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Effect of Endotoxin on Pyruvate Kinase Activity in Mouse Liver

IRVIN S. SNYDER, MICHAEL DETERS, AND JAMES INGLE
Department of Microbiology, College of Medicine, University of Iowa, Iowa City, Iowa 52240

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The pyruvate kinase (PK) activity of mouse liver increases after injection of endotoxin. It decreases in animals given cortisone alone and remains essentially unchanged in those given cortisone and endotoxin at the same time. CCl_4 causes an increase in liver PK activity, but neither it nor endotoxin changes the activity of muscle PK. Addition of octonoate to liver homogenates inhibits the activity of liver PK. These results suggest that the rapid depletion in liver glycogen after administration of endotoxin or CCl_4 may be related to increased PK activity. Induction of tolerance does not prevent the increase in liver PK activity in challenged animals.

A number of metabolic effects produced by endotoxins have been studied in attempts to understand the host alterations caused by these lipopolysaccharides. Carbohydrate metabolism has been known for many years to be altered in animals after an injection of endotoxin (6, 10, 22). More recently Berry et al. (4) have shown that injection of a lethal dose of killed *Salmonella typhimurium* results in a reduction of liver glycogen and almost a complete loss in total body carbohydrate. Cortisone not only protects mice against the lethality of endotoxin but also prevents the total loss of glycogen. Other studies by these workers suggested that endotoxin-treated animals are unable to convert body proteins into carbohydrate (5). Subsequent investigations of phosphoenolpyruvate carboxykinase (PEPCK), an enzyme important in synthesis of glycogen, showed that induction of this enzyme by cortisone was inhibited by endotoxin (3). Woods et al. (21) and Giger (7) have shown increased glycolysis in cells exposed to endotoxin.

More recently Shands et al. (13) presented data indicating that the major defect in glucose metabolism in BCG-vaccinated mice given endotoxin may be in synthesis of glucose from noncarbohydrate sources. This interpretation would agree with the data on PEPCK presented by Berry and co-workers (3).

Gluconeogenesis has as its major energy barrier the synthesis of phosphoenolpyruvate. Once this compound is available, it is converted into glucose without much difficulty. The reason pyruvate kinase (PK) is important to this process is that it reverses gluconeogenesis and converts phosphoenolpyruvate (PEP) to pyruvate. PK, therefore, occupies a pivotal position in gluconeogenesis. In our experiments we suggest that an increase in activity of liver PK among mice given endotoxin plays a role in glycogen loss.

MATERIALS AND METHODS

Mice. Male Swiss Webster mice weighing 20 ± 2 g were used (Sutter Farms, Springfield, Mo.). Oxytetracycline hydrochloride (Terramycin, Pfizer Inc., Brooklyn, New York) was added to the drinking water for 2 days after delivery of the animals from the dealer, and they were used experimentally 5 days later. All mice were used 16 hr after experimental treatment and were fasted during this time.

Endotoxin. Heat-killed cells of *S. typhimurium* strain SR-11, suspended in nonpyrogenic saline, were used in all experiments (1). Doses of this crude type of endotoxin were administered intraperitoneally as 1:6 dilutions of the heat-killed cell preparation. The median lethal dose (LD_{50}) was calculated by the method of Reed and Muench (12) and was found to be approximately 10^{10} cells.

In preliminary experiments, the effect of endotoxin (Difco) prepared from *S. typhimurium* by the Westphal method was used to determine its effect on the systems employed in the study. The results were essentially the same as those obtained with heat-killed cells. Thus, heat-killed cells were used as the source of endotoxin throughout this study.

Production of tolerance. Mice were made tolerant to the endotoxin by a schedule of daily intraperitoneal injections of heat-killed cells (2). The animals received 0.1, 0.1, 0.2, 0.2, 0.4, and 0.4 LD_{50} doses on successive days; each dose was administered in 0.5-ml amounts. These animals were used experimentally 48 hr after the last injection. Each group of animals was tested for tolerance by injection of an LD_{50} of heat-killed cells. Observations of these mice were made for 3 days.

Injections. Sterile nonpyrogenic saline was used as a diluent for all injectable substances (Macbiek Company, Cambridge, Mass.). One LD_{50} of endotoxin was contained in 0.5 ml of nonpyrogenic saline.

Cortisone acetate (The Upjohn Company, Kalamazoo, Mich.) was injected subcutaneously into the interscapular region in 0.5-ml amounts containing 5 mg of the hormone.

CCl_4 (Fisher Scientific Co., Fair Lawn, N.J.) was injected subcutaneously into the interscapular region in 0.2-ml amounts.

Control animals received 0.5 ml of nonpyrogenic saline.

Measurement of PK. The livers from mice sacrificed by cervical dislocation were quickly weighed, and approximately 1 g of tissue was added to a homogenizing tube containing 7 ml of cold 0.15 M KCl. The tissue was homogenized for 1 to 2 min by using a Tri-R stirrer. The homogenate was centrifuged at 100,000 × *g* for 30 min. The supernatant fluid was removed, placed at 4 C, and assayed for PK activity by the method of Weber et al. (20). Adenosine diphosphate, PEP (potassium salt), β-diphosphopyridine nucleotide, and lactic dehydrogenase were purchased from Sigma Chemical Company. The changes in optical density at 340 nm were recorded by using a Gilford spectrophotometer. Readings were made every 30 sec for at least 3 min. Calculations were made from the portion of the enzyme curve which was linear. The assay was done at room temperature. The protein content of the enzyme preparation was determined by the Folin-Ciocalteau method (8). The enzyme activity is expressed as the micromolar PEP per minute per milligram of tissue protein.

In an experiment relating enzyme activity of homogenates to dry weight, 3 ml of the liver homogenate was dried at 90 C for 18 hr. The dried homogenates were weighed and corrected for the KCl content. The enzyme activity of these preparations is expressed as micromolar PEP converted per minute per milligram (dry weight) of homogenate. All experimental values represent the mean value obtained on at least eight experimental animals.

Statistical method. The significance of the differences in the mean responses was determined by the *t* test.

All values for the activity of PK are given as the mean ± the standard error of the mean.

RESULTS

Influence of endotoxin on pyruvate kinase activity. Mice were inoculated with endotoxin alone, cortisone alone, and cortisone and endotoxin simultaneously. The activity of PK was followed with time. The values in Fig. 1 are expressed as percentage changes when compared with uninoculated fasted control animals. All animals were starved for 16 hr before sacrifice. The activity of PK was increased in mice given heat-killed cells at the first time-period assayed (2 hr) and reached a maximum at around 16 hr. The PK activity in animals receiving cortisone or cortisone and endotoxin was lower in the fasted controls. At 20 hr, the values for both these groups approached normal.

Table 1 shows the activity of PK in animals 4 and 16 hr after inoculation of endotoxin. The increases at both time intervals are statistically significant. PK activity is increased 36% above the values obtained with fasted control mice 4 hr after injection and 64% above normal fasted values by 16 hr.

The PK activity of blood was not altered in endotoxin-injected animals. Both values were 0.45 units. Compared with the PK activity of liver (Table 1) and muscle (Table 3), the activity of the enzyme in blood is low.

To establish whether the increased PK activity in the liver cytosol was a reflection of the enzyme activity in liver homogenates, a comparison of PK activity in homogenates and cytosol was done. Livers were removed from mice after 16 hr of experimental treatment and homogenized. One half of the homogenate was centrifuged at 100,000 × *g*, and the enzyme activity in the supernatant fluid was determined. The corresponding homogenate was also assayed for enzyme

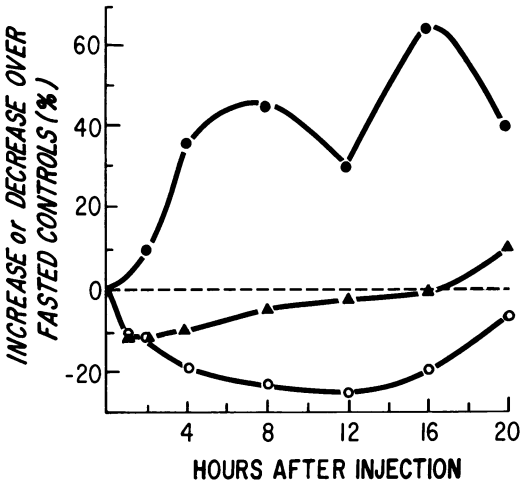

FIG. 1. *Changes in liver pyruvate kinase after treatment. Symbols:* ●, *endotoxin;* △, *cortisone acetate and endotoxin;* ○, *cortisone acetate.*

TABLE 1. *Comparison of pyruvate kinase activity in mice given endotoxin*

Time after injection	Pyruvate kinase activity[a]	Per cent increase	Significance
(1) 4 Hr...	4.67 ± 0.42[b]	+36	1 vs. 2
(2) Control	3.44 ± 0.16		$P < 0.001$
(3) 16 Hr..	5.37 ± 0.35	+64	3 vs. 4
(4) Control	3.36 ± 0.16		$P < 0.001$

[a] Expressed as micromolar phosphoenolpyruvate per minute per milligram of protein.

[b] Mean ± standard error of the mean.

TABLE 2. *Comparison of pyruvate kinase activity in liver homogenates and cytosol of mice*

Experimental[a] treatment	Liver homogenate[b]	Per cent change	Cytosol[c]	Per cent change
(1) Fasted	2.70 ± 0.18		4.79 ± 0.31[d]	
(2) Endotoxin	4.14 ± 0.23	1 vs. 2 +49	7.15 ± 0.45	1 vs. 2 +53
(3) Cortisone	2.73 ± 0.14	1 vs. 3 +3	4.94 ± 0.35	1 vs. 3 +1
(4) Cortisone and endotoxin	2.48 ± 0.21	1 vs. 4 −7	4.47 ± 0.28	1 vs. 4 −8

[a] Enzyme assays were done after 16 hr of experimental treatment.
[b] Expressed as micromolar phosphoenolpyruvate per minute per milligram (dry weight).
[c] Expressed as micromolar phosphoenolpyruvate per minute per milligram of protein.
[d] Mean ± standard error of the mean.

TABLE 3. *Comparison of effect of CCl_4 on pyruvate kinase activity of liver and muscle*

Tissue	Experimental treatment	Pyruvate kinase activity[a]	Per cent change	Significance
Liver	(1) Fasted	4.45 ± 0.41[b]		1 vs. 2 $P = <0.001$
	(2) CCl_4	6.37 ± 0.87	+29	1 vs. 3
	(3) Endotoxin	7.45 ± 0.30	+67	$P = <0.001$
Muscle	(4) Fasted	84.80 ± 2.45		4 vs. 5 NS[c]
	(5) CCl_4	85.24 ± 2.51	<1	
	(6) Endotoxin	89.92 ± 2.60	+6	4 vs. 6 NS

[a] Expressed as micromolar phosphoenolpyruvate per minute per milligram of protein.
[b] Mean ± standard error of the mean.
[c] Statistically not significant.

activity. The results (Table 2) show that the changes in PK activity for both groups were essentially unchanged. No enzyme activity was obtained with washed pellets remaining after centrifugation at 100,000 × *g*.

The measurement of PK activity in whole homogenates was not done in subsequent experiments. The sedimentation of particulate components contributes to the decrease in adsorbance, resulting from the decrease in reduced nicotinamide adenine dinucleotide, and requires periodic resuspension during the enzyme assay.

Starvation and PK activity. Several workers (9, 15) have reported that starvation causes a lowering of PK activity. This is confirmed by data which show that the activity is decreased in animals starved for 16 hr. In fed animals, the PK activity (expressed as micromolar PEP per minute per milligram of protein) was 4.7 ± 0.21 (mean ± standard error of the mean); for the starved animals, the PK activity was 3.84 ± 0.34. Since mice given heat-killed *S. typhimurium* or endotoxin (17) fail to eat, it is important to observe that fasting changes PK activity in a direction opposite that seen in Table 1.

Influence of CCl_4 on PK activity. Previously we have shown that CCl_4 causes a loss of liver glycogen and sensitizes mice to the lethal effects of endotoxin (14). In addition, Berry et al. (4) have shown that muscle glycogen is affected somewhat less than liver glycogen in animals injected with endotoxin. To determine if one might relate glycogen loss to increased PK activity, 0.2 ml of CCl_4 was inoculated into the scapular area of mice, and 16 hr after inoculation the mice were sacrificed. Table 3 shows that levels of liver enzyme are increased after CCl_4 injection but to a smaller degree than in endotoxin-poisoned animals. Muscle PK activity is not increased by treatment with CCl_4 or endotoxin. Muscle contains much more PK than does liver when compared on a weight basis.

Tolerant state and PK activity. Berry and Smythe (2) have shown convincingly that in mice made tolerant to endotoxin the levels of liver tryptophan oxygenase are not decreased by inoculation of endotoxin. The effects of tolerance on the levels of PK are shown in Table 4. The activity of the enzyme in nontolerant mice challenged with endotoxin increased 35% over control values. Tolerant mice, however, showed an increase of 21% over controls. Induction of tolerance causes

TABLE 4. *Liver pyruvate kinase activity in control and tolerant mice challenged with endotoxin*

Treatment and challenge	Pyruvate kinase activity[a]	Per cent change	Significance
(1) Nontolerant saline	3.58 ± 0.23[b]		1 vs. 2 $P < 0.001$
(2) Nontolerant endotoxin	4.85 ± 0.23	1 vs. 2 +35	
(3) Tolerant saline	5.08 ± 0.21	1 vs. 3 +45	1 vs. 3 $P < 0.001$
(4) Tolerant endotoxin	6.18 ± 0.67	3 vs. 4 +21	1 vs. 4 $P < 0.001$ 3 vs. 4 $P < 0.001$

[a] Expressed as micromolar phosphoenolpyruvate per minute per milligram of protein.
[b] Mean ± standard error of the mean.

TABLE 5. *Percentage of inhibition of pyruvate kinase in vitro*

Experimental treatment	Octonoate ($M \times 10^{-3}$)			
	0	4.8	9.6	19.2
Cortisone	0	0	56	87
Cortisone & endotoxin	0	28	72	88
Endotoxin	0	0	53	84
None	0	25	75	83

a 45% increase in the activity of PK when compared with nontolerant mice (Table 4, lines 1 and 3). To determine if the animals were tolerant, groups of mice were challenged with an LD_{50} of heat-killed cells and observed for 3 days. Tolerance was indeed established as indicated by survival of all 10 challenged mice. With nontolerant mice, three of six survived. Similar enzyme alterations were obtained in animals made tolerant by injection of heat-killed *S. typhimurium* and challenged with *S. marcescens* endotoxin.

Inhibition of PK by a free fatty acid. Weber et al. (18) have shown that free fatty acids can play a role in regulation of carbohydrate metabolism. To determine if octonoate could inhibit the enzyme from our experimental animals, octonoate at several concentrations was added to liver homogenates and incubated for 30 min before assay for PK. Different concentrations of octonoate were added to liver homogenates from fasted animals, and from animals given endotoxin, cortisone, or endotoxin and cortisone 16 hr before sacrifice. The percentage of reduction in PK activity (Table 5) shows that the increased enzyme activity observed after injection of endotoxin can be decreased by a free fatty acid. This suggests that the increased activity of PK might be related to a decrease in the level of those substances which play a role in controlling the activity of this enzyme.

DISCUSSION

A striking metabolic change that occurs in mice after injection of endotoxin is the loss of liver glycogen and the almost total depletion of body carbohydrate. Whether this loss of carbohydrate is due to an increase in glycolysis or impaired gluconeogenesis has been unclear. Berry and coworkers (3, 4) and Shands et al. (13) suggested that the carbohydrate loss is due to the failure of the endotoxin-poisoned animals to convert gluconeogenic intermediates into glycogen. Specifically, Berry et al. (3) have shown that endotoxin prevents induction of PEP carboxykinase by cortisone, thus confirming by direct enzyme assay impaired conversion of glyconeogenic intermediates to PEP.

Our work suggests that the dramatic loss in carbohydrate may be due not only to a decrease in synthesis of PEP but to an accelerated loss of this crucial compound due to increased PK activity. Woods et al. (21) found that endotoxin exerts an insulin-like effect on glycolytic processes, and Pieroni and Levine (11) discovered that injection of endotoxin at sublethal doses with insulin results in death of mice. These data tend to confirm our work.

The ability of cortisone acetate to prevent the increase in PK activity elicited by endotoxin is consistent with the demonstration of the protection of mice against the lethality of endotoxin (4). Weber et al. (19) showed that glucocorticoids do not induce PK, and cortisone is known to maintain glycogen levels in mice given endotoxin (3, 4).

Snyder et al. (14) demonstrated that injection of CCl_4 not only increases the susceptibility of mice to endotoxin and stimulates PK, but that it lowers the activity of liver tryptophan oxygenase and causes a loss of glycogen.

A difference in the effect of endotoxin and CCl_4 on PK in muscle and liver has been demonstrated, the liver enzyme being more easily affected than the muscle enzyme. The absence of a significant change in enzyme activity in the muscle of animals injected with endotoxin or CCl_4 may be related to the different properties of this enzyme. Tanaka et al. (15) demonstrated two types of PK, an M (muscle) and L (liver) type. The more dramatic loss of liver glycogen as compared with muscle glycogen shown by Berry et al. (4) may be related

to the differences in the muscle and liver types of PK.

The inability to maintain the activity of PK in the endotoxin-tolerant mouse is surprising. But if the rate of gluconeogenesis is increased by induction of tolerance, then one might expect maintenance of glycogen in the presence of higher enzyme activity. Induction of tolerance causes a greater increase in enzyme activity than did injection of endotoxin into tolerant animals.

The importance of PK as a key enzyme in glycolysis has been well demonstrated (18). Weber and associates (18) have shown that the ratio of the sum of PEPCK and pyruvate carboxylase to PK is 0.04. Thus, the importance of PK control during gluconeogenesis is obvious. Under conditions of gluconeogenesis, the activities of PEPCK and pyruvate carboxylase increase, whereas the activity of PK decreases. If the mechanisms for control of activity are impaired, net synthesis of glycogen cannot occur. Our data and that of others (3, 13) indicate that the loss of glycogen in animals given endotoxin is due not only to an increased activity of PK but also to inability of the host to synthesize glycogen from intermediates.

The mechanism by which endotoxin causes an increase in activity of PK is unknown. Preliminary experiments indicate that increased activity is not the result of a direct interaction between enzyme and endotoxin. However, addition of endotoxin to liver homogenates does cause a more rapid loss in glycogen than is observed in the absence of endotoxin. Inhibition of adenosine triphosphatase activity by endotoxin is the only example of a direct enzyme-endotoxin inhibition (16).

Weber and associates (19) demonstrated several mechanisms which regulate PK activity. Inhibition in vitro of PK by octonoate agrees with the results of Weber et al. (18). This suggests that a similar approach to study of the metabolic control mechanisms in endotoxin-treated animals may be meaningful. The effects of endotoxin on these control mechanisms are under study in our laboratory.

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