Development and regulation of ketogenesis in hepatocytes isolated from newborn rats

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The development of fatty acid metabolism was studied in isolated hepatocytes from newborn rats. Ketone-body production from oleate is increased 6-fold between 0 and 16 h after birth. This increase is related to an enhanced β -oxidation rather than to a channelling of acetyl-CoA from the tricarboxylic acid cycle to ketone-body synthesis. The increase in oleate oxidation is not related to a decreased esterification rate, as the latter is already low at birth and does not decrease further. At birth, lipogenic rate is 2–3-fold lower than in fed adult rats and it decreases to undetectable values in 16 h-old rats. A 90% inhibition of lipogenesis in hepatocytes of newborn rats (0 h) by glucagon and 5-(tetradecyloxy)-2-furoic acid does not lead to an increased oxidation of non-esterified fatty acids. This suggests that the inverse relationship between lipogenesis and ketogenesis in the starved newborn rat is not responsible for the switch-on of fatty acid oxidation at birth. Moreover, ketogenesis from octanoate, a medium-chain fatty acid the oxidation of which is independent of carnitine acyltransferase, follows the same developmental pattern at birth as that from oleate.

In the rat foetus, the main oxidizable substrate is glucose supplied by the mother via the placenta. Non-esterified fatty acids cannot readily cross the placental barrier (Koren & Shafrir, 1964), and the capacity of the liver to oxidize non-esterified fatty acids and to form ketone bodies is very low *in utero* (Drahota *et al.*, 1964; Augenfeld & Fritz, 1970). In contrast, after birth fat is the main energy substrate. Indeed, the newborn rat receives 69% of its energy intake as fat and only 6% as carbohydrate from milk. The concentration of blood ketone bodies increases from around 0.2 mM at birth to 2 mM 16–24 h later (Snell & Walker, 1973; Wapnir *et al.*, 1973; Foster & Bailey, 1976; Yeh & Zee, 1976; Ferré *et al.*, 1978a). However, the increase in ketogenesis does not seem to be simply regulated by the increase in plasma non-esterified fatty acids and hepatic carnitine concentrations observed after suckling (Robles-Valdes *et al.*, 1976; Ferré *et al.*, 1978a, 1981). It has been suggested that the sensitivity of carnitine acyltransferase I (EC 2.3.1.21) to malonyl-CoA, which decreases after birth in the rat, might be involved, together with a potential decrease in malonyl-CoA concentration, in the rapid switch-on of ketogenesis (Saggerson & Carpenter, 1982).

To elucidate the factors that bring about the

increase in the rate of ketogenesis during the first 24 h of life we have used hepatocytes isolated from starved newborn rats at 0, 6 and 16 h after birth. We have particularly studied whether a malonyl-CoA-mediated mechanism could explain the switch-on of this pathway after delivery.

Materials and methods

Animals

Female rats of the Wistar strain were used. Adult rats weighed between 200 and 250 g. The rats were fed *ad libitum* on laboratory chow UAR-103 (carbohydrate 65%, protein 24%, fat 11% of total energy). They were housed at 24°C with light from 06:00 to 20:00 h. The starved adult rats had their food removed for 48 h. Newborn rats were delivered by Caesarean section in the morning of day 22 of gestation and they were used either immediately or placed in an incubator (Heraeus, Hanau, West Germany) in which the temperature was 37°C and relative humidity 70%, and starved for 6 or 16 h.

Use of starved newborn rats rather than suckling rats allows quantitative estimation of oxidative capacity from measurements of [$1\text{-}^{14}\text{C}$]oleate. Indeed, the liver of starved newborn rats is devoid of endogenous triacylglycerols (Ferré *et al.*, 1978b),

whereas the liver of suckling newborn rats contains large amounts of triacylglycerols. However, *in vivo*, the developmental pattern of the capacity to synthesize ketone bodies is similar in both suckling and starved newborn rats given a triacylglycerol emulsion (Ferré *et al.*, 1978a).

Hepatocyte isolation

Adult and newborn rats were anaesthetized with Nembutal (30 and 15 mg/kg body wt. respectively). Hepatocytes from adult rats were prepared by the method of Berry & Friend (1969) as modified by Krebs *et al.* (1974). Hepatocytes from newborn rats were prepared and their viability was assessed by procedures described previously (Ferré *et al.*, 1981).

Ketogenesis

Hepatocytes (1×10^6 – 2×10^6) were incubated in oxygenated (O_2/CO_2 , 19:1) Krebs–Henseleit (1932) bicarbonate medium, pH 7.4, at 37°C for 60 min in a final volume of 4 ml, containing 0.4 mM-oleate or 2 mM-octanoate, 3% (w/v) albumin (defatted and dialysed) and 1 mM-carnitine, as it has been shown that carnitine is lost during the isolation of hepatocytes (Christiansen & Bremer, 1976).

Metabolism of [$1\text{-}^{14}C$]oleate

For the studies of oleate metabolism, $1 \mu Ci$ of [$1\text{-}^{14}C$]oleate was added to 4 ml of medium containing 0.4 mM-oleate, 3% defatted albumin and 1 mM-carnitine. For the oxidation studies the flasks were sealed with rubber caps containing plastic centre wells; incubation was ended by adding 0.5 ml of 40% (w/w) $HClO_4$ through the rubber cap. Then 0.4 ml of 1 M-Hyamine hydroxide was injected into the centre wells. The flasks were shaken for a further 45 min at room temperature to trap $^{14}CO_2$, after which the centre wells were placed in plastic vials and assayed for radioactivity in a liquid-scintillation counter. The flask contents were transferred to tubes, and after centrifugation (700 g for 10 min), the supernatant fraction was neutralized with 5 M-KOH. The neutralized extracts were analysed for acetoacetate and D-3-hydroxybutyrate by enzymic procedures and for the ^{14}C radioactivity in total acid-soluble products.

For the esterification studies, incubation was ended by centrifugation for 35 s at 5000 g to separate cells from the medium. Chloroform/methanol (2:1, v/v; 20 vol.) was added to the cell pellet and the lipids were extracted as described by Folch *et al.* (1957). The radioactivity in the chloroform phase was used as a measure of the [$1\text{-}^{14}C$]oleate converted into esterified fats; indeed, it has been shown in similar experimental conditions that esterified fats are not released in appreciable amounts into the incubation medium (Whitelaw & Williamson, 1977).

Owing to the fact that in the newborn the number of hepatocytes isolated is low, (1) esterification was measured on a batch of cells different from that used in oxidation studies and (2) [$1\text{-}^{14}C$]oleate uptake could not be reliably measured by standard techniques; thus [$1\text{-}^{14}C$]oleate uptake was calculated as the sum of [$1\text{-}^{14}C$]oleate oxidation and esterification.

Lipogenesis

Hepatocytes (3×10^6 – 5×10^6) were incubated in Krebs–Henseleit bicarbonate medium, pH 7.4, gassed with O_2/CO_2 (19:1) at 37°C in a final volume of 2 ml containing 0.4 mM-sodium oleate, 3% albumin (defatted and dialysed), 10 mM-lactate, 1 mM-pyruvate and 1 mM-carnitine. After incubation for 30 min, 250 μCi of 3H_2O was added. After a further 30 min, incubation was ended by centrifugation at 5000 g for 35 s. Labelled fatty acids were extracted from the cell pellet as described by Stansbie *et al.* (1976).

Glucagon and 5-(tetradecyloxy)-2-furoic acid

In some experiments, glucagon (1 $\mu g/ml$) and 5-(tetradecyloxy)-2-furoic acid (0.1 mM) were added at the beginning of the incubation. 5-(Tetradecyloxy)-2-furoic acid was dissolved in acetone and the solution added to the empty incubation flask. It was then evaporated to dryness under a stream of nitrogen and the incubation medium was added.

Measurement of radioactivity

This was done by adding 10 ml of Unisolve (Koch–Light Laboratories) to samples and counting in a liquid-scintillation counter (Kontron, MR 300, France).

Metabolite determinations

Acetoacetate and D-3-hydroxybutyrate were determined enzymically as described by Williamson *et al.* (1962).

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.). Pig glucagon was obtained from Novo (Copenhagen, Denmark). 5-(Tetradecyloxy)-2-furoic acid was a gift from Dr. A. Richardson (Merrell National Laboratories, Cincinnati, OH, U.S.A.). [$1\text{-}^{14}C$]Oleate and 3H_2O were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Calculations

Rates are expressed as means $\pm$ S.E.M., per g dry wt. Despite a contamination of hepatocytes by haematopoietic cells, the latter represented only 1%

of the total weight of the cells. As fatty acid oxidation has never been reported to occur in haematopoietic cells, the measurements are considered to represent essentially the metabolism of fatty acids in the hepatocyte population.

Statistics

Statistically significant differences were tested by Student's *t* test. Results are presented as means $\pm$ S.E.M. for the numbers of experiments. Flasks were run in duplicate for each experimental condition.

Results and discussion

Ketogenesis from oleate in isolated hepatocytes from newborn and adult rats

Ketone-body production from 0.4 mM-oleate increased 6-fold between 0 and 16 h after birth. The rate of ketogenesis in 16 h-old newborn rats was higher than that in 48 h-starved adult rats (Fig. 1) and was similar to that in 16 h-suckling newborn rats (Ferré *et al.*, 1981).

Thus it is clear that the increasing ketonaemia observed *in vivo* in the newborn rat (Snell & Walker, 1973; Wapnir *et al.*, 1973; Foster & Bailey, 1976; Yeh & Zee, 1976; Ferré *et al.*, 1978a) resulted from an increased hepatic capacity for ketone-body synthesis. As suggested by studies *in vivo* (Ferré *et al.*, 1978a), availability of non-esterified fatty acids is not the only factor responsible for the increase in ketone-body production observed at birth: although

hepatocytes of newborn rats were incubated with 0.4 mM-oleate at the three stages studied, ketogenesis was 6-fold higher 16 h after birth than at delivery.

Since milk contains large amounts of carnitine, liver carnitine concentration increases after the first suckling period (Robles-Valdes *et al.*, 1976; Ferré *et al.*, 1978a), and it was suggested that carnitine was an important factor in the triggering of ketogenesis at birth. Several arguments from previous studies *in vivo*, as well as from the present work, suggest that this hypothesis has to be reconsidered. Firstly, carnitine concentration increases in liver 2 h after birth, whereas hyperketonaemia is reached 12 h after birth (Ferré *et al.*, 1978a). Secondly, rats fed with triacylglycerol emulsion alone or with triacylglycerol emulsion supplemented with carnitine reach the same degree of hyperketonaemia (Ferré *et al.*, 1978a). Thirdly, although hepatocytes from newborn rats were incubated with 1 mM-carnitine at the three stages studied, ketogenesis was 6-fold higher at 16 h after birth than at delivery.

Metabolism of [1-¹⁴C]oleate in isolated hepatocytes from newborn and adult rats

The developmental pattern of production of labelled acid-soluble products from [1-¹⁴C]oleate (Table 1) was similar to that of ketone-body production (Fig. 1), assuming that 1 μ mol of oleate generates 4.5 μ mol of ketone bodies. This quantitative comparison is possible because there is no detectable production of ketone bodies in absence of added fatty acid (results not shown). This suggests

Fig. 1. Ketogenesis from 0.4 mM-oleate and 2 mM-octanoate in isolated hepatocytes from newborn and adult rats. Isolated hepatocytes from 0, 6 h- and 16 h-old starved newborn rats and fed (F) or 48 h-starved (S) adult rats were incubated with 0.4 mM-oleate or 2 mM-octanoate. The results are expressed in μ mol of ketone bodies formed/h per g dry wt. and are the means $\pm$ S.E.M. for four or five experiments: **P* < 0.01 compared with starved adults.

Table 1. *Uptake and metabolism of 0.4 mM-[1-¹⁴C]oleate in isolated hepatocytes from newborn and adult rats*

Isolated hepatocytes from 0, 6 h- and 16 h-old starved newborns and fed or 48 h-starved adult rats were incubated with 0.4 mM-[1-¹⁴C]oleate. Total oxidation is taken as the sum of acid-soluble products and CO₂. Esterification and oxidation were measured in different batches of cells. Uptake is taken as the sum of esterification and oxidation values. Results are expressed in $\mu\text{mol/h}$ per g dry wt. and are means $\pm$ S.E.M. for four or five experiments.

Type of rat	Acid-soluble products	CO ₂	Total oxidation	Esterification	Uptake
Newborn, 0 h	22 $\pm$ 1	4.0 $\pm$ 0.3	26 $\pm$ 2	24 $\pm$ 1	50
Newborn, 6 h	52 $\pm$ 3	6.0 $\pm$ 0.5	58 $\pm$ 3	28 $\pm$ 3	86
Newborn, 16 h	131 $\pm$ 10	20 $\pm$ 1	151 $\pm$ 10	16 $\pm$ 1	167
Adult, fed	24 $\pm$ 2	11 $\pm$ 1	35 $\pm$ 2	—	—
Adult, starved	99 $\pm$ 13	8 $\pm$ 1	107 $\pm$ 13	—	—

that acid-soluble products are essentially ketone bodies when the cells are incubated in the absence of lactate.

¹⁴CO₂ production from [1-¹⁴C]oleate increased 5-fold between 0 and 16 h after birth (Table 1). Thus the increased ketogenic capacity after birth is linked to an increased fatty acid oxidation and not to a redirection of acetyl-CoA from the tricarboxylic acid cycle to ketone-body synthesis: (1) the increase in ketone-body production can be compared in quantitative terms with the increase in oleate oxidation (Fig. 1 and Table 1); (2) production of ¹⁴CO₂ from [1-¹⁴C]oleate does not decrease, but increases after birth (Table 1); (3) CO₂ production from fatty acids never exceeds 15% of total fatty acid oxidation. At birth the rates of esterification and oxidation were nearly similar (Table 1), but whereas oxidation rate was increased 5-fold in 16 h-old newborns, esterification was decreased by only 33% (Table 1). This suggests that the increased [1-¹⁴C]oleate oxidation does not result from a decreased capacity of esterification. [1-¹⁴C]Oleate uptake estimated as the sum of both esterification and oxidation is increased 3-fold between 0 and 16 h after birth, but this increase can be quantitatively accounted for by the increased oxidation. In the adult rat, during the fed-starved transition, the increase in β -oxidation is attended by a similar decrease in fatty acid esterification leading to an unchanged hepatic fatty acid uptake (Mayes & Felts, 1967), a situation quite different from the present one. The rise in oleate uptake in the hepatocytes from newborn rats may suggest the existence of a limited intracellular transport capacity for fatty acids or a low acyl-CoA synthetase activity, which would increase rapidly thereafter, or a limited capacity for esterification. The present study does not allow us to discriminate between these hypotheses. However, palmitoyl-CoA synthetase activity does not increase between 0 and 12 h after birth in the starved rat (Bailey, 1981), suggesting that this enzyme is not the factor triggering the increase in fatty acid oxidation observed in the present study.

Rate of lipogenesis in isolated hepatocytes from newborn and adult rats

In the adult rat, it has been reported by McGarry *et al.* (1977, 1978a) that malonyl-CoA, a key intermediate in the pathway of lipogenesis, inhibits fatty acid oxidation by decreasing the activity of carnitine acyltransferase I (EC 2.3.1.21). As malonyl-CoA concentrations are directly related to the lipogenic rate (McGarry *et al.*, 1978b; McGarry & Foster, 1979), this implies that there is an inverse relationship between lipogenesis and ketogenesis. Moreover, during the fed-starved transition in the adult rat, there is a decreased sensitivity of carnitine acyltransferase I to malonyl-CoA, which might contribute to the increase in fatty acid oxidation (Cook *et al.*, 1980; Ontko & Johns, 1980; Bremer, 1981; Saggerson & Carpenter, 1981). In order to know whether such a mechanism could account for the switch-on of hepatic ketogenesis after birth in the rat, we have measured the rate of lipogenesis from ³H₂O in the presence of lipogenic precursor (10 mM-lactate) and of 0.4 mM-oleate and carnitine (1 mM). The rate of lipogenesis is 60% lower in isolated hepatocytes from newborn rat than in hepatocytes from fed adult rats (Table 2), thus confirming previous studies *in vivo* (Lorenzo *et al.*, 1981; Pillay & Bailey, 1982). The rate of lipogenesis decreases to undetectable values in 16 h-old starved newborns (Table 2). The inverse relationship between lipogenesis and fatty acid oxidation after birth in the starved newborns, coupled to the fact that the sensitivity of hepatic carnitine acyltransferase I to malonyl-CoA decreases between 0 and 24 h after birth (Saggerson & Carpenter, 1982), may suggest that the switch-on of hepatic ketogenesis after birth is mediated by malonyl-CoA. Owing to the low amount of hepatocytes available in newborn rats, a direct measurement of [malonyl-CoA] in neonatal-rat hepatocytes was not feasible. Thus we have tested the above hypothesis in an indirect way. The addition of glucagon to incubated hepatocytes from fed adult rats has been shown to decrease

Table 2. *Lipogenesis and [1-¹⁴C]oleate oxidation in hepatocytes from newborn and fed adult rats in the presence of 10 mM-lactate*

Isolated hepatocytes of 0, 6h- and 16h-old starved newborns and fed adult rats were incubated with 0.4 mM-[1-¹⁴C]oleate, 10 mM-lactate and 1 mM-carnitine. Oleate oxidation is expressed as $\mu\text{mol/h}$ per g dry wt., and lipogenesis as μmol of ³H₂O incorporated into lipids/30 min per g dry wt. These two rates were measured on different batches of cells. Glucagon (1 $\mu\text{g/ml}$) and 5-(tetradecyloxy)-2-furoic acid (0.1 mM) (inhibitors) were added as indicated. Results are the means $\pm$ S.E.M. for four or five experiments. For other experimental details, see the Materials and methods section.

Type of rat	In- hibitors	Acid- soluble products	CO ₂	Lipogenesis
Newborn, 0h	—	14.2 $\pm$ 0.1	2.6 $\pm$ 0.1	6.0 $\pm$ 1.3
	+	12.3 $\pm$ 0.4	2.4 $\pm$ 0.1	0.6 $\pm$ 0.1
Newborn, 6h	—	54 $\pm$ 6	11 $\pm$ 1	1.5 $\pm$ 0.2
Newborn, 16h	—	138 $\pm$ 5	36 $\pm$ 3	Undetectable
Adult, fed	—	10 $\pm$ 1	9 $\pm$ 1	14.9 $\pm$ 0.7

markedly the rate of lipogenesis and [malonyl-CoA] and simultaneously to increase fatty acid oxidation and ketogenesis (McGarry *et al.*, 1978b). The addition of glucagon into incubated hepatocytes from newborn rats (0h) decreased the rate of lipogenesis by 50% (5.6 ± 0.6 to $2.7 \pm 0.6 \mu\text{mol}$ of ³H₂O incorporated/30 min per g dry wt., $n=6$), but did not increase fatty acid oxidation or ketogenesis from 0.4 mM-oleate (results not shown). However, as hepatic carnitine acyltransferase I is very sensitive to the inhibition by malonyl-CoA in the newborn (0h) (Saggerson & Carpenter, 1982), glucagon might have lowered [malonyl-CoA] insufficiently in newborn-rat hepatocytes to suppress fatty acid oxidation. We have then incubated the hepatocytes from newborn rats (0h) with glucagon and 5-(tetradecyloxy)-2-furoic acid, which in the adult rat is a potent inhibitor of lipogenesis at the level of mitochondrial citrate transport and acetyl-CoA carboxylase (Panek *et al.*, 1977; Benito & Williamson, 1978; McCune & Harris, 1979; McGarry & Foster, 1979). In the presence of glucagon and 5-(tetradecyloxy)-2-furoic acid, the rate of lipogenesis is suppressed by 90% in hepatocytes from newborn rats (0h) (Table 2), but there is no increase in oleate oxidation and ketogenesis. This suggests that there is no reciprocal relationship between lipogenesis and ketogenesis, which might explain the rapid switch-on of fatty acid oxidation at birth in the rat. In the adult rat, a linear relationship between lipogenesis and [malonyl-CoA] has been documented (McGarry *et al.*, 1978b; McGarry & Foster, 1979). In the newborn, this relation might not hold

because of potential alterations in the relative activities of acetyl-CoA carboxylase and fatty acid synthetase. In the present experiments, as the inhibition of lipogenesis by glucagon and 5-(tetradecyloxy)-2-furoic acid occurs before the step catalysed by fatty acid synthetase (Panek *et al.*, 1977; McGarry *et al.*, 1978b; McCune & Harris, 1979), it is likely that malonyl-CoA in newborn-rat hepatocytes has been decreased in parallel with the fall in the rate of lipogenesis; thus this suggests that malonyl-CoA is not the principal factor responsible for the switch-on of hepatic fatty acid oxidation in the newborn rat.

Ketogenesis from octanoate in isolated hepatocytes from newborn and adult rats

In the newborn rat, octanoate oxidation seems to be independent from the step catalysed by carnitine acyltransferase. Indeed, octanoate oxidation is not suppressed by 2-tetradecylglycidic acid (Ferré *et al.*, 1981; Turlan *et al.*, 1983), a potent inhibitor of carnitine acyltransferase (Tutwiler & Ryzlak, 1980), and is independent of carnitine (Ferré *et al.*, 1981).

Ketone-body production from 2 mM-octanoate increases 6-fold between 0 and 16h after birth in hepatocytes from starved newborn rats and is higher at this time than in 48h-starved adult rats (Fig. 1). Thus the changes in the rate of ketogenesis from octanoate presumably indicate alterations in metabolic regulation at sites including octanoyl-CoA formation, β -oxidation and the hydroxymethylglutaryl-CoA pathway, although the increase in the maximal activity of the last-mentioned after birth is small in comparison with the dramatic increase in ketogenesis (Ferré *et al.*, 1978a).

Conclusion

There is a large increase in hepatic fatty acid oxidation and ketogenesis from both oleate and octanoate during the 16h after birth. In contrast with the adult liver, in which ketogenesis is mainly regulated at the carnitine acyltransferase step, the switch-on of ketogenesis at birth seems to involve one or several other metabolic steps.

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