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Induction of ketogenesis and fatty acid oxidation by glucagon and cyclic AMP in cultured hepatocytes from rabbit fetuses

Evidence for a decreased sensitivity of carnitine palmitoyltransferase I to malonyl-CoA inhibition after glucagon or cyclic AMP treatment

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The effects of pancreatic hormones and cyclic AMP on the induction of ketogenesis and long-chain fatty acid oxidation were studied in primary cultures of hepatocytes from fetal and newborn rabbits. Hepatocytes were cultivated during 4 days in the presence of glucagon (10^{-6} M), forskolin (2×10^{-5} M), dibutyryl cyclic AMP (10^{-4} M), 8-bromo cyclic AMP (10^{-4} M) or insulin (10^{-7} M). Ketogenesis and fatty acid metabolism were measured using [1-^{14}C]oleate (0.5 mM). In hepatocytes from fetuses at term, the rate of ketogenesis remained very low during the 4 days of culture. In hepatocytes from 24-h-old newborn, the rate of ketogenesis was high during the first 48 h of culture and then rapidly decreased to reach a low value similar to that measured in cultured hepatocytes from term fetuses. A 48 h exposure to glucagon, forskolin or cyclic AMP derivatives is necessary to induce ketone body production in cultured fetal hepatocytes at a rate similar to that found in cultured hepatocytes from newborn rabbits. In fetal liver cells, the induction of ketogenesis by glucagon or cyclic AMP results from changes in the partitioning of long-chain fatty acid from esterification towards oxidation. Indeed, glucagon, forskolin and cyclic AMP enhance oleate oxidation (basal, 12.7 ± 1.6; glucagon, 50.0 ± 5.5; forskolin, 70.6 ± 5.4; cyclic AMP, 77.5 ± 3.4% of oleate metabolized) at the expense of oleate esterification. In cultured fetal hepatocytes, the rate of fatty acid oxidation in the presence of cyclic AMP is similar to the rate of oleate oxidation present at the time of plating (85.1 ± 2.6% of oleate metabolized) in newborn rabbit hepatocytes. In hepatocytes from term fetuses, the presence of insulin antagonizes in a dose-dependent fashion the glucagon-induced oleate oxidation. Neither glucagon nor cyclic AMP affect the activity of carnitine palmitoyltransferase I (CPT I). The malonyl-CoA concentration inducing 50% inhibition of CPT I (IC_{50}) is 14-fold higher in mitochondria isolated from cultured newborn hepatocytes (0.95 μM) compared with fetal hepatocytes (0.07 μM), indicating that the sensitivity of CPT I decreases markedly in the first 24 h after birth. The addition of glucagon or cyclic AMP into cultured fetal hepatocytes decreased by 80% and 90% respectively the sensitivity of CPT I to malonyl-CoA inhibition. In the presence of cyclic AMP, the sensitivity of CPT I to malonyl-CoA inhibition in cultured fetal hepatocytes is very similar to that measured in cultured hepatocytes from 24-h-old newborns.

INTRODUCTION

In the newborn rat or rabbit, a physiological hyperketonaemia develops during the first day after birth as the result of an increased capacity for hepatic long-chain fatty acid oxidation and ketogenesis [1–4]. This postnatal emergence of hepatic fatty acid oxidation seems partly regulated by a decrease in the sensitivity of carnitine palmitoyltransferase I (CPT I) to inhibition by malonyl-CoA [2,5].

It has been shown recently that when the plasma insulin/glucagon molar ratio was decreased in the rabbit fetus by prolonging the gestation with progesterone, the rate of fatty acid oxidation was enhanced in hepatocytes from post-mature rabbit fetuses as the result of a 75% fall in the sensitivity of CPT I to malonyl-CoA inhibition [6]. This suggests that the increase in plasma glucagon and the fall in plasma insulin, which occur spontaneously at birth in the newborn of different species including the rabbit [7,8], could be involved in the postnatal development of hepatic fatty acid oxidation and ketogenesis. However, no direct evidence for such a hormonal control is available in newborn mammals.

In the adult rat, several studies have shown that glucagon and cyclic AMP both increase the capacity for hepatic fatty acid oxidation and ketogenesis (for reviews, see [9–11]). However, the maximal ketogenic rates produced by glucagon in the liver cells from fed rats remain 30–75% lower than the rates of ketogenesis measured in hepatocytes from fasting or diabetic animals [9,12]. Moreover, it was shown using permeabilized hepatocytes from adult rats that short-term exposure to glucagon failed to affect the activity and sensitivity of CPT I to malonyl-CoA inhibition [13]. In contrast, insulin antagonizes the ketogenic effects of glucagon in isolated [12] or cultured [14] hepatocytes from fed rats, whereas insulin has marginal effects [15,16] or no effect [12,17] on basal fatty acid oxidation when present as the

Abbreviations used: CPT, carnitine palmitoyltransferase; MEM, minimal essential Hanks medium, PBS, phosphate-buffered saline.

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only hormone. However, all of these regulatory processes have been described in mature adult liver cells, and it was questionable whether such a hormonal control would also occur in fetal hepatocytes which have never expressed these metabolic functions.

The aim of the present study was to test, using cultured fetal and neonatal hepatocytes, whether the decrease in the plasma insulin/glucagon molar ratio that occurs at birth in the rabbit [7] could be responsible for the development of fatty acid oxidation, ketogenesis and decreased sensitivity of CPT I to malonyl-CoA inhibition in the rabbit liver [2].

MATERIALS AND METHODS

Animals

Rabbit does from the New Zealand White strain were fed *ad libitum* on a pelleted laboratory chow (% of energy: 33% protein, 55% carbohydrate, 12% fat) and had free access to water. Term in this strain is 32 days post-coitum. Fetuses were delivered by caesarean section in the morning of day 32 of gestation (term fetus). Hepatocytes were isolated from livers within 10 min of delivery (term fetus) or 24 h after birth in newborns maintained unfed at 36 °C in an humidified incubator (70% relative humidity).

Hepatocyte monolayer cultures

Hepatocytes from three to eight animals were prepared simultaneously by the reverse perfusion technique described previously [18], except that the perfusion medium was Hepes buffer (pH 7.6) containing 20 mM-glucose. The tissue-dissociating solution was Hepes-buffered medium containing 0.025% collagenase and 5 mM-$CaCl_2$. Cell viability, estimated by Trypan Blue exclusion, was always greater than 85%.

The isolated hepatocytes were suspended in 3 ml of arginine-free Minimal Essential Hanks Medium (MEM) to avoid contamination by fibroblast-like cells [19]. The medium was supplemented with penicillin (10 units/ml), streptomycin (100 μg/ml), kanamycin (50 μg/ml), amphotericin B (2.5 μg/ml), bovine serum albumin (1 mg/ml), ornithine (0.4 mM), dexamethasone (10^{-7} M) and fetal calf serum (10%, v/v). The hepatocyte suspension was plated either in 25 cm^2 culture flasks (2×10^6 cells/flask) or in 75 cm^2 plastic Petri dishes (10^7 cells/dish). A period of 4 h was allowed for cell attachment. Then, the non-adhering haematopoietic cells were removed and the medium was replaced by arginine- and serum-free MEM containing carnitine (1 mM) and various concentrations of glucagon, insulin, forskolin or cyclic AMP derivatives, in the absence or in the presence of theophylline (10^{-3} M). Duplicate flasks or dishes were used for all experimental conditions. The cultures were maintained at 37 °C in an incubator equilibrated with CO_2/air (1:19). The medium was changed every 24 h and the experiments were performed 24, 48, 72 or 96 h after the beginning of the culture.

Isolation of mitochondria from cultured hepatocytes

After 48 h of culture, hepatocytes from ten Petri dishes (75 cm^2) were washed twice with ice-cold phosphate-buffered saline (PBS; 137 mM-NaCl/2.7 mM-KCl/21.8 mM-Na_2MPO_4/1.4 mM-NaH_2PO_4, pH 7.6) and were scraped off with a rubber policeman. After centrifugation (2 min, 1500 *g*) the cell pellet was homogenized in 2 ml of HEMS medium (10 mM-Hepes/210 mM-mannitol/60 mM-sucrose/0.1 mM-EDTA, pH 7.4). Mitochondria were isolated on a discontinuous density gradient of iso-osmotic Percoll ($d = 1.044$, 1.062 and 1.095) [20]. The 2 ml homogenate was layered on the gradient and centrifuged at 46000 *g* for 45 s in a Sorvall (RC5C) centrifuge. The mitochondrial fraction was collected, washed with HEMS buffer and resuspended to a final protein concentration of 3 mg/ml.

Measurement of fatty acid oxidation, fatty acid esterification and ketogenesis

The rate of ketogenesis was measured daily in the absence of exogenous substrate (endogenous ketogenesis) or after addition of sodium oleate (0.5 mM). Long-chain fatty acid oxidation and esterification were determined 24 h after the addition into the culture medium of [1-^{14}C]oleate (0.5 mM) bound to defatted albumin (fraction V, fatty-acid free). After 24 h of incubation, the culture medium was transferred into 25 ml conical flasks, which were sealed with rubber caps and which contained plastic centre wells. Then, $HClO_4$ (4% final concn.) was added through the rubber cap and $^{14}CO_2$ production, acid-soluble products and unlabelled ketone bodies were determined as previously described [1]. Preliminary experiments have shown that under neutral pH conditions, most of the $^{14}CO_2$ remains in the culture medium, since less than 10% of total $^{14}CO_2$ production was lost into the atmosphere. Then the hepatocyte monolayers were rapidly rinsed twice with ice-cold PBS (pH 7.6). The cells were scraped off with a rubber policeman and homogenized in 4 ml of cold PBS for protein and triacylglycerol determinations. The cell lipids were extracted with chloroform/methanol (2:1, v/v) and labelled triacylglycerol from chloroform/methanol extracts was separated by t.l.c. as described previously [1]. In some experiments, [^{14}C]triacylglycerols were measured in the culture medium according to the method described above.

Determination of the activity and sensitivity of CPT I to malonyl-CoA inhibition

The method used was that described previously [2]. The palmitoyl-CoA concentration was 80 μM and the malonyl-CoA concentration varied between 0.01 and 150 μM. In all experiments ($n = 20$), the formation of palmitoylcarnitine was almost completely suppressed (89 ± 2%) by the highest malonyl-CoA concentration (150 μM), suggesting that good membrane integrity existed in the mitochondrial preparation, and that only CPT I activity was measured without any significant contribution of CPT II.

Analytical methods

Acetoacetate, β-hydroxybutyrate and triacylglycerol were determined using enzymic methods [21]. Proteins were determined by the method of Bradford [22] with ovalbumin as standard. For the determination of cyclic AMP concentration, the cells from duplicate dishes (75 cm^2) were pooled and homogenized in 5% trichloroacetic acid (4% w/v). An aliquot of the trichloroacetic acid supernatant was rinsed with water-saturated diethyl ether and then freeze-dried. The freeze-dried extracts were reconstituted with 50 mM-Tris/4 mM-EDTA buffer (pH 7.4). Cyclic AMP was assayed by radioimmunoassay [23].

Chemicals

All enzymes, substrates and cofactors were obtained from Boehringer Corp. (Meylan, France). Fatty acid-free albumin, oleate, L-carnitine, palmitoyl-CoA, antibiotics, dibutyryl cyclic AMP, 8-bromo cyclic AMP, forskolin and theophylline were purchased from Sigma (St. Louis, MO, U.S.A.). [1-^{14}C]Oleate, [^{3}H]carnitine and the cyclic [^{3}H] AMP assay kit were from Amersham International (Amersham, Bucks., U.K.). Amphotericin B, fetal calf serum and arginine-free MEM were obtained from Eurobio (Paris, France). Dexamethasone was from Merck Sharp & Dohme (Paris, France) and pig glucagon and human insulin were from Novo (Paris, France).

Statistics

Results are expressed as means ± S.E.M. Statistical analyses were performed using Student's unpaired *t* test.

RESULTS AND DISCUSSION

Carnitine (1 mM) was systematically added after cell attachment to avoid a limitation in the rate of fatty acid oxidation due to a loss of carnitine during the preparation of isolated hepatocytes [24] and/or the absence of its precursor (butyrobetaine) in the culture medium.

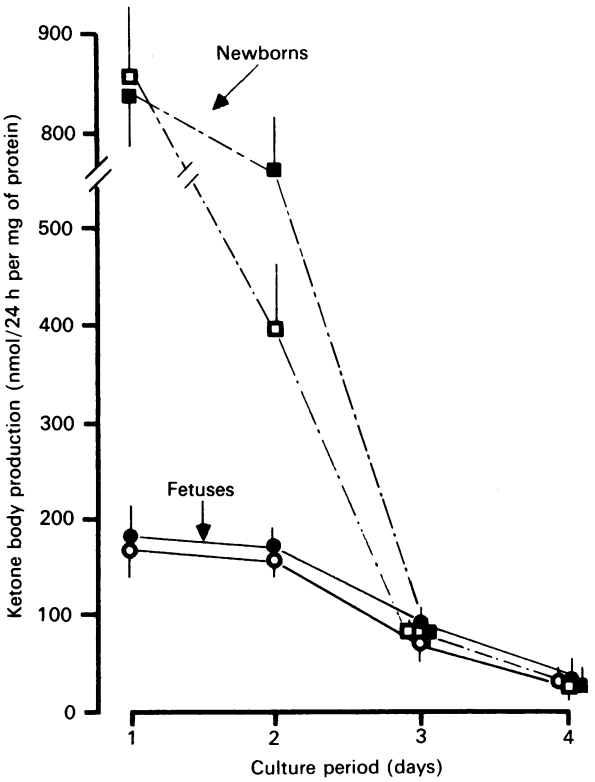

Fig. 1. Time course of ketone body production in cultured hepatocytes from term fetuses or from 24-h-old newborns

After cell attachment, hepatocytes were incubated in a serum-free medium for the period of time indicated, either in the absence (endogenous, ○, □) or in the presence for 24 h of 0.5 mM-oleate (●, ■). The rate of ketogenesis was determined every 24 h and expressed as nmol/24 h per mg of cell protein. Values are means ± S.E.M. of duplicate flasks from four to six different cultures.

Time course of ketone body production

In hepatocytes from term fetuses, the low rate of ketogenesis during the first 4 days of culture (Fig. 1) does not result from a deficiency in fatty acid availability, since the addition of exogenous oleate does not affect ketone body production (Fig. 1). In contrast, the high rates of endogenous ketogenesis in hepatocytes from newborn animals decrease by 50% on the second day of culture (Fig. 1). This is probably due to the 50% fall in triacylglycerol concentration between the first and second days of culture (from 0.20 ± 0.03 to 0.11 ± 0.03 mg/mg of cell protein; $n = 6$; $P < 0.01$), since the addition of 0.5 mM-oleate restored a high rate of ketogenesis (Fig. 1). On the third and fourth days of culture, the rate of ketone body production in newborn hepatocytes rapidly approached the low value found in cultured fetal hepatocytes, even in the presence of exogenous oleate (Fig. 1).

Effects of glucagon, cyclic AMP and various treatments on the rate of ketogenesis in cultured hepatocytes from 24-h-old newborns

It has been shown recently that specific liver function or enzyme activities can be maintained for up to 3 weeks when hepatocytes are cultured in the presence of an appropriate hormonal mixture (glucagon, insulin and dexamethasone) [25]. Although the culture conditions used in the present work were different (composition of the medium, absence of extracellular matrix, etc.), the capacity to perform efficient ketogenesis was lost after 2 days in culture despite the presence of dexamethasone, glucagon or cyclic AMP (Fig. 2). Similarly, the presence

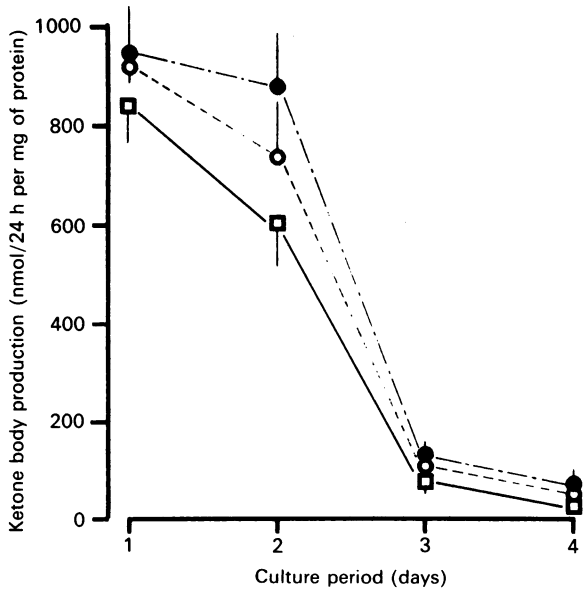

Fig. 2. Effect of glucagon or cyclic AMP on ketone body production in cultured hepatocytes from 24-h-old newborns

After cell attachment, the culture medium was removed every 24 h and replaced by a medium containing 0.5 mM-oleate bound to defatted albumin in the absence (□) or in the presence of either glucagon (10^{-6} M; ○) or dibutyryl cyclic AMP (10^{-4} M; ●) for the time indicated. The rate of ketogenesis was determined every 24 h and expressed as nmol/24 h per mg of protein. Results are means ± S.E.M. of duplicate flasks from four to six different cultures.

in the culture of 10% fetal calf serum or the addition of 2% dimethyl sulphoxide, which ensure the maintenance of differentiated function during long-term culture [26–29], was unable to sustain a high rate of ketogenesis (results not shown). As previously reported for specific liver function in cultured hepatocytes from newborn rats (reviewed in [30]), the metabolic functions must be studied within 48 h of cell plating.

Consequently, the following experiments were performed during the first 48 h of culture in the presence of 0.5 mM-oleate to avoid differences due to changes in endogenous fatty acid availability which are secondary to different initial cellular triacylglycerol concentrations (fetuses, 0.44 ± 0.06; newborns, 0.20 ± 0.03 mg/mg of protein; $n = 6$; $P < 0.01$).

Effects of glucagon and cyclic AMP on induction of ketogenesis in cultured hepatocytes from term fetuses

Although the addition of glucagon or cyclic AMP to fetal cells markedly enhances the rate of ketogenesis with 0.5 mM-oleate as substrate, (2–6-fold; Fig. 3), this rate still remains 35–55% lower than in cultured newborn hepatocytes (Fig. 2). Such a stimulation of ketogenesis has previously been found in adult rat liver during short-term or long-term exposure to glucagon or cyclic AMP [31–34]. However, the present data are the first to show that glucagon or cyclic AMP are able to induce ketogenesis in hepatic cells which have never expressed such a metabolic function.

The data of Fig. 3 also underline that a 48 h exposure to glucagon or cyclic AMP is necessary to obtain a maximal rate of ketogenesis in cultured fetal hepatocytes. Moreover, as the efficiency of glucagon and cyclic AMP could be limited by cyclic AMP degradation, theophylline, an inhibitor of phosphodiesterase [35], has been added to the culture medium. As shown in Table 1, theophylline increases the rate of basal ketogenesis in hepatocytes from term fetuses, whereas it does not affect significantly the rate of ketogenesis in newborn hepatocytes. Similar data have been found with isobutylmethylxanthine (5×10^{-4} M), another phosphodiesterase

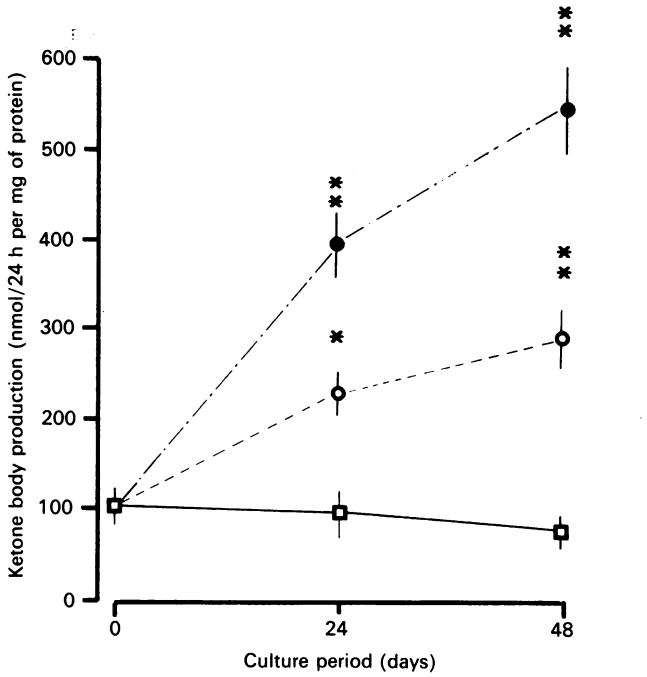

Fig. 3. Effect of glucagon and cyclic AMP on ketone body production in cultured hepatocytes from term fetuses

For experimental design and hormone concentrations, see the legend to Fig. 2. The rate of ketogenesis was measured 24 or 48 h after the beginning of the culture, in the absence (□) or in the presence of glucagon (○), or dibutyryl cyclic AMP (●). Results are means ± S.E.M. of duplicate flasks from six cultures. $*P < 0.05$ and $**P < 0.01$ when compared with control data.

Table 1. Effect of theophylline addition on the rate of ketone body production in hepatocytes from term fetuses and 24-h-old newborns after 48 h in culture

After cell attachment, hepatocytes were incubated in the absence or in the presence of either glucagon (10^{-6} M), dibutyryl (Bt_2) cyclic AMP (10^{-4} M when added alone or 10^{-5} M when added with glucagon), 8-bromo cyclic AMP (10^{-4} M) or forskolin (2×10^{-5} M). Each of these cell incubations was performed both in the absence and in the presence of theophylline (10^{-3} M). After 24 h of culture, the medium was removed and replaced by a medium containing each of the added products indicated above and supplemented with 0.5 mM-oleate. The rate of ketogenesis was determined 24 h after oleate addition and expressed as nmol/24 h per mg of protein. Results are means ± S.E.M. of duplicate flasks from six to eight different cultures. $*P < 0.001$ compared with basal conditions (absence or presence of theophylline). $\dagger P < 0.01$ when compared with the identical condition but performed in the absence of theophylline.

Additions	Theophylline	Liver source...	Ketogenesis (nmol/24 h per mg of protein) Term fetus	24-h-old newborn
None	−		71 ± 7	609 ± 117
	+		170 ± 18†	763 ± 114
Glucagon	−		307 ± 24*	692 ± 83
	+		571 ± 42*†	705 ± 64
Bt_2 cyclic AMP	−		546 ± 95*	909 ± 76
	+		1014 ± 52*†	1051 ± 66
Bt_2 cyclic AMP + glucagon	+		1220 ± 205*	–
8-Bromo cyclic AMP	−		1136 ± 130*	–
Forskolin	+		866 ± 65*	–

inhibitor, both in cultured hepatocytes from adult rats [16] and in the present study (results not shown). By contrast, theophylline markedly potentiates the effects of glucagon and cyclic AMP in cultured fetal rabbit hepatocytes (Table 1), leading to ketone body production rates similar to those found in cultured newborn hepatocytes (Table 1). Similar effects are obtained (Table 1) by using either 8-bromo cyclic AMP, which is a cyclic AMP analogue which is resistant to degradation [36], or forskolin, an activator of the catalytic subunit of adenylate cyclase [37].

Effect of glucagon and cyclic AMP on the metabolic fate of oleate in cultured rabbit hepatocytes

The presence of an uncontrolled concentration of endogenous fatty acids released from triacylglycerol stores in cultured liver cells does not permit accurate determination of intracellular specific activity of the labelled oleate. However, if glucagon or cyclic AMP increase the mobilization of triacylglycerol stores, this would induce a dilution of the intracellular specific activity of the [1-^{14}C]oleate, thus leading to an underestimation rather than an overestimation of the rate of oleate oxidation. Moreover, the total amount of [1-^{14}C]oleate which is metabolized is similar in fetal and neonatal hepatocytes and is not affected by the hormone treatment (Table 2). This allows a valid comparison of the metabolic distribution of labelled oleate into CO_2, acid-soluble products or triacylglycerols.

The sum of the radioactivity recovered in each fraction is a valid reflection of oleate uptake, as previously shown in freshly isolated hepatocytes from newborn rats or pigs [4,38]. This suggests that fatty acid uptake is unaffected by hormone treatment. This is in agreement with previous observations performed in perfused adult rat liver [31,39] or in isolated newborn pig hepatocytes [38].

In 48 h-cultured hepatocytes from fetuses or newborns, the partioning of oleate between esterification and oxidation in basal conditions (Table 2) is similar to that found in freshly isolated hepatocytes [1]. As previously reported for ketone body production (Table 1), the addition of theophylline increases significantly the rate of basal production of acid-soluble products in hepatocytes from term fetuses, whereas it fails to affect this rate in newborn hepatocytes (Table 2). Exposure to glucagon or cyclic AMP for 48 h increases by 240 and 360% respectively acid-soluble products in term fetal hepatocytes (Table 2), with half-maximal stimulation obtained respectively at 8×10^{-9} M and 6×10^{-6} M (Fig. 4). In addition, oleate esterification decreases by 40–75% (Table 2). It is noteworthy that the percentage of triacylglycerol exported into the medium is constant (about 15–20% of total triacylglycerol) whatever the origin of hepatocytes or the culture treatment. These results are in agreement with studies on isolated perfused adult rat liver, showing that the output of triacylglycerol is inversely related to the activity of the fatty acid oxidation pathway [40–41]. These results suggest that the secretion of triacylglycerols by cultured hepatocytes is functional at birth and that the accumulation of triacylglycerol in the fetal rabbit liver *in vivo* [1] could result from a low rate of long-chain fatty acid oxidation rather than to a defect in triacylglycerol exportation. However such a conclusion needs further investigation.

The reciprocal relationship between esterification and oxidation of long-chain fatty acids after glucagon or

Table 2. [1-^{14}C]Oleate metabolism in hepatocytes from term fetuses and 24-h-old newborns after 48 h in culture

For experimental details and concentrations of added products, see Table 1. [1-^{14}C]Oleate (0.35 μCi/flask; 0.5 mM) was added 24 h after the beginning of the culture. The metabolic distribution of ^{14}C was determined 24 h later. [1-^{14}C]Oleate metabolized represents the sum of the radioactivity recovered in CO_2, acid-soluble products, and cellular and secreted triacylglycerols. Results are means ± S.E.M. of six to ten cultures performed in duplicate. $*P < 0.01$ when compared with cultures performed in the presence of theophylline alone. $\dagger P < 0.05$ and $\dagger\dagger P < 0.01$ when compared with controls. $\ddagger P < 0.01$ when compared with term fetuses cultivated in similar conditions.

Liver source	Additions	$10^{-3} \times$ [1-^{14}C]Oleate metabolized (d.p.m./24 h per mg of protein)	Metabolic distribution (% of [1-^{14}C]oleate metabolized)			
			CO_2	Acid-soluble products	Triacylglycerols	
					Cellular	Medium
Term fetus	None	33.6 ± 1.1	1.9 ± 0.3	12.7 ± 1.6	66.0 ± 2.0	19.4 ± 1.7
	Theophylline	32.7 ± 1.2	2.0 ± 0.2	21.4 ± 1.4††	58.5 ± 3.1†	18.1 ± 0.8
	Glucagon + theophylline	32.0 ± 2.4	2.7 ± 0.3	50.0 ± 5.5*	40.5 ± 4.1*	6.8 ± 2.2*
	Bt_2 cyclic AMP + theophylline	35.9 ± 2.2	2.1 ± 0.5	77.5 ± 3.4*	16.2 ± 3.9*	4.2 ± 0.9*
	Forskolin + theophylline	33.3 ± 1.7	2.6 ± 0.4	70.6 ± 5.4	21.2 ± 4.1*	5.6 ± 1.4*
	Bt_2 cyclic AMP + glucagon + theophylline	34.5 ± 0.6	2.3 ± 0.3	75.7 ± 3.0*	16.8 ± 3.4	5.2 ± 0.6*
24-h-old newborn	None	32.0 ± 2.7	2.9 ± 0.2	72.1 ± 10‡	18.7 ± 0.9‡	6.3 ± 1.4‡
	Theophylline	34.8 ± 1.6	2.6 ± 0.2	76.4 ± 5.2‡	16.6 ± 1.8‡	4.4 ± 0.9‡
	Glucagon + theophylline	36.4 ± 3.1	2.9 ± 0.7	80.5 ± 6.1‡	13.0 ± 1.6‡	3.6 ± 0.6
	Bt_2 cyclic AMP + theophylline	37.1 ± 2.5	3.2 ± 0.9	85.1 ± 2.6	8.9 ± 1.8*	2.8 ± 0.8

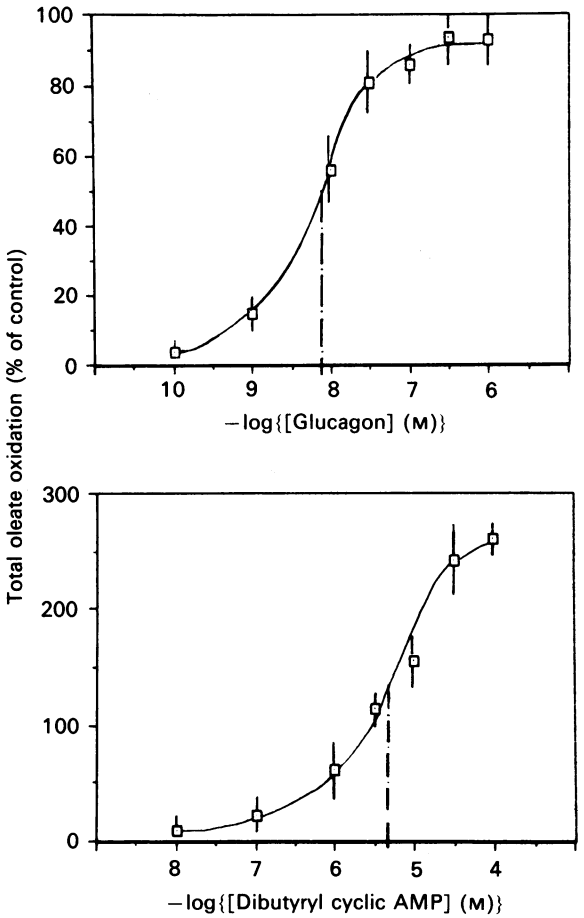

Fig. 4. Dose–response curves of oleate oxidation in the presence of glucagon and cyclic AMP in cultured hepatocytes from term fetuses

After cell attachment, hepatocytes were incubated with the concentrations of glucagon or cyclic AMP indicated, in the presence of theophylline (10^{-3} M). After 24 h the medium was replaced by a medium of the same composition to which was added 0.5 mM-[1-^{14}C]oleate. Total oleate oxidation (CO_2 + acid-soluble products) was measured between 24 and 48 h of culture and expressed as a percentage of the control value with theophylline alone (see Table 2). Values are means ± S.E.M. of duplicate flasks from four different cultures.

cyclic AMP treatment has been described previously in isolated or cultured hepatocytes from adult rats [12,42] or from chickens [43]. Glucagon or cyclic AMP are known to decrease the glycerol 3-phosphate concentration in isolated or cultured hepatocytes [34,44], which in turn could limit long-chain fatty acid esterification and thus increase the channelling of oleate towards oxidation. Indeed, the stimulation of intracellular triacylglycerol store mobilization by glucagon or cyclic AMP treatment in cultured fetal hepatocytes (basal, 0.34 ± 0.03; glucagon, 0.22 ± 0.02; cyclic AMP, 0.17 ± 0.01 mg/mg of cell protein; $n = 5$) would probably provide a sufficient amount of glycerol to re-esterify the oleate taken up by cultured fetal hepatocytes. Moreover, long-term exposure to glucagon did not affect the capacity for triacylglycerol synthesis in cultured hepatocytes from adult rats [45,46]. Although this must be verified in cultured fetal hepatocytes, these data suggest that the regulation of oleate metabolism by glucagon or cyclic AMP is mainly located at the level of oxidation.

As previously mentioned for ketone body production (Table 1), the addition of 10^{-6} M-glucagon to cultured fetal hepatocytes does not allow the rate of oleate oxidation to reach that found in cultured newborn hepatocytes (Table 2). This does not result from a deficiency of the adenylate cyclase system *per se*, since forskolin produces a maximal stimulation of ketogenesis and oleate oxidation in cultured fetal hepatocytes (Tables 1 and 2). By contrast, this result is probably due to the reduced efficiency of glucagon to increase adequately the intracellular cyclic AMP concentration (basal, 2.1 ± 0.3; glucagon, 3.3 ± 0.1; forskolin, 4.4 ± 0.2 pmol/mg of protein; $n = 7$) as previously reported in fetal rabbit [47] and rat [48] hepatocytes. This argument is strengthened by the fact that the addition of both 10^{-6} M-glucagon and 10^{-5} M-cyclic AMP (half-maximal effect, Fig. 4) leads to rates of ketogenesis and oleate oxidation similar to those found in cultured newborn hepatocytes (Tables 1 and 2). This lack of a maximal response to glucagon may result either from the reduced glucagon receptor number reported in fetal rat or rabbit liver [47,48], or from down-regulation of glucagon receptor number due to prolonged exposure of the hepatocytes to high glucagon concentrations [49].

Effect of insulin on oleate oxidation in cultured hepatocytes

In hepatocytes from term fetuses cultured in the presence of 10^{-8} M-glucagon, a concentration of hormone producing a half-maximal stimulation of oleate oxidation (Fig. 4), insulin antagonizes in a dose–dependent manner the effect of glucagon, with a physiological half-maximal efficiency at 2×10^{-9} M (Fig. 5). When glucagon-induced

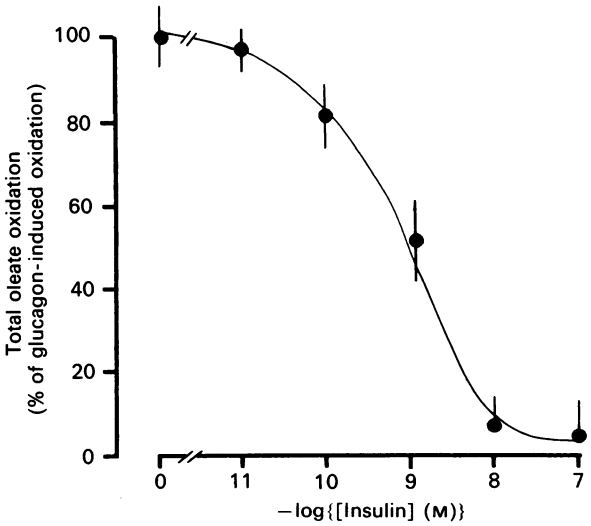

Fig. 5. Dose–response curve of oleate oxidation in the presence of insulin in cultured hepatocytes from term fetuses

Insulin at the indicated concentrations was added during cell attachment and was present for the entire 48 h of culture. After cell attachment, glucagon (10^{-8} M) and theophylline (10^{-3} M) were added. Total oleate oxidation (CO_2 + acid-soluble products) was measured between 24 and 48 h of culture and expressed as a percentage of glucagon-induced oleate oxidation. Results are means ± S.E.M. of four different cultures.

Table 3. Effect of glucagon and cyclic AMP on the activity and sensitivity of CPT I to malonyl-CoA inhibition

For experimental design and hormone concentrations, see the legend to Fig. 2. After 48 h in culture, the hepatocytes from ten Petri dishes (75 cm^2) were collected and mitochondria were isolated as described in the Materials and methods section. CPT I activity was measured in the presence of 80 μM-palmitoyl-CoA and 1 mM-carnitine. The term IC_{50} refers to the concentration of malonyl-CoA required for 50% inhibition of CPT I activity. Results are means ± S.E.M. of five to seven different cultures. *$P < 0.01$ when compared with basal conditions. †$P < 0.01$ when compared with term fetuses.

Liver source	Addition	Activity (munits/mg of protein)	IC_{50} (μM)
Term fetus	None	0.96 ± 0.21	0.07 ± 0.01
	Glucagon	0.92 ± 0.13	0.39 ± 0.09*
	Bt_2 cyclic AMP	0.93 ± 0.18	0.79 ± 0.13*
24-h-old newborn	None	1.42 ± 0.25	0.95 ± 0.12†

oleate oxidation is totally inhibited by insulin, this results in a complete re-direction of oleate towards esterification (73 ± 9% of total oleate metabolized; $n = 3$). By contrast, insulin (10^{-7} M) inhibits by no more than 35 ± 7% ($n = 4$) the high rate of oleate oxidation in hepatocytes from 24-h-old newborns, with a half-maximal effect at an insulin concentration of 10^{-9} M. This decrease in the maximal response to insulin, with a similar half-maximal effectiveness, suggests that the number of hepatic insulin receptors rather than their affinity could be decreased in newborn rabbit hepatocytes, as previously reported [47]. This hepatic 'insulin resistance' in newborn rabbit could reflect a situation *in vivo* as previously described for hepatic glucose production in the suckling newborn [50].

Effect of glucagon or cyclic AMP on the activity and sensitivity of CPT I to malonyl-CoA inhibition

As shown in Table 3, the activity of CPT I is 40% higher in mitochondria isolated from newborn rabbit hepatocytes after 48 h of culture than in those isolated from fetal hepatocytes. These activities of CPT I are very close to those found in mitochondria isolated from whole rabbit liver [2]. It seems unlikely that this small difference in the CPT I activity between fetal and neonatal rabbit hepatocytes could be responsible for the 5-fold higher rate of oleate oxidation in cultured newborn hepatocytes compared with fetal ones. Moreover, the addition of glucagon or cyclic AMP to cultured fetal hepatocytes had no effect on the activity of CPT I (Table 3). These data are in agreement with recent studies showing that short-term (60 min) exposure to glucagon had marginal [51] or no [13] effect on the activity or on the malonyl-CoA sensitivity of CPT I [13] in permeabilized hepatocytes from adult rats. This could be due to the short period of glucagon exposure, since it was shown that changes in the sensitivity of CPT I to malonyl-CoA inhibition were observed only after 4–12 h treatment of diabetic rats with insulin [52,53]. Indeed, the addition of glucagon or cyclic AMP for 48 h to cultured fetal hepatocytes increases by 330% and 740% respectively the IC_{50} for malonyl-CoA (Table 3). These results confirm indirect evidence from experiments *in vivo* showing that the fall in the plasma insulin/glucagon ratio *in utero* induced by prolongation of gestation leads to a 75% decrease in the sensitivity of CPT I to malonyl-CoA inhibition in mitochondria isolated from post-term rabbit fetuses [6]. The mechanism by which glucagon and cyclic AMP modulate the sensitivity of CPT I to malonyl-CoA inhibition and/or the role of malonyl-CoA concentration itself remain to be elucitated.

In conclusion, this work shows that: (1) the metabolic characteristics of freshly isolated hepatocytes (ketone body production, metabolic fate of long-chain fatty acids, activity and sensitivity of CPT I to malonyl-CoA inhibition) are maintained during the first 2 days of primary hepatocyte culture; (2) the addition of glucagon or cyclic AMP to cultured fetal hepatocytes, i.e. cells which have never expressed these metabolic functions, induces an increase in the capacity for long-chain fatty acid oxidation and ketogenesis; (3) the effects of glucagon are more marked in the presence of insulin deficiency; and (4) a part of the glucagon- or cyclic AMP-induced fatty acid oxidation could be due to a huge decrease in the sensitivity of CPT I to malonyl-CoA inhibition. This strongly suggests that the neonatal changes in plasma insulin and glucagon concentrations are primarly responsible for the emergence of ketogenesis and fatty acid oxidation in newborn liver.

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