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Action of Bacterial Endotoxin and Lipid A on Mitochondrial Enzyme Activities of Cells in Culture and Subcellular Fractions

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Escherichia coli O127:B8 lipopolysaccharide (LPS), prepared by the Westphal procedure, caused a marked decrease in the activities of mitochondrial malate dehydrogenase, succinate dehydrogenase, and adenylate kinase in African green monkey kidney (Vero) cells and primary cultures of mouse liver cells within 2 h after exposure to 10 μg of LPS/ml of culture medium. These three enzyme activities leaked into the supernatant fraction, and cytochrome oxidase activity was lost from the mouse liver mitochondrial particulate fraction within 45 min after exposure to 10 μg of LPS/mg of protein. Loss of malate dehydrogenase activity from isolated mitochondria was also accelerated by LPS from *E. coli* O26:B6 (Boivin preparation) or *Salmonella typhosa* O901 (Westphal preparation), and by lipid A from *Salmonella minnesota* or *Shigella sonnei*. In addition, LPS and lipid A inhibited state 3 respiration by isolated mitochondria with attendant loss of respiratory control, but adenosine 5'-diphosphate/O ratios were relatively unchanged. Impaired mitochondrial function is an early event after exposure to biologically relevant amounts of LPS or lipid A.

We have described previously direct effects of bacterial endotoxin (lipopolysaccharide, LPS) on established cell cultures (A. McGivney and S. G. Bradley, RES J. Reticuloendothel. Soc., in press). LPS at 10 μg/ml of culture medium causes a marked decrease in mitochondrial enzyme activities in African green monkey kidney (Vero) cells within 2 to 4 h; this loss correlates temporally with leakage of these enzyme activities from the mitochondrial particulate fraction. No serum was added to the cells that had been grown in continuous culture during exposure to LPS; therefore, the observed changes were not due to hormones or humoral mediators. Moreover, the involvement of an antibody-mediated process was minimized. In this investigation, the effects described for Vero cells treated with LPS have been compared to those for mouse primary liver cell cultures.

LPS has been shown by others to elicit alterations of mitochondrial function in animals (9) and subcellular fractions (19). Mela et al. (21) injected *Escherichia coli* LPS into rats intraperitoneally and removed the livers after onset of endotoxic shock. They isolated mitochondria and observed changes in respiratory control ratios (RCR) and adenosine 5'-diphosphate (ADP)/O values as compared to normal mitochondria. Greer et al. (11) have reported decreased RCR values when isolated rat liver mitochondria were treated with LPS. Harris et al. (12) observed loss of respiratory control in beef heart mitochondria treated with *Bordetella bronchiseptica* LPS isolated by the Boivin method. However, these alterations in mitochondrial function could not be elicited with other preparations of LPS.

We have examined the effects of LPS on the activities of various mitochondrial enzymes in both established cultures of Vero cells and in mouse primary liver cell cultures. Decreased mitochondrial enzyme activity was dependent upon time of exposure of the cells to LPS and the dose of LPS. This decrease was correlated with leakage of enzyme activity from the mitochondrial fraction. In addition, swelling of the mitochondria exposed to LPS and loss of respiratory control in mitochondrial preparations indicate that the mitochondrial membrane is damaged by LPS. These perturbations of mitochondrial function occurred after exposure of the mitochondria to different preparations of LPS and to purified lipid A preparations and was not unique to one type of LPS.

MATERIALS AND METHODS

Maintenance of African green monkey kidney cells. African green monkey kidney (Vero) cells in continuous culture were obtained from Byron K. Murray of this institution. The cells were cultivated and

maintained in modified Eagle minimal essential medium (MEM) with Earle balanced salt solution, containing L-glutamine as previously described (McGivney and Bradley, in press).

Preparation of mouse primary liver cells. Adult C3H/HeDub mice weighing 18 to 23 g were obtained from Flow Research Laboratories, Dublin, Va. The animals were acclimated to their new environment for 1 week before experimentation and were allowed free access to food (Purina Lab Chow, Purina Ralston Co., St. Louis, Mo.) and water. The mice were maintained on a 12-h diurnal light schedule.

The peritoneal cavity of the mice, killed by cervical dislocation, was opened, and a 25-gauge needle attached to a bottle of 0.1 M phosphate-buffered saline (pH 7.4) was inserted into the apex of the heart. The perfusion medium was allowed to flow into the heart causing swelling of the vena cava, which was then severed. The perfusion medium at unit gravity was allowed to flow through the heart until the liver and kidneys were free of blood. Five ml of 0.05% collagenase (*Clostridium histolyticum* type 1, Sigma Chemical Co., St. Louis, Mo.) was pumped with a 5-ml syringe and 25-gauge needle through the heart. The liver was removed, cut into cubes (2 mm/side) and placed into the collagenase-hyaluronidase solution. The livers were incubated in a water bath for 45 min at 37°C. After 45 min, the floating cells were removed, and the remaining liver tissue was treated with the enzyme solution for another 30 min at 37°C. After 30 min, the cells were pooled, filtered twice through gauze, dispensed into tubes, and centrifuged at 30 × *g* for 5 min. The final sediment was suspended in MEM to give a population density that formed a monlayer in a T-25 flask.

Treatment of cells. Vero and mouse primary liver cell cultures were exposed to *E. coli* O127:B8 LPS (Westphal preparation from Difco Laboratories, Detroit, Mich.) in MEM lacking added serum. The cells were incubated in this medium 2 h at 37°C. After the 2-h incubation, the cells were removed from the flasks. The Vero cells were released with 0.25% trypsin after rinsing twice with 0.02% ethylenediaminetetraacetate (EDTA) in 0.1 M phosphate buffer containing 0.15 M NaCl. Five milliliters of 0.1 M phosphate buffer containing 0.15 M KCl (pH 7.0) was added to each T-25 flask to suspend the cells. The liver cells were removed with a 10-ml pipette after releasing any adherent cells by scraping with a rubber policeman. Both cell suspensions were then centrifuged at 500 × *g* for 15 min. The supernatant fluid was discarded, and the sediment was suspended in 10 ml of 0.1 M phosphate buffer (pH 7.0) containing 0.15 M KCl. The cells were disrupted by passing a motor-driven Teflon pestle six times through the cell suspension in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 500 × *g* for 10 min to remove nuclei and intact cells (6). The sediment was discarded, and the supernatant fluid was centrifuged at 10,000 × *g* for 10 min. The sediment was suspended, washed twice, and retained as the mitochondrial fraction (23). The supernatant fluid was saved and corresponds to the supernatant fraction in Tables 1, 2, and 3 only.

Preparation of untreated mitochondrial fractions. The cells were harvested and homogenized in the same manner as in the whole-cell experiments described above (Table 4), or the livers, taken directly from the animals, were gently homogenized with a Teflon pestle in a loosely fitting tube. The homogenate was filtered through gauze to remove any intact tissue. The cells were recovered by centrifugation of this filtrate at 30 × *g* for 5 min. The sediment was suspended in 0.1 M phosphate buffer containing 0.15 M KCl, and the cells were disrupted as described above. Both cell homogenates from perfused or nonperfused livers were centrifuged at 500 × *g* for 10 min. The sediment was discarded, and the supernatant material was centrifuged at 10,000 × *g* for 10 min. The sediment from this centrifugation was suspended in 20 mM phosphate buffer (pH 7.4) supplemented with 0.3 M sucrose and 0.5 mM EDTA and washed twice until no malate dehydrogenase could be measured in the supernatant fluid. The final pellet was suspended in 15 ml of this phosphate buffer (pH 7.4) and distributed in equal portions into test tubes. The fractions were incubated in this medium with or without LPS or lipid A for 45 min, except where indicated. After incubation, the fractions were centrifuged at 10,000 × *g* for 10 min, and the supernatant fluid was assayed for leakage of mitochondrial enzyme activities. Contaminating lysosomal enzyme activities were also measured.

Enzyme assays. Hexokinase (EC 2.7.1.1), a cytoplasmic enzyme, was measured using glucose-6-phosphate dehydrogenase as a coupling enzyme by a modification of the method of Lazarus et al. (17). Mitochondrial malate dehydrogenase (EC 1.1.1.38) activity was measured in the reverse direction by using oxaloacetate as substrate (24). Three other mitochondrial enzymes were assayed. Succinate dehydrogenase (EC 1.3.99.1) was assayed by the method of Slater and Bonner (25), adenylate kinase (EC 2.7.4.3) activity, coupled to hexokinase and glucose-6-phosphate dehydrogenase, was measured by a modification of the method of Colowick and Kalckar (5), and cytochrome oxidase (EC 1.9.3.1) activity was measured with ferrocytochrome *c* as substrate (26). Contaminating lysosomal β-glucuronidase (EC 3.2.1.31) was measured using phenolphthalein-glucuronide as substrate by the method of Talalay et al. (27). Protein was determined by the method of Lowry et al. (18), with crystalline bovine serum albumin as the standard.

The specific activities of the enzymes are expressed in the following units: hexokinase and adenylate kinase as nmoles of nicotinamide adenine dinucleotide phosphate (NADP) reduced/min per mg of protein; malate dehydrogenase as nmoles of NADH oxidized/min per mg of protein; succinate dehydrogenase as nmoles of succinate utilized/min per mg of protein; cytochrome oxidase as *k*/min (first order rate constant) per mg of protein; and β-glucuronidase as μg of phenolphthalein released/min per mg of protein.

Mitochondrial swelling. The 10,000 × *g* sediment was diluted in 0.25 M sucrose to a concentration of 1 mg of mitochondrial protein per ml and added to a cuvette. The following reaction mixture was then added: 10 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.5, 50 mM sucrose, 5 mM $MgCl_2$, and 10 mM KCl. Various concentrations of either sodium oleate or *E. coli* O127:B8 LPS were added to the cuvette. The change in optical density at 25°C at 520

nm was measured every 15 s for 2 min, and a change in absorbance per min was calculated (14). A mitochondrial preparation with no sodium oleate or LPS was used as a control.

Cytochromes. The difference absorption spectra between the reduced and oxidized state of the cytochromes in mitochondrial fractions were measured at room temperature with 10 mM glutamate as substrate. Concentrations were calculated by the procedure of Chance and Hess (4). The following extinction coefficients were used: cytochrome *b*, $\Delta E_{562-575}$ = 17.9 $mM^{-1}cm^{-1}$; cytochrome aa_3, $\Delta E_{605-630}$ = 16.5 $mM^{-1}cm^{-1}$; and cytochrome *c*, $\Delta E_{550-540}$ = 19.0 $mM^{-1}cm^{-1}$.

Mitochondrial respiration. Oxygen uptake by mitochondria was measured at 30°C with a Gilson oxygraph equipped with a Clark Electrode (Gilson, Middletown, Wis., Oxygraph K-IC). The medium containing 3 mM mannitol, 3.5 mM potassium phosphate buffer (pH 7.4), 10 mM $MgCl_2$, 3.5 mM KCl, 0.33 mM EDTA, 4 mg of dialyzed crystalline bovine serum albumin, and 1.0 mg of mitochondrial protein was allowed to equilibrate for 2 min (13). LPS or lipid A at the indicated concentrations was added before the substrate, 1.4 mM L-glutamate. After approximately 2 min of substrate oxidation, 0.3 mM ADP was added to the reaction mixture. RCR were computed as nanogram atoms of oxygen consumed per minute per milligram of mitochondrial protein in the presence of ADP (respiratory state 3) to the nanogram atoms of oxygen consumed per minute per milligram of mitochondrial protein in the absence of ADP (state 4). Coupling was measured as the ratio of μmoles of ADP utilized to microatoms of O_2 consumed. A control sample with no LPS was incubated for 5 min, and the RCR and ADP/O values were determined.

Other endotoxic preparations. *E. coli* O26:B6 LPS, isolated by the Boivin procedure, and *Salmonella typhosa* O901 LPS, isolated by the Westphal procedure, were obtained from Difco Laboratories. *Salmonella minnesota* R595 lipid A was prepared by Nelda Marecki as described previously (20). *Shigella sonnei* lipid A from phase I was a gift from Tivador Kontrohr, Institute of Microbiology, University Medical School, Pécs, Hungary.

Statistical analysis. In Tables 1 to 7, the values for the controls without LPS are expressed as the means ± standard deviation for the specific activities determined in three independent experiments. The values for the experimental samples are the means ± standard deviations of the relative enzyme activities, expressed as the percentage of the corresponding sample without LPS. Statistical analyses were performed by using Student's *t* test; significant differences between mean values are expressed as *P* values.

RESULTS

Activities of malate dehydrogenase, succinate dehydrogenase, and adenylate kinase, three mitochondrial enzymes, were decreased in the mitochondrial fraction of both Vero and mouse primary liver cells exposed to LPS for 2 h in MEM lacking serum. However, the activity of hexokinase, a cytoplasmic enzyme, was not altered (Table 1).

The decreased activity in liver cells of all three mitochondrial enzymes was dependent on time of incubation of the cells with LPS. No decrease in malate dehydrogenase, succinate dehydrogenase, or adenylate kinase was seen at 1 h, but a marked decrease was seen after 2, 3, or 4 h of exposure to LPS (Table 2). Hexokinase activity did not change with time. This decrease in mitochondrial enzyme activity in primary liver cells was also dependent on the amount of LPS added to the cell cultures. At 5 μg of LPS per ml of culture medium, no decrease in mitochondrial enzyme activity was detected. However, at 7.5 μg of LPS per ml of culture medium, a marked decrease in succinate dehydrogenase was observed. At 10, 15, and 25 μg of LPS per ml of culture medium, all three mitochondrial enzyme activities decreased (Table 3).

Enzyme activity leaked extensively from isolated mitochondria exposed to LPS. Accumulation of malate dehydrogenase and succinate dehydrogenase activities in the supernatant fraction was substantial when the mitochondrial preparations from both perfused (Table 4) and nonperfused (Table 5) livers were incubated for 45 min with 10 μg of LPS per mg of protein. In

TABLE 1. *Altered mitochondrial enzyme activities of Vero and mouse primary liver cells treated with LPS*[a]

Cell	LPS[b] (μg/ml)	Enzyme activities (%)			
		Hexokinase[c]	Malate dh[d]	Succinate dh[d]	Adenylate kinase[d]
Vero	None	100 (75 ± 11)	100 (136 ± 14)	100 (172 ± 10)	100 (25 ± 5)
	10	78 ± 14	51 ± 2[e]	55 ± 4[e]	50 ± 3[e]
Liver	None	100 (243 ± 23)	100 (127 ± 7)	100 (249 ± 12)	100 (146 ± 13)
	10	82 ± 4	39 ± 7[e]	48 ± 5[e]	56 ± 8[e]

[a] Two hours of incubation at 37°C.
[b] *E. coli* O127:B8(W).
[c] Supernatant fraction; specific activities ± standard deviation in parentheses.
[d] Granular fraction; specific activity ± standard deviation in parentheses. dh, Dehydrogenase. β-Glucuronidase activity: 0.34 μg of phenolphthalein released per min per mg of protein.
[e] Significantly different from the control without LPS at $P < 0.05$.

TABLE 2. *Dependence of enzyme activities on time of incubation of primary liver cell cultures with LPS*[a]

Time (h)	LPS (μg/ml)	Enzyme activities (%)			
		Hexokinase	Malate dh	Succinate dh	Adenylate kinase
0	None	100 (318 ± 24)	100 (128 ± 6)	100 (241 ± 14)	100 (138 ± 34)
	10	99 ± 8	103 ± 10	111 ± 6	94 ± 8
1	None	100 (298 ± 13)	100 (141 ± 12)	100 (260 ± 22)	100 (142 ± 7)
	10	108 ± 12	92 ± 8	108 ± 11	102 ± 5
2	None	100 (386 ± 24)	100 (149 ± 25)	100 (243 ± 25)	100 (145 ± 8)
	10	93 ± 15	60 ± 19[b]	79 ± 8	60 ± 3[b]
3	None	100 (308 ± 35)	100 (130 ± 19)	100 (240 ± 41)	100 (142 ± 24)
	10	84 ± 10	34 ± 6[b]	54 ± 2[b]	58 ± 3[b]
4	None	100 (314 ± 33)	100 (185 ± 28)	100 (215 ± 21)	100 (135 ± 17)
	10	89 ± 12	45 ± 5[b]	65 ± 4[b]	69 ± 3[b]

[a] See footnotes from Table 1. β-Glucuronidase activity: 0.22 μg of phenolphthalein released per min per mg of protein.
[b] Significantly different from the control without LPS at $P < 0.05$.

TABLE 3. *Dependence of enzyme activities in primary liver cell cultures on dose of LPS*[a]

Dose (μg/ml)	Enzyme activities (%)			
	Hexokinase	Malate dh	Succinate dh	Adenylate kinase
0	100 (212 ± 18)	100 (234 ± 27)	100 (203 ± 18)	100 (141 ± 25)
5.0	96 ± 8	91 ± 8	96 ± 6	96 ± 11
7.5	89 ± 6	72 ± 12	51 ± 8[b]	89 ± 10
10.0	97 ± 8	29 ± 10[b]	48 ± 7[b]	80 ± 7
15.0	108 ± 5	46 ± 5[b]	47 ± 11[b]	61 ± 7[b]
25.0	81 ± 9	43 ± 3[b]	27 ± 7[b]	51 ± 5[b]

[a] Two hours of incubation at 37°C. See footnotes from Table 1. β-Glucuronidase activity: 0.64 μg of phenolphthalein released per min per mg of protein.
[b] Significantly different from the control without LPS at $P < 0.05$.

TABLE 4. *Leakage of enzyme activities from the mitochondrial fractions of perfused mouse liver cells*

Time (min)	LPS (μg/mg)[a]	Enzyme activities in supernatant fraction[b] (%)	
		Malate dh	Succinate dh
15	None	100 (22 ± 8)	100 (16 ± 5)
	10	126 ± 12	131 ± 18
30	None	100 (25 ± 7)	100 (15 ± 4)
	10	156 ± 9	167 ± 18
45	None	100 (28 ± 11)	100 (15 ± 4)
	10	200 ± 25[c]	247 ± 29[c]

[a] *E. coli* O127:B8(W). β-Glucuronidase activity in mitochondrial fraction: 0.81 μg of phenolphthalein released per min per mg of protein.
[b] Specific activities ± standard deviation in parentheses. dh, Dehydrogenase.
[c] Significantly different from the control without LPS at $P < 0.05$.

addition, the supernatant fraction of the mitochondrial preparations of nonperfused liver cells was assayed for leakage of adenylate kinase, and the granular fraction was assayed for loss of cytochrome oxidase activity. Accumulation of adenylate kinase activity in the supernatant and apparent loss of cytochrome oxidase from the granular fraction was observed (Table 5). LPS at 5 μg/mg of mitochondrial protein did not accelerate leakage of malate dehydrogenase, succinate dehydrogenase, or adenylate kinase. However, at 10 μg of LPS per mg of mitochondrial protein, a marked leakage of malate dehydrogenase activity was observed. At 20, 30, and 50 μg of LPS per mg of mitochondrial protein, an enhanced leakage of malate dehydrogenase, succinate dehydrogenase, and adenylate kinase was observed (Table 6).

The leakage of malate dehydrogenase from the mitochondria occurred when four other endotoxic preparations of LPS were used. *E. coli* O26:B6 LPS, isolated by the Boivin procedure, and *S. typhosa* O901, isolated by the Westphal procedure, caused an increased leakage of malate dehydrogenase activity at 10 μg of LPS per mg of mitochondrial protein. In addition, lipid A from *S. sonnei* phase I (2 μg/mg of mitochondrial protein) and that from *S. minnesota* R595 (10 μg/mg of mitochondrial protein) caused an increased leakage of malate dehydrogenase (Table 7).

LPS added to mitochondria at increasing concentrations caused a swelling of the mitochondria at the same rate as sodium oleate, although the extent of the swelling elicited by LPS was

TABLE 5. *Leakage of enzyme activities with time from mouse liver mitochondrial fractions treated with LPS*

Time (min)	LPS (μg/mg)[a]	Enzyme activities[b] (%)				
		Supernatant fraction			Granular fraction	
		Succinate dh	Adenylate kinase	Malate dh	Malate dh	Cytochrome oxidase
15	None	100 (22 ± 2)	100 (18 ± 2)	100 (10 ± 3)	100 (193 ± 19)	100 (0.69 ± 0.08)
	10	95 ± 11	99 ± 7	92 ± 8	95 ± 8	92 ± 7
30	None	100 (25 ± 4)	100 (19 ± 5)	100 (21 ± 5)	100 (190 ± 21)	100 (0.66 ± 0.11)
	10	96 ± 3	94 ± 8	172 ± 6[c]	80 ± 7	35 ± 4[c]
45	None	100 (32 ± 10)	100 (26 ± 3)	100 (30 ± 3)	100 (178 ± 18)	100 (0.70 ± 0.13)
	10	182 ± 10[c]	172 ± 9[c]	206 ± 10[c]	78 ± 12	46 ± 5[c]

[a] *E. coli* O127:B8(W).

[b] Specific activities ± standard deviation in parentheses. β-Glucuronidase activity in mitochondrial fraction: 0.72 μg of phenolphthalein released per min per mg of protein. dh, Dehydrogenase.

[c] Significantly different from the control without LPS at $P < 0.05$.

TABLE 6. *Leakage of enzyme activities from mouse liver mitochondrial fractions treated with various doses of LPS*[a]

Dose (μg/mg)[b]	Enzyme activities in supernatant[c] (%)		
	Malate dh	Succinate dh	Adenylate kinase
0	100 (8.7 ± 3)	100 (12.4 ± 3.2)	100 (25 ± 6)
5	139 ± 11	103 ± 10	102 ± 8
10	216 ± 15[d]	158 ± 5	154 ± 10
20	193 ± 18[d]	198 ± 11[d]	180 ± 5[d]
30	351 ± 29[d]	258 ± 9[d]	206 ± 11[d]
50	335 ± 33[d]	311 ± 32[d]	247 ± 16[d]

[a] Forty-five minutes of incubation at 37°C. β-glucuronidase activity in mitochondrial fraction: 0.48 μg of phenolphthalein released per min per mg of protein.

[b] *E. coli* O127:B8(W) LPS; micrograms per milligram of mitochondrial protein.

[c] Specific activities ± standard deviation in parentheses. dh, Dehydrogenase.

[d] Significantly different from the control without LPS at $P < 0.05$.

TABLE 7. *Leakage of malate dehydrogenase activity from mouse liver mitochondrial fractions treated with lipid A preparations*[a]

Endotoxic material	Dose (μg/mg of protein)	Malate dh activity[b] (%)
S. minnesota lipid A	0	100 (8.3 ± 2.4)
	1	127 ± 10
	2	114 ± 28
	5	125 ± 21
	10	204 ± 17[c]
	25	198 ± 9[c]
S. sonnei lipid A	1	150 ± 16
	2	204 ± 22[c]
	5	177 ± 22[c]
	10	283 ± 30[c]
	25	540 ± 74[c]
E. coli O26:B6 LPS (B)	0	100 (12 ± 3.2)
	5	82 ± 10
	10	170 ± 19[c]
	25	208 ± 30[c]
	50	233 ± 26[c]
	100	283 ± 21[c]
S. typhosa O901 LPS (W)	0	100 (8.3 ± 2.4)
	5	124 ± 8
	10	175 ± 11[c]
	25	236 ± 14[c]
	50	207 ± 28[c]
	150	219 ± 17[c]

[a] Forty-five minutes of incubation at 37°C.

[b] Specific activities ± standard deviation in parentheses. dh, Dehydrogenase.

[c] Significantly different from the control without LPS at $P < 0.05$.

less than that elicited by sodium oleate (Fig. 1). *E. coli* O127:B8 LPS at 100 μg/mg of mitochondrial protein, however, was unable to elicit an alteration in the differential absorption spectrum of the cytochromes with glutamate as substrate. In the presence or absence of LPS, the concentration of reduced cytochrome aa_3 (605 nm) was 0.11 nmol/mg of mitochondrial protein, reduced cytochrome *b* (563 nm) was 0.18 nmol/mg of mitochondrial protein, and reduced cytochrome *c* (552 nm) was 0.21 nmol/mg of mitochondrial protein.

E. coli O127:B8 LPS added to the mitochondrial fraction at a concentration of 10 μg/ml of mitochondrial protein 10 min before the substrate glutamate caused a loss of respiratory control (RCR); that is, the RCR decreased from 3 to 1 (Table 8). Lipid A from *S. sonnei* caused a loss of RCR at 10 μg of lipid A per mg of mitochondrial protein within 5 min. Lipid A from *S. minnesota* was less active, but did cause a significant reduction in RCR in 10 min at 10 μg of lipid A per mg of mitochondrial protein. The loss of respiratory control was also dependent on LPS concentration. As the amount of *E. coli* O127:B8 LPS was increased, a greater re-

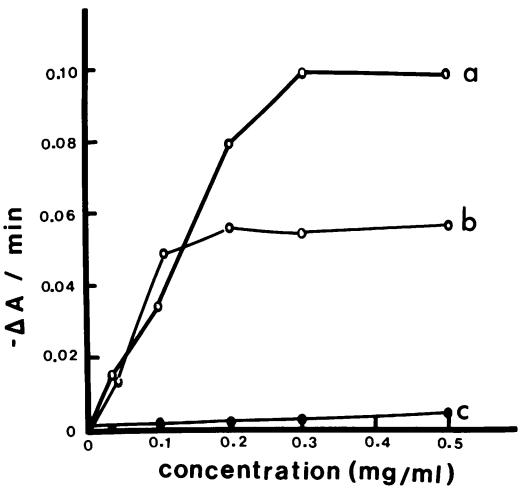

FIG. 1. *Swelling of mouse liver mitochondria incubated with sodium oleate (a), E. coli O127:B8 LPS (b), and the corresponding control without oleate or LPS (c).*

duction in RCR was observed as compared to the control at 5 min (Table 9). LPS at 10 μg/mg of mitochondrial protein caused a reduction in RCR from 2.5 to 1.5. On the other hand, 2 μg of lipid A isolated from *S. sonnei* caused a reduction in RCR of 2.8 to 1.5 after 5 min of exposure; 5 μg reduced the RCR to 1.3, and 10 μg of lipid A reduced the RCR to 1.0. *S. minnesota* lipid A at 10 μg/mg of mitochondrial protein reduced the RCR from 2.8 to 1.9 after 5 min of exposure. Throughout the respiration studies, no significant alteration was seen in the ADP/O values.

DISCUSSION

Succinate dehydrogenase activity is reduced in muscle and liver of rabbits treated with LPS in vivo (16) and in established cultures of Vero cells, primary mouse liver cell cultures, and isolated mouse liver mitochondria exposed to LPS in vitro. The changes elicited by LPS on cells in culture occur within 2 h, with 10 μg of LPS per ml, and in the absence of added serum. These results established that the effect of LPS on succinate dehydrogenase activity in whole animals, cell cultures, or subcellular fractions does not obligatorily involve hormones, humoral mediators, antibody, or complement.

In cultured cells and subcellular fractions treated with LPS, the activities of malate dehydrogenase and adenylate kinase also decrease. Succinate dehydrogenase is located in the inner membrane of the mitochondrion, adenylate kinase is located in the intermembrane space, and malate dehydrogenase is in the mitochondrial matrix (8); accordingly, LPS affects three components of the mitochondrion simultaneously. Our results indicate that LPS is acting on the integrity of the mitochondrion rather than on a particular site in electron transport or oxidative phosphorylation. Several additional observations support this proposal: (i) cytochrome oxidase, another enzyme in the inner membrane, leaks from the LPS-treated mitochondria; (ii) LPS causes mitochondrial swelling; and (iii) the differential cytochrome spectrum generated with glutamate as the substrate is not altered by large concentrations of LPS. The mechanism by which LPS alters the integrity of the mitochon-

TABLE 8. *Time-dependent loss of respiratory control by mouse liver mitochondria exposed to LPS or lipid A*

Time	Endotoxic prep[a]	State 3[b]	State 4[b]	RCR	ADP/O
0	None	68.4	25.1	2.7	2.6
	E. coli O127:B8 LPS	63.2	27.4	2.3	2.5
	S. sonnei lipid A	68.2	24.1	2.8	2.8
	S. minnesota lipid A	53.9	17.5	3.1	2.7
2	None	68.4	24.1	2.8	2.8
	E. coli O127:B8 LPS	42.2	23.0	1.8	2.6
	S. sonnei lipid A	55.8	21.5	2.6	2.6
	S. minnesota lipid A	58.9	26.3	2.2	2.6
5	None	68.4	26.3	2.6	2.5
	E. coli O127:B8 LPS	35.9	24.1	1.5	2.5
	S. sonnei lipid A	26.3	26.3	1.0	
	S. minnesota lipid A	57.8	26.3	2.1	2.8
10	None	59.9	21.0	2.8	2.3
	E. coli O127:B8 LPS	25.2	25.2	1.0	
	S. sonnei lipid A	22.0	22.0	1.0	
	S. minnesota lipid A	48.4	30.5	1.5	2.4

[a] A 10-μg/mg amount of mitochondrial protein.
[b] Expressed as nanograms of oxygen consumed per minute per milligram of mitochondrial protein.

TABLE 9. *Concentration-dependent loss of respiratory control by mouse liver mitochondria exposed to LPS or lipid A*

Endotoxic prep	Dose (μg/mg)	State 3[b]	State 4[b]	RCR	ADP/O
E. coli O127:B8 LPS	0	63.2	25.1	2.5	2.3
	5	44.5	29.7	1.5	2.4
	10	35.9	24.1	1.5	2.3
	25	26.8	19.0	1.3	2.4
	50	19.6	19.6	1.0	
S. sonnei lipid A	0	68.2	24.1	2.8	2.8
	1	59.4	21.5	2.7	2.6
	2	44.5	29.7	1.5	2.5
	5	31.5	24.1	1.3	2.4
	10	26.3	26.3	1.0	
	25	21.0	21.0	1.0	
S. minnesota lipid A	0	58.9	21.0	2.8	2.7
	1	55.8	18.0	3.0	2.8
	2	50.3	17.5	2.8	2.7
	5	42.9	18.0	2.4	2.4
	10	50.3	26.3	1.9	2.8
	25	39.0	20.3	1.9	2.8

[a] Five minutes of incubation at 30°C.
[b] Expressed as nanograms of oxygen consumed per minute per milligram of mitochondrial protein.

drion is not resolved. LPS binds to a variety of artificial and natural membranes; however, Berry (1) has suggested that LPS may elicit metabolic changes indirectly in cells containing no sequestered LPS. Although LPS affects the integrity of isolated mitochondria, our data do not rule out the possibility that perturbation of the plasma membrane or other indirect actions of LPS are the determinative early event(s) in vivo.

LPS inhibits the ADP stimulation of respiration by isolated mouse liver mitochondria with glutamate as substrate (state 3), but has little effect on respiration without added ADP (state 4). Uncoupling agents stimulate state 4 respiration; therefore, upcoupling does not seem to be a principal mechanism of action of LPS. Mela et al. (21) and Greer et al. (11) have reported previously that LPS causes loss of respiratory control in whole animals and isolated rat liver mitochondria. DePalma et al. (7) and Moss et al. (22) reported changes in the rate of oxidation of some, but not all, substrates in rat liver mitochondria treated with LPS. It should be noted that the vast majority of studies by previous workers have used excessive LPS concentrations.

Greer and Melazzo (10), Moss et al. (22), Kato (15), and Mela et al. (21) have reported that ADP/O ratios are decreased in LPS-treated mitochondria. We did not observe significant reduction of ADP/O ratios in isolated mouse liver mitochondria treated with LPS or lipid A. Accordingly, we conclude that impaired coupling of oxidative phosphorylation by LPS is secondary to other interactions between LPS and a mitochondrial constituent.

Harris et al. (12) reported that LPS from *Bordetella* inhibited substrate oxidation, decreased the RCR, and reduced ADP/O ratios in rat liver mitochondria. Harris et al. did not obtain similar results with other LPS preparations. We have established that accelerated mitochondrial leakage and impaired substrate respiration are elicited with three different LPS preparations and two different lipid A preparations. Kato (15) used 100 μg of lipid A per mg of mitochondrial protein to reduce RCR and ADP/O values, whereas we observed lowered RCR values with as little as 2 μg of *S. sonnei* lipid A per mg of mitochondrial protein. Our data establish that altered substrate oxidation is a target of LPS and of the lipid A toxophore, in particular.

Berry (1) has suggested that changes in mitochondria are secondary to other endotoxic effects because of the timing and doses used by previous workers. Although other workers (10, 15, 21, 22) have used LPS concentrations of more than 100 μg/mg of mitochondrial protein, mitochondrial damage can be detected within minutes with less than 10 μg/mg of mitochondrial protein. For a 25-g mouse, this corresponds to a dose of 50 μg of LPS, which is well within the usual experimental range (2). The hepatocytes of a mouse contain a total of about 3×10^{10} mitochondria and about 2×10^{14} respiratory assemblies consisting of NADH dehydrogenase, succinate dehydrogenase, and cytochromes *b*, *c*, c_1, *a*, and a_3. Mice rendered hyperreactive to LPS by mithramycin are killed by quantities of less than 1 μg (3), which is insufficient to supply enough lipid A residues to block more than a few percent of the respiratory assemblies. Accordingly, LPS must act at the organellar level or as an inhibitor of an enzyme or effector present in low concentrations.

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LITERATURE CITED

1. **Berry, L. J.** 1977. Bacterial toxins. CRC Crit. Rev. Toxicol. **5**:239–318.
2. **Berry, L. J., D. S. Smythe, and L. S. Colwell.** 1966. Inhibition of inducible liver enzymes by endotoxin and actinomycin D. J. Bacteriol. **92**:107–115.
3. **Bradley, S. G., A. C. Adams, and M. C. Smith.** 1975. Potentiation of the toxicity of mithramycin by bacterial lipopolysaccharide. Antimicrob. Agents Chemother. **7**: 322–327.
4. **Chance, B., and B. Hess.** 1959. Metabolic control mechanisms. I. Electron transfer in the mammalian cell. J. Biol. Chem. **234**:2404–2412.
5. **Colowick, S. P., and H. M. Kalckar.** 1943. The role of myokinase in transphosphorylation. I. The enzymatic phosphorylation of hexoses by adenyl pyrophosphate. J. Biol. Chem. **148**:117–137.
6. **de Duve, C., B. C. Pressman, R. Gianetto, R. Wattieux, and F. Appelmans.** 1955. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat liver tissues. Biochem. J. **60**:604–617.
7. **DePalma, R. G., Y. Harano, A. V. Robinson, and W. D. Holder.** 1970. Structure and function of mitochondria in hemorrhage and endotoxemia. Surg. Forum **21**: 3–6.
8. **Ernster, L., and B. Kuylenstierna.** 1969. Structure, composition, and function of mitochondrial membranes. FEBS Symp. **17**:5–31.
9. **Fonnesu, A., and C. Severi.** 1956. Oxidative phosphorylation in mitochondria from livers showing cloudy swelling. J. Biophys. Biochem. Cytol. **2**:293–306.
10. **Greer, G. G., and F. H. Melazzo.** 1975. *Pseudomonas aeruginosa* lipopolysaccharide: an uncoupler of mitochondrial oxidative phosphorylation. Can. J. Microbiol. **21**:877–883.
11. **Greer, G. G., N. A. Epps, and W. J. Vail.** 1973. Interaction of lipopolysaccharides with mitochondria. II. Effects of magnesium ions on toxicity of a rough lipopolysaccharide. J. Infect. Dis. **128**:724–729.
12. **Harris, R. A., D. C. Harris, and D. E. Green.** 1968. Effects of *Bordetella* endotoxin upon mitochondrial

respiration and energized processes. Arch. Biochem. Biophys. **128**:219-230.
13. **Higgins, E. S., H. Seibel, W. Friend, and K. S. Rogers.** 1978. Heterogeneity of renal mitochondria of the rat. Proc. Soc. Exp. Biol. Med. **158**:595-598.
14. **Hunter, F. E.** 1967. High amplitude swelling and shrinking. Methods Enzymol. **10**:689-696.
15. **Kato, M.** 1972. Site of action of lipid A on mitochondria. J. Bacteriol. **112**:268-275.
16. **Kun, E., and C. P. Miller.** 1948. Effects of bacterial endotoxin on carbohydrate metabolism of rabbits. Proc. Soc. Exp. Biol. Med. **67**:221-225.
17. **Lazarus, W. R., A. H. Ramel, Y. M. Rustan, and E. A. Barnard.** 1966. Yeast hexokinase. I. Preparation of the pure enzyme. Biochemistry **5**:4003-4016.
18. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. **193**:265-275.
19. **Mager, J., and E. Theodor.** 1957. Inhibition of mitochondrial respiration and uncoupling of oxidative phosphorylation by fractions of *Shigella paradysenteriae* type III somatic antigen. Arch. Biochem. Biophys. **67**: 169-177.
20. **Marecki, N. M., S. G. Bradley, A. E. Munson, and D. C. Drummond.** 1975. Effect of bacterial lipopolysaccharide, lipid A, and concanavalin A on lethality of 5-fluorouracil for mice. Toxicol. Appl. Pharmacol. **31**:83-92.
21. **Mela, L., L. V. Bacalzo, Jr., and L. D. Miller.** 1969. Defective oxidative metabolism of rat liver mitochondria in hemorrhagic and endotoxin shock. Am. J. Physiol. **220**:571-577.
22. **Moss, G. S., P. O. Erve, and W. Schumer.** 1969. Effect of endotoxin on mitochondrial respiration. Surg. Forum **20**:24-25.
23. **Schneider, W. C.** 1948. Intracellular distribution of enzymes. III. The oxidation of octanoic acid by rat liver fractions. J. Biol. Chem. **176**:259-266.
24. **Siegel, A., and R. J. Bing.** 1966. Plasma enzyme activity in myocardial infarction in dog and man. Proc. Soc. Exp. Biol. Med. **91**:604-607.
25. **Slater, E. C., and W. D. Bonner.** 1952. The effect of fluoride on the succinic oxidase system. Biochem. J. **52**: 185-196.
26. **Smith, L.** 1955. Spectrophotometric assay of cytochrome c oxidase. Methods Biochem. Anal. **2**:427-434.
27. **Talalay, R., W. H. Fishman, and C. Huggins.** 1946. Chromogenic substrates. II. Phenolphthalein glucuronic acid as a substrate for the assay of glucuronidase activity. J. Biol. Chem. **166**:757-772.