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EFFECT OF LIPOPOLYSACCHARIDE ON THE PHYSICAL CONFORMATION OF THE ERYTHROCYTE CYTOSKELETAL PROTEINS

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Summary

Red blood cell deformability is important for effective circulation in the capillaries. It is known that red cell deformability is significantly reduced during septic shock. Surface to volume ratio, physical effects of the cytoskeletal proteins and the fluidity of lipid bilayer are some of the important intrinsic factors that regulate this mechanical function. Alterations in the physical conformation of cytoskeletal proteins in septic conditions could significantly alter their function. In this study, erythrocytes in whole blood were treated with lipopolysaccharide, the outer covering of Gram-negative bacteria released during Gram-negative sepsis. Electron paramagnetic resonance spectroscopy in conjunction with a protein-specific maleimide nitroxide spin label covalently bound to cytoskeletal proteins was used to investigate the resulting changes occurring in the physical state of cytoskeletal proteins in isolated membranes. Treatment of red blood cells with a lipopolysaccharide concentration as low as 40 μg/mL of blood solution for 90 minutes showed a significant decrease in the relevant EPR parameter ($p<0.01$) of the spin label bound to subsequently isolated membranes, suggestive of a decreased segmental motion of the spin label and an increase in cytoskeletal protein-protein interactions. These results suggest a marked conformational alteration in the cytoskeletal proteins induced by the lipopolysaccharide and may explain, in part, the marked reduction in red blood cell deformability during septic shock. Bacterial lipopolysaccharide does not exert most of its effects on the host directly, but rather elicits the production of host factors that leads to complex septic shock. Leukocytes, endothelial tissue and many other cells release these mediators. Leukocytes are thought to be a particularly important source of such mediators, including cytokines (tumor necrosis factor, interleukins, etc.), oxygen free radicals, proteases, and hydrolyses. In order to characterize the possible mechanism by which the lipopolysaccharide acts on the physical state of the erythrocyte cytoskeleton, erythrocytes void of leukocytes and plasma were treated with lipopolysaccharide. The relevant EPR parameter showed no significant change over the control value. These results indicate that the leukocytes and their factors are responsible for the rearrangements seen in the cytoskeletal proteins of the erythrocyte membrane.

Key Words: lipopolysaccharide, sepsis, cytoskeletal proteins

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Septic shock is a potentially lethal complication that can develop due to a bacterial infection. The incidence of this disorder has risen from 74 cases per 100,000 people in 1979 to 176 cases per 100,000 people in 1987 in the USA (1). The definition of septic shock has been an issue of considerable debate and a consensus has been reached only recently suggesting that an increase in heart and respiratory rate, vasodilatation, and increased leukocyte count are the criteria (2). Bacterial components that have been identified to cause the complex septic cascade leading to biochemical, hemodynamic and physiological alterations are lipopolysaccharides (LPS), the outer covering of the Gram-negative bacteria, peptidoglycans, the elementary cell wall components of Gram-positive bacteria, and exotoxins of Gram-positive bacteria (3). Activation of the plasma kallikrein system, production of prostaglandins (4), vasoactive kinins (5), and cytokines (3) are characteristics of septic shock.

Red blood cell deformability is critical for effective microcirculatory function. Impaired red cell deformability has been shown to alter the hemodynamics of blood flow in diseases such as diabetes, hemolytic and sickle cell anemia. Patients with septic shock display similar impaired hemodynamics, and it is believed that altered red cell mechanics may be partly responsible for reduced tissue oxygen delivery observed during septic shock (6-8).

The erythrocyte membrane consists of a protein meshwork on its cytoplasmic face which is connected by noncovalent protein linkages to the transmembrane proteins that are intercalated to various extents in the membrane lipid bilayer. The principal component of cytoskeletal proteins is spectrin, which is attached to the membrane proteins at two junctions. Band 4.1 links the spectrin network to the transmembrane protein glycophorin (9-11), while ankyrin (Band 2.1 protein) links the protein network to the Band 3 transmembrane protein in another junctional complex (12). Spectrin is largely responsible for the structural stability and regulation of cellular deformability (13). Mohandas et al., 1986 (14), showed that spectrin molecules are in a folded conformation in an unstressed state but undergo linear extensions on application of fluid forces. On reversible deformation, they rearrange with uncoiling and unwinding. Any changes in the structural conformation of these proteins or a change in the cytoskeletal components may adversely affect the cellular deformability that is so important for the passage of the cell through the microcapillaries and for effective oxygen transport.

In previous studies we have shown that viscoelastic properties of the erythrocyte membrane are altered following treatment of whole blood with lipopolysaccharide (LPS) (15), and we also observed that a significant decrease in the erythrocyte membrane lipid bilayer fluidity occurred following treatment of whole blood with LPS (16). Prior investigations from our laboratory have shown that changes in the structural conformation of one component of the membrane can affect the structure and function of the other components (17-23). Thus, it is conceivable that decreases in erythrocyte membrane lipid fluidity that we observed following LPS addition to whole blood (16) may contribute to changes in the protein-protein interactions of the transmembrane and cytoskeletal proteins.

Macrophages and polymorphonuclear neutrophils (PMNs) release many mediators that play an important role in septic shock. Such mediators include cytokines, prostaglandins, amines, proteases, and lysosomal hydrolyses. Most of the mediators affect the function of diverse target cells at minute concentrations. Cytokines, for example, are capable of mediating a wide range of biological effects, many of which are essential for the metabolic, hemodynamic, immunologic and wound-healing responses to injury and infection. The continued production of these mediators even after the infection has been countered, will result in the attack of host cells and tissues.

We and other investigators have shown that red blood cell deformability remained unaltered in the absence of the plasma components (15,24), and out experiments with the lipid order and motion show no alterations in the lipid fluidity in the absence of plasma components and leukocytes (16). In view of the importance of leukocyte activity during septic shock, in this study red blood cells void of leukocytes and plasma were incubated with LPS and changes in their protein cytoskeletons were compared with those isolated from red blood cells incubated with LPS in whole blood. A protein-specific nitroxide spin label covalently bound to the sulfhydryl groups of cytoskeletal proteins was used to study the physical state of cytoskeletal proteins in subsequently isolated membranes.

Materials and Methods

Blood Preparation

Six male or female Sprague-Dawley rats each weighing between 200-250 g were used for experiments. The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (65 mg/kg i.p.). Blood was drawn by cardiac puncture and collected in heparinized vacutainers. A minimum of 10 cc of blood was drawn from each rat, and the rat was then sacrificed by a injection of KCl. The procedures were approved by the University of Kentucky Institutional Animal Care Committee.

Lipopolysaccharide Stock Solution Preparation

Lipopolysaccharide from *E. Coli* (Serotype: 0111:B4, Sigma Chemical Co.) was available in 5 mg vials and was mixed with phosphate buffered saline (pH = 7.4) to make solutions of various concentrations. Three batches of lipopolysaccharide stock solution were prepared at described below:

Batch 1: 5 mg of lipopolysaccharide + 100 mL of phosphate buffered saline

Batch 2: 5 mg of lipopolysaccharide + 62.5 mL of phosphate buffered saline

Batch 3: 5 mg of lipopolysaccharide + 14.5 mL of phosphate buffered saline

The final concentrations of the batch solutions prepared were 50, 80, and 340 μg LPS/mL of PBS, respectively.

LPS Treatment and Erythrocyte Ghosts Preparation

Whole Blood Studies: whole blood from each rat was divided into four equal aliquots; three aliquots were used to prepare samples by adding 0.5 mL of lipopolysaccharide of three different concentrations to 0.5 mL of whole blood to make final concentrations of 25, 40, and 170 μg LPS per mL of blood solution; each suspension was then incubated for 90 minutes at 4°C. The blood sample for the baseline/control was incubated for 90 minutes with 0.5 mL of PBS per 0.5 mL of whole blood. The LPS-treated blood and the control samples were used to prepare membrane ghosts. To isolate ghosts, approximately 5 mL aliquots of LPS-treated blood were placed in 30 mL plastic Corex tubes and washed three times with PBS (pH = 8) in a refrigerated centrifuge at 1500 × g for 5 minutes at 4°C. The washed cells free of plasma and leukocytes were lysed in 5 mM sodium phosphate buffer (5P8, pH = 8) for 30 minutes at 4°C. The lysed cells were washed four to five times with 5P8 at 27,000 × g for 10 minutes at 4°C to remove all the hemoglobin to make membrane ghosts.

Leukocyte-Free Blood Studies: Whole blood was centrifuged at 1500 × g for 5 minutes at 4°C and was washed with PBS (pH = 7.4). The leukocyte-free blood was divided into two aliquots; 0.5 mL of packed erythrocytes from one aliquot was incubated with 0.5 mL of PBS to serve as a baseline. The other aliquot was used for LPS incubation. Half a mL of packed erythrocytes were incubated with 0.5 mL of LPS to make a final concentration of 170 μg per mL of erythrocytes. The samples were mixed using a vortex and incubated for 90 minutes at 4°C. The incubated cells were lysed and membrane ghosts were prepared as described above.

Protein Spin Labeling

The cytoskeletal proteins of the erythrocyte membrane ghosts were spin labeled with N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl) maleimide (MAL-6) as previously described (20).

Approximately 90% of the spin label covalently binds to spectrin, while the rest binds to Band 3, Band 2.1, and other high molecular weight proteins. The relevant electron paramagnetic resonance (EPR) spectroscopy parameter derived from these experiments with the piperidine maleimide spin label on proteins was the ratio of spectral amplitudes of motionally determined weakly immobilized sites to the spectral amplitude of strongly immobilized sites (W/S ratio) of the low field line ($M_I = +1$). Changes in the W/S ratio provide insight into the perturbations in the normal conformation of cytoskeletal proteins (17-23). Previous studies in our laboratory showed that alterations in cytoskeletal protein ultrastructure that led to decreased protein-protein interactions after addition of hemin (21), polyphosphates (23), or increase in dimeric state of spectrin (18) loosen the conformation state of spectrin resulting in a decreased hindrance for the spin label to move, and yielding an increased W/S ratio. Other studies showed, in contrast, that alterations in the cytoskeleton leading to increased protein-protein interactions observed by the addition of spermine (22) or an increase in tetrameric state (18) resulted in increased steric hindrance of the spin label, leading to a decreased W/S ratio. Barber et al. (25) confirmed that the spectra obtained from the spin labeling of erythrocyte ghosts with MAL-6 by this procedure resulted in highly reproducible spectra.

Electron Paramagnetic Resonance Spectroscopy

EPR spectroscopy was performed with a protein concentration of 2.5-3.0 mg/mL in a flat quartz cell made for aqueous solutions. The Bruker ESP 300 spectrometer was equipped with computerized data acquisition and analysis capabilities. The measurement mode parameters for spectral acquisition were a microwave frequency = 9.5 GHz, microwave power = 10 mW, modulation frequency = 100 KHz, modulation amplitude = 0.481 G and a time constant of 1.28 ms.

Data Analysis

Single factor repeated measures ANOVA and post hoc tests was used for statistical analysis of whole blood experiments. The Student's t-test with a was used for testing the statistical significance of the leukocyte free-blood experiments.

Results

The physical state of the cytoskeletal proteins of erythrocyte membranes was monitored by EPR using a protein specific nitroxide spin label (MAL-6). As previously mentioned, investigators using selective isolation experiments or antibodies to MAL-6 have shown that 90% of the spin label binds to spectrin, the major cytoskeletal protein (20). Since MAL-6 is membrane-permeable and consequently would perferentially bind to the high levels of SH groups in hemoglobin in the cytoplasm of intact cells (20), it was necessary to spin label isolated membranes [ghosts] (i.e., erythrocyte plasma membranes were isolated from LPS-treated whole blood or LPS-treated washed intact red blood cells). Figure 1 shows a typical EPR spectrum, and measurements used for the W/S ratio, the relevant EPR parameter calculated as a strong indicator of the physical state of the cytoskeletal proteins.

Tables I and II summarize the effect of LPS on the physical conformation of the cytoskeletal proteins of subsequently isolated ghosts following LPS addition to either whole blood or to washed erythrocytes, respectively. The treatment of whole blood with LPS of three different concentrations was followed by subsequent washings to remove plasma, multiple washings of the ghosts after lysis, overnight spin labeling of the membrane proteins and additional subsequent washings to remove excess spin label. In spite of these many washings, the W/S ratio was significantly decreased (Table I, $p<0.01$) over the tested range of concentrations suggesting that there is a permanent increase in protein-protein interactions of cytoskeletal proteins and/or integral proteins and the peripheral proteins following LPS treatment of whole blood. Significant effects of lipopolysaccharide occurred at LPS concentrations of 40 and 170 μg per mL of blood solutions when compared with the control (Table I, $p<0.01$). The maximum decrease in the W/S ratio was

observed with a LPS concentration of 40 μg/mL of blood solution (mean % of control ±S.D. = 91.4 ± 0.892). No significant effect was observed at an LPS concentration of 25 μg per mL.

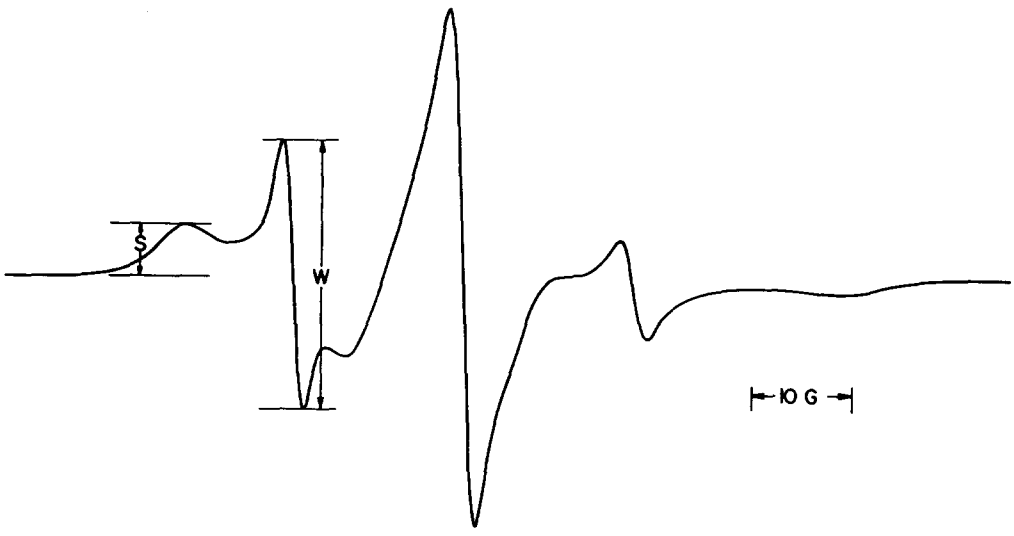

FIG. 1

Typical EPR spectrum of MAL-6 in erythrocyte membranes. S- and W- sites are indicated (see text).

TABLE I

Effect of Lipopolysaccharide Addition to Whole Blood on the Physical Conformation of Erythrocyte Membrane Skeletal Proteins in Subsequently Isolated Ghosts.

LPS Concentration μg/mL	W/S Ratio % of Control ± S.D.	Number of Rats N	Statistical Significance Assigned p-value < 0.05
0	100	6	---
25	98.0 ± 2.064	6	n.s.
40	91.4 ± 0.892	6	<0.01
170	93.4 ± 1.95	6	<0.01

The experiments with the leukocyte-free blood showed no significant alteration in the W/S ratio of the control/baseline with LPS treated samples (Table II), suggesting that leukocytes are important in the observed effects with whole blood.

TABLE II

Effect of Lipopolysaccharide Addition to Leukocyte-Free, Washed Erythrocytes on the Physical Conformation of Erythrocyte Membrane Skeletal Proteins in Subsequently Isolated Ghosts.

LPS Concentration μg/mL	W/S Ratio % of Control ± S.D.	Number of Rats N	Statistical Significance Assigned p-value < 0.05
0	100	6	---
170	100.5 ± 0.657	6	n.s.

Discussion

The results of this study suggesting a LPS-induced, permanent decrease in motion of cytoskeletal proteins in membrane ghosts isolated from red blood cells in whole blood strongly support the findings of our previous studies which showed impaired red cell deformability associated with increased erythrocyte membrane elasticity (15) and significant alterations in order and motion of the erythrocyte membrane lipids on treatment of whole blood with LPS (16). In previous investigations involving microcirculatory disorders, alterations in red blood cell deformability have been strongly related to molecular changes in the protein cytoskeleton (7,26). Here we suggest that the increase in protein-protein interactions of the cytoskeletal proteins on exposure to LPS may be partly responsible for the reduced red blood cell deformability. Wyse and Butterfield (22) showed a 30 to 50% decrease in the W/S ratio when erythrocytes were treated with spermine, a naturally occurring polyamine known to strengthen cytoskeletal protein-protein interactions. Although the magnitude of change in W/S ratio following LPS treatment is relatively small in comparison to changes in structural conformations seen by the addition of spermine, it should be recalled that the effect of lipopolysaccharide-induced alterations was permanent, significant, and reproducible after many washes of whole blood and isolated membranes.

A conceptual protein model (22) can be used to explain the increased protein-protein interactions. The decrease in W/S ratio may be either due to formation of higher order protein molecules resulting in reduced number of weakly immobilized sites or due to a complete transformation of the protein resulting in more strongly immobilized sites. Such alterations in the structural conformation of the protein cytoskeleton (spectrin, Band 2.1, Band 4.1) have been known to affect its interactions with the transbilayer proteins (Band 3 and glycophorin) and membrane lipids (9,27). This suggests that a coupled effect of reduced lipid motion diffusion (16) and an increase in protein-protein interactions (this work) may be the cause of reduced erythrocyte deformability seen during septic shock (15).

In our investigations, it is evident that lipid motion and order and protein interactions of erythrocyte membrane are affected on exosure to LPS. Cellular biochemical compositions such as ATP levels, cGMP's, cAMP's, Ca^{2+} and Mg^{2+} levels are important intrinsic factors that contribute to effective cellular deformation and possible alterations in their compositions during septic shock should be considered. Oxygen free radicals are secondary mediators in septic shock. Investigators have shown that oxygen radicals stimulate intracellular proteolysis and lipid peroxidation by independent mechanisms in erythrocytes (28). Filtration studies of erythrocytes treated with reactive oxygen species such as superoxide ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH\cdot$) have shown reduced erythrocyte membrane elastance (29,30). The

alterations we have seen in membrane elastance (15), lipid fluidity (16) and conformation of the protein cytoskeleton (this work) conceivably may be an effect of the reactive oxygen species released during septic shock.

The mechanism(s) of LPS interactions with erythrocyte membrane may either be specific or nonspecific. Specific interactions may involve lipids acting as LPS receptors (31), while nonspecific interactions may involve activation of plasma components (monocytes and PMNs) that release mediators which act on the erythrocyte membrane (1,29,32,33). Neter et al. (31) indicated that the red cell lipid bilayer may serve as receptors for the recognition of LPS and has provided evidence that the initial step is the specific attachment of LPS to the erythrocyte membrane. However the results of our EPR experiments with lipids (16) as well as proteins show that LPS-induced changes in red blood cell membrane properties are absent without plasma and leukocytes; these findings support a mechanism involving non-specific interactions. Among others, possible non-specific interactions include: (a) binding of LPS to leukocyte receptors leading to cytokine release and its subsequent action on red blood cells; and (b) LPS binding to the LPS binding protein (LBP) in the blood plasma, which subsequently binds to the CD14 receptor on macrophages, activating them to release mediators that act on red blood cells. Our studies are currently unable to distinguish between the possibilities since both would alter the physical state of the membranes that we ultimately examined. Therefore, further studies are warranted to elucidate the mechanism of and mediators involved in LPS-induced alterations in the physical state of cytoskeletal proteins in erythrocytes.

Acknowledgments

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