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Effect of treatment *in vivo* of rats with bacterial endotoxin on fructose 2,6-bisphosphate metabolism and L-pyruvate kinase activity and flux in isolated liver cells

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The effect of treatment of rats with bacterial endotoxin on fructose 2,6-bisphosphate (Fru-2,6-P_2) metabolism was investigated in isolated liver cells prepared from 18 h-starved animals. The results obtained support the hypothesis that a stimulation of 6-phosphofructo-1-kinase (PFK-1) activity and an inhibition of fructose-1,6-bisphosphatase (Fru-1,6-P_2ase) may be one mechanism underlying the inhibition of gluconeogenesis from lactate and pyruvate by endotoxin. We suggest that the stimulation of PFK-1 and inhibition of Fru-1,6-P_2ase activity is the result of a 2–3-fold increase in Fru-2,6-P_2. The latter is not due to changes in the total activity or phosphorylation state of the bifunctional 6-phosphofructo-2-kinase (PFK-2)/fructose-2,6-bisphosphatase, but appears to be the result of a decrease in the cytosolic concentration of phosphoenolpyruvate (PEP), an inhibitor of PFK-2 activity. The effect of endotoxin is resistant to the presence of glucagon, which has comparable effects in cells prepared from both control and endotoxin-treated animals. The mechanism by which endotoxin treatment of the rat decreases PEP and gluconeogenesis remains to be established. However, it does not involve alterations in either the total activity or the phosphorylation state of pyruvate kinase, nor does it involve increased flux through this enzyme in the intact cell, which is in fact decreased in this model of septic shock. It is suggested that the decreased flux may result from a lower rate of formation of PEP, suggesting that the prime lesion in sepsis is an inhibition of one or more of the steps leading to PEP formation.

INTRODUCTION

Septic shock is a pathological state which results from infection by Gram-negative bacteria and the release of endotoxins from the bacterial cell wall (Wardle, 1979). One of the major features of endotoxic shock is a transient hyperglycaemia followed by a prolonged hypoglycaemia, which can be mimicked by administration of endotoxin to the intact animal, but not by treatment of isolated hepatocytes with the endotoxin (La Noue *et al.*, 1968; Williamson *et al.*, 1970; Filkins & Cornell, 1974; Wolfe *et al.*, 1977; Filkins & Buchanan, 1977). It is now evident that the hypoglycaemic phase is the result of a depression of hepatic gluconeogenesis (Williamson *et al.*, 1970; Filkins & Cornell, 1974; Filkins & Buchanan, 1977; Knowles *et al.*, 1986, 1987). From cross-over studies using perfused livers from both normal and endotoxin-treated animals, Williamson *et al.* (1970) suggested that the effect of endotoxin treatment could be explained by an increased degree of cycling at the pyruvate kinase and PFK-1/Fru-1,6-P_2ase futile cycles in the pathway. More recent work by Knowles *et al.* (1986) has confirmed that the inhibition of gluconeogenesis is indeed accompanied by an increase in substrate cycling at the PFK-1/Fru-1,6-P_2ase step in the pathway, and in the absence of significant changes in the activities of effectors or of other enzymes thought to be rate-limiting for the pathway, it was suggested that this was the major site at which endotoxin exerted its effect. Although no mechanism was reported, it is well established that both PFK-1 and Fru-1,6-P_2ase are known to be reciprocally regulated by the intracellular concentration of Fru-2,6-P_2, the concentration of which is determined by the activity of the bifunctional PFK-2/Fru-2,6-P_2ase (reviewed by Van Schaftingen, 1987). More recent studies by Miller and co-workers have demonstrated that freeze-clamped livers removed from rats treated with bacterial endotoxin exhibit a 2–3-fold increase in Fru-2,6-P_2 (Miller *et al.*, 1989), whereas in sepsis induced by caecal ligation and puncture a 20% decrease in Fru-2,6-P_2 has been reported under conditions in which the rate of glucose synthesis from lactate was inhibited by 50% (Ardawi *et al.*, 1989). However, liver cell Fru-2,6-P_2 concentrations *in vivo* are known to be influenced by ambient hormonal and substrate concentrations which can result in sustained or even increased rates of gluconeogenesis despite the inhibition of gluconeogenesis observed at any given hormone/substrate combination (Spitzer *et al.*, 1985; Knowles *et al.*, 1986, 1987). The aim of this study was to investigate the effects of endotoxaemia on the concentrations of Fru-2,6-P_2 in isolated hepatocytes under defined hormone/substrate conditions, to correlate any changes with changes in gluconeogenesis, and to determine whether changes in the PFK-2/Fru-2,6-P_2ase enzymes were responsible for the effects observed.

EXPERIMENTAL

Materials

Glucagon, enzymes, $NaH^{14}CO_3$ and the lipopolysaccharide (trichloroacetic acid extract of *Salmonella typhimurium*) were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Collagenase (230 units/mg) was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Other reagents were of AnalaR grade or similar from BDH Chemicals, Poole, Dorset, U.K., or Sigma.

Animals

Male Sprague–Dawley rats (180–220 g) were used for all experiments. The animals were housed in a controlled environ-

Abbreviations used: Glc-6-*P*, glucose 6-phosphate; Fru-2,6-P_2, fructose 2,6-bisphosphate; Fru-1,6-P_2, fructose 1,6-bisphosphate; Fru-6-*P*, fructose 6-phosphate; Fru-2,6-P_2ase, fructose-2,6-bisphosphatase (EC 3.1.3.46); Fru-2,6-P_2ase, fructose-1,6-bisphosphatase (EC 3.1.3.11); PFK-1, 6-phosphofructo-1-kinase (EC 2.7.1.11); PFK-2, 6-phosphofructo-2-kinase (EC 2.7.1.105); PEP, phosphoenolpyruvate.

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ment exposed to a 12 h-lightish-dark cycle and maintained on standard laboratory chow and water *ad libitum*. At 16:00 h on the day preceding the cell preparation, the rats were injected with either endotoxin (4 mg of lipopolysaccharide from *Salmonella typhimurium*/kg body wt. intraperitoneally) or pyrogen-free saline. The rats were fasted overnight and the hepatocytes prepared at 10:00 h next morning. In general, the endotoxic rats showed characteristic responses such as raised fur, dysentery and lack of movement. Plasma urea levels were significantly ($P < 0.05$) elevated in these animals (13.2 ± 2.7 mM, $n = 9$) compared with controls (4.1 ± 0.4 mM, $n = 9$). Only rats showing increased plasma urea were considered to be endotoxic.

Preparation and incubation of hepatocytes

Liver cells were prepared by collagenase digestion as described previously (Allan *et al.*, 1983). The isolated hepatocytes were resuspended in Krebs–Ringer buffer (Krebs & Henseleit, 1932) to a final concentration of 40 mg wet wt./ml. In general, the cells were incubated in 125 ml plastic Erlenmeyer flasks with 18 mM-lactate/2 mM-pyruvate as substrates under an atmosphere of O_2/CO_2 (19:1) in a rotatory shaking water bath (30 cycles/min) at 37 °C for 15 min. Samples were then removed for the determination of glucose and ATP, and then glucagon (0.1 μM final concn.) or vehicle was added and the incubation continued for a further 15 min before samples were removed for the determination of Fru-2,6-P_2, PFK-2/Fru-2,6-P_2ase activities and glucose. When pyruvate kinase assays were to be carried out, samples were removed 5 min after addition of glucagon. Cell viability was assessed by measuring total cellular ATP, which was 2.64 ± 0.26 and 2.22 ± 0.21 ($n = 7$) nmol/mg wet wt. for control and endotoxin-treated cells respectively. These values are consistent with those reported previously for cells incubated under similar conditions (Schwenke *et al.*, 1981; Allan & Titheradge, 1984; Knowles *et al.*, 1987).

Flux measurements

PFK-1 flux was determined by measuring the release of ^3H_2O from D-[3-^{3}H]glucose (Bontemps *et al.*, 1978; Hue, 1980). Cells were incubated with 5 mM-[3-^{3}H]glucose (sp. radioactivity 0.025 μCi/μmol), 10 mM-lactate and 1.0 mM-pyruvate for 1 h and the [^{3}H]glucose was separated from ^3H_2O as described by Bontemps *et al.* (1978).

Pyruvate kinase flux was measured by the incorporation of $NaH^{14}CO_3$ into glucose and lactate plus pyruvate by a modification of the method of Rognstad (1975). The cells were incubated in Krebs–Ringer buffer containing 20 mM-Hepes, pH 7.4, 18 mM-lactate/2 mM-pyruvate and 25 mM-$NaH^{14}CO_3$ (sp. radioactivity 0.3 μCi/μmol). The flasks were gassed with 100% O_2 to prevent dilution of the label. The reaction was terminated after 15 min by addition of 2.7 M-$HClO_4$ (10% v/v). The extracts were gassed with CO_2 to remove unincorporated label and then centrifuged (800 *g*, 15 min at 4 °C). The incorporation of label into [1-^{14}C]lactate and [1-^{14}C]pyruvate was measured as $^{14}CO_2$ released during incubations with lactate oxidase and pyruvate oxidase. The neutralized cell extracts were incubated for 3 h at 37 °C with an equal volume of conversion mixture containing 160 mM-KH_2PO_4, pH 8.4, 1.57 mM-$MnCl_2$, 0.02 mM-FAD, 0.1 mM-pyridoxal phosphate, 4.7 units of catalase/ml and either a mixture of lactate oxidase and pyruvate oxidase (final concentrations 0.3 unit/ml), pyruvate oxidase alone or 0.9% NaCl containing 1 mg of BSA/ml. This allowed the measurement of lactate, pyruvate and background radioactivity respectively. The incubation was terminated by addition of 0.25 vol. of 2.7 M-$HClO_4$ and 0.25 vol. of $NaHCO_3$ (120 mM). The samples were incubated for a further 90 min at 4 °C and the $^{14}CO_2$ released was collected by trapping in 0.25 ml of methylbenzethonium hydroxide [50% (v/v) in methanol]. The results were corrected for incomplete trapping by the use of [1-^{14}C]lactate and [1-^{14}C]pyruvate standards. For measurement of labelled glucose, 400 μl of the neutralized extracts was incubated for 1 h with 120 μl of a conversion mixture containing 1.5 mM-$(NH_4)_2SO_4$, 10 mM-$MgCl_2$, 10 mM-ATP, 10 mM-Tris/HCl, pH 7.0, and 5 mM-glucose in the absence and presence of hexokinase (0.154 unit/ml). Then 400 μl of this mixture was added to 0.4 g of a mixture of Dowex 50W-8X and Amberlite IRA 68 (1:1, w/w), diluted with 500 μl of water and left shaking for 1 h. Then 300 μl of the sample was counted for radioactivity in a Beckmann LS 2800 scintillation counter, and the radioactivity in the glucose was determined from the difference in radioactivity in the samples incubated with and without hexokinase. Parallel incubations were carried out with [U-^{14}C]glucose standards to correct for the efficiency of both the conversion assay and recovery of glucose.

Measurement of enzymes and metabolites

Pyruvate kinase was measured after $(NH_3)_2SO_4$ precipitation by the method of Garrison *et al.* (1979). The PFK-2 activity of the bifunctional PFK-2/Fru-2,6-P_2ase enzyme was measured after $(NH_4)_2SO_4$ precipitation by the method of El-Maghrabi *et al.* (1982). Fru-2,6-P_2ase was measured by monitoring the disappearance of Fru-2,6-P_2 (Van Schaftingen *et al.*, 1982).

For measurement of metabolites, cells were fractionated into cytosolic and mitochondrial fractions by the low-temperature digitonin method of Brocks *et al.* (1980). For measurements of adenine nucleotides, 20% (v/v) methanol was added to the $HClO_4$ layer to inhibit any residual adenylate kinase activity. Metabolite contents of the cellular compartments were calculated as described by Knowles *et al.* (1987). Glc-6-*P*, Fru-6-*P*, glycerol 3-phosphate and PEP were measured as described by Lowry & Passonneau (1972). AMP was measured by ion-pair reversed-phase h.p.l.c. as described by Ingebretsen *et al.* (1982) on a system Gold high-performance chromatograph (Beckman, High Wycombe, Bucks., U.K.). ATP was determined fluorimetrically by the method of Lowry & Passonneau (1972) or by luminescence (Stanley & Williams, 1969). Collection, treatment of samples and measurement of Fru-2,6-P_2 was as described by Van Schaftingen (1984). D-Glucose was measured by the glucose oxidase method (Bergmeyer & Bernt, 1972). Plasma urea was determined as described by Fawcett & Scott (1960) with the modification of Chaney & Marbach (1962).

Results are presented as means ± S.E.M. with the numbers of different cell preparations given in parentheses. Statistical analysis of results was carried out with a pooled *t* test for comparison between control and endotoxin-treated cells and a paired *t* test for comparison of the effects of glucagon in control and endotoxin-treated cells.

RESULTS AND DISCUSSION

Effect of endotoxin on gluconeogenesis and PFK-1 substrate cycling

Table 1 confirms that chronic endotoxin treatment of the rat depresses gluconeogenesis from a mixture of lactate plus pyruvate. The magnitude of the response (41% decrease) is comparable with the previously observed decrease measured under similar conditions (Filkins & Cornell, 1974; Knowles *et al.*, 1987). The effect of endotoxin treatment was independent of the presence of 0.1 μM-glucagon, the hormone producing a comparable stimulation in cells prepared from both control and endotoxin-treated animals ($P < 0.05$). The depression of gluconeogenesis was accompanied by an increase in the isotopic flux through PFK-1 as measured by the detritiation of [3-^{3}H]glucose. Addition of glucagon produced the expected decrease in PFK-1

Table 1. Effect of endotoxin treatment on gluconeogenesis and PFK-1 flux

Isolated hepatocytes were isolated from control and endotoxin-treated rats, and glucose output and PFK-1 flux were measured as described in the Experimental section. $*P < 0.05$, $***P < 0.001$ for difference between control and endotoxic cells; $\dagger P < 0.05$, $\dagger\dagger P < 0.01$ for difference between cells incubated in the absence and presence of 0.1 μM-glucagon.

Treatment	Glucose synthesis (μmol/h per g wet wt.)	PFK-1 flux (μmol/h per g wet wt.)
Control	91±3 (10)	9.7±1.2 (6)
+Glucagon	110±4 (10)††	6.2±0.6 (6)†
Endotoxin	54±1 (10)***	13.0±0.6 (6)*
+Glucagon	76±4 (10)††*	8.6±1.1 (6)†

flux in control cells and caused a similar inhibition of flux in the endotoxin-treated cells (31 % as compared with 33 %), such that the effect of the endotoxin on flux was maintained in the presence and absence of glucagon (the mean increase being 49 % and 42 % in the absence and presence of the hormone respectively). This confirms and extends the previous observations by Knowles *et al.* (1987).

Effect of endotoxin on Fru-2,6-P_2 metabolism

Table 2 shows the effect of endotoxin treatment of the rat on the Fru-2,6-P_2 content and PFK-2 activity in isolated hepatocytes incubated in the presence of lactate plus pyruvate. Chronic treatment of the rats with endotoxin produced a significant increase (140 %) in the concentration of Fru-2,6-P_2. This is similar to the increase observed in the liver *in vivo* by Miller *et al.* (1989), but contrasts to the study by Ardawi *et al.* (1989), where sepsis was induced by caecal ligation and puncture. This result indicates that the response to the lipopolysaccharide is resistant to cell preparation and suggests that the response to direct endotoxin administration *in vivo* is not simply the result of changes in plasma concentrations of circulating hormones and substrates (Spitzer *et al.*, 1985; Knowles *et al.*, 1986, 1987), but is an effect of the endotoxin at the level of the liver. Incubating the cells in the presence of 0.1 μM-glucagon produced the expected decrease in the activator in cells prepared from both groups of animals. The magnitude of the response to the hormone was comparable in both the control and endotoxic cells, the concentration being decreased to 39 % and 41 % of their respective levels. These data would suggest that the effects of glucagon and endotoxin on Fru-2,6-P_2 metabolism are independent, thus explaining the maintenance of the effects of endotoxin on PFK-1 flux in the presence of a glucagon challenge (Table 1).

Several reports have indicated that the livers of animals suffering from septic shock show an increased intracellular concentration of hexose phosphates (Miller *et al.*, 1989; Ardawi *et al.*, 1989), which is known to influence the PFK-2 and Fru-2,6-P_2ase activity, such that Fru-2,6-P_2 concentrations are increased (Hers & Van Schaftingen, 1982). Although the time course for this increase does not appear to show a good correlation with the onset of the elevation of Fru-2,6-P_2 formation (Miller *et al.*, 1989), no evidence was presented as to whether it was important for the maintenance of the effect over 18 h. To address this problem, cells were incubated with 20 mM-glucose to increase the intracellular concentrations of hexose phosphates, and as a result to increase the level of Fru-2,6-P_2 (Hue *et al.*, 1981; Claus *et al.*, 1982). Inclusion of glucose in the incubation medium increased the concentration of Fru-2,6-P_2 5-fold in the control cells (Table 2). In the endotoxic cells there was only a 3.3-fold increase; however, the absolute change in Fru-2,6-P_2 after addition of glucose was comparable. The effect of glucose and endotoxin was therefore additive. Challenging the cells with glucagon decreased the levels of Fru-2,6-P_2 back to those in cells incubated in the absence of glucose but in the presence of glucagon, irrespective of whether the cells were prepared from control or endotoxin-treated cells. This indicates that the effect of glucose is fully reversible by the cyclic AMP-dependent phosphorylation of PFK-2/Fru-2,6-P_2ase and precludes the possibility that the effect of endotoxin treatment is the sole result of an increase in hexose phosphates.

Miller *et al.* (1989) have suggested that the effect of endotoxin treatment may be the result of a decreased phosphorylation of the bifunctional PFK-2/Fru-2,6-P_2ase. Therefore we have measured the PFK-2 and Fru-2,6-P_2ase activities in both control and endotoxin-treated cells. Table 2 shows that at 5 mM-Fru-6-P, which is reported to be saturating for PFK-2 (El-Maghrabi *et al.*, 1982), there was no significant effect of endotoxin treatment of the cells, suggesting that the expression of the enzyme is not altered in this model of septic shock. Similarly no effect was apparent at sub-saturating concentrations, indicating that the phosphorylation state of the enzyme was unaltered. The addition of glucagon as a positive control inhibited both the maximal enzyme activity and the activity at sub-saturating concentrations of Fru-6-P by approx. 50 % in both control and endotoxic cells. This agrees with previous observations after treatment of hepatocytes with glucagon and phosphorylation of PFK-2 (Van Schaftingen *et al.*, 1981) and confirms that the effects of glucagon and endotoxin treatment on Fru-2,6-P_2 metabolism are independent of each other. Measurements of Fru-2,6-P_2ase activity also failed to demonstrate any change in activity upon treatment of the animals with the lipolysaccharide, the K_m and $V_{max.}$ for the enzyme being K_m 0.076±0.013 μM, $V_{max.}$ 3.38±0.31 nmol/min per g of liver (3), and K_m 0.084±0.009 μM, $V_{max.}$ 4.21±0.23 nmol/min per g of liver (3), for cells prepared from control and endotoxin-treated animals respectively. Inclusion of 2.5 mM-phosphate in the assay also failed to reveal any effect

Table 2. Effect of endotoxin treatment on Fru-2,6-P_2 metabolism

Cells were incubated with 18 mM-lactate/2 mM-pyruvate. Glucose was added to a final concentration of 20 mM. $*P < 0.05$ for difference between control and endotoxic cells; $\dagger P < 0.05$ for difference between cells incubated in the absence and presence of 0.1 μM-glucagon.

Treatment	Fru-2,6P_2 (nmol/g wet wt.)		PFK-2 (nmol/min per g wet wt.)	
	−Glucose	+Glucose	0.5 mM-Fru-6-P	5 mM-Fru-6-P
Control	0.98±0.23 (11)	4.82±1.09 (7)	0.45±0.12 (8)	1.34±0.17 (8)
+Glucagon	0.38±0.11 (11)†	0.40±0.09 (7)†	0.18±0.07 (8)†	0.69±0.13 (8)†
Endotoxin	2.32±0.42 (12)*	7.72±0.60 (7)*	0.44±0.12 (8)	1.12±0.23 (8)†
+Glucagon	0.98±0.18 (12)*†	1.12±0.16 (7)*†	0.19±0.06 (8)†	0.55±0.11 (8)†

Table 3. Effect of endotoxin on the distribution of metabolites in isolated hepatocytes

The cells were incubated with 18 mM-lactate plus 2 mM-pyruvate and fractionated as described in the Experimental section. *$P < 0.05$ for difference between control and endotoxic cells.

	Content (nmol/mg wet wt.)					
	Control			Endotoxin		
Metabolite	Total	Mitochondria	Cytosol	Total	Mitochondria	Cytosol
ATP	2.09±0.19 (10)	0.49±0.07 (10)	1.59±0.15 (10)	1.69±0.16 (11)	0.41±0.05 (11)	1.28±0.13 (11)
AMP	0.18±0.03 (6)	0.09±0.02 (6)	0.09±0.02 (6)	0.17±0.08 (4)	0.08±0.03 (4)	0.09±0.04 (4)
Glc-6-*P*	0.05±0.01 (4)	–	0.05±0.01 (4)	0.06±0.01 (4)	–	0.06±0.01 (4)
Fru-6-*P*	0.014±0.002 (3)	–	0.014±0.004 (3)	0.015±0.003 (4)	–	0.015±0.003 (4)
PEP	0.24±0.05 (5)	0.11±0.05 (5)	0.14±0.03 (5)	0.19±0.04 (3)	0.15±0.03 (5)	0.06±0.01* (3)

of endotoxin treatment, the kinetic parameters being K_m 0.128±0.020 μM, $V_{max.}$ 4.02±0.36 nmol/min per g of liver (3), and K_m 0.145±0.017 μM, $V_{max.}$ 4.34±0.30 nmol/min per g of liver (3), for cells prepared from control and endotoxin-treated animals respectively. These results suggest that the derangement in Fru-2,6-P_2 metabolism must arise from either changes in the intracellular levels of allosteric effectors of PFK-2/Fru-2,6-P_2ase, e.g. AMP, citrate, PEP (Van Schaftingen *et al.*, 1981) or glycerol 3-phosphate (Claus *et al.*, 1982), or an as yet unidentified mediator, rather than changes in the phosphorylation state of the protein.

Effect of endotoxin treatment of the rat on effectors of PFK-2/Fru-2,6-P_2ase

Table 3 shows the effect of endotoxin treatment on the intracellular distribution of a number of metabolites known to be involved in the regulation of the bifunctional PFK-2/Fru-2,6-P_2ase. In contrast with the studies *in vivo* (Miller *et al.*, 1989; Ardawi *et al.*, 1989), there was no effect of sepsis on the cytosolic hexose phosphate concentrations in cells incubated with lactate plus pyruvate alone. This confirms that the increase in Fru-2,6-P_2 was not the result of an increase in Fru-6-*P* after endotoxin treatment leading to activation of PFK-2 and inhibition of Fru-2,6-P_2ase. Except for PEP, no significant changes in the cytosolic concentrations of any of the other metabolites measured were observed. The level of PEP in the cytosol fell by 60% in cells prepared from endotoxin-treated animals, whereas that in the mitochondria remained unchanged. Assuming a cytosolic volume of 1.34 μl/mg dry wt. (Knowles *et al.*, 1987), this corresponds to a change in the cytosolic PEP concentration from 0.37 mM to 0.15 mM in the control and endotoxic cells respectively. Changes in PEP within this concentration range are known to cause a marked inhibition of PFK-2 activity (Van Schaftingen *et al.*, 1981), and therefore the decrease in cytosolic concentration observed in sepsis would explain the rise in Fru-2,6-P_2 and increased flux through PFK-1. A similar fall in total PEP has previously been observed in cross-over studies in perfused livers from rats treated with a lethal dose of endotoxin (Williamson *et al.*, 1970). The concentration of glycerol 3-phosphate was below the level of detection under the incubation conditions used. Similar low levels of glycerol 3-phosphate have been found in hepatocytes prepared from starved animals unless incubated with dihydroxyacetone, glycerol or ethanol (Claus *et al.*, 1982) and suggests that any changes in concentration after endotoxin treatment are unlikely to be of significance (Claus *et al.*, 1982; Van Schaftingen, 1984). Similarly, we have previously shown that endotoxin treatment of the rat has no effect on the cytosolic concentration of citrate when incubated with lactate plus pyruvate as the substrate (Knowles *et al.*, 1987). These results suggest that, although alterations in Fru-2,6-P_2 metabolism may explain the increased cycling at the PFK-1 step, this effect is secondary to alterations in the rate of formation or degradation of PEP.

Effect of endotoxin treatment on pyruvate kinase activity

The cross-over studies of Williamson *et al.* (1970) showed a decrease in both glucose output and PEP levels in perfused livers obtained from endotoxin-treated rats when compared with livers from normal animals. It was suggested that an increased flux through pyruvate kinase might be responsible for the decrease in glucose synthesis, the increased pyruvate kinase activity decreasing the available PEP for glucose synthesis. Consistent with this hypothesis, Snyder and co-workers found that pyruvate kinase activity measured in mouse liver after injection of either endotoxin or bacteria was increased by 40–60% (Snyder, 1971; Snyder *et al.*, 1971), although this has not been confirmed in rat liver (Miller *et al.*, 1989). Therefore we have measured the effects of endotoxin treatment of the rat on pyruvate kinase activity in extracts prepared by $(NH_4)_2SO_4$ precipitation to remove allosteric effectors. The effects are compared with those of glucagon as a positive control. The results are shown in Table 4. The total activity measured in the presence of 5 mM-PEP and 20 μM-Fru-1,6-P_2 was not significantly changed by endotoxin treatment of the animals, in either the presence or the absence of glucagon. Similarly there was no change in the activity ratio during endotoxic shock, whereas glucagon treatment significantly decreased the activity ratio and increased the K_m for PEP, the mean values being 1.7 mM and 2.2 mM for control cells in the presence and absence of glucagon and 1.9 mM and 2.3 mM for cells prepared from endotoxin-treated animals in the presence and absence of glucagon respectively. This is further evidence to indicate that the effects of endotoxin are independent of changes in enzyme phosphorylation. Although these data indicate that there are no changes in the activity in the extracted enzyme, this does not preclude a change in flux through the enzyme in intact

Table 4. Effect of endotoxin on pyruvate kinase activity and flux in isolated hepatocytes

Total pyruvate kinase activity was measured in the presence of 20 μM-Fru-1,6-P_2. The activity ratio is expressed as the ratio of the activity of the enzyme measured at 1.0 mM-PEP to the total activity. Pyruvate kinase flux was measured over a 15 min incubation period as described in the Experimental section. Results shown are means ± S.E.M. for 6 different cell preparations: *$P < 0.05$ for difference between control and endotoxic cells; †$P < 0.05$ for difference between cells incubated in the absence and presence of 0.1 μM-glucagon.

	Control		Endotoxin	
		+Glucagon		+Glucagon
Total activity (μmol/min per g wet wt.)	7.9±0.8	7.1±1.2	8.0±0.9	7.2±0.4
Activity ratio	0.23±0.04	0.11±0.03†	0.17±0.03	0.09±0.08†
Pyruvate kinase flux (μmol/g wet wt.)	11.5±2.7	4.5±0.3†	5.1±0.3*	4.5±0.5
Glucose flux (μmol/g wet wt.)	29.1±4.0	35.4±6.1†	13.9±4.0*	22.6±6.2†
Partitioning (glucose/lactate+pyruvate)	3.1±0.5	8.3±1.9†	2.8±0.8	5.1±1.6†

liver cells. It is clearly established that Fru-1,6-P_2 is a potent allosteric activator of pyruvate kinase (Ljungstrom *et al.*, 1974), and an increased concentration of Fru-1,6-P_2 has been noted in both the endotoxic perfused liver (Williamson *et al.*, 1970) and in the livers of animals in which sepsis was induced by caecal ligation and puncture (Ardawi *et al.*, 1989), presumably as a result of the increases in Fru-2,6-P_2 and PFK-1 flux. Therefore the rate of flux through pyruvate kinase in the presence of 18 mM-lactate plus 2 mM-pyruvate was also measured (Table 4). The incorporation of label into glucose was also measured in these experiments, and this showed the typical pattern observed when glucose was analysed chemically as in Table 1, with endotoxin treatment decreasing glucose labelling by approx. 50% and glucagon stimulating the incorporation of label into glucose in cells prepared from both control and endotoxic animals. The ratio of glucose output/lactate plus pyruvate indicates the partitioning of [1-^{14}C]PEP between gluconeogenesis and pyruvate kinase. Endotoxin treatment resulted in an inhibition of pyruvate kinase flux rather than the expected stimulation; however, it did not affect the partitioning, suggesting that the decreased flux through pyruvate kinase was largely the result of a decreased formation of [1-^{14}C]PEP rather than any effect of endotoxin treatment on the enzyme itself or its allosteric effectors. In contrast, glucagon treatment significantly increased this ratio in control cells, the decreased pyruvate kinase flux matching the increase in glucose production. There was a similar increase in the ratio in the endotoxic cells after glucagon treatment; however, the change in glucose output could not be accounted for solely by a change in pyruvate kinase flux, suggesting that other sites may be more important, i.e. the decreased cycling at the PFK-1/Fru-1,6-P_2ase step or an increased formation of PEP. Confirmation that pyruvate kinase flux was not increased in septicaemia was obtained by measuring lactate formation from 10 mM-glycerol or 10 mM-dihydroxyacetone. With neither substrate was there any significant change in lactate production (results not shown), suggesting that, when PEP is generated by the oxidation of these substrates rather than by the action of pyruvate carboxylase and PEP carboxykinase, no change in pyruvate kinase flux occurs.

Conclusions

These results confirm and extend the original work of Knowles *et al.* (1987) and demonstrate that an increased flux through PFK-1 is a potential site of action of endotoxin which may contribute to the inhibition of gluconeogenesis observed during endotoxic shock. The observed 2-fold increase in liver cell Fru-2,6-P_2 provides a mechanism for the increased cycling, and demonstrates that the increased Fru-2,6-P_2 observed by Miller *et al.* (1989) in the liver *in vivo* was not merely a consequence of changes in circulating hormones or substrates. The elevation of Fru-2,6-P_2 does not appear to originate from changes in the expression of the bifunctional PFK-2/Fru-2,6-P_2ase or in the phosphorylation state of the protein, but as a result of a decreased cytosolic concentration of PEP, an inhibitor of PFK-2. The effect of glucagon on both glucose output and Fru-2,6-P_2 metabolism was not significantly different in cells from control and endotoxin-treated animals, suggesting that there is no impairment of the cyclic AMP-mediated pathway during sepsis.

The prime lesion during sepsis would appear to be to decrease the cytosolic concentration of PEP. This does not appear to be the result of an increased flux through pyruvate kinase, although PFK-1 flux is increased and an elevation in Fru-1,6-P_2 has been previously reported (Williamson *et al.*, 1970; Ardawi *et al.*, 1989). This would imply an inhibition of the PEP-generating steps in the pathway, e.g. pyruvate entry into the mitochondria, pyruvate carboxylase activity or PEP carboxykinase activity. The activity of the latter has been shown to be decreased in liver of mice by antagonizing its induction by glucocorticoids (Berry, 1977, and references therein); however, studies in the rat have been unable to demonstrate any change in activity under conditions where gluconeogenesis is inhibited (La Noue *et al.*, 1968; Knowles *et al.*, 1987; Miller *et al.*, 1989). Similarly, no change in pyruvate carboxylase activity has been demonstrated in cells permeabilized with saponin, although the latter does not necessarily preclude a change in flux through the enzyme in intact cells or *in vivo* (Knowles *et al.*, 1987). Work is needed to investigate these possibilities.

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