Glutamine ameliorates lipopolysaccharide-induced cardiac dysfunction by regulating the toll-like receptor 4/mitogen-activated protein kinase/nuclear factor-κB signaling pathway

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Abstract. The inflammatory response of sepsis induced by lipopolysaccharide (LPS) may result in irreversible cardiac dysfunction. Glutamine (GLN) has a multitude of pharmacological effects, including anti-inflammatory abilities. Previous studies have reported that GLN attenuated LPS-induced acute lung injury and intestinal mucosal injury. The present study investigated whether GLN exerts potential protective effects on LPS-induced cardiac dysfunction. Male Sprague-Dawley rats were divided into three groups (15 rats per group), including the control (saline-treated), LPS and LPS+GLN groups. Pretreatment with 1 g/kg GLN was provided via gavage for 5 days in the LPS+GLN group, while the control and LPS groups received the same volume of normal saline. On day 6, a cardiac dysfunction model was induced by administration of LPS (10 mg/kg). After 24 h, the cardiac functions of the rats that survived were detected by echocardiography and catheter-based measurements. The serum levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 were detected by enzyme-linked immunosorbent assay, while the mRNA levels of toll-like receptor (TLR)4, TNF-α, IL-1β and IL-6 were examined by reverse transcription-quantitative polymerase chain reaction. The protein expression of TLR4, mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) were also determined by western blot analysis. The results of echocardiography and catheter-based measurements revealed that GLN treatment attenuated cardiac dysfunction. GLN treatment also attenuated the serum and mRNA levels of the pro-inflammatory cytokines. In addition, the protein levels of TLR4, phosphorylated (p)-extracellular signal-regulated kinase, p-c-Jun N-terminal kinase and p-P38 were reduced upon GLN pretreatment. Furthermore, GLN pretreatment resulted in decreased activation of the NF-κB signaling pathway. In conclusion, GLN has a potential therapeutic effect in the protection against cardiac dysfunction mediated by sepsis through regulating the TLR4/MAPK/NF-κB signaling pathway.

Introduction

Sepsis is defined as a systemic inflammatory response syndrome that is caused by infection. Despite advances in the management of septic shock, it remains the primary cause of mortality in intensive care units (1). Lipopolysaccharide (LPS) from gram-negative bacteria is the primary cause of severe sepsis (2). LPS enhances the inflammatory reaction by activating cells associated with inflammation, and causing the synthesis and release of associated pro-inflammatory cytokines, and ultimately the inflammatory response of sepsis may lead to irreversible cardiac dysfunction (2). Cardiac dysfunction is a frequently occurring, serious complication of sepsis, which is correlated with the mortality of septic patients (3). Compared with septic patients without cardiac dysfunction, patients with cardiac dysfunction exhibit an evidently increased mortality rate (3). Therefore, it is urgent to identify an effective treatment for this dysfunction in septic patients.

Toll-like receptor (TLR)-4 is a member of the TLR family, which serves a vital role in the innate immune system and may be activated by LPS (4). TLR4 and its associated mitogen-activated protein kinase (MAPK) and nuclear factor-κB signaling pathways have been demonstrated to be correlated with LPS-induced inflammation (5,6). An increased level of cytokines, including tumor necrosis factor-α (TNF-α),...
interleukin (IL)-1β and IL-6, aggravates the development and progression of the septic myocardial dysfunction (7). The downregulation of TLR4-mediated MAPK and NF-xB signaling pathways inhibits the LPS-induced inflammatory response, and provides a protective effect in various organs against sepsis (5,6). Previous studies have reported that glutamine (GLN), as the most abundant free amino acid in the human body, is able to regulate the immune response induced by LPS, attenuate the release of cytokines and alleviate LPS-induced acute liver, renal, intestinal mucosal and lung injuries (8-10). However, the effect and underlying mechanisms of GLN in LPS-induced cardiac dysfunction remains unclear.

In order to investigate whether GLN has a potential protective effect against cardiac dysfunction, the present study established an LPS-induced sepsis model in Sprague-Dawley rats and attempted to identify the possible underlying mechanisms. It was hypothesized that GLN may be used against LPS-induced cardiac dysfunction by blocking the activation of the TLR4/MAPK and NF-xB signaling pathways.

Materials and methods

Animals and cardiac dysfunction model. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (11), and approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University (Wuhan, China). A total of 45 specific pathogen-free male Sprague-Dawley rats (8-10 weeks-old; 180-220 g) were purchased from the Hunan SJA Laboratory Animal Co., Ltd. (Changsha, China). All animals were housed in a light-controlled room with a 12 h light/dark cycle, at a temperature of 25°C and a humidity of 45-50%. All animals had free access to food and water for 1 week prior to the commencement of the study to allow them to acclimatize to the laboratory environment. Rats were randomly divided into three groups (15 rats per group), including the control, LPS and LPS+GLN groups. Rats in the LPS+GLN group were intragastrically administered with GLN (cat. no. V900419; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at a dose of 1 g/kg once per day for 5 days, while rats in the control and LPS groups received the same volume of normal saline. On day 6, cardiac dysfunction was induced by a single intraperitoneal injection of 10 mg/kg LPS (cat. no. L2880; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at a dose of 50 mm/s at the mid-papillary muscle level from the LV M-mode tracing. The LV end-systolic diameter (LVESD), LV end-diastolic dimension L (LVEDD) and fractional shortening (FS) were also measured. Hemodynamic variables were analyzed using a microtip transducer catheter (SPR-839; Millar, Inc., Houston, TX, USA) and a Millar Pressure-Volume system (PMVS-400; Millar, Inc.). The maximal rate of pressure development (dP/dt max), and minimal rate of pressure decay (dP/dt min) were then processed with the PVAN data analysis software LabChart version 7.3.7 (Millar, Inc.; ADInstruments, Dunedin, New Zealand).

Analysis of blood samples by ELISA. Following the collection of echocardiography and catheter-based measurements, blood was collected from the retro orbital plexus in the rats eyes. All blood samples were centrifuged at 4,200 x g for 10 min at room temperature, and the supernatants were subsequently collected and stored at -20°C. The levels of TNF-α (cat. no. KRC3011), IL-1β (cat. no. BMS630) and IL-6 (cat. no. KRC0061) in the blood samples were measured using ELISA kits (eBioscience; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocols.

Histological examination. The animals were sacrificed following the collection of the blood samples and the hearts were excised, washed with 10% KCl and fixed with 10% formalin. Next, the heart tissues were embedded in paraffin and then cut transversely close to the apex to visualize the left and right ventricles. Subsequently, 4-5-µm cardiac tissue sections were stained with hematoxylin and eosin (HE) for histopathological examination, and the pathological changes were evaluated under a light microscope.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In order to investigate the relative mRNA expression of B-type natriuretic peptide (BNP), TLR4, TNF-α, IL-1β and IL-6, pulverized heart tissues were homogenized and total RNA was isolated using TRIzol reagent (cat. no. 15596-026; Invitrogen; Thermo Fisher Scientific, Inc.). A DU 730 Series UV/Vis spectrophotometer (Beckman Coulter, Inc., Brea, CA, USA) was used to determine the quality of RNA using the A260/A280 ratio. Total mRNA (2 μg of each sample) was reverse transcribed using a PrimeScript™ RT reagent kit (cat. no. RR047A; Takara Bio, Inc., Otsu, Japan). According to the manufacturer's protocol, PCR amplification was conducted using a SYBR® Green Master Mix kit (cat. no. RR820A; Takara Bio, Inc.) and a 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The thermocycling conditions for PCR were as follows: Initial denaturation step at 95°C for 30 sec; followed by 40 cycles of denaturation at 95°C for 5 sec, annealing at 60°C for 34 sec; and a dissociation stage at 95°C for 15 sec, followed by 60°C for 1 min and 95°C for 15 sec. The primers used for amplification of the respective genes are listed in Table I. All results were normalized against GAPDH gene expression by using the 2^-ΔΔCq method (14).
**Western blotting.** In order to determine the activation state of the TLR4/ MAPK/NF-κB signaling pathway, the nuclear and cytoplasmic proteins were extracted from the cardiac tissues. Cardiac tissues were harvested and homogenized on ice for 15 min using a radioimmunoprecipitation assay lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology, Haimen, China), a Complete Protease Inhibitor Cocktail (cat. no. 4693159001; Roche Diagnostics, Basel, Switzerland) and PhosStop Phosphatase Inhibitor Cocktail (cat. no. 4906837001; Roche Diagnostics). The homogenates were subsequently collected and transferred to microcentrifuge tubes for centrifugation at 12,000 x g for 30 min at 4°C. The supernatant was collected and the protein concentration of samples was measured using a BCA kit (cat. no. 23227; Thermo Fisher Scientific, Inc.). The nuclear proteins were extracted using Nuclear and Cytoplasmic Extraction Reagents (cat. no. P0027; Beyotime Institute of Biotechnology), according to the manufacturer's protocol. A total of 50 µg of protein was loaded per lane, subjected to 10% SDS-PAGE and subsequently transferred onto polyvinylidene difluoride membranes (IPFL00010; EMD Millipore, Billerica, MA, USA). The membranes were subsequently blocked with 5% non-fat milk for 1 h at room temperature and washed with PBS 3 times for 5-10 min each time. The membranes were incubated overnight at 4°C with the following primary antibodies directed against TLR4 (dilution, 1:1,000; cat. no. 14358), total extracellular signal-regulated kinase 1/2 (dilution, 1:1,000; ERK1/2; cat. no. 4695), phosphorylated (p-)ERK1/2 (dilution, 1:2,000; cat. no. 4370), total p38 (dilution, 1:1,000; cat. no. 8690), p-p38 (dilution, 1:1,000; cat. no. 4511), total c-Jun N-terminal kinase (dilution, 1:100; JNK; cat. no. 9592), p-JNK (dilution, 1:1,000; cat. no. 4668), p65 (dilution, 1:1,000; cat. no. 8242), IkBa (dilution, 1:1,000; cat. no. 4814) and GAPDH (dilution, 1:1,000; cat. no. 4693), as additional loading controls for nuclear protein expression analysis at 24 h after LPS administration. The results revealed that LVEDD, and LVESD were significantly decreased (Fig. 1A and B). These results suggest that LPS aggravated cardiac dysfunction. Pretreatment of the rats with GLN resulted in a significant decrease in the level of LVEDD and LVESD compared with the control group, whereas the dP/dt max, dP/dt min and FS were significantly increased (Fig. 1A and B). These results suggest that LPS aggravated cardiac dysfunction. Pretreatment of the rats with GLN resulted in a significant decrease in the level of LVEDD and LVESD compared with the LPS group, and also caused a significant increase in the dP/dt max, dP/dt min and FS. These results indicate that treatment with GLN has a beneficial effect on the impaired cardiac function in LPS-induced sepsis in rats. As an indicator of cardiac function, the mRNA level of BNP in the LPS+GLN group was significantly decreased compared with the LPS only group, but still significantly increased compared with the control group.

**Table I. List of primer sequences used in quantitative polymerase chain reaction.**

| Gene   | Forward            | Reverse            |
|--------|--------------------|--------------------|
| GAPDH  | GACATGCCGCTTGGAGAAAC | AGCCCAAGATGCCTTTAAGT |
| TLR4   | TTATCCAGACCGTGGTGTG  | CCCACTCGAGGTAGGTTT  |
| BNP    | CTCAAAGGACCAAGGCCCCTA | TAAAACACCTCAGGCCCTGC |
| TNF-α  | AGCATGATCCGAGATGTGGA | ATCTGAGTGAGGGTCGTCG |
| IL-1β  | CCTGTGTGATGAAAGACGCC | TATGTCCCGACATTGTGCTT |
| IL-6   | GTGGCCTCTTGGGACTGATG | TACTGTCCTGTTGCTGGTTT |

TLR, Toll-like receptor; TNF, tumor necrosis factor; IL, interleukin; BNP, B-type natriuretic peptide.

**Statistical analysis.** Data are expressed as the means ± standard deviation. Differences among groups were determined by two-way analysis of variance followed by a post hoc Tukey's test. Comparisons between two groups were performed by an unpaired Student's t-test. Kaplan-Meier curves were used to analyze the mortality rate of the three groups and the log-rank test was used to compare the survival distributions of two groups and determine the efficacy of GLN treatment. All data were analyzed by SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA), and P<0.05 was considered to be an indicator of a statistically significant difference.

**Results**

**GLN improves the impaired cardiac function induced by LPS.** To evaluate the protective effect of GLN pretreatment on the progression of LPS-induced cardiac dysfunction, the rats were assessed by echocardiography and hemodynamic analysis at 24 h after LPS administration. The results revealed that LVEDD, and LVESD were significantly increased in the LPS group compared with the control group, whereas the dP/dt max, dP/dt min and FS were significantly decreased (Fig. 1A and B). These results suggest that LPS aggravated cardiac dysfunction. Pretreatment of the rats with GLN resulted in a significant decrease in the level of LVEDD and LVESD compared with the LPS group, and also caused a significant increase in the dP/dt max, dP/dt min and FS. These results indicate that treatment with GLN has a beneficial effect on the impaired cardiac function in LPS-induced sepsis in rats. As an indicator of cardiac function, the mRNA level of BNP was also investigated (Fig. 1C). The results demonstrated that the mRNA level of BNP in the LPS+GLN group was significantly decreased compared with the LPS only group, but still significantly increased compared with the control group.
GLN alleviates the inflammation infiltration and the expression levels of inflammatory cytokines. Compared with the control group, the LPS rats demonstrated numerous necrotic alterations in the myofibril and neutrophil granulocyte infiltration, which were confirmed by HE staining. The necrotic alterations in the myofibrils and neutrophil granulocyte infiltration were attenuated in the LPS+GLN group compared with the LPS only group (Fig. 2A). RT-qPCR revealed that the mRNA expression levels of inflammatory cytokines, including TNF-α, IL-1β and IL-6, in the LPS+GLN group were significantly decreased compared with the LPS only group, however, they were still significantly increased compared with the control group (Fig. 2B). Similarly, the levels of inflammatory cytokines in the serum were also markedly reduced in the LPS+GLN group compared with the LPS only group and significantly higher compared with the control group (Fig. 2C). These results suggest that GLN may alleviate inflammation and the expression levels of inflammatory cytokines induced by LPS.

GLN downregulates the expression of TLR4 protein and mRNA. Subsequent to stimulation for 24 h with LPS, the level of TLR4 protein expression was evidently higher compared with that in the control group. By contrast, upon pretreatment with GLN, TLR4 protein expression was significantly decreased compared with the LPS only group, however, TLR4 protein expression was still significantly higher in the LPS+GLN group compared with the control group (Fig. 3A and B). This trend was also detected in the level of TLR4 mRNA expression (Fig. 3C).

GLN inhibits the activation of the MAPK and NF-κB signaling pathways. In order to elucidate the molecular mechanisms underlying the anti-inflammatory effect of GLN, the activation state of the TLR4 downstream signaling pathways, including MAPK and NF-κB pathways, were examined. As revealed in Fig. 4A and B, following the activation of TLR4 induced by LPS, the protein expression levels of p-ERK, p-P38 and p-JNK were significantly increased in the LPS group compared with the control group. GLN pretreatment regulated the MAPK signaling pathway by significantly decreasing the LPS-induced expression of p-ERK, p-P38 and p-JNK compared with the LPS only group. The protein expression levels of p-ERK, p-P38 and JNK in the LPS+GLN group were also significantly increased compared with the control group. Similarly, the protein expression of nuclear p65 (normalized to Lamin B1 protein expression) was significantly increased in the LPS group compared with the control group, but significantly decreased in the LPS+GLN group compared with the LPS only group (Fig. 4C and D). The expression of nuclear p65 in the LPS+GLN group was still significantly increased compared with the control group. Conversely, the protein expression of IκBα was significantly decreased in the LPS group compared with the control group, but was significantly increased in the LPS+GLN group compared with the LPS group. The expression of IκBα in the LPS+GLN group was still significantly decreased compared with the control group. These results suggest that the activation of the MAPK and NF-κB signaling pathways may be partially inhibited by GLN.

GLN pretreatment decreases the mortality of rats with sepsis induced by LPS. Kaplan-Meier survival curves (Fig. 5) demonstrated that the rats with LPS-induced sepsis without GLN pretreatment presented a 24 h mortality rate of up to 40%. However, GLN pretreatment reduced the 24 h mortality rate

Figure 1. GLN treatment improved cardiac function as observed by echocardiography and pressure-volume analysis. (A) GLN improved the LPS-induced cardiac dysfunction by normalizing the LVEDD, LVED and FS. (B) Cardiac function data of hemodynamic parameters, including dP/dt max and dP/dt min, were normalized by GLN pretreatment. (C) BNP mRNA expression was examined using reverse transcription-quantitative polymerase chain reaction analysis. *P<0.05 vs. the control group; #P<0.05 vs. the LPS group. GLN, glutamine; LPS, lipopolysaccharide; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic dimension; FS, fractional shortening; dP/dt max, maximal rate of pressure development; dP/dt min, minimal rate of pressure decay; BNP, B-type natriuretic peptide.
to 13.3% of the sepsis group rats. The result of the log-rank test revealed a statistically significant difference between the LPS group and the LPS+GLN group ($\chi^2=8.769; P=0.012$).

After 24 h, the rats were examined by echocardiography and...
Discussion

Sepsis is a common condition with a high mortality rate; however, no effective anti-sepsis treatments are currently available for this condition (15,16). The cardiovascular system is frequently affected by sepsis and cardiac dysfunction, as it has been widely observed in patients with severe sepsis (17). As a primary innate immune receptor, TLR4 can be stimulated by LPS and participated in the sepsis-induced acute myocardial dysfunction (18). GLN has been proven to be beneficial against LPS-induced acute liver, renal, intestinal mucosal and lung injury. Therefore, the present study investigated whether GLN exerted a beneficial effect on LPS-induced myocardial dysfunction. It was observed that, compared with the LPS group, GLN pretreatment normalized the echocardiography and hemodynamic indices, and had a positive effect on reducing the mortality rate of rats. These data suggest that GLN is an effective cardioprotective agent in septic rats.

Numerous studies have demonstrated that macrophages served a key role in the progression of LPS-induced sepsis, since macrophages release various pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6 (19-21). Among these, TNF-α leads to inflammation and tissue damage, and the level of TNF-α is positively correlated with mortality in patients with septic shock (22). IL-1β is a type of pro-inflammatory cytokine that enhances the activation of inflammatory cells and amplifies the systemic inflammatory response (23). As an important pleiotropic cytokine in the initial stage of inflammation, IL-6 has the effect of mediating a variety of inflammatory responses, and can reflect the degree of inflammation and disease (24,25). Upon stimulation with LPS, macrophages generate these inflammatory cytokines, and persistent release of TNF-α, IL-1β and IL-6 can aggravate sepsis and even lead to multiple organ failure (26). After 24 h of LPS stimulation,
the mRNA expression and serum levels of inflammatory cytokines were markedly increased in the present study. However, GLN was able to reduce the expression and release of TNF-α, IL-1β and IL-6, thus protecting against LPS-induced cardiac dysfunction by inhibiting the production of inflammatory cytokines.

TLR4 is one of the most studied members of the TLR family, it is a major receptor and signal transduction molecule for the identification of LPS, and serves an important role in mediating the pathophysiological effects of LPS (27). In addition, TLR4 is responsible for the recognition of LPS and for the initiation of sepsis (28). Subsequent to stimulation by LPS, the mRNA and protein expression levels of TLR4 were significantly increased in the current study. The results also revealed that GLN was able to normalize these expression levels of TLR4, suggesting that GLN may be a potential target for the treatment of sepsis.

MAPK is induced by the activation of TLR4, and several studies have reported that the MAPK signaling pathway was involved in the pathogenesis of septic shock (29-31). MAPKs constitute one of the major kinase families associated with immune defense and inflammatory reaction. Mitogen-activated protein kinase phosphatase-1 is a natural negative regulator of MAPKs, and subsequent to its knockdown, mice presented an increased level of pro-inflammatory cytokines, such as TNF-α and IL-6, and exhibited a marked increase in mortality (32). Furthermore, ERK1/2, JNK and p38 are three major subfamilies of MAPK signaling. Several studies of systemic inflammation have demonstrated that MAPKs are key mediators promoting the production of inflammatory cytokines during sepsis (33,34). It has also been observed that the inhibition of the p38 MAPK-dependent mechanism is able to significantly reduce the mortality associated with sepsis in rats with endotoxic shock (35). Additionally, the activation of MAPK signaling increases the synthesis of TNF-α, IL-1β and IL-6. These pro-inflammatory cytokines, in turn, promote further activation of MAPK (26).

The synthesis of inflammatory cytokines also mainly depends on the activation of NF-kB (26). Under normal conditions, NF-kB and IκBα form a complex, which is present in the cytoplasm in an inactive form, the degradation of IκBα can be induced by the activation of MAPK signaling pathway, which then leads to activation of NF-kB and nuclear translocation (36,37). However, the activation of TLR4 also leads to the activation of NF-kB, increasing the expression of inflammatory cytokines TNF-α, IL-1β and IL-6 in the serum and heart tissue samples (38,39). Furthermore, the activation of NF-kB led to an increase in oxidative stress levels and aggravation of cardiac dysfunction in type II diabetes (40).

Previous studies reported that NF-kB serves a key role in the development of sepsis and septic shock, while inhibition of TLR4-mediated signaling reduced the mortality rate of septic mice (41,42). In LPS-treated rats, the activity of NF-kB was increased, while suppression of the NF-kB activity had a beneficial effect on LPS-induced myocardial dysfunction (43). In the present study, it was identified that the NF-kB and MAPK signaling pathways were involved in regulating inflammatory processes following stimulation by LPS. However, treatment with GLN significantly decreased the protein expression levels of p-ERK, p-P38, p-JNK and nuclear NF-kB p65, whereas the expression of IκBα was significantly increased. Therefore, it is suggested that GLN may alleviate cardiac dysfunction by partially preventing the activation of MAPK/NF-κB signaling pathway.

In conclusion, GLN pretreatment effectively improved cardiac dysfunction and reduced the mortality rate induced by LPS in rats. The underlying mechanism of its action may be associated with the inhibition of the TLR4/MAPK/NF-κB signaling pathway. GLN may provide a potentially effective therapy for cardiac dysfunction in patients with sepsis.

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