SHORT-TERM EFFECTS OF INSULIN AND GLUCAGON ON LIPID SYNTHESIS IN ISOLATED RAT HEPATOCYTES

Covariance of acetyl-CoA carboxylase activity and the rate of ^3H_2O incorporation into fatty acids

Math J. H. GEELEN, Anton C. BEYNEN, Renata Z. CHRISTIANSEN+*, Mimi J. LEPREAU-JOSE+ and David M. GIBSON+

Laboratory of Veterinary Biochemistry, State University of Utrecht, The Netherlands and +*Department of Biochemistry, Indiana University School of Medicine, Indianapolis, IN 46202, USA*

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1. Introduction

The rate of fatty acid synthesis in cultures or suspensions of isolated hepatocytes has been shown to be stimulated with added insulin [1–3] and depressed with glucagon [1–7]. Insulin acting alone does not change basal cAMP levels but does partially dampen glucagon-stimulated cAMP spikes [8.9]. Although added cAMP mimicks the glucagon effect in vitro [4,10–12] it cannot be assumed that fatty acid synthesis is uniquely controlled through changes in intracellular cAMP concentrations. The activity of acetyl-CoA carboxylase (EC 6.4.2.1), long considered the limiting enzyme in fatty acid synthesis, is decreased in liver in vivo following addition of glucagon [13] or in liver slices [14] and primary cultures of hepatocytes [7] following in vitro addition of cAMP. This was substantiated by the observation [5] that in liver steady-state levels of malonyl-CoA, the product of acetyl-CoA carboxylation, were rapidly depressed to 1/4 normal following glucagon injection into intact rats. On the basis of this finding acetyl-CoA carboxylase was considered to be the focal point of glucagon signalling even though the activity of acetyl-CoA carboxylase, per se, as assayed in the liver cytosol, did not seem to be affected. Attempts to correlate the insulin enhancement of fatty acid synthesis in isolated hepatocytes [1–3] with changes in the activity of acetyl-CoA carboxylase have not been reported.

We present evidence here that, in short-term incubations of rat hepatocyte suspensions with either glucagon or insulin, acetyl-CoA carboxylase activity (in cell homogenates) was covariant with the rate of fatty acid synthesis as measured by incorporation of ^3H_2O (in the intact hepatocytes) provided that: (a) precautions were taken to prepare, incubate and homogenize hepatocytes at 25–37°C [14–16]; (b) the activity of acetyl-CoA carboxylase was measured in the absence of added citrate.

2. Methods and materials

Parenchymal liver cells (hepatocytes) were obtained from male Wistar rats which had free access to a stock pelleted diet and water. Cells were prepared and purified by the method in [17], except that the operational temperature was maintained at 37°C throughout. Cell viability (dye exclusion) was routinely >95%. Glucose (10 mM) was added to the perfusion buffer and to all buffers subsequently employed in the isolation procedure to minimize glycogenolysis [4]. Incubations of hepatocytes (45–50 mg wet wt/ml) were carried out in Krebs-bicarbonate buffer [18] (pH 7.4, final vol. 30 ml), supplemented with 10 mM glucose [4] and with fatty acid-free bovine serum albumin (35 mg/ml), in sealed glass Erlenmeyer flasks (250 ml) under 95% oxygen, 5% carbon dioxide at 37°C in a Dubnoff metabolic incubator (90 osc./min).

* On leave from the Institute for Medical Biochemistry, University of Oslo, Oslo, Norway

For optimal rates of fatty acid synthesis 10 mM lactate was included in the incubation medium [4].

Two sets of incubation flasks were run simultaneously. From the first set duplicate 2.0 ml aliquots were removed at 15 min intervals for acetyl-CoA carboxylase assay in homogenates of the hepatocytes (see below). In the second set ^3H_2O was added (0.3 mCi/ml) at 0 time and duplicate 2.0 ml aliquots were subsequently removed (at the same time intervals) for measuring the incorporation rate of ^3H into fatty acids. Lipogenesis in these aliquots was terminated by addition of 0.5 ml 2 N perchloric acid to 2 ml cell suspension. The precipitate was extracted twice for total lipids with methanol:chloroform (2:1, v/v) as in [20]. Benzene was added before evaporation of the chloroform under nitrogen [4]. Total lipids were saponified at 75°C with 0.3 M NaOH in 90% (v/v) methanol. The non-saponifiable fraction was extracted and discarded. Fatty acids were extracted in petroleum ether, the solvent evaporated under nitrogen, and the residue dissolved in scintillation fluid and counted for radioactivity. Addition of benzene (before complete evaporation of the solvent) reduced 0 time controls to background levels of radioactivity since traces of labelled water were removed more effectively as an azeotropic mixture [4].

Aliquots of the isotope-free hepatocyte incubation mixtures (2.0 ml in duplicate) for measuring acetyl-CoA carboxylase activity were centrifuged at room temperature for 90 s at 100 × *g*. The pellets were resuspended with 1.0 ml buffer (K_2HPO_4, 85 mM; KH_2PO_4, 9 mM; and DTT, 1 mM; pH 7.4) and homogenized at room temperature with a 15 s burst of a Polytron tissue homogenizer at full speed. Aliquots of the homogenate (0.1 ml) were immediately added to 0.28 ml of a buffered, citrate-free medium and preincubated for 30 min pior to the acetyl-CoA carboxylase assay. The total preincubation mixture contained: K_2HPO_4, 22.4 mM; KH_2PO_4, 2.4 mM; DTT, 4.2 mM; Tris, 79 mM; $MgCl_2$, 10 mM; and albumin, 0.8 mg/ml; pH 7.5. Reagents for the acetyl-CoA carboxylase assay were then added (0.12 ml) so that the final concentrations were the same as in [21] (except for the omission of citrate): ATP, 2 mM; acetyl-CoA, 0.2 mM; $NaHCO_3$, 10 mM; and $NaH^{14}CO_3$ (1.0 μCi) in final vol. 0.50 ml. Acetyl-CoA-dependent $H^{14}CO_3^-$ fixation into acid-stable ^{14}C activity (malonyl-CoA) [22] was measured over a 5 min interval at 37°C [21]. All values for acetyl-CoA carboxylase activity were corrected for acetyl-CoA-independent CO_2 fixation. Protein was determined by the Lowry method [23].

Nucleotides were obtained from Sigma Chemical Co., St Louis, and radioactive reagents from New England Nuclear, Boston. Bovine serum albumin (Sigma, fraction V, essentially fatty acid-free) was dialysed extensively against Krebs-bicarbonate buffer before addition to the various media [24].

3. Results and discussion

The effects of insulin and glucagon on the synthesis of fatty acids were investigated in suspensions of isolated hepatocytes, incubated in Krebs-bicarbonate buffer. The results shown in fig.1 may be summarized as follows:

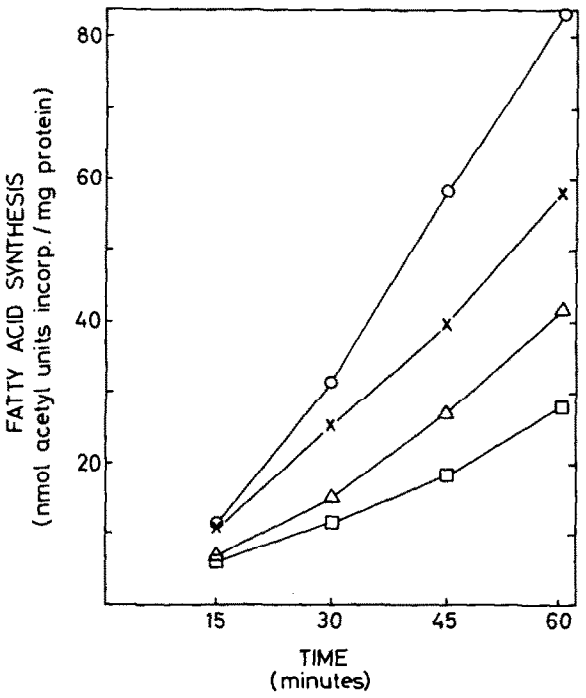

Fig.1. Effect of insulin and glucagon on fatty acid synthesis. Hepatocytes were incubated as in section 2 with ^3H_2O for the indicated periods of time. The incorporation of ^3H_2O into fatty acids was converted to acetyl equivalents by the factor 1.15 [19]. All points are the means of duplicate determinations. Control (X); insulin, 85 nM (○); glucagon, 25 nM (□); insulin plus glucagon (△).

(i) Fatty acid synthesis in hepatocytes isolated as in [17] is stimulated by insulin (85 nM) even in the absence of fetal calf serum (cf. [1]) or without special handling (cf. [3]);
(ii) Glucagon (25 nM) inhibits fatty acid synthesis;
(iii) Hepatic fatty acid synthesis is subject to rapid hormonal regulation, i.e., within 30 min after hormone addition.

The activity of acetyl-CoA carboxylase was measured in homogenates of hepatocytes, which had been preincubated in the presence or absence of insulin or glucagon. When cells were homogenized at 0°C and the activity of acetyl-CoA carboxylase was measured in the 100 000 × *g* cytosol, following its preincubation in the presence of 20 mM citrate, a time-dependent correlation between enzyme activity and rate of fatty acid synthesis from ^3H_2O in intact hepatocytes could not be established (fig.2A). However, when cells were

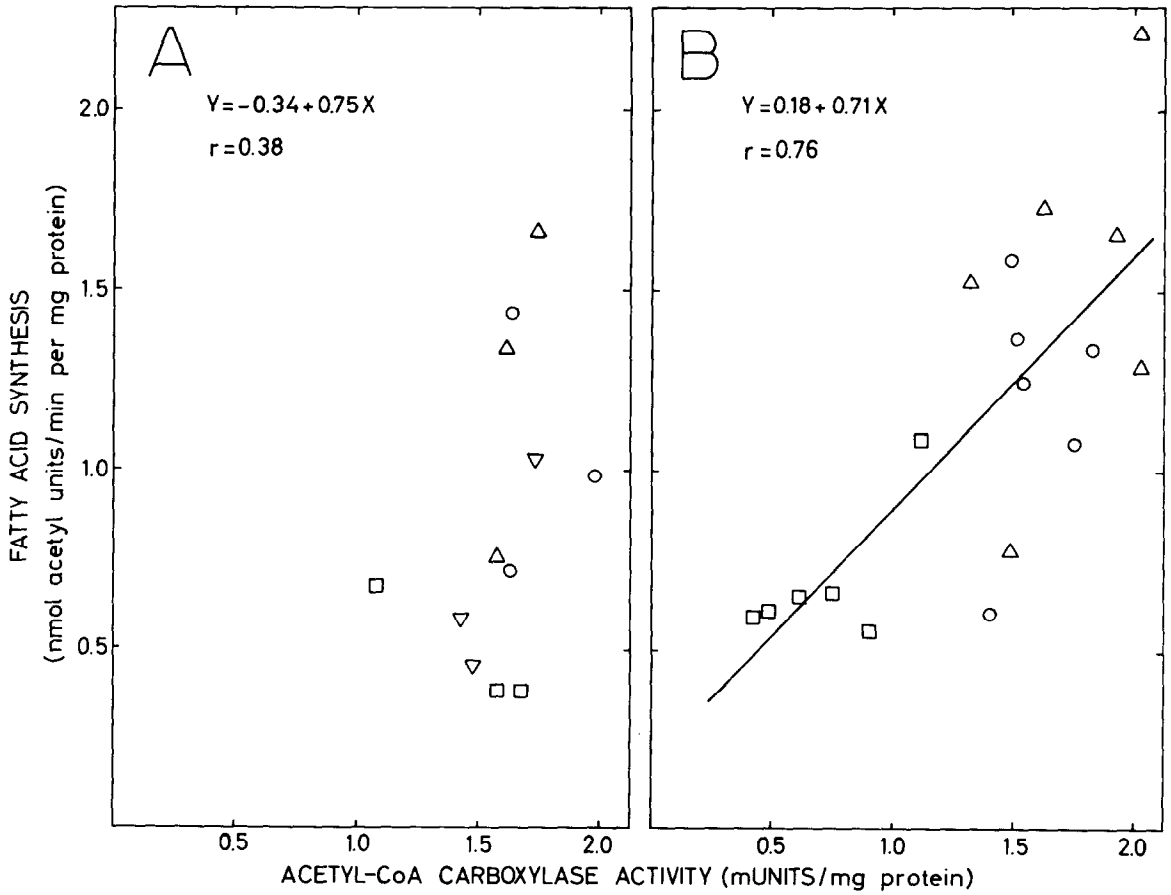

Fig.2. Covariance of the rate of fatty acid synthesis and the activity of acetyl-CoA carboxylase. Panel A: homogenates were prepared as in [21] and preincubated in the presence of 20 mM citrate; Panel B: homogenates were prepared as in [15] and preincubated in the absence of added citrate. Control (○); glucagon, 25 nM (□); insulin, 85 nM (△); insulin plus glucagon (▽). In the course of a 60 min (panel A) to 90 (panel B) min incubation period, similar to the experiment in fig.1, samples were taken at 15, 30 and 60 min (panel A) or every 15 min (panel B) for determining the rate of fatty acid synthesis (nmol acetyl units/min/mg protein) in intact hepatocytes and the activity of acetyl-CoA carboxylase (mU/mg protein) in cell homogenates. All points are the means of duplicate determinations in case of fatty acid synthesis and quadruplet determinations in case of acetyl-CoA caroboxylase activity, in each assay. ^3H_2O incorporation and acetyl-CoA carboxylase activity were determined as in section 2. A mU of acetyl-CoA carboxylase activity is equivalent to 1.0 nmol $H^{14}CO_3^-$ fixed/min at 37°C under the conditions of the assay and corrected for acetyl-CoA-independent bicarbonate fixation (section 2). The incorporation of ^3H_2O into fatty acids was converted to acetyl equivalents by the factor 1.15 [19].

homogenized as in [15], i.e., at 37°C instead of 0°C, and the crude homogenate was immediately used for the assay instead of a 100 000 × *g* supernatant, then the activity of acetyl-CoA carboxylase, measured after 30 min preincubation of the homogenate at 37°C in the absence of added citrate, was covariant with the rate of fatty acid synthesis in the intact hepatocytes (fig.2B). Under these conditions glucagon diminished both the activity of acetyl-CoA carboxylase and the rate of lipogenesis, whereas insulin increased the enzyme activity as well as the rate of lipogenesis, relative to the endocrine-free baseline (fig.3). These results indicate that addition of citrate to the preincubation mixture apparently overrides whatever change was effected by incubation of hepatocytes with insulin or glucagon. Perhaps for this reason no changes were observed [5] in liver acetyl-CoA carboxylase activity after glucagon administration. In this context it is of interest to note that high citrate concentrations are stated [25] to prevent the in vivo inactivation of acetyl-CoA carboxylase by phosphorylation.

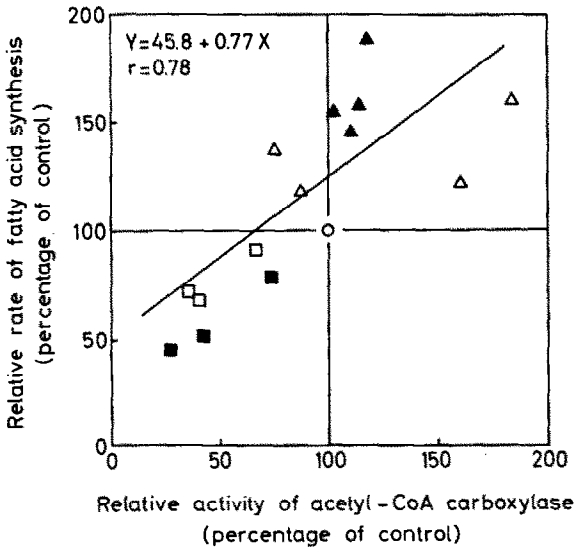

Fig.3. Correlation between the hormone-determined rate of fatty acid synthesis and the activity of acetyl-CoA carboxylase. The rate of fatty acid synthesis over 30 min intervals as well as the activity of acetyl-CoA carboxylase are expressed relative to the endocrine-free control (100%). Control (○); insulin, 85 nM (△); glucagon, 25 nM (□). Open symbols 30 min incubations; closed symbols 60 min incubations. Four different cell preparations.

The covariance of fatty acid synthesis and acetyl-CoA carboxylase in response to glucagon and insulin would suggest that this rat hepatocyte system would be useful in resolving the several mechanisms proposed for the short-term regulation of acetyl-CoA carboxylase [26–33].

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