Metabolism
Clinical and Experimental

VOL 46, NO 4 APRIL 1997

Time-Dependent Effects of Insulin on Lipid Synthesis in Cultured Fetal Rat Hepatocytes: A Comparison Between Lipogenesis and Glycogenesis

H. M'Zali, C. Guichard, M. Lavau, and C. Plas

The lipogenic effect of insulin was studied in 18-day-old fetal rat hepatocytes after 2 to 3 days of culture in the presence of glucocorticoids when an acute stimulatory effect of insulin on glycogenesis was present. The rate of [1-^{14}C]-acetate incorporation into lipids measured for 4 hours was much higher than with [U-^{14}C]-glucose (30 *v* 3.8 nmol/h/mg protein). The stimulatory effect of insulin on lipid labeling remained weak (1.2-fold) and contrasted with its striking stimulatory effect on [U-^{14}C]-glucose incorporation into glycogen (fourfold). When lipid labeling was assessed in longer experiments, increasing acetate concentrations in the medium stimulated the incorporation rate of [1-^{14}C]-acetate into lipids (3.5-fold from 1 to 5 mmol/L after 36 hours) and decreased that of [U-^{14}C]-glucose (by twofold). The stimulatory effect of insulin on the rate of lipid labeling developed with both precursors from 12 to 36 hours after insulin exposure (by ~twofold) independently of acetate concentration and was not glucocorticoid-dependent, contrary to the glycogenic response. Addition of a glucose load simultaneously with insulin increased the stimulation of lipogenesis when measured with [U-^{14}C]-glucose (twofold to 3.7-fold). Besides contributing to an accumulation of larger and numerous lipid droplets in the cells, insulin increased fatty acid synthase activity by 26%, whereas malic enzyme was not affected. Thus, insulin-dependent lipogenesis in cultured fetal hepatocytes appears to be mostly regulated by a long-term mechanism, contrary to the glycogenic effect of insulin.

THE IMPORTANCE OF THE LIVER as a lipogenic organ is well known. After ingestion of a high-carbohydrate diet, the liver stores some carbohydrate as glycogen and transforms the rest into fatty acids. Insulin has been shown to be required for dietary induction of hepatic lipogenic enzymes in previously fasted diabetic rats refed with a high-carbohydrate, fat-free diet[1] and in young rats during the suckling-weaning transition.[2] Also, in the rhesus monkey, chronic fetal hyperinsulinemia leading to insulin levels similar to those observed in human infants of diabetic mothers enhances hepatic lipogenic enzyme activities.[3,4] Investigations on the regulation of lipogenic enzymes by nutritional factors and insulin have led researchers to distinguish two types of mechanisms according to the necessary time to exert an effect.[5,6] On the other hand, studies in isolated and cultured adult hepatocytes have shown that hormonal factors, particularly insulin, are implicated in short- and long-term regulation of hepatic lipogenesis.[7,8]

During the perinatal period in the rat, storage of liver glycogen in late gestation and its mobilization at birth occur under the influence of hormonal factors such as insulin and glucagon,[9] a situation that can be mimicked in cultured fetal hepatocytes.[10] Also, an inverse relationship between liver glycogen and lipid syntheses around birth has been described.[11,12] A previous study has shown an increase in glycogenesis in cultures of term fetal hepatocytes after a 24-hour period of exposure to insulin, which was accompanied by an increase in fatty acid synthase activity.[13] Chick embryo hepatocytes cultured in a chemically defined medium have provided a particularly useful system to study the regulation of lipogenesis by substrates and hormones.[6] In these cells, triiodothyronine stimulates the transcription of fatty acid synthase, with this effect being amplified by insulin and insulin-like growth factor-I.[14] Cultured fetal rat hepatocytes, which are suitable to characterize the early and time-dependent stimulatory effect of insulin on glycogen synthesis,[15] were used in the present investigation to study the effect of the hormone on lipid synthesis. To compare lipogenesis and glycogenesis, incorpora-

From the Laboratoire Biologie-Odontologie, Université Paris 7, and Unité de Recherches sur la Physiopathologie de la Nutrition, Institut National de la Santé et de la Recherche Médicale U-177, Institut Biomédical des Cordeliers, Paris; and Département de Biochimie, Institut de Biologie, Université de Tizi-Ouzou, Algérie, France.

Submitted November 28, 1994; accepted November 16, 1996.

Supported by Contrat de recherche externe Grant No. 897009 from the Institut National de la Santé et de la Recherche Médicale and by the Fondation pour la Recherche Médicale.

Address reprint requests to C. Plas, DSc, Laboratoire Biologie-Odontologie, Université Paris 7, Institut Biomédical des Cordeliers, 15, rue de l'Ecole de Médecine, 75270 Paris Cedex 06, France.

0026-0495/97/4604-0001$03.00/0

tion of [1-^{14}C]-acetate and [U-^{14}C]-glucose into lipids and glycogen was measured during time-variance experiments in the presence and absence of insulin. A stimulatory effect of insulin on lipid synthesis from both precursors, which was hardly seen after 4 hours, developed following prolonged exposure to the hormone. This was independent of the presence of glucocorticoids, contrary to the glucocorticoid-dependent development of the glycogenic effect of insulin, a response already fully expressed early. The long-term stimulation of lipogenesis by insulin was accompanied by an increase in cellular lipid content and a stimulation of fatty acid synthase activity.

MATERIALS AND METHODS

Chemicals

[1-^{14}C]-acetic acid, sodium salt (50 to 60 mCi/mmol) was purchased from Amersham (Buckinghamshire, UK) and [U-^{14}C]-glucose (3.5 mCi/mmol) from Dupont-NEN (Mississauga, Canada). Insulin was a gift from Eli Lilly Laboratories (Indianapolis, IN). The source of other chemicals has been specified previously.[15]

Culture Procedure

Primary cultures of hepatocytes were obtained from 18-day-old Sprague-Dawley rat fetuses (Iffa credo, Lyon, France) as described previously.[6] After mild trypsin treatment, the isolated cells were plated on a collagen substratum to which only the hepatocytes adhered, and after 6 hours, nonadhering hematopoietic cells were removed. At this step and after 18 hours, the culture medium (1 mL/dish) was replaced. The basal medium consisted of NCTC 109 medium[17] at a concentration of 5.5 mmol/L glucose and 0.36 mmol/L acetate and containing 10 mmol/L HEPES, pH 7.4. It was supplemented or not with 10% (vol/vol) fetal calf serum, 10 μmol/L cortisol, 8 mmol/L glucose, and various concentrations of acetate as indicated for each experiment.

Lipid Studies

Different labeled precursors were used to measure lipid synthesis: [1-^{14}C]-acetate (1.0 μCi/mL) supplemented with unlabeled acetate to produce various final concentrations in the medium; and [U-^{14}C]-glucose (1.0 μCi/mL) in the presence or absence of an 8-mmol/L glucose load (final concentration in the medium, 13.5 mmol/L). Acetate content in the culture medium was measured by enzymatic method after conversion of acetic acid to acetyl coenzyme A (CoA) (Boehringer, Mannheim, Germany). Glucose concentration in the culture medium was determined by the glucose oxidase procedure after deproteinization with $Ba(OH)_2/ZnSO_4$. Different sets of labeling procedures were performed: in short-labeling experiments, [1-^{14}C]-acetate or [U-^{14}C]-glucose was added to the medium at day 2 of culture together with insulin (10 nmol/L) or its HCl vehicle (2.5 μmol/L), and cultures were incubated for periods up to 4 hours; in long-labeling experiments, labeled precursors and agents to be tested were introduced after 18 hours of culture at the time of medium renewal, and cultures were incubated for periods up to 48 hours; and in continuous-labeling experiments, [1-^{14}C]-acetate or [U-^{14}C]-glucose was added to the medium at the start of culture to produce a final activity of 0.2 μCi/μmol, which was maintained throughout the 3-day culture period, with insulin (10 nmol/L) and/or a glucose load (8.0 mmol/L) being present from 18 hours of culture. After completion of the incubation, the medium was removed and the cells were washed with cold phosphate buffered saline (PBS). Total lipids were extracted with chloroform, methanol, and water,[18] and radioactivity was assessed from dried aliquots of chloroform extracts. The results are expressed per milligram protein, with protein content measured according to the method of Lowry et al.[19]

Fatty acid synthase activity was determined as described by Guichard et al[20] by measuring malonyl CoA–dependent oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) at 35°C.[21] Malic enzyme activity was assayed by the method of Ochoa[22] as previously reported.[23] For the data reported herein, 1 U enzyme activity is defined as the amount of enzyme needed to catalyze the oxidation or reduction of 1 nmol NADPH or nicotinamide adenine dinucleotide phosphate (NADPH) per minute at 35°C, with enzyme activities expressed in units per milligram protein.

Staining with Nile red, a selective fluorescent dye for intracellular lipid droplets,[24] was performed on cells grown on glass cover slips using the culture conditions described earlier for long-term experiments in the presence and absence of 10 nmol/L insulin from 18 hours. After 48 hours of hormone exposure, cells were washed rapidly with ice-cold PBS and transferred to culture dishes containing PBS. They were then stained with Nile red (100 ng/mL) and kept for 15 minutes at room temperature in the absence of light. After washing with PBS to remove excess dye, cells were viewed using a Leitz Orthoplan (Leica, St Gall, Switzerland) fluorescence microscope (excitation, 450 to 490 nm; emission > 520 nm). Paired micrographs were taken with Kodak TMAX-film (3200 ASA; Eastman Kodak, Rochester, NY). Intracellular neutral lipid droplets were quantified in representative fields using a Quantimet 570 image analysis system (Leica).

Glycogen Studies

Glycogen labeling in the presence of [U-^{14}C]-glucose and glycogen content were measured as previously described.[16] Short- and continuous-labeling experiments were conducted in parallel with the lipid studies already described.

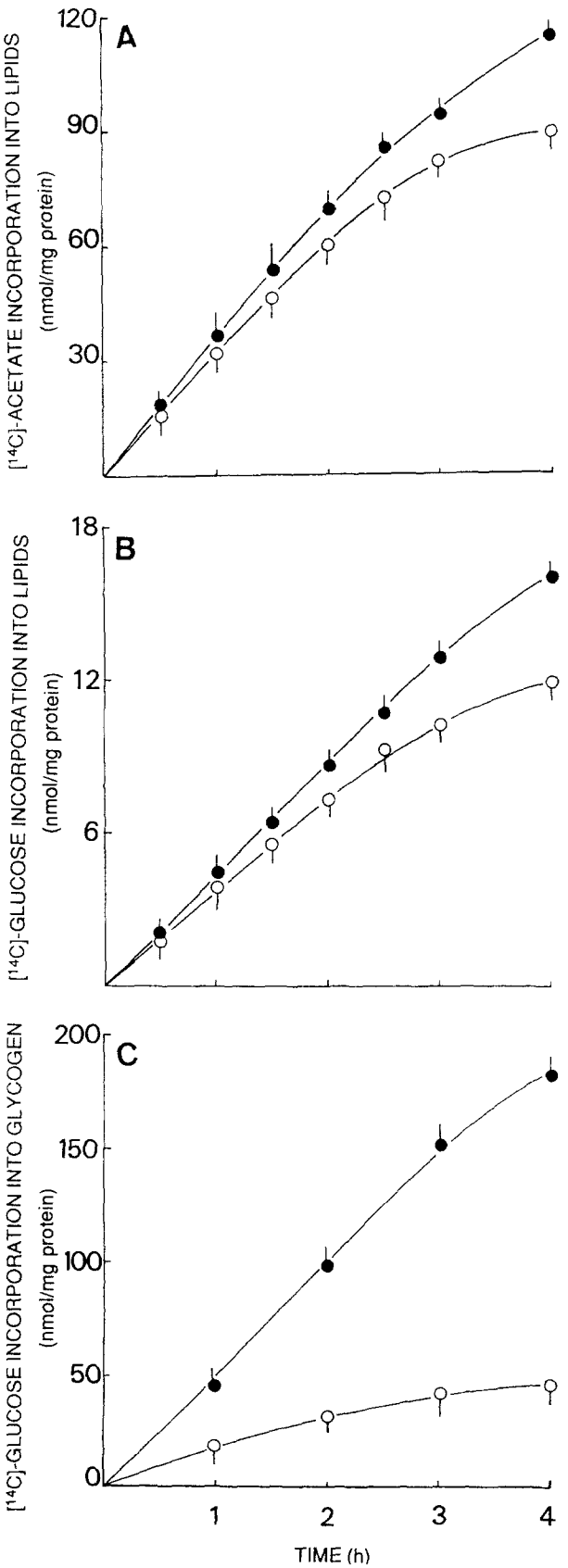
Presentation of Data

Each protocol involved at least three experiments performed with different cell preparations. Data are presented as the mean ± SD for the number of independent experiments (n). For statistical analyses, Student's *t* test for paired samples was used, with treated cultures and the corresponding controls for n independent experiments. To express the insulin or glucose load response, a stimulation index (SI) was used, defined as the following ratio: nanomoles of precursor in lipids (or in glycogen) per milligram cell protein in treated cultures divided by nanomoles of precursor in lipids (or in glycogen) per milligram cell protein in control cultures. The cell population of a culture dish was on the order of 0.6×10^6 hepatocytes, which corresponds to 230 μg protein and 1.6 mg wet liver.

RESULTS

Short-Term Insulin Effect on [1-^{14}C]-Acetate and [U-^{14}C]-Glucose Incorporation Into Lipids and Glycogen

The time course for incorporation of [1-^{14}C]-acetate into lipids was first measured in fetal rat hepatocytes grown for 2 days in the presence of cortisol after 10 nmol/L insulin was added or not to the conditioned medium containing 0.2 mmol/L acetate. Total lipid labeling developed linearly for the first 3 hours, and the rate was 28.3 ± 1.1 nmol [1-^{14}C]-acetate/h/mg protein (n = 3) in the absence of insulin (Fig 1A). The insulin effect was hardly detectable 1 hour after its addition, with lipid labeling being slightly stimulated after 2 hours (SI, 1.19 ± 0.02; n = 4). Insulin at 100 nmol/L had the same results (SI, 1.15 ± 0.04; n = 3). When insulin was added in the presence of higher acetate concentrations up to 25 mmol/L, the hormone stimulatory effect on short-term [1-^{14}C]-acetate incorporation into lipids remained weak (Fig 2). The distribution of [1-^{14}C]-acetate between lipids and glycogen was also investigated by



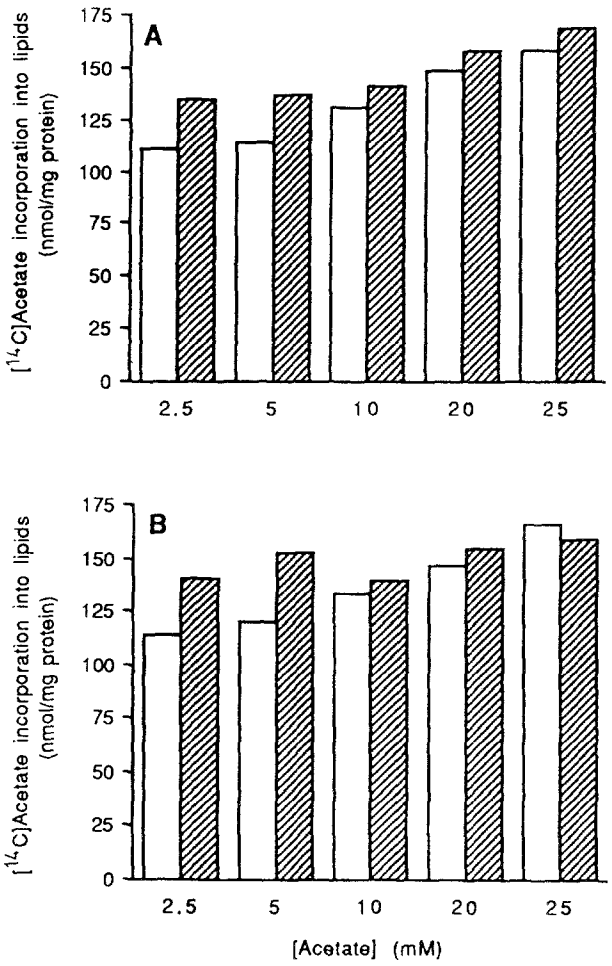


Fig 2. Effect of medium acetate concentrations on basal and insulin-stimulated [1-^{14}C]-acetate incorporation into lipids. Cells were incubated at 37°C with or without 10 nmol/L insulin in the absence (A) or presence (B) of an 8-mmol/L glucose load, and lipid synthesis was determined after exposure to [1-^{14}C]-acetate (1.0 μCi/mL) for 4 hours at the various acetate concentrations indicated. Incorporation of [1-^{14}C]-acetate into total lipids in the absence (□) and presence (▨) of insulin is shown.

parallel labeling measurements of both products. As expected, the low amount of radioactivity incorporated from [1-^{14}C]-acetate into glycogen, which after 2 hours represented only 1% to 2% of the radioactivity incorporated into lipids regardless of whether insulin was present, likely corresponded to exchange reactions.

Incorporation of [U-^{14}C]-glucose into lipids was then studied in the conditioned medium containing 4 mmol/L glucose. In the presence and absence of insulin, labeling occurred linearly for

Fig 1. Time course of lipid and glycogen labeling in the presence of [1-^{14}C]-acetate or [U-^{14}C]-glucose. At day 2, cells were incubated at 37°C up to 4 hours with [1-^{14}C]-acetate or [U-^{14}C]-glucose (1.0 μCi/mL) in the presence of 10 nmol/L insulin or vehicle in conditioned medium containing 0.20 mmol/L acetate and 4 mmol/L glucose. Lipid labeling from [1-^{14}C]-acetate (A) or [U-^{14}C]-glucose (B) and glycogen labeling from [U-^{14}C]-glucose (C) in the presence of insulin (1) and in the absence of the hormone (m) are shown. Each symbol corresponds to the mean ± SD obtained with triplicate cultures.

at least 3 hours, and the rate was 3.7 ± 0.1 nmol glucose/h/mg protein (n = 3) in the absence of the hormone (Fig 1B). A slight stimulatory effect of insulin detected after 1 hour did not change thereafter (SI, 1.19 ± 0.02 after 3 hours; n = 4). Basal and insulin-stimulated time courses were thus similar to those observed with [1-^{14}C]-acetate, yet with a much lower rate of lipid labeling. In parallel cultures, incorporation of [U-^{14}C]-glucose into glycogen was also measured (Fig 1C). The insulin stimulatory effect was clear as early as 1 hour and developed with time (SI, 2.12 ± 0.1 and 4.23 ± 0.32 after 1 and 4 hours, respectively; n = 7). Moreover, glycogen labeling largely exceeded [U-^{14}C]-glucose incorporation into lipids (by 3.3-fold and 11-fold after 4 hours in the absence and presence of insulin, respectively).

Effect of Various Acetate Concentrations on [1-^{14}C]-Acetate or [U-^{14}C]-Glucose Incorporation Into Lipids

The effect of insulin on [1-^{14}C]-acetate incorporation into lipids was investigated during longer incubation periods up to 36 hours by varying the acetate concentration in the medium. Lipid labeling increased for 20 hours and then reached a plateau at the lowest acetate concentration, ie, 1 mmol/L (Fig 3A). Between 20 and 36 hours, lipid labeling increased with 2.50 and 5 mmol/L acetate, with a clear insulin stimulatory effect shown after 36 hours at all acetate concentrations (SI, 1.35 ± 0.02 at 5 mmol/L acetate; n = 3). When using [U-^{14}C]-glucose, lipid labeling increased during the first 20 hours and remained constant thereafter at 2.50 and 5 mmol/L acetate in the absence of insulin, whereas it increased throughout the incubation at 0.36 mmol/L acetate (Fig 3B). The presence of insulin produced a stimulatory effect on lipid labeling mostly after 36 hours at all acetate concentrations (SI, 1.49 ± 0.03 at 0.36 mmol/L acetate; n = 3). Thus, acetate dose dependency showed after 36 hours a progressive increase of [1-^{14}C]-acetate incorporation into lipids from 1 to 5 mmol/L acetate (Fig 3C). By contrast, a more important [U-^{14}C]-glucose incorporation into lipids was obtained at low acetate concentration, with incorporation not being modified between 2.50 and 5 mmol/L. The stimulatory effect of insulin, whatever the precursor, did not vary greatly with acetate concentration.

Saturation of the incorporation of [1-^{14}C]-acetate into lipids with the lower concentration of 1 mmol/L acetate could be attributed to the consumption of acetate by the cells. This consumption was examined during 48 hours under culture conditions using acetate supplementation or renewal of the medium, which permitted acetate concentrations to vary from 5 to 10 mmol/L. The results showed that a 5-mmol/L acetate concentration when the medium was not supplemented for the 48-hour period seemed to be insufficient, since an important part of acetate (60%) was consumed by the cells; yet acetate consumption became saturated with further acetate supplies up

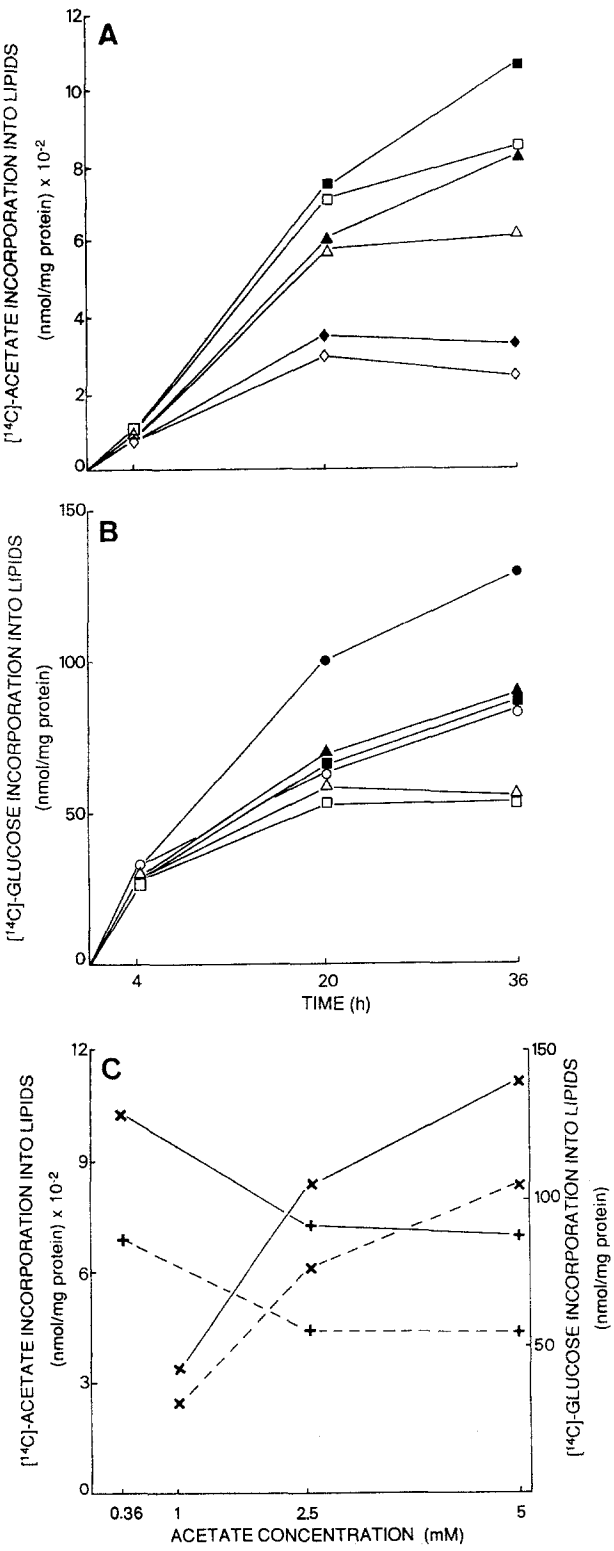


Fig 3. Effect of various medium acetate concentrations on [1-^{14}C]-acetate and [U-^{14}C]-glucose incorporation into lipids. At day 2, medium was replaced and cells were incubated at 37°C up to 36 hours with [1-^{14}C]-acetate (0.2 μCi/μmol) (A) or [U-^{14}C]-glucose (0.2 μCi/μmol) (B) at various acetate concentrations. Labeling of total lipids in the presence of 0.36 (●, ○), 1 (◆, ◇), 2.50 (▲, △), and 5 mmol/L (■, □) acetate in the presence (filled symbols) and absence (empty symbols) of insulin is represented. (C) Results obtained after 36 hours are expressed as a function of acetate concentration. Incorporation of [1-^{14}C]-acetate (×) and [U-^{14}C]-glucose (+) into total lipids in the presence (—) and absence (----) of 10 nmol/L insulin is shown. A representative experiment is shown where SD values for measurements of triplicate cultures were in all cases < 15% of the mean values shown.

Table 1. Acetate Consumption by the Cells During Long Incubation Periods

Culture Conditions	Addition	Acetate Consumption (μmol/48 h/ culture	Lipid Synthesis (nmol/mg protein)
5 mmol/L acetate at 18 h	No insulin added	3.12 ± 0.29	367.6 ± 35.0
	10 nmol/L insulin	3.37 ± 0.30	488.1 ± 50.2*
5 mmol/L acetate at 18 h, medium renewed after 24 h	No insulin added	7.55 ± 0.65	553.8 ± 52.3
	10 nmol/L insulin	7.72 ± 0.69	778.4 ± 69.4*
5 mmol/L acetate at 18 h, medium supplemented with 5 mmol/L acetate after 24 h	No insulin added	7.33 ± 0.67	621.5 ± 59.1
	10 nmol/L insulin	7.42 ± 0.65	954.0 ± 87.0*
10 mmol/L acetate at 18 h	No insulin added	7.44 ± 0.70	665.4 ± 62.7
	10 nmol/L insulin	7.55 ± 0.67	1,011.4 ± 98.3*

NOTE. Cells were incubated as from 18 hours with [1-^{14}C]-acetate under the different culture conditions indicated in the presence or absence of 10 nmol/L insulin for a further 48-hour period, during which acetate specific activity was maintained at the same value (0.2 μCi/μmol). Acetate concentration in the medium was measured after 24 and 48 hours, and [1-^{14}C]-acetate incorporation into lipids was determined at the end of incubation. Values are the mean ± SD from data obtained with triplicate cultures.

*$P < .01$ *v* corresponding control in the absence of insulin.

to 10 mmol/L (30%) (Table 1). The presence of insulin did not significantly modify consumption of acetate whatever the culture conditions tested. Basal and insulin-stimulated lipid synthesis was enhanced when the medium was enriched with acetate from 5 to 10 mmol/L (~twofold), whereas the stimulation of lipid synthesis by insulin was not greatly modified (SI, 1.30 to 1.50).

Long-Term Insulin Effect on Lipogenesis and Glycogenesis and Effect of Glucocorticoids

The effect of insulin was also investigated in continuous-labeling experiments in the presence of [1-^{14}C]-acetate and [U-^{14}C]-glucose during 3 days of culture, with 10 nmol/L insulin and/or 8.0 mmol/L glucose added at 18 hours. This led to different lipogenesis time courses. [1-^{14}C]-acetate incorporation into lipids increased during the first 48 hours of culture and tended to reach a plateau between 54 and 66 hours in the absence of insulin regardless of whether a glucose load was present (Fig 4A). A clear insulin stimulation of lipid labeling was shown between 30 and 54 hours, whereas the concomitant presence of a glucose load had a slight beneficial effect during the entire incubation period. When [U-^{14}C]-glucose was used, the stimulatory effect of insulin appeared as early as 30 hours (Fig 4B). The same situation was observed with a glucose load alone, and additive effects were present when both agents were added together. The increase in lipid labeling between 18 and 66 hours was fourfold in the presence of insulin and reached 6.4-fold in the presence of both agents, with a corresponding increase in untreated cultures of 2.4-fold.

In parallel experiments, the effect of insulin and a glucose load was studied on [U-^{14}C]-glucose incorporation into glycogen and glycogen content for 48 hours (Table 2). Insulin and a glucose load increased the radioactivity present in glycogen to the same extent (SI, 2.75 ± 0.12 and 2.66 ± 0.15, respectively; n = 3). Simultaneous addition of both agents gave a stimulatory effect more important than in the presence of either agent alone (SI, 7.30 ± 0.35; n = 3). Glycogen content was increased 2.5-fold in the presence of insulin, 1.5-fold in the presence of a glucose load, and 4.5-fold in the presence of both agents. The glycogen content appeared less stimulated in the presence of a

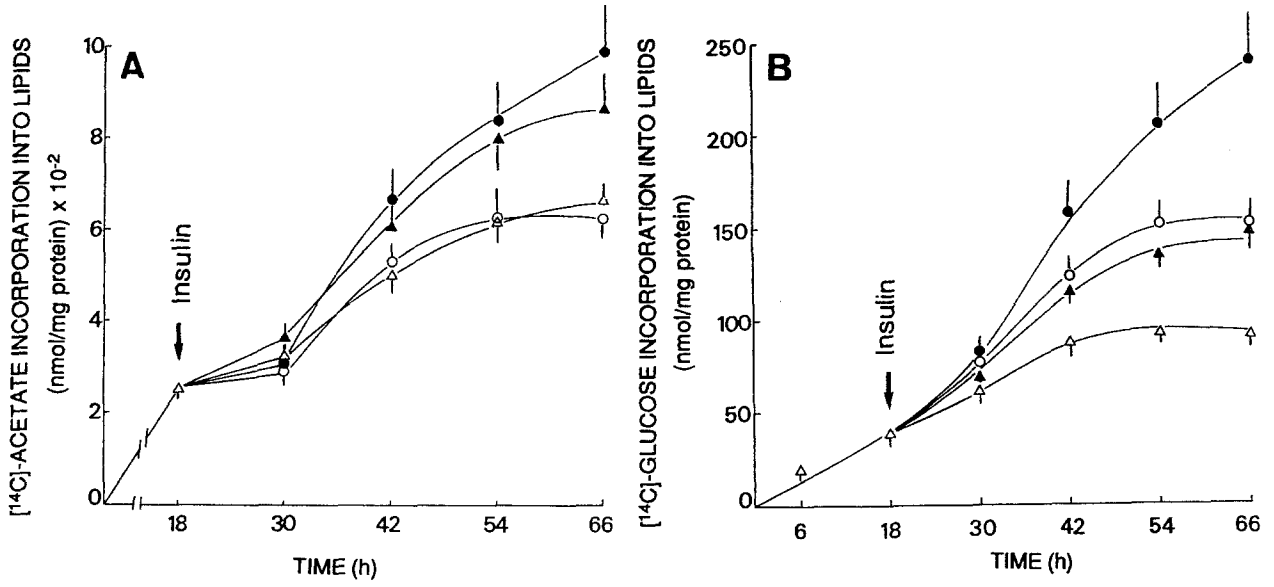


Fig 4. Effect of insulin and/or a glucose load on [1-^{14}C]-acetate and [U-^{14}C]-glucose incorporation into lipids during long incubation periods. [1-^{14}C]-acetate (5 mmol/L) or [U-^{14}C]-glucose (5.5 mmol/L) was added at the start of culture to produce a final activity of 0.2 μCi/μmol, which was maintained throughout the 3-day culture period. After renewal of medium at 18 hours, 10 nmol/L insulin or vehicle and/or 8 mmol/L glucose (final concentration in medium, 13.5 mmol/L) was added, and cells were incubated for a further 48 hours. Lipid labeling from [1-^{14}C]-acetate (A) and [U-^{14}C]-glucose (B) in the presence (●, ○) or absence (▲, △) of a glucose load and in the presence (filled symbols) or absence (empty symbols) of 10 nmol/L insulin is shown. Each symbol corresponds to the mean ± SD obtained with triplicate cultures.

Table 2. Long-Term Insulin Effect on [U-^{14}C]-Glucose Incorporation Into Glycogen and Glycogen Content (nmol/mg protein)

Additions	[U-^{14}C]-Glucose Incorporation Into Glycogen	Glycogen Content
No insulin added	180 ± 21	452 ± 48
10 nmol/L insulin	495 ± 49	1,132 ± 100
8 mmol/L glucose	479 ± 55	690 ± 70
10 nmol/L insulin + 8 mmol/L glucose	1,313 ± 126	2,048 ± 198

NOTE. [U-^{14}C]-glucose was added at the start of culture to produce a final activity of 0.2 μCi/μmol, which was maintained throughout the 3-day culture period. After renewal of medium at 18 hours, 10 nmol/L insulin or vehicle and/or 8 mmol/L glucose (final concentration in the medium, 13.5 mmol/L) were added and cells were incubated for a further 48 hours. [U-^{14}C]-glucose incorporation into glycogen and glycogen content are the mean ± SD for 3 experiments performed with different cell preparations.

glucose load than with insulin, contrary to the results obtained for glycogen labeling. This was due to differences in the relative contribution of glucose to glycogen formation.[25,26]

In these experiments, cortisol (10 μmol/L) was always present in the culture medium. As glucocorticoids are known to be necessary for development of the insulin glycogenic response in cultured fetal rat hepatocytes,[27] it was of interest to compare the effect of glucocorticoids on [1-^{14}C]-acetate incorporation into lipids and on glycogen content in the presence of 5 mmol/L acetate. Labeled precursors and insulin were added after 18 hours at the time of medium renewal, and cultures were incubated for a further 48 hours. [1-^{14}C]-acetate and [U-^{14}C]-glucose incorporation into lipids was slightly decreased when cells were grown in the absence of glucocorticoids, whereas the stimulatory effect of insulin on lipogenesis was similar in the presence and absence of glucocorticoids (SI, 1.34 ± 0.15 and 1.28 ± 0.14, respectively; n = 3) (Fig 5A). [U-^{14}C]-glucose incorporation into lipids showed a comparable pattern. In parallel cultures, glycogen content and the insulin glycogenic response were strikingly dependent on the presence of glucocorticoid (Fig 5B). This situation was identical regardless of whether the medium was supplemented with 5 mmol/L acetate. On the other hand, the effect of the deprivation of fetal calf serum during 48 hours after the initial 18-hour period in its presence was tested in terms of lipid synthesis. In the absence of serum, incorporation of both [1-^{14}C]-acetate and [U-^{14}C]-glucose into lipids was increased under basal and insulin-stimulated conditions (Table 3). This serum effect could be due to the fact that a metabolite originating from a precursor present in the serum diluted the radioactivity of the label and decreased lipid labeling independently of insulin. As to the stimulatory effect of insulin on lipid labeling, it was enhanced significantly for both precursors when the medium was deprived of serum (1.58 ± 0.19 *v* 1.32 ± 0.17, n = 4, $P < .05$, in the presence of [1-^{14}C]-acetate).

Long-Term Insulin Effect on Fatty Acid Synthase and Malic Enzyme Activities

The effect of insulin on lipogenic enzyme activities, ie, fatty acid synthase and malic enzyme, was investigated in cultures grown in the presence of glucocorticoids after a 48-hour period in the presence and absence of insulin as described for Fig 4. Under such conditions, insulin enhanced the cytosolic protein content by 20% with no modification of total proteins. This could not be due to differences in cytosolic protein degradation, which has been shown to be unaffected by insulin in cultured fetal hepatocytes.[28] Insulin increased fatty acid synthase specific activity by 26%, whereas a glucose load (15 mmol/L) had no effect, with the stimulatory effect of the simultaneous presence of insulin and a glucose load being similar to that obtained with insulin alone (Table 4). Malic enzyme specific

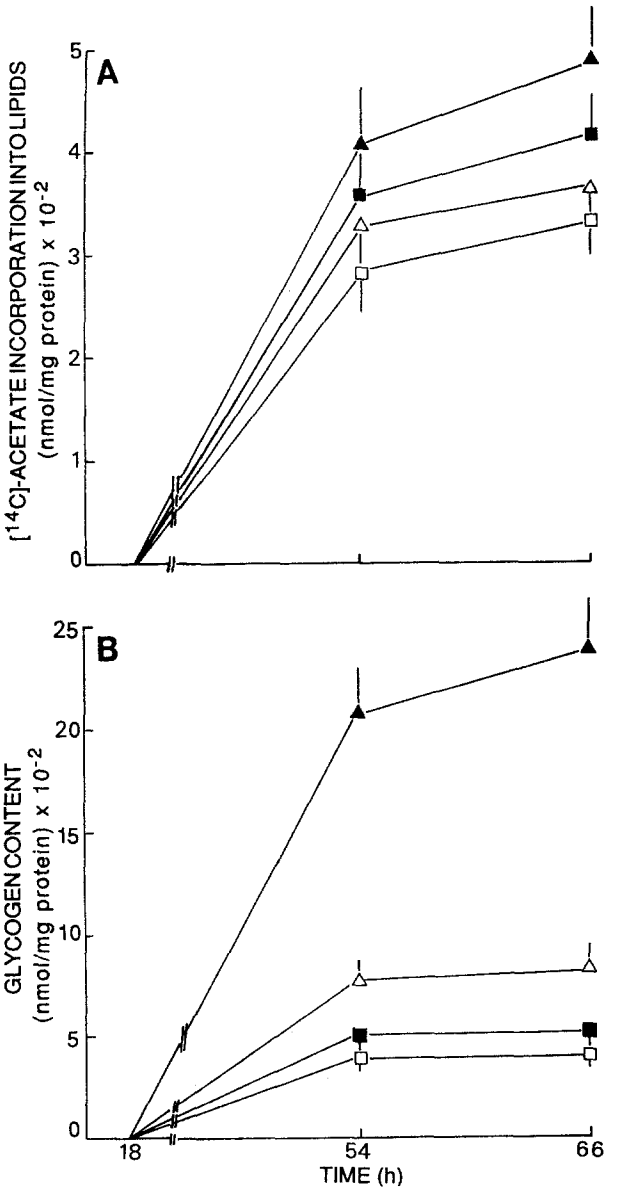


Fig 5. Compared effects of glucocorticoids in lipogenesis and glycogenesis. Two parallel sets of culture were grown in the presence or absence of glucocorticoids. After renewal of medium at 18 hours, cells were incubated with [1-^{14}C]-acetate (5 mmol/L, 0.2 μCi/μmol) for a further 48 hours, with insulin (10 nmol/L) present or not from 18 hours. Lipid labeling from [1-^{14}C]-acetate (A) and glycogen content (B) in cells grown in the presence (▲, △) or absence (■, □) of 10 μmol/L cortisol and incubated in the presence (filled symbols) or absence (empty symbols) of 10 nmol/L insulin are shown. Each symbol corresponds to the mean ± SD obtained with triplicate cultures.

Table 3. Effect of Fetal Calf Serum on Basal and Insulin-Stimulated [1-^{14}C]-Acetate and [U-^{14}C]-Glucose Incorporation Into Lipids (nmol/mg protein)

Media	[U-^{14}C]-Acetate Incorporation Into Lipids	[U-^{14}C]-Glucose Incorporation Into Lipids
Medium containing serum		
No insulin added	367.6 ± 35.2	175.5 ± 16.2
10 nmol/L insulin	488.1 ± 50.1	228.7 ± 20.4
Medium deprived of serum		
No insulin added	428.0 ± 41.7	283.7 ± 30.2
10 nmol/L insulin	661.3 ± 59.3	417.7 ± 41.5

NOTE. After 18 hours of culture in the presence of glucocorticoids and fetal calf serum, medium was replaced by a medium deprived or not of serum but always supplemented with glucocorticoids. At this time, [1-^{14}C]-acetate (5 mmol/L, 0.2 μCi/μmol) or [U-^{14}C]-glucose (5.5 mmol/L, 0.2 μCi/μmol) together with 10 nmol/L insulin or vehicle were added and cells were incubated for a further 48 hours. Values are the mean ± SD from data obtained with triplicate cultures.

activity was not modified in the presence of insulin or a glucose load, whereas the combination of the two agents increased this activity by 20%.

Insulin Effect on Cellular Fluorescence Produced by Nile Red Staining

It was of interest to measure in parallel experiments the neutral lipid content of fetal hepatocytes. Cell staining with Nile red, a fluorescent dye that provides resolution of cytoplasmic neutral lipid droplets,[24] showed striking differences in the fluorescence distribution between fetal hepatocytes incubated in the presence and absence of insulin (Fig 6). When estimated by an image-analysis system, cells were found to display larger and more numerous neutral lipid droplets in the presence of insulin: the number of lipid droplets expressed per arbitrary representative unit of cell culture surface was 87 ± 10 and 56 ± 7 (n = 4) in the presence and absence of insulin, respectively, with a corresponding mean droplet area of 110 ± 23 and 72 ± 10 mm^2.

Table 4. Long-Term Insulin Effect on Fatty Acid Synthase and Malic Enzyme Activities

Additions	Fatty Acid Synthase Activity (U/mg protein)	Malic Enzyme Activity (U/mg protein)	Protein Cytosolic Content (μg/culture)
No insulin added	4.1 ± 0.9	18.8 ± 5.5	97.7 ± 9.9
10 nmol/L insulin	5.2 ± 1.0†	18.0 ± 3.8	118.3 ± 6.8*
15 mmol/L glucose	4.4 ± 0.5	19.6 ± 6.6	103.7 ± 9.5
10 nmol/L insulin + 15 mmol/L glucose	5.3 ± 1.4*	22.5 ± 8.1†	122.0 ± 9.7*

NOTE. After 18 hours of culture, 10 nmol/L insulin and/or 15 mmol/L glucose was added to the incubation medium and cells were incubated up to 66 hours. Fatty acid synthase and malic enzyme activities were determined. Values for enzyme activities are the mean ± SD for 3 experiments, with 1 U enzyme activity defined as the amount of enzyme needed to catalyze oxidation or reduction of 1 nmol NADPH or NADP per minute at 35°C. Total protein content was 332 ± 26 and 318 ± 29 μg/culture in the presence and absence of insulin, respectively.

*$P < .05$, †$P < .01$: significant difference from cultures without additions.

DISCUSSION

Lipid synthesis was studied in primary cultures of 18-day-old fetal rat hepatocytes using two different labeled precursors: [1-^{14}C]-acetate and [U-^{14}C]-glucose. [1-^{14}C]-acetate has been shown to be a useful tool to measure lipogenesis and its stimulation by insulin in adult hepatocytes.[7,8] [U-^{14}C]-glucose was of interest for the study of the relationship between lipid and glycogen synthesis, as the latter is known to be strongly stimulated by insulin in cultured fetal hepatocytes.[15,26] The results obtained corresponded to an apparent incorporation of acetate and glucose into lipids, since ^{14}C label from such exogenous substrates can be diluted by endogenous substrate carbon.[29] The rate of lipid labeling with [1-^{14}C]-acetate was much higher than with [U-^{14}C]-glucose, a result previously obtained with liver slices from term rat fetus.[30]

Comparison between lipogenesis and glycogenesis in cultured fetal hepatocytes during short incubation periods showed that [1-^{14}C]-acetate was only incorporated into lipids, whereas incorporation of [U-^{14}C]-glucose was much more pronounced into glycogen than into lipids. With both precursors, the short-term insulin lipogenic response remained weak. A similar result has been obtained for [1-^{14}C]-acetate incorporation into lipids in isolated hepatocytes from term fetuses.[31] In contrast to these investigators, we did not find any increase in the insulin stimulatory effect on lipid labeling at high acetate concentrations (≤25 mmol/L). Such a discrepancy may be due to the different hepatocyte "in vitro" systems and ages of fetuses. As for glycogenesis, in agreement with previous studies,[15] a strong stimulatory effect of insulin was maximally expressed in cultured fetal hepatocytes after 2 hours of incubation, showing a short-term insulin responsiveness much more marked on glycogenesis than on lipogenesis.

For longer periods of exposure, the rate of lipid labeling with both precursors varied when increasing the acetate concentration in the medium, yet with a progressive increase for [1-^{14}C]-acetate and a decrease for [U-^{14}C]-glucose. It follows that differences in the apparent efficiency of the two labeled precursors were less pronounced at low acetate concentrations. The stimulatory effect of insulin developed from 20 to 48 hours of hormone exposure whatever the precursor. During long incubation periods, the concentration of 5 mmol/L acetate in the medium could be rate-limiting for [1-^{14}C]-acetate incorporation into lipids. According to the different methods of acetate supplementation used, an increase in lipid synthesis was obtained without an increase in the stimulatory effect of insulin. A similar long-term insulin stimulatory effect has been shown on [1-^{14}C]-acetate incorporation in cultured adult hepatocytes incubated for 20 hours with insulin in the presence of 5 mmol/L acetate.[8,32] It is not known whether concentrations in fetal plasma are within the range of those (0.10 to 0.50 mmol/L) found in adult rat blood.[33,34]

The pronounced stimulatory effect of insulin on lipid labeling after 48 hours of hormone exposure was associated with an increase in the number and size of lipid droplets. The higher cellular neutral lipid content could be accounted for by an increased lipid synthesis, although a decreased lipoprotein secretion in response to insulin could intervene, as shown in cultured adult hepatocytes.[35] The long-term effect of insulin on lipogenesis in cultured fetal hepatocytes should be linked to an

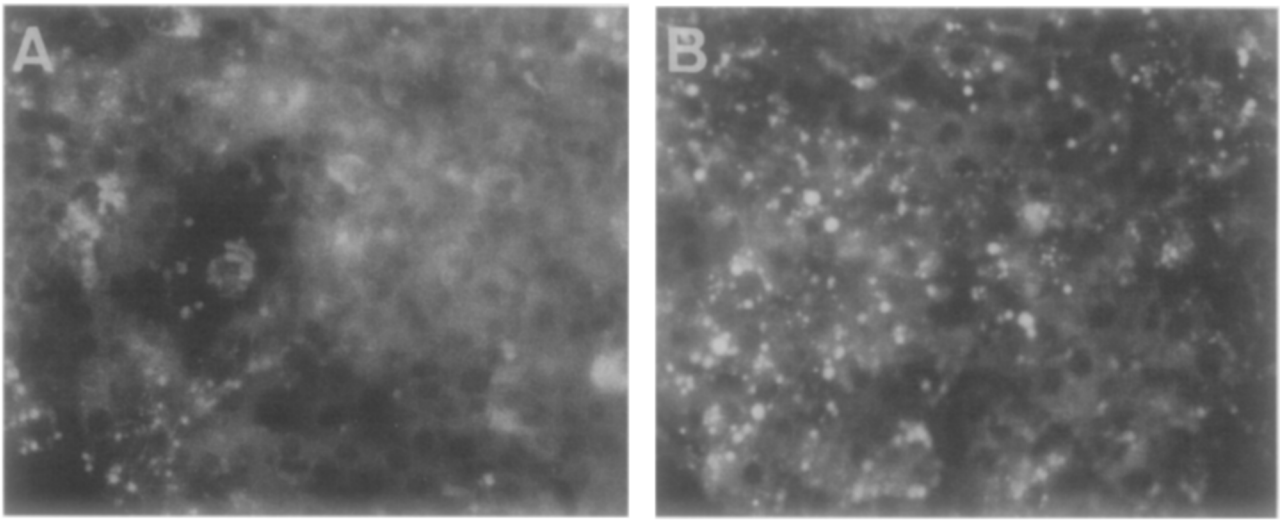


Fig 6. Effect of insulin on Nile red fluorescence of cultured fetal hepatocytes. Cells incubated under the same conditions as in Fig 3 were grown on glass cover slips and stained with Nile red (100 ng/mL). After staining, cells were examined with a Leitz Orthoplan fluorescence microscope. Representative fields of cells incubated in the absence (A) or presence (B) of 10 nmol/L insulin and photographed at identical microscope and camera settings are shown.

increase in the activity of some key lipogenic enzymes. The activity of fatty acid synthase, a multifunctional enzyme, was increased either by insulin or by insulin together with a glucose load, but not by a glucose load alone. By contrast, glucose has been found to enhance stimulation of the enzyme activity in the presence of insulin in cultured term fetal and adult hepatocytes.[13,36,37] Malic enzyme plays an important role by providing NADPH for lipogenesis (together with the active pentose phosphate pathway) and participates with insulin in the coordinated regulation in response to nutritional factors in adult liver.[1,5] In this study, an increase in malic enzyme activity was observed only in the simultaneous presence of insulin and a glucose load. Malic enzyme activity has been found to be scarcely detectable in the rat fetus,[38,39] and insulin in the presence of glucocorticoids has a low stimulatory effect on the enzyme protein content in cultured fetal rat hepatocytes.[40] Another key lipogenic enzyme, acetyl CoA carboxylase, catalyzes the first necessary step of fatty acid biosynthesis. This enzyme is concerned with a short-term regulation by allosteric effectors and phosphorylation-dephosphorylation in adult liver.[6,41] It has been shown to be activated by insulin in isolated adult rat hepatocytes, in which fatty acid synthase is not subject to acute hormonal regulation.[42] The activity of acetyl CoA carboxylase was barely detectable in extracts of cultured fetal hepatocytes (H. M'Zali, unpublished observation, July 1996), in accordance with the fact that the activity of this enzyme has been shown to be low and rate-limiting in the fetal liver.[12,43]

Insulin regulation of the key lipogenic enzymes at a pretranslational and transcriptional level has been the object of many important studies.[6] In cultured adult rat hepatocytes, insulin has been shown to regulate total RNA and poly(A)$^+$ RNA content, as well as expression of specific mRNAs.[44] In chick embryo hepatocytes in culture, insulin amplifies the triiodothyronine-induced enzyme activity together with the level and rate of transcription of mRNA for both fatty acid synthase and malic enzyme, but has little or no effect by itself.[6,14] Levels of malic enzyme mRNA, which was barely detectable throughout the neonatal rat period,[45] have been found to be increased in cultured fetal hepatocytes by insulin when added in combination with triiodothyronine.[40] The enzymatic equipment could be specific to the fetal liver with regard to lipid metabolism.[46] For example, fatty acid synthesis is active in fetal liver despite the extremely low levels of mRNA-S14 encoding for a protein involved in liver lipogenesis in adult rats and at weaning.[39]

The situation for lipogenesis regulation in cultured fetal hepatocytes differs from that described for adult isolated and cultured hepatocytes,[7,8] since their ability for a rapid insulin response on lipogenesis was poor. Also, the insulin responses characteristic of adult hepatocytes, ie, stimulation of lactate production,[47] stimulation of the amino acid transport A system,[48,49] and inhibition of protein degradation,[50] are not present in fetal hepatocytes.[26,28,31,50-52] Glucocorticoids did not influence the pattern of insulin regulation of lipogenesis in cultured fetal hepatocytes. This was at variance with the situation for glycogenesis, inasmuch as exposure to glucocorticoids is known to allow development of an acute glycogenic response to insulin,[27] proving the presence of a functional insulin receptor at these stages. The situation particular to cultured fetal hepatocytes requiring a delay for insulin to stimulate lipogenesis and accompanied by stimulation of fatty acid synthase activity could be due to incomplete maturation of key lipogenic liver enzymes in late gestation. The results of the present study show that the anabolic insulin action was exerted first on glycogenesis and then on lipogenesis during fetal hepatocyte development in culture.

ACKNOWLEDGMENT

This research work is taken from a Doctorat ès Sciences submitted by H. M'Zali and supervised by Prof B. Azzout. We thank E. Marie-Rose for skillful secretarial services. We wish to acknowledge the encouragement and help of Professor N. Forest, in whose laboratory part of this study was performed.

REFERENCES

1. Nepokroeff CM, Lakshmanan MR, Ness GC, et al: Coordinate control of rat liver lipogenic enzymes by insulin. Arch Biochem Biophys 162:340-344, 1974
2. Girard J, Ferré P, Pégorier J-P, et al: Adaptations of glucose and fatty acid metabolism during perinatal period and suckling-weaning transition. Physiol Rev 72:507-562, 1992
3. McCormick KL, Susa JB, Widness JA, et al: Chronic hyperinsulinemia in the fetal rhesus monkey. Effects on hepatic enzymes active in lipogenesis and carbohydrate metabolism. Diabetes 28:1064-1068, 1979
4. Susa JB, Gruppuso PA, Widness JA, et al: Chronic hyperinsulinemia in the fetal rhesus monkey: Effects of physiologic hyperinsulinemia on fetal substrates, hormones, and hepatic enzymes. Am J Obstet Gynecol 150:415-420, 1984
5. Gibson DM, Lyons RT, Scott DF, et al: Synthesis and degradation of the lipogenic enzymes of rat liver. Adv Enzyme Regul 10:187-204, 1972
6. Hillgartner FB, Salati LM, Goodridge AG: Physiological and molecular mechanisms involved in nutritional regulation of fatty acid synthesis. Physiol Rev 75:47-67, 1995
7. Beynen AC, Vaartjes WJ, Geelen MJH: Opposite effects of insulin and glucagon in acute hormonal control of hepatic lipogenesis. Diabetes 28:828-835, 1979
8. Caro JF, Amatruda JM: The regulation of lipid synthesis in freshly isolated and primary cultures of hepatocytes from fasted rats: The primary role of insulin. Metabolism 31:14-18, 1982
9. Ktorza A, Bihoreau M-T, Nurjhan N, et al: Insulin and glucagon during the perinatal period: Secretion and metabolic effects on the liver. Biol Neonate 48:204-220, 1985
10. Menuelle P, Plas C: Variations in the antagonistic effects of insulin and glucagon on glycogen metabolism in cultured foetal hepatocytes. Biochem J 277:111-117, 1991
11. Benito M, Lorenzo M, Medina JM: Relationship between lipogenesis and glycogen synthesis in maternal and foetal tissues during late gestation in the rat. Effect of dexamethasone. Biochem J 204:865-868, 1982
12. Pillay D, Bailey E: Perinatal lipogenesis in the liver and brown adipose tissue of the rat. Int J Biochem 14:511-517, 1982
13. Roncero C, Lorenzo M, Fabregat I, et al: Rates of lipogenesis in fetal hepatocytes in suspension and in primary culture: Hormonal effects. Biochim Biophys Acta 1012:320-324, 1989
14. Stapleton SR, Mitchell DA, Salati LM, et al: Triiodothyronine stimulates transcription of the fatty acid synthase gene in chick embryo hepatocytes in culture. Insulin and insulin-like growth factor amplify that effect. J Biol Chem 265:18442-18446, 1990
15. Plas C, Menuelle P, Moncany MLJ, et al: Time dependence of the glycogenic effect of insulin in cultured fetal hepatocytes. Diabetes 28:705-712, 1979
16. Plas C, Chapeville F, Jacquot R: Development of glycogen storage ability under cortisol control in primary cultures of rat fetal hepatocytes. Dev Biol 32:82-91, 1973
17. Evans VJ, Bryant JC, Kerr HA, et al: Chemically defined media for cultivation of long-term cell strains from four mammalian species. Exp Cell Res 36:439-474, 1964
18. Bligh EG, Dyer WJ: A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911-917, 1959
19. Lowry OH, Rosebrough NJ, Farr AL, et al: Protein measurement with the Folin phenol reagent. J Biol Chem 193:265-275, 1951
20. Guichard C, Dugail I, Le Liepvre X, et al: Genetic regulation of fatty acid synthetase expression in adipose tissue: Overtranscription of the gene in genetically obese rats. J Lipid Res 33:679-687, 1992
21. Halestrap AP, Denton RM: Insulin and the regulation of adipose tissue acetyl-coenzyme A carboxylase. Biochem J 132:509-517, 1973
22. Ochoa S: "Malic" enzyme from pigeon liver and wheat germ. Methods Enzymol 1:739-753, 1955
23. Bazin R, Lavau M: Development of hepatic and adipose tissue lipogenic enzymes and insulinemia during suckling and weaning onto a high-fat diet in Zucker rats. J Lipid Res 23:839-849, 1982
24. Greenspan P, Mayer EP, Fowler SD: Nile red: A selective fluorescent stain for intracellular lipid droplets. J Cell Biol 100:965-973, 1985
25. Menuelle P, Buc HA, Plas C: Differences between glucose and insulin stimulation of glycogenesis in cultured fetal hepatocytes. Biochim Biophys Acta 928:332-340, 1987
26. Menuelle P, M'Zali H, Forest N, et al: Compared roles of glucose, galactose and fructose as glycogen precursors during the acute response to insulin in cultured foetal hepatocytes. Int J Biochem 20:777-782, 1988
27. Plas C, Nunez J: Role of cortisol on the glycogenic effect of glucagon and on the glycogenic response to insulin in fetal hepatocyte culture. J Biol Chem 251:1431-1437, 1976
28. Peavy DE, DeSante DC: Protein degradation in cultured fetal hepatocytes. Absence of an inhibitory effect of insulin. Biochem J 267:671-677, 1990
29. Bottomley S, Garcia-Webb P: Rat hepatocyte lipogenesis and insulin-stimulated lipogenesis: Comparison of metabolite effects and methods of measurement. Biochem Int 14:751-758, 1987
30. Ballard FJ, Hanson RW: Ranges in lipid synthesis in rat liver during development. Biochem J 102:952-958, 1967
31. Miller JD, Sinha MK, Sperling MA, et al: Insulin stimulates amino acid and lipid metabolism in isolated fetal rat hepatocytes. Pediatr Res 20:609-612, 1986
32. Amatruda JM, Danahy SA, Chang CL: The effects of glucocorticoids on insulin-stimulated lipogenesis in primary cultures of rat hepatocytes. Biochem J 212:135-141, 1983
33. Buckley BM, Williamson DM: Origins of blood acetate in the rat. Biochem J 166:539-545, 1977
34. Majchrowicz E, Hunt WA: Effect of ethanol on the disappearance of acutely administered acetate from the blood in rats. Biochem Pharmacol 27:128-130, 1978
35. Bartlett SM, Gibbons GF: Short- and long-term regulation of very low-density lipoprotein secretion by insulin, dexamethasone and lipogenic substrates in cultured hepatocytes. A biphasic effect of insulin. Biochem J 249:37-43, 1988
36. Spence JT, Pitot HC: Induction of lipogenic enzymes in primary cultures of rat hepatocytes. Relationship between lipogenesis and carbohydrate metabolism. Eur J Biochem 128:15-20, 1982
37. Giffhorn-Katz S, Katz NR: Carbohydrate-dependent induction of fatty acid synthase in primary cultures of rat hepatocytes. Eur J Biochem 159:513-518, 1986
38. Taylor CB, Bailey E, Bartley W: Changes in hepatic lipogenesis during development of the rat. Biochem J 105:717-722, 1967
39. Perez-Castillo A, Schwartz HL, Oppenheimer JH: Rat hepatic mRNA-S14 and lipogenic enzymes during weaning: Role of S14 in lipogenesis. Am J Physiol 253:E536-E542, 1987
40. Molero C, Benito M, Lorenzo M: Regulation of malic enzyme gene expression by nutrients, hormones, and growth factors in fetal hepatocyte primary cultures. J Cell Physiol 155:197-203, 1993
41. Geelen MJH, Harris RA, Beynen AC, et al: Short-term hormonal control of hepatic lipogenesis. Diabetes 29:1006-1022, 1980
42. Witters LA, Moriarity D, Martin DB: Regulation of hepatic acetyl coenzyme A carboxylase by insulin and glucagon. J Biol Chem 254:6644-6649, 1979
43. Lockwood EA, Bailey E, Taylor CB: Factors involved in changes in hepatic lipogenesis during development of the rat. Biochem J 118:155-162, 1970

44. Hsu C-J, Kimball SR, Antonetti DA, et al: Effects of insulin on total RNA, poly(A)$^+$ RNA, and mRNA in primary cultures of rat hepatocytes. Am J Physiol 263:E1106-E1112, 1992

45. Mann DJ, Bailey E: Pre-translational control of hepatic malic enzyme expression during the development of the rat. Biochem J 279:407-412, 1991

46. Fain JN, Scow RO: Fatty acid synthesis in vivo in maternal and fetal tissues in the rat. Am J Physiol 210:19-25, 1966

47. Probst I, Jungermann K: Short-term regulation of glycolysis by insulin and dexamethasone in cultured rat hepatocytes. Eur J Biochem 135:151-156, 1983

48. Kletzien RF, Pariza MW, Becker JE, et al: Induction of amino acid transport in primary cultures of adult rat liver parenchymal cells by insulin. J Biol Chem 251:3014-3020, 1976

49. Fehlmann M, Le Cam A, Freychet P: Insulin and glucagon stimulation of amino acid transport in isolated rat hepatocytes. Synthesis of a high affinity component of transport. J Biol Chem 254:10431-10437, 1979

50. Hopgood MF, Clark MG, Ballard FJ: Protein degradation in hepatocyte monolayers. Effects of glucagon, adenosine 3′:5′-cyclic monophosphate and insulin. Biochem J 186:71-79, 1980

51. Handlogten ME, Kilberg MS: Transport system A is not responsive to hormonal stimulation in primary cultures of fetal rat hepatocytes. Biochem Biophys Res Comm 108:1113-1119, 1982

52. Bismut H, Poggi-Bach J, Plas C: Consumption and production of amino acids by insulin-responsive cultured fetal rat hepatocytes: The particular case of serine. Biol Neonate 62:37-46, 1992