Hormonal regulation of rat foetal lipogenesis in brown-adipocyte primary cultures

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Insulin stimulates lipogenesis by 100 % for 5 h by a covalent modulation of acetyl-CoA carboxylase, and by 200 % for 24 h by increasing malic enzyme and fatty acid synthase enzymic activities in brown-adipocyte primary cultures. At short times, noradrenaline and isoprenaline decrease lipogenesis. However, phenylephrine and glucagon have no effect. At long times, dexamethasone inhibits lipogenesis. This effect is precluded in the presence of insulin. Progesterone and tri-iodothyronine, alone or in the presence of insulin, produce a stimulation of the rates of lipogenesis.

INTRODUCTION

The rates of lipogenesis increase during the last 2 days of gestation in rat foetal brown adipose tissue (Pillay & Bailey, 1982). After birth, rates of lipogenesis sharply decrease and initiation of thermogenesis occurs (Benito *et al.*, 1984; Nicholls & Locke, 1984).

In addition, we have investigated the short-term and the long-term regulation of lipogenesis in foetal brown adipose tissue and brown adipocytes at 22 days of gestation. Thus insulin produces a slight increase in the rates of lipogenesis, and noradrenaline inhibits their rates, mainly by a β -adrenergic mechanism in isolated brown adipocytes. The presence of glucagon in the cell incubation medium had no effect (Roncero *et al.*, 1987). In addition, administration of progesterone through the mother increases the rates of lipogenesis *in vivo* and in isolated brown fat-cells (Roncero *et al.*, 1987). Thus short-term regulatory studies have been carried out in brown fat-cells and the long-term regulatory studies have been done indirectly on brown adipose tissue.

Accordingly, the aim of this work was to study the short-term and the long-term hormonal regulation of lipogenesis under the same suitable experimental conditions: primary cultures of foetal brown fat-cells.

EXPERIMENTAL

Isolation, culture and characterization of cells

Interscapular brown-fat tissue was taken from 22-day foetuses of Wistar rats for the material of primary cultures. After removal under sterile conditions, the tissue was minced, placed in a 20 ml sterile bottle containing 100 mM-Hepes/4 % (w/v) albumin isolation buffer (Né Chad *et al.*, 1983) with 2 mg of collagenase/ml and incubated at 37 °C for 45–60 min in a shaking water bath at 100 strokes/min. Every 5 min the bottle was shaken for 10 s in an automatic mixer, to facilitate tissue digestion. After collagenase treatment the tissue was filtered through a 100 μ m-pore-size nylon mesh. The free cells were collected at the bottom of a sterile plastic tube by centrifugation at 80 g for 5 min, and washed once with the isolation buffer. The procedure produced approx. 25×10^6 – 35×10^6 cells/g of foetal brown adipose

tissue, and cell viability, determined by Trypan Blue exclusion, was over 90 %.

The isolated cells were plated in 60 mm-diam. plastic dishes containing 2.5 ml of Eagle's medium modified with Earle's salts, glutamine and 20 mM-Hepes, supplemented with 10 % (v/v) foetal-calf serum, 120 μ g of penicillin/ml, 100 μ g of streptomycin/ml, 50 μ g of gentamicin/ml and 25 units of nystatin/ml (supplied by Antibioticos S.A., Madrid, Spain). Each dish was inoculated with 0.8×10^6 – 1.2×10^6 cells, and the primary culture was kept at 37 °C under an atmosphere of 5 % CO₂ in air with 80 % humidity in a cell incubator. At 20 h after the inoculation of the cells, the attached monolayer was washed twice with Hanks' balanced salt solution to remove any red cells or cell debris remaining in the culture, and fresh culture medium was added, the culture medium then being changed every 48 h.

Brown-adipose-tissue cells were grown for 5 days until they formed a confluent monolayer, and at that time foetal-calf serum was omitted from the culture medium and cells were cultured for a further 3, 5 or 24 h in the presence or absence of hormones. Cells were observed under light microscopy routinely, showing a characteristic spindle shape for the primary cultures of brown adipocytes, as described by Sugihara *et al.* (1983). Oil-Red O staining of cells (Elks & Manganiello, 1985) at different culture periods confirmed the presence of typical multilocal brown-adipocyte lipid droplets. Foetal brown adipocytes in a confluent monolayer were finally characterized by the localization of the uncoupling protein, a specific mitochondrial protein of this tissue, by an immunohistochemical technique. The rabbit anti-(uncoupling protein) serum was generously given by Dr. E. Rial (Dundee, U.K.). Bound antibody was detected by sheep anti-(rabbit IgG) conjugated with peroxidase, and developed with 3-amino-9-ethylcarbazole, as described by Fernández *et al.* (1987). Coloured cells expressing uncoupling protein could easily be distinguished from fibroblasts, which comprised less than 10 % of the total cells.

Determination of lipid synthesis

Foetal brown adipocytes cultured as described above were supplemented with 0.5 mg of fatty acid-free bovine

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serum albumin/ml and 3.125 mCi of $^3\text{H}_2\text{O}$ per plate, and incubated for 3 h. Cells were harvested after being detached from the plates by addition of 0.3 ml of 200 mM-EDTA for 5 min and collected in plastic vials. Lipogenesis was measured by the method described by Harris (1975). An external-standard curve for quenching correction was used for measurement of radiolabelling.

DNA was determined in duplicate non-radioactive plates by a fluorimetric method (Labarca & Paigen, 1980) with calf thymus DNA as standard. Lipid synthesis was expressed as nmol of $^3\text{H}_2\text{O}$ incorporated into fatty acids/h per mg of DNA.

Determination of enzymic activities

After washing with 20 mM-EDTA in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hanks' balanced salt solution, the cell monolayer was scraped off, sonicated for 30 s at 1.5 mA, in 1 mM-EDTA/1 mM-dithiothreitol/0.25 M-saccharose/25 mM-Tris/HCl (pH 7.5) for acetyl-CoA carboxylase and malic enzyme determinations, and in 1 mM-dithiothreitol/1 mM- MgCl_2 /0.1 mM-EDTA/10% (v/v) glycerol/0.1 mM- NaH_2PO_4 (pH 7.2) for fatty acid synthase determination, and centrifuged at 12000 g for 4 min. The supernatant was used for measurement of enzymic activities.

Acetyl-CoA carboxylase was measured as described by Kelley *et al.* (1986). Two types of activities ('I' and 'C') are referred to below. The 'initial' activity ('I') is measured immediately after sonication in the presence of 100 mM-NaF. The 'citrate-activated' activity ('C') is measured in a sonicated homogenate obtained in the absence of NaF and after 30 min incubation in the presence of sodium citrate (20 mM), MgCl_2 (20 mM), dithiothreitol (1 mM), bovine serum albumin (0.5 mg/ml), Tris/HCl (50 mM), pH 7.5. Consequently, the fraction of the enzyme in the active form is expressed as 'I/C', as previously described (Zammit & Corstorphine, 1982). Malic enzyme and fatty acid synthase were measured spectrophotometrically, as described by Ochoa *et al.* (1948) and Nepokroeff *et al.* (1975) respectively. Protein was determined by the method of Bradford (1976), with bovine serum albumin as standard. Enzymic activities were expressed as nmol/min per mg of protein.

RESULTS AND DISCUSSION

Rates of foetal lipogenesis and lipogenic enzymic activities in brown-adipocyte primary cultures: effect of insulin

Dose/response curves for rates of lipogenesis to insulin (0.4–400 nM) for 5 or 24 h are shown in Fig. 1. Insulin (present for 5 h) increases the rates of lipogenesis linearly from 0.4 to 4 nM (50% and 100% respectively) as compared with the control value observed in its absence. Addition of higher concentrations of insulin (40 and 400 nM) does not increase the lipogenic flux in brown-adipocyte primary cultures (Fig. 1). These results show that the rates of lipogenesis in response to insulin (at short time) are much higher than that observed in isolated brown-fat cells subjected to the hormone for 1 h (16–26%, depending on the substrate studied) (Roncero *et al.*, 1987), and seem to indicate repair of insulin receptors (throughout the time of culture) damaged by proteinase contamination of collagenase during the brown-fat-cell isolation procedure (Seglen, 1976). In addition, insulin (present for 24 h) increases the rates of lipogenesis linearly from 0.4 to 40 nM (100% and 200%

Fig. 1. Dose/response curves to insulin for rates of lipogenesis

Results are means $\pm$ S.E.M. ($n = 8-15$) and represent nmol of $^3\text{H}_2\text{O}$ incorporated into fatty acids/h per mg of DNA. Values for insulin present for 5 h (●) or for 24 h (▲) that are significantly different by Student's *t* test from those for controls in the absence of insulin are shown: ** $P < 0.001$.

Table 1. Acetyl-CoA carboxylase activity in foetal brown-adipocyte primary cultures: effect of insulin (5 h)

For details see the Experimental section. Enzyme activities are expressed as nmol/min per mg of protein. I/C (%) represents the fraction of the enzyme in the active form. Results are means $\pm$ S.E.M. ($n = 4$). Values that are significantly different by the Student's *t* test from those obtained for their controls are shown by: * $P < 0.05$.

	Initial activity (I)	Citrate-activated (C)	I/C (%)
Control	1.28 $\pm$ 0.22	1.81 $\pm$ 0.14	69 $\pm$ 5
Insulin (40 nM) (5 h)	1.78 $\pm$ 0.21*	1.83 $\pm$ 0.32	98 $\pm$ 1*

respectively) as compared with the control value observed in its absence. Insulin (400 nM) does not produce any further increase in the rates of lipogenesis (Fig. 1).

Half-maximal stimulation of lipogenesis by insulin is produced at 0.4 nM concentration, regardless of the time during which the hormone is present in the culture (Fig. 1). However, the saturating effect on stimulation of lipogenesis is produced at 4 nM by insulin present in the culture for 5 h, and at 40 nM by insulin present for 24 h, indicating a possible increment of insulin degradation in primary cultures submitted to very high insulin concentrations for 24 h.

On the other hand we have investigated the effect of insulin on acetyl-CoA carboxylase (Table 1) and on malic enzyme and fatty acid synthase enzymic activities (Table 2) in primary cultures. Insulin (5 h) increases the 'initial' activity (I) of acetyl-CoA carboxylase, without any significant change in the 'citrate-activated' activity

Table 2. Malic enzyme and fatty acid synthase activities in foetal brown-adipocyte primary cultures: effect of insulin

For details see the Experimental section. Enzyme activities are expressed as nmol/min per mg of protein. Results are means $\pm$ S.E.M. ($n = 7-10$). Values that are significantly different by Student's t test from those for no insulin added are shown by: ** $P < 0.001$.

Period of treatment	Hormone added ...	Malic enzyme (nmol/min per mg)		Fatty acid synthase (nmol/min per mg)	
		None	Insulin (40 nM)	None	Insulin (40 nM)
5 h		28.2 $\pm$ 2.3	26.0 $\pm$ 2.3	1.19 $\pm$ 0.20	1.38 $\pm$ 0.29
24 h		32.3 $\pm$ 1.5	50.2 $\pm$ 5.7**	1.20 $\pm$ 0.03	1.82 $\pm$ 0.14**

Table 3. Short-term regulation of lipogenesis in foetal brown-adipocyte primary cultures

For details see the Experimental section. Results are means $\pm$ S.E.M. ($n = 5-16$) and are expressed as nmol of $^3\text{H}_2\text{O}$ incorporated into fatty acids/h per mg of DNA. Values that are significantly different by Student's t test from those for no hormones added are shown by: * $P < 0.05$.

Hormones added	Lipogenesis
None	356 $\pm$ 14
Glucagon (1 μM)	352 $\pm$ 7
Noradrenaline (0.1 μM)	316 $\pm$ 10*
Noradrenaline (10 μM)	305 $\pm$ 6*
Isoprenaline (10 μM)	297 $\pm$ 8*
Phenylephrine (0.1 μM)	336 $\pm$ 18
Propranolol (100 μM) + phenylephrine (10 μM)	344 $\pm$ 24

(C). Thus insulin enhances by 30 % the fraction of the enzyme in the active form (I/C) (Table 1). However, insulin has no effect on both malic enzyme and fatty acid synthase activities at short times (5 h) (Table 2). Accordingly, these results indicate that insulin works on lipogenesis at short times by mechanisms independent of malic enzyme or fatty acid synthase, involving stimulation of acetyl-CoA carboxylase (Table 1), as suggested in brown adipose tissue *in vivo* (McCormack *et al.*, 1986). In addition, insulin may be increasing glucose transport to the brown cells, as suggested by Ferré *et al.* (1986), and, accordingly, glucose availability for lipogenesis. Moreover, insulin increases by 50 % both malic enzyme and fatty acid synthase activities at long times (24 h) as

compared with values found in the absence of insulin (Table 2). Thus insulin stimulates lipogenesis at long times by further mechanisms involving malic enzyme and fatty acid synthase activation, presumably by an increase in the amount of both enzyme proteins.

Short-term regulation of foetal lipogenesis in brown-adipocyte primary cultures

The short-time lipogenic response to hormones in brown-adipocyte primary cultures is shown in Table 3. The presence of 1 μM -glucagon (3 h) has no effect on the rates of lipogenesis observed in brown-cell primary cultures. These results are consistent with those found in isolated brown fat-cells, where glucagon had no effect on the rates of lipogenesis (Roncero *et al.*, 1987), or on cyclic AMP accumulation in the cells or on the rate of lipolysis studied (Elliott *et al.*, 1984), confirming that glucagon plays no role in the regulation of lipogenesis in brown adipocytes.

Conversely, the presence of 0.1 μM - or 10 μM -noradrenaline during 3 h decreases lipogenesis in primary cultures, by 12 % or 14 % (Table 3). These results are consistent with the noradrenergic stimulation of thermogenesis in brown adipose tissue at birth (Nicholls & Locke, 1984). In addition, we have tested a β -adrenergic agonist (10 μM -isoprenaline), an α_1 -adrenergic agonist (0.1 μM -phenylephrine) and an α_1 -adrenergic agonist in the presence of a β -blocker (10 μM -phenylephrine + 100 μM -propranolol) on the rates of lipogenesis in primary cultures (Table 3). Isoprenaline decreases the rates of lipogenesis by 16 %, but phenylephrine alone or in the presence of propranolol did not change their rates (Table 3). These results show that the inhibitory effect of noradrenaline on lipogenesis in brown-adipocyte primary cultures is mainly exerted through a β -adrenergic

Table 4. Long-term regulation of lipogenesis in foetal brown-adipocyte primary cultures

For details see the Experimental section. Results are means $\pm$ S.E.M. ($n = 10-15$) and are expressed as nmol of $^3\text{H}_2\text{O}$ incorporated into fatty acids/h per mg of DNA. Values that are significantly different by Student's t test from those for no hormones added in the presence or in the absence of insulin are shown by: * $P < 0.05$; ** $P < 0.001$.

Hormones added ...	Lipogenesis			
	None	Progesterone (3.2 μM)	Tri-iodothyronine (10 μM)	Dexamethasone (0.1 μM)
None	348 $\pm$ 9	381 $\pm$ 16*	479 $\pm$ 14**	160 $\pm$ 10**
Insulin (40 nM)	885 $\pm$ 53	1044 $\pm$ 32*	1326 $\pm$ 90**	934 $\pm$ 61

mechanism. In addition, the noradrenergic inhibition of lipogenesis in primary cultures shown in Table 3 is significantly lower than that observed in isolated brown fat-cells (Roncero *et al.*, 1987), suggesting a possible loss of β -noradrenergic cell response during the time of culture, related to a gradual conversion of brown into white fat-cells observed in long-period primary cultures (Sugihara *et al.*, 1983).

Long-term regulation of foetal lipogenesis in brown-adipocyte primary cultures

The long-time lipogenic response to hormones in brown-adipocyte primary cultures is shown in Table 4. Dexamethasone ($0.1 \mu\text{M}$) decreases the rates of lipogenesis by 50% as compared with those values obtained in its absence. However, in the presence of 40 nM-insulin, dexamethasone did not produce any inhibitory effect on lipogenesis in primary cultures (Table 4). These results are in agreement with the inhibition of brown-adipose-tissue lipogenesis *in vivo* observed in newborn rats after dexamethasone treatment (Benito *et al.*, 1984). In addition, it has been suggested that dexamethasone may work on lipogenesis *in vivo* through inhibition of glucose utilization by extra-hepatic tissues (Benito *et al.*, 1982). Accordingly, the presence of insulin at saturating concentration might be increasing glucose transport in brown-adipocyte primary cultures, avoiding the inhibitory effect on lipogenesis by dexamethasone seen above in its absence (Table 4).

The presence of $3.2 \mu\text{M}$ -progesterone (for 24 h) increases the rates of lipogenesis in brown-adipocyte primary cultures, by 10%. When insulin was present, progesterone increases lipogenesis by 18% (Table 4). These results are consistent with those obtained in foetal brown adipose tissue *in vivo* from 24 h progesterone-treated rats (Roncero *et al.*, 1987). However, the stimulatory effect of progesterone on lipogenesis in primary cultures seen above (Table 4) is significantly much lower than that observed in brown adipocytes from foetuses treated with progesterone *in situ* (Roncero *et al.*, 1987). Thus our results seem to indicate that progesterone may play an indirect regulatory role in foetal brown-fat lipogenesis (through an increased food intake and substrate availability to the tissues) rather than a direct effect on the lipogenic flux in that tissue.

Finally, the presence of tri-iodothyronine for 24 h increases the rates of lipogenesis in brown-adipocyte primary cultures, by 38% in the absence of 40 nM-insulin and by 50% in its presence (Table 4). These results are consistent with the stimulatory effect of thyroid hormones on brown-fat lipogenesis *in vivo* in newborn rats (Benito *et al.*, 1984) and in 4-week-old rats (Gnoni *et al.*, 1983). In addition, the presence of tri-iodothyronine (24 h) in the culture medium increases significantly ($P < 0.01$) the fatty acid synthase activity in the absence ($1.37 \pm 0.06 \text{ nmol/min per mg of protein}$) or in the presence of insulin ($2.11 \pm 0.07 \text{ nmol/min per mg of protein}$) as compared with their respective controls (see Table 2). No effect of tri-iodothyronine was observed on malic enzyme activity under the same conditions. Thus the stimulatory effect on lipogenesis observed in primary cultures submitted to tri-iodothyronine for 24 h, alone or in the presence of insulin, may be explained by a direct induction of the genetic expression of fatty acid synthase.

In conclusion, insulin plays a direct role on the regulation of lipogenesis in brown-adipocyte primary cultures at short and long times. At short times, insulin increases lipogenesis by a mechanism independent of induction of lipogenic enzymes, involving a covalent modulation of acetyl-CoA carboxylase. At long times, insulin works on lipogenesis through an activation of malic enzyme and fatty acid synthase enzymic activities, probably owing to an induction of content of both enzyme proteins.

At short times, noradrenaline inhibits lipogenesis in brown-cell cultures mainly through a β -adrenergic mechanism, glucagon not being involved in the short-term regulation of brown-adipocyte lipogenesis.

At long times, dexamethasone inhibits lipogenesis in brown-adipocyte primary cultures. This inhibition is precluded by the presence of insulin at saturating concentration, suggesting that dexamethasone inhibition of lipogenesis is mediated by a decrease in glucose transport to the brown cells. In addition, progesterone does not seem to regulate directly the rates of lipogenesis in brown adipose tissue. Conversely, thyroid hormones play a direct role in the regulation of lipogenic flux, alone or in the presence of insulin, presumably by the induction of the fatty acid synthase content.

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