Changes in Erythrocyte Membrane Fluidity by Endotoxin in Rats

Yoshitami KADOTA, Tetsuro KAMADA*,
Nozomu YOSHIMURA and Shogo OTSUJI*

The effect of endotoxin on fluidity and lipid composition of the erythrocyte membrane was studied in rats following the intraperitoneal administration of endotoxin (30 mg·kg^{-1} body weight). Erythrocyte membrane fluidity measured with 16-stearic acid spin label (16-SAL) was significantly decreased in the endotoxin-treated rats as compared with control. A decrease of lysophosphatidylcholine in the membrane lipid was evident in the endotoxin-treated rats. The cholesterol to phospholipid molar ratios and other phospholipid fractions did not differ significantly in the two groups.

The levels of plasma β-glucuronidase activity and lipoperoxide were significantly increased in the endotoxin-treated rats when compared to controls. There were significant correlations between the parameter of 16-SAL in erythrocytes and plasma β-glucuronidase activities or lipoperoxide from both endotoxin-treated and control groups, $P < 0.005$ or $P < 0.02$ respectively.

In conclusion endotoxin decreased rat erythrocyte membrane fluidity *in vivo*. Since membrane fluidity is closely related to the vital functions of the membranes, the change described could be related to the abnormality of cell membrane functions in endotoxin shock state.
(Key words: membrane fluidity, erythrocytes, endotoxin, lipoperoxide, rat)

(Kadota Y, Kamada T, Yoshimura N, et al.: changes in erythrocyte membrane fluidity by endotoxin in rats. J Anesth 6:145–152, 1992)

The effects of endotoxin on biological membrane have gained increased attention with respect to the initiation of various pathophysiologic responses at the cellular level in the endotoxin shock[1–3]. Endotoxic lipopolysaccharide is known to possess a high affinity for the biological membrane, altering the lipid bilayer structure and its functions[1,2,4–7]. We reported previously that endotoxin decreased the fluidity of erythrocyte membrane *in vitro*[8], suggesting a direct interaction of endotoxin with the lipid bilayer of erythrocyte membrane. Fluidity is one of the parameters which represent the dynamic physical state of the membrane, especially that of the lipid bilayer, and it is closely related to maintain cell membrane functions such as permeability to ions and metabolites, membrane-bound enzyme activity, and hormone-receptor behavior[9–11].

We now report the effects of endo-

Department of Anesthesiology and Laboratory Medicine, Faculty of Medicine, Kagoshima University, Kagoshima, Japan*

Address reprint requests to Dr. Yoshimura: Department of Anesthesiology, Faculty of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima, 890 Japan

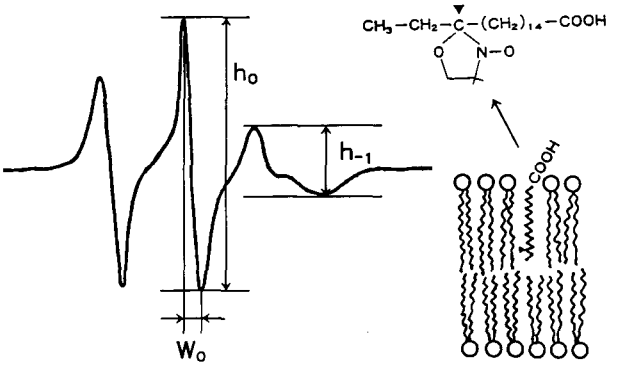

Fig. 1. Chemical formula and representative ESR spectrum of 16-SAL embedded in the lipid bilayer of the membrane.
W_0: mid-field peak width; h_0: mid-field peak height; h_{-1}: high-field peak height; SAL: stearic acid spin label.

toxin on circulating erythrocyte membrane *in vivo* by measuring its fluidity and lipid composition in rats 6 hours after the administration of endotoxin.

To evaluate the severity of shock and the extent of damage produced at the cellular level, we also measured the levels of plasma β-glucuronidase activity and lipoperoxide, since these two factors are believed to increase in the presence of damage to the cell membranes[12,13].

Materials and Methods

A total of 17 male Wistar rats aged 10 weeks and weighing 290 to 390g was maintained on a standard laboratory chow and water ad libitum. They were divided into two groups at random. The average body weight of the two groups was 338 ± 29g (n=8) and 320 ± 18g (n=9) (mean ± SD) each. After fasting for 24 hrs, endotoxin (Escherichia coli, 0127 B-8, Difco Laboratories) dissolved in 1.0 ml physiological saline was administered intraperitoneally to the former group of rats (endotoxin-treated) at a dose of 30 $mg \cdot kg^{-1}$ bw. The latter group (control) was given 1.0 ml of physiological saline. In the endotoxin-treated rats, we observed piloerection, reduced motion and hypothermia several hours after endotoxin administration. Six hours after the administration, the animals were decapitated and the blood collected. In the preliminary experiment, we found the severe crenation of erythrocytes after anesthetizing the rats with barbiturate or ether. Therefore, we decapitated the animals without anesthetics in accordance with Guide for Animal Experimentation, Faculty of Medicine, Kagoshima University. After decapitation, the blood from the carotid and jugular vein was immediately collected and heparinized.

Erythrocytes were separated from the plasma by centrifugation (1,200 × g for 10 min), and washed three times with phosphate-buffered saline (PBS, pH 7.4, 310 mOsm) with elimination of the buffy coat at each washing.

Erythrocyte membrane fluidity

A stearic acid spin label, 16-SAL (16-doxylstearate spin label), was purchased from the Aldrich Chemical Co., Inc. (Milwaukee, Wis). This agent is a stearic acid analogue with a nitroxide radical ring at the carbon position 16th (counted from the carboxyl group of acyl-chain) (fig. 1). It is thought to be incorporated into the lipid bilayer perpendicularly; its motion reflects the surrounding microenvironment of the lipid bilayer referred to as the membrane "fluidity"[14].

The incorporation of this spin label into the erythrocyte membrane was accomplished by the method previously described with a small modification[15]. Briefly, 2 μl of 16-SAL stock solution (5 $\mu g \cdot \mu l^{-1}$ in ethanol) were diluted

Table 1. Cholesterol/phospholipid molar ratios and phospholipid classes of erythrocyte membranes in control and endotoxin treated rats

	n	C/PL	lyso-PC	SM	PC	PS	PE (mol%)
Control	9	0.74 ± 0.02	4.12 ± 0.69	11.93 ± 0.89	40.78 ± 2.23	18.12 ± 1.47	25.06 ± 1.63
Endotoxin	8	0.72 ± 0.05	3.52 ± 0.38*	11.68 ± 0.94	40.37 ± 2.95	17.89 ± 1.35	26.55 ± 1.62

C/PL: cholesterol to phospholipid molar ratio, lyso-PC: lysophosphatidylcholine, SM: sphingomyelin, PC: phosphatidyl-choline, PS: -serine, PE: -ethanolamine. *: $P < 0.05$ vs control.

Data are expressed as mean ± SD.

with 400 μl of PBS and the same volume of erythrocyte suspension in PBS (Ht = 50%) was added. After incubation for 20 min at 35°C, erythrocytes were washed three times and packed by centrifugation at 1,200g for 20 min and used immediately for measuring electron spin resonance (ESR). ESR spectra were obtained at 35°C controlled by a variable temperature controller (JES-VT-3A2) on a JEOL X-band spectrometer, Model JES FE1X (JEOL Ltd., Tokyo, Japan).

The motion parameter (τ_0) was calculated as follows from the formula of Henry and Keith[16], where W_0 is the mid-field peak width, h_0 the mid-field peak height, h_{-1} the high-field peak height (fig. 1) and the constant $K = 6.5 \times 10^{-10}$ (seconds):

$$\tau_0 = K \cdot W_0[(h_0/h_{-1})^{1/2} - 1]$$

A greater value of membrane fluidity, i.e., a greater freedom of motion of spin labels in the double membrane, is associated with smaller value for the motion parameter.

Erythrocyte membrane lipids

Erythrocyte membrane lipids were extracted with isopropanol : chloroform (11 : 7 by vol) followed by chloroform : methanol (2 : 1 by vol), according to a modification of the method of Rose and Oklander[17]. The antioxidant, butylated hydroxytoluene (BHT), was added to the solvent used for extraction at a concentration of 50 mg·l^{-1}. The total amount of phospholipids in the extract was measured by the method of Bartlett[18]. Phospholipid classes were quantitatively separated by thin-layer chromatography on a HPTLC plate (silica gel 60 precoated plate, Merck, Germany) activated at 200°C for 2 hrs with a developing solvent of chloroform : methanol : acetic acid : water (25 : 14 : 4 : 1 by vol), containing BHT at a concentration of 50 mg·l^{-1}. Individual phospholipid classes were quantitated by measuring their inorganic phosphate after scraping each fraction[18]. Cholesterol was measured by the method of Zak[19]. The cholesterol to phospholipid (C/PL) molar ratios were calculated from a mixture containing known amounts of the two lipids.

Plasma β-glucuronidase activity

Plasma β-glucuronidase activity was measured by the method of Kato et al.[20] with p-nitrophenyl β-D-glucopyranosiduronate. One unit of glucuronidase represents the activity at which 1 μg of p-nitrophenol per 100 ml per hour is released from p-nitrophenyl β-D-glucopyranosiduronate.

Plasma lipoperoxide level

The plasma level of lipoperoxide was measured by the fluorometric method of Yagi[21] and expressed as nmoles of malondialdehyde per ml.

Statistical analysis

Data are presented as mean ± standard deviation. The significance of the differences between groups was tested by the Mann-Whitney test. The effi-

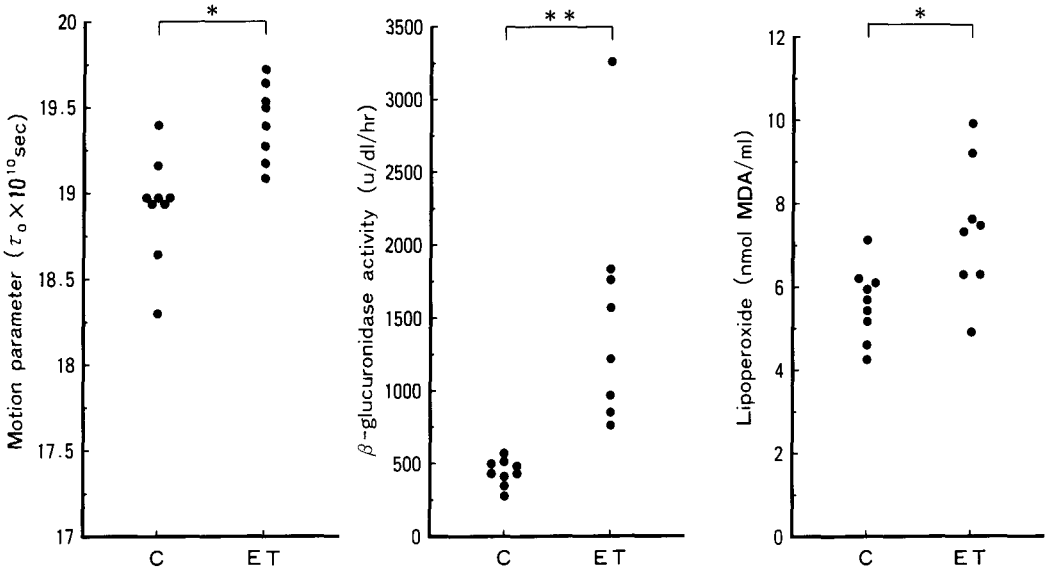

Fig. 2. Changes in erythrocyte membrane fluidity (motion parameter of 16-SAL), plasma β-glucuronidase activity and lipoperoxide following endotoxin administration.

C: control rats (n=9); ET: endotoxin-treated rats (n=8); SAL: stearic acid spin label; MDA: malondialdehyde; *: $P < 0.01$, **: $P < 0.001$ vs control rats.

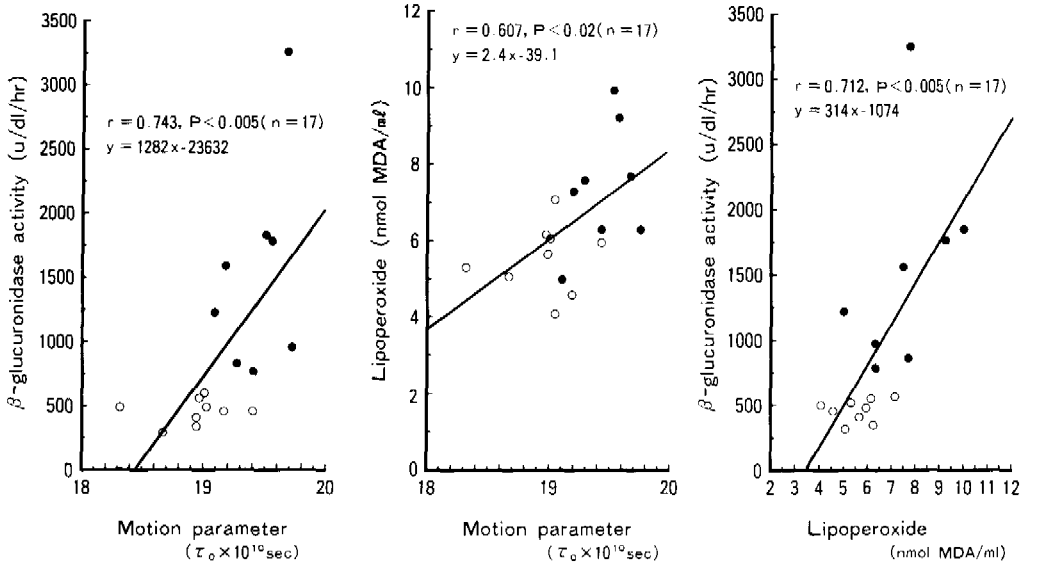

Fig. 3. The relationships among erythrocyte membrane fluiditiy (motion parameter of 16-SAL), plasma β-glucuronidase activity and lipoperoxide in rats.

○: control rats (n=9); ●: endotoxin-treated rats (n=8); SAL: stearic acid spin label; MDA: malondialdehyde.

ciency of the correlation was tested by Spearman's test. The criterion for statistical significance was a *P* value less than 0.05.

Results

1. Membrane fluidity

The values of the motion parame-

ter of 16-SAL increased significantly in the endotoxin-treated rats as compared to controls (19.44 ± 0.23 vs. 18.96 ± 0.31, fig. 2), indicating that erythrocyte membrane fluidity was decreased by endotoxin.

2. Cholesterol and phospholipid classes of the erythrocyte membranes

Lysophosphatidylcholine (lyso-PC) was significantly decreased in the endotoxin-treated rats (table 1). However, the change did not correlate with the values of motion parameter. The other phospholipid classes and C/PL molar ratios did not differ significantly between the groups.

3. Plasma β-glucuronidase activity

The activities of plasma β-glucuronidase were significantly increased in the endotoxin-treated rats as compared to the controls (1533 ± 810 vs 457 ± 93, fig. 2). This activity correlated significantly with the values of motion parameter of both endotoxin-treated and control groups ($r=0.743$, $P < 0.005$, fig. 3). But the correlation was not statistically significant in each group.

4. Plasma lipoperoxide level

The plasma levels of lipoperoxide were significantly increased in the endotoxin-treated rats as compared to controls (7.47 ± 1.61 vs. 5.61 ± 0.91, fig. 2). The levels were significantly correlated with the values of motion parameter of 16-SAL from both groups ($r=0.607$, $P < 0.02$, fig. 3), but the correlation did not show a statistical significance in the individual groups. There was also a significant correlation between the level of lipoperoxide and β-glucuronidase activity from both groups ($r=0.712$, $P < 0.005$, fig. 3) but not in the individual groups.

Discussion

Our data showed that administration of endotoxin decreased membrane fluidity and altered the lipid composition of erythrocyte membranes in rats. The decrease in membrane fluidity was consistent with the results reported by Liu et al. for liver cell membranes after the administration of endotoxin[3]. Membrane fluidity represents physical property of lipid bilayer of the membranes, and is known to be an important factor affecting membrane functions such as permeability of ions and metabolites, membrane bound enzyme activity, and hormone receptor behavior[9–11]. Therefore, the change in membrane fluidity observed possibly affects these membrane functions in erythrocytes. The difference in membrane fluidity between the endotoxin-treated and control rats, although statistically significant, was relatively small. Nevertheless, this change is worth considering, since it is reported that even a slight alteration in membrane fluidity can lead to definite changes in membrane enzyme activities[22].

Several studies have shown that endotoxin administration produces diverse biologic effects such as activation of the immune system, hypotension, hypoglycemia and disseminated intravascular coagulation[23]. The mechanism underlying these effects is unknown, but it may be related to the endotoxin-induced alteration of cell membrane structure and functions[1–8]. Changes in vital membrane functions, such as a decrease in Na^+-K^+ATPase activity, the altered Na^+-Ca^{++} exchange system[24], and impaired functions of glucocorticoid-[25] and insulin-receptors[26], have reportedly been produced by endotoxin. The lipid bilayer structure is common in any biological membranes, it is therefore possible that membrane changes similar to

erythrocytes occur in other cell membranes, affecting the functions of the cells. It is plausible that these alterations are related to changes in membrane fluidity[9–11].

In this study, elevated levels of plasma β-glucuronidase activity and lipoperoxide were evident in the endotoxin-treated rats. The former is a lysosomal enzyme that is believed to be released from damaged or ruptured lysosomes[12], and the latter is formed through the peroxidation of unsaturated fatty acids rich in cell membranes[13]. Therefore, the increases are suggestive of membrane damage caused by the administration of endotoxin. The significant correlations between fluidity in erythrocyte membranes and plasma β-glucuronidase activity or lipoperoxide support this concept.

As to the mechanism for the decrease in membrane fluidity in the endotoxin-treated rats, a direct effect of endotoxin on membrane fluidity can be presented as one possibility. We previously reported that endotoxin decreased erythrocyte membrane fluidity *in vitro*[8]. Endotoxin is suggested to be incorporated into cell membranes, inserting its lipid A portion into the membrane lipid bilayer[27], and disturbing the flexibility of phospholipid acyl chains[5,6,8]. However, the concentration of endotoxin which affected membrane fluidity was much higher than that expected in the present *in vivo* experiment.

Another possibility is that the decreased membrane fluidity reflects secondary changes in membrane structure induced by endotoxin administration. Membrane lipid composition is an important factor in regulating membrane fluidity[9]. Lyso-PC has been reported to increase membrane fluidity *in vitro*[28]. Although our results failed to demonstrate a correlation between the contents of lyso-PC and the fluidity, the decrease in lyso-PC content in the endotoxin-treated rats might be responsible for the decreased membrane fluidity *in vivo*. The mechanism of the decrease in lyso-PC is unknown.

Lipid peroxidation is known to decrease fluidity by forming cross-links with membrane components and/or by consuming polyunsaturated acyl chains in the membranes[10,29,30]. Levels of plasma lipoperoxide do not necessarily reflect lipid peroxidation in erythrocyte membranes, but Koster et al. reported an increased lipoperoxide in erythrocyte membranes following incubation with peroxidized linoleic acid[31]. Therefore, it is possible that lipid peroxidation also occurrs in the erythrocyte membranes from endotoxin-treated rats, decreasing the membrane fluidity.

Several other lipid-soluble mediators in addition to lipoperoxide have been found increased in plasma during endotoxin shock, among them prostaglandin derivatives and platelet activating factor are important[32]. These lipid mediators may also affect erythrocyte membrane fluidity, since they are known to be incorporated into lipid bilayer[33,34], perturbing its structure.

In conclusion, a decrease in erythrocyte membrane fluidity was found in endotoxin-treated rats. Since membrane fluidity is intimately connected with the vital functions of the cell membranes, the changes described in this study may impair the cell membrane functions, contributing to the pathogenesis of the endotoxin shock.

(Received Jun. 17, 1991, accepted for publication Sep. 6, 1991)

References

1. Hawiger J, Hawiger A, Steckley S, et al: Membrane changes in human platelets induced by lipopolysaccharide endotoxin. Br J Haematol 35:285–299, 1977
2. Davies M, Stewart-Tull DES, Jackson DM: The binding of lipopolysaccha-

ride from Escherichia coli to mammalian cell membranes and its effect on liposomes. Biochim Biophys Acta 508:260–276, 1978
3. Liu MS, Ghosh S, Yang Y: Change in membrane lipid fluidity induced by phospholipase A activation: a mechanism of endotoxic shock. Life Sci 33:1995–2002, 1983
4. Shands JW: Affinity of endotoxin for membranes. J Infect Dis 128 (Suppl): S197–S201, 1973
5. Onji T, Liu MS: Effect of E coli endotoxin on the leakage of ^{14}C-sucrose from phosphatidylcholine liposomes. Circ Shock 8:403–410, 1981
6. Liu MS, Onji T, Snelgrove NE: Changes in phase transition temperature of phospholipids induced by endotoxin. Biochim Biophys Acta 710:248–251, 1982
7. Larsen NE, Sullivan R: Interaction between endotoxin and human monocyte: characteristics of the binding of ^{3}H-labeled lipopolysaccharide and ^{51}Cr-labeled lipid A before and after the induction of endotoxin tolerance. Proc Natl Acad Sci USA 81:3491–3495, 1984
8. Kadota Y: Effects of endotoxin on erythrocyte membrane fluidity in rat. Jpn Anaesth J Rev 1:207–209, 1986
9. Cooper RA: Abnormalities of cell-membrane fluidity in the pathogenesis of disease. N Engl J Med 297:371–377, 1977
10. Chapman D, Benga G: Biomembrane fluidity -studies of model and natural biomembranes, Biological Membranes. vol 5. Edited by Chapman D. New York, Academic Press, 1984, pp. 1–56
11. Shinitzky M: Membrane fluidity and receptor function, Biomembranes 12 Membrane fluidity. Edited by Morris K, Lionel AM. New York, Plenum Press, 1984, pp. 585–601
12. Lefer AM: Lysosomes in the pathogenesis of circulatory shock, Handbook of Shock and Trauma vol 1. Edited by Altura BM, Lefer AM, Schumer W. New York, Raven Press, 1983, pp. 291–308
13. Kappus H, Sies H: Toxic drug effects associated with oxygen metabolism: redox cycling and lipid peroxidation Experientia 37:1233–1358, 1981
14. Keith AD, Sharnoff M, Cohn GE: A summary and evaluation of spin labels used as probes for biological membrane structure. Biochim Biophys Acta 300:379–419, 1973
15. Kamada K, Otsuji S: Lower levels of erythrocyte membrane fluidity in diabetic patients. Diabetes 32:585–591, 1983
16. Henry SA, Keith AD: Membrane properties of saturated fatty acid mutants of yeast revealed by spin labels. Chem Phys Lipids 7:245–265, 1971
17. Rose HG, Oklander M: Improved procedure for the extraction of lipids from human erythrocytes. J Lipid Res 6:428–431, 1965
18. Bartlett GR: Phosphorus assay in column chromatography. J Biol Chem 234:466–468, 1959
19. Zak B: Simple rapid microtechnic for serum total cholesterol. Am J Clin Pathol 27:583–588, 1957
20. Kato K, Yoshida K, Tsukamoto H, et al: Synthesis of p-nitrophenyl β-D-glucopyranosiduronic acid and its utilization as a substrate for the assay of β-glucuronidase activity. Chem Pharmacol Bull 8:239–242, 1960
21. Yagi K: A simple fluorometric assay for lipoperoxide in blood plasma. Biochem Med 15:212–216, 1976
22. Gordon LM, Sauerheber RD, Esgate JA, et al: The increase in bilayer fluidity of rat liver plasma membranes achieved by the local anesthetic benzyl alcohol affects the activity of intrinsic membrane enzymes. J Biol Chem 255:4519–4527, 1980
23. Hinshaw LB, Beller-Todd BK, Archer LT, et al: Septic endotoxemia, Handbook of Shock and Trauma vol 1. Edited by Altura BM, Lefer AM, Schumer W. New York, Raven Press, 1983, pp. 425–435
24. Liu MS: Mechanisms of myocardial membrane alterations in endotoxin shock: Roles of phospholipase and phosphorylation. Circ Shock 30:43–49, 1990
25. Li F, Xu RB: Changes in canine leukocyte glucocorticoid receptors

during endotoxin shock. Circ Shock 26:99–105, 1988
26. Holley DC, Spitzer JA: Insulin action and binding in adipocytes exposed to endotoxin *in vitro in vivo*. Circ Shock 7:3–12, 1980
27. Hammerling U, Westphal O: Synthesis and use of O-stearoyl polysaccharides in passive hemagglutination and hemolysis. Eur J Biochem 1:46–50, 1967
28. Weltzien HU: Cytolytic and membrane-perturbing properties of lysophosphatidylcholine. Biochim Biophys Acta 559:259–287, 1979
29. Rice-Evans C, Hochstein P: Alterations in erythrocyte membrane fluidity by phenylhydrazine-induced peroxidation of lipids. Biochem Biophys Res Comm 100:1537–1542, 1981
30. Bruch RC, Thayer WS: Differential effect of lipid peroxidation on membrane fluidity as determined by electron spin resonance probes. Biochim Biophys Acta 733:216–222, 1983
31. Koster JF, Slee RG, Rutten-Van Beysterveld CCM, et al: The effect of (1300H) linoleic acid on human erythrocytes and on erythrocyte ghosts. Biochim Biophys Acta 754:238–242, 1983
32. Lefer AM: Significance of lipid mediators in shock states. Circ Shock 27:3–12, 1989
33. Valone FH, Goetzl EJ: Specific binding by human polymorphonuclear leucocytes of the immunological mediator 1-0-hexadecyl/octadecyl-2-acetyl-sn-glycero-3-phosphorylcholine. Immunology 48:141–149, 1983
34. Ray TK, Dutta-Roy AK, Sinha AK: Regulation of insulin receptor activity of human erythrocyte membrane by prostaglandin E_1. Biochim Biophys Acta 856:421–427, 1986