Epidermal growth factor counteracts the glycogenic effect of insulin in parenchymal hepatocyte cultures

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Rat parenchymal hepatocytes in monolayer culture were used to study the metabolic effects of epidermal growth factor (EGF) and insulin on ketogenesis, gluconeogenesis and glycogen metabolism. EGF, unlike insulin, did not inhibit ketogenesis from palmitate or gluconeogenesis from pyruvate in hepatocyte cultures. It also had no effect on these pathways in the presence of insulin. In contrast, EGF potently counteracted the stimulation of [14 C]pyruvate incorporation into glycogen by insulin, and also glycogen deposition from both gluconeogenic precursors and glucose. The EGF concentration causing half-maximal effect was about 0.1 nM. The anti-glycogenic effect of EGF was observed after both long-term (24 h) and short-term (1 h) exposure to EGF, and was more marked in the presence of insulin than in its absence. EGF did not displace bound insulin, suggesting that it neither competes for the insulin receptor nor affects the affinity of the receptor for insulin. EGF did not alter cellular cyclic AMP; and inhibition of cyclic AMP phosphodiesterase activity did not prevent the anti-glycogenic effect of EGF. In liver-derived dividing epithelial cells, Hep-G2 cells and fibroblasts, which have no capacity for gluconeogenesis, EGF did not counteract the stimulatory effect of insulin on [14 C]glucose incorporation into glycogen, and in the epithelial cells EGF increased [14 C]glucose incorporation into glycogen. The counter-effect of EGF on the glycogenic action of insulin in parenchymal hepatocytes may be due to a direct effect on glycogen metabolism or to an interaction with the post-receptor events in insulin action.

INTRODUCTION

Epidermal growth factor (EGF) is a 53-amino-acid polypeptide which can act as a mitogen in a variety of cell types [1,2], and it also exerts a variety of acute metabolic effects, which do not appear to be related to mitogenic stimulation. The membrane receptor for EGF, like that of other growth factors [3,4] and also insulin [5,6], has tyrosine-specific protein kinase activity. The liver has high-affinity receptors for EGF, with tyrosine kinase activity [7–9]. Both EGF-receptor number and tyrosine kinase activity are decreased during starvation in rat liver [10], suggesting that EGF may have a physiological role in liver. EGF, like insulin, increases DNA synthesis in liver *in vivo* [11,12] and in cultured liver cells [13–15]. Several metabolic similarities, but also some differences, between EGF and insulin action in liver have been reported. In hepatocyte cultures, EGF, like insulin, induces the synthesis of glucose-6-phosphate dehydrogenase (G6PDH) [16], but, unlike insulin, it does not increase amino acid transport [17]. In freshly isolated hepatocyte suspensions, EGF, like insulin, stimulates fatty acid synthesis [18,19] and the phosphorylation of ATP citrate lyase and acetyl-CoA carboxylase [18], and it activates glycogen synthase at high glucose concentration [20]. EGF also activates glycogen synthase in fibroblasts and epidermoid carcinoma cells [21]. The aim of the present study was to examine how far the metabolic similarities between EGF and insulin extend in liver. Our results show that, unlike insulin, EGF does not affect ketogenesis and gluconeogenesis, but it counteracts

the glycogenic effect of insulin in parenchymal hepatocytes. A preliminary report of this work has been presented [22].

MATERIALS AND METHODS

Materials

EGF (from mouse submaxillary gland), insulin (pig) and phorbol 12-myristate 13-acetate were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dexamethasone phosphate was a gift from Merck, Sharp & Dohme (Hoddesdon, Herts., U.K.). [14 C]Pyruvate and cyclic AMP radioimmunoassay kits were from New England Nuclear (Boston, MA, U.S.A.). 125 I-A14-insulin was from Novo Research Institute (Bagsvaerd, Denmark). The materials used for cell isolation and culture and other reagents were as reported previously [23,24].

Isolation and monolayer culture of rat parenchymal hepatocytes

Parenchymal hepatocytes were isolated from male Wistar rats (180–200 g body wt.) by collagenase perfusion of the liver [25]. Cell viability estimated by Trypan Blue exclusion was > 90%. The hepatocytes were suspended in Minimum Essential Medium (MEM with Earle's salts) supplemented with 5% (v/v) heat-inactivated fetal-bovine serum, and plated in 25 cm² culture flasks [23]. After cell attachment (4 h) the medium was replaced with 5 ml of serum-free MEM containing dexamethasone (10 nM) and the test hormones or other

Abbreviations used: EGF, epidermal growth factor; G6PDH, glucose-6-phosphate dehydrogenase (EC 1.1.1.49); MEM, Minimal Essential Medium; IBMX, 3-isobutyl-1-methylxanthine.

additions, each in duplicate flasks. The change to serum-free medium was designated zero time. The flasks were equilibrated with 5% CO₂ in air at 37 °C. The medium was changed either after 21 h or at the time indicated. In the 'long-term' experiments EGF was added at 0 and 21 h, whereas in the 'short-term' experiments EGF was added at 21 h only. Unless indicated otherwise, measurements of rates of metabolic flux were performed between 22 and 24 h after the designated zero time.

Determination of gluconeogenesis, ketogenesis and incorporation of [¹⁴C]pyruvate into glycogen in parenchymal hepatocyte cultures

After 22 h (1 h after the change of medium) the medium was supplemented with 0.5 mM-[3-¹⁴C]pyruvate (0.2 Ci/mol), 0.5 mM-palmitate, 1 mM-L-carnitine and 7.5 mg of bovine serum albumin/ml [23,24], and the flasks were incubated at 37 °C for a further 2 h after substrate addition. The medium was then decanted, and 1 ml was rapidly deproteinized with HClO₄ (0.33 M) for determination of pyruvate, lactate, acetoacetate, 3-hydroxybutyrate [23] and [¹⁴C]glucose [26]. The hepatocyte monolayers were rapidly rinsed twice with ice-cold phosphate-buffered (0.1 M, pH 7.4) saline (150 mM-NaCl) and then solubilized either in 15 mM-NaOH, for determination of total glycogen [27] and ¹⁴C incorporation into glycogen [24], or in buffer containing 50 mM-Tris, 100 mM-KCl, 20 mM-EDTA, 25 mM-KF, 0.05% (w/v) Lubrol PX, pH 7.9, for determination of G6PDH activity [23]. Cell protein was determined by the Lowry method [28].

Determination of glycogen deposition

Hepatocyte monolayers were precultured either with dexamethasone (10 nM) alone or with the additional presence of insulin (10 nM). After about 16 h, the medium was changed to fresh medium supplemented with test substrates and hormones (in triplicate), and the monolayers were incubated at 37 °C for a further 30–90 min. Control flasks were terminated at zero time. On termination of the incubations the monolayers were washed three times and extracted as described above.

Insulin binding

Insulin binding in freshly isolated hepatocyte suspensions was determined at 30 °C as described in [29], except that bacitracin and gentamicin were omitted from the medium.

Cyclic AMP determination

The effects of EGF on cellular cyclic AMP were determined in freshly isolated hepatocyte suspensions and in hepatocyte monolayers precultured with dexamethasone and insulin (10 nM). Hepatocyte suspensions were incubated (3×10^6 cells in 3 ml) in 25 ml flasks, at 37 °C in a shaking water bath. After a 5 min preincubation, duplicate 300 μ l samples of cell suspension were removed and deproteinized with trichloroacetic acid (0.6 M), EGF or glucagon was then added, and further duplicate samples were taken after 5 and 10 min. In the experiments on the hepatocyte monolayers, the medium was changed after 24 h to MEM buffered with Hanks salts. EGF or glucagon was added after 1 h preincubation at room temperature. The incubations were terminated

after 0, 5 or 10 min by removal of the medium and extraction of the monolayers with trichloroacetic acid. The deproteinized acid extracts were washed with diethyl ether, freeze-dried, and cyclic AMP was determined by a radioimmunoassay kit according to the manufacturer's instructions.

Experiments with liver cell lines

Cultures of epithelial cells and fibroblasts were obtained from human liver. An end-lobe section (25 g) from an organ donor (female, 22 years) was perfused by a two-step perfusion, as for rat liver [25]. After dissociation of the liver, the cell suspension was sedimented at 50 g for 5 min and washed six times. The cells were inoculated in MEM containing 5% (v/v) fetal-bovine serum. Dividing epithelial and fibroblast cells were cloned and grown in Dulbecco's MEM containing 5% fetal-bovine serum. The epithelial cells were characterized by cytokeratin staining, by using Labsystem's PKK1 monoclonal antibody to cytokeratin polypeptides, by indirect immunoperoxidase staining. The epithelial cells were passaged every 4 days and the fibroblasts every 8 days. The cells were split 1:5 at each passage. Hep-G2 cells (human hepatoma; [30]) were grown in Dulbecco's MEM with 5% fetal-bovine serum, and passaged every 5–6 days. Experiments on these three cell types were performed 3 or 4 days after passaging. The medium was changed to serum-free MEM buffered with Hanks salts and containing 10 mM-[U-¹⁴C]glucose (0.1 Ci/mol) and EGF or insulin as described. The flasks were incubated at 37 °C for 3 h. The incubations were terminated as described above for parenchymal hepatocytes: the medium was collected for determination of lactate and pyruvate [23] and the cell monolayers were extracted for determination of glycogen [27] and protein [28].

Expression of results and calculations

Parenchymal hepatocyte cultures. Preliminary studies showed that, after addition of 0.5 mM-pyruvate and 0.5 mM-palmitate, the accumulation of ketone bodies (acetoacetate and 3-hydroxybutyrate) and the decrease in the concentrations of pyruvate plus lactate determined at 30, 60 and 120 min were linear. Pyruvate metabolism represents the decrease in pyruvate plus lactate and ketogenesis the increase in acetoacetate plus 3-hydroxybutyrate, determined from the difference in concentrations at the start of the incubation and after 2 h, expressed as nmol of substrate metabolized or product formed/h per mg of cell protein. Gluconeogenesis and glycogen synthesis, determined from the incorporation of [¹⁴C]pyruvate into glucose and glycogen, respectively, are expressed in nmol of pyruvate incorporated/h per mg of cell protein. Cellular glycogen is expressed in nmol of glucosyl units/mg of cell protein.

Liver cell lines. The incorporation of [U-¹⁴C]glucose into glycogen is expressed as nmol of glucose incorporated/3 h per mg of cell protein. Glycolysis was estimated from the accumulation of lactate in the medium and is expressed as nmol of lactate formed/mg of cell protein. The accumulation of pyruvate was less than 3% of lactate formation.

Results are expressed as means $\pm$ S.E.M. for duplicate flasks from the numbers of cultures indicated. Statistics was by Student's *t* test, either paired or unpaired.

Table 1. Long-term effects (24 h) of EGF and insulin on hepatocyte monolayer cultures

Hepatocyte monolayers were cultured with 10 nM-dexamethasone and either EGF (10 nM) or insulin (10–100 nM) as indicated below. After 21 h the medium was changed to fresh medium containing the same hormones as at zero time. Pyruvate metabolism and formation of ketone bodies (acetoacetate + 3-hydroxybutyrate) were determined between 22 and 24 h. Cellular glycogen was determined at 24 h (see the Materials and methods section). Rates are expressed as nmol of substrate metabolized or product formed/h per mg of cell protein. Cellular glycogen is expressed as nmol of glucosyl units/mg of cell protein. Values are means $\pm$ S.E.M. for duplicate flasks from the numbers of cultures indicated in parentheses. Statistics (*t* test): **P* < 0.05 versus control with no additions; †*P* < 0.0005 versus insulin alone.

Additions	Cellular glycogen (nmol/mg of protein)	Pyruvate incorporation (nmol/h per mg of protein) into:		Total pyruvate metabolism (nmol/h per mg of protein)	Ketone-body formation (nmol/h per mg of protein)
		Glycogen	Glucose		
None	28 $\pm$ 3 (8)	0.46 $\pm$ 0.06 (10)	130 $\pm$ 12 (7)	254 $\pm$ 27 (6)	313 $\pm$ 14 (7)
EGF	22 $\pm$ 3 (6)	0.28 $\pm$ 0.03 (6)	123 $\pm$ 16 (5)	242 $\pm$ 32 (5)	322 $\pm$ 24 (5)
Insulin	115 $\pm$ 10* (8)	3.52 $\pm$ 0.53* (10)	89 $\pm$ 9* (7)	216 $\pm$ 16 (6)	263 $\pm$ 14* (7)
Insulin + EGF	37 $\pm$ 3† (8)	0.56 $\pm$ 0.07† (8)	100 $\pm$ 10 (7)	201 $\pm$ 24 (6)	271 $\pm$ 23 (6)

RESULTS

Long-term (24 h) effects of EGF on hepatocyte cultures

After cell attachment (zero time) the hepatocyte monolayers were cultured in serum-free medium, containing 10 nM-dexamethasone, and either insulin or EGF as indicated in Table 1. Glycogen synthesis, gluconeogenesis and ketogenesis were determined between 22 and 24 h, and cellular glycogen was determined at 24 h (see the Materials and methods section). Dexamethasone (10 nM) was used throughout this study because it potentiates the anti-ketogenic, anti-gluconeogenic and glycogenic effects of insulin [24]. In hepatocyte monolayers cultured with insulin, the cellular glycogen content was 4 times that of the controls. EGF counteracted the effect of insulin on cellular glycogen (Table 1). The rates of pyruvate metabolism (decrease in pyruvate plus lactate in medium), gluconeogenesis ($[^{14}\text{C}]$ pyruvate incorporation into glucose) and ketogenesis (formation of acetoacetate and 3-hydroxybutyrate) were decreased by insulin, but were unaffected by EGF in either the presence or the absence of insulin (Table 1). Insulin increased the incorporation of $[^{14}\text{C}]$ pyruvate into cellular glycogen 8-fold. This effect of insulin was counteracted by EGF (Table 1). Since EGF had no effect on $[^{14}\text{C}]$ pyruvate incorporation into glucose, its effects on $[^{14}\text{C}]$ pyruvate incorporation into glycogen are probably specific for glycogen metabolism, as opposed to gluconeogenesis. The effects of EGF concentration on the rate of $[^{14}\text{C}]$ pyruvate incorporation into glycogen and the cellular glycogen content in hepatocyte monolayers cultured with insulin are shown in Fig. 1. The [EGF] causing half-maximal inhibition was about 0.1 nM.

Short-term effects of EGF on hepatocyte glycogen

In the above experiments cellular glycogen was determined 24 h after the first exposure to EGF. To examine the short-term effects of EGF under similar substrate conditions, the monolayers were precultured for 21 h with dexamethasone (10 nM) and insulin (10 nM), the medium was then changed to fresh medium without or with 10 nM-EGF; 0.5 mM-pyruvate was added after 1 h, and the incubation were terminated after a further 2 h. The glycogen content was significantly lower

Fig. 1. Dose-response of EGF on glycogen metabolism in hepatocyte monolayers cultured with insulin

Hepatocyte monolayers were cultured with 10 nM-dexamethasone, 100 nM-insulin and the concentrations of EGF indicated. The medium was changed after 21 h for fresh medium containing the same hormones as at zero time. The rate of $[3\text{-}^{14}\text{C}]$ pyruvate incorporation into glycogen (nmol/h per mg of protein) was determined between 22 and 24 h, and cellular glycogen (nmol of glucosyl units/mg of protein) was determined at 24 h (see the Materials and methods section). Values are means $\pm$ S.E.M. for duplicate flasks from three cultures.

Table 2. Short-term effects of EGF and insulin on glycogen deposition in hepatocyte cultures

Hepatocytes isolated from either fed or starved rats were cultured with either 10 nM-dexamethasone (Dex) alone or with additional 10 nM-insulin (Ins) as indicated. After 16–20 h the medium was changed to fresh medium containing the substrates and hormones indicated. Incubations were terminated either immediately after the change of medium or after 90 min incubations with the substrates and hormones indicated. Cellular glycogen was determined on termination of the incubations, and is expressed in nmol of glucosyl units/mg of cell protein; values represent means $\pm$ S.E.M. for triplicate flasks. The values in parentheses represent the difference in glycogen content before and 90 min after substrate addition.

Expt. no. ... State of rat ... Cells precultured with ...	Glycogen (nmol of glucosyl units/mg of cell protein)				
	1 Fed Dex	2 Fed Dex + Ins	3 Fed Dex + Ins	4 Fed Dex + Ins	5 Fasted Dex + Ins
Glycogen before substrate addition	76.8 $\pm$ 1.8	274.8 $\pm$ 4.8	138.0 $\pm$ 7.2	84.7 $\pm$ 1.0	159.0 $\pm$ 2.4
Glycogen 90 min after addition of:					
(a) 5 mM-Pyruvate	91.2 $\pm$ 7.8 (14)	333.0 $\pm$ 2.4 (58)		85.6 $\pm$ 0.4 (0.9)	231.6 $\pm$ 2.4 (73)
+ Insulin	114.6 $\pm$ 1.2 (38)	366.6 $\pm$ 1.8 (92)		98.5 $\pm$ 1.7 (14)	264.6 $\pm$ 6.6 (106)
+ EGF	81.6 $\pm$ 6.0 (4.8)	267.0 $\pm$ 3.0 (–8)		–	202.2 $\pm$ 6.6 (43)
+ Insulin + EGF	77.4 $\pm$ 1.8 (0.6)	293.4 $\pm$ 2.4 (19)		80.2 $\pm$ 0.3 (–5)	221.4 $\pm$ 1.8 (62)
(b) 5 mM-Lactate			156.0 $\pm$ 3.0 (18)		191.4 $\pm$ 1.2 (32)
+ Insulin			172.8 $\pm$ 4.2 (35)		232.2 $\pm$ 2.4 (73)
+ EGF			–		172.2 $\pm$ 2.4 (13)
+ Insulin + EGF			145.8 $\pm$ 11.4 (8)		159.0 $\pm$ 1.8 (0)
(c) 15 mM-Glucose			154.2 $\pm$ 1.2 (16)	95.9 $\pm$ 1.7 (11)	236.4 $\pm$ 6.6 (77)
+ Insulin			221.4 $\pm$ 10.8 (83)	108.9 $\pm$ 3.0 (24)	253.8 $\pm$ 3.0 (95)
+ EGF			–	–	213.6 $\pm$ 11 (55)
+ Insulin + EGF			132.0 $\pm$ 1.2 (–6)	80.4 $\pm$ 1.9 (–4)	146.4 $\pm$ 2.4 (–13)
(d) 30 mM-Glucose	184.2 $\pm$ 1.2 (107)		269.4 $\pm$ 6.6 (131)		349.8 $\pm$ 4.8 (191)
+ Insulin	237.0 $\pm$ 11.4 (160)		313.2 $\pm$ 19.2 (175)		340.8 $\pm$ 13.2 (181)
+ EGF	155.4 $\pm$ 3.0 (79)		–		226.2 $\pm$ 7.8 (67)
+ Insulin + EGF	190.8 $\pm$ 23.4 (113)		228.0 $\pm$ 1.8 (90)		248.4 $\pm$ 9.6 (89)

($P < 0.0001$) when EGF was added at 21 h than in the controls without EGF (82 ± 5 versus 142 ± 7 nmol of glucosyl units/mg of protein; means $\pm$ S.E.M., $n = 8$). When EGF was added at zero time and 21 h, the glycogen content at 24 h was lower ($P < 0.01$) than when EGF was added at 21 h only (39 ± 3 versus 63 ± 5 nmol of glucosyl units/mg of protein, $n = 6$).

The effects of EGF on glycogen deposition were determined with high concentrations of either gluconeogenic precursors (5 mM-lactate or -pyruvate) or glucose (15 or 30 mM; Table 2). Hepatocyte monolayers were precultured with dexamethasone (10 nM) or with the additional presence of 10 nM-insulin. After 16 h the medium was replaced with fresh medium (MEM, containing 5 mM-glucose) supplemented with test substrates, either with no added hormones or with insulin and/or EGF. Control flasks were terminated at 16 h (for determination of cellular glycogen before substrate addition), the rest of the flasks were terminated 30, 60 or 90 min after substrate addition. When the medium (containing 5 mM-glucose) was not supplemented with either gluconeogenic substrates or additional glucose, there was no significant glycogen deposition during 90 min. In contrast, significant glycogen deposition occurred with 0.5 mM-pyruvate or the substrates indicated in Table 2. Preliminary time-course experiments showed that for all substrates there was a lag phase during the initial 30 min; thereafter glycogen deposition was either linear between 30 and 90 min or slightly lower

or higher during 30–60 min than during 60–90 min. Table 2 shows the results of 90 min incubations. With the high substrate concentrations, rates of glycogen deposition were very variable in different preparations (as reported in other studies [31]), and results of different experiments are therefore presented separately. A stimulatory effect of insulin on glycogen deposition was observed with pyruvate, lactate and 15 mM-glucose, but the percentage stimulatory effect of insulin was lower with 30 mM-glucose. In all situations where insulin increased glycogen deposition, EGF counteracted the effect of insulin. EGF also decreased glycogen deposition in the absence of insulin (Table 2). In experiments where incubations were terminated after 60 min, a similar counteraction of the insulin effect by EGF was observed, as shown in Table 2 for 90 min incubations. These results suggest that EGF elicits an effect on glycogen synthesis or degradation within 60 min.

Inhibition of cyclic AMP phosphodiesterase

To investigate whether changes in cyclic AMP phosphodiesterase activity might be involved in the short-term effects of EGF, the effects of a cyclic AMP phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine (IBMX), were examined. IBMX was used at a concentration of 0.2 mM, because this concentration increases the cyclic AMP response to glucagon in hepatocyte cultures. Hepatocytes were cultured with either insulin or proinsulin (which exerts similar meta-

Table 3. Short-term effects of EGF on hepatocyte monolayers cultured with insulin and proinsulin for 24 h

Hepatocyte monolayers were cultured with insulin (100 nM) or proinsulin (1 μ M) in the absence or presence of the phosphodiesterase inhibitor IBMX (0.2 mM). The same hormones were added at 21 h, without (–) or with (+) the additional presence of 10 nM-EGF as indicated. Gluconeogenesis (pyruvate incorporation into glucose) and ketogenesis were determined between 22 and 24 h. Cellular glycogen (nmol of glucosyl units/mg of protein) was determined at 24 h. Values are means $\pm$ S.E.M. of duplicate flasks from two cultures. The effects of EGF as a percentage of the respective controls (+/–) are shown in parentheses.

Additions present for 24 h	Cellular glycogen (nmol/mg of protein)		Gluconeogenesis (nmol/h per mg of protein)		Ketogenesis (nmol/h per mg of protein)		
	EGF present between 21 and 24 h ...	—	+	—	+	—	+
None		47 ± 7	48 ± 3 (102)	78 ± 4	78 ± 6 (100)	244 ± 20	284 ± 16 (116)
IBMX		34 ± 12	26 ± 8 (76)	228 ± 6	214 ± 9 (94)	312 ± 12	288 ± 8 (92)
Insulin		160 ± 7	100 ± 8 (63)	58 ± 6	72 ± 8 (124)	183 ± 13	202 ± 17 (110)
Insulin + IBMX		97 ± 11	65 ± 2 (67)	104 ± 8	118 ± 8 (113)	177 ± 20	196 ± 16 (111)
Proinsulin		165 ± 10	91 ± 6 (55)	54 ± 6	52 ± 6 (96)	174 ± 8	158 ± 4 (91)
Proinsulin + IBMX		75 ± 23	43 ± 8 (57)	110 ± 4	128 ± 14 (116)	171 ± 20	172 ± 19 (101)

bolic effects to insulin, though at higher concentrations [23]), either without or with 0.2 mM-IBMX. After 21 h, fresh medium was added containing the same test additions as at zero time, but without or with 10 nM-EGF. Pyruvate and palmitate metabolism was determined between 22 and 24 h. The cellular glycogen content (at 24 h), in monolayers incubated with either insulin or proinsulin, was lower in the presence of IBMX (Table 3). EGF decreased glycogen deposition in monolayers cultured with insulin or proinsulin, irrespective of the presence of IBMX (Table 3). These results suggest that the inhibitory effect of EGF on glycogen deposition (or the stimulatory effect on glycogen breakdown) is unlikely to be due to inhibition of cyclic AMP phosphodiesterase activity. In the presence of IBMX, the inhibitory effects of insulin and proinsulin on gluconeogenesis and ketogenesis were more marked (55% and 57% respectively) than in its absence (26 and 25% respectively) in both percentage and absolute terms (Table 3), but EGF had no effect on these pathways in the presence of IBMX.

Cyclic AMP in hepatocyte suspensions and cultures

The effects of EGF on cellular cyclic AMP were determined in freshly isolated hepatocyte suspensions and in hepatocyte monolayers cultured with 10 nM-dexamethasone and 10 nM-insulin for 24 h. At 5 or 10 min after EGF addition there was no significant change in cyclic AMP in either cultures [0 min, 23 $\pm$ 2; 5 min, 21 $\pm$ 4; 10 min, 21 $\pm$ 2 (pmol/mg of protein)] or suspensions [0 min, 21 $\pm$ 2; 5 min, 17 $\pm$ 2; 10 min, 23 $\pm$ 2 (pmol/10⁶ cells)]. In the same experiments, 100 nM-glucagon caused a 6–8-fold increase in cyclic AMP [cultures; 0 min, 23 $\pm$ 1; 5 min, 96 $\pm$ 9; 10 min, 127 $\pm$ 20 (pmol/mg of protein); suspensions: 0 min, 20 $\pm$ 3; 5 min, 154 $\pm$ 9; 10 min, 129 $\pm$ 21 (pmol/10⁶ cells)].

Displacement of insulin binding

To investigate whether the counter-effect of EGF on the glycolytic action of insulin might be due to competition for the insulin receptor, the effects of EGF on displacement of ¹²⁵I-insulin binding were examined in hepatocyte suspensions (Fig. 2). EGF at concentrations

Fig. 2. Competitive displacement of cell-bound ¹²⁵I-insulin by EGF, insulin and proinsulin in hepatocyte suspensions

Freshly isolated hepatocyte suspensions were incubated with ¹²⁵I-insulin and the concentrations of EGF (▲), proinsulin (○) and insulin (●) indicated. Results represent the bound radioactivity at the hormone concentrations indicated as a percentage of the total binding (tracer only). Values are means $\pm$ S.E.M. of duplicate incubations from three experiments.

up to 100 nM did not displace bound insulin, indicating that it does not compete for the insulin receptor. Proinsulin, unlike EGF, displaced bound insulin at concentrations which stimulate glycogen synthesis [23].

G6PDH activity in hepatocyte cultures

EGF increases G6PDH in hepatocyte monolayers cultured in medium containing fetal bovine serum and 1 μ M-dexamethasone [16]. In the present study, in hepatocytes cultured in serum-free medium, containing 10 nM-dexamethasone and 10 nM-insulin, EGF (added at zero time) caused a dose-dependent increase in G6PDH activity (determined after 24 h): control, 6.75 $\pm$ 0.22; 0.01 nM-EGF, 7.24 $\pm$ 0.17; 0.1 nM-EGF, 8.57 $\pm$ 0.26;

Table 4. Short-term effects of EGF and insulin on [U-¹⁴C]glucose incorporation into glycogen in epithelial, fibroblast and Hep-G2 cells derived from human liver

The epithelial, fibroblast and Hep-G2 cells were cultured as described in the Materials and methods section. The short-term effects of EGF (10 nM) and insulin (100 nM) on glycogen synthesis were determined during a 3 h incubation with 10 mM-[U-¹⁴C]glucose (0.1 Ci/mol). Glycogen synthesis is expressed as nmol of glucose incorporated into glycogen/mg of cell protein. Cellular glycogen determined on termination of the 3 h incubation is expressed as nmol of glucosyl units/mg of cell protein. Glycolysis represents the increase in lactate in the medium during 3 h, expressed as μ mol of lactate/mg of protein. Values are means $\pm$ S.E.M. for the numbers of cultures indicated: *values that are significantly different (*t* test, *P* < 0.05) from the control with no additions.

Additions Cell line... No. of cultures...	[U- ¹⁴ C]Glucose incorporation to glycogen (nmol/mg of protein)			Cellular glycogen (nmol of glucosyl units/mg of protein)			Glycolysis (μ mol of lactate/mg of protein)		
	Epithelial 5-7	Fibroblast 3	Hep-G2 5	Epithelial 5-7	Fibroblast 3	Hep-G2 5	Epithelial 4-6	Fibroblast 3	Hep-62 5
None	16.9 $\pm$ 3.0	11.7 $\pm$ 0.7	4.8 $\pm$ 1.1	236 $\pm$ 34	720 $\pm$ 136	128 $\pm$ 9	9.7 $\pm$ 0.7	10.3 $\pm$ 0.9	1.9 $\pm$ 0.3
EGF	22.5 $\pm$ 2.9*	16.6 $\pm$ 2.8	5.0 $\pm$ 1.3	243 $\pm$ 34	635 $\pm$ 122	147 $\pm$ 27	10.2 $\pm$ 0.5	11.1 $\pm$ 1.1	2.0 $\pm$ 0.4
Insulin	26.2 $\pm$ 6.3*	20.8 $\pm$ 5.0	5.3 $\pm$ 1.8	253 $\pm$ 41	707 $\pm$ 223	136 $\pm$ 23	10.1 $\pm$ 0.8	10.9 $\pm$ 1.0	2.1 $\pm$ 0.4
Insulin + EGF	26.4 $\pm$ 4.1*	20.6 $\pm$ 3.7*	5.9 $\pm$ 1.2*	266 $\pm$ 33	607 $\pm$ 154	146 $\pm$ 25	10.3 $\pm$ 0.4	9.9 $\pm$ 0.6	2.1 $\pm$ 0.3

1 nM-EGF, 11.2 $\pm$ 0.34; 10 nM-EGF, 11.2 $\pm$ 0.33 (munits/flask; means $\pm$ S.E.M., *n* = 4). A similar effect of EGF on G6PDH was observed in the absence of insulin or dexamethasone (results not shown). No significant effect of EGF was observed after 3 h exposure to EGF. The half-maximal effective [EGF] for stimulation of G6PDH (0.1 nM) was similar to its anti-glycogenic effects (Fig. 1). A similar half-maximal effective [EGF] was observed by Yoshimoto and co-workers [16] for stimulation of G6PDH, but higher half-maximal effective concentrations have been reported for activation of glycogen phosphorylase (5 nM; [32]) and for stimulation of glycogen synthase (9 nM; [20]) in freshly isolated hepatocyte suspensions.

Effects of EGF and insulin on liver-derived cell lines

Since EGF has been shown to activate glycogen synthase in a transformed epidermoid cell line (A431 carcinoma) and in 3T3 fibroblasts [21], we examined the effects of EGF and insulin on glycogen synthesis in cell lines derived from human liver. Three cell types were examined: epithelial cells (characterized by cytokeratin staining); fibroblasts (that did not stain for either cytokeratin or factor VIII); and the human hepatoma cell line, Hep-G2 [30]. None of these cell types had any gluconeogenic capacity or fructose biphosphatase activity (L. Agius, unpublished work), and therefore [¹⁴C]glucose rather than [¹⁴C]pyruvate was used as glyco-genic substrate. The incorporation of [¹⁴C]glucose into glycogen was increased, by insulin and EGF separately, in the epithelial cells, and by insulin and EGF in combination, in the three cell types (Table 4). The increased label incorporation into glycogen by EGF, alone or in combination with insulin, in these cell lines contrasts with the counter-effect of EGF on the insulin stimulation of glycogen synthesis in parenchymal hepatocyte cultures. Unlike in parenchymal hepatocytes, increased [¹⁴C]incorporation into glycogen was not associated with net glycogen deposition. The rate of glycolysis was very high in the three cell types and not further increased by EGF or insulin (Table 4).

DISCUSSION

A variety of 'insulin-like' metabolic effects of EGF on liver cells have been reported, including: long-term induction of G6PDH in hepatocyte cultures [16]; acute stimulation of fatty acid synthesis and phosphorylation of acetyl-CoA carboxylase and ATP citrate lyase [18]; increased [¹⁴C]glucose incorporation into glycogen at high [glucose] [20]; suppression of the cyclic AMP response to glucagon and suppression of glycogen phosphorylase activation by glucagon and α -agonists in hepatocyte suspensions [20]. Effects of EGF on hepatocytes that are not insulin-like have also been observed. These include activation of glycogen phosphorylase in hepatocyte suspensions [32], inhibition of glucagon-stimulated amino acid transport [17], and suppression of insulin-stimulated acetate incorporation into lipid in cultured hepatocytes [16]. The results of the present study show that: (1) EGF does not mimic the effects of insulin on gluconeogenesis and ketogenesis; (2) EGF counteracts the glycogenic effect of insulin in cultured hepatocytes, incubated with either gluconeogenic precursors or 15 mM-glucose. These findings contrast with the report by Bosch and co-workers [20] that EGF mimics the glycogenic effect of insulin in hepatocyte suspensions.

EGF has been shown to increase glycogen synthase activity in A431 cells (an epidermoid carcinoma cell line), in 3T3 fibroblasts [21] and in freshly isolated hepatocyte suspensions incubated with a high [glucose] [20]. The effects of EGF on glycogen synthase in these two studies is probably mediated by different mechanisms. In the former study [21], EGF increased glycogen synthase activity assayed in the absence of glucose 6-phosphate (glucose 6-phosphate-independent activity) but not in its presence (total activity); but in the latter study [20], EGF caused a parallel increase in glucose 6-phosphate-independent and total activity. EGF caused a 3-fold increase in glycogen phosphorylase activity in hepatocyte suspensions [31]. The activation of glycogen phosphorylase by EGF is probably mediated by the increase in cytosolic Ca²⁺ [20,31]. The overall effect of EGF on glycogen metabolism would be expected to depend on

the relative effects on glycogen synthase and glycogen phosphorylase. The present observation, that EGF counteracts the glycogenic effect of insulin in hepatocyte cultures, might be due to a stimulation of glycogen phosphorylase by EGF or to a counteraction of the activation of glycogen synthase by insulin.

The synthase is regulated by hormones that control its phosphorylation state [33]. There are at least seven phosphorylation sites [34] on serine or threonine residues [34,35]. Insulin activates glycogen synthase by decreasing its phosphorylation state [34,36]. In muscle, insulin dephosphorylates three sites (3a, 3b, 3c), that are phosphorylated by a cyclic-AMP- and Ca^{2+} -independent kinase (kinase 3), but it does not dephosphorylate the sites phosphorylated by cyclic-AMP-dependent kinase (1a, 1b, 2 [34]). If EGF phosphorylates the same sites that are dephosphorylated by insulin, it might counteract the stimulatory effect of insulin.

The anti-glycogenic effect of EGF in parenchymal hepatocytes was observed after short-term (1 h) as well as long-term (24 h) exposure. The absence of an increase in cyclic AMP in response to EGF in freshly isolated and cultured hepatocytes rules out a cyclic-AMP-mediated inhibition of glycogen synthesis or activation of glycogen breakdown, and the failure of EGF to stimulate gluconeogenesis and ketogenesis in hepatocyte cultures is also consistent with the lack of an increase in cyclic AMP. The failure of the cyclic AMP phosphodiesterase inhibitor to abolish the anti-glycogenic effect of EGF suggests that the effects of EGF are not mediated by changes in cyclic AMP phosphodiesterase activity. The acute metabolic effects of EGF may be mediated by increased phosphatidylinositol breakdown with the formation of diacylglycerol, an activator of protein kinase C, and inositol triphosphate which may be responsible for the increase in Ca^{2+} [37]. Phorbol ester (phorbol 12-myristate 13-acetate; 100 nM), an activator of protein kinase C [38], which has been shown to inactivate glycogen synthase in hepatocyte suspensions [39–41], had similar metabolic effects on hepatocyte cultures to those of EGF (L. Agius & M. H. Chowdhury, unpublished work), suggesting that the effects of EGF and phorbol ester on glycogen metabolism might involve similar mechanisms. The effects of EGF on glycogen metabolism may be the combined result of a rise in cytosolic $[\text{Ca}^{2+}]$ and activation of protein kinase C.

The finding that EGF increased ^{14}C glucose incorporation into glycogen in dividing epithelial cells and fibroblasts suggests that these cells respond similarly to epithelial A431 cells and 3T3 fibroblasts [20]. The fibroblasts and epithelial cells had very high glycolytic rates. EGF can stimulate glucose transport in fibroblasts [42], and it also stimulates glycolysis in quiescent fibroblasts [43]. The rates of lactate production in the epithelial cells and fibroblasts in the present study were 4 times higher than in quiescent 3T3-fibroblasts [43]; this may account for the failure of EGF to stimulate glycolysis in these cells. The increase in ^{14}C incorporation into glycogen may be due to activation of glycogen synthase or to stimulation of glucose transport [42]. Unlike the studies with parenchymal hepatocytes, the increase in ^{14}C incorporation into glycogen was associated with little (if any) glycogen deposition, suggesting that it may represent label exchange rather than net synthesis.

The studies with parenchymal hepatocytes raise the question whether the more marked anti-glycogenic effect

of EGF in the presence of insulin than in its absence might be due to an interaction of EGF with the post-receptor events in insulin action, rather than (or in addition to) a direct effect on glycogen synthase or glycogen phosphorylase. The counteraction of the glycogenic effects of insulin by EGF and phorbol ester may be analogous to previous observations on lipogenesis. In hepatocyte cultures, EGF suppresses the stimulation of acetate incorporation into lipid by insulin, but has no effect on lipogenesis in the absence of insulin [16], and, in adipocytes, phorbol esters inhibit insulin-stimulated lipogenesis, but not the basal rate of lipogenesis in the absence of insulin [44]. Phorbol esters, through activation of protein kinase C, increase serine and threonine phosphorylation of the insulin and EGF receptors, thereby blocking tyrosine phosphorylation of these receptors in response to their ligands [45,46]. One may speculate that EGF might phosphorylate serine or threonine residues on the insulin receptor, thereby blocking tyrosine phosphorylation of the receptor in response to insulin. If this were the case, it would imply that different mechanisms are involved in mediating the glycogenic effects of insulin and its anti-ketogenic and anti-gluconeogenic effects, since the former, but not the latter, are counteracted by EGF.

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