The effect of treatment of the rat with bacterial endotoxin on gluconeogenesis and pyruvate metabolism in subsequently isolated hepatocytes

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The effect of treatment of rats with bacterial endotoxin on gluconeogenesis and the flux through pyruvate kinase, phosphoenolpyruvate carboxykinase (PEPCK), pyruvate carboxylase and pyruvate dehydrogenase (PDH) was measured in isolated hepatocytes, prepared from animals starved for 18 h, incubated in the presence of 1 mM pyruvate. The lipopolysaccharide reduced gluconeogenesis by 50% and lowered the flux through pyruvate kinase, PEPCK and pyruvate carboxylase by comparable amounts. There was no effect of endotoxaemia on PDH flux, indicating that the lowered rate of gluconeogenesis is not

the result of a redistribution of pyruvate metabolism between oxidation and carboxylation. The results confirm that a stimulation of pyruvate kinase activity following treatment with lipopolysaccharide is not involved in the inhibition of gluconeogenesis, but that the effect resides at the level of phosphoenolpyruvate formation. The most favoured mechanism for the inhibition of glucose synthesis is via an inhibition of PEPCK and subsequent feedback inhibition of pyruvate carboxylase, although a secondary effect at the level of the mitochondria and pyruvate carboxylase cannot be excluded.

INTRODUCTION

Septicaemia caused by Gram-negative organisms or insult with endotoxin characteristically results in a profound hypoglycaemia and impairment of hepatic gluconeogenesis, despite the prevailing hyperglucagonaemia and high plasma cortisol levels [1–7]. Various sites in the gluconeogenic pathway have been suggested as contributing to the impairment of gluconeogenesis following treatment *in vivo* with endotoxin. Cross-over studies in perfused livers from both normal and endotoxin-treated animals have suggested that the effect of the endotoxin resides at the two futile cycles involved in the conversion of pyruvate into phosphoenolpyruvate (PEP) and fructose 1,6-bisphosphate into fructose 6-phosphate [2]. A stimulation of 6-phosphofructo-1-kinase activity has been demonstrated by the measurement of the detritiation of [3-³H]glucose in isolated hepatocytes [7,8] and this has been ascribed to an increase in the concentration of fructose 2,6-bisphosphate [8,9]. However, in the absence of any changes in the activities of either 6-phosphofructo-2-kinase or fructose-2,6-bisphosphatase [8], it has been proposed that the increase in fructose 2,6-bisphosphate concentration is secondary to a fall in the concentration of cytosolic PEP, suggesting that the primary lesion in sepsis resides at the level of the first futile cycle, i.e. the conversion of pyruvate into PEP [8]. The latter could be explained by either a decrease in the activity of the PEP-generating steps, e.g. an inhibition of pyruvate carboxylase or phosphoenolpyruvate carboxykinase (PEPCK) activity, or an increase in pyruvate kinase activity. Investigations into the effects of endotoxin on these enzymes have been inconclusive. An increase in pyruvate kinase activity has been reported to occur following endotoxin administration in mice [10,11]; however, several studies have failed to confirm this in rats [7–9] and measurements of pyruvate kinase flux in hepatocytes have also failed to demonstrate an increase [7,8]. Endotoxin administration has

been shown to prevent the cortisol- or glucagon-dependent induction of PEPCK [12–14]. However, the failure to demonstrate a significant decrease in total PEPCK activity under conditions in which gluconeogenesis is still severely impaired suggests that other mechanisms may be important [1,7,9]. Similarly, no change in pyruvate carboxylase activity has been demonstrated in hepatocytes permeabilized with saponin [7], although the latter does not necessarily preclude a change in flux through the enzyme in intact cells or *in vivo*. The aim of this study was to re-investigate the effects of endotoxin treatment on the enzymes involved in this first futile cycle by measuring the flux through the enzymes at physiological levels of substrate. The effect of endotoxin treatment on pyruvate dehydrogenase (PDH) flux was also examined as competition between PDH and pyruvate carboxylase for pyruvate could occur, and it is evident that the activity of both skeletal muscle and liver PDH may be enhanced by inflammation and sepsis under certain conditions [15].

EXPERIMENTAL

Materials

Lipopolysaccharide (a trichloroacetic acid extract from *Salmonella typhimurium*), collagenase, [U-¹⁴C]glucose and NaH¹⁴CO₃ were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). [1-¹⁴C]Pyruvate and [2-¹⁴C]pyruvate were from NEN [Du Pont (U.K.) Ltd., Stevenage, Herts, U.K.]. All other chemicals were of AnalaR grade or similar from BDH Chemicals (Enfield, Middx., U.K.) or from Sigma.

Preparation and incubation of hepatocytes

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

environment exposed to a 12 h light–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/kg body wt. intraperitoneally) or pyrogen-free saline. The rats were fasted overnight and the hepatocytes prepared at 11:00 h next morning. The hepatocytes were prepared as in [16] and resuspended to a final concentration of 12 mg wet wt./ml in a modified Krebs–Henseleit buffer containing 25 mM NaHCO₃, 20 mM Hepes, pH 7.4, and 1.5 % (w/v) defatted albumin and were gassed with 100 % oxygen. The hepatocytes were kept on ice before the start of the incubations.

Pyruvate-kinase-flux measurements

Pyruvate kinase flux (J_{PK}) and the gluconeogenic flux ($J_{glucose}$) were measured by the incorporation of NaH¹⁴CO₃ into glucose, pyruvate and lactate as described previously [16]. Incubations were started by the addition of 1.25 ml of hepatocytes to an equal volume of modified Krebs–Henseleit buffer containing 20 mM Hepes, pH 7.4, 1.5 % (w/v) defatted albumin, 25 mM NaH¹⁴CO₃ (specific radioactivity 0.15 μ Ci/ μ mol) and 2.0 mM pyruvate as substrate. Triplicate incubations were carried out in 25 ml Erlenmeyer flasks for 15 min with the order of the incubations randomized with respect to the cell preparations. The incubations were stopped by the addition of 10 % (v/v) 2.7 M HClO₄ and the level of incorporation of label into glucose, pyruvate and lactate was determined [16]. J_{PK} is expressed in terms of nmol of PEP converted into lactate and pyruvate/15 min per mg of cell wet wt. $J_{glucose}$ is expressed in terms of nmol of PEP converted into glucose/15 min per mg of cell wt. The flux through PEPCK (J_{PEPCK}) was calculated as the sum of $J_{glucose}$ and J_{PK} [16,17].

PDH and pyruvate carboxylase flux measurements

The flux through pyruvate carboxylase (J_{PC}) and PDH (J_{PDH}) was measured as described previously [18,19], with the modifications described in [16]. J_{PDH} was calculated from the total release of ¹⁴CO₂ from [1-¹⁴C]pyruvate oxidation after correction for ¹⁴CO₂ released via the first turn of the tricarboxylic-acid cycle and through the reactions of malic enzyme and PEPCK [16,18,19]. Tricarboxylic-acid-cycle activity was estimated from the release of ¹⁴CO₂ from [2-¹⁴C]pyruvate [18]. A factor (F) was derived from the incorporation of label into ([pyruvate] + [lactate] + [glucose])/[glucose] in the pyruvate kinase experiments and this was used to calculate the contribution of ¹⁴CO₂ released by malic enzyme and PEPCK to the total [1-¹⁴C]pyruvate oxidation ([1-¹⁴C]pyruvate incorporated into glucose $\times$ F) [16,18,19]. From studies using perfused livers, it has been suggested that between 15 and 55 % of the ¹⁴CO₂ formed during oxidative reactions may be re-incorporated [20]. This could lead to a substantial underestimation of J_{PDH} if it were to occur in the above study. However, the work of Agius and Alberti [19] indicates that, with the low cell concentrations employed in both their study and ours, this is not a major problem and that re-incorporation of metabolically formed ¹⁴CO₂ leads to an underestimation of total ¹⁴CO₂ output of less than 5 %. Therefore no attempt was made to account for ¹⁴CO₂ recycling in the calculation of J_{PDH} . The ATP concentration was determined in the suspension of cells plus medium following deproteinization using 2.7 M HClO₄ [10 % (v/v)] by fluorimetric analysis [21].

The results are expressed as the means $\pm$ S.E.M., with the number of different cell preparations given in parentheses.

Statistical analysis was performed using two-way analysis of variance.

RESULTS

Effect of endotoxin treatment on metabolite flux through pyruvate kinase and PEPCK

Previous studies have suggested that treatment of the rat with endotoxin does not increase the flux through pyruvate kinase in subsequently isolated hepatocytes [7,8]. However, no studies have been carried out with physiological concentrations of substrate. Table 1 shows the effect of endotoxin shock on the incorporation of ¹⁴CO₂ into pyruvate and lactate (J_{PK}), and glucose ($J_{glucose}$). J_{PEPCK} was calculated as the sum of the flux of ¹⁴CO₂ into (glucose + lactate + pyruvate) [16,17]. Endotoxin treatment significantly decreased J_{PEPCK} and decreased the incorporation of label into both glucose, and lactate plus pyruvate. The extent of the inhibition of glucose synthesis (50 %) in the endotoxin-treated cells agrees favourably with previously published values using a similar model [3,7,8]. The results indicate that there was no significant alteration in partitioning of the PEP formed between gluconeogenesis and pyruvate kinase; the $J_{glucose}/J_{PEPCK}$ and J_{PK}/J_{PEPCK} ratios being unaffected by endotoxin treatment. This confirms the previous observations, using non-physiological concentrations of substrate, that pyruvate kinase activity is not increased following endotoxin treatment [7–9], but that flux is in fact decreased as a result of lowered availability of PEP [8]. These results confirm the suggestion that the major effect of endotoxin treatment on the control of gluconeogenesis is at the level of PEP formation [8]; however, the small percentage difference in the decrease in J_{PEPCK} (43 %) compared with $J_{glucose}$ (50 %) may reflect the increased cycling at the level of 6-phosphofructo-1-kinase [7,8].

Effect of endotoxin treatment on pyruvate metabolism

Table 2 shows that incorporation of [1-¹⁴C]pyruvate into glucose was decreased by 54 % in cells prepared from endotoxin-treated animals. This agrees favourably with the measurements of absolute gluconeogenic flux in Table 1. Since the incorporation of [1-¹⁴C]pyruvate into glucose is determined by the gluconeogenic rate and the dilution of the label in the oxaloacetate

Table 1 Effect of endotoxin treatment on J_{PK} with 1.0 mM pyruvate as the substrate

Hepatocytes were prepared from control and endotoxin-treated animals as described in the Experimental section and incubated with NaH¹⁴CO₃ plus 1.0 mM pyruvate as the substrate. After 15 min the reaction was terminated and incorporation of ¹⁴CO₂ into pyruvate, lactate and glucose was determined. J_{PK} , $J_{glucose}$ and J_{PEPCK} were measured as described in the Experimental section. Results are expressed as nmol of [¹⁴C]PEP incorporated/15 min per mg wet wt. of cells, with the number of cell preparations in each group given in parentheses. * $P < 0.05$, ** $P < 0.01$.

	Incorporation of [¹⁴ C]PEP (nmol of [¹⁴ C]PEP/15 min per mg wet wt. of cells)	
	Control	Endotoxin- treated cells
$J_{glucose}$	20.6 $\pm$ 2.5 (5)	10.2 $\pm$ 0.9 (5)**
J_{PK}	15.1 $\pm$ 2.0 (4)	10.0 $\pm$ 1.1 (4)*
J_{PEPCK}	35.4 $\pm$ 3.9 (4)	20.0 $\pm$ 3.7 (4)*
($J_{glucose}/J_{PEPCK}$) %	57.3 $\pm$ 2.8 (4)	52.3 $\pm$ 4.8 (4)
(J_{PK}/J_{PEPCK}) %	52.3 $\pm$ 4.8 (4)	47.7 $\pm$ 4.7 (4)

Table 2 Effect of endotoxin treatment on [1-¹⁴C]pyruvate and [2-¹⁴C]pyruvate metabolism

Hepatocytes were prepared from control and endotoxin-treated animals as described in the Experimental section and incubated with 1.0 mM [1-¹⁴C]pyruvate or [2-¹⁴C]pyruvate as the substrate. The fluxes through malic enzyme and PEPCK, PDH (J_{PDH}) and pyruvate carboxylase (J_{PC}) were measured as described in the Experimental section. Results are expressed as nmol/15 min per mg wet wt of cells, with the number of cell preparations in each group given in parentheses. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Flux	Control	Endotoxin-treated cells
Incorporation of [1- ¹⁴ C]pyruvate into glucose	11.0 ± 0.9 (5)	5.1 ± 0.5 (5)**
Oxidation of [1- ¹⁴ C]pyruvate	29.5 ± 2.5 (5)	15.8 ± 1.9 (5)**
Oxidation of [2- ¹⁴ C]pyruvate	6.2 ± 0.7 (4)	2.4 ± 0.4 (4)*
Total [1- ¹⁴ C]pyruvate metabolized	50.9 ± 3.5 (4)	27.4 ± 2.3 (4)***
F factor	1.76 ± 0.07 (4)	1.96 ± 0.19 (4)
¹⁴ CO ₂ lost due to malic enzyme and PEPCK	19.3 ± 1.3 (4)	10.0 ± 1.4 (4)***
J_{PC}	44.8 ± 2.8 (4)	22.4 ± 2.8 (4)***
J_{PDH}	6.1 ± 1.7 (4)	5.0 ± 0.4 (4)

pool, it is evident from this that the dilution of the oxaloacetate label is not significantly different between the control and endotoxin-treated cells. Endotoxin treatment also significantly decreased the oxidation of [1-¹⁴C]pyruvate to ¹⁴CO₂ by 53%. This is the result of a loss of ¹⁴CO₂ via PDH, plus that lost following conversion of the [1-¹⁴C]pyruvate to [1-¹⁴C]oxaloacetate, via the first turn of the tricarboxylic acid cycle or through the action of malic enzyme and PEPCK. The loss of ¹⁴CO₂ owing to the activities of malic enzyme and PEPCK and the flux through the tricarboxylic acid cycle were similarly reduced by 52% and 61% respectively following endotoxin treatment. The use of [2-¹⁴C]pyruvate or [3-¹⁴C]pyruvate to measure the contribution of tricarboxylic-acid-cycle activity to [1-¹⁴C]oxidation has been criticized on the basis that it contains an indeterminable contribution from PEPCK activity [22]. Therefore the apparent decrease in tricarboxylic-acid-cycle flux in cells prepared from endotoxin-treated rats may be an artifact of the decreased flux through PEPCK. To explore this possibility, 0.1 mM 3-mercaptopicolinate, a specific inhibitor of PEPCK [23], was incubated with the control cells to reduce flux through PEPCK and gluconeogenesis to a similar extent to that observed in cells prepared from endotoxin-treated animals. Despite the fact that J_{PEPCK} was reduced by 31%, no significant inhibition of [2-¹⁴C]pyruvate oxidation was observed (results not shown). Similar results are also evident from the results of Myles et al., who measured [3-¹⁴C]pyruvate oxidation [24]. Therefore the contribution from PEPCK activity to either [2-¹⁴C]pyruvate or [3-¹⁴C]pyruvate oxidation is negligible and unlikely to be a major contributing factor to the observed decrease in tricarboxylic-acid-cycle flux noted following endotoxin treatment of the rat. The diminished ¹⁴CO₂ output via malic enzyme, PEPCK and the tricarboxylic-acid-cycle reactions quantitatively accounted for the lowering of total ¹⁴CO₂ output, such that there was no significant effect of endotoxaemia on the flux through PDH. This agrees favourably with the previous observation that severe chronic sepsis has no significant effect on either total PDH or the percentage of the enzyme in the active form in liver [15]. In contrast, pyruvate carboxylase activity was reduced by 50%, such that there was a partitioning of pyruvate metabolism away from pyruvate carboxylation towards pyruvate oxidation and a large decrease in the $J_{PC}:J_{PDH}$ ratio from 7.3 to 4.5 in the cells prepared from control and endotoxin-treated animals respectively. It is evident that the inhibition of pyruvate metabolism and

gluconeogenesis is not the result of gross changes in cell viability, as the total cell-ATP content was found not to be significantly different [3.0 ± 0.5 ($n = 5$) and 3.0 ± 0.4 ($n = 8$) nmol/mg wet wt. for cells prepared from control and endotoxin-treated animals respectively]. This agrees with previously published results for hepatocytes prepared under similar conditions [7,8].

DISCUSSION

From the results presented above, it is evident that treatment of the rat with bacterial endotoxin decreases $J_{glucose}$ and J_{PK} by inhibiting the forward arm of the pyruvate-PEP futile cycle at either pyruvate carboxylase, PEPCK, or both. As the flux through pyruvate carboxylase, the first step in the pathway, is significantly diminished, with all subsequent steps showing a similar percentage decrease in flux, this suggests that with 1.0 mM pyruvate as the substrate the site of action of endotoxin resides predominantly at the level of pyruvate carboxylase. However, measurement of pyruvate carboxylase activity in permeabilized cells has failed to show any effect of endotoxin treatment [7], and of the known effectors, we have been unable to demonstrate any change in the mitochondrial ATP concentration and the ATP/ADP ratio in this model of sepsis [8], while the observed change in the mitochondrial glutamate concentration would be expected to increase rather than decrease flux through the enzyme [7]. Similarly it is evident that the decreased J_{PC} is not due to a significantly increased flux of pyruvate through PDH, diverting the substrate away from pyruvate carboxylase. Inhibition of pyruvate carboxylation by a decrease in acetyl CoA is a possibility as there are a number of reports which suggest that both sepsis and bacterial endotoxin lower hepatic ketogenesis [25,26] and the concentration of acetyl CoA and the acetyl CoA/CoA ratio [27]. However, these results have not been found universally following treatment of the rat with bacterial endotoxin [2,7]. This suggests that the prime locus of the effect may reside at the level of PEPCK. This is supported by the fact that gluconeogenesis from substrates which are independent of pyruvate carboxylase activity, e.g. asparagine, glutamine and proline, are also similarly inhibited following endotoxin treatment of the rat [7]. In addition, the cytosolic oxaloacetate concentration has been shown to rise following treatment with lipopolysaccharide [17], which is consistent with a decreased rate of utilization by PEPCK. It is suggested that an inhibition of PEPCK may explain the decreased flux through pyruvate carboxylase as a result of feedback inhibition, possibly via the increased mitochondrial and cytosolic oxaloacetate concentrations [7]. Evidence for such a feedback mechanism can be derived from the use of 3-mercaptopicolinate to inhibit PEPCK activity. We have found that incubation of hepatocytes with 0.1 mM 3-mercaptopicolinate to inhibit J_{PEPCK} and gluconeogenesis by 31%, also results in a comparable 34% inhibition of J_{PC} (results not shown). Similar calculations can be applied to the study of Myles et al. [24], which confirms a decreased flux through pyruvate carboxylase following inhibition of PEPCK activity. The mechanism underlying the inhibition of PEPCK activity remains to be established. It is accepted that endotoxin can prevent the cortisol- or glucagon-dependent induction of PEPCK and decrease the amount of mRNA for PEPCK [12-14]; however, measurements of PEPCK activity in liver homogenates [9], liver slices [1], or isolated hepatocytes [7] following endotoxin treatment have failed to show any significant change in activity under the conditions described above for the flux experiments. This suggests the involvement of a short-term regulator of the existing PEPCK activity. It has been proposed that an increase in intracellular Ca²⁺ may activate PEPCK as a result of a redistribution of Fe²⁺ [28] or Mn²⁺ [29]. A decrease in

cytoplasmic Ca^{2+} has been observed following endotoxin treatment of the rat [30]; however, there is no evidence for a decrease in the intracellular concentration of either Fe^{2+} or Mn^{2+} . We have recently demonstrated that 2-oxoglutarate can act as a competitive inhibitor of cytosolic PEPCK in liver and have proposed that the hormonal activation of 2-oxoglutarate dehydrogenase and the consequent fall in cytoplasmic 2-oxoglutarate concentration could be responsible for the increased conversion of oxaloacetate into PEP and glucose by both glucagon and Ca^{2+} -dependent hormones [31]. Therefore the finding that tri-carboxylic-acid-cycle activity is lowered following endotoxin treatment, together with the observation of a raised cytosolic 2-oxoglutarate concentration in hepatocytes isolated from endotoxin-treated animals [7], may provide a mechanism for the inhibition of PEPCK activity and the decreased flux through PEPCK and gluconeogenesis.

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