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IS THERE A GLOXYLATE CYCLE IN THE LIVER OF THE FETAL GUINEA PIG?

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SUMMARY

Studies on the effect of the inhibitor of fatty acid oxidation (+)-octanoylcarnitine on the perfused liver of the 48-51 days fetal guinea pig indicate that the oxidation of endogenous fatty acids is a major source of carbon for the citric acid cycle and for synthesis of hexose. Consistent with this the liver can convert isocitrate to glyoxylate and glyoxylate to malate and may therefore operate a glyoxylate cycle allowing the net production of sugars from acetyl-CoA.

Fatty acid metabolism in the fetal liver is unusual in that high rates of fatty acid synthesis can occur at times when the plasma non-esterified fatty acid concentration may be comparatively high (1-4). Moreover, despite an active pentose pathway providing NADPH to maintain fatty acid synthesis (3, 5, 6) the glucose uptake to sustain that pathway is comparatively low because of the presence of alternative substrates such as fatty acids, acetate, lactate or alanine (7). To sustain the pentose phosphate pathway the fetal liver appears to use the gluconeogenic pathway to generate glucose 6-phosphate (7, unpublished observations). However this leaves short-chain and long-chain fatty acids as one of the other main sources of carbon for biosynthesis. Only in plants, bacteria and nematodes has it been convincingly shown that they can sustain such a role because of the presence of the glyoxylate cycle (8-13). Thus in the present study the role of fatty acid oxidation in providing the carbon

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source for hexose production is investigated and tentative evidence for the existence of a glyoxylate cycle in the fetal liver is presented.

METHODS

Guinea pigs of the Dunkin-Hartley strain were used in this study. They were mated, reared and time of conception was estimated as described previously (14). Fetuses were used between days 48-51 of gestation. The methods of liver perfusion of freeze-clamping and of the determination of intracellular metabolite concentrations have been described (15, 16). The livers were perfused with 0.2 mM-[U-^{14}C]glucose; increasing the glucose concentrationin the medium above 0.1 to 0.2 mM did not increase glucose uptake and these low concentrations were required to measure accurately the uptake. Glucose 6-phosphate specific activity in the liver was determined by decarboxylation at the 1-carbon position. For this 1 ml of neutralised $HClO_4$ extract of the fetal liver was incubated with 1 ml of 50 mM-triethanolamine-HCl, pH 7.5, 0.05 ml of 20 mg/ml-$NADP^+$, 0.1 ml of 0.6 M $NaHCO_3$, 1.2 units of glucose 6-phosphate dehydrogenase and 0.1 unit of 6-phosphogluconate dehydrogenase. The incubation was carried out for 3 h in a sealed Warburg flask with a centre well containing 0.2 ml of 5 M-NaOH. At the end of the incubation 0.2 ml of 5 M-H_2SO_4 was added. Trapped $^{14}CO_2$ was converted to $Ba^{14}CO_3$ with barium acetate and radioactivity determined by counting in Aquasol (New England Nuclear) in a Phillips PW 4510 liquid scintillation counter. 6-Phosphogluconate specific activity was determined as above but omitting glucose 6-phosphate dehydrogenase from the incubation. The counts due to 6-phosphogluconate were subtracted from the total to determine those from glucose 6-phosphate. The 6-phosphogluconate dehydrogenase used was selected from several batches so as to contain little glucose 6-phosphate dehydrogenase activity and give little significant breakdown of glucose 6-phosphate during the decarboxylation of 6-phosphogluconate.

For the incubation studies fetal liver was homogenised with 4 vol of 50 mM-triethanolamine-HCl, pH 7.5, containing 0.2 mM-sucrose, 0.2 mM-dithiothreitol and 1 mM-EDTA. For the assay of isocitrate conversion to glyoxylate the reaction mixture contained 7.5 mM-tris-HCl, pH 7.0, 4.0 mM $MgCl_2$, 0.2 mM-dithiothrietol and 5 mM-[1,5-^{14}C]isocitrate. The incorporation was started by the addition of isocitrate and after incubation at 30°C for 5-15 min was stopped by the addition of 0.4 M-$HClO_4$. For the assay of glyoxylate conversion to malate the reaction mixture contained 100 mM-tris-HCl, pH 7.7, 10 mM-$MgCl_2$, 1.5 mM-[1-^{14}C] glyoxylate, 0.2 mM-acetyl-CoA, 1 mM-acetylphosphate and 2 units of phosphotransacetylase. The incorporation was started by the addition of glyoxylate and continued at 30°C for 5 to 15 min, then stopped with 0.4 M-$HClO_4$.

For both incubation systems described above the $HClO_4$ extracts were neutralised with KOH and the organic acids separated on Dowex 1-X8(Cl^-), 200-400 mesh, essentially as described by von Korff (17). The fractions containing glyoxylate

and malate were collected so as to minimise cross-contamination. The derived malate and glyoxylate fractions were both treated with 0.1 ml of 50 mM-p-nitrophenylhydrazine in 10 M-H_2SO_4 and after shaking for 60 min at room temperature the glyoxylate-p-nitrophenylhydrazone was extracted three times with 1 ml of ethylacetate. The aqueous sample was neutralised with KOH. Aqueous and ethylacetate fractions were counted for ^{14}C-malate and ^{14}C-glyoxylate respectively in Aquasol in a Phillips PW 4510 liquid scintillation counter. Cross contamination was 2% and was corrected for in each case. Correction was made for recovery.

RESULTS

The perfused liver of fetal guinea pigs of 48-51 days of gestation oxidised 0.5 mM-[U-^{14}C] palmitic acid to CO_2. The palmitic acid virtually abolished glucose uptake (Table 1). Perfusion with 0.5 mM-(+)-octanoylcarnitine blocked the oxidation of palmitate by the perfused liver and increased glucose uptake to a value higher than that with glucose alone (Table I).

Table I: Fatty acid oxidation by the perfused liver of the 48-51 days fetal guinea pig

	control	+0.5 mM-palmitate	+1 mM-palmitate and 0.5 mM-(+)-octanoylcarnitine
$^{14}CO_2$-production (nmol/min per g)	-	36.3 ± 18.4	5.7 ± 3.2**
O_2-consumption (umol/min per g)	0.44 ± 0.08	0.53 ± 0.10	0.49 ± 0.07
Glucose uptake (nmol/min per g)	36.2 ± 8.3	5.9 ± 3.6***	79.3 ± 17.6***

Fetal livers were perfused in a non-recirculating system via the umbilical vein with Krebs' bicarbonate buffer containing 0.2 mM-glucose and the additional substrates listed above. The perfusions were carried out for 30 min and the data refers to the final 10 min when the steady-state had been achieved. Results are means ± S.D. of 6 experiments. P values for comparison of palmitate perfused against controls, or palmitate plus (+)-octanoylcarnitine perfused against palmitate are: **, <0.01; ***, <0.001.

Table II: The effect of (+)-octanoylcarnitine on the concentration of intracellular metabolite in the perfused liver of 48-51 days fetal guinea pigs

	concentration (nmol/g)	
	control	plus 0.5 mM-(+)-octanoylcarnitine
citrate	78 ± 17	11.5 ± 4.9***
2-ketoglutarate	60 ± 10.4	4.9 ± 2.7***
malate	25.3 ± 7.8	3.6 ± 1.8**
6-phosphogluconate	4.1 ± 1.9	0.6 ± 0.25**
glucose 6-phosphate	66 ± 19	36.9 ± 10.6*
	specific activity (nmol/nmol)	
glucose 6-phosphate	0.20 ± 0.05	0.65 ± 0.14***
6-phosphogluconate	0.13 ± 0.07	0.21 ± 0.10

Livers were perfused in a non-recirculating system via the umbilical vein with Krebs' bicarbonate buffer containing 0.2 mM [U-^{14}C]glucose for 30 min. At the end of the perfusion the liver was freeze-clamped with tongs cooled in liq. N_2 and metabolites were determined in $HClO_4$ extracts. P values for comparison of controls with (+)-octanoylcarnitine treated livers are: *, <0.05; **, <0.01; ***, <0.001.

The effect of (+)-octanoylcarnitine on intracellular metabolite concentration and specific activity are shown in Table II. The specific activity for glucose 6-phosphate in fetal livers perfused with [U-^{14}C]glucose is normally low and does not change significantly between 10 and 60 min of perfusion. Despite depressing the glucose 6-phosphate concentration (+)-octanoylcarnitine caused a substantial increase in the glucose 6-phosphate specific activity (Table II) consistent with the increase in glucose uptake. Exogenous palmitate did not influence glucose 6-phosphate specific activity in the presence or absence of (+)-octanoyl-

carnitine (results not shown). In the presence of (+)-octanoyl-carnitine there was a substantial fall in the concentration of citric acid cycle intermediates in the perfused liver (Table II).

The results in Table I and II indicate that fatty acid breakdown, largely using endogenous fatty acids, contributes significantly to the flux of carbon into glucose 6-phosphate. This is responsible for the low specific activity of glucose 6-phosphate (glycogen concentration at this time is < 0.2 mg/g and glycogen breakdown makes a small contribution only to the glucose 6-phosphate pool). In the presence of (+)-octanoyl-carnitine (an inhibitor of fatty acid oxidation (18)) the gluconeogenic flux appears to be inhibited causing an increased glucose uptake and increased glucose 6-phosphate specific activity. This interpretation requires a pathway from acetyl CoA to triose-phosphates.

Homogenates of the fetal liver converted [1,5-^{14}C] isocitrate into ^{14}C-glyoxylate at a rate that was almost linear for 10 min. This was apparently inhibited by acetyl-CoA, phosphoenolpyruvate and EGTA (Table III). The homogenates also converted [1-^{14}C] glyoxylate into ^{14}C-malate at a rate that was linear for 5-10 min. It required acetyl-CoA and an acetyl-CoA-regenerating system and was inhibited by EGTA (Table III).

DISCUSSION

The fetal liver has the problem of sustaining high rates of biosynthesis at a time when glucose consumption is probably low (7). In the guinea pig this occurs when hepatic stores of glycogen are very low (<0.2 mg/g) although there are stores of triglyceride (4). At the same time the perfused liver of the fetal guinea pig is capable of high rates of lactate production

Table III: Conversion of isocitrate to glyoxylate and of glyoxylate to malate by homogenates of liver of 48-51 days fetal guinea pigs

	Rate of conversion (μmol/min per g of liver)	
Incubation time	5 min	10 min
[1,5-^{14}C]isocitrate to ^{14}C-glyoxylate		
Complete system	0.63 ± 0.21	0.55 ± 0.24
plus 1 mM-acetyl-CoA	0.27 ± 0.13	-
plus 10 mM-phosphoenolpyruvate	0.13 ± 0.05	0.07 ± 0.04
plus 10 mM-EGTA	0.05 ± 0.02	0.03 ± 0.014
[1-^{14}C] glyoxylate to ^{14}C-malate		
complete system	0.45 ± 0.19	0.39 ± 0.27
minus acetyl-CoA and regenerating system	0.05 ± 0.026	0.06 ± 0.03
plus 10 mM-EGTA	0.041± 0.023	0.033± 0.019

For the conversion of isocitrate to glyoxylate the incubation system contained 75 mM-tris-HCl, pH 7.0, 4 mM-$MgCl_2$, 0.2 mM-dithiothreitol, 5 mM-[1,5-^{14}C]isocitrate and 0.1 ml of a 20% (w/v) liver homogenate in a final vol. of 3 ml. For the conversion of glyoxylate to malate the incubation system contained 100 mM-tris-HCl, pH 7.7, 10 mM-$MgCl_2$, 0.2 mM-acetyl-CoA, 1 mM-acetyl phosphate, 2 units of phosphotransacetylase and 0.05 ml of a 20% (w/v) liver homogenate in a final volume of 1 ml. Incubations were at 30°C and were stopped with 0.4 M-$HClO_4$. ^{14}C-glyoxylate and malate were extracted as described in the Methods section.

from endogenous substrates. An insight into how the fetal liver copes with these problems is possibly indicated by the presence of the gluconeogenic enzymes when little glucose synthesis occurs (19, 20). Recent experiments have suggested that they may be involved in the production of glucose 6-phosphate to maintain the activity of the pentose phosphate pathway. The low specific activity of glucose 6-phosphate in the fetal guinea pig livers perfused with [U-^{14}C]glucose is consistent with this and suggests

that substantial flux of endogenous substrate into glucose 6-phosphate occurs. If this is an acceptable explanation then the ability of (+)-octanoylcarnitine to increase glucose 6-phosphate specific activity while depressing the concentration suggests that fatty acids are the main endogenous substrate. Clearly (+)-octanoylcarnitine inhibited fatty acid oxidation by the fetal liver and its marked effect on citric acid cycle intermediates indicates that fatty acid oxidation provides the main source of carbon to sustain the cycle.

Tissues of higher animals are thought not to be able to convert acetyl-CoA into sugars (21). Bacterial and plant tissues do so when metabolising lipid by using the glyoxylate cycle (8-13). Recently it has been shown that toad bladder contains the necessary activities required for a glyoxylate cycle (22). The present results provide tentative evidence that the fetal guinea pig liver possesses the ability to operate a glyoxylate cycle and that this could be involved in the conversion of fatty acids into sugars to sustain the pentose phosphate pathway and the supply of NADPH for biosynthesis.

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