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Susceptibility of Mitochondria from Endotoxin-Resistant Mice to Lipopolysaccharide[1] (40722)

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Gram-negative bacterial lipopolysaccharide (LPS) impairs mitochondrial function when injected into animals (1, 2), added to cells in culture (2, 3), or added to subcellular fractions (1–3). Succinate dehydrogenase (EC 1.3.99.1) activity is decreased and ADP-stimulated respiration with glutamate as the substrate is inhibited by LPS (1, 3–5). These changes are elicited by biologically relevant amounts of LPS within a few hours under conditions in which the involvement of humoral mediators or hormones is minimized (2, 3, 5). Although LPS affects the activity of isolated mitochondria, it is not definitively established that the interaction of LPS with the mitochondrion per se is the causal early event in endotoxicosis (1, 4). It is conceivable that LPS acts first on the plasma membrane, thereby triggering several diverse series of reactions (6). In order to identify the site of some of the early determinative alterations elicited by LPS, we have examined the effects of LPS on liver cells in primary culture and subcellular fractions of liver from LPS susceptible mice, from mice rendered resistant to endotoxin by prior treatment with LPS and from the genetically LPS-refractory C3H/HeJ mouse. The activities of isolated mitochondria from normal and endotoxin refractory mice were impaired by LPS whereas mitochondrial activities of cells from endotoxin refractory mice were not altered by LPS added to liver cells in culture.

Materials and methods. Adult male C3H/HeDub mice weighing 18 to 23 g were obtained from Flow Research Animals, Inc., Dublin, Virginia, and comparable male C3H/HeJ mice were obtained from Jackson Laboratories, Bar Harbor, Maine. The animals were acclimated to their new environment for 1 week prior to use and were allowed free access to food (Purina Lab Chow, Purina Ralston Co., St. Louis, Mo.) and water. C3H/HeDub mice were rendered resistant to endotoxin by four intraperitoneal injections of LPS: 1 mg/kg on Day 0, 2 mg/kg on Days 1 and 2, 4 mg/kg on Day 3. The treated mice were used on Day 4. The endotoxin used throughout was *Escherichia coli* 0127:B8 LPS, isolated by the Westphal procedure (Difco, Laboratories, Detroit, Mich.).

Primary liver cell cultures were prepared as described previously (5). The livers were perfused with 0.1 *M* phosphate buffered saline and then dispersed with 0.05% collagenase (*Clostridium histolyticum* Type 1, Sigma Chemical Co., St. Louis, Mo.) and 0.1% hyaluronidase (bovine testes, Type 1, Sigma). The freed liver cells were harvested and suspended in modified Eagle's medium to give a population density that formed a monolayer in a T-25 flask. These cell cultures were exposed to LPS for 2 hr at 37°C. The treated liver cells were harvested and disrupted by passing a motor-driven Teflon pestle 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. The supernatant fluid was centrifuged at 10,000*g* for 10 min to sediment the mitochondria (3).

Mitochondria were also isolated from liver samples taken directly from animals. The isolated mitochondrial fractions were washed twice until malate dehydrogenase (EC 1.1.1.38) could no longer be detected in the supernatant fluid. Leakage of mitochondrial enzymes was measured in sam-

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ples exposed to LPS for 45 min at 37°C (5). Oxygen uptake by mitochondria was measured at 30°C with a Gilson oxygraph equipped with a Clark Electrode (Gilson, Middletown, Wisc.). The reaction mixture contained 3 m*M* mannitol, 3.5 m*M* potassium phosphate buffer, pH 7.4, 10 m*M* $MgCl_2$, 3.5 m*M* KCl, 0.33 m*M* EDTA, 4 mg dialyzed crystalline bovine serum albumin, and 1 mg mitochondrial protein. The substrate was 3.3 m*M* L-glutamate. Respiratory control ratios (RCR) were expressed 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 mitochondrial protein in the absence of ADP (state 4).

Mitochondrial malate dehydrogenase (EC 1.1.1.38) activity was measured in the reverse direction using oxaloacetate as the substrate (7). Succinate dehydrogenase (EC 1.3.99.1) was assayed according to the method of Slater and Bonner (8). Adenylate kinase (EC 2.7.4.3) activity, coupled to hexokinase and glucose-6-phosphate dehydrogenase, was measured according to a modification of the method of Colowick and Kalckar (9). Protein was determined by the method of Lowry et al. (10). The specific activities of the enzymes were expressed in the following units: malate dehydrogenase as nanomoles of NADH oxidized per minute per milligram of protein; succinate dehydrogenase as nanomoles of succinate utilized per minute per milligram of protein and adenylate kinase as nanomoles of NADP reduced per minute per milligram of protein.

Results. Activities of malate dehydrogenase, succinate dehydrogenase, and adenylate kinase were decreased in the mitochondrial fraction of C3H/HeDub liver cells exposed to 10 or 25 μg LPS/ml for 2 hr in medium lacking serum. The activities of these three enzymes were not reduced in liver cells from endotoxin tolerant C3H/HeDub mice exposed to 10 or 25 μg LPS/ml in cell culture medium (Table I). Similarly, malate dehydrogenase activity was not reduced in liver cells from C3H/HeJ mice exposed to 10 μg LPS/ml of cell culture medium.

LPS caused a concentration-dependent loss of malate dehydrogenase activity into the supernatant fluid of mitochondrial samples from normal C3H/HeDub mice, from tolerant C3H/HeDub mice, and from C3H/HeJ mice. Succinate dehydrogenase and adenylate kinase activities also leaked into the supernatant fluid from mitochondrial samples of LPS susceptible and LPS refractory mice (Table II).

LPS added to the mitochondrial fraction at 10 μg/mg mitochondrial protein 10 min before the substrate, glutamate, caused a loss of respiratory control for samples prepared from LPS susceptible and LPS refractory mice (Table III). The loss of respiratory control was dependent on LPS concentration. Throughout the respiration studies, no significant alteration in the ratio of micromoles of ADP utilized to microatoms O_2 consumed (ADP/O) was observed (data not shown).

TABLE I. EFFECTS OF LPS ON THE MITOCHONDRIAL ENZYME ACTIVITIES OF PERFUSED MOUSE LIVER CELLS OF NORMAL AND ENDOTOXIN-TOLERANT C3H/HeDub MICE AND C3H/HeJ MICE[a]

LPS[b] (μg/ml)	Enzyme activities in granular fraction[c]						
	Malate dehydrogenase			Succinate dehydrogenase		Adenylate kinase	
	Normal	Tolerant	C3H/HeJ	Normal	Tolerant	Normal	Tolerant
0	234 ± 27	153 ± 14	186 ± 11	203 ± 18	220 ± 16	141 ± 25	186 ± 9
10	68 ± 7[c]	130 ± 8	188 ± 24	97 ± 7[c]	167 ± 10	113 ± 8	160 ± 6
25	100 ± 3[c]	130 ± 13	not done	55 ± 4[c]	178 ± 19	72 ± 6[c]	186 ± 19

[a] Liver cells incubated for 2 hr at 37°C in modified Eagle's medium without added serum.
[b] *E. coli* 0127:B8 (Westphal).
[c] Significantly different from the untreated control at $P < 0.05$. Means ± standard deviations, as determined for three independent experiments.

TABLE II. LPS-ENHANCED LEAKAGE OF ENZYME ACTIVITIES FROM MITOCHONDRIAL FRACTIONS OF NORMAL OR ENDOTOXIN-TOLERANT C3H/HeDub MICE AND C3H/HeJ MICE[a]

LPS[b] (μg/mg)	Enzyme activities in supernatant (%)[c]					
	Malate dehydrogenase			Succinate dehydrogenase		Adenylate kinase
	Normal	Tolerant	C3H/HeJ	Normal	Tolerant	Normal	Tolerant
0	100 (9 ± 3)	100 (18 ± 3)	100 (18 ± 4)	100 (12 ± 3)	100 (16 ± 4)	100 (25 ± 6)	100 (19 ± 5)
10	216 ± 15[d]	177 ± 12[d]	151 ± 7[d]	158 ± 5[d]	131 ± 9[d]	154 ± 10[d]	158 ± 12[d]
25	272 ± 23[d]	216 ± 14[d]	193 ± 21[d]	228 ± 10[d]	163 ± 14[d]	193 ± 8[d]	140 ± 20
50	335 ± 33[d]	223 ± 19[d]	232 ± 25[d]	311 ± 32[d]	182 ± 13[d]	247 ± 16[d]	221 ± 15[d]

[a] 45 min incubation at 37°C.
[b] *E. coli* 0127:B8 (Westphal).
[c] Specific activities ± standard deviation in parentheses.
[d] Significantly different from the control without LPS at $P < 0.05$, as determined for three independent experiments.

TABLE III. LPS INDUCED LOSS OF RESPIRATORY CONTROL IN LIVER MITOCHONDRIA FROM NORMAL OR ENDOTOXIN TOLERANT C3H/HeDub MICE AND C3H/HeJ MICE[a]

Source of mitochondria	LPS[b] (μg/mg)	State[c]		RCR
		3	4	
C3H/HeDub Normal	0	59.9	21.0	2.8
C3H/HeDub Normal	10	25.2[d]	25.2	1.0[d]
C3H/HeDub Tolerant	0	41.4	21.5	1.9
C3H/HeDub Tolerant	10	19.5[d]	19.5	1.0[d]
C3H/HeJ	0	62.0	22.9	2.7
C3H/HeJ	10	33.9[d]	23.2	1.5[d]

[a] LPS was added 10 min before the substrate: oxygen uptake was measured at 30°C.
[b] *E. coli* 0127:B8 (Westphal).
[c] ng oxygen consumed/min/mg protein with 3.3 m*M* glutamate as the substrate (state 4) and with glutamate and ADP (state 3).
[d] Statistically different from the untreated control at $P < 0.05$. Data represent three independent experiments.

Discussion. Spleen cells and peritoneal exudate cells from normal mice, produce elevated levels of lysosomal enzymes after being exposed to LPS in cell cultures (11). Clearly, LPS is able to act on a wide range of cell types including B-lymphocytes, T-lymphocytes, macrophages, granulocytes and platelets (2, 4). In addition, liver cells from normal mice have impaired mitochondrial activity after being exposed to LPS in cell culture (5). The changes in liver cells in culture, elicited by LPS, occur in medium lacking serum thereby minimizing the involvement of hormones, humoral mediators and effectors, and antibody. It seems clear from our results that LPS can act directly on liver cells in primary cultures.

Liver cells from endotoxin refractory mice, whether rendered resistant by prior treatment with LPS, or genetically resistant, exhibited normal mitochondrial activity even after being exposed to LPS. Resistance to LPS, therefore, is a cellular attribute. Mitochondria from endotoxin-refractory mice, however, are susceptible to LPS, indicating that resistance is proba-

bly not an organellar attribute. Several investigators (1, 2) have reported that cells of C3H/HeJ mice and C3H/HeN mice bind LPS equally well. These results indicate that, although binding of LPS should be a prerequisite for a response, binding is not by itself sufficient to cause a response (1). Resistance in the C3H/HeJ mouse may reflect altered internalization of LPS or impaired triggering of crucial responses on the plasma membrane (1, 2). Alternatively, resistance in the C3H/HeJ mouse may involve impaired transmission of the toxophore or effector through the cytoplasm to the ultimate target (2). Tolerance elicited by repeated prior exposure to LPS probably involves antibody that neutralizes the endotoxin molecule (12) thereby impairing its capability to interact with the plasma membrane.

Attempts to determine the primary mechanism of action of bacterial endotoxin in whole animals are beset with a myriad of complications. A number of reports show direct effects of LPS on cells in culture and on subcellular particles (2, 4). One of the earliest changes elicited by LPS on cells in cultures is altered mitochondrial activity (3, 5). The mechanism by which LPS alters the integrity of the mitochondrion is not known. Although LPS affects the integrity of isolated mitochondria, our data do not rule out the possibility that perturbation of the plasma membranes or other indirect actions of LPS are the determinative early event(s) *in vivo*.

Summary. Liver cells from normal C3H/HeDub mice have reduced activities of mitochondrial succinate dehydrogenase (EC 1.3.99.1), malate dehydrogenase (EC 1.1.1.38), and adenylate kinase (EC 2.7.4.3) after being exposed to 10 μg *Escherichia coli* 0127:B8 lipopolysaccharide (Westphal preparation) per milliliter of culture medium lacking serum for 2 hr at 37°C. Liver cells from endotoxin-refractory mice, whether rendered resistant by prior treatment with LPS, or from the genetically resistant C3H/HeJ mouse, exhibited normal mitochondrial activity after being exposed to LPS. Isolated mitochondria from endotoxin susceptible and endotoxin-refractory mice were sensitive to LPS indicating that resistance is a cellular and not an organellar attribute.

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