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Glucan phosphate inhibits HMGB-1 release from rat myocardial H9C2 cells in sepsis via TLR4/NF- κ B signal pathway

Abstract

Purpose: The effect of glucan phosphate (GP) on the release of HMGB-1 from rat myocardial cells (H9C2) during lipopolysaccharide-induced sepsis, and the underlying mechanisms, were investigated.

Methods: H9C2 cells were divided into three groups: normal; lipopolysaccharide (LPS) (1 mg/ml LPS); and, LPS+GP (2 mg/ml GP). Western blot was used to determine toll-like receptor 4 (TLR4) levels, and electrophoretic mobility-shift assays (EMSA) was used to determine nuclear factor- κ B (NF- κ B) activity 3, 6 and 9 h after treatment. HMGB-1 mRNA levels in cultured cells were determined by real-time PCR and supernatant HMGB-1 protein levels were evaluated by ELISA at 12, 24, 36 and 48 h after treatment. Following the transfection of H9C2 cells with Ad5-I κ B α , which inhibits NF- κ B activity, TLR4, NF- κ B and HMGB-1 levels were determined.

Results: Intracellular TLR4 levels and NF- κ B activity in LPS and LPS+GP groups increased 3-9 h after stimulation, but the increased levels of TLR4 and elevated activity of NF- κ B were significantly lower in the LPS+GP group *vs.* the LPS group. HMGB-1 mRNA levels in both LPS and LPS+GP groups, increased gradually from 24 h after stimulation, but the increase was more obvious in the LPS group *vs.* the LPS+GP group. Supernatant HMGB-1 levels in the LPS and LPS+GP groups increased gradually from 9 h after stimulation, and also increased markedly in the LPS group. After the inhibition of NF- κ B activity, LPS-induced HMGB-1 release decreased significantly ($p < 0.05$).

Conclusion: GP can attenuate HMGB-1 release from rat myocardial H9C2 cells in LPS-induced sepsis by inhibiting the activation of NF- κ B.

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Sepsis involves the release and subsequent interaction of various cytokines and inflammatory mediators, which cause uncontrolled inflammatory response and immune function disorders; eventually resulting in septic shock, multiple organ dysfunction syndrome and death [1,2].

High mobility group box-1 (HMGB1), an intracellular DNA-binding protein, is widely distributed in the nucleus and cytoplasm of almost types of cells [3] and can stabilize nucleosomes as an important transcription factor [4]. Recent studies have shown that HMGB-1, an important damage-associated molecular pattern (DAMP) and an inflammatory indicator, is detected in necrotic or damaged tissues and is secreted by activated macrophages [5]. Studies have shown that application of anti-HMGB-1 antibodies can lead to sepsis and acute lung injury 24 h after cecal ligation and puncture in rats [4], indicating that HMGB-1 is a regulatory factor in the pathogenicity of sepsis. Additional experiments *in vitro* found that HMGB-1 proinflammatory responses were mainly mediated by the receptor for advanced glycation endproducts and toll-like receptor (TLR) 2, 4 and 9, thereby resulting in the activation of NF- κ B for inducing various proinflammatory genes [9-13]. It is still unclear whether TLR4-mediated NF- κ B activation exerts a regulatory effect on the release of HMGB-1 during sepsis.

Previous studies have demonstrated that sepsis and septic shock can cause cardiac dysfunction through the activation of NF- κ B [10]. Glucan phosphate (GP), a carbohydrate ligand, can regulate the innate immunity and proinflammatory signalling pathway [11], as well as down-regulate the elevated level of TLR4 induced by sepsis and the activation of NF- κ B; therefore, we investigated the association between the effect of GP on HMGB1 release induced by LPS from *in vitro* rat H9C2 cells and the activation of NF- κ B pathway mediated by TLR4.

Materials and Methods

LPS and GP solutions

LPS (Sigma) was dissolved in DMEM medium (Gibco) to produce a 1 mg/ml solution. The mixture was sterilized with a 0.22 mm diameter filter and refrigerated at 4°C, before being diluted to 0.1 µg/ml. The GP solution (Sigma) was diluted with sterile saline to the final concentration of 2 mg/ml.

Cell culture and experimental groups

Rat H9C2 cells were cultured in DMEM medium containing fetal bovine serum (Gibco) and incubated in 5% CO₂ at 37°C.

The cultured cells were divided into three groups: normal; LPS; and, LPS+GP groups. In the LPS group, cells were administrated a concentration of 0.1 µg/ml *Escherichia coli* LPS; while cells in the LPS+GP group were pretreated for 1 h with GP (1 µg/ml) 1h before LPS treatment. After LPS treatment, detection was performed at the indicated times.

Cell transfection

H9C2 cells were transfected with recombinant adenovirus Ad-I κ B α (containing unmutated I κ B α gene from the Chinese population) or Ad5-GFP (construction for a control) using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Following the mixing of the plasmids and transfection reagent, the mixture was added to cultured cells in serum-free medium. After 6 h, the medium was replaced with complete medium to continue culturing.

Detection of TLR4 levels with Western blot

After the cells or tissues were washed with PBS, lysis buffer was added, the mixture was placed on ice for 20 min and the cells were ruptured via sonication. The supernatant was obtained after 15,000 rpm centrifugation for 20 min at 4°C, and the protein quantification was performed using a BCA protein quantification kit (Thermo). The objective proteins were collected for electrophoresis, then grafted onto a membrane, washed with TBS buffer and sealed at room temperature. Rabbit polyclonal antibody against TLR4 (Cell Signaling Technology) and mouse monoclonal antibody against GAPDH (Cell Signaling Technology), which served as primary antibodies, were incubated with the samples prior to development with ECL.

Detection of HMGB1 mRNA levels with RT-PCR

Total RNA was extracted for reverse transcription using a total RNA extraction kit (BioTeke) according to manufacturer's instructions. Rat HMGB1 and GAPDH primers (internal reference) were designed using Premier4.1 software, and synthesized (Sangon Biotech). The forward primer for HMGB-1 was 5'-CCCGGATGCTTCTGTCAACTTC-3' and the reverse primer was 5'-TAACGAGCCTTGTCAGCC TITG-3', with the amplification product of ~120 bp in length. The forward primer for GAPDH was 5'-ATGGCCTTCCGTGTGCC-3' and the reverse primer was 5'-GCGGCACGTCAGATCC-3' with amplification product of ~53 bp. RT-PCR amplification was then performed using qRT-PCR kit (TaKaRa) and SYBR@Premix (Roche), in accordance with the manufacturer's instructions.

After the reaction was finished, Ct values of the melting curve and amplification curve were recorded, and the data was calculated based on $2^{-\Delta\Delta C_t}$ method; $2^{-\Delta\Delta C_t} > 2$ was considered statistically significant.

Detection of the activity of NF- κ B with biotin-labeled EMSA probe

Nuclear components were extracted with nuclear extraction kit (Activemotif) according to manufacturer's instructions. Electrophoretic mobility-shift assays (EMSA) were performed to detect NF- κ B activity in nuclear protein using biotin-labeled probe (Roche). Following the formation of the protein-double-stranded oligonucleotide probe compounds, the above compounds were subjected to electrophoresis in non-denaturing polyacrylamide gel, and then grafted to a membrane in the precooled $0.5\times$ TBE. After the grafted membrane was removed, the mixture was washed in a container containing buffer. After removal of the buffer, the container was placed on an oscillator containing a blocking solution for a slight concussion, and sealed for 20 min at room temperature.

Following the addition of HRP enzyme-labelled streptavidin, the mixture was incubated for 60 min at room temperature and the membrane was washed with buffer (3×5 min). With the formulation of reaction substrate, a chemiluminescence imaging system was used for image-forming exposures.

Detection of the level of supernatant HMGB-1 by ELISA

The medium of the two experimental groups was collected sterilely via filtration, and HMGB-1 levels were detected using an ELISA kit, according to the manufacturer's instructions. All samples had duplicates in the same batch.

Statistical analysis

All statistical analyses were performed using SPSS version 20.0 software. Experimental data were recorded dependently at the indicated time in all three experimental groups, and expressed as $\bar{x} \pm s$ through the normality test and Mauchly's test of sphericity. Based on two-factor repeated measures analysis of variance, the differences were analyzed using the t-test between the two groups, while the difference between two time points was also compared using the t-test; $\alpha = 0.05$ was considered as the overall significance level, and Bonferroni correction method was the reference for inspection level of pairwise comparison to adjust.

Results

Attenuation of the increased level of TLR4 expression and elevated NF- κ B activity in LPS-induced H9C2 cells after pretreatment with GP

As shown in Figure 1, the expression of TLR4 in rat myocardial cells increased gradually during 0-9 h stimulation with LPS (Western blot), but the differences at 6 h and 9 h were not significant. TLR4 expression in the LPS+GP group was markedly lower than that in LPS group at the same time point (Figure 1).

As shown in Figure 2, NF- κ B activity in H9C2 cells increased gradually during LPS-stimulation (EMSA test) when compared with the normal group. NF- κ B activity was decreased significantly after pretreatment with GP when compared with the LPS group (Figure 2).

Attenuation of the increased level of HMGB-1 mRNA in LPS-induced H9C2 cells after pretreatment with GP

As shown in Figure 3, RT-PCR results showed that HMGB-1 mRNA levels in H9C2 cells did not change 0~12 h after stimulating in the LPS and LPS+GP groups. HMGB-1 mRNA levels in the LPS group were significantly higher than in the LPS+GP group 24, 36 and 48 h after stimulating ($2^{-\Delta\Delta C_t} > 2$; $p < 0.01$). HMGB-1 mRNA levels in the LPS + GP group at 24, 36 and 48 h after stimulating did not increase significantly compared with the levels at 0~12 h ($2^{-\Delta\Delta C_t} < 2$; $p > 0.05$; Figure 3).

Attenuation of the elevated level of HMGB-1 release from LPS-induced H9C2 cells after pretreatment with GP

As shown in Figure 4, ELISA results showed that supernatant HMGB-1 levels in the LPS group increased gradually 6-48 h after stimulating and were significantly higher than in the LPS+GP and normal groups ($p < 0.01$), suggesting the extracellular HMGB-1 levels were increased significantly (Figure 4).

Attenuation of LPS-induced HMGB-1 release after the suppression of NF- κ B activity

To explore the key role of NF- κ B in the release of HMGB-1, H9C2 cells were transfected with the adenovirus Ad5-I κ B α , which expresses an I κ B α mutant that inhibits NF- κ B activity (with Ad5-GFP as a control). As shown in Figure 5, supernatant HMGB-1 levels in Ad5-I κ B α -transfected H9C2 cells decreased significantly 6-48 h after stimulating when compared with the Ad5-GFP-transfected control.

FIGURE 1. Inhibitory effect of GP on the over-expression of TLR4 induced by LPS in H9C2 cell.

At 3, 6 and 9 h after LPS stimulation, TLR4 expression was significantly up-regulated in the LPS group when compared with the control group. TLR4 expression level in the LPS+GP group was higher than that in the control group, but lower than that in LPS group at the corresponding time.

0 h: control group; L: LPS group; L+GP: LPS+GP group.

FIGURE 2. Inhibitory effect of GP on the activity of NF-κB induced by LPS in H9C2 cell.

At 3, 6 and 9 h after LPS stimulation, NF-κB activity was significantly higher in the LPS group when compared with the control group, and NF-κB activity group was also higher in the LPS+GP than that in the control group, but lower than that in the LPS group at the corresponding time.

N: control group; L: LPS group; L+GP: LPS+GP group.

FIGURE 3. Inhibitory effect of GP on HMGB-1 over-transcription induced by LPS in H9C2 cell.

At 24, 36 and 48 h after LPS stimulation, HMGB-1 over-transcription was significantly up-regulated in H9C2 cells in the LPS group when compared with the control group, and the transcription level were not significantly different between the LPS+GP and control groups, but were lower than that in the LPS group at the corresponding time.

0 h: control group; N: normal group; L: LPS group; L+GP: LPS+GP group; ** $p < 0.01$.

FIGURE 4. Inhibitory effect of GP on the over-expression of HMGB-1 induced by LPS in H9C2 cell.

At 24, 36 and 48 h after LPS stimulation, HMGB-1 expression was significantly up-regulated in H9C2 cell supernatant in the LPS group when compared with the control group, and its expression level was not significantly different between the LPS+GP and control groups, but was lower than that in the LPS group at the corresponding time.

N: control group; L: LPS group; L+GP: LPS+GP group; $n = 3$, ** $p < 0.01$.

Discussion

As a widespread disease, sepsis is accompanied by systemic inflammatory response syndrome (SIRS), and great attention has been paid to its ability to affect heart function [17]. The onset of sepsis primarily results from a variety of infections, especially bacterial infections. A major component of the cell wall of Gram-negative bacteria, LPS can trigger the production of a variety of inflammatory mediators and the activation of cytokines by binding to the corresponding receptors, resulting in shock, multiple organ failure and even death in severe cases. In mice, sepsis has been shown to lead to severe cardiac dysfunction [18].

The innate immune system is the first line in the body's defense against microbial pathogen invasion, and the TLR family plays a key role in this defense system. So far, 11 kinds of human TLRs have been identified, of which TLR1, 2, 4, 5 and 6 exist on the cell membrane, TLR1, 2, 4 and 6 recognize lipids and TLR4 specifically recognizes LPS on the cell wall of Gram-negative bacteria (LPS receptors) [19]. The

FIGURE 5. Reduction of LPS-induced HMGB-1 over-expression via inhibiting NF- κ B activity.

The level of HMGB-1 in H9C2 cell supernatant was significantly increased at 24, 36, 48 h after Ad5-GFP+LPS treatment compared with the control group, while its level in the Ad5-I κ B α +LPS group was significantly lower than that in the Ad5-GFP + LPS group at the corresponding time point.

N: control group; L+Ad5-GFP: LPS+Ad5-GFP group; L+Ad5-I κ B α : LPS+Ad5-I κ B α group; $n = 3$, ** $p < 0.01$.

combination of TLR4 and its specific ligand can activate the innate immune response, resulting in immunological cascade.

In this study, we induced sepsis by treatment with LPS and determined that TLR4 expression levels in LPS-induced H9C2 cells were significantly increased as compared with the normal group. Furthermore, an increase in the activity of NF- κ B is also a sign of inflammation and this study showed that NF- κ B activity in LPS-induced H9C2 cells was significantly higher than that in the normal group, indicating that the inflammatory response was successfully induced [20].

Previous studies have confirmed that HMGB1 plays a crucial role in the pathological lesions of organs in sepsis and that HMGB-1 antibody binding to HMGB-1 can significantly reduce organ damage in rats with severe sepsis, followed by improvement in rat survival [16]. Subjected to various stimuli from pathological factors, some innate immune cells, including macrophages or dendritic cells, can release HMGB-1. In the present study, we found that sepsis can cause an increase in supernatant HMGB-1 release from myocardial cells. Previous studies have shown that GP can attenuate the cardiac dysfunction in response to sepsis/septic shock via the

PI3K/Akt-mediated signalling pathways [21] and regulate cell signalling pathways via the dectin-1-mediated TLR2 signalling pathway [22]. In this study, we found that GP pretreatment significantly attenuated the increased TLR4 expression, NF- κ B activity and HMGB-1 mRNA expression and its supernatant levels, in LPS-induced H9C2 cells, indicating that GP participated in innate immunity and numerous pathological processes [11]. Moreover, the signalling pathway of the LPS-induced increase in HMGB-1 release may be an important mechanism of GP protection for cardiac function, and after further inhibition of NF- κ B activity, HMGB-1 release levels induced by LPS decreased significantly. These data suggest that GP regulates the HMGB-1 release from myocardial cells, possibly by inhibiting the activity of NF- κ B; therefore, regulation effects of GP on HMGB-1 release may prove useful in the alleviation of cardiac dysfunction in response to severe septic pathological processes and improvement in the survival and quality of life of patients.

In conclusion, TLR4-mediated NF- κ B signalling pathway plays a vital role in HMGB-1 release resulting from severe sepsis, and attenuation of cardiac function damage can be achieved by suppressing HMGB-1 release. Further studies need to be carried out to determine whether GP inhibition of NF- κ B activity can attenuate HMGB-1 release from myocardial cells in severe sepsis and thereby improve the survival of patients.

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