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HYDROXYL RADICAL GENERATION AND MEMBRANE FLUIDITY OF ERYTHROCYTES TREATED WITH LIPOPOLYSACCHARIDE

YUJI HINO, RYUKICHI KUMASHIRO, MICHIO SATA, JUICHI NISHI, RYOHEI OGURA, and KYUICHI TANIKAWA

Second Department of Medicine, Department of Medical Biochemistry, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830, Japan

The effect of lipopolysaccharide (LPS) and/or bile acids on rat erythrocyte membranes was studied *in vitro*. Addition of LPS isolated from *E. coli* (J5 mutant) into the erythrocyte resulted in the decrease of membrane fluidity as determined by spin labelling using electron paramagnetic resonance (EPR). This was accompanied by membrane fragility. It was found that hydroxyl radicals were generated from erythrocytes treated with LPS by using DMPO spin trapping. However, pretreatment of erythrocytes with taurine-conjugated bile acids was found to modify the membrane response induced by LPS. Taurocholic acid (TCA) and tauroursodeoxycholic acid (TUDCA) prevented the decrease of membrane fluidity induced by LPS, and, as a result, the membrane integrity was maintained although no significant changes were observed in the amount of hydroxyl radicals produced by LPS addition. However, taurochenodeoxycholic acid (TCDCA) exhibited little beneficial effect on the dynamic properties and the function of the erythrocyte membranes, although the hydroxyl radical declined markedly in the erythrocytes.

Therefore, it is suggested that TCA and TUDCA have a protective effect against LPS-induced membrane fragility by modulating membrane fluidity.

KEY WORDS: lipopolysaccharide (LPS), taurine-conjugated bile acids, electron paramagnetic resonance (EPR), hydroxyl radical, membrane fluidity.

INTRODUCTION

Lipopolysaccharide (LPS) located in the outer membranes of gram-negative bacteria is known to be a macromolecular complex composed of amphipathic subunits. Pathophysiological reactions induced by LPS have been reported by many investigators[1-5]. In cases of hepatic reticuloendothelial system dysfunction induced by severe liver injury or obstructive jaundice, a high concentration of LPS appears in the blood stream. Under these clinical conditions, especially in obstructive jaundice, the concentrations of serum bile acids are markedly increased, with a taurine or glycine conjugated form being dominant[6]. Therefore, the question of whether the conjugated-bile acids can modify the dynamic properties of membranes affected by LPS is discussed in the present study.

All correspondence to Yuji Hino, Second Department of Medicine, Kurume University School of Medicine, Asahi-machi, Kurume 830, Japan.

MATERIALS AND METHODS

Washed erythrocytes obtained from venous blood of Wistar male rats weighing about 200 g were incubated with various concentrations LPS solution or taurine-conjugated bile acids in Ca^{2+}, M^{2+}, free phosphate buffered saline (PBS) (pH 7.4) for 1 h. Furthermore, to observe the interaction of LPS and taurine-conjugated bile acids on erythrocyte membranes, erythrocytes were pretreated with taurine-conjugated bile acids and were incubated again with LPS for 1 h. These erythrocytes were used in the following experiments.

5-Doxyl stearic acid (5-DSA) was used as a spin label agent to measure membrane fluidity. 5-DSA was stored in a freezer at −80°C, as a stock solution containing 0.2 mg/ml in ethanol. The stock solution (50 μl) was placed in a small test tube and dried to a thin film under flow of nitrogen gas. One-hundred microliters of washed erythrocytes non-treated or treated with LPS and/or taurine-conjugated bile acids in two and a half ml of Ca^{2+}, Mg^{2+} free phosphate buffered saline (PBS) (pH 7.4) were put into the test tube as mentioned above and incubated for 10 min at 37°C with gentle shaking. The erythrocytes were then washed with PBS to remove the free spin labels and centrifuged at 1870 g for 5 min. The resultant pellet was transferred to a disposable glass capillary (50 μl) and one end of the capillary was sealed with clay. The EPR cavity was kept at 37°C with a variable temperature controller, and the EPR spectrum was obtained by a JOEL X-band spectrometer model JES-FE 3X. Order parameter S was calculated from the EPR spectrum according to the formula of Gaffney[7].

Membrane fragility of erythrocytes was determined by the release of lactate dehydrogenase (LDH) from the erythrocytes to the incubation mediums[8].

Spin trapping methods using EPR spectrometry were used to detect the generation of oxygen radicals[9]. In the present study, the spin trapping agent, 5,5-dimetyl-pyrroline-N-oxide (DMPO) was used. The following procedures were carried out to detect the kinds of oxygen radicals produced from rat erythrocytes when LPS was added to the erythrocyte. LPS was placed into a test tube containing 0.134 μmol of DMPO, 2 mM diethylenetriamine pentaacetic acid (DETAPAC) in 0.1 M sodium phosphate buffer (pH 7.4) and 80 μl of packed erythrocytes for a total volume of 200 μl. Subsequently, the reaction mixture was quickly transferred to a 160 μl EPR flat cell, and was put into the EPR cavity. EPR spectra of the spin adducts were immediately recorded by a JOEL, X-band spectrometer at 100 kHz of magnetic field modulation. The magnetic field was set at 3350 gauss, field scan 50 gauss, modulation frequency 100 kHz, modulation amplitude 1.0 gauss, receiver gain 2×10^3, time constant 0.1 s, scan time 2 min and microwave power was 8 mW.

RESULTS

As shown in Figure 1, the order parameter(S) increased slightly following treatment of erythrocytes with LPS at concentrations over 500 μg/ml. On the other hand, the treatment of erythrocytes with TCDCA increased the membrane fluidity markedly at concentrations over 0.3 mM, as shown in Figure 2. Addition of either TCA or TUDCA to the erythrocytes had little influence on the membrane fluidity at concentrations below 3.0 mM. Therefore, in the following studies, the conjugated bile acids were used at two-thirds of the maximum concentration which had no effect on the

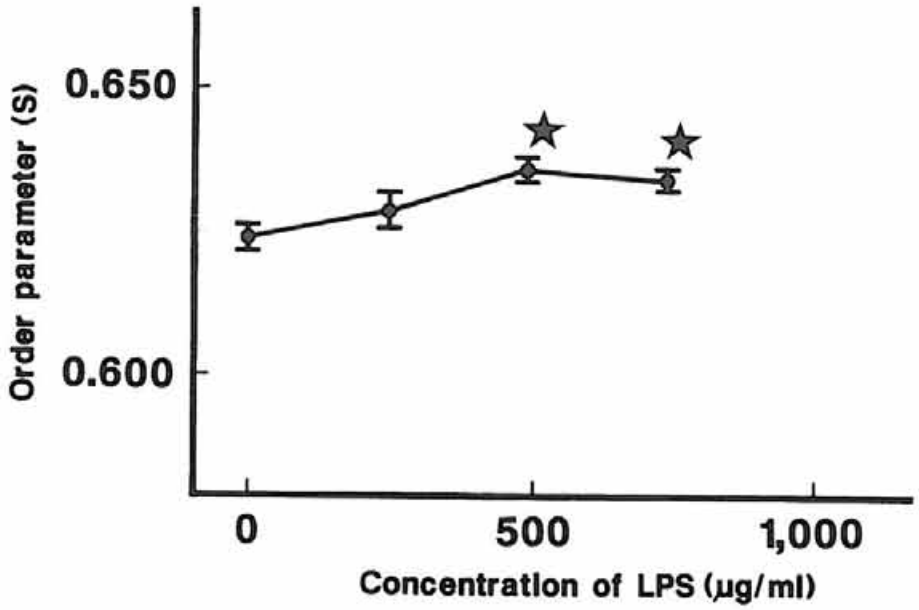

FIGURE 1 Effects of LPS on order parameter(S) of 5-DSA in rat erythrocyte membranes. Values are given as mean ± SD for 6 rats. Significantly different from control, ★ $P < 0.05$.

membrane fluidity, that is, the concentrations of TCDCA, TCA and TUDCA were 0.2 mM, 2.0 mM and 2.0 mM respectively.

The effects of LPS and bile acids on membrane integrity of the erythrocytes were examined. The fragility was found to increase in erythrocytes treated with LPS at concentrations of 500 μg/ml, while the treatment of erythrocytes with 0.2 mM TCDCA, 2.0 mM TCA and 2.0 mM TUDCA resulted in no remarkable changes of membrane fragility (data not shown).

Membrane fluidity was examined to determine the effect of pretreating the erythrocytes with taurin-conjugated bile acids. Table 1 shows that 2.0 mM TCA and 2.0 mM TUDCA prevented the decrease of erythrocyte membrane fluidity caused by LPS. However, 0.2 mM TCDCA did not have this preventative effect. Similar results were obtained to those regarding membrane integrity as described in Figure 3.

Oxygen radicals were determined by EPR on the LPS-treated erythrocytes in order to examine the involvement of oxygen radicals in LPS-induced membrane damages. Figure 4 shows that the EPR spectrum was detected in the presence of both erythrocytes and LPS at the concentration of 500 μg/ml. A dose dependent decline of the EPR spectrum of DMPO-OH adduct was detected after pretreatment of erythrocytes with TCDCA. However, the pretreatment of erythrocytes with 2.0 mM TCA or TUDCA had no effect on the generation of oxygen radicals as shown in Figure 5.

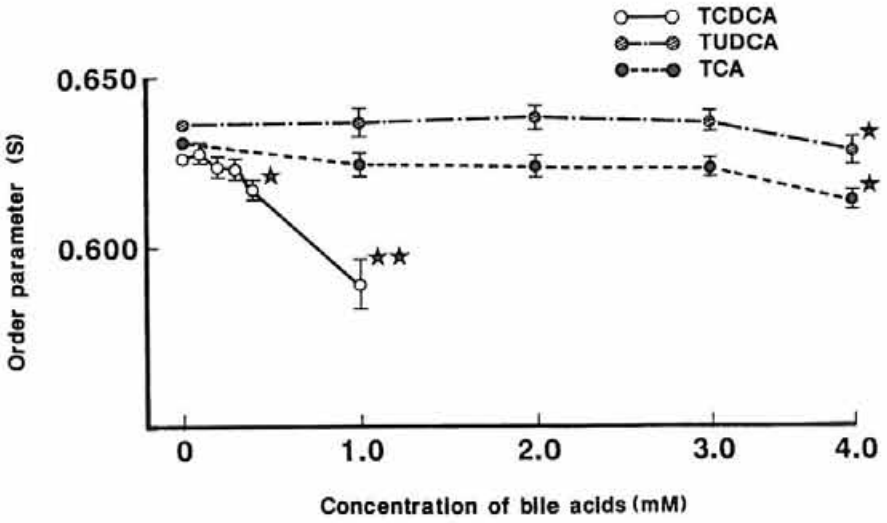

FIGURE 2 Effects of bile acids on order parameter (S) of 5-DSA in rat erythrocyte membranes. Values are given as mean ± SD for 6 rats.
★ $P < 0.05$ as compared to the control groups.
★★ $P < 0.01$ as compared to the control groups.
TCDCA: taurochenodeoxycholic acid.
TUDCA: tauroursodeoxycholic acid.
TCA : taurocholic acid.

DISCUSSION

Under severe cholestatic conditions such as obstructive jaundice, endotoxin with diverse physiological effects has been identified at high incidence in the systemic circulation due to decreased reticuloendothelial function and to increased LPS absorption from the intestine[10-11]. Moreover, it is well known that free bile acids are increased in systemic circulation and easily conjugated with either taurine or glycine in the liver[6]. Therefore, it is noteworthy to discuss the interaction between

TABLE 1
Order parameter (S) of 5-DSA in rat erythrocyte membranes

	Order parameter (S)
Control	0.624 ± 0.005
LPS	0.630 ± 0.002[a]
LPS + TCDCA	0.628 ± 0.007
LPS + TUDCA	0.621 ± 0.004[b]
LPS + TCA	0.613 ± 0.007[b]

Values are given as mean ± SD for 6 rats.
a: $P < 0.05$ as compared to the control groups.
b: $P < 0.05$ as compared to the LPS groups.
LPS : 500 μg/ml lipopolysaccharide.
TCDCA: 0.2 mM taurochenodeoxycholic acid
TUDCA: 2.0 mM tauroursodeoxycholic acid.
TCA : 2.0 mM taurocholic acid.

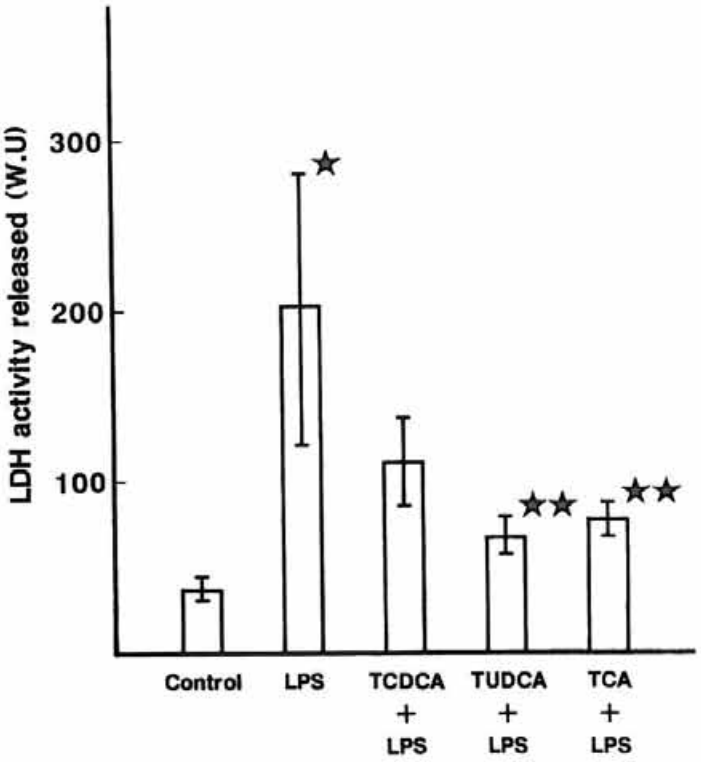

FIGURE 3 Effects of pretreatment of bile acids on LPS-induced membrane dysfunction.
★ $P < 0.01$ as compared to the control groups.
★★ $P < 0.05$ as compared to the LPS groups.
LPS : 500 μg/ml lipopolysaccharide.
TCDCA: 0.2 mM taurochenodeoxycholic acid.
TUDCA: 2.0 mM tauroursodeoxycholic acid.
TCA : 2.0 mM taurocholic acid.

LPS and taurine-conjugated bile acids on the cell membranes. In the present study, rat erythrocytes were used as a model of plasma membranes because no influence of intracellular organella was involved. It was discovered that the membrane fluidity of rat erythrocytes was decreased significantly following addition of LPS at concentrations over 500 μg/ml. This results suggested that LPS altered the dynamic properties of the surface layer of the erythrocyte membranes since the 5-doxyl stearic acid (5-DSA) used as a spin labelling agent reflects the physical response in the hydrophilic region of membranes. Moreover, a marked membrane fragility of erythrocytes was found following treatment with LPS. Benedetto *et al.*[12] have demonstrated that there is a possibility of direct interaction through the hydrophobic bond between lipid A of LPS and phospholipid in artificial membranes. Taurine-conjugated bile acids may have an influence on the effect of LPS on erythrocyte membranes because taurine-conjugated bile acids affect the dynamic properties of the outer layer of the membranes as does LPS. In fact, pretreatment of erythrocytes with taurocholic acid (TCA) or tauroursodeoxycholic acid

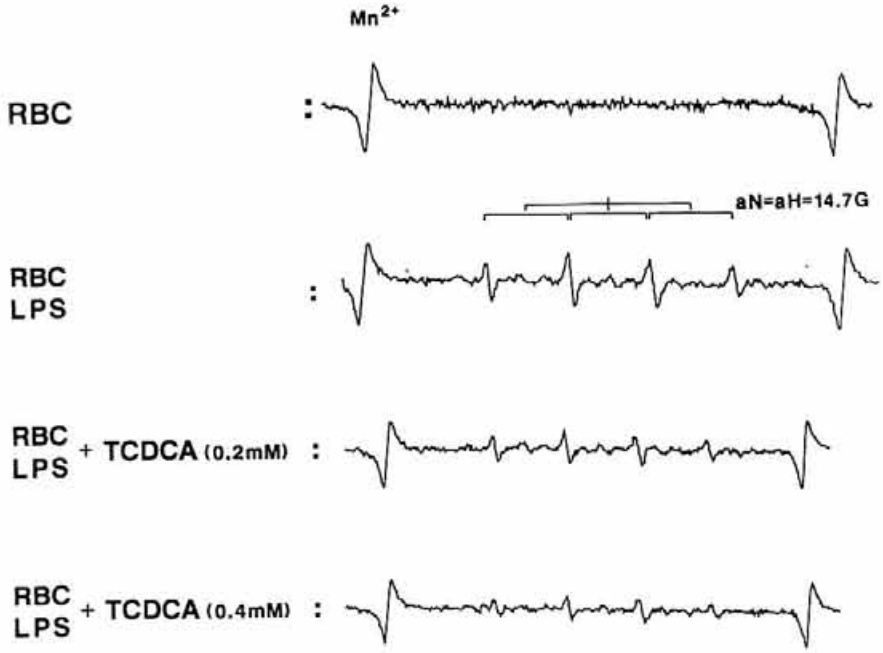

FIGURE 4 Effects of TCDCA on free radical produced by LPS in rat erythrocytes.
LPS : 500 μg/ml lipopolysaccharide.
TCDCA: taurochenodeoxycholic acid.

(TUDCA) had a protective effect against the decrease of membrane fluidity induced by LPS. In striking contrast, the pretreatment of erythrocytes with taurochenodeoxycholic acid (TCDCA) showed no effect on the membrane disorder induced by LPS treatment. These results indicate that TCA and TUDCA may help to neutralize the decreased membrane fluidity. Similar results were obtained regarding the membrane fragility. As for uptake of these taurine-conjugate bile acids into the membranes *in vitro,* the strength is in the order, TCA > TUDCA > TCDCA[13]. It seems reasonable to consider that taurine-conjugated bile acids have to be present in the membranes in large amounts to exhibit the protective effect against LPS-induced membrane disturbance. As demonstrated by Oelberg *et al.*[14], about 60% of the cholic acid taken up by human erythrocytes remains in the membrane fraction. It is supposed, therefore, that the protective effects of taurine-conjugated bile acids on erythrocyte membranes depend, in part, on their permeability to the membrane.

In the present study, oxygen radicals were also determined by EPR in LPS-treated rat erythrocytes. Hydroxyl radicals were detected in the presence of both erythrocytes and LPS, and enhancement of the radical by LPS was found in a dose-dependent manner. In 1983, Bannister *et al.*[15] reported the production of hydroxyl radicals in erythrocytes treated with adriamycin, and suggested that stimulation of hexose monophosphate shunt by adriamycin, resulting in the formation of hydrogen peroxide, may contribute to the generation of hydroxyl radicals. However, the detailed mechanism of hydroxyl radical generation in erythrocytes treated with LPS

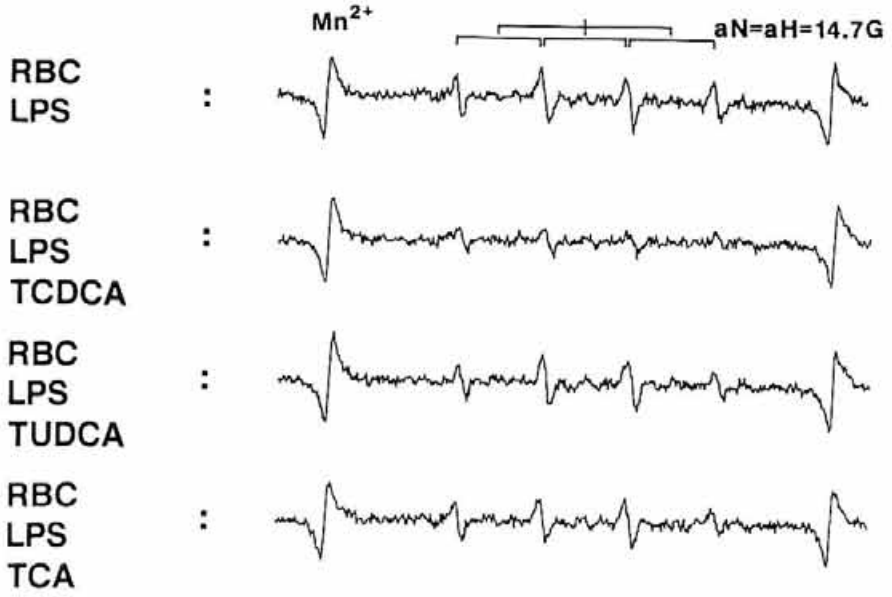

FIGURE5 EPR spectra obtained by addition of LPS into rat erythrocytes pretreated with bile acids.
LPS : 500 μg/ml lipopolysaccharide.
TCDCA: 0.2 mM taurochenodeoxycholic acid.
TUDCA: 2.0 mM tauroursodeoxycholic acid.
TCA : 2.0 mM taurocholic acid.

was not defined in the present studies. The generation of the hydroxyl radical was reduced dose-dependently by pretreating the erythrocytes with TCDCA, suggesting the possibility that TCDCA functioned, in part, as a scavenger of hydroxyl radicals or inhibited their production. On the other hand, pretreatment with TUDCA or TCA showed little influence on the oxygen radical. In the present study, it was shown that there is no correlation between the protective effects and the quenching action to hydroxyl radicals due to pretreatment with taurine-conjugated bile acids in erythrocytes treated with LPS. In 1987, Carp *et al.*[16] demonstrated that oxygen radicals including hydroxyl radicals attack the membrane lipid or protein of hepatocytes, resulting in membrane dysfunction. But judging from the present results, the hydroxyl radicals produced in erythrocytes treated with LPS seem to contribute little to LPS-induced membrane fragility at least in this study.

In conclusion, the possibility should be emphasized that the action by bile acids, especially by TCA and TUDCA, of normalizing the membrane fluidity may help to maintain the membrane integrity and to bring about cytoprotection in severe liver injury or obstructive jaundice with systemic endotoxemia.

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