Glucan phosphate attenuates myocardial HMGB1 translocation in severe sepsis through inhibiting NF-κB activation

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Glucan phosphate attenuates myocardial HMGB1 translocation in severe sepsis through inhibiting NF-κB activation. Am J Physiol Heart Circ Physiol 301: H848–H855, 2011. First published June 3, 2011; doi:10.1152/ajpheart.01007.2010.—Myocardial dysfunction is a major consequence of septic shock and contributes to the high mortality of sepsis. High-mobility group box 1 (HMGB1) serves as a late mediator of lethality in sepsis. We have reported that glucan phosphate (GP) attenuates cardiac dysfunction and increases survival in cecal ligation and puncture (CLP)-induced septic mice. In the present study, we examined the effect of GP on HMGB1 translocation from the nucleus to the cytoplasm in the myocardium of septic mice. GP was administered to mice 1 h before induction of CLP. Sham-operated mice served as control. The levels of HMGB1, Toll-like receptor 4 (TLR4), and NF-κB binding activity were examined. In an in vitro study, H9C2 cardiomyoblasts were treated with lipopolysaccharide (LPS) in the presence or absence of GP. H9C2 cells were also transfected with Ad5-IκBα mutant, a super repressor of NF-κB activity, before LPS stimulation. CLP significantly increased the levels of HMGB1, TLR4, and NF-κB binding activity in the myocardium. In contrast, GP administration attenuated CLP-induced HMGB1 translocation from the nucleus to the cytoplasm and reduced CLP-induced increases in TLR4 and NF-κB activity in the myocardium. In vitro studies showed that GP prevented LPS-induced HMGB1 translocation and NF-κB binding activity. Blocking NF-κB binding activity by Ad5-IκBα attenuated LPS-induced HMGB1 translocation. GP administration also reduced the LPS-stimulated interaction of HMGB1 with TLR4. These data suggest that attenuation of HMGB1 translocation by GP is mediated through inhibition of NF-κB activation in CLP-induced sepsis and that activation of NF-κB is required for HMGB1 translocation.

high-mobility group box 1: myocardium; nuclear factor-κB

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MATERIALS AND METHODS

Experimental animals. Age- and weight-matched male ICR/HSD mice were obtained from Harlan Sprague Dawley (Indianapolis, IN). The TLR4 deleted strain (C57BL/10ScCr) and wild-type (WT; C57BL/10ScSn) mice were obtained from Jackson Laboratory (Bar Harbor, ME) as described previously (13). The mice were maintained in the Division of Laboratory Animal Resources at East Tennessee State University. The experiments outlined in this article conform with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996). All aspects of the animal care and experimental protocols were approved by the East Tennessee State University Committee on Animal Care.

GP. Water soluble GP was prepared and chemically characterized in our laboratory as previously described (34). We have previously demonstrated that GP will increase long-term survival in CLP-induced sepsis (30, 31), improves cardiac function in CLP-induced sepsis, rescues TLR4 shoc (n = 9), and decreases myocardial injury in response to ischemia-reperfusion (IR) injury (16).

CLP polymicrobial sepsis model. Cecal ligation and puncture was performed to induce sepsis in mice as previously described (9, 30, 31). In brief, the mice were anesthetized by isoflurane inhalation and ventilated with room air using a rodent ventilator. A midline incision was made on the anterior abdomen, and the cecum was exposed and ligated with a 4-0 suture. Two punctures were made through the holes. The abdomen was then closed. Sham surgically operated mice were subjected to CLP polymicrobial sepsis model. Sham surgical operation served as sham control. Mice that were not subjected to surgery were sham operated and kept with the Guide for the Care and Use of Laboratory Animals published with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996). All aspects of the animal care and experimental protocols were approved by the East Tennessee State University Committee on Animal Care.

Immunohistochemistry. Immunohistochemistry (IHC) was performed to examine TLR4 and HMGB1 expression in heart sections using a specific anti-TLR4 antibody (Ruslan Medzhitov, Yale University) or anti-HMGB1 antibody (BD, Bioscience) as described previously (9). In brief, hearts from each group were harvested and one section was immersion-fixed in 4% buffered paraformaldehyde, embedded in paraffin, cut at 5 μm, and stained with specific anti-TLR4 or anti-HMGB1 (8–11). Three slides from each block were evaluated with brightfield microscopy.

Isolation of nuclear and cytoplasmic proteins. Nuclear and cytoplasmic proteins were isolated as described in previous studies from our laboratory (15, 16). In brief, heart or cell samples were incubated with 400 μl of ice-cold hypotonic buffer containing protease and phosphatase inhibitors (15, 16) on ice for 20 min, vortexed for 30 s after addition of 25 μl of 10% Nonidet P-40, and centrifuged for 1 min at 4°C in an Eppendorf centrifuge. Supernatants containing cytoplasmic proteins were collected and stored at −80°C. The pellets were suspended in an ice-cold hypotonic salt buffer containing protease and phosphatase inhibitors, incubated on ice for 30 min, mixed frequently, and centrifuged for 15 min at 4°C. The supernatants were collected as nuclear extracts and stored at −80°C. The concentration of total protein in the samples was determined by the Pierce protein assay reagent (Pierce Chemical, Rockford, IL).

Immunoprecipitation. Approximately 800 μg of cellular protein was immunoprecipitated with 2 μg of antibodies to TLR4 (Ruslan Medzhitov, Yale University) for 1 h at 4°C followed by the addition of 15 μl of protein A/G-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) as previously described (8, 16). The precipitates were washed four times with lysis buffer and subjected to immunoblotting with anti-HMGB1 antibody (BD, Bioscience).

Western blot. Immunoblots were performed as described previously (9, 11, 15, 16). In brief, the cellular proteins were separated by SDS-PAGE and transferred onto Hybond ECL membranes (Amersham Pharmacia). The same membranes were analyzed using a specific anti-TLR4 antibody (Ruslan Medzhitov, Yale University) for 1 h at 4°C followed by the addition of 15 μl of protein A/G-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) as previously described (8, 16). The precipitates were washed four times with lysis buffer and subjected to immunoblotting with anti-HMGB1 antibody (BD, Bioscience).

Statistical analysis. Data are expressed as means ± SE. Comparisons of data between groups were made using one-way ANOVA, and Tukey’s procedure for multiple range tests were performed. A P value of < 0.05 was considered to be significant.

RESULTS

GP attenuated CLP-induced increases in cytoplasmic HMGB1 levels in the myocardium. We have previously shown that GP administration significantly attenuated cardiac dysfunction and increased survival in CLP-induced sepsis (9). Neutralization of HMGB1 has been shown to protect mice against lethal endotoxemia and sepsis (28, 29), as well as endotoxin-induced acute lung injury (28, 29). To examine the effect of GP on HMGB1
translocation from the nucleus to the cytoplasm in the myocardium following CLP, we analyzed the HMGB1 distribution in the heart tissues by IHC and immunoblot. IHC examination showed that HMGB1 staining is mainly located in the nuclei of cardiac myocytes and endothelium in sham controls (Fig. 1A). In contrast, cytoplasmic HMGB1 staining was increased in CLP-induced septic mice. GP treatment attenuated CLP-induced increases in the cytoplasmic HMGB1 staining. Data obtained from immunoblots (Fig. 1, B and C) was consistent with the IHC observation. CLP-induced septic mice showed significantly decreased levels of nuclear HMGB1 and increased levels of cytoplasmic HMGB1 in the myocardium. GP treatment significantly attenuated the translocation of HMGB1 from the nucleus to the cytoplasm in the myocardium in response to fulminating sepsis.

GP attenuated CLP-induced increases in TLR4 expression and NF-κB binding activity in the myocardium. It has been reported that TLR4-deficient mice are resistant to endotoxic shock and show preserved cardiac function following LPS administration (3, 18, 20, 21). We investigated whether GP will affect TLR4 expression in the myocardium of CLP mice. Figure 2A shows that CLP-induced sepsis significantly increased the levels of TLR4 in the myocardium compared with sham control (0.81 ± 0.11 vs. 0.30 ± 0.09). IHC staining showed that increased TLR4 expression occurred primarily in cardiac myocytes in the hearts of CLP mice (Fig. 2B). GP treatment prevented CLP-induced increases in the levels of myocardial TLR4 (0.27 ± 0.02 vs. 0.81 ± 0.11; Fig. 2, A and B).

NF-κB is an important transcription factor in the TLR4 signaling pathway and plays a critical role in the pathophysiology of sepsis/septic shock (1, 30). We examined the effect of GP on NF-κB binding activity in the myocardium following CLP. As shown in the Fig. 2C, NF-κB binding activity is low in the myocardium of sham control but was significantly increased in CLP-induced septic mice. In contrast, administration of GP blunted the increase in the levels of NF-κB binding activity compared with untreated CLP mice (Fig. 2C).

TLR4 deficiency attenuated CLP-induced HMGB1 translocation from the nucleus to the cytoplasm in the myocardium. GP treatment attenuated CLP-induced activation of TLR4-mediated NF-κB binding activity (Fig. 2). To investigate the role of TLR4 in mediating HMGB1 translocation, we examined the levels of HMGB1 in the nuclei and the cytoplasm in the myocardium of TLR4−/− mice after CLP. Figure 3, A and B, shows that the levels of HMGB1 in both TLR4−/− and WT mice were decreased compared to WT mice (Fig. 3, A and B).
mice were higher in the nuclei and lower in the cytoplasm. After CLP, the levels of HMGB1 were significantly decreased in the nuclei and increased in cytoplasm in WT mice. In contrast, CLP did not significantly decrease the levels of HMGB1 in the nuclei or increase HMGB1 in the cytoplasm in heart tissues from TLR4−/− mice, suggesting that TLR4 plays a role in mediating HMGB1 translocation in the myocardium.

Fig. 2. GP administration attenuated CLP-induced increases in the levels of Toll-like receptor 4 (TLR4) and NF-κB binding activity in the myocardium. GP was administrated to mice by intraperitoneal injection 1 h before the mice were subjected to CLP. Surgically operated mice served as sham control. Twelve hours after CLP, hearts were harvested. Cellular proteins and tissue sections were prepared. A: Western blot analysis of TLR4 levels in the myocardium. B: immunohistochemistry staining TLR4 in the heart sections. C: EMSA of NF-κB binding activity was measured in nuclear proteins isolated from heart tissues. There were 6 mice in each group. Representative blots are shown. *P < 0.01 compared with indicated groups.
GP administration prevented LPS-induced NF-κB activation in H9C2 cells. H9C2 cells were treated with GP 15 min before LPS stimulation. Thirty minutes after LPS administration, cells were harvested and nuclear proteins were isolated for NF-κB binding activity by EMSA. As shown in Fig. 4, LPS stimulation resulted in a significant increase in NF-κB binding activity compared with control. GP administration, however, prevented LPS-induced NF-κB activation.

GP attenuated LPS-induced HMGB1 translocation to the cytoplasm in cardiac myoblasts H9C2. We performed in vitro experiments to examine the mechanisms by which GP may attenuate HMGB1 translocation from the nucleus to the cytoplasm. Cardiac myoblasts H9C2 were treated with LPS for 16 h in the presence or absence of GP. The cells were harvested, and the nuclear and cytoplasmic proteins were isolated. The levels of HMGB1 in the nucleus and cytoplasm were examined by Western blot, respectively. Figure 5A shows that HMGB1 is mainly present in the nucleus in untreated H9C2 cells. After LPS stimulation, the cytoplasmic levels of HMGB1 were significantly increased compared with untreated cells (Fig. 5B), suggesting that HMGB1 translocated from the nucleus into the cytoplasm. In GP-treated cells, the levels of HMGB1 in the cytoplasm were similar to untreated control cells. However, the cytoplasmic levels of HMGB1 in GP-treated cells in the presence of LPS were significantly lower compared with LPS-treated cells alone (Fig. 5B). The data suggest that glucan administration significantly attenuated LPS-induced HMGB1 translocation from the nucleus to the cytoplasm.

Inhibition of NF-κB activation attenuated HMGB1 translocation following LPS challenge. To examine whether activation of NF-κB will regulate the translocation of HMGB1, we transfected H9C2 cells with Ad5-IκBα mut, which is a super-suppressor of NF-κB activation (17). Ad5-GFP served as the vector control (17). Twenty-four hours after transfection, the cells were stimulated with LPS for 16 h and harvested for isolation of the nuclear and cytoplasmic proteins. Figure 6, A and B, shows that LPS stimulation significantly induced HMGB1 translocation from the nucleus to the cytoplasm compared with untreated cells. Ad5-GFP transfection did not affect LPS-induced HMGB1 translocation. However, transfection of the Ad5-IκBα mut attenuated LPS-induced HMGB1 translocation as evidenced by maintaining high levels of HMGB1 in the nuclear fraction.

**GP administration prevented LPS-induced NF-κB activation in cardiac myoblasts H9C2 in vitro.** It is well known that LPS can stimulate NF-κB activation. We examined the effect of GP on LPS-induced NF-κB activation in H9C2 cells. H9C2 cells were treated with GP 15 min before LPS stimulation. Thirty minutes after LPS administration, cells were harvested and nuclear proteins were isolated for NF-κB binding activity by EMSA. As shown in Fig. 4, LPS stimulation resulted in a significant increase in NF-κB binding activity compared with control. GP administration, however, prevented LPS-induced NF-κB activation.

**GP attenuated LPS-induced HMGB1 translocation to the cytoplasm in cardiac myoblasts H9C2.** We performed in vitro experiments to examine the mechanisms by which GP may
administration significantly attenuated CLP-induced HMGB1 translocation and decreased sepsis-induced NF-κB binding activity in the myocardium. In vitro studies demonstrated that blocking NF-κB binding activity prevented LPS-induced HMGB1 translocation from the nucleus to the cytoplasm. These results suggest that GP modulation of HMGB1 translocation in the myocardium could be via its inhibition of NF-κB binding activity. Modulation of HMGB1 translocation may be a mechanism by which GP attenuates cardiac dysfunction and increases survival outcome in sepsis (9, 30, 31).

It has been clearly demonstrated that HMGB1 plays a critical role in mediating organ damage in severe sepsis (2, 14, 28). Neutralization of HMGB1 with anti-HMGB1 antibodies attenuated organ damage and improved survival of severe septic mice (2, 14, 28), suggesting that HMGB1 is a mediator of organ damage in severe sepsis. We observed in the present study that sepsis induces HMGB1 translocation from the nucleus to the cytoplasm in the myocardium. Interestingly, GP treatment also increased the levels of HMGB1 in the cytoplasm.
that affect HMGB1 translocation or perturbed HMGB1, which prevented production of unknown mediators of shock. For example, GP inhibited CLP-induced activation of HMGB1 translocation in the myocardium during sepsis/septic shock. Therefore, there are two important mechanisms by which GP preserves cardiac function in septic mice. We have previously shown that GP induced protection against myocardial ischemic injury through rapid modulation of cellular signaling pathways (16). GP treatment switched the signaling from deleterious, such as TLR4-mediated NF-κB activation, to protective signaling, such as activation of the PI3K/Akt signaling pathway (16). GP also attenuated cardiac dysfunction in CLP-induced septic mice (9). Collectively, these data suggest that prevention of CLP-induced HMGB1 translocation in the myocardium could be an important mechanism by which GP preserves cardiac function in septic mice.

In vitro studies have shown that HMGB1 can be actively released from innate immune cells such as macrophages and mature dendritic cells after these cells are challenged with pathogen associated molecular patterns. HMGB1 is also released passively by damaged cells, such as necrosis (4, 25, 28). How GP modulated HMGB1 translocation in the myocardium during sepsis/septic shock is unclear. We have observed in the present study that GP treatment significantly attenuated CLP-induced increases in NF-κB binding activity in the myocardium. TLR4 deficiency also prevented CLP-induced HMGB1 translocation in the myocardium. These data suggest that the TLR4-mediated NF-κB activation pathway determines the translocation of HMGB1 during sepsis. Therefore, there are several possibilities that may explain how GP attenuated HMGB1 translocation in the myocardium during sepsis/septic shock. For example, GP inhibited CLP-induced activation of NF-κB, which prevented production of unknown mediators that affect HMGB1 translocation or perturbed HMGB1 translocation through an epigenetic mechanism. In addition, GP may activate its receptor, Dectin-1, resulting in modulation of cellular signaling. Indeed, recent studies have demonstrated that glucan-induced cellular signaling pathway is mediated through a Dectin-1-dependent TLR2-mediated signaling pathway (5). We have observed that TLR4 deficiency prevented CLP-induced HMGB1 translocation, suggesting that TLR4 contributes to HMGB1 translocation during sepsis. We also performed in vitro experiments using cardiac myoblasts. We observed that LPS stimulation resulted in the translocation of HMGB1 from the nucleus to the cytoplasm. LPS also significantly increased TLR4 levels and NF-κB binding activity in the cells. Interestingly, inhibition of NF-κB binding activity by transfecting Ad5-IκBα mutant significantly attenuated LPS-induced HMGB1 translocation in cardiac myoblasts. Similarly, GP administration blunted LPS-induced increases in NF-κB binding activity and decreased LPS-induced HMGB1 translocation. The data suggest that HMGB1 translocation could be regulated by the TLR4-mediated NF-κB activation pathway and that GP attenuated the translocation of HMGB1 through blunting TLR4/NF-κB signaling in CLP mice.

We have previously reported that GP administration significantly blunted NF-κB binding activity both in septic mice (30) and in ischemic hearts (16) through interuption of TLR4/MyD88 interaction (16). HMGB1 has been demonstrated to be an endogenous ligand of TLR4 (23, 24). In the present study, we examined the interaction between HMGB1 and TLR4 following LPS stimulation in the presence or absence of GP. We observed that LPS stimulation increased the association between HMGB1 and TLR4, suggesting that HMGB1 could, in turn, activate the TLR4-mediated NF-κB signaling pathway. In GP-treated cells, the association between HMGB1 and TLR4 was significantly reduced compared with LPS-treated cells. The data suggest that disruption of the interaction between HMGB1 and TLR4 could be important for preventing NF-κB activation by HMGB1 via TLR4. Our observations suggest that TLR4-mediated NF-κB activation plays a role in the translocation of HMGB1 from the nucleus to the cytoplasm. Cytoplasmic HMGB1 may promote NF-κB activation, via a TLR4-dependent mechanism in the myocardium, which could contribute to cardiac dysfunction in CLP-induced sepsis (9). Our

Fig. 7. GP reduced the association of HMGB1 with TLR4 following LPS stimulation. H9C2 cells were treated with GP for 1 h followed by LPS stimulation for 16 h. The cells were harvested, and cellular proteins were isolated for immunoprecipitation with specific anti-TLR4 antibody followed by immunoblot with specific anti-HMGB1 antibody. There were 3 replicates. A representative blot is shown above the graphs. *P < 0.05 compared with indicated groups.

Recent studies suggest that HMGB1 may interact with RAGE and TLR2 and/or TLR4 (23, 24, 27). We observed that CLP significantly increased the expression of TLR4 and NF-κB binding activity in the myocardium. In GP-treated CLP mice, the levels of TLR4 and NF-κB binding activity were significantly reduced in the myocardium, suggesting that glucan-reduced translocation of HMGB1 in the myocardium is through modulation of the TLR4/NF-κB signaling pathway. To evaluate our hypothesis, we employed TLR4-deficient mice to examine the role of TLR4 in HMGB1 translocation. We observed that TLR4 deficiency prevented CLP-induced HMGB1 translocation, suggesting that TLR4 contributes to HMGB1 translocation during sepsis. We also performed in vitro experiments using cardiac myoblasts. We observed that LPS stimulation resulted in the translocation of HMGB1 from the nucleus to the cytoplasm. LPS also significantly increased TLR4 levels and NF-κB binding activity in the cells. Interestingly, inhibition of NF-κB binding activity by transfecting Ad5-IκBα mutant significantly attenuated LPS-induced HMGB1 translocation in cardiac myoblasts. Similarly, GP administration blunted LPS-induced increases in NF-κB binding activity and decreased LPS-induced HMGB1 translocation. The data suggest that HMGB1 translocation could be regulated by the TLR4-mediated NF-κB activation pathway and that GP attenuated the translocation of HMGB1 through blunting TLR4/NF-κB signaling in CLP mice.
data also suggest that it is possible to pharmacologically inhibit myocardial HMGB1 translocation in severe sepsis and that this may result in attenuation of cardiac dysfunction and improved outcome.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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