pathways (Moore et al., 2007; Huang et al., 2011). These G protein–independent pathways have been attributed to the regulation of myoccardial hypertrophy and apoptosis (Métyáé et al., 2005; Dorn, 2009; Huang et al., 2011) and to the promotion of a cardio-protective 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., 2017), proliferation of rat smooth muscle (Zhang et al., 2017), proliferation of rat smooth muscle (Zhang et al., 2017), proliferation of rat smooth muscle (Zhang et al., 2017), 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, V8679) was from Sigma-Aldrich. V1aR-selective antagonist (SR49059) was from Tocris Bioscience (Minneapolis, MN), paroxetine hydrochloride (cat no. 1500218) was purchased from Albemarle, the adenosine containing β-galactosidase was a gift from Dr. Yihin Wang (University of California, Los Angeles), and the adeno-GRK2-Ct virus was obtained from Dr. Walter Koch (Temple University, Philadelphia, PA). Both NF-kB luciferase (E8491) and Renilla (E2231) were purchased from Promega (Madison, WI). Anti-P-NF-kB(S536) antibody was from Cell Signaling Technology (no. 3033; Danvers, MA); antibody for GRK2 (Sc-13143), NF-kB (Sc-109), and GAPDH (Sc-25778) were from Santa Cruz Biotechnology, Dallas, TX.

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

Transient Transfection of NF-kB Promoter Luciferase Reporter Gene and Measurement of NF-kB-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 plasms. 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, Somerville, NJ).

Quantitative PCR for Measurement of IL-6. NRCFs with a density of 1 × 10⁴ 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-dithiocarbamic acid (PTDC), 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 reaction mixture according to the recommended conditions in the Quantitect SYBR PCR kit (Qiagen, Valencia, CA) to allow for quantitative detection of the PCR product. The primer sequences for IL-6 were 5′-ACT-CCA-TCT-GCC-CTT-CA-3′ (sense) and 5′-ACT-CCA-TCT-GCC-CTT-CA-3′ (antisense); IL-6 accession no. NM_012589. The primer sequences for GAPDH were 5′-TTC-AAT-GGC-ACA-GTC-AAG-GC-3′ and 5′-TCA-CAT-TG-ACT-GTTCCC-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 for AVP with 0–10 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 (GraphPad Software, Inc., San Diego, CA). Comparison of means ± S.E.M. was conducted with one- or two-way ANOVA followed by Tukey’s post hoc comparison test.
mRNA induced by AVP stimulation. Similarly, overexpression of GRK2-ct (Fig. 2B, upper panel) efficiently suppressed the AVP-induced IL-6 mRNA production (Fig. 2B). These results suggest that GRK2 is involved in the AVP-induced IL-6 production.

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

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

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).

Discussion

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

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

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 (Packirisamy 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-κB signaling pathway in immune and nonimmune cells. It is has been shown that GRK5 directly interacts with NF-κB p105 (one of the IκB members) (Patial et al., 2009) and inhibits Toll-like receptor-4–induced IκB 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-κB 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-κB 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-κB 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-κB signaling.

It is a well established concept that NF-κB, a redox-sensitive transcription factor, is a key transcription factor that regulates inflammatory factor, including IL-6 and other cytokines. NF-κB has been implicated in the initiation and progression of pathogenesis in myocardium fibrosis (Neves et al., 2005; Kawano et al., 2006). NF-κB 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-κB signaling, evidenced by NF-κB p65 phosphorylation and activation as shown with the pharmacologic approach abolished the effects of AVP on the production of IL-6, suggesting that NF-κB 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.

**Fig. 4.** GRK2 is necessary for AVP-evoked NF-κB signaling. (A) Pretreatment of POTH abolished the AVP-evoked NF-κB activation. Cells were transfected with plasmid containing NF-κB luciferase for 6 hours; cells were starved overnight and further stimulated with 1 μM AVP for 24 hours. The cellular lysates were done for activation of luciferase expression. Data were expressed as mean ± S.E.M. of four separate experiments. **P < 0.01 versus control; # P < 0.05; ## P < 0.01 versus AVP alone. (B) Inhibition of GRK2 with overexpression of GRK2-CT abolished the AVP-induced IL-6 expression. Data were expressed as mean ± S.E.M. of three separate experiments. **P < 0.01 versus control; ## P < 0.01 versus AVP alone.

**Fig. 5.** V1A receptor mediates AVP-induced NF-κB activation and IL-6 production. (A) V1A receptor subtype-selective blockers efficiently inhibited AVP-induced IL-6 production in NRCFs. After the starved cells were pretreated with 1–1000 μM of V1A receptor blocker SR45059 for 1 hour, cells were further stimulated with 0.1 μM of AVP for 6 hours. (B) SR49059 blocked the AVP-induced NF-κB activity in dose-dependent manner. Average data were from three to six separate experiments. **P < 0.01 versus control; ***P < 0.01 versus AVP alone. It was noted that 1 μM of SR 49059 has no effects on the basal level of either IL-6 expression or NF-κB luciferase activation.
cells. Structurally, the \( V_1A \)R has a conserved DRY motif in its second intracellular loop, and its C-terminus contains multiple serine or threonine residues that can be phosphorylated by GRKs (Hawtin, 2006). Our previous study in H9c2 cells has shown that AVP enhances cell survival via a GRK2/β-arrestin-1/ERK1/2-dependent pathway (Zhu et al., 2013) and that \( V_1A \)R blocker SR45049 abolished AVP-induced NRCF proliferation (Chen et al., 2017) and smooth muscle cell proliferation, although the \( V_1A \)R also exists in A7R5 rat aorta smooth muscle (Zhang et al., 2016). Vasopressin receptor and oxytocin receptor belong to the same subfamily of the GPCR superfamily (Mayasich and Clarke, 2016), and AVP is a partial agonist of the oxytocin receptor (Ramos et al., 2016). Thus, AVP-regulated inflammation should distinguish \( V_1A \)R from oxytocin receptor. In the present study, the data further demonstrated that \( V_1A \)R-selective blocker abolished AVP-induced IL-6 production and NF-κB signaling, suggesting that AVP \( V_1A \)R mediates AVP-evoked inflammation in NRCFs.

In summary, AVP induces IL-6 production of NRCFs by activating \( V_1A \)R signaling via a GRK2/NF-κB pathway. These findings provide a possible molecular mechanism for inflammation that occurs in heart failure and other cardiac conditions.

Acknowledgments

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

Author Contributions

Participated in research design: Xu, Sun, Zhu.
Conducted experiments: Xu, Sun, Wang, Ni, Zhao.
Performed data analysis: Sun, Xu, Zhu.
Wrote or contributed to the writing of the manuscript: Xu, Sun, Zhu.

References


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