The characteristics and site of inhibition of gluconeogenesis in rat liver cells by bacterial endotoxin

Stimulation of phosphofructokinase-1

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The characteristics and site of inhibition of gluconeogenesis by endotoxin were investigated in liver cells isolated from control and endotoxin-treated rats. Endotoxin treatment was associated with inhibition (40–50%) of gluconeogenesis from lactate plus pyruvate over a range of concentrations of substrate and of oleate and with or without glucose or glucagon. Similar inhibition was observed with asparagine, proline, glutamine, alanine and a substrate mixture, but not with glycerol, glyceraldehyde, dihydroxyacetone or endogenous substrates. There was no change in cellular ATP content or in the rates of ketogenesis or ureogenesis from asparagine, proline or glutamine. Other effects on isotopic fluxes, metabolite contents, enzyme activities and control coefficients were consistent with the suggestion that the effects of endotoxin on gluconeogenesis are exerted at the level of phosphofructokinase-1, and not at phosphoenolpyruvate carboxykinase, pyruvate kinase, pyruvate carboxylase or glucokinase.

INTRODUCTION

Endotoxin is likely to be of crucial importance in the pathogenesis of Gram-negative septic shock in man, because (1) endotoxin injections mimic the clinical features of sepsis and (2) antibodies to the central core of endotoxin attenuate the mortality and morbidity in Gram-negative septicaemia ([1–3], and references therein). A prominent effect of endotoxin is the observed impairment of glucose homeostasis ([4], and references therein) and it has been suggested that the consequent hypoglycaemia plays an important role in the development of the lethal shock state [5–7].

We have shown [4] that the changes in glucose homeostasis are probably caused by increased glucagon secretion, blockade of the effects of glucocorticoids, and inhibition of basal gluconeogenesis and/or increased basal glucose utilization. The hypoglycaemic phase of endotoxic shock is probably a consequence of diminished gluconeogenesis, since it has been reported that both perfused livers [8] and isolated liver cells [9,10] from rats treated with endotoxin (at LD₅₀ doses) exhibit significantly decreased rates of gluconeogenesis from lactate. Endotoxin is known to decrease the activity of the enzyme phosphoenolpyruvate carboxykinase (PEPCK) in the livers of mice by antagonizing its induction by glucocorticoids (reviewed in [11]). Because of the important regulatory role postulated for PEPCK in gluconeogenesis [12,13], it has been inferred that this decrease in PEPCK by endotoxin is the primary cause of the inhibition of gluconeogenesis; a role for increases in pyruvate kinase activity has, however, also been suggested [14,15].

To determine the site and characteristics of inhibition of gluconeogenesis by endotoxin *in vivo*, we have carried

out a systematic series of experiments comparing the metabolic properties of liver cells isolated from control and endotoxin-treated rats; the results demonstrate that gluconeogenesis is inhibited by a stimulation of phosphofructokinase-1 (PFK-1), and not by effects at PEPCK or pyruvate kinase.

MATERIALS AND METHODS

Sources of materials

All radiolabelled compounds were from Amersham International, Amersham, Bucks., U.K. Bovine serum albumin (fraction V) was from Park Scientific, Northampton, U.K., and the charcoal used to treat it (Norit GSX) was from Norit Clydesdale Co., Glasgow, Scotland, U.K. The lipopolysaccharide used was prepared by trichloroacetic acid extraction of *Salmonella typhimurium*, and was obtained from Sigma Chemical Co., Poole, Dorset, U.K. Dow Corning 550 and dinonyl phthalate were from BDH Chemicals, Poole, Dorset, U.K. Enzymes were obtained from BCL, Lewes, Sussex, U.K., or Sigma. Other reagents were of analytical grade or the highest grade commercially available and were obtained from the above suppliers.

Animals

Male Sprague–Dawley rats (180–220 g) were used throughout. They were fed *ad libitum* on a standard laboratory diet (CRM; Labsure, Poole, Dorset, U.K.) until 16:00 h on the day before use. At this time they were given an intraperitoneal injection of either endotoxin [1 ml/kg body wt. of lipopolysaccharide (from *Salmonella typhimurium*; 5 mg/ml) in pyrogen-free saline) or vehicle alone, and were then starved overnight; this protocol is based on that of Filkins & Cornell [10].

Abbreviations used: PEPCK, phosphoenolpyruvate carboxykinase (EC 4.1.1.32); PFK-1, phosphofructokinase-1 (6-phosphofructo-1-kinase) (EC 2.7.1.11).

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Preliminary experiments showed that the effect of this protocol was somewhat variable. Most rats showed characteristic responses to the endotoxin, particularly a relative lack of movement and raised fur, and these animals had substantially raised plasma urea concentrations (controls 4.7 ± 0.2 mM, endotoxin 10.9 ± 1.5 mM; means $\pm$ S.E.M. for 13 and 8 rats respectively); liver cells isolated from such rats exhibited a substantial inhibition of gluconeogenesis from lactate plus pyruvate, as described below in the Results and discussion section. However, a small number of rats ($< 10\%$) died, and a further group of rats (approx. 10%) failed to respond in any of the ways described. Routinely, therefore, a small sample of blood was taken during the operation to perfuse the liver; liver cells from endotoxic animals were not used unless the plasma urea concentration determined on this sample was appreciably above the control value.

Preparation and incubation of liver cells

Isolated liver cells were prepared essentially as described previously [16], between 09:00 and 11:00 h; the perfusion medium was Ca^{2+} -free Krebs buffer [17] containing 0.05% (w/v) collagenase. The isolated liver cells were washed three times by centrifugation (50 g, 2 min) in Krebs buffer containing 1.25 mM- Ca^{2+} , and were finally resuspended in Krebs buffer containing 2.5 mM- Ca^{2+} and 2% (w/v) bovine serum albumin (defatted as described in [18], and then gel-filtered through Sephadex G-25).

Isolated liver cells were incubated at 37°C in silicone-treated glass scintillation vials under O_2/CO_2 (19:1), and were shaken at 100 cycles/min in a reciprocating water bath (Mickle Engineering, Gomshall, Surrey, U.K.). The liver cell concentration was approx. 3 mg dry wt./ml, except in experiments with low lactate plus pyruvate concentrations, in which it was 1.5–3 mg dry wt./ml, and in experiments to determine cellular and mitochondrial metabolites, in which it was approx. 50 mg dry wt./ml. For determinations of total metabolites in the incubation (glucose, urea, lactate, pyruvate and ketone bodies), incubations were terminated by addition of 0.1 vol. of 20% (w/v) HClO_4 and cooling on ice. For determinations of total cellular metabolites, 1 ml of cell suspension was layered over 300 μl of a 'silicone oil' (Dow Corning 550/dinonyl phthalate, 3:1, v/v) and the cells were centrifuged (10 s, 13000 g) through the oil into 150 μl of 12% HClO_4 . For determinations of mitochondrial metabolites, fractionation of the liver cells was carried out essentially as described in [19]: 1 ml of cell suspension was added to 8.5 ml of ice-cold fractionation medium (0.25 M-sucrose, 20 mM-Mops, 3 mM-EDTA, 4 mg of digitonin/ml, pH 7.0 at 0°C) and the whole was thoroughly mixed by decantation into an ice-cold silicone-treated glass centrifuge tube containing 1 ml of 'silicone oil' (Dow Corning 550/dinonyl phthalate, 2:1, v/v) over 0.5 ml of 12% HClO_4 . Then, 30 s after the addition of cells to fractionation medium, the permeabilized cells were centrifuged (30 s, 2500 g) through the 'silicone oil' into the HClO_4 . All HClO_4 extracts were incubated on ice for at least 5 min before centrifugation (30 s, 13000 g) to remove the precipitated material. Samples of HClO_4 supernatant were stored at -20°C for determinations of glucose, lactate, pyruvate, ketone bodies or urea; these were stable under these conditions for several weeks,

whereas pyruvate and the ketone bodies were found to be unstable in neutralized extracts stored at -20°C . For determinations of ATP, glutamate, 2-oxoglutarate, malate and aspartate, samples of HClO_4 supernatant were neutralized with 0.5 M-triethanolamine/2 M-KOH, the insoluble KClO_4 was removed by centrifugation (1 min, 13000 g), and the supernatants were stored at -20°C .

Metabolite assays

D-Glucose was determined with glucose oxidase [20]. L-Lactate, L-glutamate and D-3-hydroxybutyrate were assayed in 0.4 M-hydrazine/12 mM-EDTA, pH 9.0, with the respective dehydrogenases [21]. Standard spectrophotometric assays were used to determine pyruvate and acetoacetate [22], citrate [23], 2-oxoglutarate [24] and urea [25]. L-Aspartate and L-malate were determined radiometrically after conversion into oxaloacetate [26]. ATP was determined by using firefly luciferin-luciferase [27].

Other assays

Cellular pyruvate carboxylation was assayed essentially as described in [28], except that (a) liver cells were preincubated in Krebs buffer [17] containing 2% bovine serum albumin, 10 mM-lactate and 1 mM-pyruvate for 20 min, and (b) saponin (200 $\mu\text{g}/\text{ml}$) was used to permeabilize the cells. PEPCK was assayed at 37°C in the physiological direction in extracts of isolated liver cells as previously described [29,30].

Aspartate aminotransferase was assayed at 37°C in a medium containing 100 mM-potassium phosphate (pH 7.5), 12 units of malate dehydrogenase/ml, 0.16 mM-NADH, 0.5 mM-2-oxoglutarate, 1.5 mM-L-glutamate, 50 μl of liver extract and various concentrations of L-aspartate. The liver extracts were obtained by homogenization of liver in 10 vol. of 0.25 M-sucrose/20 mM-Mops/3 mM-EDTA (pH 7.0 at 0°C) at $0-4^\circ\text{C}$, centrifugation (750 g for 10 min) to remove debris and nuclei, and then further centrifugation (12000 g for 10 min) to remove the mitochondrial fraction; the supernatant material was designated cytosolic aspartate aminotransferase, and the pellet material, solubilized in homogenization medium containing 0.1% (v/v) Triton X-100, mitochondrial aspartate aminotransferase. The K_i for amino-oxyacetate was determined in the presence of 0.5 mM- and 5.0 mM-L-aspartate, 0–1 μM -amino-oxyacetate (cytosolic enzyme), or 0.05 mM- and 0.50 mM-L-aspartate, 0–5 μM -amino-oxyacetate (mitochondrial enzyme). Amino-oxyacetate was a non-competitive inhibitor with respect to aspartate, with K_i values of 0.4 μM (cytosolic enzyme) and 1.3 μM (mitochondrial enzyme).

Fluxes from D-[2- ^3H]- and D-[3- ^3H]-glucose to $^3\text{H}_2\text{O}$, as measurements of the fluxes through glucokinase and PFK-1 respectively [31–33], were determined by incubation of labelled glucose (5 mM, 0.15 $\mu\text{Ci}/\text{ml}$) with liver cells; separation of [^3H]glucose from $^3\text{H}_2\text{O}$ in extracts in 2% HClO_4 was carried out by ion-exchange chromatography on Amberlite CG400 (borate form) [32]. Fluxes from D-[U- ^{14}C]glucose to $^{14}\text{CO}_2$ were determined by trapping the $^{14}\text{CO}_2$ formed in centre wells containing 0.25 ml of 2-phenethylamine (50%, v/v, in methanol), as previously described [34]; ^{14}C in lactate was determined after ion-exchange chromatography on Amberlite CG400 (acetate form) [35,36]. Pyruvate kinase fluxes

Table 1. Effect of endotoxin treatment on gluconeogenesis from different substrates in isolated liver cells

Liver cells were incubated for 30 min with various substrates (10 mM unless specified); rates of glucose production were then measured over the next 30 min. Results are means $\pm$ S.E.M. for the numbers of independent preparations shown in parentheses. Significance of differences from control: * $P < 0.05$, ** $P < 0.005$.

Substrates	Gluconeogenesis (nmol/h per mg dry wt.)	
	Control	Endotoxic
None (endogenous)	8 $\pm$ 2.2 (5)	9 $\pm$ 1.1 (5)
Lactate (10 mM) + pyruvate (1 mM)	263 $\pm$ 16 (6)	161 $\pm$ 16 (6)**
Alanine	131 $\pm$ 6 (3)	97 $\pm$ 9 (3)*
Asparagine	97 $\pm$ 13 (3)	55 $\pm$ 6 (3)*
Proline	154 $\pm$ 2 (3)	85 $\pm$ 15 (3)*
Glutamine	137 $\pm$ 6 (3)	71 $\pm$ 12 (3)**
Glycerol	235 $\pm$ 17 (4)	211 $\pm$ 18 (5)
Glyceraldehyde	185 $\pm$ 11 (3)	153 $\pm$ 19 (3)
Dihydroxyacetone	548 $\pm$ 61 (6)	649 $\pm$ 129 (3)

Scheme 1. Scheme of gluconeogenesis from various substrates

The numbered steps are (1) glucose-6-phosphatase, (2) glucokinase, (3) fructose-1,6-bisphosphatase, (4) PFK-1, (5) PEPCK, (6) pyruvate kinase and (7) pyruvate carboxylase. The horizontal broken line represents the mitochondrial inner membrane; the subscript m denotes mitochondrial metabolites and the subscript c denotes cytosolic metabolites. Broken arrows indicate reactions of more than one step. Extra abbreviations used: Fru, fructose; Glc6P, glucose 6-phosphate; Mal, malate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; Pyr, pyruvate.

during gluconeogenesis from lactate/pyruvate (10 mM:1 mM) were determined from the incorporation of ^{14}C (as $\text{H}^{14}\text{CO}_3^-$) into glucose and lactate, and were corrected for ^{14}C reutilization by determination of the partition of ^{14}C from L-[U- ^{14}C]alanine between lactate, glucose and CO_2 [35,36].

Calculation and expression of results

Results are expressed as a function of cellular dry weight; this is approx. 27% of the wet weight [37]. The dry weight was determined by centrifugation (2 min, 150 g) of portions of cell suspension and of incubation medium in preweighed 2 ml glass vials, aspiration of the supernatants, and drying of the pellets overnight at 110 °C before reweighing; the dry weight of cells was taken as the difference between the results for the cell suspension and for the medium. For the calculation of metabolite contents of cellular compartments after cell fractionation, the spaces of compartments and the extracellular or extramitochondrial 'carry-down' of medium through the 'silicone oil' were determined by using $^3\text{H}_2\text{O}$ and [U- ^{14}C]sucrose. The spaces obtained were: after separation of cells from medium, intracellular space $1.34 \pm 0.09 \mu\text{l}/\text{mg}$ dry wt., extracellular carry-down $0.66 \pm 0.07 \mu\text{l}/\text{mg}$ dry wt., after digitonin fractionation, intramitochondrial $0.15 \pm 0.06 \mu\text{l}/\text{mg}$ dry wt., extramitochondrial carry-down $1.59 \pm 0.09 \mu\text{l}/\text{mg}$ dry wt. (all means $\pm$ S.E.M. for experiments with four liver cell preparations).

Data are given as means $\pm$ S.E.M. from the given numbers of liver cell preparations, or as means $\pm$ S.D. from single preparations. All assays were carried out in triplicate. Statistical differences were assessed by Student's t test.

RESULTS AND DISCUSSION

Characteristics of the inhibition of gluconeogenesis by endotoxin

Filkins & Cornell [10] have reported that injection of rats with LD_{50} doses of endotoxin causes an inhibition of gluconeogenesis from lactate, pyruvate and alanine in subsequently isolated liver cells. The data in Table 1 show that a low dose of endotoxin also causes inhibition of gluconeogenesis from lactate plus pyruvate and alanine, as well as from asparagine, proline and glutamine; Table 1 also shows that this effect is to some extent substrate-specific, because gluconeogenesis from glycerol, glyceraldehyde, dihydroxyacetone, and from endogenous substrates is unaffected. The pathways of gluconeogenesis from these substrates are outlined in Scheme 1.

The degree of inhibition of gluconeogenesis from lactate plus pyruvate was approximately constant over a range of substrate concentrations, and was independent of the presence of a maximally effective concentration of glucagon (Fig. 1). The inhibition was also relatively constant over a range of oleate concentrations (Fig. 2). Gluconeogenesis from a substrate mixture (lactate, pyruvate, amino acids and oleate) was substantially increased by the inclusion of glycerol (0.25–10 mM); the rates of gluconeogenesis in liver cells from endotoxin-treated rats remained 40–50% lower than those in cells from controls over the range of glycerol concentrations (Fig. 3). Gluconeogenesis from glycerol is therefore only unaffected by endotoxin pre-treatment when the glycerol

Fig. 1. Effects of substrate concentration and glucagon on the inhibition of liver cell gluconeogenesis by endotoxin treatment of donor rats

Liver cells from control (○, ●) or endotoxin-treated (□, ■) rats were preincubated for 15 min; lactate+pyruvate (at a molar ratio of 10:1) was then added, either alone (○, □) or with glucagon (final concn. 0.1 μ M) (●, ■). Rates of glucose production over the next 30 min were then determined. Results shown are means $\pm$ S.E.M. from experiments with three or four separate liver cell preparations. Rates of gluconeogenesis by endotoxic cells were significantly lower than those by control cells under all conditions ($P < 0.05$). Significance of effects of glucagon by a paired t test are shown: * $P < 0.05$, ** $P < 0.005$.

is presented to the cells as the sole substrate. Figs. 1–3 therefore show that the effects of endotoxin treatment on gluconeogenesis are likely to be manifest *in vivo*, since they are observed over the normal physiological range of plasma concentrations of lactate plus pyruvate and of non-esterified fatty acid, as well as at physiological concentrations of glycerol in the presence of other substrates. They also demonstrate that the effect of endotoxin is on the ' V_{max} ' of gluconeogenesis rather than on the ' K_m ' for substrates.

Site of inhibition of gluconeogenesis by endotoxin

Inhibition of gluconeogenesis cannot be attributed to general liver cell damage, because the cells from endotoxin-treated rats had ATP contents no lower than those from control animals: control 8.01 ± 0.32 , endotoxin 8.49 ± 0.64 nmol of ATP/mg dry wt. (both means $\pm$ S.E.M. from six independent experiments). Ureogenesis from endogenous substrates or from asparagine, glutamine or proline, and ketogenesis over a range of oleate concentrations, were also unaffected by treatment of the liver cell donors with endotoxin (results not shown), confirming that endotoxin treatment has no effect on the viability of the isolated liver cells.

A plausible hypothesis is that the observed net

Fig. 2. Effect of oleate concentration on gluconeogenesis from lactate+pyruvate by liver cells from control and endotoxin-treated rats

Liver cells from control (●) or endotoxin-treated (■) rats were incubated for 30 min in the presence of lactate (10 mM), pyruvate (1 mM) and a range of concentrations of oleate, and rates of glucose production were then determined over the next 30 min. Results shown are means $\pm$ S.E.M. from experiments with three to six separate liver cell preparations. Significance of inhibition by endotoxin treatment: * $P < 0.05$, ** $P < 0.005$.

Fig. 3. Effects of glycerol concentration on gluconeogenesis from a substrate mixture by liver cells from control and endotoxin-treated rats

Isolated liver cells from control (●) or endotoxin-treated (■) rats were incubated for 30 min in the presence of lactate (10 mM), pyruvate (1 mM), oleate (0.5 mM) and a physiological mixture of amino acids [38] before the addition of glycerol (0–10 mM); rats of glucose production were then determined over the next 30 min. Results shown are means $\pm$ S.E.M. for experiments with three separate liver cell preparations. Significance of inhibition by endotoxin treatment: * $P < 0.05$, ** $P < 0.005$.

inhibition of liver cell gluconeogenesis is a consequence of increased rates of flux through one or more of the glycolytic steps in the pathway: pyruvate kinase, PFK-1

Fig. 4. Effects of endotoxin treatment on glycolytic ^{14}C flux in liver cells

Isolated liver cells from control (○) or endotoxin-treated (●) rats were incubated for up to 2 h in the presence of $[\text{U-}^{14}\text{C}]$ glucose ($0.02 \mu\text{Ci/ml}$; 5 mM) and in the presence (a) or absence (b) of lactate (10 mM) plus pyruvate (1 mM) before determination of ^{14}C in CO_2 and lactate as described in the Materials and methods section. The data shown are each means $\pm$ S.E.M. from experiments with three or four liver cell preparations. Significance of effect of endotoxin treatment: * $P < 0.05$.

or glucokinase (see Scheme 1). Measurements in cells of the rates of incorporation of ^{14}C into lactate and CO_2 from $[\text{U-}^{14}\text{C}]$ glucose supported this hypothesis, these rates being more than 4 times faster in the endotoxic liver cells (Fig. 4); this increase was observed both with glucose (5 mM) alone and with glucose (5 mM), lactate (10 mM) and pyruvate (1 mM) together.

Bontemps *et al.* [32] have shown that the rate of detritiation of $[2\text{-}^3\text{H}]$ glucose by rat liver cells can be used to determine the rate of glucose phosphorylation. Figs. 5(a) and 5(b) show that the rate of glucose phosphorylation is essentially constant in liver cells from control and endotoxin-treated animals. In contrast, the rates of detritiation of $[3\text{-}^3\text{H}]$ glucose, taken as an indication of flux through PFK-1 [31,33,39], were 3–4-fold higher in liver cells from endotoxic rats than in control cells, whether with glucose alone (Fig. 5d) or with glucose, lactate and pyruvate together (Fig. 5c). The net rates of gluconeogenesis/glycolysis under these conditions are shown in Table 2; gluconeogenesis from lactate plus pyruvate was lower in the presence of 5 mM-glucose than in its absence (Table 1), the difference being close to the measured glucokinase flux (100 nmol/h per mg dry wt.). Endotoxin treatment resulted in an inhibition of gluconeogenesis proportionately similar to that seen in the absence of glucose; in the presence of lactate plus pyruvate the increased PFK-1 flux presumably causes a decrease in the concentrations of hexose 6-phosphates, and therefore a decrease in glucose-6-phosphatase flux (Scheme 1, step 1). There was little net glycolysis in the presence of glucose alone, indicating that under these

Fig. 5. Effects of endotoxin treatment on the detritiation of $[2\text{-}^3\text{H}]$ glucose and $[3\text{-}^3\text{H}]$ glucose by liver cells

Isolated liver cells from control (○) or endotoxin-treated (●) rats were incubated for up to 2 h in the presence of $[2\text{-}^3\text{H}]$ glucose (a, b) or $[3\text{-}^3\text{H}]$ glucose (c, d) at $0.15 \mu\text{Ci/ml}$ (5 mM) in the presence (a, c) or absence (b, d) of lactate (10 mM) plus pyruvate (1 mM) before determination of ^3H in $^3\text{H}_2\text{O}$ as described in the Materials and methods section. The data shown are means $\pm$ S.E.M. for experiments with four liver cell preparations. Significance of effect of endotoxin treatment: * $P < 0.05$.

conditions the fructose 1,6-bisphosphate formed from fructose 6-phosphate by PFK-1 is quantitatively converted back into fructose 6-phosphate by fructose bisphosphatase. The increased flux of ^{14}C from glucose to lactate plus CO_2 is therefore caused by increases in the specific radioactivities of fructose 1,6-bisphosphate and phosphoenolpyruvate. These results are consistent with the idea that the inhibition of gluconeogenesis and increased glycolytic carbon flow in liver cells from endotoxic animals are caused by a stimulation of flux through PFK-1, and not of flux through glucokinase.

Snyder and co-workers have proposed a role for increases in pyruvate kinase activity in the inhibition of glucose synthesis [14,15]. Three lines of evidence suggest that this is not the mechanism of the inhibition of

Table 2. Effect of endotoxin treatment on gluconeogenesis in the presence of glucose in isolated liver cells

Liver cells were incubated in the presence of the substrates shown for 2 h. Results are all means $\pm$ S.E.M. for four independent preparations. Significance of difference from control: * $P < 0.01$.

Substrates	Glucose production (nmol/h per mg dry wt.)	
	Control	Endotoxic
Glucose (5 mM) + lactate (10 mM) + pyruvate (1 mM)	154 $\pm$ 18	86 $\pm$ 5*
Glucose (5 mM)	-4 $\pm$ 13	-9 $\pm$ 2

gluconeogenesis observed here: (1) rates of flux through pyruvate kinase during gluconeogenesis from lactate plus pyruvate were not increased in liver cells from endotoxic rats [40,41]: control 140 $\pm$ 34 (4), endotoxin 80 $\pm$ 14 (4) nmol of pyruvate formed/h per mg dry wt.; (2) rates of formation of lactate from asparagine, proline, glutamine or glycerol were not increased in liver cells from endotoxic rate (results not shown); (3) the inhibition of gluconeogenesis from lactate plus pyruvate was proportionally very similar whether glucagon was present or not (Fig. 1), even though glucagon is thought to inhibit flux through pyruvate kinase substantially (reviewed in [40]).

A variety of experimental approaches have implicated pyruvate carboxylation as an important controlling step in gluconeogenesis from lactate and pyruvate ([28], [41] and references therein); this step could therefore be proposed as a plausible locus for the action of endotoxin. Table 1, however, shows that endotoxin treatment not only inhibits gluconeogenesis from substrates (lactate plus pyruvate, alanine) which require pyruvate carboxylase for their conversion into glucose, but also inhibits that from substrates (asparagine, proline, glutamine) which do not (Scheme 1). In addition, rates of pyruvate carboxylation in liver cells permeabilized with saponin (as described in the Materials and methods section) were not decreased in liver cells from endotoxin-treated rats: control 5.9 $\pm$ 1.12 nmol/min per mg dry wt., endotoxic 6.3 $\pm$ 1.07 nmol/min per mg dry wt. (means $\pm$ S.E.M. from experiments with five and three liver cell preparations respectively). Furthermore, it would be predicted that an inhibition of pyruvate carboxylase should decrease the concentrations of intermediates after this step in the gluconeogenic pathway from lactate; Table 3 shows that the content of aspartate in the cytosol was substantially increased in the cells from endotoxin-treated animals, as are the calculated cytosol and mitochondrial contents of oxaloacetate, whether the cells were incubated in the presence of 0.5 mM-oleate or not. These results are all inconsistent with pyruvate carboxylase being the primary site of inhibition of gluconeogenesis.

Another suggestion is that the inhibition of hepatic gluconeogenesis by treatment with endotoxin *in vivo* is caused by a decrease in the activity of PEPCK, as a consequence of glucocorticoid antagonism. However, the

Table 3. Metabolite contents of isolated liver cells from control and endotoxin-treated rats during gluconeogenesis from lactate + pyruvate

Isolated liver cells were preincubated with or without oleate (0.5 mM) for 10 min before addition of lactate (10 mM) and pyruvate (1 mM) and incubation for a further 5 min. Samples (1 ml) of cell suspension were then fractionated as described in the Materials and methods section, for the determination of mitochondrial and of total cellular metabolites respectively. The data shown are means $\pm$ S.E.M. for determinations on three or four liver cell preparations. The oxaloacetate values were calculated by assuming that the reactants of aspartate aminotransferase are close to equilibrium [42]. Significance of differences from control: * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$.

Metabolite	Cytosol (nmol/mg dry wt.)				Mitochondria (nmol/mg dry wt.)			
	-Oleate		+Oleate		-Oleate		+Oleate	
	Control	Endotoxic	Control	Endotoxic	Control	Endotoxic	Control	Endotoxic
Aspartate	0.16 $\pm$ 0.02	0.64 $\pm$ 0.10**	0.29 $\pm$ 0.02	0.75 $\pm$ 0.07***	0.034 $\pm$ 0.008	0.040 $\pm$ 0.012	0.023 $\pm$ 0.007	0.043 $\pm$ 0.008
Glutamate	1.74 $\pm$ 0.38	4.60 $\pm$ 0.47**	2.00 $\pm$ 0.38	4.99 $\pm$ 0.74*	0.71 $\pm$ 0.03	0.39 $\pm$ 0.05**	0.54 $\pm$ 0.06	0.43 $\pm$ 0.10
2-Oxoglutarate	0.62 $\pm$ 0.40	1.74 $\pm$ 0.31	0.13 $\pm$ 0.23	1.28 $\pm$ 0.44	0.58 $\pm$ 0.13	0.99 $\pm$ 0.13	0.51 $\pm$ 0.10	0.66 $\pm$ 0.13
Malate	0.33 $\pm$ 0.03	0.31 $\pm$ 0.08	0.62 $\pm$ 0.19	0.78 $\pm$ 0.14	0.06 $\pm$ 0.009	0.04 $\pm$ 0.012	0.17 $\pm$ 0.046	0.09 $\pm$ 0.026
Citrate	1.34 $\pm$ 0.44	1.56 $\pm$ 0.24			0.80 $\pm$ 0.18	0.27 $\pm$ 0.24		
Oxaloacetate (calculated)	0.009	0.037	0.003	0.029	0.004	0.015	0.003	0.010

Table 4. Activity of PEPCK, and the flux control coefficients of PEPCK and aspartate aminotransferase for gluconeogenesis in liver cells from control and endotoxin-treated rats

Isolated liver cells were preincubated for 15 min before the addition of inhibitors (0–30 μM -3-mercaptopycolinate or -amino-oxyacetate), followed 1 min later by lactate (10 mM) + pyruvate (1 mM); rates of glucose production were determined over the following 60 min, or samples of incubations were used to provide liver cell extracts for PEPCK assay (as described in the Materials and methods section) after 30 min. The initial slopes of the inhibitor titration curves $(\partial J / \partial [I])_{I \rightarrow 0}$ and the rates of gluconeogenic flux J were inserted into the equation relating to the non-competitive inhibitors' K_i values and the flux control coefficients C_J [45]:

$$(C_J)_{I \rightarrow 0} = -\frac{K_i \left(\frac{\partial J}{\partial [I]} \right)_{I \rightarrow 0}}{J}$$

The K_i of 3-mercaptopycolinate for PEPCK was taken to be 3 μM [46] and that of amino-oxyacetate for aspartate aminotransferase to be 0.4 μM (see the Materials and methods section). The data are means $\pm$ S.E.M. for determinations on the numbers of liver cell preparations given in parentheses. Significance of difference from control: * $P < 0.05$.

	Control	Endotoxic
PEPCK activity ($\mu\text{mol/min}$ per g wet wt.)	2.41 $\pm$ 0.13 (3)	2.75 $\pm$ 0.25 (3)
Control coefficients for gluconeogenesis		
PEPCK	0.057 $\pm$ 0.010 (3)	0.034 $\pm$ 0.004 (4)
Aspartate aminotransferase	0.052 $\pm$ 0.010 (5)	0.015 $\pm$ 0.003 (4)*

activities of PEPCK in liver cells isolated from control and endotoxic rats were found to be not significantly different (Table 4), despite the substantial inhibition of gluconeogenesis from lactate plus pyruvate measured concurrently. One might maintain that the activity of PEPCK was not fully expressed within the liver cells after endotoxin treatment, as, for example, by changes in the concentrations of metal ions or of the PEPCK-ferroactivator protein [43]. Therefore the control coefficient [44–46] of PEPCK for gluconeogenesis from lactate plus pyruvate in liver cells from control and endotoxin-treated rats was determined, because inhibition at this point should increase this parameter. The data in Table 4 show that this control coefficient, determined by inhibitor titration with 3-mercaptopycolinate [46,47], is decreased in endotoxic cells; this is consistent with inhibition of gluconeogenesis at some other locus, and not at the reaction catalysed by PEPCK.

Similar inhibitor-titration experiments with amino-oxyacetate as an inhibitor of aspartate aminotransferase [48,49] show that the aminotransferase also has a low control coefficient for gluconeogenesis from lactate plus pyruvate; this coefficient is again lower in the endotoxic liver cells, as expected if the pathway is inhibited at some other locus (Table 4).

Stimulation of PFK-1 as the primary site of inhibition of gluconeogenesis

Figs. 5(c) and 5(d) show that there is increased PFK-1

flux in liver cells from endotoxin-treated rats. As discussed above, this should cause a decrease in the flux through glucose-6-phosphatase, so that the net gluconeogenic flux is decreased through both the glucose-6-phosphatase/glucokinase and fructose bisphosphatase/PEPCK cycles. It would therefore be predicted that the concentrations of intermediates preceding the fructose bisphosphatase/PEPCK cycle should increase: Table 3 shows that aspartate and oxaloacetate concentrations are indeed increased in endotoxic liver cells. Since pyruvate kinase flux is not increased in endotoxic cells, there must be a decrease in pyruvate carboxylase flux for there to be a decrease in net gluconeogenesis from lactate plus pyruvate or alanine (see Scheme 1, steps 5, 6 and 7). This may be brought about by the over 3-fold increase in mitochondrial oxaloacetate observed in endotoxic cells (Table 2); Groen *et al.* [41] have shown that such large increases in mitochondrial oxaloacetate (in their experiments occasioned by inhibition of PEPCK) can substantially decrease flux through pyruvate carboxylase. Thus a stimulation of flux through PFK-1 can adequately explain the observed inhibition of gluconeogenesis from lactate plus pyruvate and alanine, as well as that from asparagine, proline and glutamine. It is not clear why no inhibition was observed with dihydroxyacetone, glyceraldehyde or glycerol as sole substrates (Table 1). The fact that inhibition is observed from glycerol in the presence of other substrates, however (Fig. 3), suggests some possible explanations: (a) the presence of a mitochondrial substrate may be necessary for production of the effector which stimulates PFK-1; (b) the initial metabolism of the trioses may give rise to an effector of the fructose bisphosphatase/PEPCK cycle which prevents the stimulation of PFK-1 flux, and which can be removed efficiently only when a mitochondrial substrate is present; (c) PFK-1 may have a much lower control coefficient for gluconeogenesis from these substrates: the control may, for example, instead reside in triose phosphorylation or fructose bisphosphatase; in this case a stimulation of PFK-1 flux would have little effect on gluconeogenic flux.

We do not know how the pretreatment of rats with endotoxin causes the observed stimulation of PFK-1 flux. PFK-1 is subject to many regulatory mechanisms (reviewed in [33] and [50]): changes in V_{max} , in different nutritional and hormonal states, phosphorylation/dephosphorylation, and the allosteric effects of fructose 2,6-bisphosphate, ATP, citrate, phosphoenolpyruvate, phosphocreatine, AMP, P_i and fructose 1,6-bisphosphate; of these mechanisms, we can only eliminate a decrease in allosteric inhibition of the enzyme because of a decreased citrate concentration, because the cytosolic citrate concentration is not decreased (Table 2).

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