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Rapid Report

Effect of endotoxin on lipid order and motion in erythrocyte membranes

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Electron paramagnetic resonance employing a lipid-specific spin label has been used to investigate the molecular effects of endotoxin on the physical state of bilayer lipids in rat erythrocyte membranes. When added at a concentration as low as 40 μg/ml to whole blood (plasma plus leukocytes present), decreased membrane lipid motion was found in subsequently washed and spin-labeled intact erythrocytes ($P < 0.02$). However, if endotoxin were added to washed, plasma plus leukocyte-free intact erythrocytes, no change in the motion of the spin label was found, suggesting that plasma-soluble substances and/or leukocytes are required to produce the change in the physical state of lipids. The decreased lipid motion found in these studies is discussed with reference to the known decreased deformability of endotoxin-treated red cells and to the pathogenesis of sepsis.

Endotoxins, also known as somatic O-antigens, are lipopolysaccharide (LPS) complexes derived from the cell wall of Gram-negative bacteria. LPS form complexes of relatively large particle sizes due to aggregation of their amphiphilic structures: hydrophilic polysaccharide bound to hydrophobic lipid. They are generally composed of three typical regions: region I: the O-specific polysaccharide chain; region II: the R-specific core polysaccharide composed of the inner and outer core, and region III: lipid A [1]. Administration of endotoxin to experimental animals elicits a complex array of biochemical and physiological alterations which are characteristic of gram-negative sepsis. These include hematological changes, the production of vasoactive kinins, the activation of the plasma kallikrein system, the release of prostaglandins, the induction and release of cytokines, and marked alterations in hemodynamic status [1].

Sepsis has often been defined as the presence of pathogenic microorganisms and their toxins in the bloodstream. It is estimated that as many as 200,000 cases of sepsis occur in the United States annually with a mortality of 20–50%, making septicemia the 13th leading cause of death in the United States [2]. Recent reports have suggested that multiple organ system failure and other septic-related complications may be secondary to microcirculatory alterations [2]. Red blood cells (RBCs) have been shown to become less deformable during infection [2,3]. The sequence of this alteration is thought to be the inability of the RBC to alter its shape to traverse capillaries which results in a reduction of blood flow through the microcirculation, decreasing oxygen delivery to the tissues and leading to end-organ damage.

Clinically, many infections are caused by Gram-negative bacilli. *Escherichia coli* is the most commonly isolated pathogen, followed by *Klebsiella-Enterobacter* species. Hence, research efforts have been directed towards *E. coli* endotoxin in attempts to understand the pathophysiological mechanisms of sepsis. The decrease of RBC deformability was observed in the in vitro addition of *E. coli* endotoxin into blood, when assessed with either the micropipette aspiration technique or microfiltration methods [3–6]. Recent studies showed that the effects of endotoxin on certain blood cells are mediated by plasma-soluble substances such as cytokines, including tumor neurosis factor (TNF) [7], and that free radical processes may be involved [8–11]. Godin [12] reported that the hypotonic stability of erythrocytes decreases under the action of endotoxins, accompanied by the comparable changes in lipid composition in both human and rat RBCs. This suggests a

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possible modulatory role of membrane lipids in governing the molecular consequences of membrane-endotoxin interaction. In order to test this possibility, and to assess the role of plasma-soluble substances in the physical state of membrane lipids, electron paramagnetic resonance investigations employing a lipid-specific spin label have been performed.

The spin label 5-doxylstearic acid (5-NS) was obtained from Aldrich. *E. coli* lipopolysaccharide (0111:34) was obtained from Sigma Chemical Company. All other chemicals were of the highest purity available.

Blood was obtained from rats by heart puncture into heparinized tubes following procedures approved by the University of Kentucky Institutional Animal Care Committee, immediately placed on ice, and processed within 30 min of collection.

When washed cells were required, intact cells were isolated by centrifugation at 4°C at $600 \times g$ and three subsequent resuspensions and washings in PBS buffer [150mM NaCl/5mM sodium phosphate, pH8.0]. The buffy coat was carefully removed.

Endotoxin addition. Endotoxin was added in separate experiments to either whole blood (plasma, including leukocytes, present) or washed intact cells (plasma absent). In order to achieve the desired final concentrations of endotoxin in blood, four separate batches of endotoxin solution were made, each containing 5 mg endotoxin in variable amounts of PBS solution.

In the studies of whole blood, 0.5 ml of whole blood were incubated at 4°C for 90 min with 0.5 ml of the appropriate batch to afford pre-samples of concentration 1 (25 μg endotoxin/ml), concentration 2 (40 μg/ml) and concentration 3 (170 μg/ml). After the 90-min incubation, the plasma (plus leukocytes) was removed by washing with PBS leaving washed intact cells. These latter plasma plus leukocyte-free cells were all then labeled with 5-NS as described previously [13–21]. In separate studies, endotoxin was added to washed, intact cells for 90 min at 4°C. Endotoxin concentrations of 25 and 175 μg/ml were used. These cells were then spin-labeled with 5NS as previously described [13–22].

EPR spectra were recorded on a Bruker 300 EPR spectrometer equipped with computerized acquisition and analysis capabilities and located in a constant temperature and humidity room. Typical parameters for acquiring spectra were: 26mW microwave power, 0.32G modulation amphitude of a 100-kHz modulation frequency, and a scan width of 100G. All studies were performed in a blind fashion, i.e., the experimenter did not know the identity of the samples until after all measurements and calculations were completed.

To gain insight into possible alterations in the physical state of bilayer lipids on rat erythrocyte membranes caused by endotoxin, EPR employing the lipid-specific spin label 5-NS was used. The order and motion of the membrane lipids were assessed by measurement of the half-width at half-height (HWHH) of the $M_I = 1$ low-field resonance line of the 5-NS spectrum [22] as shown in Fig. 1. Mason et al. [22] demonstrated that the HWHH was more sensitive to small changes in molecular motion than measurement of spectral extrema. This increased sensitivity is due to the fact that with increased (decreased) lipid order and decreased (increased) lipid motion, line narrowing (broadening) occurs before changes in extrema separation (used in calculations of the order parameter [22]) take place. Additionally, unlike the measurements that lead to the order parameter, HWHH is independent of the polarity of the local microenvironment in which the nitroxide moiety of 5-NS is found [13,14,22]. Consistent with theoretical models [23,24], changes in HWHH have their basis in the rate of reorientation of the principal axis of 5-NS between parallel and perpendicular orientations relative to the normal to the erythrocyte membrane surface, analogous to linewidth changes associated with chemical exchange phenomena. The more rigid the lipid bilayer, the slower the reorientation rate of the spin label and the more narrow the HWHH. EPR studies employing the HWHH parameter have been used to investigate membrane interactions of pharmacological agents [15], environmental toxins [16], cell metabolites [17–19], transmembrane signaling events [20], and membrane alterations in pathological states [13,14,21].

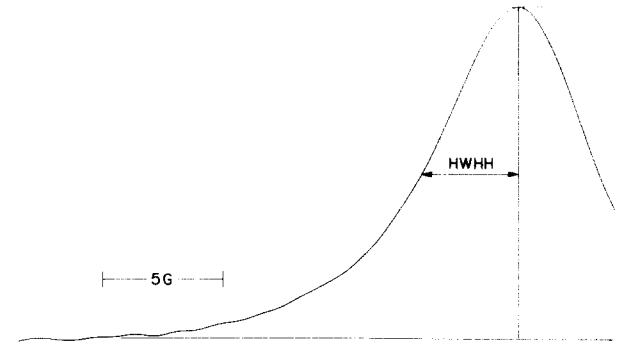

Fig. 1. Half-width at half-height of the $M_I = +1$ low-field EPR line of 5-NS in intact erythrocytes. EPR spectrometer conditions are indicated in the text.

In blind studies, addition of endotoxin to whole blood (i.e., plasma plus leukocytes were present) followed by subsequent washings of the cells and spin labeling of the washed, plasma-free erythrocytes led to a dose-dependent decrease in HWHH of the spin label (Table I). Endotoxin at a concentration as low as 40 μg/ml yielded a statistically significant decrease in HWHH ($P < 0.02$), consistent with the concept that endotoxin decreases erythrocyte membrane lipid fluidity when added to whole blood. In contrast, even at the highest concentration employed (170 μg/ml) no

TABLE I

Effect of endotoxin addition to whole blood (plasma and leukocytes present) on the physical state of the membrane lipids of subsequently washed and spin labeled erythrocytes [a]

[a] Mean ± standard deviations are presented. [b] Half-width at half-height of the low field line of 5-NS spectra. [c] *P*-value was assessed by a Student's two-tailed *t*-test. [d] Not significant.

Concentration of endotoxin μg/ml	HWHH [b] (% of control)	*N*	*P* [c]
0	100	6	-
25	98.5 ± 2.88	5	ns [d]
40	97.0 ± 2.33	6	< 0.02
170	94.2 ± 3.03	6	< 0.005

change in HWHH was found if endotoxin were added to plasma plus leukocyte-free, washed erythrocytes (Table II), suggesting that plasma-soluble substance(s) and/or leukocytes are required to produce an endotoxin-induced alteration in erythrocyte membrane lipid order and motion. The magnitude of the changes in HWHH are consistent with significant membrane alterations observed in previous EPR studies of membranes perturbed by differences in composition and temperature or upon interaction of membrane-active substances (reviewed in [13,14,21]).

The importance of alterations in the microcirculation of red blood cells in the clinical manifestations of sepsis has been documented recently [2,3], and erythrocytes are reportedly less deformable in sepsis [3–6]. The results of the current study (Table I) suggest that the molecular basis of this reduced deformability in sepsis may involve increased lipid rigidity. The effects of endotoxin observed here require plasma-soluble substances and/or leukocytes, and the latter may be very important as a source of cytokines [3]. Consistent with the EPR results of the current study (Table II), Anderson and co-workers demonstrated that cell deformability was altered by endotoxin only in the presence of plasma and leukocytes but not upon addition of endotoxin to washed, plasma-free erythrocytes (unpublished observations).

TABLE II

Effect of endotoxin addition to washed, plasma plus leukocytes-free erythrocytes subsequently spin labeled with 5-NS [a]

[a] Mean ± standard deviations are presented. [b] Half-width at half-height of the low field line of 5-NS spectra. [c] *P*-value was assessed by a Student's two-tailed *t*-test. [d] Not significant.

Concentration of endotoxin μg/ml	HWHH [b] (% of control)	*N*	*P* [c]
0	100	7	-
25	99.98 ± 2.63	7	ns [d]
170	103.7 ± 5.22	7	ns [d]

Other workers suggest free radical processes are involved in endotoxin-caused membrane alterations [8–11]. Free radical oxidation of unsaturated sites on acyl chains of phospholipids would be expected to lead to cross-linking of adjacent lipids and consequent decreased lipid motion. This would be expected to decrease HWHH of 5-NS, an expectation observed in the current study (Table I).

As noted, the HWHH parameter is more sensitive than the order parameter [22]. This may explain why in an earlier EPR study of the effect of endotoxin on RBC lipids using 5-NS, Hino et al. [25], using the order parameter to analyze the EPR spectra observed a decreased lipid motion only at endotoxin levels higher than 500 μg/ml. This result should be compared to our finding of decreased lipid motion at endotoxin levels of 40 μg/ml (Table I).

Although the results of the current research suggest a role for the lipid bilayers in endotoxin-induced alterations in RBC deformability, cell flexibility is governed by several factors including the physical state of cytoskeletal proteins. In order to obtain a more complete understanding of the role of endotoxin on RBC deformability, studies of endotoxin's effect on the physical state of cytoskeletal proteins will be required. Such studies are currently in progress.

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