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Protective effect of phosphatidylserine blockade in sepsis induced organ dysfunction

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ABSTRACT

Background: Phosphatidylserine is usually an intracellularly oriented cell membrane phospholipid. Externalized phosphatidylserine on activated cells is a signal for phagocytosis. In sepsis, persistent phosphatidylserine exposure is also a signal for activation of the coagulation and inflammatory cascades. As such, phosphatidylserine may be a key molecule in sepsis induced cellular and organ injury. We hypothesize that phosphatidylserine blockade provides a protective effect in sepsis induced organ dysfunction.

Methods: Sepsis was induced in adult female rats using an endotoxin model. Diannexin, a homodimer of annexin A5, was administered for phosphatidylserine blockade. Rats were allocated to control ($n = 5$), sepsis ($n = 6$), or sepsis and phosphatidylserine blockade ($n = 9$) groups. Gut, pulmonary, renal, and hematologic dysfunctions were evaluated by mesenteric microvascular fluid leak, partial pressure of oxygen, serum creatinine, activated clotting time, and glomerular fibrin deposition, respectively.

Results: Rats in the sepsis group demonstrated gut, renal, and hematologic dysfunction. Phosphatidylserine blockade reversed signs of gut dysfunction and mesenteric microvascular leak ($P < .01$). In addition, phosphatidylserine blockade corrected systemic coagulopathy, as measured by activated clotting time ($P = .03$) and glomerular fibrin deposition ($P = .008$). There was no difference in renal dysfunction ($P = .1$) or pulmonary dysfunction in any of the groups ($P = .6$).

Conclusion: In sepsis, phosphatidylserine blockade had a protective effect on gut dysfunction and coagulopathy. Increased phosphatidylserine exposure may be a key mediator of organ dysfunction and coagulopathy during sepsis. These data may provide insights into novel treatment options for septic patients.

Introduction

Sepsis is a complex disease process with a prolonged inflammatory and anti-inflammatory mechanism. Although aimed initially at clearance of infection, this ultimately can result in collateral tissue injury, extensive morbidity, and death. Dysregulation of inflammatory mediators, coagulation, fibrinolysis, and compliment are presumed to underlie the impaired tissue oxygenation, endothelial dysfunction, and cellular death present in sepsis.[1] At the cellular level, signaling molecules on the plasma membrane seem to play a pivotal role in disease activation and perpetuation.[2]

In normal physiologic states, plasma membrane phospholipids are maintained in an asymmetric distribution, with anionic phospholipids concentrated in the intracellular membrane. In particular, the phospholipid phosphatidylserine (PS) is localized almost exclusively to the inner leaflet of the lipid bilayer.[3] Its orientation is maintained via energy dependent aminophospholipid translocases (flippase and floppase).[4–6] However, with cellular aging or stress, activation of nonspecific lipid transporters (scramblases) results in loss of the lipid gradient and prolonged extracellular PS exposure on the outer leaflet of the lipid bilayer.[7–9]

During homeostasis, PS exposure is an important apoptotic signal to rid the body of senescent and damaged cells, avoiding spillage of cellular contents and inflammatory milieu.[4,8] However, in sepsis, PS exposure is aberrantly up-regulated on cell surfaces throughout the body, including endothelial cells, platelets, erythrocytes, neutrophils, lymphocytes, and extra cellular microparticles. The degree and persistence of extracellular PS exposure

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activates the body's inflammatory and coagulation cascades and is presumed to underlie much of the organ injury inflicted during sepsis.[4,10–12]

A potent endogenous blocker of PS signaling is annexin A5. A member of the annexin family, annexin A5 is a phospholipid binding protein with selective affinity for PS.[13] The 73.1kDa homodimer of annexin A5, Diannexin, was synthesized to allow for a longer half-life and enhanced PS binding affinity compared with endogenous annexin A5.[14,15] Previous studies have demonstrated the ability of Diannexin to bind externalized PS residues and reduce the inflammatory mediated tissue damage in ischemia reperfusion injury[16–18] and transplant models.[19–21]

In ischemia reperfusion injury, endothelial dysfunction and inflammatory signaling promote extravasation of fluid into the interstitium predisposing to multi organ dysfunction in the critically ill. We have previously reported reduced microvascular leak in ischemia reperfusion injury with PS blockade.[18] Considering the significance of microvascular dysfunction in both ischemia reperfusion injury and sepsis, we sought to probe the role of PS externalization in sepsis. Therefore, we hypothesized that PS blockade with Diannexin would provide a protective effect against sepsis induced organ dysfunction. To test this, we examined the outcome of PS blockade on lipopolysaccharide (LPS)-induced gut, renal, pulmonary, and coagulation dysfunction.

Methods

Animal and solution preparation

All studies were approved by an Institutional Committee for the use of animals and complied with institutional animal research protocols. All animals were allowed food and water ad libitum. Animal preparation and Ringer's solution have been described previously.[22]

In brief, the Ringer's solution was prepared daily in distilled deionized water containing, in mM, 135 NaCl, 4.6 KCl, 2.46 $MgSO_4$, 5.0 NaHCO3, 5.5 dextrose, 9.03 HEPES salt, and 11.04 HEPES acid (Research Organics, Cleveland, OH). A 1% bovine serum albumin (BSA) Ringer's perfusate was prepared by adding BSA (BSA crystallized, Sigma, St. Louis, MO) to the Ringer's solution.

Adult female Sprague-Dawley rats (225–300 g; Hilltop Lab Animals Inc., Scottsdale, PA), were anesthetized with subcutaneous sodium pentobarbitol (55 mg/kg body weight). Female rats were used to minimize potential sex related variability in biologic response. The femoral vessels were cannulated and venous blood obtained for study serology. The mesentery was exposed via a midline incision and the bowel positioned on an inverted microscope (Diaphot, Nikon; Melville NY). The animal's mesentery was bathed continuously in Ringer's solution and body temperature was maintained at 37°C throughout the study period. All rats were allowed a 30-minute stabilization period before administration of treatments.

Sepsis protocol

Rats were allocated to 1 of 3 experimental groups: sepsis ($n = 6$), sepsis with phosphatidylserine blockade ($n = 9$), and control ($n = 5$). Sepsis was induced using a well-accepted endotoxin sepsis murine model.[23] This method was employed over the infection model (cecal ligation and puncture) given its compatibility with obtaining accurate microvascular leak (L_p) measurements. Its limitations are previously described.[23–25] In brief, after obtaining baseline serology and L_p measurements, sepsis was induced with LPS at a constant infusion rate of 0.65 mg/h. Infusions were run during a 4-hour time period. In the PS blockade group, endotoxin sepsis was induced in similar fashion. PS blockade was subsequently accomplished 20 minutes after initiation of LPS infusion by the administration of Diannexin blockade, 400 μg/kg. Diannexin redosing (400 μg/kg) was administered at hour 2 of the 4-hour study period. Dosing parameters were based on a phase II trial using Diannexin in kidney transplantation (NCT00615966). In this phase II trial, administration of Diannexin up to 400 μg/kg was not associated with adverse events in recipients and showed reduced dialysis days and incidence of delayed graft function.[19] Doses of ≤1,000 μg/kg have been shown to be safe in animal studies.[21] For control animals, all steps were carried out in the same manner as the sepsis experimental group, except in lieu of LPS, infusion was performed with 1% BSA Ringer's perfusate at 0.65 mg/h. To evaluate our study hypothesis, evidence of gut, renal, and pulmonary dysfunction and coagulopathy were evaluated in each group. Serology and L_p measurements were conducted in the same manner for all groups.

Gut dysfunction (microvascular leak, L_p)

Single vessel L_p (microvascular leak, hydraulic conductivity) was determined using the modified Landis micro-occlusion technique.[26] The assumptions and limitations of this technique have been previously described.[27] Mesenteric post capillary venules, 20 to 30 μm in diameter and at least 400 μm in length, were identified based on flow patterns. Vessels with no evidence of leukocyte adherence or side branches were chosen. The vessels were cannulated with micropipettes attached to a water manometer to control hydrostatic perfusion pressure. Initial cell velocity (dl/dt) was obtained by recording marker cell position as a function of time. Transmural water flux per unit area (Jv/S) was calculated using the equation $Jv/S = (dl/dt)(r/2l)$, where r is the capillary radius and l is the initial distance between the marker cell and the occluded site. L_p was determined using a modified version of Starling's equation of fluid filtration: $L_p = (Jv/S)(1/Pc)$, where Pc is the capillary hydrostatic pressure. L_p was calculated from the slope of the regression of Jv/S on Pc derived from several occlusions at 3 different perfusate pressures. Control studies that document the stability of this model over time, and after multiple recannulations of the vessels, have been reported.[22,28,29]

In all study animals, microvessels were perfused with 1% BSA Ringer's solution for 10 minutes before obtaining baseline microvascular leak rates. L_p was subsequently measured at 1-hour intervals throughout the infusion period to assess the effect of PS blockade during endotoxemia.

Renal, pulmonary dysfunction

Venous and arterial blood was obtained after induction of anesthesia to establish baseline creatinine (Cr) and partial pressure of oxygen (PaO_2) parameters. Venous and arterial sampling was repeated after termination of infusion. Post infusion creatinine and PaO_2 levels were obtained. To evaluate renal dysfunction, percentage changes from baseline serum Cr were measured in each study group. Percentage change in PaO_2 from baseline was measured in each study group to evaluate pulmonary dysfunction. Laboratory values were measured using Abbot Point of Care i-STAT System (Abbott Point of Care Inc., Princeton, NJ) per manufacture protocol. Serum was centrifuged for 20 minutes at 2,000×g and stored at −20°C.

Coagulopathy

Hematologic dysfunction was evaluated using 2 parameters, activated clotting time (ACT) and glomerular fibrin deposition. As

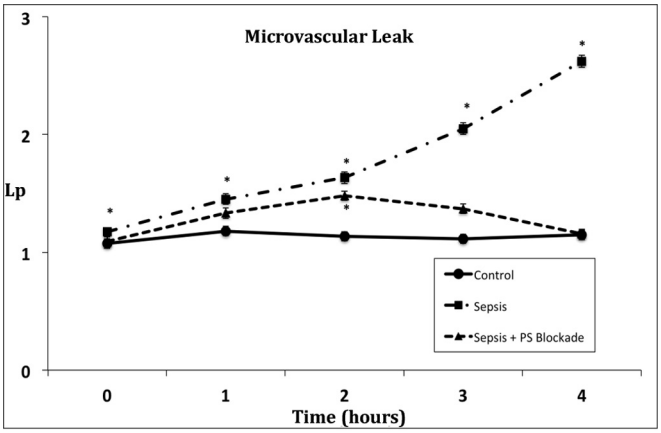

Fig 1. Phosphatidylserine (PS) blockade protects against gut dysfunction (Microvascular Leak Rate, *Lp)* in sepsis. Diannexin blockade (400 μg/kg) was administered 20 minutes after initiation of LPS infusion and redosed at 2 hours. L_p increased >2-fold from baseline in the sepsis group. L_p returned to baseline levels with PS blockade. *Lp* units = 10^{-7} cm·s $^{-1}$·cmH_2O $^{-1}$. *P < .01 versus control. Error bars indicate SEM.

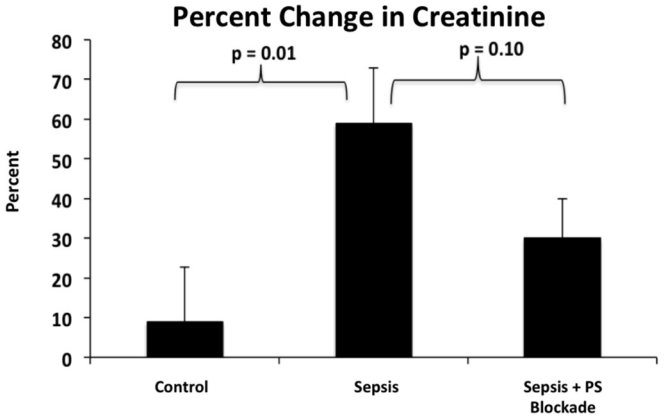

Fig 2. Effect of PS blockade on renal dysfunction. Renal dysfunction represented by percentage change from baseline creatinine (Cr) levels. Septic rats demonstrated a 59% increase from baseline Cr levels (P = .01 versus control). With PS blockade (Diannexin 400 μg/kg), Cr levels increased 40%; however this did not reach statistical significance (P = .1 versus control). Error bars indicate SEM.

described earlier, ACT was measured from venous blood obtained immediately after induction of anesthesia and was compared to blood samples obtained at termination of the infusion period. At termination of the study period, rats were killed and renal tissue was collected and processed with hematoxylin and eosin stain. Fibrin deposition was examined using light microscopy at 40× magnification. One hundred consecutive glomeruli were evaluated per study animal, and proportions of glomeruli with fibrin deposition were tabulated. Glomerular fibrin deposition assessment of coagulopathy has been previously described.[30]

Statistical analysis

Data are presented as mean ± standard error of the mean, except when fold increases or percentage change are reported. Statistical analysis was performed with paired *t* test and analysis of variance assuming normal distribution. *Lp* values are represented as mean ± standard error of the mean 10^{-7} cm·s $^{-1}$·cmH_2O $^{-1}$.

Results

Effect of PS blockade on gut dysfunction

PS blockade with Diannexin attenuated the endotoxin induced increase in mesenteric L_p. In the sepsis group, LPS infusion for 4 hours increased L_p more than 2-fold from baseline (L_p = 1.17 ± 0.03 to 2.62 ± 0.2; P < .01). With PS blockade, L_p increased during the first 2 hours of infusion (from 1.16 ± 0.01 to 1.48 ± 0.01); however, unlike the sepsis group, L_p decreased to baseline levels at 4 hours (P < .01). Control animals showed no change from baseline L_p rates (Fig 1).

Effect of PS blockade on renal dysfunction

PS blockade demonstrated a progression toward improvement of sepsis-induced renal impairment. Renal dysfunction was evident in septic rats with mean Cr levels demonstrating a 59% increase from baseline levels (0.53–0.88 mg/dL, P = .01). In the sepsis and PS blockade group, Cr levels increased 40% from baseline, from 0.5 to 0.70 mg/dL, compared with the sepsis group, however this did not reach statistical significance (P = .1). Control animals showed no change from baseline Cr levels (Fig 2).

Effect of PS blockade on pulmonary dysfunction

Pulmonary dysfunction, as measured by arterial blood oxygenation (PaO_2), did not change throughout the study period in the sepsis, blockade, or control groups (P = .6, data not shown).

Effect of PS blockade on coagulopathy

PS blockade mitigated sepsis-induced coagulopathy. Dysfunction of coagulation was noted in the sepsis group. In septic rats, ACT increased 19% from baseline, whereas control animals showed an 8% decrease from baseline ACT (P = .04). PS blockade prevented elevation of ACT among study rats. As in the control group, ACT in the PS blockade group decreased 8% from baseline (P = .03; Fig 3).

PS blockade also prevented glomerular fibrin deposition owing to sepsis. Coagulopathic glomerular fibrin deposition was evident in histology from septic rats with increased glomerular fibrin deposition from control values of 20% ± 4.6% glomeruli involvement to 99.5% ± 0.5% in sepsis (P < .001). A protective effect was noted with PS blockade, as glomerular fibrin deposition improved from 99.5% ± 0.5% glomeruli involvement in sepsis alone to 20% ± 9.8% with PS blockade in sepsis (P = .008; Fig 4).

Discussion

Sepsis develops as a result of a complex, dysregulated host immune response leading to cellular dysfunction, tissue injury, and ultimately organ dysfunction.[2] Sepsis-related microvascular and endothelial dysfunction is presumed to perpetuate tissue edema, cellular hypoxia, and tissue ischemia, resulting in a vicious cycle leading to multiple organ failure and even death. PS exposure on the exterior surface of the bilipid cellular membrane seems to be an important signaling molecule in this cascade of events.[4,7,10–12] Diannexin, the homodimer of annexin A5, is known to avidly bind and block externalized PS signaling.[14–17] Therefore, we sought to evaluate whether Diannexin-induced PS blockade would demonstrate a protective effect on organ dysfunction from septic insult. Our hypothesis was that PS blockade with Diannexin would protect against sepsis-induced organ dysfunction.

We found that PS blockade reversed gut dysfunction in our sepsis model, with correction of sepsis-induced mesenteric endothelial leak back to baseline levels. In sepsis, profound alterations in the endothelium occur resulting in extravascular leak

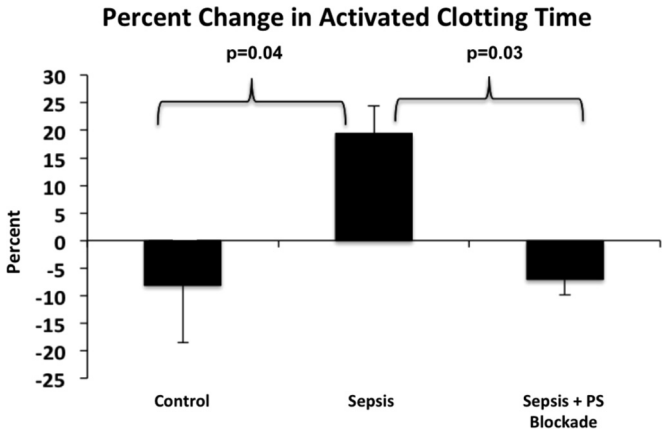

Fig 3. PS blockade corrects prolongation of ACT in sepsis. ACT in controls decreased 8% from baseline. However, ACT increased 19% from baseline in septic rats ($P = .04$ versus controls), whereas PS blockade (Diannexin 400 μg/kg) prevented prolonged ACT among study animals. ACT in the PS blockade group decreased 8% from baseline similar to control animals ($P = .03$ versus septic rats). Error bars indicate SEM.

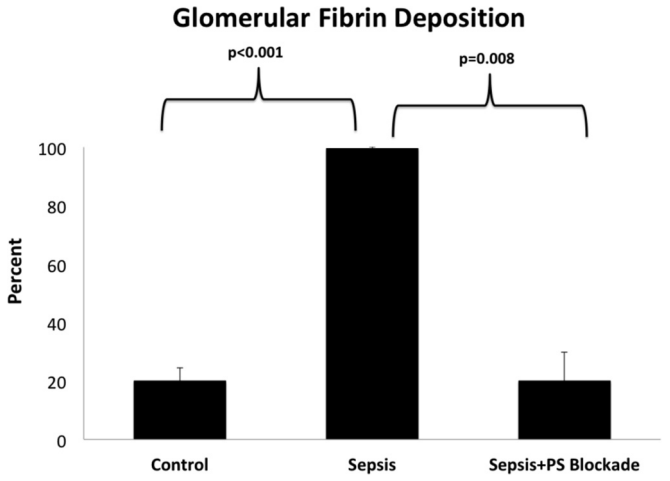

Fig 4. PS blockade prevents coagulopathic glomerular fibrin deposition. Glomeruli stained with hematoxylin and eosin were examined using light microscopy at 40× magnification for fibrin deposition. In sepsis, glomeruli demonstrated 99.5% fibrin deposition ($P < .001$ versus controls). With PS blockade (Diannexin 400 μg/kg), glomeruli demonstrated 20% fibrin deposition ($P = .008$ versus sepsis group). Error bars indicate SEM.

and gut edema. Fluid extravasation into the gut mesentery leads to gut edema, delayed transit time, and impaired barrier function.[31] Our study demonstrates, in vivo, the ability to prevent LPS-induced gut dysfunction with PS blockade. In sepsis, endothelial leak results from disassembly of intercellular junctions by cytokines and other inflammatory mediators.[32] Marked increase in PS externalization on endothelial cells is presumed to be a key signaling molecule inducing this inflammatory endothelial injury.[10,33] Diannexin blockade seems to suppress leukocyte adhesion, inflammatory infiltrate, and thrombin generation,[14,20] all of which contribute to ongoing endothelial damage in sepsis, and may be the reasons for the improvement in endothelial leak with PS blockade.

In the PS blockade group we initially observed a mild increase in L_p. However, L_p corrected to basal levels by study termination. Likely, this was due to the timing of dosing Diannexin after initiating LPS infusion. Because Diannexin was administered 20 minutes after LPS infusion, rat mesenteric endothelium was exposed to the effects of LPS without PS blockade. This has important therapeutic implications because it suggests the benefit derived from PS blockade is not reliant on administration before LPS exposure. PS blockade obtained early in LPS exposure may correct reversible organ dysfunction and provide an ongoing protective benefit. Other studies have reported similar findings in murine hepatic ischemia reperfusion injury[34] and endotoxemia[35] with delayed Diannexin administration.

We also observed the ability of PS blockade to prevent serologic and histologic evidence of LPS-induced coagulopathy. In sepsis, early thrombin generation is considered a pivotal event in tissue injury via microvascular fibrin deposition, thrombosis, and tissue ischemia.[11] Persistent externalization of PS on endothelium triggers prothrombinase assembly and activation of the coagulation cascade. In addition, externalized PS on platelet microparticles triggers thrombin formation and subsequent coagulopathy in sepsis.[10,11] In the sepsis group, ACT was increased corresponding to ongoing coagulopathy. With PS blockade, we demonstrated a protective effect against septic induced coagulopathy. Furthermore, despite extensive glomeruli fibrin deposition in our sepsis rats, PS blockade mitigated fibrin deposition. In coagulopathic states, intravascular fibrin clogs the microvasculature resulting in tissue ischemia and injury. Our study supports the role of PS blockade in attenuating sepsis-induced microvascular fibrin occlusion and resultant tissue ischemia.

The therapeutic effect of PS blockade with Diannexin has predominately been studied in the setting of renal, hepatic, and pancreatic ischemia reperfusion injury,[16,20,34] solid organ transplant,[14,19–21] and muscle flaps.[17] During hypoxia, PS is externalized on the cellular membrane of endothelial cells, hematologic cells, and microvesicles shed from injured cells.[36,37] PS residues provide attachment sites for leukocytes, activated platelets, prothrombinase complexes and secretory forms of phospholipase A2. Overall, this initiates generation of thrombin, compliment, and inflammatory lipid mediators leading to microvascular thrombotic occlusion, endothelial injury, and edema.[10,11] Diannexin binding to exposed PS mitigates all these effects.

In this study, attenuation of renal dysfunction with PS blockade was not observed. Studies examining renal reperfusion injury and transplantation have described the ability of Diannexin PS blockade to decrease renal ischemic injury.[16,19] In particular, Diannexin inhibition in mouse ischemia reperfusion injury decreased proximal tubule damage and leukocyte influx,[16] and in marginal donor renal transplant recipients demonstated reduced rates of delayed graft function and hemodialysis days.[19] Although our results failed to demonstrate a clear renal protective benefit with PS blockade, we did not directly evaluate renal injury. Serum creatinine is limited as a marker for renal injury given many renal and nonrenal factors influencing its level in the blood. Serum levels can be elevated during catabolic states such as sepsis, volume depletion, and other causes of decreased renal perfusion independent of kidney injury. Morever, acute changes in creatinine lag behind renal recovery.[38,39] We suspect these inherent limitations of serum creatinine contributed to our study results.

In summary, dysregulation of the host immune and hematologic response underlies multiple organ dysfunction in sepsis. Externalization of PS on the outer leaflet of the lipid bilayer appears to be a key mediator via multiple pathways. PS mediated activation of inflammatory cytokines, the coagulation cascade and complement, triggers concomitant microvascular thrombosis, hypoxia, endothelial leak, edema and tissue injury.[10,32] We have demonstrated that PS blockade with Diannexin mitigates gut dysfunction and the coagulopathy induced by sepsis. Our findings suggest a potential therapeutic target to prevent organ injury in critically ill septic patients.

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Conflict of interest/Disclosures

The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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