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ENDOTOXIN (*PROTEUS MIRABILIS* O14)-INDUCED CHANGES IN HUMAN RED BLOOD CELL MEMBRANE PROPERTIES

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Endotoxin (lipopolysaccharide, LPS) isolated from *Proteus mirabilis* (serogroup O14) strain induced alteration in red blood cell membrane properties. Using spin labeling method, lipid membrane fluidity and physical state of membrane protein and were examined. The osmotic fragility of red blood cells was also studied. A slight increase in lipid membrane fluidity was indicated by 5-doxylstearic acid after LPS treatment. Higher concentrations of endotoxin induced an increase in fluidity measured with 12-doxyl derivatives of fatty acids. As significant dose-dependent increase in h_w/h_s ratio of 4-maleimido-2,2,6,6-tetramethylpiperidine-1-oxyl (MSL) suggested conformational changes in membrane proteins or increase of their mobility. These results indicate a dissociation of membrane cytoskeleton and changes mainly in the spectrin-actin complex. A no significant increase in osmotic fragility of red blood cells due to LPS treatment was found. These results suggest that endotoxin induces alterations in membrane components mainly in protein structure.

INTRODUCTION

Endotoxins (lipopolysaccharide; LPS) are components of the cell wall of Gram-negative bacteria and play an important role in the development of septic shock (Morrison & Ryan, 1987; Bone, 1991; Rietschel, Kirikae, Schade, Mamat, Schmidt, Loppnow, Ulmer, Zahringer, Seydel, Di Padova, Schreier & Brade, 1994). LPS released from bacteria induces a variety of pathophysiological responses such as hypertension, fever, myocardial depression and multiple-organ failure in an affected person. Endotoxin can initiate activation of macrophages, monocytes and endothelial cells. These cells can release numerous mediators such as tumor necrosis factor α (TNFα), platelet activating factor (PAF), interleukin-1 (IL-1) and IL-6 and arachidonic acid metabolites. There is considerable evidence that neutrophils and macrophages play a critical role in the pathogenesis of endotoxin. LPS can stimulate neutrophils via CD14 and the LPS-binding protein to express CR3 activity responsible for neutrophil adhesion (Wright, Ramos, Hermanowski, Rockwell & Detmers, 1991; Weingarten, Sklar, Mathison, Omidi, Ainsworth, Simon, Ulevitch & Tobias, 1993). Activated neutrophils increase oxygen consumption and produce oxygen species such as superoxide, hydrogen peroxide, hydroxyl radicals and hypochlorous acid which can destroy normal cells (Bone, 1991; Parillo, 1993; Rosen, Pou, Ramos, Cohen & Britigan, 1995). ROS play a critical role in the pathogenesis of multiple organ injury. The endotoxin as an amphiphilic molecule can interact with cell membrane unspecifically via hydrophobic interactions (Jacobs, Yeh & Price, 1990; Jackson, James, Rowlands & Mile, 1992).

In this paper the effect of *Proteus mirabilis* O14 endotoxin on the plasma membrane in whole human red blood cells as well as in isolated membranes was studied using spin labeling and spectrophotometric methods. The membrane lipid fluidity, physical state of membrane proteins and osmotic fragility of erythrocytes were examined.

MATERIALS AND METHODS

Spin labels: 5-doxylstearic acid (5-DS), methyl 12-doxylstearate (12-DS), 16-doxylstearic acid

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(16-DS) and 4-maleimido-2,2,6,6-tetramethyl-piperidine-1-oxyl (maleimide spin label, MSL) were from Sigma Chemical Co. All other chemicals of analytical grade were purchased from POCh (Gliwice, Poland).

The *Proteus mirabilis*, classified in the O14 serogroup, was from the bacterial strain collections of the Centre of Microbiology and Virology Polish Academy of Sciences Łódź, Poland. LPS was extracted by phenol-water procedure and purified by nuclease treatment and ultracentrifugation as described previously (Kaca, Roth & Levin, 1994).

Human blood samples from 8 healthy donors were obtained from an outpatient clinic of a local hospital.

Blood was centrifuged and erythrocytes were washed three times with cold phosphate-buffered saline (PBS), pH 7.4 at 4°C. The packed cells were suspended at a hematocrit of 50%.

The red blood cell membranes were prepared by the method of Dodge, Mitchell and Hanahan (1963) with a modification. RBC were lysed by hypotonic lysis using 20 mmol/l sodium phosphate buffer pH 7.4 at 4°C. The ghosts were successively washed with 20 mmol/l, 10 mmol/l and 5 mmol/l phosphate buffer, pH 7.4. The final concentration of proteins in membrane suspensions was approx. 3 mg/ml.

Red blood cells were incubated with three concentrations of LPS (0.1, 0.5 and 1 mg/ml) for 1 h at 37°C. After washing with 10 mmol/l phosphate buffer, 5-doxylstearic acid, methyl 12-doxylstearate and 16-doxylstearic acid were introduced from stock ethanol solutions into red blood cell suspension (final concentration of ethanol did not exceed 0.05%) and incubated for 0.5 hr at room temperature.

Isolated RBC membranes were labeled with MSL. Spin label was introduced using a stock solution (0.1 mol/l) of MSL in ethanol into membrane suspension (30 μg of MSL per 1 mg of protein) and incubated for 1 hr at 4°C. Several washings with cold phosphate buffer removed the unbound spin label until the EPR signal in supernatant disappeared. The whole procedure was performed at 4°C.

For osmotic fragility measurements erythrocyte suspension was added to solutions containing increasing concentrations of sodium chloride. The absorbance of supernatants was measured in a Pharmacia-LKB spectrophotometer at 540 nm. The extent of hemolysis was calculated from equation:

$$H = \frac{A_x - A_c}{A_{100} - A_c}$$

where: H is the extent of hemolysis, A_x, absorbance of the sample, A_c, absorbance of the control, and A_{100} absorbance after complete hemolysis.

EPR spectra were recorded in a Bruker ESP-300E (X-band) spectrometer operating at 9.73 GHz with a 100 Hz modulation frequency at room temperature.

Statistical analysis included the calculation of means ± S.D. The normal distribution of data was confirmed using the Shapiro-Wilk's test. The significance of differences was estimated by the Tukey's test for multiple comparisons.

RESULTS AND DISCUSSION

Endotoxins consist of hydrophilic heteropolysaccharide and a covalently bound lipid component, termed lipid A (Riettschel *et al.*, 1994). Lipid A represents the endotoxic center of lipopolysaccharide. LPS can interact with cell membrane via specific coupling directly to a membrane-bound receptor (CD14) or indirectly to the LPS-binding protein, which transfers LPS to CD14. LPS as a hydrophobic molecule can also interact with membrane via hydrophobic interactions. The interaction of endotoxin with membrane may influence on the state of order of hydrocarbon chains in lipids leading to fluidisation or rigidification and changes in membrane potential.

In this paper focused on the action of endotoxin on red blood cell membrane in whole cell as well as in isolated membranes. Using the spin-labeling method we examined lipid membrane fluidity and state of membrane proteins. For estimation of lipid membrane fluidity we applied three doxyl derivatives of fatty acids (5-DS, 12-DS and 16-DS), in which paramagnetic residue is attached to different carbon atoms in the carbohydrate chain. Three spin labels used give information from different depth of the lipid bilayer. The h_{+1}/h_0 ratio (where h_{+1} and h_0 are the heights of low-field line and middle line of the spectra, respectively) was calculated from the spectra. This parameter is a semi-quantitative measure of acyl chain mobility corresponding to lipid bilayer fluidity (Morrisett, Pownall, Plumlee, Smith, Zahner, Esfahani & Wakil, 1975; Stuhne-Sekalec & Stanacev, 1978). A slight an increase in the h_{+1}/h_0 ratio was observed for 5-doxylstearic acid in LPS-treated erythrocytes (Fig. 1). However, a significant increase in the h_{+1}/h_0 ratio of 12-DS was observed for higher

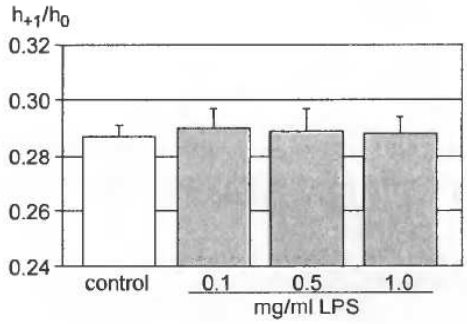

Fig. 1. Effect of endotoxin on the h_{+1}/h_0 ratio of 5-doxyl-stearic acid in red blood cell (no statistically significant changes; n = 6)

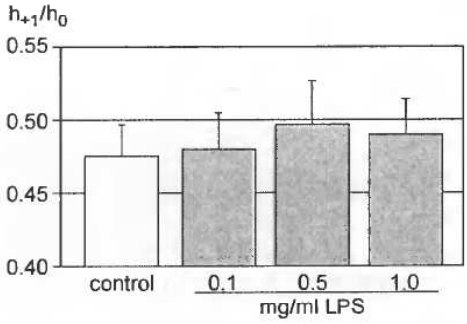

Fig. 2. Changes in the h_{+1}/h_0 ratio of methyl 12-doxyl-stearate in endotoxin treated red blood cells (no statistically significant changes; n = 7)

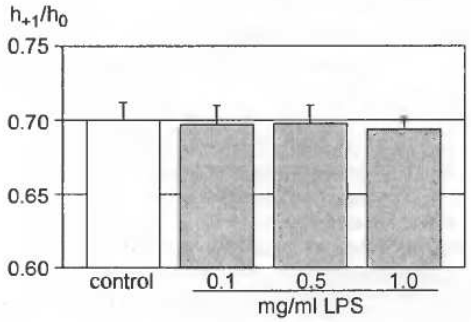

Fig. 3. Effect of endotoxin on the h_{+1}/h_0 ratio of 16-doxylstearic acid incorporated to RBC (no statistically significant changes; n = 8)

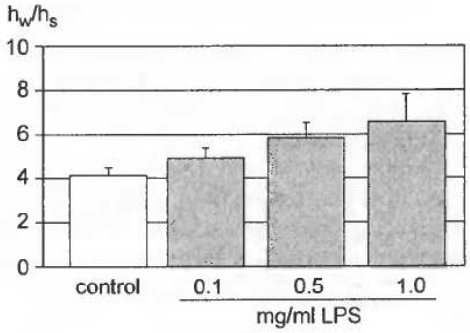

Fig. 4. Alterations in the h_w/h_s ratio of MSL in isolated RBC ghosts treated with LPS (control vs. 0.1 mg/ml, $P < 0.05$; control vs. 0.5 mg/ml and 1 mg/ml, $P < 0.0002$; n = 15)

concentrations of endotoxin (Fig. 2). An increase in lipid fluidity was found in human red blood cells treated with endotoxin from *Proteus mirabilis* O29 and S 1959 (Gwoździński, Pieniążek, Sudak & Kaca, 2000). On the other hand a diminished lipid fluidity (indicated by 5-DS) was observed in rat red blood cells treated with the same concentration of LPS from *E. coli* for higher doses (Hino, Kumashiro, Sata, Nihi, Ogura & Tanikawa, 1993). These authors suggested that LPS changes dynamic properties of the surface layer of the erythrocyte membrane. A decrease in lipid motion was found in erythrocytes in whole blood after LPS treatment but in isolated RBC a slight increase in lipid fluidity was observed (Butterfield, 1994).

For determination of physical state of membrane protein the maleimide spin label was applied. At physiological pH maleimide reacts mainly with the thiol groups of proteins (Berliner, 1983). The EPR spectrum of MSL attached to intact human red blood cells membranes consists of a weakly immobilised (narrow-line, h_w) and a strongly immobilised (broad-line, h_s) components. The h_w/h_s ratio is a very sensitive measure of the physical state of proteins in the membrane (Butterfield, Sun, Bellary, Arden & Anderson, 1982; Fung, 1983). We found a significant increase in the h_w/h_s ratio in membranes treated with all concentration of endotoxin (Fig. 4). The changes in h_w/h_s reflect alterations in protein conformation or disposition of spin labeled fragments of proteins after LPS treatment. It has been shown that more than 75% of the total amount of attached spin labels is bound to the spectrin-actin complex in the erythrocyte membrane (Fung, 1983). The changes obtained in this study may be due to dissociation of this complex. It seems that the action of endotoxin concerns rather proteins than lipids. In the previous study we have shown that hemoglobin is one of LPS-binding proteins (Kaca *et al.*, 1994). On the other hand endotoxin O14 induced a slight decrease of osmotic fragility of red blood cells (Fig. 5).

Alterations in the plasma membrane induced by LPS O14 suggest a lower effectiveness in its ac-

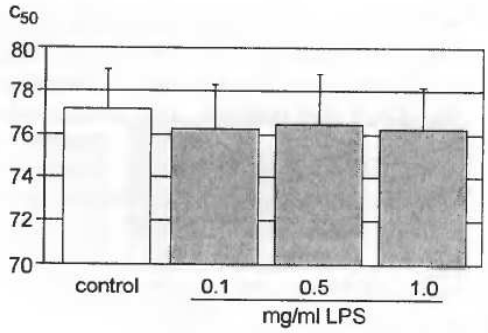

Fig. 5 Osmotic fragility in control and LPS-treated red blood cells (expressed as a concentration of NaCl in which 50% of red blood cells were lysed) (no statistically significant changes; n = 7)

tion on membrane in comparison to LPS O29 or OX19. These differences may be due to the differences of the polysaccharide chain structure of *P. mirabilis* LPS studied.

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