Interactions *in vivo* between Oxidation of Non-Esterified Fatty Acids and Gluconeogenesis in the Newborn Rat

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Metabolic interactions between fatty acid oxidation and gluconeogenesis were investigated *in vivo* in 16-h-old newborn rats under various nutritional states. As the newborn rat has no white adipose tissue, starvation from birth induces a low rate of hepatic fatty acid oxidation. Hepatic gluconeogenesis is inhibited in the starved newborn rat when compared with the suckling rat, which receives fatty acids through the milk, at the steps catalysed by pyruvate carboxylase and glyceraldehyde 3-phosphate dehydrogenase. These inhibitions are rapidly reversed by triacylglycerol feeding. Inhibition of fatty acid oxidation by pent-4-enoate in the suckling animal mimics the effect of starvation on the pattern of hepatic gluconeogenic metabolites. It is concluded that, in the newborn rat *in vivo*, hepatic fatty acid oxidation can increase the gluconeogenic flux by providing the acetyl-CoA necessary for the reaction catalysed by pyruvate carboxylase and the reducing equivalents (NADH) to displace the reversible reaction catalysed by glyceraldehyde 3-phosphate dehydrogenase in the direction of gluconeogenesis.

Studies conducted previously in this laboratory have shown that concentrations of plasma and liver non-esterified fatty acids are very low in the newborn rat starved from birth (Girard *et al.*, 1973). This is different from the adult rat, which responds to starvation by mobilization of its fat stores; the newborn rat is unable to do this because of a virtual absence of white adipose tissue (Hahn & Novak, 1975). The starved newborn rat rapidly becomes hypoglycaemic owing to a low rate of gluconeogenesis compared with the suckling rat (Girard *et al.*, 1973, 1975). The low rate of gluconeogenesis in the starved newborns is not due to a deficiency in enzymes of the gluconeogenic pathway, since the effect of starvation is rapidly reversed by triacylglycerol feeding (Ferré *et al.*, 1978). In these conditions, triacylglycerol feeding, which increases plasma non-esterified fatty acids as well as their hepatic oxidation, strongly stimulates the rate of gluconeogenesis (Ferré *et al.*, 1978). Moreover, the administration of sodium pent-4-enoate, a compound which inhibits fatty acid oxidation (Corredor *et al.*, 1967; Senior & Sherratt, 1969) to suckling newborn rats inhibits gluconeogenesis and mimics the effects of starvation (Pégorier *et al.*, 1977).

In perfused livers of adult rats, various workers have shown a stimulation of gluconeogenesis on addition of large amounts of fatty acids (Söling *et al.*, 1969; Williamson *et al.*, 1968, 1969; Menahan

& Wieland, 1969; Parrilla *et al.*, 1976), as well as a decreased rate of gluconeogenesis on addition of inhibitors of fatty acid oxidation (Williamson *et al.*, 1968, 1970; Menahan & Wieland, 1969; Toews *et al.*, 1970). The main mechanisms suggested for the action of fatty acids on gluconeogenesis are: the generation of extra ATP to support gluconeogenesis, the production of acetyl-CoA, an obligatory cofactor for pyruvate carboxylase (EC 6.4.1.1) (Utter & Keech, 1963), and the provision of reducing equivalents, transferred from the mitochondria to the cytosol to meet the requirements of the glyceraldehyde 3-phosphate dehydrogenase reaction (EC 1.2.1.12). This transfer is necessary for oxidized substrates such as pyruvate or alanine. There do not, however, appear to be any convincing experiments *in vivo* which demonstrate a stimulatory effect of fatty acid oxidation on gluconeogenesis under physiological conditions (for a review, see Ruderman *et al.*, 1969).

The aim of the present work was to elucidate the mechanisms involved in the stimulation of gluconeogenesis by fatty acid oxidation *in vivo* in the newborn rat.

Materials and Methods

Animals

Rats of Wistar strain were used. They were all born between 16 and 18 h on day 22 of gestation.

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The groups of rats that were starved from birth or fed with triacylglycerol emulsion were delivered by Caesarean section after neck dislocation of the mother and placed at 37°C and 70% relative humidity. Suckling rats were born naturally on day 22 of gestation and left with the mother at nest temperature (35°C) until the time of experiment.

Triacylglycerol feeding

Starved rats (13 h old) were fed by using a thin and flexible catheter (Intramedic PE; Clay Adams, Parsippany, NJ, U.S.A.) either with 20 µl of 0.9% NaCl/g body wt. or with 20 µl of a triacylglycerol emulsion [10% (w/v) glycerol-free, soya-bean oil fat emulsified with egg-yolk phospholipids (1.2, w/v); Vitrum, Stockholm, Sweden]/g body wt.; the exact composition is described elsewhere (Ferré *et al.*, 1978). After feeding, the pups were immediately placed at 37°C for 3 h.

Sodium pent-4-enoate injection

Litters of 16h-old suckling rats were divided into two groups. One was injected subcutaneously with sodium pent-4-enoate (pH 7.4) at a dose of 2 mg/g body wt. and the other was injected with the same volume of 0.9% NaCl. The pups were then separated from the mother and placed at 37°C and 70% relative humidity. They were studied 2.5 h later, as the maximum effects of pent-4-enoate are observed at this time (Pégorier *et al.*, 1977).

Liver sampling

The liver was rapidly removed from decapitated newborn rats and freeze-clamped between metal blocks previously cooled in liquid nitrogen (Wollenberger *et al.*, 1960). The average time elapsing between decapitation and freeze-clamping was less than 5 s. The frozen liver was weighed, pulverized in a mortar, extracted with HClO₄ and neutralized as described by Williamson *et al.* (1967).

Metabolite analysis

The following metabolites were determined in the neutralized extracts: acetyl-CoA (Pearson, 1965); ATP (Lamprecht & Trautschold, 1963); ADP and AMP (Adam, 1963); glucose and glucose 6-phosphate (Slein, 1963); dihydroxyacetone phosphate (Bücher & Hohorst, 1963); glycerol 3-phosphate (Hohorst, 1963a); D-3-hydroxybutyrate and acetoacetate (Williamson *et al.*, 1962); lactate and pyruvate (Hohorst *et al.*, 1959); L-malate (Hohorst, 1963b); phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate (Czok & Eckert, 1963). Oxaloacetate was calculated by the formula:

$$[\text{Oxaloacetate}] = \frac{[\text{pyruvate}] \cdot [\text{malate}] \cdot k_{\text{MDH}}}{[\text{lactate}] \cdot k_{\text{LDH}}}$$

where k_{MDH} and k_{LDH} represent respectively the equilibrium constants of lactate dehydrogenase (EC 1.1.1.27) and malate dehydrogenase (EC 1.1.1.37) (Hawkins *et al.*, 1973).

Statistics

Results are expressed as means $\pm$ S.E.M. Statistical significance was determined by using the Wilcoxon rank test (Wilcoxon, 1947). As oxaloacetate values are calculated, no S.E.M. is given for this.

Special chemicals

All enzymes and coenzymes were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. Pent-4-enoic acid was purchased from K & K Laboratories, Plainview, NY, U.S.A.

Fig. 1. Crossover plot showing the effect of starvation on hepatic metabolite concentrations in 16h-old newborn rats

Newborn rats (16 h old) starved from birth (○) are compared with suckling rats (●) of the same age. The liver was removed from the animals and immediately freeze-clamped. For further details see the Materials and Methods section. Values shown are the means $\pm$ S.E.M. for 10 to 25 determinations. The concentration of each metabolite in the liver of suckling rats, taken as control, is given in µmol/g wet wt.: lactate, 1.0; pyruvate, 0.074; oxaloacetate (calculated), 0.008; malate, 0.45; phosphoenolpyruvate, 0.13; 2-phosphoglycerate, 0.050; 3-phosphoglycerate, 0.33; dihydroxyacetone phosphate, 0.022; glucose 6-phosphate, 0.14; glucose, 4.2. The gluconeogenic flux, given in µmol of glucose formed from lactate/h per 100 g body wt., is taken from Girard *et al.* (1975); control value is 22.

Results

Effect of starvation on liver metabolite concentration in vivo

When the concentrations of liver metabolites of 16h-old newborn rats, starved from birth, are expressed as percentages of the values obtained in suckling rats of the same age, two reverse crossovers are observed (Fig. 1): between pyruvate and oxaloacetate and between 3-phosphoglycerate and dihydroxyacetone phosphate. As the gluconeogenic flux is decreased in the starved group (Girard *et al.*, 1975), the two crossover points can be considered as limiting steps along the gluconeogenic pathway. The first crossover suggests an inhibition of the reaction catalysed by pyruvate carboxylase. In this situation, hepatic acetyl-CoA, which has been shown to be an obligatory cofactor for this enzyme (Utter & Keech, 1963), is markedly decreased (Table 1).

As dihydroxyacetone phosphate is in near-equilibrium with glyceraldehyde 3-phosphate via

triose phosphate isomerase (EC 5.3.1.1), the second crossover indicates an inhibition of glyceraldehyde 3-phosphate dehydrogenase or, as 1,3-bisphosphoglycerate was not measured, of 3-phosphoglycerate kinase (EC 2.7.2.3). To differentiate between these two potential sites of inhibition, we have measured the cofactors involved in these two enzymic steps, namely [ATP] and the [ATP]/[ADP] ratio for the reaction catalysed by 3-phosphoglycerate kinase and the cytosolic oxidation/reduction state for the reaction catalysed by glyceraldehyde 3-phosphate dehydrogenase, as NADH is the co-substrate for this reaction when the flux is oriented in the direction of glucose formation. Starvation does not induce a change in liver [ATP] or in the [ATP]/[ADP] ratio (Fig. 1 and Table 1). On the other hand, the decreases in liver [lactate]/[pyruvate] ratio, [glycerol 3-phosphate]/[dihydroxyacetone phosphate] ratio as well as in the [D-3-hydroxybutyrate]/[acetoacetate] ratio, indicate a limited availability of reducing equivalents (Table 1).

Table 1. Concentrations of hepatic metabolites in 16h-old rats treated in various ways

For full details, see the Materials and Methods section. Values shown are the means $\pm$ s.e.m., with the numbers of experiments indicated in parentheses. Significant differences were determined by the Wilcoxon (1947) rank test: * $P < 0.01$ when compared with suckling animals; ** $P < 0.01$ when compared with starved animals.

Liver metabolites ($\mu\text{mol/g}$ wet. wt.)	Suckling pups	Animals starved from birth	Animals starved from birth and fed at 13h with triacylglycerol	Suckling pups injected with sodium pent-4-enoate
Lactate	1000 $\pm$ 100 (16)	730 $\pm$ 110 (25)	1000 $\pm$ 100 (23)	2369 $\pm$ 308* (6)
Pyruvate	74 $\pm$ 4 (16)	83 $\pm$ 4 (25)	70 $\pm$ 5 (27)	232 $\pm$ 40* (6)
Lactate/pyruvate	13 $\pm$ 1 (16)	8.8 $\pm$ 0.8* (25)	15 $\pm$ 1** (23)	10 $\pm$ 1* (6)
Glycerol 3-phosphate	254 $\pm$ 11 (19)	116 $\pm$ 18* (16)	310 $\pm$ 40** (12)	27 $\pm$ 10* (6)
Dihydroxyacetone phosphate	22 $\pm$ 2 (14)	14 $\pm$ 1* (12)	23 $\pm$ 2** (10)	18 $\pm$ 2 (6)
Glycerol 3-phosphate/ dihydroxyacetone phosphate	11.3 $\pm$ 1 (14)	8.3 $\pm$ 1* (12)	13.5 $\pm$ 2** (10)	1.7 $\pm$ 0.2* (6)
Acetoacetate	840 $\pm$ 50 (16)	186 $\pm$ 11* (21)	653 $\pm$ 73** (12)	236 $\pm$ 21* (6)
D-3-Hydroxybutyrate	876 $\pm$ 70 (16)	133 $\pm$ 16* (21)	610 $\pm$ 62** (12)	129 $\pm$ 18* (6)
D-3-Hydroxybutyrate/ acetoacetate	1.00 $\pm$ 0.07 (16)	0.72 $\pm$ 0.07* (21)	0.95 $\pm$ 0.07 (12)	0.56 $\pm$ 0.08* (6)
ATP	2.0 $\pm$ 0.1 (6)	1.92 $\pm$ 0.03 (16)	1.85 $\pm$ 0.10 (7)	1.85 $\pm$ 0.04 (6)
ADP	0.85 $\pm$ 0.06 (6)	0.75 $\pm$ 0.04 (16)	0.7 $\pm$ 0.1 (7)	1.1 $\pm$ 0.1* (6)
AMP	0.16 $\pm$ 0.02 (6)	0.15 $\pm$ 0.02 (16)	0.19 $\pm$ 0.02 (7)	0.24 $\pm$ 0.02* (6)
ATP/ADP	2.4 $\pm$ 0.2 (6)	2.5 $\pm$ 0.1 (16)	2.6 $\pm$ 0.2 (7)	2.64 $\pm$ 0.14* (6)
Acetyl-CoA	33 $\pm$ 2 (8)	14 $\pm$ 1* (16)	27 $\pm$ 1** (10)	19 $\pm$ 3* (6)

Effect of triacylglycerol feeding on concentrations of liver metabolites in starved newborn rats

The concentrations of liver metabolites of 16h-old rats, starved until 13h and then fed with the triacylglycerol emulsion, are expressed as percentages of the values for starved rats of the same litters (Fig. 2). As described previously (Ferré *et al.*, 1978), the ingestion of the triacylglycerol emulsion increases fatty acid oxidation by the liver, as shown by the increase in hepatic ketone bodies (Table 1), and enhanced gluconeogenic flux. In these conditions,

Fig. 2. Crossover plot showing the effects of triacylglycerol feeding on hepatic metabolite concentrations in 16h-old newborn rats

Newborn rats (16h old) fed at 13h with 100mg of a triacylglycerol emulsion (○) are compared with 16h-old starved rats (●). The liver was removed from the animals and immediately freeze-clamped. For further details see the Materials and Methods section. Values shown are the means $\pm$ S.E.M. for 10 to 25 determinations. The concentration of each metabolite in the liver of starved rats, taken as control, is given in $\mu\text{mol/g}$ wet wt.: lactate, 0.73; pyruvate, 0.083; oxaloacetate (calculated), 0.005; malate, 0.18; phosphoenolpyruvate, 0.15; 2-phosphoglycerate, 0.07; 3-phosphoglycerate, 0.35; dihydroxyacetone phosphate, 0.014; glucose 6-phosphate, 0.09; glucose, 0.98. The gluconeogenic flux, given in μmol of glucose formed from lactate/h per 100g body wt., is taken from Ferré *et al.* (1978) and Girard *et al.* (1975); control value is 4.6.

two forward crossovers are observed: one between pyruvate and oxaloacetate, and the other, although less clear, between 3-phosphoglycerate and dihydroxyacetone phosphate. The first one is accompanied by an increase in hepatic [acetyl-CoA] and suggests an activation of pyruvate carboxylase. The large increase in liver [malate] could result from this activation together with the more reduced mitochondrial milieu, as indicated by the increase in the [D-3-hydroxybutyrate]/[acetoacetate] ratio (Table 1), which will tend to drive the near-equilibrium reaction catalysed by malate dehydrogenase towards the formation of malate. For the second crossover site, it is not possible to differentiate clearly between an effect on glyceraldehyde phosphate dehydrogenase or 3-phosphoglycerate kinase. However, there is no increase in liver [ATP] or [ATP]/[ADP] ratio (Table 1). On the contrary, there is a large increase in the [lactate]/[pyruvate] and [glycerol 3-phosphate]/[dihydroxyacetone phosphate] ratios (Table 1), indicating a reduced cytosolic state. Triacylglycerol feeding, which leads to increased fatty acid oxidation, stimulates the two steps that were limiting gluconeogenesis in the starved animals.

Effect of pent-4-enoate on concentrations of liver metabolites in suckling newborn rats

To substantiate the conclusion that there is a direct relationship *in vivo* between fatty acid oxidation and gluconeogenesis in the suckling animal, oxidation was inhibited with sodium pent-4-enoate (Pégorier *et al.*, 1977). Injection of sodium pent-4-enoate produces a pattern of liver metabolites in suckling pups which is very similar to that obtained in starved pups (compare Figs. 1 and 3). There are two backward crossovers, one between pyruvate and oxaloacetate and the other between 3-phosphoglycerate and dihydroxyacetone phosphate, a decrease in the liver [lactate]/[pyruvate], [glycerol 3-phosphate]/[dihydroxyacetone phosphate] and [D-3-hydroxybutyrate]/[acetoacetate] ratios and a decrease in liver [acetyl-CoA] (Fig. 3 and Table 1). Although there is no change in liver [ATP], there is a significant decrease in the [ATP]/[ADP] ratio, but of small magnitude (Table 1).

Discussion

In a previous study (Ferré *et al.*, 1978) the importance of fat in the maintenance of a high rate of gluconeogenesis was emphasized. Comparison of the pattern of metabolites from suckling and triacylglycerol-fed rats, expressed as percentage of that in the starved newborn rats (Fig. 2), indicates that triacylglycerol feeding mimics the effect of suckling on gluconeogenesis in the absence of any change in plasma glucagon (Ferré *et al.*, 1978), suggesting a

Fig. 3. Crossover plot showing the hepatic metabolite concentrations after injection of sodium pent-4-enoate in suckling newborn rats

Suckling rats (16h old) were injected subcutaneously with NaCl or sodium pent-4-enoate. Liver was removed 2.5h later and freeze-clamped. For further details see the Materials and Methods section. Values shown are the means $\pm$ S.E.M. for six to ten determinations. The concentration of each metabolite in the liver of suckling rats injected with NaCl is given in $\mu\text{mol/g}$ wet wt.: lactate, 1.18; pyruvate, 0.04; oxaloacetate (calculated), 0.008; malate, 0.465; phosphoenolpyruvate, 0.066; 2-phosphoglycerate, 0.02; 3-phosphoglycerate, 0.196; dihydroxyacetone phosphate, 0.022; glucose 6-phosphate, 0.140; glucose, 4.0. The gluconeogenic flux, given in μmol of glucose formed from lactate/h per 100g body wt., is taken from Pégrier *et al.* (1977). Control value is 11.1.

direct effect of fatty acid on hepatic gluconeogenesis. Inhibition of fatty acid oxidation with pent-4-enoate in suckling rats gives a pattern of liver metabolites similar to that observed during starvation (Fig. 3), despite a large increase in plasma glucagon (Pégrier *et al.*, 1977), providing direct evidence for the role of fatty acid oxidation in promoting gluconeogenesis.

Fatty acid oxidation and pyruvate carboxylation

The results presented in Figs. 1–3 suggest that fatty acid oxidation has a stimulatory effect on

pyruvate carboxylation, although calculation of hepatic oxaloacetate is not entirely satisfactory because of compartmentation of malate. Changes in malate, however, were large, and these are unlikely to be due to the small changes in mitochondrial redox state as indicated by the [D-3-hydroxybutyrate]/[acetoacetate] ratios. The apparent stimulation of pyruvate carboxylation by fatty acid oxidation could be due either to a direct effect on pyruvate carboxylase (EC 6.4.1.1), or to increased availability of pyruvate secondary to an inhibition of pyruvate dehydrogenase (EC 1.2.4.1), or to an activation of pyruvate transport across the mitochondrial membrane. In the present experiments [pyruvate] does not increase significantly with stimulation of gluconeogenesis (Fig. 2) and the administration of dichloroacetate, which activates pyruvate dehydrogenase (McAllister *et al.*, 1973), does not affect hepatic gluconeogenic flux in suckling newborn rats (Pégrier *et al.*, 1977). These facts would seem to rule out an effect of fatty acids on pyruvate dehydrogenase as being mainly responsible for the stimulation of gluconeogenesis. On the other hand, an enhanced pyruvate transport across the mitochondrial membrane, driven by increased acetoacetate output (Dennis *et al.*, 1978), cannot be ruled out. It has been shown in perfused liver that there is a good correlation between hepatic [acetyl-CoA], an obligatory effector of pyruvate carboxylase (Utter & Keech, 1963), and the rate of pyruvate carboxylation (Williamson *et al.*, 1968, 1969, 1970; Toews *et al.*, 1970). [Acetyl-CoA] was low in the starved newborn rats or those injected with pent-4-enoate and increased on feeding triacylglycerols or on suckling (Figs. 1–3).

Fatty acid oxidation and glyceraldehyde 3-phosphate dehydrogenase

The metabolite profiles (Figs. 1–3) suggest an interaction between fatty acid oxidation and the glyceraldehyde 3-phosphate dehydrogenase or 3-phosphoglycerate kinase reactions. However, it is unlikely that 3-phosphoglycerate kinase is the step involved, as there is no significant difference in the [ATP] or [ATP]/[ADP] ratio between starved and suckling or triacylglycerol-fed rats and only a decrease of small magnitude in the [ATP]/[ADP] ratio in the group injected with pent-4-enoate. On the contrary, there are large changes in the [lactate]/[pyruvate] and [glycerol 3-phosphate]/[dihydroxyacetone phosphate] ratios (Table 1), indicating a limited availability of reducing equivalents when fatty acid oxidation is impaired. Fatty acid oxidation could thus provide the reducing equivalents necessary for the reaction catalysed by glyceraldehyde phosphate dehydrogenase in the direction of gluconeogenesis. However, this conclusion is limited by the fact that it is based on indirect assumptions,

since the true reactants of glyceraldehyde phosphate dehydrogenase, namely glyceraldehyde 3-phosphate and 1,3-bisphosphoglycerate, are not measured owing to their very low concentration in the liver. The decreased [dihydroxyacetone phosphate] in the starved or pent-4-enoate-injected animals will in turn depress gluconeogenesis by decreasing the availability of substrate for subsequent reactions. The enolase (EC 4.2.1.11) and phosphoglyceromutase (EC 2.7.5.3) reactions appear to be near equilibrium, since the mass-action ratios of these two enzymes calculated from the [phosphoenolpyruvate]/[2-phosphoglycerate] and [2-phosphoglycerate]/[3-phosphoglycerate] ratios remain fairly constant in various experimental groups (results not shown) and close to the values given in the literature (Newsholme & Start, 1973). Therefore [phosphoenolpyruvate], [2-phosphoglycerate] and [3-phosphoglycerate] move in the same direction on perturbation of gluconeogenic flux. At first sight, it is somewhat surprising that [oxaloacetate] and [malate] are low, despite inhibition at the glyceraldehyde 3-phosphate dehydrogenase step, but this is probably due to the fact that phosphoenolpyruvate carboxykinase is not subject to marked product inhibition (Williamson *et al.*, 1969).

Conclusion

These experiments demonstrate that under physiological conditions fatty acids can increase the rate of gluconeogenesis in the newborn rat by stimulating the steps catalysed by pyruvate carboxylase and glyceraldehyde 3-phosphate dehydrogenase.

The main reason why the newborn rat is so sensitive to changes in the exogenous supply of fatty acids is that it lacks white adipose tissue at birth and thus, unlike the adult, it is an excellent model for studying interactions between fatty acid oxidation and gluconeogenesis.

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