PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 161, 255–259 (1979)

# Energy Metabolism and $Na^+,K^+$ Redistribution in Human Erythrocytes Treated with Lipopolysaccharide Endotoxin (40531)[1]

CHARLES H. WALLAS,[2] JOHN R. WARREN,[3] AND MARK M. KOWALSKI

*Department of Pathology, Northwestern University Medical School, Chicago, Illinois 60611*

Mild alkaline hydrolysis of bacterial lipopolysaccharide endotoxin (LPS) reduces the molecular size of LPS and alters the fatty acid composition of the lipid A toxophore (1, 2). Despite these physical and chemical changes, LPS treated by mild alkaline hydrolysis demonstrates enhanced mitogenicity and toxicity for murine B-lymphocytes cultured *in vitro* without serum (2). A common LPS configuration is thought to initiate both *in vitro* lymphocyte mitogenicity and *in vivo* pyrogenicity (3). Therefore, *in vitro* interactions with mammalian cells of LPS mildly hydrolyzed at alkaline pH are relevant to possible mechanisms of endotoxicosis. One such derivative prepared by subjection of LPS to mild hydrolysis in 0.25 *N* NaOH at 37° (h-LPS) has been demonstrated to strongly adsorb to erythrocytes (4) and stimulate an increase in intracellular $Na^+$ and decrease in intracellular $K^+$ content of the erythrocytes (5). Three possible mechanisms of action can be proposed to explain the effects of h-LPS on erythrocyte intracellular cation concentrations. First, h-LPS might interact with and thereby inhibit the $Na^+,K^+$-dependent adenosine triphosphatase (ATPase) of red cell membranes. Indeed, LPS has been reported to inhibit $Na^+,K^+$-ATPase activity of liver plasma membrane fragments (6) and $K^+$-ATPase activity of leukocyte homogenates (7). Second, it is well established that LPS can markedly stimulate the rate of aerobic and anaerobic glycolysis by cells (8). Since the h-LPS-treated erythrocytes were maintained in glucose-free buffer in our previous experiments (5), it is possible that h-LPS directly stimulates glycolysis in red cells with a depletion of cellular adenosine triphosphate (ATP) and thus diminution of $Na^+,K^+$-ATPase activity. Third, h-LPS perhaps increases the permeability of red cell membranes to $Na^+$ and $K^+$.

To examine the possibility of direct h-LPS actions on the ATPase-dependent transport of $Na^+$ out of and $K^+$ into erythrocytes, the effects of glucose, adenosine, and ouabain on the intracellular ATP content and $Na^+,K^+$ concentrations of h-LPS-treated red cells were directly measured. In addition, to ascertain if h-LPS might directly stimulate glycolysis, effects of h-LPS on the rate of glycolysis by intact erythrocytes and fragment erythrocytes were compared. The results of these experiments are reported in this communication.

*Materials and methods.* The h-LPS was prepared from *Escherichia coli* R127:B8 LPS (Difco Laboratories, Detroit, Mich.) as previously described (5). Human erythrocytes were obtained from 2- to 5-day-old blood unsuitable for transfusion due to increased plasma bilirubin level. The erythrocytes were washed three times in phosphate-buffered saline (PBS), pH 7.4, treated with h-LPS (100–200 μg/ml) as described previously (5), and finally washed three times in PBS prior to incubation studies. Untreated (control) and h-LPS-treated erythrocytes were incubated at 22° at a 2% packed cell volume (PCV) in PBS and sampled periodically for measurement of intracellular sodium ($Na_i$), potassium ($K_i$), and ATP concentrations (5, 9). Erythrocyte ATP levels were artificially elevated in some experiments prior to treatment with h-LPS by a 2-hr incubation at 37° at a PCV of 20% in an isotonic medium containing 23 m*M* sodium chloride, 96 m*M* sodium phosphate, 20 m*M* glucose, and 10 m*M* adenosine at pH 7.4. To determine the rate of glycolysis by control and h-LPS-treated erythrocytes, the cells were incubated at 37° at a 20% PCV in medium containing 135 m*M* sodium chloride,

[1] This study was supported in part by Public Health Service Grant RR-05370.

[2] Present address: Department of Pathology, Vanderbilt University Medical Center, Nashville, Tenn. 37232.

[3] All reprint requests should be sent to Dr. Warren at Northwestern University.



0037-9727/79/070255-05$01.00/0

5 m*M* potassium chloride, 20 m*M* glycylglycine, 1.2 m*M* sodium phosphate, and 10 m*M* glucose at pH 7.5. Samples were taken periodically over 2 hr and processed for measurement of lactate production as previously described (10). The rate of lactate production was also measured for erythrocytes which were fragmented as follows. Erythrocytes were washed three times in isotonic medium containing 20 m*M* Tris base, 10 m*M* sodium chloride, 90 m*M* potassium chloride, 5 m*M* potassium phosphate, and 34 m*M* tetramethylammonium chloride at pH 8.0. After the addition of 25 m*M* nicotinamide, the erythrocytes were fragmented by four cycles of freezing and thawing in an acetone–dry-ice bath. The erythrocyte fragments were suspended at a final hemoglobin concentration of 25 g/100 ml in pH 7.4 PBS containing 10 m*M* glucose and 4.5 m*M* ATP with or without 200 μg of h-LPS per milliliter. Samples were taken periodically during a 30-min incubation at 37° for measurement of lactate production (10).

*Results.* Human erythrocytes exposed for 1 hr to h-LPS and then incubated for 24 hr in PBS without added h-LPS demonstrated an increase in $Na_i$ and a decrease in $K_i$ associated with a marked fall in ATP level (Table I). However, when glucose was present during the 24-hr incubation in PBS, the increase in $Na_i$ and decrease in $K_i$ of h-LPS-treated erythrocytes were 50% smaller while ATP was maintained at the level observed for control (untreated) cells (Table I). The relationship of the intracellular ATP of h-LPS-treated cells to $Na_i$ and $K_i$ concentrations was further examined utilizing erythrocytes with ATP content artificially elevated by a short incubation in isotonic medium enriched with glucose, adenosine, and inorganic phosphate. The h-LPS-treated cells with artificially elevated ATP content resembled untreated cells (either with or without an elevated ATP content) by their ability to decrease $Na_i$ and increase $K_i$ during incubation in PBS without added h-LPS (Fig. 1). This contrasts sharply with h-LPS-treated cells without artificially elevated ATP content which demonstrated a steady increase in $Na_i$ and decrease in $K_i$ (Fig. 1). The data reported in Fig. 1 were obtained in a single experiment using the same preparation of human erythrocytes. The

TABLE I. EFFECT OF GLUCOSE AND OUABAIN ON $Na_i$, $K_i$, AND ATP CONCENTRATIONS OF UNTREATED AND h-LPS-TREATED ERYTHROCYTES

| | Incubation conditions[a] | | |
|---|---|---|---|
| | PBS | Glucose | Glucose and ouabain |
| $Na_i$ | | | |
| Control | 16.5 | 11.1 | 20.4 |
| treated[b] | 25.8 | 15.7 | 30.7 |
| $\Delta Na_i$[c] | +9.3 | +4.6 | +10.3 |
| $K_i$ | | | |
| Control | 70.6 | 75.7 | 69.2 |
| treated[b] | 59.2 | 71.0 | 56.9 |
| $\Delta K_i$[c] | −11.4 | −4.7 | −12.3 |
| ATP | | | |
| Control | 0.81 | 1.60 | 1.56 |
| treated[b] | 0 | 1.57 | 1.58 |

[a] Incubation for 24 hr in PBS or in PBS containing 20 m*M* glucose or glucose plus 0.1 m*M* ouabain. Concentrations of $Na_i$, $K_i$, and ATP were determined as millimoles per liter erythrocytes. Each reported value for $Na_i$, $K_i$, or ATP concentration represents the mean of duplicate determinations with variation in duplicate determinations of $Na_i$ and $K_i$ less than 1.5% of the mean and of ATP less than 3.5% of the mean. The data were obtained in a single experiment using the same preparation of erythrocytes.

[b] Treated with 200 μg of h-LPS per milliliter.

[c] Values for $\Delta Na_i$ and $\Delta K_i$ were calculated for values of $Na_i$ and $K_i$ of h-LPS treated erythrocytes relative to the values of $Na_i$ and $K_i$ of untreated erythrocytes.

ability of h-LPS-treated erythrocytes with artificially elevated ATP content to decrease $Na_i$ and increase $K_i$ during a 24-hr incubation in PBS without added h-LPS was confirmed by repeated experiments ($n = 5$) using different preparations of red cells. High intracellular levels of ATP, therefore, protected h-LPS-treated cells against the loss of $K_i$ and gain of $Na_i$. Importantly, ouabain completely abolished the protective effect of glucose addition against $K_i$ loss and $Na_i$ gain by h-LPS-treated erythrocytes (Table I). The inhibitory action of ouabain reported in Table I was observed in repeated experiments ($n = 5$) using different preparations of h-LPS-treated red cells. Since ouabain is a strong inhibitor of $Na^+,K^+$-dependent ATPase activity, it appears that glucose and ATP are serving as energy sources for the ATPase-dependent transport of $Na^+$ and $K^+$ across the membranes of h-LPS-treated cells. When the ATP content of h-LPS-treated cells is adequate, active cation transport is maintained, and changes in $Na_i$ and $K_i$ concentrations are

thereby diminished.

These results strongly suggest that h-LPS adsorbed to erythrocytes does not alter cation concentrations of the cells by direct inhibition of ATPase-dependent cation transport. Information obtained on the inhibitory effect of bacterial LPS on ATPase activity of broken cell fragments (6, 7) would appear to have little or no relevance to LPS effects on ATPase activity localized to the internal membrane face of intact cells.

A significant stimulatory effect of h-LPS on the rate of glycolysis by intact human erythrocytes was observed. The results of a single experiment are shown in Fig. 2A in which the rate of lactate production was compared for untreated and h-LPS-treated intact erythrocytes. In repeated experiments ($n = 4$) h-LPS-treated intact erythrocytes demonstrated a glycolytic rate of 3.30 ± 0.6 mmole lactate/liter erythrocytes/hour (mean ± SE) as compared to a rate of 2.00 ± 0.41 mmole lactate/liter erythrocytes/hour for untreated intact erythrocytes ($P < 0.05$). To test whether h-LPS is capable of directly stimulating glycolysis, the erythrocytes were fragmented and the fragments then incubated both with and without h-LPS. Results of the single experiment with erythrocyte fragments reported in Fig. 2B would seem to indicate inhibition rather than stimulation by h-LPS of glycolysis by fragmented erythrocytes. However, in repeated experiments ($n = 4$) the rates of glycolysis by fragments incubated without h-LPS (6.09 ± 2.97 mmole/liter erythrocytes/hour) and by fragments incubated with h-LPS (4.73 ± 2.56 mmole/liter erythrocytes/hour) were not significantly different ($P > 0.1$). In none of the four separate experiments did lactate production by fragments incubated with h-LPS exceed lactate production by fragments incubated without h-LPS. It appears, therefore, that h-LPS does not significantly increase the rate of glycolysis by erythrocyte fragments. The increased rate of glycolysis induced in intact erythrocytes by h-LPS is thus probably not a direct effect of h-LPS but is rather secondary to some other h-LPS-induced change in the erythrocytes.

*Discussion.* The data reported here indicate that h-LPS does not inhibit the ATPase-dependent transport of $K^+$ into or of $Na^+$ out of the human erythrocyte. Furthermore, even

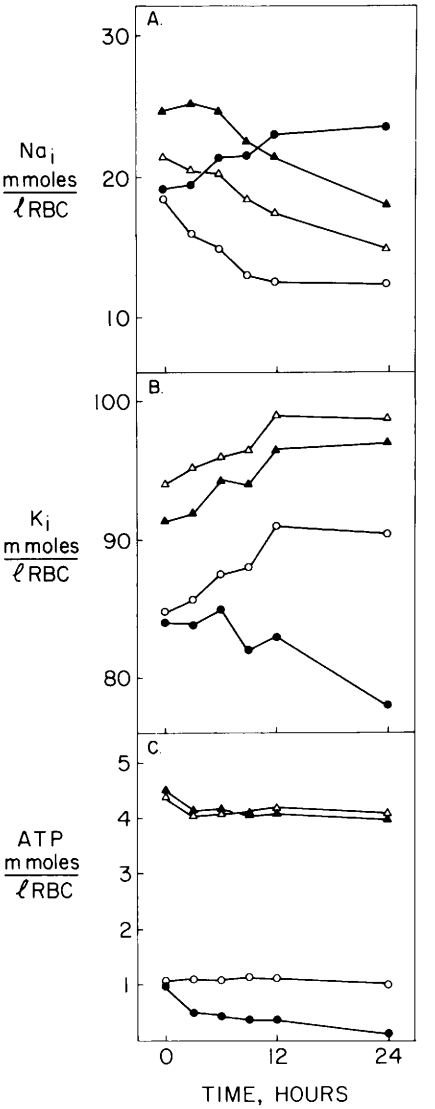


FIG. 1. Relationship of $Na_i$ and $K_i$ concentrations (Panels A and B, respectively) to the ATP content (Panel C) of h-LPS-treated and untreated human erythrocytes. Erythrocytes treated for 1 hr with 100 μg of h-LPS per milliliter are indicated by the closed symbols and untreated erythrocytes by the open symbols. Circles indicate values obtained with erythrocytes without artificially elevated ATP content. Values obtained with erythrocytes in which ATP content was artificially elevated by prior incubation in isotonic medium containing sodium phosphate, adenosine, and glucose are indicated by a triangle. The time of incubation of h-LPS-treated or untreated erythrocytes in PBS is shown on the abscissa. Each point represents the mean of duplicate determinations with variation in duplicate determinations of $Na_i$ and $K_i$ less than 1.5% of the mean and of ATP less than 3.5% of the mean.

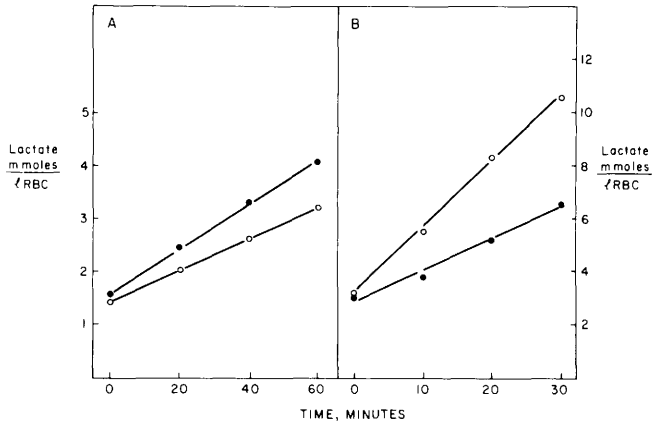


FIG. 2. Effect of h-LPS on lactate production by intact (Panel A) and fragmented (Panel B) human erythrocytes. In panel A, closed circles indicate lactate production by erythrocytes treated with 200 μg/ml of h-LPS and open circles lactate production by untreated erythrocytes. In panel B, lactate production by erythrocyte fragments incubated with 200 μg/ml h-LPS is indicated by closed circles and lactate production by erythrocytes incubated without h-LPS by open circles. Each point represents the mean of duplicate determinations with variation in duplicate determinations less than 3.5% of the mean.

though h-LPS does stimulate glycolysis in intact erythrocytes, h-LPS appears not to act directly on glycolysis by erythrocyte fragments. Increased glycolysis by intact red cells treated with h-LPS thus seems to be a secondary effect of h-LPS on the cells. An increase in the erythrocyte membrane permeability toward $Na^+$ and $K^+$ remains as an attractive possibility for the primary action of h-LPS on red cells. The $Na^+$ influx and $K^+$ efflux resulting from an increase in membrane permeability would markedly stimulate the membrane-bound $Na^+,K^+$-ATPase activity. Such an increase in $Na^+,K^+$-ATPase activity would result in the depletion of intracellular ATP observed for h-LPS-treated erythrocytes incubated in PBS without artificially elevated ATP levels (Table I and Fig. 1). Furthermore, the increased requirement for ATP would explain the accelerated rate of glycolysis detected for intact h-LPS-treated cells (Fig. 2). It must be stressed that even when high intracellular ATP levels are maintained throughout the 24-hr incubation of h-LPS-treated cells, the treated cells still contain more $Na^+$ and less $K^+$ than the untreated cells (Table I, Fig. 1). This is best explained as an inability of the membrane-bound ATPase to completely keep pace with the large amount of $Na^+$ and $K^+$ leaking through the highly permeable membranes of the h-LPS-treated cells.

Biologically active variants of bacterial LPS have been demonstrated to strongly inhibit the agglutination of human erythrocytes (11) and rodent nucleated cells (12) by the lectin concanavalin A without blocking lectin-binding to the cells. Furthermore, glycolipid mR595 bound to rat nucleated cells has been found to block the formation of caps by fluorescent concanavalin A bound to the cells (12). Cell-bound LPS thus appears to have a marked effect on plasma membrane structure in the vicinity of concanavalin A receptor proteins. Membrane regions of cells containing concanavalin A receptor proteins are thought to be involved in ion permeation through membranes, including $Na^+$ and $K^+$ (13). Effects of LPS on cation fluxes in intact cells should now be directly measured. Furthermore, possible relationships of LPS-induced cation redistributions to the activation of cell metabolism and function should be evaluated.

*Summary.* High intracellular concentrations of ATP have been observed to protect human erythrocytes against the loss of $K^+$ and gain of $Na^+$ induced by h-LPS. This protection was abolished by ouabain, indicating that red cells treated with h-LPS are competent for cation transport mediated by $Na^+,K^+$-dependent ATPase. In addition, the failure of h-LPS to stimulate glycolysis by fragmented erythrocytes suggests that the in-

creased glycolytic rate of intact h-LPS-treated red cells is a secondary effect of h-LPS. Thus, the cation redistribution induced by h-LPS appears not to be due to a primary effect of h-LPS on red cell energy metabolism. Since h-LPS demonstrates neither an obvious inhibitory effect on $Na^+,K^+$-ATPase activity nor a direct stimulating effect on erythrocyte glycolysis, it is proposed that a primary action of h-LPS on human erythrocytes is to increase plasma membrane permeability toward $Na^+$ and $K^+$. The cation redistribution resulting from increased membrane permeability would explain the secondary increase in energy metabolism observed for the h-LPS-treated intact cells. Additional studies are now required on rapid effects of h-LPS on transmembrane fluxes of cations in intact mammalian cells, and the possible role played by such effects in the biological activation of cells by h-LPS.

1. Lüderitz, O., Galanos, C., Lehmann, V., Nurminer, M., Rietschel, E. T., Rosenfelder, G., Simon, M., and Westphal, O., J. Infect. Dis. **128,** S17 (1973).
2. Goodman, G. W., and Sultzer, B. M., Infect. Immunol. **17,** 205 (1977).
3. Elin, R. J., Sandberg, A. L., and Rosenstreich, D. L., J. Immunol. **117,** 1238 (1976).
4. Ciznar, I., and Shands, J. W., Jr., Infect. Immunol. **4,** 362 (1971).
5. Warren, J. R., Kowalski, M. M., and Wallas, C. H., Infect. Immunol. **17,** 389 (1977).
6. Utili, R., Abernathy, C. O., and Zimmerman, H. J., J. Infect. Dis. **136,** 583 (1977).
7. Tenney, S. R., and Rafter, G. W., Arch. Biochem. Biophys. **126,** 53 (1968).
8. Woods, M. W., Landy, M., Whitby, J. L., and Burk, D., Bacteriol. Rev. **25,** 447 (1961).
9. Wallas, C. H., Brit, J. Haematol. **27,** 145 (1974).
10. Wallas, C. H., Brit. J. Haematol. **26,** 71 (1974).
11. Warren, J. R., and Kowalski, M. M., Exp. Cell Res. **107,** 462 (1977).
12. Brailovsky, C. A., Nigam, V. N., and Pesant, S., Exp. Cell Res. **109,** 389 (1977).
13. Averdunk, R., Biochem. Biophys. Res. Commun. **70,** 101 (1976).

Received November 27, 1978. P.S.E.B.M. 1979, Vol. 161.