Glucan phosphate attenuates cardiac dysfunction and inhibits cardiac MIF expression and apoptosis in septic mice

Tuanzhu Ha,1 Fang Hua,1 Daniel Grant,1 Yeling Xia,1 Jing Ma,1 Xiang Gao,4 Jim Kelley,2 David L. Williams,1 John Kalbfleisch, I. William Browder, Race L. Kao, and Chuanfu Li1

Departments of 1Surgery and 2Internal Medicine, and 3Section of Medical Education, East Tennessee State University, Johnson City, Tennessee; and 4Animal Model Research Center, Nanjing University, Nanjing, China

Submitted 30 November 2005; accepted in final form 5 May 2006

CARDIOVASCULAR DYSFUNCTION is a major consequence of septic shock and contributes to the high morbidity and mortality of sepsis (11, 12, 23). Current wisdom implies that, after severe injury or infectious challenge, some patients respond by activating proinflammatory signaling pathways and overexpressing inflammatory mediators that result in a systemic inflammatory response that culminates in severe shock, multiorgan failure, and death (34). Despite extensive investigation, the cellular and molecular mechanisms that mediate myocardial dysfunction during septic shock have remained elusive. Furthermore, developing effective methods for preventing and/or treating sepsis-induced cardiovascular dysfunction has proven to be difficult. A growing body of evidence suggests that there is a link between the innate immune response and myocardial dysfunction in several important disease states, including ischemia-reperfusion (I/R) injury (13), congestive heart failure (21), and septic shock (12).

Glucan phosphate (GP) is a (1→3)-β-d-linked glucose ligand that has been reported to modulate innate immunity and proinflammatory signaling in sepsis (46–49). We have reported that GP will significantly increase long-term survival (43), downregulate sepsis-induced expression of Toll-like receptor 4 (TLR-4) (44), and blunt tissue NF-κB (43) and NF-IL-6 (43) activation in a murine model of cecal ligation and puncture (CLP)-induced polymicrobial sepsis. Several groups (26, 28, 35) have reported that TLR-4-mediated NF-κB activation contributes to myocardial injury in response to I/R injury. We have reported that GP administration dramatically reduces myocardial damage in response to I/R injury (28). The mechanisms of glucan-induced cardioprotection involve decreased association of TLR-4 with myeloid differentiation factor-88 (MyD88), inhibition of I/R-associated kinase and IKK-β activity, and decreased NF-κB activity (28). In addition, GP increased tyrosine phosphorylation of the TLR-4 transmembrane domain, resulting in increased phosphoinositide 3-kinase (PI3K)/Akt activity in the myocardium, which correlated with decreased cardiac myocyte apoptosis after I/R (28). We have also shown that GP increases long-term survival in CLP sepsis via a PI3K/Akt-dependent mechanism (45). On the basis of these data, we hypothesized that GP may exert a protective effect on cardiovascular function during septic shock.

Macrophage migration inhibitory factor (MIF) is a neuropoietic and inflammatory mediator that has been reported to play a critical role in sepsis-induced multiple organ failure and immune homeostasis (8). Increased levels of circulating MIF have been observed in septic animals and in patients with septic shock (6, 7). MIF is thought to play a role in host response to endotoxin via modulation of TLR-4 expression (37, 38). In support of this concept, neutralization of MIF with specific antibody or through MIF gene deletion results in protection from lethal endotoxemia and septic shock (5, 6). In addition, MIF has been implicated as an initiating factor in myocardial inflammatory responses, cardiac myocyte apoptosis, and cardiac dysfunction during sepsis (11, 16). It is possible, therefore, that modulation of MIF expression in the myocardium could result in the improvement of cardiac dysfunction induced by septic shock. In the present study, we evaluated...
left ventricular (LV) function in CLP-induced sepsis in the presence or absence of GP treatment. We observed that GP administration attenuated LV dysfunction in CLP-induced sepsis. GP treatment also inhibited myocardial MIF expression, activated PI3K/Akt, and decreased cardiac myocyte apoptosis.

MATERIALS AND METHODS

Experimental animals. Age- and weight-matched male ICR/HSD mice were obtained from Harlan Sprague Dawley (Indianapolis, IN). The mice were maintained in the Division of Laboratory Animal Resources at East Tennessee State University (ETSU). The experiments outlined in this paper conform to 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 ETSU Committee on Animal Care.

Glucan phosphate. We selected GP for this study because we have previously demonstrated that GP will increase long-term survival in CLP sepsis (43–45), and it decreases myocardial injury in response to I/R (28). Water-soluble GP was prepared and chemically characterized in our laboratory as previously described (22, 47).

CLP polymicrobial sepsis model. CLP was performed to induce sepsis in mice as previously described (3, 42, 52). Briefly, 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 cecum with an 18-gauge needle, and feces were extruded from the holes. The abdomen was then closed. Sham surgically operated mice served as the surgical control group. Mice that were not subjected to surgery or anesthesia served as the normal controls. For the treatment group, the animals were administered GP at 40 mg/kg body wt by intraperitoneal injection 1 h before surgery. This dose of GP has been shown to be effective in increasing survival of septic animals (43) and protecting the myocardium from I/R injury (28). There were six groups, with 4–8 mice in each group: normal control (N), sham surgery control (S), CLP, N + GP, S + GP, and CLP + GP.

In separate experiments, a less severe model of CLP sepsis was employed in combination with fluid resuscitation. GP (40 mg/kg) was administered to the experimental mice 1 h before surgical operation. CLP was performed as described above. A single puncture was made through the cecum with a 20-gauge needle, and feces were extruded from the hole. After surgical operation, a single dose of resuscitative fluid (lactated Ringer solution, 50 ml/kg body wt) was immediately administered by subcutaneous injection. There were six groups, which were the same as described above.

Experimental protocols. Mice were subjected to CLP at time 0, and 6 h after CLP, cardiac function measurements were performed as described previously (17, 20). To examine the effects of GP on the expression of MIF and cardiac myocyte apoptosis, hearts were harvested and washed free of blood with ice-cold phosphate buffered saline. A single heart tissue section (5 mm) was taken from each heart at the same anatomical location, immersion fixed in 4% buffered paraformaldehyde, embedded in paraffin, cut at 5 μm, and stained with an antibody directed against activated caspase-3 or MIF (17, 18). Three slides from each block were evaluated with brightfield microscopy.

Western blot. Cytoplasmic proteins were isolated from heart tissues, and immunoblots were performed as described previously (18, 27–30). Briefly, the cellular proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred onto Hybond ECL membranes (Amersham Pharmacia, Piscataway, NJ). The ECL membranes were incubated with appropriate primary antibody [anti-phospho-Akt (anti-phospho-GSK-3β (anti-Ser9), anti-GSK-3β (Cell Signaling Technology, Beverly, MA), anti-Akt, and anti-MIF (Santa Cruz Biotechnology)], respectively, followed by incubation with peroxidase-conjugated second antibodies (Cell Signaling Technology). The membranes were analyzed by the ECL system (Amersham Pharmacia). The same membranes were stripped and reprobed with anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Biodesign, Saco, ME) as loading controls. The signals were quantified by scanning densitometry and computer-assisted image analysis.

Hemodynamic measurements. Mice were anesthetized with isoflurane inhalation and ventilated with room air using a rodent ventilator. A microconductance pressure catheter (Millar Instruments, Houston, TX) was positioned in the LV via the right carotid artery for continuous registration of LV pressure-volume loops (17, 20) using the PowerLab system (AD Instruments, Colorado Springs, CO). A cuvette calibration method was used to convert the conductance voltage into volume units by filling nonconductive cuvettes of known diameter with heparin-treated mouse blood. Parallel conductance from surrounding structures was determined by intravenous (external jugular vein) injection of a small bolus (15 μl) of hypertonic saline (15% NaCl). All measurements were performed while ventilation was turned off momentarily. Indices of systolic and diastolic cardiac performance were derived from LV pressure-volume data obtained at steady state. Cardiac output, ejection fraction, stroke volume, and stroke work were chosen as indices of cardiac function.

Statistical analysis. The figures present group mean levels and corresponding SE. Analysis of variance (ANOVA) and the Kruskal-Wallis (KW) procedure were used to assess differences between the six group means and six group medians (KW). Specific comparisons of interest (S vs. CLP and CLP vs. CLP + G) were judged by the least significant difference test and the t-test (when ANOVA was significant) and by the Mann-Whitney U-test when a normal distribution was not indicated (using residuals and the Anderson-Darling test). Probability levels of 0.05 and smaller are used for reporting in the figures.

RESULTS

GP prevented cardiac dysfunction in septic mice. In vivo cardiac function was measured 6 h after the mice were subjected to CLP by using the Millar pressure-volume conductance system. As shown in Fig. 1A, the levels of end-systolic volume were significantly reduced in CLP-induced septic shock mice without fluid resuscitation. CLP-induced septic shock also resulted in significant suppression of cardiac function as evidenced by reduction of stroke work by 47.7% and cardiac output by 41.1%, respectively, compared with sham control. In addition, end-systolic pressure (in mmHg) was decreased by 15.1%, maximal rate of change in LV pressure...
GLUCAN PHOSPHATE PREVENTS CARDIAC DYSFUNCTION IN SEPSIS

Fig. 1. Glucan phosphate (G) administration prevented left ventricle (LV) dysfunction in cecal ligation and puncture (CLP)-induced septic mice. Glucan phosphate was administrated to mice by intraperitoneal injection 1 h before mice were subjected to CLP. Surgically operated mice served as sham control. A: experimental mice did not receive fluid resuscitation; B: experimental mice were given fluid resuscitation. Six hours after CLP, LV hemodynamic parameters were examined. There were 4–8 mice in each group. *P < 0.05 compared with age-matched respective sham (S) control; #P < 0.05 compared with CLP group. N, normal; dP/dt\text{\text{\text{\text{max}}}}, maximal rate of change in LV pressure.

(dP/dt\text{\text{\text{\text{max}}}}; in mmHg/s) by 25.7%, and ejection fraction by 11.7% in the CLP group compared with sham control. After GP administration, end-systolic volume and end-diastolic volume were maintained at normal levels. GP treatment prevented sepsis-induced cardiac dysfunction. When compared with the CLP group, GP administration increased cardiac output by 53.8%, ejection fraction by 11.7%, dP/dt\text{\text{\text{\text{max}}}} (in mmHg/s) by 49.8%, and stroke work by 58.6%, respectively. Cardiac function in glucan-treated CLP mice was not significantly different from normal or sham control animals.

Figure 1B shows that fluid resuscitation immediately after surgery maintained circulating blood volume in CLP-induced septic mice as evidenced by the levels of end-systolic volume, which were not reduced compared with sham control. However, CLP-induced sepsis with fluid resuscitation still resulted in cardiac dysfunction, which showed a similar pattern when compared with CLP-induced septic mice without fluid resuscitation (Fig. 1A). It was also noted that the effect of glucan was similar in the presence or absence of fluid resuscitation.

**GP prevented increased MIF expression in myocardium of CLP-induced septic mice.** Neutralization of MIF has been shown to reverse endotoxin-induced myocardial dysfunction in an experimental rat model (11). To examine the effect of GP on the expression of MIF in the myocardium of septic mice, we analyzed the expression of MIF in the hearts by immunoblot and immunohistochemistry. As shown in Fig. 2A, the levels of MIF in the myocardium were significantly increased by 88.3% in CLP mice compared with sham control (0.98 ± 0.12 vs. 0.52 ± 0.10%). In GP-treated mice, the levels of MIF in the myocardium were not significantly different from normal or sham controls (Fig. 2A). Immunohistochemical examination showed increased expression of MIF in cardiac myocytes of CLP mice (Fig. 2B). GP treatment prevented increased MIF expression in cardiac myocytes from CLP mice (Fig. 2B).
GP inhibited sepsis-induced cardiac myocyte apoptosis. Cardiac myocyte apoptosis plays a major role in cardiac dysfunction (24, 25, 41). Therefore, we examined the effect of GP administration on cardiac myocyte apoptosis in septic mice with the use of the TUNEL assay. Figure 3A shows that cardiac myocyte apoptosis was significantly increased (7.8-fold) in the myocardium of septic mice compared with sham control (39.63 ± 2.04 vs. 4.49 ± 0.80%). The percentage of apoptotic myocytes was significantly increased in the myocardium of septic mice compared with sham control (39.63 ± 2.04 vs. 4.49 ± 0.80%).

![Graph showing levels of MIF (MIF/GAPDH) in different groups.](image)

**Fig. 2.** Glucan phosphate administration prevented increased expression of myocardial migration inhibitory factor (MIF) in septic mice. Glucan phosphate was administrated to mice by intraperitoneal injection 1 h before mice were subjected to CLP. Six hours after CLP, hearts were harvested. Cellular proteins were isolated from hearts, and expression of MIF was examined by Western blot analysis with specific antibodies (A). Immunohistochemistry was performed for examination of MIF with specific antibody in heart tissue sections embedded in paraffin (B). There were 4–8 mice in each group. *P < 0.05 compared with age-matched respective sham control; #P < 0.05 compared with CLP group.
cells in GP-treated CLP mice was also significantly increased compared with the controls; however, the increase was significantly less than in the CLP mice. Activation of caspase-3 is an established marker for apoptotic cells. As shown in Fig. 3B, caspase-3 activity was increased in the myocardium of septic mice as evidenced by immunohistochemistry with specific anti-cleaved caspase-3 antibody when compared with sham control. We observed decreased caspase-3 activity in the myocardium of GP-treated CLP septic mice compared with the untreated CLP mice (Fig. 3B).

Glucan phosphate prevented the decrease in phospho-Akt and phospho-GSK-3β in myocardium of septic mice. Activation of the PI3K/Akt signaling pathway has been shown to prevent cardiac myocyte apoptosis (15, 51). We have demonstrated that GP increases PI3K/Akt activity in ischemic rat hearts and that the increase in PI3K/Akt activation correlates...
with decreased myocardial apoptosis (28). We have previously shown that GP increased long-term survival in CLP sepsis via a PI3K/Akt-dependent mechanism (45). To examine the effect of GP on the activation of PI3K/Akt in the myocardium of septic mice, we examined the levels of phospho-Akt. Figure 4A shows that the levels of the phospho-Akt were reduced in the myocardium of CLP mice compared with sham controls. In contrast, GP treatment prevented the sepsis-induced decrease in myocardial phospho-Akt levels (Fig. 4A). The levels of phospho-Akt in the myocardium of GP-treated CLP mice were significantly higher than in the CLP group and not significantly different from sham controls.

**DISCUSSION**

An important finding in the present study is that GP administration attenuated LV cardiac dysfunction in CLP sepsis. GP attenuation of cardiac dysfunction positively correlated with increased PI3K/Akt activity, decreased MIF expression, and reduction of cardiac myocyte apoptosis in septic mice. These results suggest that activation of PI3K/Akt, inhibition of MIF expression, and reduced cardiac myocyte apoptosis in the myocardium by GP could explain, in part, the mechanisms of improved cardiac function in sepsis.

The septic shock model induced by CLP in the present study is a hypodynamic sepsis model that is characterized by reduced levels of end-systolic volume and cardiac output. The hypovolemia during sepsis is usually caused by vasodilatation due to inflammatory cytokines, resulting in maldistribution of blood...
flow and myocardial depression. Adequate fluid resuscitation, therefore, is one of the keystones in the management of septic shock. In the present study, we have observed that, after fluid resuscitation, the levels of end-systolic volume in CLP animals were maintained at the control levels, indicating that fluid resuscitation significantly improved circulating blood volume. However, cardiac output in CLP mice was still significantly decreased compared with that in sham control, suggesting that CLP-induced septic shock results in significant myocardial suppression independent of fluid status. Tao et al. (39) have shown that cardiac function was significantly reduced in CLP mice with fluid resuscitation. Albuszies et al. (1) reported that a combination of fluid resuscitation and norepinephrine resulted in significantly increased cardiac output in CLP-induced septic mice. Collectively, these data suggest that prevention of cardiac dysfunction could be an important strategy in management of septic shock.

Clinical and experimental studies have shown that myocardial dysfunction is an early and fatal complication of septic shock (11, 12, 23, 39) and that the TLR-4-mediated NF-κB dysregulation is an early and fatal complication of septic shock. In the present study, we have observed that GP administration significantly increased survival in CLP mice (43) and the mechanisms involved downregulating the expression of TLR-4 and blunting NF-κB activation in the lung, liver, and spleen (44). Therefore, we postulated that GP administration could also improve myocardial function in the septic mouse. To evaluate our hypothesis, we examined cardiac function in CLP-induced sepsis with or without GP treatment. We observed that the cardiac function was significantly depressed in untreated CLP mice. In GP-treated CLP mice, however, cardiac function was maintained at control levels. We have previously shown that GP administration significantly blunted NF-κB activation both in septic mice (43) and in ischemic hearts (28). NF-κB is a critical transcription factor in TLR-mediated signaling pathways and plays a critical role in regulation of the expression of a number of genes, including inflammatory cytokines such as TNF-α and IL-1-β, which have been shown to suppress cardiac function synergistically during sepsis (10). Unfortunately, anti-TNF-α or anti-IL-1-β therapy did not result in increased survival in patients with septic shock (12). Furthermore, we have reported that glucan treatment in CLP mice did not result in significant changes in serum cytokine levels, even though survival outcome was increased (45). Therefore, it is likely that the improved cardiac function observed in septic animals treated with GP is mediated by mechanisms that are independent of inflammatory cytokine expression.

Recent studies have shown that MIF is expressed in the myocardium (11, 16) and that MIF neutralization by anti-MIF antibody reversed endotoxin or burn injury-induced cardiac dysfunction (11, 50). Anti-MIF treatment also protected TNF-α knockout mice, which were sensitive to CLP and succumbed quickly to uncontrolled infection from lethal peritonitis induced by CLP (11). The septic TNF-α knockout mice were protected even if the treatment was started 8 h after the onset of bacterial peritonitis (11). In the present study, we observed an inverse relationship between cardiac function and myocardial MIF levels in sepsis. Specifically, cardiac function was significantly depressed, whereas myocardial MIF expression was significantly increased in the CLP mice. In contrast, GP-treated septic animals showed normal cardiac function and myocardial MIF levels that were equivalent to the untreated controls. These data suggest that GP preserved cardiac function in septic mice while preventing upregulation of MIF expression in the myocardium. The mechanism(s) by which GP prevented myocardial MIF expression are unclear. Recent studies suggest that IL-1-β-induced MIF synthesis by human endometrial stromal cells is mediated via NF-κB activation, because blockade of NF-κB translocation into the nucleus significantly inhibited MIF secretion (9). MIF also regulates TLR-4 expression (37, 38). Activation of the TLR-4 signaling pathway leads to NF-κB activation (37, 38). In addition, MIF-deficient macrophages were found to be hyporesponsive to LPS stimulation due to downregulation of TLR-4 expression (37, 38). In our previous studies, we have reported that GP blunted TLR-4 upregulation and inhibited NF-κB activation in CLP sepsis. Therefore, it is possible that the effect of GP on MIF expression in the myocardium may involve modulation of sepsis-induced TLR-4 and NF-κB signaling.

Cardiac myocyte apoptosis plays an important role in cardiac dysfunction (28). Numerous studies have shown that apoptosis plays a significant role in the morbidity and mortality associated with sepsis (4). By way of example, prevention of apoptosis with caspase inhibitors significantly improved survival in murine CLP-induced sepsis (10, 24). Support for this concept can also be found in the work of Bommhardt et al. (4). These investigators reported that mice that constitutively overexpress active Akt in their lymphocytes showed decreased lymphocyte apoptosis, a T-helper type 1 cytokine propensity, and a marked improvement in survival outcome in response to CLP sepsis (4). We have previously shown that CLP-induced sepsis significantly increased apoptosis in the lung and spleen (45). In the present study, we observed that cardiac myocyte apoptosis was significantly increased in septic mice. GP administration significantly reduced cardiac myocyte apoptosis and decreased caspase-3 activity in the myocardium of the septic mice. In addition, GP prevented the decrease in expression of Bel-2 in the myocardium in septic mice. The results were consistent with our previous observation that GP significantly decreased splenocyte apoptosis and caspase-3 activity in CLP-induced septic mice (45). Recent studies suggested that death receptor-mediated apoptotic signaling contributes to septic shock-induced apoptosis (41). For example, caspase-8 activity was significantly increased in the myocardium of LPS-induced cardiac dysfunction (24) and in vivo delivery of caspase-8 or Fas small interfering RNA improved the survival of septic mice (41). Interestingly, stimulation of TLRs can result in apoptosis by triggering proapoptotic signaling (2, 19, 31) and blocking TLR signaling by transfection of dominant negative MyD88 or dominant negative Fas-associated death-domain protein reduced cell death (19). These observations suggest that death receptor-mediated signaling is involved in TLR-mediated apoptosis. We have observed that overexpression of TLR-2 and TLR-4 contributed to apoptosis (14) and that GP administration significantly reduced I/R-mediated cardiac myocyte apoptosis through modulation of the TLR-4-mediated signaling pathway (28). GP administration also reduced the expression of TLR-4 in the tissues of CLP mice (44). Thus we speculate that GP treatment reduces cardiac myocyte apoptosis by modulating TLR-4-mediated apoptotic signaling pathways in the myocardium of septic mice.
Activation of the PI3K/Akt signaling pathway has been shown to prevent apoptosis and promote cell survival (15, 51). We have reported that inhibition of PI3K/Akt by wortmannin significantly increased apoptosis and resulted in a change in the distribution of splenocyte apoptotic profiles in CLP sepsis (45). We have also shown that GP mediated protection in CLP sepsis (45) and myocardial I/R injury (28) through a PI3K/Akt-dependent mechanism. In the present study, we observed that GP prevented the decrease in myocardial phospho-Akt levels by which GP exerts its cardioprotective effect.

In summary, GP administration attenuated cardiac dysfunction in CLP sepsis. The mechanisms by which GP attenuated cardiac function include activation of Akt, inhibition of MIF expression, and reduction of cardiac myocyte apoptosis. The present study also indicates that increased expression of MIF and cardiac myocyte apoptosis in the myocardium could contribute to the depression of cardiac function in CLP-induced sepsis. Future studies are needed to determine whether specific blocking of MIF expression will prevent septic shock-induced cardiac dysfunction and whether treatment with GP after sepsis has been initiated will prevent cardiac dysfunction. In addition, studies will be needed to determine the molecular mechanisms by which GP exerts its cardioprotective effect.

REFERENCES


10. Fan W, Ha T, Li Y, Ozment-Skelton T, Williams DL, Kelley J, Browder IW, and Li C. Glucan treatment also resulted in increased myocardial phosphorylation of GSK-3β. Phosphorylation of Akt at Ser473 activates the enzyme, whereas phosphorylation of GSK-3β at Ser9 results in its inactivity (32). The data showed that glucan treatment activates myocardial Akt and inactivates myocardial GSK-3β. These changes in Akt/GSK-3β activity correlate with decreased myocardial apoptosis and improved cardiac function in CLP sepsis (45).


