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Effect of Alkali-Treated Lipopolysaccharide on the Intracellular Cations of Human Erythrocytes

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The adsorption to human erythrocytes of *Escherichia coli* lipopolysaccharide treated by mild alkaline hydrolysis (h-LPS) stimulated an increase in the intracellular Na^+ concentration and a decrease in the intracellular K^+ concentration of the erythrocytes. Erythrocytes treated by h-LPS remained responsive to the membrane adenosine triphosphatase inhibitors ouabain and ethacrynic acid, indicating that h-LPS did not alter erythrocyte cations by depleting energy intermediates or uncoupling energy metabolism from active cation transport. The h-LPS-treated erythrocytes became non-agglutinable by the lectin concanavalin A prior to the development of changes in intracellular cations. In addition, h-LPS-treated erythrocytes demonstrated a threefold-greater cation response to ethacrynic acid than the untreated erythrocytes; this greater response was probably due to local membrane effects by h-LPS on the ethacrynic acid-sensitive adenosine triphosphatase. It is suggested that the h-LPS-induced alteration of erythrocyte cation content was secondary to an increase in ion permeability localized to the concanavalin A receptor regions of the erythrocyte membrane, possibly combined with indirect effects of membrane-bound h-LPS on ethacrynic acid-sensitive adenosine triphosphatase.

Disk-shaped particles of lipopolysaccharide (LPS) from gram-negative bacteria can bind to mammalian erythrocyte membranes by edge attachment (8). In addition, a protein has been isolated from human erythrocyte membranes that demonstrates receptor specificity for LPS (9). However, relatively little information is available concerning specific alterations of membrane structure and/or function induced by attachment of LPS to cell surfaces. Čižnár and Shands have reported that LPS subjected to mild alkaline hydrolysis demonstrates a greatly increased affinity for the erythrocyte surface (4). In addition, excessive quantities of the alkali-treated LPS cause lysis of the erythrocytes (4). The principal chemical effect of mild alkaline hydrolysis ($\leq$0.25 N NaOH for $\leq$60 min at $\leq$56°C) is a diminution of LPS particle size (11) accompanied by release of some fatty acid and ninhydrin-positive material (7). However, treatment by mild alkaline hydrolysis results in a moderate or negligible decrease in the toxicity and pyrogenicity of *Escherichia coli* LPS (7). Release of extensive amounts of free fatty acids and detoxification occur only after prolonged alkaline hydrolysis (24 h) of LPS (11). Since LPS treated by mild alkaline hydrolysis retains significant biological activities, we have chosen the interaction of alkaline-hydrolyzed LPS (h-LPS) with human erythrocytes as a model for the effects of LPS on cell membranes. We now report that h-LPS adsorbed to human erythrocytes stimulates large decreases in the intracellular K^+ and increases of intracellular Na^+ concentrations of the erythrocytes. Increased flux of Na^+ and K^+ across the concanavalin A receptor regions of the erythrocyte membrane and indirect effects of h-LPS on ethacrynic acid-sensitive adenosine triphosphatase (ATPase) are discussed as possible mechanisms for the effect of h-LPS on erythrocyte cations.

MATERIALS AND METHODS

LPS. Lyophilized preparations of *E. coli* O127:B8 or O26:B6 LPS (Boivin) were purchased from Difco Laboratories, Detroit, Mich. The h-LPS was obtained by incubating the LPS in a 0.25 N NaOH solution for 60 min at 37°C. The NaOH, low-molecular-weight fatty acids, and ninhydrin-positive materials that were split from LPS by alkaline hydrolysis were then removed from the h-LPS preparation by dialysis against a large volume of phosphate-buffered saline (PBS) for 16 h at 4°C. Until utilized in experiments, LPS and h-LPS were stored as aqueous concentrates at 4°C in the PBS (140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, and 1 mM $NaH_2PO_4 \cdot H_2O$ titrated to pH 7.2 with 0.1 N HCl).

Treatment with h-LPS and incubation of eryth-

rocytes. Human erythrocytes were obtained from 2- to 21-day-old blood unsuitable for transfusion due to increased plasma bilirubin levels. The erythrocytes were washed three times with PBS before use in experiments. Treatment with h-LPS was accomplished by incubating the erythrocytes (2%, vol/vol) for 60 min at room temperature (22 to 24°C) in PBS containing h-LPS, followed by low-speed centrifugation of the erythrocytes into a pellet and washing of the pellet by PBS without h-LPS. The ability of h-LPS to strongly adsorb to erythrocyte membranes (4, 7) was confirmed for our conditions by observing agglutination of O127:B8 h-LPS-treated erythrocytes by *E. coli* O127:B8 antiserum (Difco) to a reciprocal dilution titer of 0.0078. For the cation content experiments, control (untreated) erythrocytes or erythrocytes treated by h-LPS were incubated at a 2% packed-cell volume for variable periods of time at room temperature in PBS. To determine Na^+ and K^+ concentrations after incubation, the erythrocytes were washed three times with chilled 150 mM tetramethylammonium chloride, and the intracellular sodium (Na_i) and potassium (K_i) concentrations were measured by flame photometry of erythrocytes diluted 1:200 in 15 mM lithium nitrate. The packed-cell volume of erythrocyte samples was determined in triplicate in microhematocrit capillary tubes. Values reported for Na_i and K_i are expressed in millimoles per liter of erythrocytes. Hemoglobin released into the medium by erythrocytes was chemically determined by the cyanmethemoglobin method (3).

Hemagglutination. Erythrocytes were suspended to 1.0% (vol/vol) in PBS and added in 125-μl portions to glass tubes containing 125 μl of PBS with variable concentrations of concanavalin A (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) and/or h-LPS. The erythrocyte suspensions were gently mixed, and a 200-μl portion was transferred from each glass tube into a microtiter plate well (V-shaped bottoms, Cooke Engineering Co., Alexandria, Va.). The microtiter plates were faced by transparent plastic tape, and the effacement of wells was evaluated after 3 h at room temperature. Complete effacement of a microtiter plate well represents hemagglutination, and the presence of a dense button of erythrocytes at the bottom of a well surrounded by a clear zone of buffer represents absence of hemagglutination.

RESULTS

Effect of h-LPS on erythrocyte cation concentrations. Human erythrocytes exposed to 400 μg of LPS per ml for 1 h, washed, and then incubated for 24 h in PBS without LPS showed no significant changes in Na_i or K_i. In contrast, erythrocytes incubated for 1 h with h-LPS at concentrations up to 200 μg/ml, washed, and then maintained in PBS without h-LPS for 24 h demonstrated a progressive increase of Na_i and decrease of K_i (Fig. 1). A linear relationship with a slope near unity was observed in 11 separate experiments for the ΔNa_i and ΔK_i of

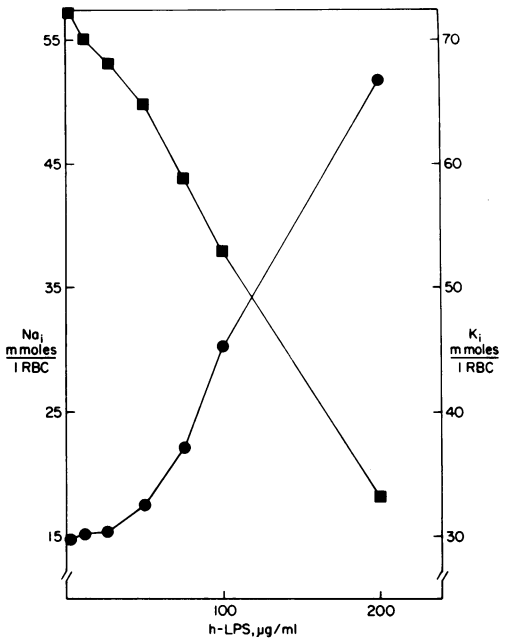

FIG. 1. *Intracellular sodium (●) and potassium (■) concentrations of human erythrocytes treated for 1 h with the indicated concentrations of h-LPS and incubated for 24 h in PBS without h-LPS.*

erythrocytes treated by 100 μg of h-LPS per ml (Fig. 2). The mean ΔNa_i by linear regression analysis of the 11 different experiments was +10.1, and the mean ΔK_i was −11.2 ($P < 0.001$). Therefore, the increase in cellular Na^+ was almost equivalent to the decrease in cellular K^+, and the Na^+-plus-K^+ content of erythrocytes treated with 100 μg of h-LPS per ml was similar to that of the control cells. However, treatment with less than 100 μg of h-LPS per ml appears to have had less effect on Na_i than on K_i (Fig. 1). Whether the lower doses of h-LPS (<100 μg/ml) have a selective action on erythrocyte K_i or whether the initial low erythrocyte Na_i concentration (Fig. 1) renders values for ΔNa_i too small for accurate measurement by flame photometry is presently undetermined.

Ouabain dose-response curves. The effect of h-LPS on Na_i and K_i of human erythrocytes was significantly greater than that observed for the membrane ATPase inhibitor ouabain (Fig. 3). In addition, similar ouabain dose-response curves were obtained for h-LPS-treated and control erythrocytes.

Enhanced effect of ethacrynic acid on h-LPS-treated erythrocytes. The membrane ATPase inhibitor ethacrynic acid stimulated an almost threefold-greater change in the intracellular cations of h-LPS-treated erythrocytes

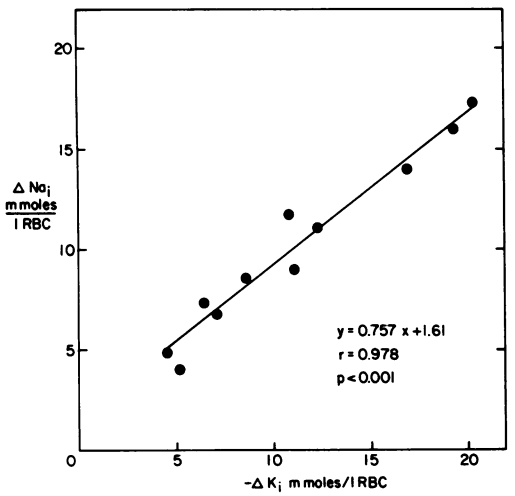

FIG. 2. *Linear regression analysis of the decrease in intracellular potassium ($-\Delta K_i$) as a function of the increase in intracellular sodium (ΔNa_i) induced by treatment of human erythrocytes with 100 μg of h-LPS per ml followed by incubation in PBS without h-LPS for 24 h. Each point represents 1 of 11 separate experiments. The value for the slope of the line is given as r, the y-intercept is given as y, and the P-value for the statistical significance of the analysis is given as P.*

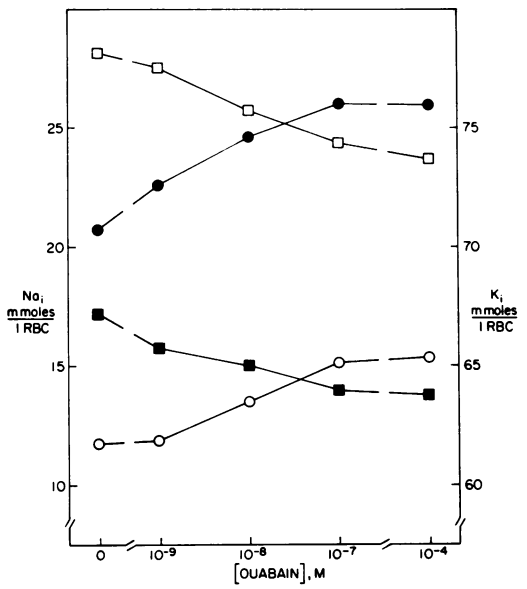

FIG. 3. *Dose-response curves for the effect of ouabain on the intracellular sodium (Na_i) concentration of h-LPS-treated (●) or control (○) erythrocytes and on the intracellular potassium (K_i) concentration of h-LPS-treated (■) or control (□) erythrocytes. Treatment of erythrocytes was with 100 μg of h-LPS per ml. Both treated and control erythrocytes were incubated for 24 h in PBS containing the indicated concentrations of ouabain.*

than in the cations of erythrocytes not treated by h-LPS (Table 1). Such enhancement was not observed for ouabain (Fig. 3).

Concanavalin A agglutinability of h-LPS-treated erythrocytes. Previously untreated erythrocytes were not agglutinated by the lectin concanavalin A in the concomitant presence of 1,000 μg of h-LPS per ml (Fig. 4B). More importantly, erythrocytes treated with h-LPS, washed with PBS, and exposed to concanavalin A without h-LPS (in buffer) were not agglutinated by concanavalin A (Fig. 4D). The inhibitory action of h-LPS on concanavalin A thus appears to be due to h-LPS bound to erythrocyte membranes and not due to excess h-LPS present in the suspension buffer. Loss of concanavalin A agglutinability was also noted immediately after treatment with 75 μg of h-LPS per ml and before significant cation changes were measurable (Table 2). During subsequent 24- to 48-h incubations in PBS, treated erythrocytes remained unresponsive to concanavalin A while intracellular cation changes developed.

Hemoglobin leakage from h-LPS-treated erythrocytes. Erythrocytes treated with progressively greater concentrations of h-LPS released increasing amounts of hemoglobin into the suspension medium (Table 3). However, only 5.9% of the erythrocytes were lysed at the highest h-LPS concentration studied.

DISCUSSION

Mild alkaline hydrolysis greatly enhances the affinity of LPS for erythrocyte membranes

TABLE 1. *Effect of ethacrynic acid on intracellular cations of untreated and h-LPS-treated erythrocytes*

Intracellular cation	Incubation condition[a]		Effect of ethacrynic acid[b]
	PBS	Ethacrynic acid	
Na_i			
Control	20.7	26.1	+5.4
Treated[c]	32.3	47.6	+15.3 (2.8)[d]
K_i			
Control	76.1	73.9	−2.2
Treated[c]	62.7	56.3	−6.4 (2.9)[d]

[a] Incubation for 24 h in PBS with or without 10^{-3} M ethacrynic acid. Concentrations of Na_i and K_i were determined as millimoles per liter of erythrocytes.

[b] The effect of ethacrynic acid is reported as the difference between Na_i (or K_i) of cells incubated with ethacrynic acid and Na_i (or K_i) of cells incubated without ethacrynic acid.

[c] Treated with 100 μg of h-LPS per ml.

[d] Values in parentheses indicate magnitude of enhanced ethacrynic acid effect on cations of treated cells as compared to that of untreated cells.

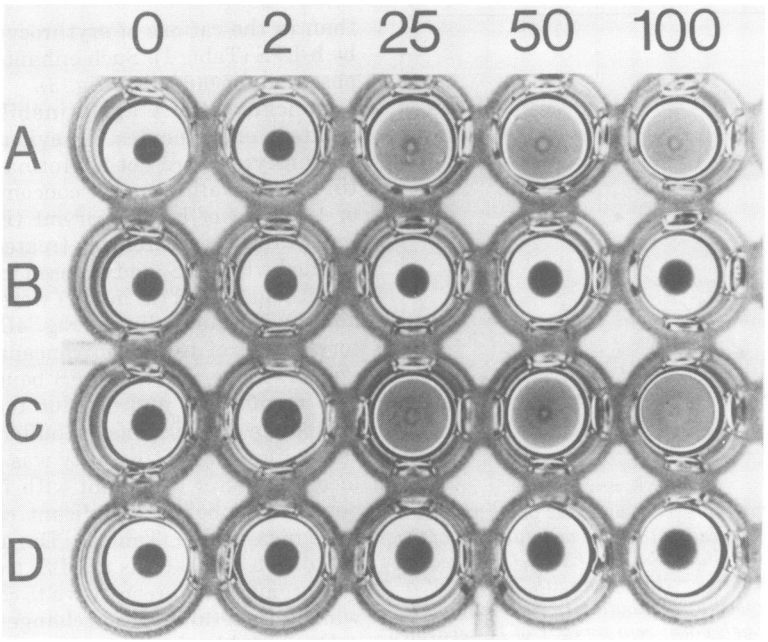

FIG. 4. *Inhibition of concanavalin A hemagglutination by h-LPS treatment of human erythrocytes. (A) Untreated erythrocytes incubated in PBS. (B) Untreated erythrocytes incubated in PBS containing 1,000 μg of h-LPS per ml. (C) Erythrocytes treated by 10 μg of h-LPS per ml followed by incubation in PBS without h-LPS. (D) Erythrocytes treated with 1,000 μg of h-LPS per ml followed by incubation in PBS without h-LPS. Numbers at the top of each vertical row indicate the concentration of concanavalin A (micrograms per milliliter) added to the incubation mixtures of that row.*

TABLE 2. *Effect of incubation on intracellular cations and concanavalin A agglutinability of erythrocytes treated with h-LPS*[a]

Incubation time (h)	ΔNa_i[b]	ΔK_i[b]	Concanavalin A agglutination[c]	
			Control	Treated
0	−1.2	−0.7	++	0
24	+2.6	−3.3	++	0
48	+3.0	−5.1	++	0

[a] Erythrocytes treated with 75 μg of h-LPS per ml and then incubated in PBS for the indicated time periods.

[b] ΔNa_i (or ΔK_i) = Na_i (or K_i) of treated cells − Na_i (or K_i) or untreated cells. The concentrations of Na_i and K_i were determined as millimoles per liter of erythrocytes.

[c] Concanavalin A agglutinability of control and h-LPS-treated erythrocytes recovered after the indicated times of incubation in PBS for determination of Na_i and K_i. ++, Complete effacement of microtiter wells by both 20 and 250 μg of concanavalin A per ml.

(4, 7). The adsorption of h-LPS to human erythrocytes induced an increase in intracellular Na^+ concentration and a decrease in intracellular K^+ concentration, dependent on the h-LPS dose (Fig. 1) and incubation time in PBS (Table 2). The h-LPS-treated erythrocytes retained both ouabain- and ethacrynic acid-sensitive ATPase activity (Fig. 3, Table 1). Thus, h-LPS bound to erythrocytes does not induce cation changes by stimulating erythrocyte metabolism (13) with rapid depletion of energy sub-

TABLE 3. *Release of hemoglobin by h-LPS-treated erythrocytes*[a]

h-LPS (μg/ml)	ΔNa_i[b]	ΔK_i[b]	Hemoglobin[c] (mg/100 ml)	Hemolysis[d] (%)
0			2.2	0.3
50	+2.9	−7.6	9.0	1.3
100	+16.0	−19.4	15.0	2.1
200	+37.4	−33.3	41.0	5.9

[a] Erythrocytes treated with the indicated concentration of h-LPS for 1 h and then incubated in PBS for 24 h.

[b] ΔNa_i and ΔK_i as defined in footnote *b* of Table 2.

[c] Hemoglobin concentration determined on cell-free suspension medium after a 24-h incubation.

[d] When 100% hemolysis was induced by treatment of erythrocytes (2%, vol/vol) with distilled water, the concentration of released hemoglobin was 700 mg/100 ml.

strates and consequent loss of ATPase-dependent ion transport or by uncoupling energy metabolism of the erythrocyte. In addition, there is no evidence that h-LPS competes with either ouabain or ethacrynic acid in stimulating erythrocyte cation changes (Fig. 3, Table 1). It is, therefore, unlikely that h-LPS interacts directly with the membrane-bound, ouabain- or ethacrynic acid-sensitive ATPases of the erythrocyte.

A previous study from this laboratory has indicated that membrane-associated h-LPS acts predominantly on the concanavalin A receptor regions of erythrocyte membranes (J. R. Warren and M. M. Kowalski, Exp. Cell Res., in press). The present study demonstrates that loss of concanavalin A agglutinability induced by membrane-bound h-LPS (Fig. 4) occurs prior to the changes in intracellular cation concentrations (Table 2). There is considerable evidence that membrane regions containing concanavalin A receptor sites are involved in the permeation of ions and small molecules through membranes. Averdunk has reported that concanavalin A treatment of mouse thymocytes increases the leak fluxes of Na^+ and K^+, with a corresponding increase in the intracellular Na^+ concentration and a decrease in the intracellular K^+ concentration (1). The principal high-affinity receptor of human erythroctyes for concanavalin A is the minor glycoprotein (the band 3 component on polyacrylamide gel electrophoresis of erythrocyte membranes in sodium dodecyl sulfate) (5). Anion transport across the erythrocyte membrane can be specifically inhibited by covalent modification of the minor glycoprotein by 4,4'-diisothiocyano-2,2'-ditritiostilbene-disulfonate (2). There is also evidence that the minor glycoprotein provides sites for the facilitated diffusion of glucose across erythrocyte membranes (6). Perturbation of the erythrocyte concanavalin A receptor regions by h-LPS could result, therefore, in an increased flux of Na^+ and K^+ (and perhaps other ions and organic solutes) across the receptor regions.

Augmentation of the ethacrynic acid effect on erythrocyte cations by h-LPS (Table 1) was unexpected. It is improbable that the greater action of ethacrynic acid was due to alteration by h-LPS of erythrocyte metabolism, since a similar enhancement was not observed for ouabain (Fig. 3). It is more likely that h-LPS modifies membrane responses to ethacrynic acid either by promoting increased binding of ethacrynic acid or by altering the responses of membrane-associated ATPase to bound ethacrynic acid. Such indirect effects of membrane-bound h-LPS on ethacrynic acid-sensitive ATPase could also be important for the h-LPS-induced cation changes. Unfortunately, no information is presently available in the literature on the effects of LPS or LPS derivatives on membrane-associated ATPase activity. Inhibition of ATPase activity on the external surfaces of intact rabbit leukocytes or in soluble leukocyte extracts by LPS has been reported (11). The relevance of this inhibition, however, to possible effects of h-LPS on ATPase activity localized to the inner membrane of human erythrocytes is at present unknown.

In summary, h-LPS bound to human erythrocytes stimulates significant alterations in the cation content of the erythrocytes and appears to do so by localized changes in membrane permeability possibly combined with indirect effects on membrane-associated, ethacrynic acid-sensitive ATPase. Large changes in Na_i and K_i were detectable in the absence of extensive hemolysis of erythrocyte suspensions (Table 3). Consequently, the alterations in membrane structure and/or function responsible for the cation changes must precede the gross erythrocyte membrane disorganization reported by Čižnár and Shands (4) at high h-LPS concentrations. The loss of cellular K^+ ions and entrance of excess Na^+ ions are thought to be linked to LPS stimulation of endogenous pyrogen production by exudate granulocytes (12). Thus, mechanisms by which h-LPS stimulates erythrocyte cation changes are relevant to the important biological property of LPS as a pyrogen. Whether membrane-mediated effects on intracellular cations might be of importance for other biological and toxic properties of LPS remains to be determined by future work.

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