

Received: 2017.05.31
Accepted: 2017.09.20
Published: 2018.05.13

Brain Natriuretic Peptide-Regulated Expression of Inflammatory Cytokines in Lipopolysaccharide (LPS)-Activated Macrophages via NF- κ B and Mitogen Activated Protein Kinase (MAPK) Pathways

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Data Interpretation D
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Source of support: Departmental sources

Background: This study aimed to investigate the effects of recombinant human brain natriuretic peptide (rhBNP) on IL-6, TNF- α , and IL-10 secretion in LPS-activated RAW 264.7 cells and human peripheral blood mononuclear cells (PBMCs) *in vitro* and to explore the related signaling pathways of the regulation mechanisms of BNP in systemic inflammatory response syndrome (SIRS).





Material/Methods: MTT assay was used to evaluate the effects of rhBNP on cell viabilities. Lipopolysaccharide (LPS) was used to induce inflammation response. The whole study was divided into 8 groups: Control, low, middle, and high concentrations of rhBNP, LPS, LPS with low, middle, and high concentrations of rhBNP. Levels of IL-6, TNF- α , and IL-10 were evaluated using the Cytometric Bead Array Kit and RT-PCR assay. Western blotting was used to test the effects of rhBNP on inflammation-related NF- κ B and MAPK pathways.

Results: Except for the concentrations ≥ 1.6 ng/mL, all concentrations of rhBNP showed little effect on cell viabilities of RAW264.7 cells and PBMCs after 24 h and 48 h, suggesting a weak cytotoxicity to cells. Expression of IL-6 and TNF- α significantly increased and expression of IL-10 significantly decreased at protein and mRNA levels after LPS treatment, and these effects were strongly inhibited in a dose-dependent manner by pretreatment of rhBNP. Similarly, the LPS-induced increase of NF- κ B and MAPK pathway phosphorylation levels were also significantly inhibited by rhBNP.

Conclusions: rhBNP can regulate expression of IL-6, TNF- α , and IL-10 in LPS-activated RAW 264.7 cells and PBMCs through inhibiting NF- κ B and MAPK pathways. These results may reveal potential causes of the increase of BNP in SIRS and may provide an experimental basis for treatment of SIRS.

MeSH Keywords: **Macrophage Activation • Natriuretic Peptide, Brain • Systemic Inflammatory Response Syndrome**

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Background

Brain natriuretic peptide (BNP), a family member of the natriuretic peptides (NPs), which also includes atrial natriuretic peptide (ANP) and C-type natriuretic peptide (CNP), has been demonstrated to be involved in several biological processes [1]. In past years, bio-functions of BNP have been gradually recognized, mainly in the cardiovascular system, such as regulating heart and renal homeostasis, as well as modulating vascular cell functions [2]. There have also been studies focusing on the effect of BNP on regulation of inflammatory mediators [3]. Our previous study in a mouse model of burn-caused systemic inflammatory response syndrome (SIRS) showed that the BNP level was significantly increased in plasma and mononuclear cells of the mouse model. Our previous study also demonstrated that treatment of RAW264.7 cells with burn serum or BNP could induce the expression of IL-12, TNF- α , and CRP secretin, and inhibit the HSF-1 signaling pathway. Silencing the BNP receptor can reverse the effect of burn serum on RAW264.7 cells [4]. All the above results indicate that SIRS following burns might be mediated by BNP, and BNP might be associated with the occurrence, development, and regulation of SIRS. However, the details of the role of BNP in SIRS are still unclear.

SIRS, which is frequently observed in hospitals and can progress rapidly to life-threatening conditions, is a fundamental host response common to traumatic or focal injury, or inflammatory challenges such as traumatic injury to the brain and spinal cord, sepsis, or burns [5,6]. Even being sterile or under control of the inciting infection, SIRS can lead to organ dysfunction induced by inflammatory cells from the circulation of invaded organs [7]. However, the mechanisms of SIRS are still incompletely understood. SIRS is considered to be a systemic immune response which is a complex pathological reaction mediated by many inflammatory mediators and immune-related cells [8]. Macrophages are primary mediators of the innate immune response to injury and infections, and can mediate the innate immune response through production of pro-inflammatory cytokines, including interleukin 6 (IL-6) and tumor necrosis factor α (TNF- α), and inhibition of anti-inflammatory cytokines such as IL-10 [9] [10,11]. Macrophages play important roles in the development of SIRS [12,13]. Studies showed that pro-inflammatory mediators, including cytokines, arachidonic acid derivatives, activated oxygen species, and proteases, can be released locally by over-activated neutrophils and monocytes/macrophages, which can further participate in development of SIRS [14,15].

Recombinant human brain natriuretic peptide (rhBNP), a man-made peptide made by gene engineering, is widely used in treatment of uncompensated heart failure, guiding fluid therapy, and so on [16]. Studies have proved that rhBNP has multiple functions in inflammatory reaction. It can increase the production of nitric oxide (NO), which regulates the inflammatory factors [17] and

inhibit oxidative stress and the nuclear factor kappa-B (NF- κ B)-dependent inflammatory/MMP-9 pathway [16], as well as reduce intestinal and acute lung injury in canine models of sepsis [18].

In the present study, we used RAW264.7 cells from a mouse macrophage cell line and human peripheral blood mononuclear cells to study the effects of rhBNP on inflammation in macrophages *in vitro* based on our previous studies. We also investigated the mechanisms of the increase of BNP in serum and mononuclear macrophages in development of systemic inflammation and tried to provide a clinical basis for control of SIRS.

Material and Methods

Cell culture and treatment

RAW264.7 cells were obtained from a mouse macrophage cell line (ATCC[®] TIB-71[™], Manassas, VA, USA), and human peripheral blood mononuclear cells were purchased from ATCC (ATCC[®] PCS-800-011[™], Manassas, VA, USA). Briefly, cells were cultured in RPMI Medium 1640 (Thermo Fisher Scientific, USA) supplemented with 10% Gibco[®] fetal bovine serum (Thermo Fisher Scientific, USA) and 100 μ g/mL penicillin-streptomycin (Sigma-Aldrich Co, USA). Cells were pretreated with different concentrations of rhBNP (0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 ng/mL) or were pretreated with 15 μ M NF- κ B pathway inhibitor BAY-11-7082 (Sigma-Aldrich, USA) or 10 μ M MAPK pathway inhibitor SP600125 (Sigma-Aldrich, USA) for 0.5 h.

Normal MTT assay [19] was used to evaluate the cell viabilities after rhBNP treatment for 24 h and 48 h to determine the 3 best concentrations (low, middle, and high). Briefly, different groups of cells were seeded (3×10^3 cells/well) in 96-stripwell plates and were cultured for 24 h or 48 h. Then, 25 μ l MTT solution (5 mg/mL) was added to each well and incubated at 37°C for 4 h. Subsequently, the supernatant was replaced with 180 μ l DMSO and the absorbance (490 nm) was evaluated by a SYNERGY-HT multiwell plate reader (Synergy-HT, Bio-Tek Instruments, Winooski, VT, USA).

Then, cells were pretreated with rhBNP under different concentrations, followed by addition of lipopolysaccharide (LPS) to induce inflammation response. The whole study was divided into 8 groups: control, low concentration of rhBNP, middle concentration of rhBNP, high concentration of rhBNP, LPS, LPS with low concentration of rhBNP, LPS with middle concentration of rhBNP, and LPS with high concentration of rhBNP.

Evaluation of inflammatory factors

Inflammatory-related factors, including IL-6, TNF- α , and IL-10, were evaluated using Cytometric Bead Array (CBA) method

by using the Human Inflammatory Cytokines CBA Kit (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. A BD™ FACS Calibur flow cytometer was used to collect data, and the results were analyzed using CBA analysis software (BD Bioscience-Pharmingen).

RT-PCR

RT-PCR assay was used to determine the expression of IL-6, TNF- α , and IL-10. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control. The RNA extraction and reverse-transcription were performed using Izadpanah et al. [20] as a reference. Real-time PCR was conducted in an ABI7500 real-time PCR instrument (Applied Biosystems) with the SYBR Premix Ex Taq (TaKaRa). Primers used in the present study were: IL-6, forward 5'-AAATCCCTCCTCGCCAATCT-3', reverse 5'-CCCTCAGGTCTTCTCCATAAA-3'; TNF- α , forward 5'-TATGGCTCAGGTCCAAC-3', reverse 5'-ACGGCTTCCCTACTTACACA-3'; IL-10, forward 5'-ACCTGCTCCACTGCCTTGCT-3', reverse 5'-GGTTGCCAAGCCTTATCGGA-3'; GAPDH, forward 5'-TGGCTCCAAGGAGTAAGAAAC-3', reverse 5'-GGCTCTCTTGTCTCAGTATC-3'.

Western blotting

Western blotting was used to test the expression of NF- κ B, p-NF- κ B, and MAPK pathway factors ERK, p-ERK1/2, JNK, p-JNK, P38, and p-P38. β -Tubulin was used as a control. Samples were extracted from the cells following standard protocols as described elsewhere [16] and were loaded on SDS-PAGE and transferred to PVDF membranes, using the iBlot Dry Blotting System (Invitrogen, Italy). The membranes were then incubated with a primary antibody following a conjugated secondary antibody. The films were scanned using the EasySee Western Blot Kit (Beijing TransGen Biotech, Beijing, China). Antibodies used in Western blotting were purchased from Cell Signaling Technology, Inc, USA.

Statistical analysis

Measurement data are expressed as mean \pm SD. Comparison between 2 groups was performed using the *t* test. Comparisons among 3 or more groups were conducted using one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. Differences were considered to be statistically significant when *P*-values were less than 0.05. All calculations were made using SPSS 18.0.

Results

Effects of different concentrations of rhBNP on cell viabilities of RAW264.7 cells and PBMCs

To investigate the effects of rhBNP on cell viabilities and determine the best concentrations for further studies, MTT assay was performed 24 h and 48 h after addition of rhBNP, respectively. As shown in Figure 1, except for the concentrations of ≥ 1.6 ng/mL, cell viabilities were more than 80% after 24 h and 48 h at all concentrations, suggesting that rhBNP had weak toxicity to RAW264.7 cells and PBMCs. According to this result, concentrations of 0.1, 0.2, and 0.4 ng/mL were selected to be the low, middle, and high concentrations for RAW264.7 cells, and concentrations of 0.2, 0.4, and 0.8 ng/mL were selected as the low, middle, and high concentrations for PBMCs in the subsequent studies.

Effects of rhBNP on LPS-induced inflammatory factors

Inflammation response was induced by LPS, and effects of rhBNP on the inflammation response were studied by evaluating the protein levels of IL-6, TNF- α , and IL-10 by the CBA method, and the expression of their mRNAs was determined using RT-PCR. Results showed that in both of these cell lines, when treated with LPS, levels of both IL-6 and TNF- α significantly increased, as well as the mRNA levels compared with the blank control, $P < 0.05$ (Figures 2, 3). Expression of IL-10 significantly decreased at protein levels after treatment with LPS compared with the blank control ($P < 0.05$). The ratio of TNF- α /IL-10 also significantly increased after LPS treatment ($P < 0.05$). However, when cells were pretreated with rhBNP under all concentrations, the above effects induced by LPS were all significantly reversed at both protein and mRNA levels in a dose-dependent manner ($P < 0.05$), which indicated that rhBNP might moderate the production of inflammatory factors.

Effects of rhBNP on NF- κ B and MAPK pathways

To further study the possible mechanisms of the inhibitory effect of rhBNP, we assessed the levels of NF- κ B and MAPK pathway-related proteins ERK1/2, p-ERK1/2, JNK, p-JNK, P38, and p-P38 in the 2 cell lines through Western blotting assay. As shown in Figure 4, after treatment with LPS, a significant up-regulating effect was observed in levels of p-NF- κ B, p-ERK1/2, p-JNK, and p-P38 compared with the blank control ($P < 0.05$), indicating the successful induction of inflammation. However, when pretreated with rhBNP, all of these increased proteins were significantly down-regulated compared with the LPS group, ($P < 0.05$). Interestingly, among all proteins, the inhibition effect of rhBNP on p-ERK was the weakest. These results suggest that the inhibitory effects of rhBNP on inflammation may be mainly through the inhibition of p-NF- κ B, p-JNK, and p-P38.

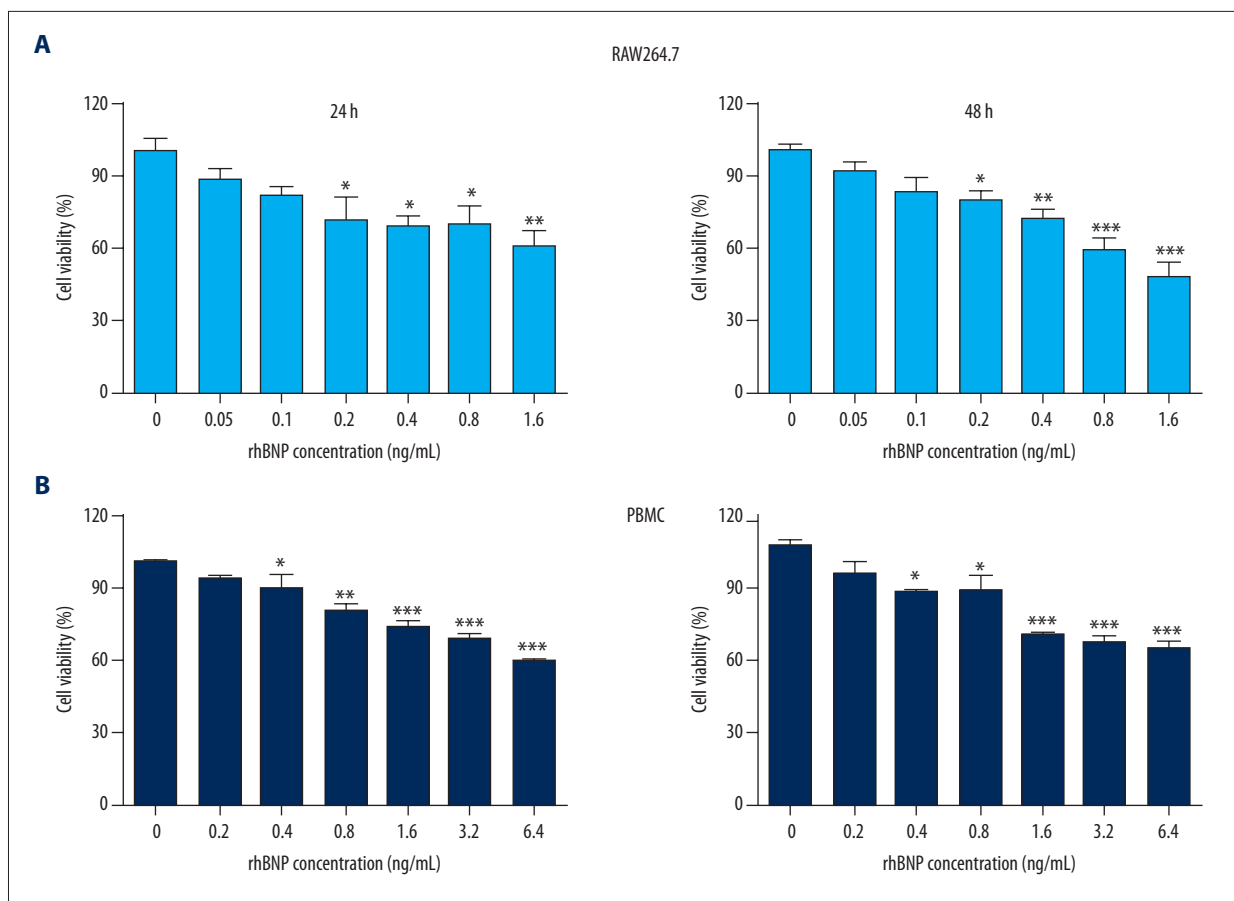


Figure 1. Cell viabilities by MTT assay for RAW 264.7 cells and PBMCs after rhBNP treatment for 24 h and 48 h. Cells were treated with rhBNP of different concentrations (0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 ng/mL). (A) Effects of different concentrations of rhBNP on viabilities of RAW 264.7 cells after 24 h and 48 h; (B) Effects of different concentrations of rhBNP on viabilities of PBMCs after 24 h and 48 h. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ versus the control group.

Effects of rhBNP on mRNA levels of IL-6 and TNF- α when treated with signaling pathway inhibitors

Two inhibitors, the NF- κ B pathway inhibitor BAY-11-7082 and MAPK pathway inhibitor SP600125, were used to pretreat RAW264.7 cells, followed by addition of LPS to further confirm the mechanism of rhBNP on inflammation. When treated with 15 μ M BAY-11-7082 or 10 μ M SP600125, levels of IL-6 and TNF- α significantly were decreased compared with the LPS group, as shown in Figure 5, indicating that NF- κ B and MAPK pathways play key roles in LPS-induced inflammation. This result further confirms that inhibitory effects of rhBNP on LPS-induced inflammation occurred through inhibition of the NF- κ B and MAPK pathways.

Discussion

Studies have demonstrated the correlation between BNP and inflammation: BNP was up-regulated in serum of patients with

sepsis and burn-induced SIRS [4,21–23] and can regulate the production of inflammatory mediators in human THP-1 macrophages [3]. However, a deeper understanding of these effects is still lacking. As an artificial product, rhBNP also showed functions on inflammation as well as its protective effects on organ injuries in diseases like lung fibroblasts [24], acute renal injury [25], acute lung injury [26], and heart failure [27–29]. However, there have been few *in vitro* studies on rhBNP in systemic inflammatory response syndrome.

In our previous study, we found that in a SIRS mouse model, the BNP level was significantly increased in plasma and mononuclear cells. We also found that treatment of RAW264.7 cells with burn serum or BNP induced the expression of IL-12, TNF- α , and CRP secretin, and inhibited the expression of HSF-1. Moreover, silencing of BNP receptor inhibited the effect of burn serum on RAW264.7 cells [4]. Thus, we hypothesized that BNP might play an important role in development of SIRS. However, the details of these effects are still unclear. In the present study, we used LPS to induce inflammation response in RAW 264.7 cells and

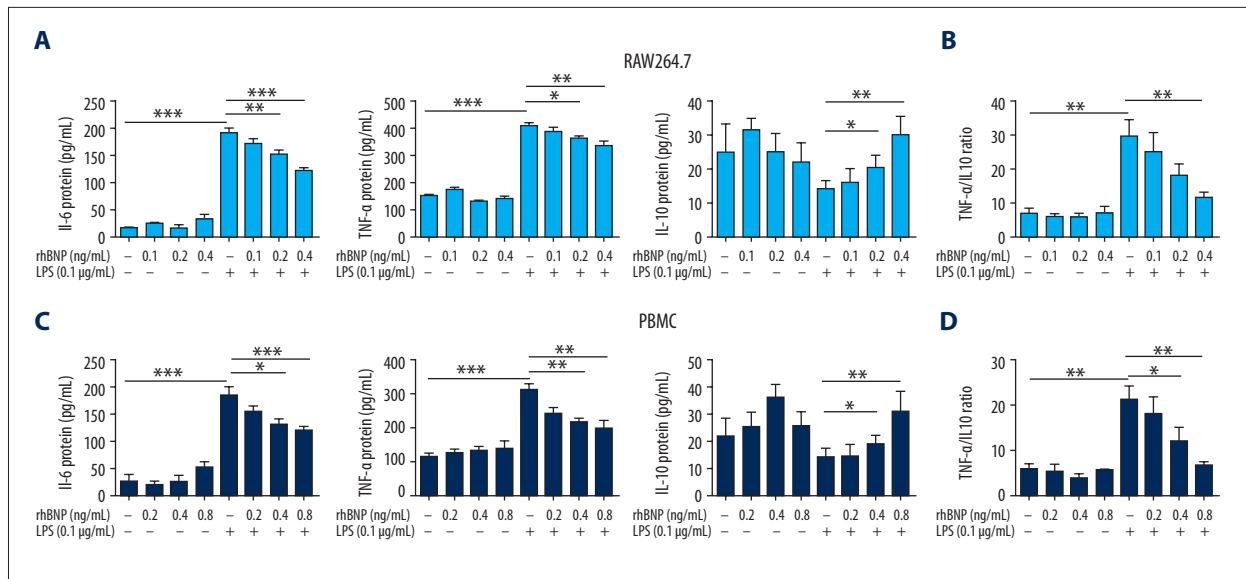


Figure 2. Effects of rhBNP on LPS-induced inflammatory factors by CAB method. (A) Effects of rhBNP on LPS-induced IL-6, TNF- α , and IL-10 in RAW 264.7 cells; (B) Effects of rhBNP on TNF- α /IL-10 ratio in RAW 264.7 cells; (C) Effects of rhBNP on LPS-induced IL-6, TNF- α , and IL-10 in PBMCs; (D) Effects of rhBNP on TNF- α /IL-10 ratio in PBMCs. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

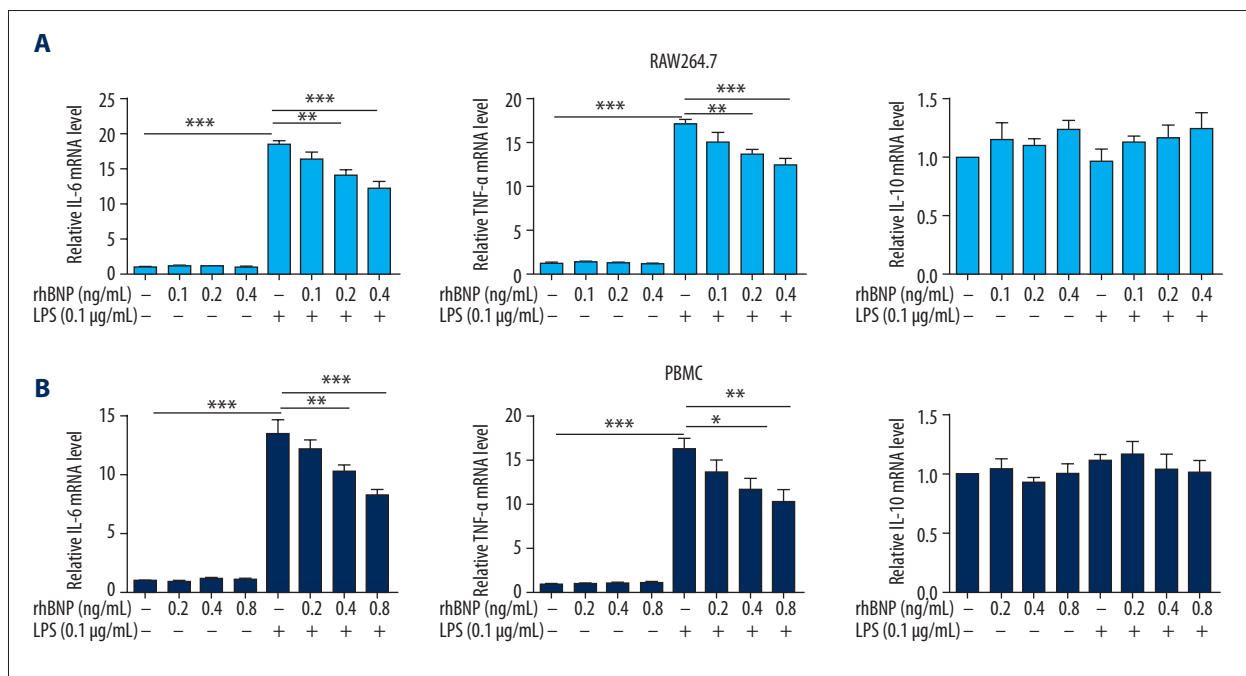


Figure 3. Effects of rhBNP on mRNAs of LPS-induced inflammatory factors by RT-PCR. (A) Effects of rhBNP on LPS-induced IL-6, TNF- α , and IL-10 in RAW 264.7 cells; (B) Effects of rhBNP on LPS-induced IL-6, TNF- α , and IL-10 in PBMCs. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

human peripheral blood mononuclear cells, and investigated the effects of rhBNP on LPS-activated cells. Because SIRS is a kind of systemic immune response, which is a complex pathological reaction mediated by inflammatory mediators, study of the effects of rhBNP on inflammatory alteration in LPS-activated cells can provide *in vitro* evidences for the role of rhBNP in SIRS.

At first, we studied the effects of rhBNP on cell viabilities of RAW 264.7 cells and PBMCs using MTT assay, and found that rhBNP showed very weak toxicity. By selecting 3 suitable concentrations of rhBNP according to the results, effects of different concentrations of rhBNP on LPS-induced inflammatory factors were investigated at protein and mRNA levels. Results showed

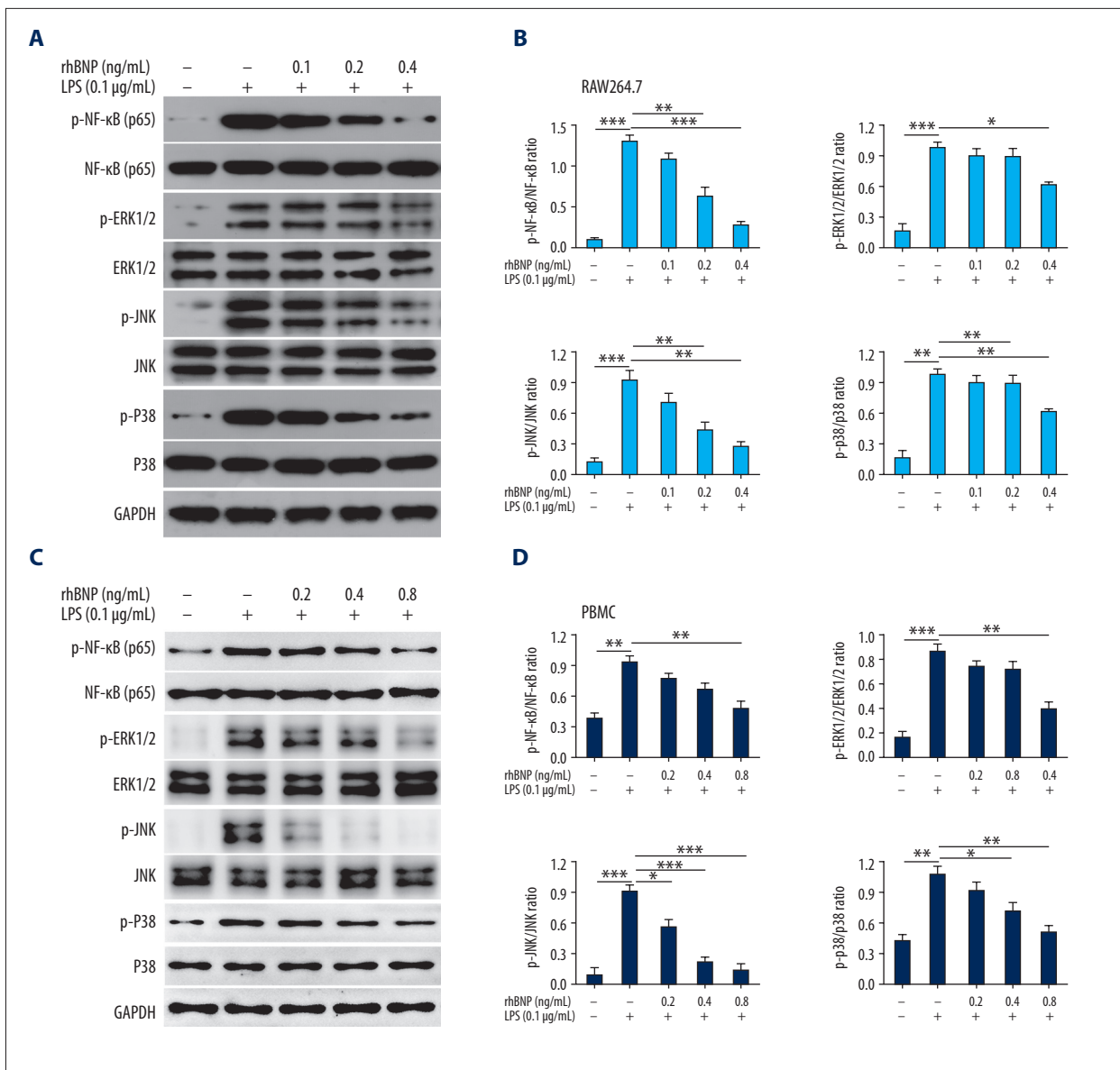


Figure 4. Western blotting analysis of NF-κB, ERK1/2, JNK, and P38 and the phosphorylation productions in RAW 264.7 cells and PBMCs. GAPDH served as a loading control. Data are representative images of 3 independent experiments. (A) Expression of NF-κB, ERK1/2, JNK, and P38 and the phosphorylation productions in RAW 264.7 cells; (B) Quantitative data of Western blotting of phosphorylation of NF-κB, ERK1/2, JNK, and P38 in RAW 264.7 cells; (C) Expression of NF-κB, ERK1/2, JNK, P38, and the phosphorylation productions in PBMCs; (D) Quantitative data of Western blotting of phosphorylation of NF-κB, ERK1/2, JNK, and P38 in PBMCs. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

that at protein and mRNA levels, rhBNP significantly inhibited the expression of IL-6 and TNF- α and promoted the expression of IL-10, in a dose-dependent manner. Song et al. investigated the protective effects of rhBNP against LPS-induced acute lung injury in dogs and found that rhBNP significantly inhibited LPS-induced serum IL-6 and TNF- α levels [25]. Chen et al. studied rhBNP in patients with acute myocardial infarction complicating congestive heart failure and found that rhBNP reduced plasma TNF- α and BNP levels [30]. Inomata et al. revealed that

blocking IL-6 led to decrease of circulating N-terminal pro-brain natriuretic peptide levels in patients with active rheumatoid arthritis [31]. A study also showed that plasma ANP and IL-6 were significantly correlated in patients with septic shock [32]. These results suggested a correlation between BNP and inflammatory factors like TNF- α and IL-6, and also indicated that rhBNP inhibits expression of inflammatory factors *in vitro* and *in vivo*. Through the above studies, we concluded that BNP had an anti-inflammation function in many diseases,

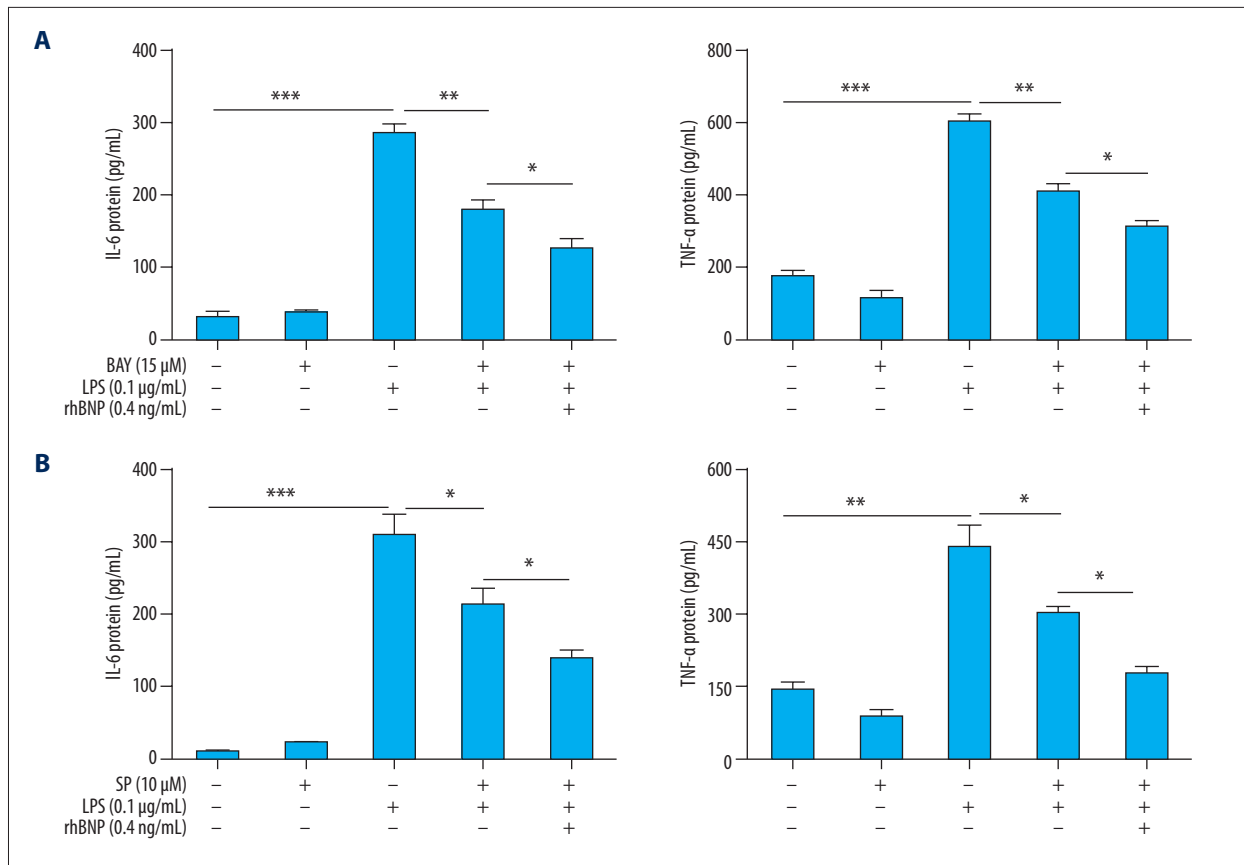


Figure 5. Effects of inhibitors of NF-κB and MAPK pathways on expression of IL-6 and TNF-α by CBA method. A. Effects of NF-κB pathway inhibitor BAY-11-7082 on expression of IL-6 and TNF-α; B. Effects of MAPK pathway inhibitor SP600125 on expression of IL-6 and TNF-α. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

including heart failure and lung injury. Our investigation further revealed that the increase of BNP in burn-induced systematic inflammation was a regulation response to inhibit the expression of inflammation factors and mediate the balance of anti-inflammatory and pro-inflammatory mediators *in vivo*.

To further investigate the mechanism of rhBNP in inhibition of inflammation, effects of rhBNP on NF-κB and MAPK pathways were studied through evaluation of related proteins. Results showed that in RAW 264.7 cells and PBMCs, LPS-induced increasing levels of phosphorylation of p-NF-κB, p-ERK1/2, p-JNK, and p-P38 were significantly inhibited by rhBNP treatment [33]. Interestingly, the inhibitory effect of rhBNP on p-ERK was the weakest in both of these 2 cell lines, which needed more investigation to reveal the mechanism. When treated with inhibitors of NF-κB and MAPK pathways, the same inhibition effects were observed at the mRNA level. These results indicated that the inhibitory effect of rhBNP on inflammation was through the inhibition of NF-κB and MAPK pathways. Several studies have reported similar results. Mezzasoma et al. showed that BNP inhibited expression of IL-1β by down-regulation of NF-κB/ERK1/2 and NALP3/ASC/caspase-1 activation in human

THP-1 monocytes [34]. Moriyama et al. demonstrated that another family member of natriuretic peptides, ANP, inhibited LPS-induced stimulation of rat microglial cells by suppressing NF-κB and AP-1 activations [35]. Chen et al. studied effects of C-type natriuretic peptide on LPS-induced endothelial activation, and found that C-type natriuretic peptide inhibited LPS-induced endothelial activation through p38, Akt, and NF-κB pathways [36]. Liang et al. demonstrated that BNP is modulated through the NF-κB pathway in cardiomyocytes [37]. All these results demonstrate that NF-κB and MAPK pathways are related to many biological process involved with BNP, and play an important role in mediating the inflammatory process in systematic inflammation. Further studies on these 2 signaling pathways involved in systematic inflammation and related diseases might provide more valuable insights in this field.

Conclusions

In conclusion, we performed an *in vitro* study to investigate effects of recombinant human brain natriuretic peptide on LPS-activated RAW 264.7 cells and PBMCs. Results showed that

rhBNP significantly inhibited the expression of IL-6 and TNF- α and promoted the expression of IL-10 through inhibiting NF- κ B and MAPK pathways. These results provide deeper insights into understanding mechanisms for occurrence and development of systemic inflammatory response syndrome, and provide clinical and experimental basis for the treatment of SIRS.

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Conflict of interest

None.