Effects of Endotoxin on Glycogenolytic Enzymes of Mouse Liver (37431)

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(Introduced by J. R. Porter)
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One of the metabolic aberrations in animals given endotoxin is a dramatic loss of liver glycogen (1–5). The exact mechanism of endotoxin action is unknown. Several workers (6–9) indicate a blockage of glyconeogenesis, whereas others have demonstrated increased levels of glycogenolytic (4) and glycolytic (10) enzymes. Recently, an *in vitro* model (11) was developed in our laboratory which permitted a sensitive, easily controlled system to study the effect of endotoxin on the glycogenolytic enzymes. The purpose of this study was to (i) use this *in vitro* system to investigate the effect of endotoxin on those enzymes responsible for the degradation of glycogen, (ii) relate *in vitro* effects to *in vivo* changes, and (iii) investigate the mechanism whereby endotoxin might cause these changes.

Materials and Methods. Endotoxin. Endotoxin extracted from *Salmonella typhimurium* by the phenol–water method of Westphal and Lüderitz was obtained commercially (Difco, Detroit, Mich.). Fresh solutions were prepared the day of experiment.

Mice. Female Swiss Webster–Cox mice (Laboratory Supply, Indianapolis, Ind.) were used in all studies. The mice were prepared and handled as described (11).

Preparation of homogenates and in vitro *system.* The livers were homogenized in KCl–EDTA and the *in vitro* system of Zwadyk and Snyder was used.

Protein determinations. Protein was measured by Folin–Ciocalteau method (12) using albumin (Pentex, Miles Laboratory, Kankakee, Ill.) as the standard.

Enzyme measurements. Amylo-1,6-glucosidase. A series of tubes containing 0.1 ml of a solution of 2% phosphorylase limit dextrin (Courtesy of Dr. B. I. Brown, Washington University, St. Louis, Mo.), 0.1 *M* histidine (Eastman Organic Chemicals, Rochester, N.Y.) pH 6.5, and 0.005 *M* EDTA were prepared (13). To each of the tubes, 30 μg of liver homogenates prepared in 0.15 *M* KCl–0.002 *M* EDTA were added. To provide the 0 time value, 0.1 ml of 0.3 *N* $Ba(OH)_2$ was added before the addition of the liver homogenate. At various times 0.2 ml of 0.3 *N* $Ba(OH)_2$ was added to a set of endotoxin and control tubes to stop the reaction. The solutions were deproteinized (14) and the glucose content was then measured with the Worthington Glucostat Special (Worthington Biochem, Freehold, N.J.). The amount of free glucose released from the limit dextrin was an indication of the amylo-1,6-glucosidase activity.

Enzyme measurements. α-Amylase. The determination of amylase was based on the iodometric method (15). Soluble starch solution (40 mg/ml) (Soluble Starch, Fisher Chem. Co., Fairlawn, N.J.) was prepared in 0.005 *M* histidine–HCl buffer pH 6.5 containing 0.006 *M* NaCl. Working solutions of iodine were prepared daily by making a 1:10 dilution of the stock solution (0.25 *M* KI, 0.0035 *M* I_2) with water.

The liver homogenate was added to 1 ml of prewarmed (37°) starch solution. The reaction was stopped after 0, 20, 30, or 60 min incubation at 37° by the addition of 1 ml of 10% (w/v) trichloracetic acid. Portions (0.03 ml) of the stopped reaction mixture were added to 5 ml of the working iodine solutions. After dilution to 15 ml with water, the OD at 550 nm was measured using a Spectronic 20 (Bausch and Lomb, Rochester, N.Y.). The OD was determined immediately because of the color loss with time. The OD was converted to micrograms of starch by referring to a standard curve.

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Enzyme activity was expressed as micrograms of starch hydrolyzed.

Enzyme measurements. Acid phosphatase. The method of Huggins and Talalay (16) was used to measure acid phosphatase. The acid phosphatase activity was recorded as micrograms of phenolphthalein released in 1 hr.

Enzyme assay. Adenosine triphosphatase (ATPase). ATPase activity was measured as phosphate released from ATP (17). Microsomes were prepared as described below except that the pellet obtained after centrifugation (50,000*g*) was resuspended in water instead of sucrose to lyse the microsomes. This preparation served as the enzyme preparation and was added to 1 ml of a prewarmed solution (37°) containing equal parts of 400 m*M* imidazole–HCl buffer pH 7.1, 10 m*M* $MgCl_2$, 40 m*M* NaCl, 80 m*M* KCl, and 10 m*M* Na_2 ATP. The reaction was stopped by adding 1 ml of the mixture to 1 ml of 10% trichloracetic acid. The released phosphate was then measured (18).

Preparation of microsomes. Mice were fasted for 18 hr, killed by cervical dislocation, and homogenates prepared as described above using sucrose in place of EDTA–KCl. Microsomes were isolated by the procedure of Brosemer and Rutter (19).

Statistics. Data were evaluated by analysis of variance (20).

Results. Measurement of enzyme levels in the in vitro *system.* Several liver enzymes were studied employing the *in vitro* system previously described in an attempt to determine their role in the accelerated glycogen loss noted after endotoxin treatment. No differences in the activity of either amylo-1-6-glucosidase or acid lysosomal glucosidase were observed between the endotoxin-treated or control homogenates. The lack of glucose phosphates in the homogenates negated the importance of phosphorylase in this system (11). If the phosphatases acted on the glucose phosphates, the results should have been observed as increased glucose in the endotoxin-treated homogenates. The absence of a difference in free glucose levels (11) also eliminated the neutral glucosidase and acid lysosomal glucosidase from further consideration. Because the major products were oligosaccharides of the maltose series, the activity of α-amylase in the control and endotoxin-treated homogenates were compared 60 min after addition of endotoxin. The homogenates were centrifuged at 50,000*g* for 50 min and the activity of the α-amylase in the supernatant fluids was measured (Table I). The supernatant fluids from endotoxin-treated homogenates contained approximately 4.5 times more specific α-amylase activity than did the supernatant fluids from the control homogenates.

Determination of mode of action of endotoxin on α-amylase. The direct effects of endotoxin on isolated microsomes was tested by addition of various concentrations of endotoxin to microsomes and incubating the mixtures for 60 min at 37° (Table II). An increase in α-amylase activity was noted in the microsomal preparations treated with endotoxin. Maximum enzyme activity was obtained with 100 μg/ml of endotoxin. Increasing the concentration of endotoxin above this level resulted in a lowering of the amylase activity. Total α-amylase was measured by dissolving the microsomal membrane with Triton X-100 (final concn 0.2%).

Since the α-amylase activity of the untreated microsomes was 39% of the total activity, the action of endotoxin could have been to stimulate the α-amylase or to release

TABLE I. Amylase Activity in Supernatant Fluids from Endotoxin-Treated[a] and Control Liver Homogenates.

Treatment	Amylase activity (μg of starch hydrolyzed)	Mg protein	Specific activity (μg starch hydrolyzed/mg)	Probability (control vs endotoxin)
None	21[b]	1.60	13.3	$p < 0.001$
Endotoxin	103[b]	1.71	59.8	

[a] Final concentration of endotoxin 300 μg/ml.

[b] Represents mean value for four different homogenates.

TABLE II. Effect of Endotoxin on Release of α-Amylase from Isolated Microsomes.

Treatment	μg of starch hydrolyzed[a]	% of total activity
Microsome + Triton X-100[R]	115	100
Microsome alone	45	39
Microsome + endotoxin (1 μg/ml)[b]	55	48
Microsome + endotoxin (25 μg/ml)	55	48
Microsome + endotoxin (50 μg/ml)	75	65
Microsome + endotoxin (100 μg/ml)	85	73
Microsome + endotoxin (500 μg/ml)	65	57
Microsome + endotoxin (1000 μg/ml)	65	57

[a] Data representative of typical experiments.
[b] ( ) = final endotoxin concentration.

the enzyme by lysing microsomal membrane. Endotoxin at concentrations from 1 to 1000 μg/ml did not increase the activity of the nonmicrosomal-bound α-amylase, indicating that the endotoxin did not have a direct effect on the enzyme.

Preliminary experiments suggested that the microsomal preparations were contaminated with lysosomes as evidenced by the presence of acid phosphatase. Thus the true action of endotoxin could have been to lyse the lysosomes which in turn caused lysis of the microsomes. To test this hypothesis the microsomal preparation was treated with endotoxin (100 μg/ml) for 60 min at 37°; the intact microsomes and lysosomes were removed by centrifugation at 50,000*g* for 50 min and the acid phosphatase activity of the supernatant fluid determined. Untreated microsomal preparations served as the control and Triton X-100 treated microsomes were used to measure total phosphatase activity. The untreated preparation had 24% of the total acid phosphatase activity, whereas the endotoxin preparation had 26% of the total activity. The microsomal preparation used in the acid phosphatase experiment was the same used for the α-amylase experiment shown in Table II.

Tenny and Rafter (21) reported that endotoxin inhibited membrane transport ATPase of leukocytes and rabbit brain. Since microsomal membranes contain ATPase, the endotoxin inhibition of microsomal membrane ATPase could have resulted in altered permeability and subsequent release of the α-amylase. To test this possibility, the effect of endotoxin on microsomal ATPase was measured. The final concentration of endotoxin, 100 μg/ml, was the same concentration which caused maximum release of α-amylase. There was no difference in ATPase activity between the control and endotoxin-treated homogenates 30 or 60 min after treatment.

Measurement of α-amylase activity in livers of mice injected with endotoxin. To determine if the endotoxin effect observed *in vitro*, occurred *in vivo*, α-amylase activity was measured in mice injected intravenously with endotoxin. Eight hours after injection of an LD_{80} of endotoxin, the mice were sacrificed, livers were removed and homogenized in 0.25 *M* sucrose. The homogenates were centrifuged at 5000*g* and the amylase activity of the supernatant fluid was measured in the presence and absence of Triton X-100. The livers of endotoxin-treated mice contain one-third of the microsomal α-amylase activity as compared with the livers of control mice indicating release of the enzyme (Table III). The total amylase content of the two groups also differed. The serum α-amylase content of endotoxin-treated mice was higher than the serum α-amylase level of the control mice (Table IV). These differences were significant as measured by analysis of variance and demonstrated that the effects of endotoxin obtained in the *in vitro* model do relate to an *in vivo* effect.

Discussion. The data presented in this paper show that endotoxin causes an increase in α-amylase activity *in vitro* and *in vivo*. The findings of this increased activity are in agreement with our previous *in vitro* data which

TABLE III. Measurement of Microsome-Associated α-Amylase Activity in Livers from Mice Injected with Endotoxin.

Treatment	α-Amylase activity (μg starch hydrolyzed)		Probability (control vs endotoxin)
	Control	Endotoxin	
Total activity Triton X-100[R] added	8.3	1.9	$p < 0.025$
Free activity no Triton X-100[R] added	3.5	1.1	$p < 0.025$
Microsomal associated enzyme (total—free)	4.8	0.8	$p < 0.025$

showed an increase in oligosaccharides (11). This increase in amylase activity resulted from the release of α-amylase from the microsomes. In order to be active, amylase must have chloride ions as an activator. Under normal conditions, there is little intracellular chloride ion. Thus, if amylase activity is increased intracellularly, cell permeability must be altered to allow increased entrance of the activator. Since the microsomal preparation was contaminated with lysosomes, the possibility existed that endotoxin interacted with lysosomes causing a release of lytic substances, *e.g.*, lipases or proteases, which in turn lysed the microsomes. Measurement of acid phosphatase or acid lysosomal glucosidase after the addition of endotoxin did not show an increase in activity over the control indicating that lysosomal damage did not occur. Other workers (22, 23) reported an *in vivo* release of lysosomal enzymes such as cathepsin by endotoxin. A subsequent report (24) showed that the site of endotoxin action was not directly on the lysosome. It was postulated that the action of endotoxin on lysosomes may be the result of endotoxin-stimulated glycolysis and oxygen consumption in intact cells causing increased acid production which produced lysosomal damage. More recent evidence (25) shows that the attachment of endotoxin to lysosomes takes place at pH 5.5. The pH of the microsomal preparation used in our experiment was 6.8, a pH which would not favor lysosomal involvement.

TABLE IV. Serum Levels of α-Amylase in Endotoxin-Treated Animals.

Treatment	α-Amylase activity (μg starch hydrolyzed/0.02 ml serum)	Probability (control vs endotoxin)
Control	45	$p < 0.025$
Endotoxin	75	

The preliminary results of this paper indicated that there was no endotoxin–microsomal ATPase interaction. This would appear to contradict the results of Tenny and Rafter (21). Other unreported results from our laboratory using a membrane bound ATPase isolated from guinea pig brain, have shown a 50% inhibition of ATPase by endotoxin. However, the concentration of endotoxin required to inhibit the guinea pig brain ATPase was 16 times the concentration of endotoxin used in the microsomal ATPase experiments. It is possible that lysis of the microsomes, prior to addition of endotoxin, released components which could have bound to the endotoxin and prevented the endotoxin from acting on membrane ATPase.

Rutter and Brosemer (15) found that with isolated rat liver cells and slices, the amylase pathway was prominent in the production of glucose. Subsequent work (26) showed that this system was not a significant pathway for the production of serum glucose in the rat. Our results indicate that endotoxin may have enhanced this degradative pathway both *in vitro* and *in vivo*.

The inability to detect phosphorylase activity *in vitro* could have been due to the use of EDTA in the homogenizing solution. The binding of magnesium by the EDTA probably accounts for the lack of glucose-1-

phosphate recovery and thus may be responsible for the inability to duplicate the results of Hamosh and Shapiro (4). These authors found an increase in phosphorylase activity in the liver of endotoxin-treated rats which they attributed to an increase in phosphorylase kinase activity rather than to a direct stimulation of phosphorylase *a*. In addition they suggested that the activation of phosphorylase may be due to some previous changes in general metabolism.

Studies are now being conducted to improve the *in vitro* model so that direct measurement of phosphorylase levels can be made. However, this study clearly shows that endotoxin can interact with microsomes *in vitro* and *in vivo* to cause the release of a glycogenolytic enzyme.

Summary. The effect of endotoxin on the glycogenolytic enzymes was determined in an *in vitro* system. No increases in activities of phosphorylase, acid lysosomal glucosidase, neutral glucosidase or amylo-1, 6-glucosidase were noted. The increased glycogenolysis was attributed to increased activity of α-amylase, which resulted in release of α-amylase from microsomes. Endotoxin did not release acid phosphatase from lysosomes suggesting that release of microsomal α-amylase was not mediated by released lysosomal enzymes. Preliminary experiments did not show inhibition of microsomal membrane-bound ATPase.

The livers of mice injected intravenously with endotoxin had one-sixth of the microsomal-associated α-amylase activity as compared with control mice, indicating *in vivo* microsomal damage. The sera of these endotoxin-treated mice had a corresponding increase in α-amylase activity over the sera of control mice.

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