Relationship between ketogenesis and gluconeogenesis in isolated hepatocytes from newborn rats

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In hepatocytes from 1-day-old rats, active gluconeogenesis occurs in parallel with active ketogenesis, although the carbon atoms of non-esterified fatty acids do not participate in glucose synthesis. Once a significant ketogenesis is established, a further increase does not enhance gluconeogenesis. Indeed, octanoate is more ketogenic than oleate, but stimulates gluconeogenesis to a similar extent.

In the starved adult rat, an active gluconeogenesis occurs in the liver concomitantly with an active oxidation of non-esterified fatty acids and a high rate of ketogenesis. Although previous studies performed *in vivo* have not demonstrated a clear-cut relationship between these two processes (Ruderman *et al.*, 1969), studies performed with isolated perfused rat liver have shown that the rate of gluconeogenesis was dependent on efficient fatty acid oxidation (Williamson *et al.*, 1969, 1970).

The neonatal period is a physiological situation in which gluconeogenesis and fatty acid oxidation are closely associated. Indeed, the newborn rat is fed with a high-fat low-carbohydrate diet, and it has to perform active gluconeogenesis to maintain normoglycaemia (Ferré *et al.*, 1977). Active fatty acid oxidation is also essential for the suckling newborn to sustain a high gluconeogenic flux (Pégorier *et al.*, 1977). Furthermore, in the starved newborn rat, which has very low concentrations of circulating non-esterified fatty acids and a low rate of hepatic fatty acid oxidation, hepatic gluconeogenesis is markedly decreased and hypoglycaemia develops (Girard *et al.*, 1973; Ferré *et al.*, 1978a). Thus the neonatal period is very convenient for the study of interrelationships between gluconeogenesis and fatty acid oxidation. In previous studies performed *in vivo*, it has been suggested that two steps in the gluconeogenic pathway [pyruvate carboxylase (EC 6.4.1.1) and glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12)] were controlled by oxidation of non-esterified fatty acids in the newborn-rat liver (Ferré *et al.*, 1979). Nevertheless, the possibility of direct participation of the carbon atoms of non-esterified fatty acids in the synthesis of glucose,

through the glyoxylate cycle (Jones, 1980) or through ω -oxidation (Wada & Usami, 1977), cannot be excluded.

Another particular aspect of lipid metabolism during the neonatal period in the rat has received little attention: the milk of the rat contains 40% medium-chain triacylglycerols (Smith *et al.*, 1968), and it has been shown that medium-chain fatty acids have a metabolic fate very different from that of long-chain fatty acids. Firstly, medium-chain fatty acids enter in the blood through the portal vein and reach the liver directly (Senior, 1968). Secondly, their entry into the mitochondria does not require the acylcarnitine transferase system, since they are activated directly inside the mitochondria. Thus it was interesting to compare in more quantitative terms the capacity of long- and medium-chain fatty acids to stimulate gluconeogenesis.

To study these problems, we have developed a technique for the isolation of newborn-rat hepatocytes and their subsequent use for metabolic studies.

Experimental

Animals

Female rats of the Wistar strains were used. Adult rats weighed between 200 and 250 g. The rats were fed *ad libitum* on breeding diet for rats (carbohydrate 65%, protein 24%, fat 11% of total energy). The starved rats had their food removed for 48 h. Two groups of newborns were used. In the first group, rats were born between 15:00 and 18:00 h on day 22 of gestation, and they were left for 16 h with the mother at animal-room temperature, 22°C. They are termed suckling newborns. In the second group, newborn rats were delivered by Caesarean section at

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21.5 days of gestation, and immediately transferred to and kept unfed for 16 h in an incubator (Heraeus, Hanau, West Germany) in which the temperature was 37°C and relative humidity 70%. They are termed unfed newborns.

Hepatocyte isolation

Adult rats were anaesthetized with sodium pentobarbital (30 mg/kg body wt.). Hepatocytes were prepared essentially by the method of Berry & Friend (1969) as modified by Krebs *et al.* (1974). Hepatocytes from newborn rats were prepared by the same method with the following modifications: livers from six newborns were perfused simultaneously to give a sufficient yield of cells; after anaesthesia of the animals (sodium pentobarbital, 1.5 mg/kg body wt.), a needle was inserted in the inferior vena cava under the kidney; the perfusion medium entered the liver by hepatic veins and left it by the portal vein, which was simply cut. The vena cava was then secured after its passage through the diaphragm. This ensured a satisfactory flow (1.5 ml/min) through the portal vein. Hepatocytes isolated by this procedure were contaminated with haematopoietic cells, still abundant in the liver 1 day after birth (Greengard *et al.*, 1972). However, haematopoietic cells were easily distinguishable from hepatocytes, owing to their 2–3-fold smaller diameter as seen by light microscopy. Thus, despite the usual contamination found (20% in number), haematopoietic cells represent only 2% of the total weight of the cells. The number and morphological viability of hepatocytes were determined with an haemocytometer by using a phase-contrast microscope.

Incubation

Hepatocytes were incubated in Krebs–Henseleit (1932) bicarbonate medium, pH 7.4, at 37°C for 60 min in a final volume of 4 ml. In preliminary experiments, it was found that glucose or ketone-body productions were linear with time during 60 min (results not shown). Incubations were performed without addition of substrates or in the presence of lactate (10 mM), oleate (0.1–2 mM) or octanoate (2 mM). In these experiments, both fatty acids were bound to defatted albumin (2%, w/v). In some experiments, L-carnitine (1 mM) was added in the incubation medium. The inhibitor of carnitine acyltransferase (EC 2.3.1.21), 2-tetradecylglycidic acid (MCN 3802), was prepared as described by Tutwiler & Delleigne (1979) and used at a final concentration of 20 μ M. Incubation was ended by adding 0.5 ml of 40% (v/v) HClO₄ to the incubation flask. Glucose, acetoacetate and D-3-hydroxybutyrate were determined in the neutralized HClO₄ extracts by enzymic methods (Slein, 1963; Williamson *et al.*, 1962). Results were expressed as means $\pm$

S.E.M., in μ mol/h per 10⁹ hepatocytes, and significant differences were tested by using the Wilcoxon (1947) rank order test.

Chemicals

All enzymes and coenzymes were obtained from Boehringer Corp. (Paris, France). Fatty-acid-free albumin, oleate and octanoate were purchased from Sigma (St. Louis, MO, U.S.A.). 2-Tetradecylglycidic acid was a gift from Dr. G. F. Tutwiler (MacNeil Laboratories, Philadelphia, PA, U.S.A.).

Results and discussion

Viability of the hepatocytes

Viability of the hepatocytes was initially tested by using Trypan Blue exclusion. Routine tests have indicated that at least 90% of the hepatocytes excluded Trypan Blue. However, this is a test of membrane integrity which does not fully correlate with the metabolic competence of the hepatocytes (Krebs *et al.*, 1979). Thus we have examined their capacity for producing glucose from lactate (Hems *et al.*, 1966). Indeed, the operation and regulation of the gluconeogenic pathway involves the integrated work of different cellular compartments, i.e. mitochondria, cytosol and microsomal fraction, and gluconeogenesis takes place to a very low extent in damaged cells. The rate of gluconeogenesis from lactate (10 mM) was 4.5-fold higher in hepatocytes from 16 h-old suckling rats ($223 \pm 17 \mu$ mol/h per 10⁹ hepatocytes, $n = 12$) than in hepatocytes from fed adult rats ($49 \pm 7 \mu$ mol/h per 10⁹ hepatocytes, $n = 6$) and it was similar to the rate measured in hepatocytes from 48 h-starved adult rats ($210 \pm 19 \mu$ mol/h per 10⁹ hepatocytes, $n = 8$). This is in agreement with studies performed with hepatocytes from 10–15-day-old suckling rats (Beaudry *et al.*, 1977; Sly & Walker, 1978). This indicates the high metabolic performance of the hepatocytes isolated from newborn rats and confirms studies *in vivo* showing that the suckling rat is dependent on active gluconeogenesis to maintain normoglycaemia (Ferré *et al.*, 1977).

Ketogenesis

It has been described that foetal hepatocytes oxidized ketone bodies (Shambaugh *et al.*, 1978), and this could lead to an underestimation of the rates of ketone-body production measured in our experiments. However, the rates of ketone-body utilization reported by Shambaugh *et al.* (1978) were negligible compared with the actual rates of ketone-body production.

In hepatocytes from 16 h-old suckling rats, the rate of ketogenesis from endogenous sources was high (Table 1). This suggests that the elevated

Table 1. *Ketone-body production in hepatocytes from 16 h-old unfed or suckling rats*

For details, see the Experimental section. Final concentrations of carnitine and 2-tetradecylglycidic acid were respectively 1 and 0.02 mM. Results are means $\pm$ S.E.M. for the numbers of experiments in parentheses. Production of ketone bodies is expressed as $\mu\text{mol/h}$ per 10^9 hepatocytes. Endogenous production is not subtracted. * $P < 0.01$ compared with endogenous ketone-body production in hepatocytes from unfed rats; † $P < 0.01$ compared with ketone-body production from oleate (1 or 2 mM) in hepatocytes from unfed rats.

Substrates added	Suckling	Unfed
None	163 $\pm$ 20* (16)	28 $\pm$ 5 (10)
None + 2-tetradecylglycidic acid	24 $\pm$ 3 (6)	—
Octanoate (2 mM) + 2-tetradecylglycidic acid	331 $\pm$ 4* (6)	—
Oleate (1 mM)	—	195 $\pm$ 12* (22)
Oleate (1 mM) + carnitine	—	275 $\pm$ 23*† (6)
Oleate (2 mM)	—	208 $\pm$ 19* (5)
Oleate (2 mM) + carnitine	—	267 $\pm$ 28*† (6)
Octanoate (2 mM)	—	430 $\pm$ 30*† (30)
Octanoate (2 mM) + carnitine	—	383 $\pm$ 32*† (6)

concentrations of blood ketone bodies measured in the suckling rat (Ferré *et al.*, 1978b) resulted from the high rate of hepatic ketogenesis rather than from a low rate of peripheral ketone-body utilization. In the hepatocytes from unfed newborn rats, the rate of ketogenesis from endogenous sources was very low (Table 1). However, when hepatocytes from unfed newborns were incubated in the presence of oleate, the rate of ketogenesis became similar to that observed in hepatocytes from suckling animals (Table 1).

The low rate of ketogenesis observed in hepatocytes from unfed animals was due to the absence of endogenous triacylglycerols and not to a decreased ketogenic capacity. This is not in agreement with the conclusion drawn by Higham *et al.* (1980), on the basis of the measurement of lower activities of acyl-CoA synthetase (EC 6.2.1.3) and hydroxymethylglutaryl-CoA synthase (EC 4.1.3.5) in the liver of unfed newborns as compared with suckling newborn rats. Thus the ketogenic capacity which progressively develops during the 16 h after birth (Ferré *et al.*, 1978b) is not induced by the high-fat diet or by the high concentrations of plasma non-esterified fatty acids, since the plasma concentration of non-esterified fatty acids remains low in unfed newborn rats, which nevertheless acquire normally the capacity to perform active ketogenesis (Girard *et al.*, 1973; Ferré *et al.*, 1978b). When hepatocytes from unfed animals are incubated in the presence of octanoate (2 mM), which provides the same amount of carbon atoms as 1 mM-oleate, the rate of ketogenesis is 2–3-fold higher than from oleate. As carnitine is lost during the preparation of isolated hepatocytes (Christiansen & Bremer, 1976) and as carnitine is required for the entry of oleate, but not of octanoate, into the mitochondria, we have investigated whether the lower rate of ketogenesis from oleate was not due to carnitine depletion of isolated hepatocytes. The addition of carnitine increased the rate of ketogenesis from oleate (1 mM,

+41%; 2 mM, +28%), but the rates of ketogenesis achieved were still 30–40% lower than the rate measured in the presence of octanoate (2 mM) (Table 1). Exogenous carnitine did not increase ketogenesis from octanoate (Table 1). This suggests that in the liver cells of the newborn rat the medium-chain fatty acids are more ketogenic than long-chain fatty acids. The oxidation of long-chain fatty acids is probably limited at the carnitine acyltransferase step, as in the adult rat.

Gluconeogenesis

In the hepatocytes from unfed newborn rats, in which ketogenesis is limited by lack of endogenous fatty acids (Table 1), the gluconeogenic rate from lactate ($64 \pm 5 \mu\text{mol/h}$ per 10^9 hepatocytes, $n = 12$) is markedly decreased compared with that in suckling animals ($223 \pm 17 \mu\text{mol/h}$ per 10^9 hepatocytes, $n = 12$). When active ketogenesis is restored by adding increasing concentrations of oleate in hepatocytes from unfed newborns, the gluconeogenic rate is increased 2.5-fold (Table 2). The maximal stimulation of gluconeogenesis from lactate is nearly achieved at 0.2 mM-oleate, which is also the concentration needed to increase ketogenesis significantly (Table 2). Although the rate of ketogenesis in the presence of octanoate (2 mM) is twice that observed with oleate (1 mM), the rate of gluconeogenesis is not further increased (Table 2). This suggests that once a sufficient rate of fatty acid oxidation has been achieved, gluconeogenesis is not sensitive to a further increase. This could explain why it has been difficult to demonstrate *in vivo* the effects of exogenous fatty acids on the rate of gluconeogenesis, since, apart from some pathological situations, endogenous fatty acid oxidation is never sufficiently diminished to observe any effect. In the absence of exogenous lactate, addition of oleate (1 mM) or octanoate (2 mM) to the hepatocytes from unfed newborn rats increased glucose production from $9 \pm 2 \mu\text{mol/h}$ per 10^9 hepato-

Table 2. *Glucose and ketone-body production in hepatocytes from 16 h-old unfed rats*

For details, see the Experimental section. Final concentrations of the substrates are: lactate 10 mM; oleate 0.1–1 mM; octanoate 2 mM. Results are means $\pm$ S.E.M. for the numbers of experiments in parentheses. Production of glucose is expressed as $\mu\text{mol/h}$ per 10^9 hepatocytes, and endogenous glucose production, 9 ± 2 ($n = 8$), is subtracted * $P < 0.01$ compared with glucose production from lactate in hepatocytes from unfed rats; † $P < 0.01$ compared with ketone-body production in absence of exogenous oleate.

Substrates	Glucose production	Ketone-body production
Lactate	64 ± 5 (12)	24 ± 6 (12)
Lactate + oleate (0.1 mM)	86 ± 5 (8)	34 ± 4 (8)
Lactate + oleate (0.2 mM)	$121 \pm 12^*$ (8)	$58 \pm 6^\dagger$ (8)
Lactate + oleate (0.4 mM)	$128 \pm 13^*$ (4)	$164 \pm 17^\dagger$ (4)
Lactate + oleate (0.6 mM)	$136 \pm 3^*$ (4)	$169 \pm 24^\dagger$ (4)
Lactate + oleate (1.0 mM)	$144 \pm 13^*$ (20)	$154 \pm 7^\dagger$ (20)
Lactate + octanoate	$164 \pm 10^*$ (6)	$345 \pm 10^\dagger$ (6)

cytes to respectively 14 ± 2 ($n = 8$) and 13 ± 2 ($n = 10$) ($P < 0.05$). In the presence of lactate (10 mM), the addition of the same amount of oleate or octanoate produced an increase in glucose production which averaged $100 \mu\text{mol/h}$ per 10^9 hepatocytes (Table 2). This indicates that the carbon atoms of long- or medium-chain fatty acids do not participate to a significant extent in the synthesis of glucose and that fatty acid oxidation increases the hepatic gluconeogenic rate essentially by stimulating specific steps of the pathway (Ferré *et al.*, 1979). The small increase in the gluconeogenic rate observed when fatty acids are added in the absence of exogenous substrate could be entirely explained by the stimulating effect of fatty acids on gluconeogenesis from endogenous precursors. When the oxidation of long-chain fatty acids is inhibited by 2-tetradecylglycidic acid ($20 \mu\text{M}$) in hepatocytes from suckling animals (Table 1), the rate of gluconeogenesis from lactate (10 mM) is decreased from $223 \pm 17 \mu\text{mol/h}$ per 10^9 hepatocytes ($n = 12$) to 109 ± 18 ($n = 8$) ($P < 0.01$). The further addition of octanoate (2 mM) restores both a high ketogenic rate (Table 1) and a normal rate of gluconeogenesis, $237 \pm 17 \mu\text{mol/h}$ per 10^9 hepatocytes ($n = 12$). This suggests that the decreased gluconeogenic rate was secondary to the decreased fatty acid oxidation and not to a toxic effect of the inhibitor used.

In conclusion, this work confirms and extends previous studies *in vivo* (Ferré *et al.*, 1978a) by showing that active fatty acid oxidation is necessary to sustain a high rate of gluconeogenesis, and that the carbon atoms of non-esterified fatty acids do not participate in glucose synthesis. In addition, it shows that the medium-chain fatty acids contained in the milk of the rat are potentially important for body fuel homeostasis: (1) medium-chain fatty acid oxidation in the liver allows a higher rate of ketone-body production than from long-chain fatty acids, and thus more ketone bodies are available for

peripheral tissue oxidative needs; (2) medium-chain fatty acids are as efficient as long-chain fatty acids in providing the necessary cofactors to sustain a high rate of gluconeogenesis. The preferential utilization of medium-chain fatty acids from the milk in the liver could allow the channelling of more long-chain fatty acids towards adipose tissues, which begin to fill as soon as 6 h after birth (Péquignot-Planche *et al.*, 1977; Cryer & Jones, 1978).

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