LIPOGENESIS IN MAINTENANCE CULTURES OF RAT HEPATOCYTES

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1. Introduction

Induction of the several enzymes in the liver cytosol catalyzing de novo synthesis of fatty acids from glucose* has been demonstrated in intact animals (for review, see [1]). When carbohydrate is provided to previously starved rats the metabolism of liver switches from a gluconeogenic-ketogenic economy to a program facilitating net glycogen and triglyceride synthesis. Induction of the lipogenic enzyme set* [1–3] which commences at least 3 h after carbohydrate alimentation, is preceded by short-term control events which include the accumulation of acetyl precursors (e.g. pyruvate and citrate) that act as positive feed-forward effectors [4,5], as well as the relative dephosphorylation of interconvertible enzymes that channel glucose into glycogenesis and lipogenesis [6,7]. Induction of the lipogenic enzymes seems to be initiated by insulin and counteracted by glucagon (via cAMP) [1,2,8].

With the advent of methods for preparing suspensions of intact hepatocytes [9–11] came the prospect of studying the sequence of events leading to the induction of the lipogenic enzymes in vitro. Although of proven value in examining short-term control [12,13], suspensions of hepatocytes from mature animals incubated in simple buffered systems have not proved useful for demonstrating insulin-dependent induction of the fatty acid synthesizing enzymes. Other approaches nevertheless have been rewarding. Alberts et al. [14] achieved net synthesis of acetyl-CoA carboxylase and fatty acid synthase in proliferating cultures of HeLa and Chang liver cells in response to insulin. Similarly, Goodridge et al. [15] demonstrated induction of malate enzyme, fatty acid synthase and citrate lyase by triiodothyronine (but not by insulin) in primary cultures of hepatocytes from neonatal chicks.

Recently Bissell et al. [16] and Bonney et al. [17] have successfully plated hepatocytes isolated from mature rats and have maintained these non-proliferating cells for days in Hams medium supplemented with fetal calf serum. The cultured hepatocytes retained morphological and metabolic characteristics of normal adult liver.

In this communication we present evidence that hepatocytes isolated from adult rats and incubated in Hams medium supplemented with fetal calf serum display an increased rate of lipogenesis in response to physiological concentrations of insulin, and that the insulin effect is blocked by low concentrations of glucagon.

*This group of enzymes includes acetyl-CoA carboxylase (EC 6.4.1.2), fatty acid synthase complex, ATP-citrate lyase (EC 4.1.3.8), malic enzyme (EC 1.1.1.40), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and glucokinase (EC 2.7.1.2).

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2. Materials and methods

Male Wistar rats weighing 250–300 g were used throughout in these studies. Animals were starved for 24 h prior to use. Isolated hepatocytes were prepared essentially according to the procedure of Ingebretsen and Wagle [11] under aseptic conditions. The isolated hepatocytes were suspended in Hams F-12 culture medium supplemented with 15% fetal calf serum and buffered with 14.5 mM bicarbonate and 12.5 mM each of 2-(*N*-morpholino) ethanesulfonic acid (MES) and *N*-Tris(hydroxymethyl)methyl-2-amino ethanesulfonic acid (TES) [17]. Four ml of the cell suspension ($1.5–2.0 \times 10^6$ cells) were added to vented plastic Petri dishes (Falcon, 60 mm) for plating [17]. The dishes were incubated in equilibrium with a 95% air and 5% CO_2 gas mixture at 37°C. Full details of our procedures for cell isolation and culturing will be described separately [18].

Synthesis of fatty acids and non-saponifiable lipids (NSL) was followed by measuring the rate of [1-^{14}C]-acetate or [^{3}H] H_2O incorporation into these lipid fractions. Assays were carried out essentially as described by Alberts et al. [14]. Aliquots were taken for a Lowry protein determination [19]. Collagenase, Type II, was obtained from Worthington; ^{14}C- and ^{3}H-labelled compounds from Amersham; culture media from FLOW; all other reagents from Baker. Insulin and glucagon were gifts from Dr Walter Shaw (Eli Lilly Laboratories, Indianapolis, Ind.).

Table 1
Influence of insulin and glucagon on fatty acid and NSL synthesis in hepatocyte cultures.

Additions		Time (h)[a]	Fatty acids	NSL
none		4	0.54 ± 0.04	1.03 ± 0.10
none		7	1.17 ± 0.09	0.89 ± 0.04
glucagon 10^{-8} M		7	0.25 ± 0.03	0.40 ± 0.04
glucagon 10^{-7} M		7	0.23 ± 0.01	0.34 ± 0.02
glucagon 10^{-6} M		7	0.23 ± 0.02	0.33 ± 0.06
insulin 0.01 μg/ml		7	2.54 ± 0.07	1.24 ± 0.01
insulin 0.01 μg/ml	+ glucagon 10^{-8} M	7	0.36 ± 0.05	0.43 ± 0.10
insulin 0.01 μg/ml	+ glucagon 10^{-7} M	7	0.39 ± 0.03	0.51 ± 0.05
insulin 0.01 μg/ml	+ glucagon 10^{-6} M	7	0.36 ± 0.04	0.18 ± 0.02
insulin 0.1 μg/ml		7	3.06 ± 0.13	0.68 ± 0.06
insulin 0.1 μg/ml	+ glucagon 10^{-8} M	7	0.49 ± 0.04	0.28 ± 0.03
insulin 0.1 μg/ml	+ glucagon 10^{-7} M	7	0.46 ± 0.08	0.25 ± 0.04
insulin 0.1 μg/ml	+ glucagon 10^{-6} M	7	0.45 ± 0.11	0.31 ± 0.07
insulin 1.0 μg/ml		7	3.11 ± 0.12	1.03 ± 0.14
insulin 1.0 μg/ml	+ glucagon 10^{-8} M	7	0.71 ± 0.18	0.52 ± 0.19
insulin 1.0 μg/ml	+ glucagon 10^{-7} M	7	0.47 ± 0.05	0.38 ± 0.09
insulin 1.0 μg/ml	+ glucagon 10^{-6} M	7	0.52 ± 0.09	0.43 ± 0.08

Cells were plated in Hams medium containing 15% fetal calf serum [17]. After 4 h medium was removed by aspiration and 4.0 ml fresh medium added together with hormones where indicated. For determination of fatty acid and NSL synthesis [1-^{14}C] acetate (2.5 μCi, final specific radioactivity of 0.96 mCi/mmole, including 0.24 mM endogenous acetate due to fetal calf serum) was added at the indicated times[a]. Both hormones and label were added in 50 μl quantities. After incubation for an additional hour medium was removed by aspiration and the cells were washed three times with ice-cold 0.15 M KCl. Finally, addition of 1.5 ml of 0.5N KOH solubilized all adherent cells. Fatty acid and NSL synthesis are expressed as nmoles of acetate incorporated/mg protein/60 min. Each point represents the average value ± S. D.[b] of three incubated plates.
[a]time of addition of [1-^{14}C] acetate
[b]standard deviations.

3. Results and discussion

Over 80% of freshly isolated hepatocytes from mature rats became firmly attached to the surface of the plastic dishes as a contiguous sheet of flattened cells within a period of 4 h, provided that the complete medium was employed. Microscopic examination and dye exclusion indicated that the adherent cells were intact [18]. A baseline rate of lipogenesis was determined, following the initial 4 h preincubation, in fresh medium supplemented with [1-^{14}C] acetate, i.e. the capacity of adherent cells to incorporate acetate into fatty acids (and NSL) was measured between the fourth and the fifth hour of the experiment (see line 1 of table 1). If insulin was added at 4 h and the hepatocyte culture incubated for an additional 3 h, acetate incorporation in the seventh hour was markedly enhanced in comparison to controls that were incubated in the absence of added insulin (compare lines 6,10 and 14 with 2, table 1). A significant rise in the rate of fatty acid synthesis was routinely observed, however, even though no insulin was added (compare line 2 with line 1, table 1). This increase may be attributed to endogenous factors in the fetal calf serum of the complete medium. Plated cells incubated for a total of 24 h still retained the capacity to respond to insulin over a 3 h period, as above, although the baseline level of lipogenesis (no added insulin) was 2–3 fold higher at 24 h than at 4 h (data not shown).

Glucagon added to hepatocyte cultures at 4 h completely prevented the elevation in the rate of lipogenesis anticipated 3 h later both in the presence and absence of added insulin (table 1). The apparent inhibition of lipogenesis obtained with glucagon between 10^{-6} to 10^{-8} M could not be overcome with any level of added insulin. There were no significant changes in the adherence of hepatocytes to the surface of culture dishes in response to insulin or glucagon in the 4 h experimental period (as reflected in the protein remaining on the plates following three washes with cold buffer at the conclusion of the lipogenesis assays).

The rate of incorporation of [1-^{14}C]acetate into the NSL fraction was followed routinely. In general this function of hepatocytes paralleled the rate of fatty acid synthesis in response to insulin and glucagon, although percent changes in the rate of NSL synthesis with and without added hormones were often less pronounced.

An effort was made to ascertain the concentrations of insulin that were suboptimal in the hepatocyte culture system between the fourth and seventh hours. In fig.1 are plotted the rates of lipogenesis observed in the seventh hour as a function of final insulin concentration. Ignoring the possibility of endogenous insulin in hepatocytes and medium the sensitive range appeared to lie between 1 and 10 ng of insulin per ml (1.7×10^{-10} to 1.7×10^{-9} M, or 25 to 250 μunits per ml). Glucagon at a concentration of 10^{-9} M exerted a significant depression in the rate of fatty acid synthesis,

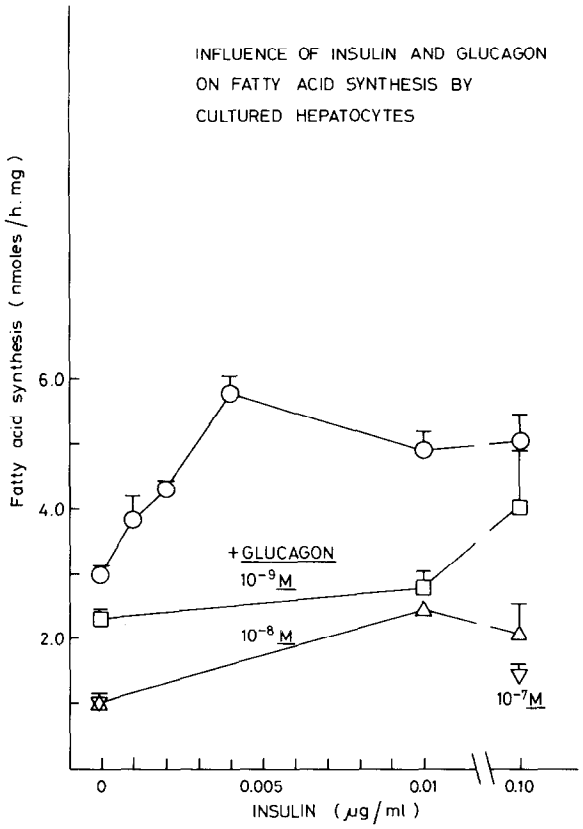

Fig.1. Influence of insulin and glucagon on fatty acid synthesis by cultured hepatocytes. Hepatocytes were plated and incubated as described in table 1. After 4 h of preincubation the medium was changed and hormones added as indicated. Insulin and glucagon were added at the same time. [1-^{14}C]-acetate was added at 7 h and incubated for an additional hour. The rate of fatty acid synthesis at 4 h was 1.56 ± 0.12 nmoles [1-^{14}C]acetate incorporated per h per mg protein. Each point is the mean of at least three separate plates. The verticle bars represent standard deviations.

the more striking dampening occurring at lower insulin concentrations. Glucagon at 10^{-10} M exhibited only marginal effects (not shown).

The stimulatory and inhibitory effects of insulin and glucagon, respectively, on fatty acid synthesis support the concept that these hormones act on the hepatocyte directly. It is significant that both insulin and glucagon affect [1-^{14}C] acetate incorporation at concentrations that compare favorably with in vivo levels of these hormones [1,20,21]. Since hepatocytes in maintenance cultures retain a sensitivity to insulin and glucagon approximating the responsiveness of liver in vivo, this preparation should be ideal for elucidating the vexing interplay of insulin and glucagon in determining hepatic cAMP levels relative to the control of lipogenesis.

A plot of the rate of fatty acid synthesis as a function of time following the addition of insulin is presented in fig.2. As in previous experiments [1-^{14}C] acetate incorporation was measured during the hour following the indicated time of incubation. The initial rapid rise in the rate of lipogenesis in response to insulin indicated that short-term control mechanisms may make a contribution to the overall enhancement of the process, e.g. interconversion of preexisting acetyl-CoA carboxylase to a more active form [22,23].

It could be argued that insulin might affect the specific radioactivity of the intracellular ^{14}C-acetyl CoA pool and thereby seemingly effect a stimulation of fatty acid (and cholesterol) synthesis. Therefore, studies were initiated using [^{3}H] H_2O incorporation into lipids as an independent index of lipogenic activity [24]. As shown in the experiment described in table 2, insulin increased the metabolic flux to fatty acids as measured by the rate of tritium incorporation in a manner similar to [1-^{14}C] acetate.

Cycloheximide at a concentration of 5×10^{-5} M [14] severely diminished the stimulation of lipogenesis by insulin (table 3). Cycloheximide had no effect on the attachment of hepatocytes in the 4 to 7 h interval of the experiment, nor did cycloheximide inhibit [1-^{14}C] acetate incorporation into lipids when added at the beginning of the 60 min lipogenesis assay (data not shown).

On the whole these results suggest that induction of lipogenic enzymes is primarily responsible for the enhanced lipogenesis observed 3 h following the provision of insulin to cultured hepatocytes. Nevertheless,

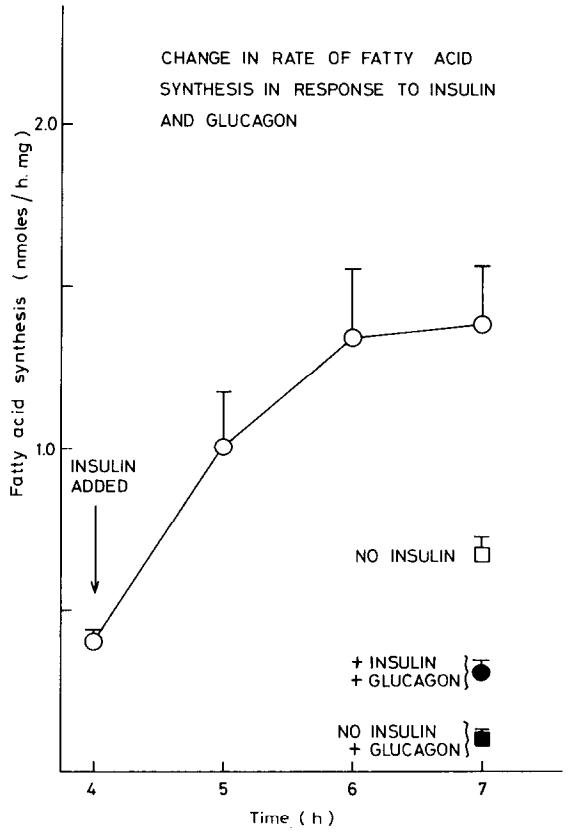

Fig.2. Change in rate of fatty acid synthesis in response to insulin and glucagon. After 4 h of preincubation the medium was changed and hormones added as indicated (insulin) 0.5 μg/ml; glucagon 10^{-6} M). At the times shown [1-^{14}C] acetate was added and incubated for an additional hour. Conditions of incubation and assay are described in table 1. Each point is the mean of at least three separate plates. The verticle bars represent standard deviations.

until changes in enzyme levels can be demonstrated by immunochemical techniques [1,14] and indirect action of cycloheximide on the flow of labelled acetate to fatty acids and NSL can not be ruled out.

The remarkable responsiveness to insulin and glucagon of freshly isolated mature hepatocytes maintained in a fully enriched medium containing fetal calf serum does not necessarily imply that a plated culture, *per se*, (i.e. populations of contiguous adherent, flattened cells) is the basis for this endowment. Recent experiments indicate that lipogenesis in suspensions of hepatocytes is influenced by insulin and glucagon wit-

Table 2
Incorporation of [^{3}H]H_2O and [1-^{14}C]acetate into fatty acid and NSL by cultures of hepatocytes

Series	Additions	Time (h)[a]	Fatty acids	NSL	n[b]
[^{3}H]H_2O	none	4	1.20 ± 0.06	0.98 ± 0.16	3
	none	7	1.87 ± 0.25	1.10 ± 0.09	3
	insulin 0.5 μg/ml	7	3.91 ± 0.18	1.53 ± 0.05	3
[1-^{14}C]acetate					
	none	4	0.21 ± 0.03	0.32 ± 0.03	2
	none	7	0.37 ± 0.07	0.28 ± 0.02	2
	insulin 0.5 μg/ml	7	1.16 ± 0.24	0.51 ± 0.03	2

Hepatocytes were plated as described in table 1 except that 10 ml of hepatocytes in suspension were added to 100-mm plates at time zero. Insulin was added at 4 h. Two separate series were studied with hepatocytes derived from a single rat: (a) [^{3}H]H_2O, 25 mCi per 10 ml; final specific radioactivity 9.9 × 10^4 dpm/μmole. (b) [1-^{14}C] acetate, 6.25 μCi per 10 ml; final specific radioactivity 2.121 × 10^6 dpm/μmole. Rates of lipogenesis are expressed in terms of nmol of acetate incorporated per h per mg of cell protein. Values for [^{3}H]H_2O incorporation were converted to acetate equivalents by multiplying with 1.15 in the case of fatty acid synthesis [24] and 1.31 for NSL synthesis [25]. Labelled lipid products were recovered as described in table 1 in both series.
[a]time of addition of label
[b]n: number of plates used for each value.

Table 3
Effect of cycloheximide on insulin-stimulated fatty acid and NSL synthesis (from [^{14}C]acetate) in cultured hepatocytes

Additions	Time (h)	Fatty acids	NSL
none	0	0.06 ± 0.01	0.32 ± 0.01
none	4	0.20 ± 0.03	0.26 ± 0.03
none	7	0.74 ± 0.07	0.28 ± 0.01
cycloheximide 5 × 10^{-5} M	7	0.35 ± 0.01	0.10 ± 0.01
insulin 0.5 μg/ml	7	1.84 ± 0.32	0.44 ± 0.01
insulin 0.5 μg/ml + cycloheximide 5 × 10^{-5} M	7	0.86 ± 0.07	0.15 ± 0.01

Addition of cycloheximide was made in 50 μl quantities. Where indicated, insulin and cycloheximide were added at four hours. Conditions were as in table 1, except for the first line where the aspirated medium was centrifuged and the pellet combined with the cells from the plate.

hin two hours after isolation provided that whole fetal calf serum is included with the incubation medium [18].

As anticipated the rates of incorporation of [1-^{14}C]-acetate and [^{3}H] H_2O into fatty acids and NSL by cultured hepatocytes obtained from 24-h starved rates were quite low [1]. By contrast, lipogenic rates reported by Berry et al. [26] for suspensions of hepatocytes isolated from well-fed rats were two orders of magnitude higher. Although the starvation state is probably the principal distinguishing feature in this comparison, differences in cell isolation and incubation conditions may also be relevant. As indicated above [18] we find that cultured, starved hepatocytes exhibit lipogenic rates that are comparable to suspensions of starved hepatocytes incubated under identical conditions. Nevertheless it remains to be proved that cultured hepatocytes retain all metabolic parameters of freshly isolated hepatocytes and of intact liver [16,17].

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