Epidermal growth factor administration decreases liver glycogen and causes mild hyperglycaemia in mice

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Several laboratories report different effects of epidermal growth factor (EGF) on glycogen metabolism in hepatocytes. The discrepancies may be attributed to differences in the experimental conditions. It is therefore important to establish the actual effect of EGF *in vivo*. Because large physiological variations of EGF concentration in plasma occur in mice, we used this species to address this question. In freshly isolated mouse hepatocytes, EGF increased glycogen degradation in a dose-dependent manner. The maximal effect (36 % increase over basal glycogenolysis) was smaller than maximal effects of classical glycogenolytic hormones like adrenaline or glucagon (more than 150 % increase over basal). This is in keeping with the smaller effect of EGF on phosphorylase *a* activity. In contrast with these hormones, EGF did not inhibit glycolysis. Thus these effects of EGF in mouse hepatocytes are similar to those recently described by us in rat hepatocytes [Quintana, Grau, Moreno, Soler, Ramírez and Soley (1995) Biochem. J. **308**, 889–894]. When administered to whole animals, EGF increased phosphorylase *a* activity, decreased the glycogen content in the liver and caused mild hyperglycaemia. Taking together the results obtained for isolated cells and for whole animals, we suggest that the glucosyl residues released from glycogen are used mostly by the liver rather than released to the circulation. This would be different from the action of the classical glycogenolytic hormones, adrenaline and glucagon.

INTRODUCTION

The liver contains a large number of epidermal growth factor (EGF) receptors [1–3]. So the liver, in both rats and mice, contributes significantly to the clearance of circulating EGF [4,5]. The liver is not only responsible for this uptake; it also responds to this peptide. EGF is a potent mitogen for hepatocytes in culture [6–8], and the role of EGF in liver regeneration after partial hepatectomy has been demonstrated in both rats and mice [9–11].

In addition to the mitogenic effect, a number of reports indicate that EGF also affects liver metabolism under non-proliferating conditions. In both perfused livers [12] and isolated hepatocytes [13] from fasted rats, EGF increases gluconeogenesis in a rapid but transient manner. Some delayed effects on gluconeogenesis were also reported [3,14,15] but they are secondary to the effect of EGF on cell redox state [13].

In hepatocytes from fed rats, it was reported that EGF, like insulin, stimulates glycogen synthesis [16]. Other reports indicate that EGF counteracts the glycogenic effect of insulin [17–19]. In the absence of added insulin, it was recently reported that EGF inhibited glycogen deposition [19]. Under different experimental conditions we have shown that EGF stimulates glycogen degradation [20]. Concerning enzyme activities, it was reported that EGF increases both glycogen synthase [16] and glycogen phosphorylase [20,21].

In addition to the effect on glycogen metabolism, it has been reported that EGF stimulates glycolysis and the pentose phosphate pathway in isolated hepatocytes [22]. We have shown that the early stimulation of glycolysis by EGF is secondary to the glycogenolytic effect [20].

The differences in the metabolic effects of EGF reported in the literature could be a consequence of differences in the experimental conditions (such as primary cultures or freshly isolated cells, or the presence or absence of glucose and other components in the incubation medium). Therefore, to understand the effects of EGF in liver glycogen metabolism *in vivo*, it is necessary to extend the studies performed in cell systems to the whole animal. Here we report that the administration of EGF to mice rapidly decreases the glycogen content of the liver and causes mild hyperglycaemia.

EXPERIMENTAL

Experiments in isolated hepatocytes

Hepatocytes were isolated from the livers of adult male Swiss CD-1 mice, fed *ad libitum*, as previously described [3] with minor modifications (Hepes concentration in washing and collagenase-containing buffers was decreased to 20 mM, and the $CaCl_2$ concentration in the collagenase-containing buffer was also decreased, to 1.25 mM). Initial cell viability measured by the Trypan Blue exclusion test was over 90 % and decreased by less than 10 % during the incubations (up to 60 min). Hormones did not affect this decrease. Isolated hepatocytes were incubated as indicated [20]. At the end of the incubation a sample of the suspension was placed into enough ice-cold $HClO_4$ to give a final concentration of 3 % (w/v). After neutralization, glucose [23], glycogen [24], lactate [25] and pyruvate [26] concentrations were determined. cAMP was determined in $HClO_4$ extracts as described [27]. Cytosolic free Ca^{2+} was measured in fura-2/AM-loaded hepatocytes as described [20]. To determine glycogen phosphorylase *a* activity, a sample was taken at the indicated times and centrifuged at 4 °C for 30 s at 10000 ***g***. The medium was discarded and the cells were immediately frozen in liquid nitrogen. Cell pellets were processed as indicated [20]. Glycogen

Abbreviation used: EGF, epidermal growth factor.

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phosphorylase *a* activity was determined in the direction of glucose 1-phosphate release from glycogen at 30 °C [28]. One unit of enzyme activity was defined as the amount of enzyme that catalysed the release of 1 μmol of glucose 1-phosphate per min.

Experiments in whole animals

Adult male Swiss CD-1 mice (45–50 g body weight), fed *ad libitum*, were anaesthetized (sodium pentobarbital, 60 mg/kg) before receiving an intravenous (at the tail vein) injection of EGF (Boehringer Mannheim) (0.25 mg/kg) or an intravenous (0.37 mg/kg) plus an intraperitoneal (1.25 mg/kg) injection of adrenaline (Sigma, St. Louis, MO, U.S.A.). Control animals received identical volumes of saline. After 10 min the blood was collected into heparinized syringes from the inferior vena cava. The liver was then immediately excised and frozen in liquid nitrogen. A sample of the liver was digested in 3 % (w/v) $HClO_4$ and the neutralized supernatant was used to determine glycogen [24]. Another sample was homogenized with 10 vol. of buffer (40 mM glycerol 2-phosphate, pH 6.8, 40 mM 2-mercaptoethanol, 10 mM NaF, 0.1 % BSA) and glycogen phosphorylase *a* was determined as indicated above. Plasma was obtained from the heparinized blood and a sample was used to determine glucose concentration [23] and another sample was processed to determine EGF concentration by ELISA as described [5].

RESULTS AND DISCUSSION

A regulated endocrine secretion of EGF has been described in mice [5,29–31]. The concentration of EGF in plasma (or serum) can rise from the basal level (about 0.1 nM) to nearly 100 nM under appropriate conditions [29,32]. We therefore used the mouse to determine the effect of EGF on liver glycogen metabolism in the whole animal. First, however, we analysed the effect of EGF on glycogen metabolism in isolated mouse hepatocytes.

EGF, adrenaline and glucagon all produced a dose-dependent increase in the rate of glycogen degradation (Figure 1). EGF also increased the output of free glucose (ED_{50} 6 nM) and the accumulation of lactate and pyruvate (ED_{50} 2 nM) (results not shown). In rat hepatocytes this early effect of EGF on glycolysis is secondary to the glycogenolytic effect [33].

Table 1 Effects of EGF, adrenaline and glucagon on glycogen degradation in isolated mouse hepatocytes

Isolated mouse hepatocytes were incubated in glucose-free medium in the absence (basal) or in the presence of 100 nM EGF, 10 μM adrenaline or 0.3 nM glucagon. At 5 min a sample was taken to determine glycogen phosphorylase *a* activity, and the incubation was continued. At 20 min (to determine glycogen and glucose) and 30 min (to determine lactate and pyruvate) further samples were taken. The initial glycogen content of the cells was 2.63 ± 0.17 μmol glucosyl units per 10^6 cells. Units of activity: phosphorylase *a*, m-units per 10^6 cells; glycogen degradation, nmol glucosyl units per 20 min per 10^6 cells; glucose release, nmol glucosyl units per 20 min per 10^6 cells; glycolysis, nmol (lactate + pyruvate) per 30 min per 10^6 cells. The results are means ± S.E.M. for ten experiments made with different cell preparations. Triplicates of every incubation condition were made in each experiment. Statistically significant differences from the basal condition were determined by using Student's paired *t*-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

	Condition			
Parameter	Basal	EGF	Adrenaline	Glucagon
Phosphorylase *a*	20 ± 1	37 ± 2***	72 ± 5***	88 ± 5***
Glycogen	729 ± 63	992 ± 74*	1874 ± 82***	1692 ± 101***
Glucose release	447 ± 33	707 ± 68**	1832 ± 98***	1620 ± 96***
Glycolysis	172 ± 13	255 ± 26**	35 ± 9***	60 ± 7***

As in rat hepatocytes [21,33], EGF stimulates phosphorylase activity in mouse hepatocytes (Table 1). As in rats, the increase in phosphorylase activity was lower when the cells were stimulated with maximal doses of EGF than with adrenaline or glucagon (Table 1). This is in keeping with the smaller increase in glycogen degradation and glucose output (Table 1).

In rat hepatocytes the effect of EGF on glycogen degradation is linked to the increase in cytosolic free Ca^{2+} [20]. In mouse hepatocytes EGF led to a moderate decrease in cAMP concentration (Table 2), and increased the cytosolic free Ca^{2+} concentration (Figure 2, top panel). Adrenaline at 10 μM [which produced a maximal stimulation of glycogen breakdown (Figure 1, middle panel)], increased the concentration of both messengers. It is known that hepatocytes contain both α_1- and β-

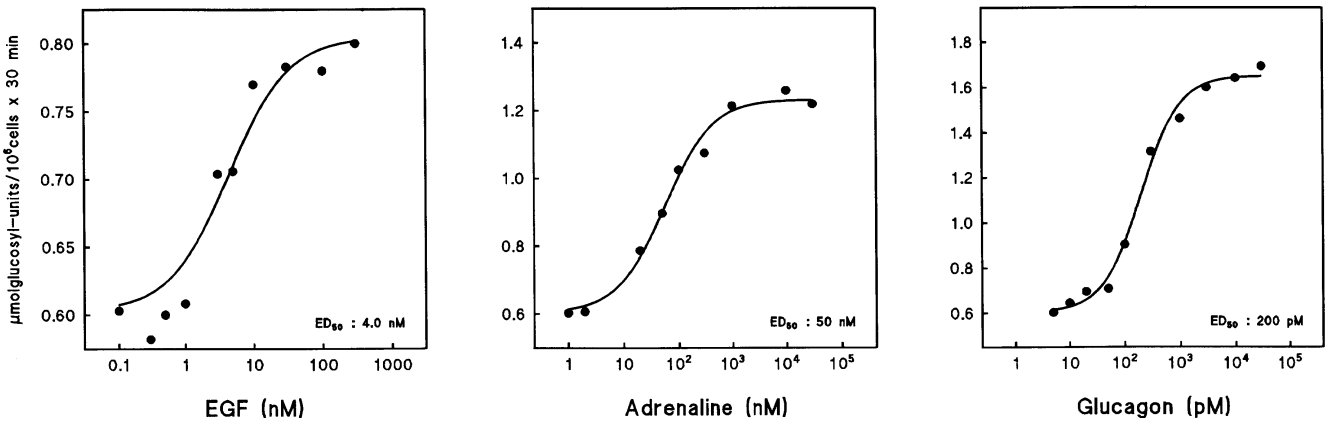

Figure 1 Dose-dependent effect of EGF, adrenaline and glucagon on glycogen degradation

Isolated mouse hepatocytes were incubated in glucose-free medium in the presence of increasing concentrations of EGF (left panel), adrenaline (middle panel) or glucagon (right panel). At 20 min a sample was taken to determine glycogen. The initial glycogen content of the cells was 2.72 μmol glucosyl units per 10^6 cells. The results are the means of duplicate values from a representative experiment. Two experiments were performed with different cell preparations.

Table 2 Effect of EGF, adrenaline and glucagon on cAMP concentration in mouse hepatocytes

Isolated mouse hepatocytes were incubated in glucose-free medium in the absence (basal) or in the presence of EGF, adrenaline or glucagon. At 5 min a sample was taken to determine cAMP concentration. Results represent the means ± S.E.M. for eight experiments made with different cell preparations. Triplicates of every incubation condition were made in each experiment. Statistically significant differences were determined using Student's paired *t*-test: *, $P < 0.05$; ***, $P < 0.001$.

Condition	[cAMP] (pmol per 10^6 cells)
Basal	6.3 ± 0.8
EGF (100 nM)	5.4 ± 0.6*
Adrenaline (10 μM)	20.1 ± 1.1***
Glucagon (0.3 nM)	17.7 ± 1.3***

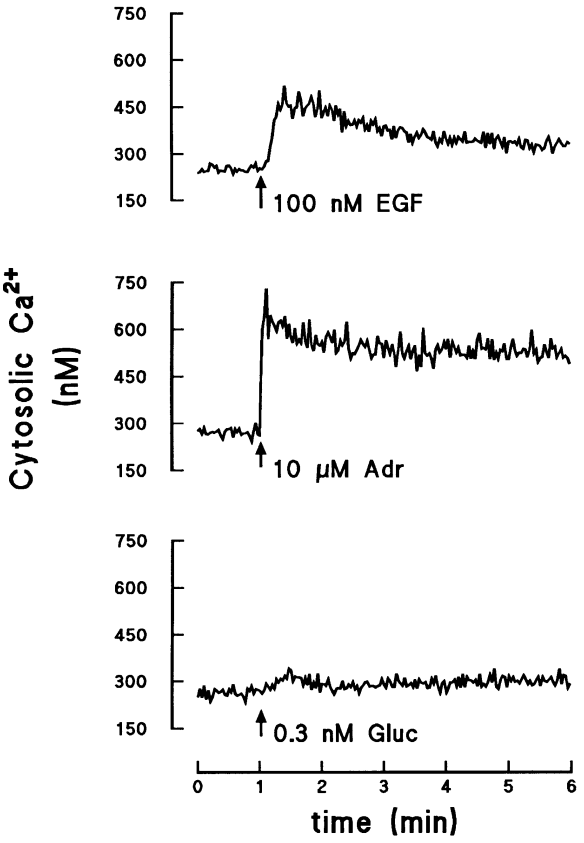

Figure 2 Effect of EGF, adrenaline and glucagon on cytosolic free Ca^{2+} concentration in mouse hepatocytes

Fura-2/AM-loaded hepatocytes were incubated at 25 °C to monitor cytosolic free Ca^{2+} concentration. At the time indicated with an arrow, EGF (top panel), adrenaline (middle panel) or glucagon (bottom panel) was added. The graphs show the results of a representative experiment. Five experiments with different cell preparations were performed with similar results.

adrenergic receptors [34]. We have observed that the increase in cAMP is the result of the interaction of adrenaline with the β-receptors and that the Ca^{2+} signal is linked to the interaction with the α_1-receptors (M. Grau, M. Soley and I. Ramírez, unpublished work). The increase in cAMP was quite low because the number of β-adrenergic receptors is small in males [34,35].

Glucagon at 0.3 nM produced a nearly half-maximal increase in glycogen degradation (Figure 1, right panel). At this concentration the increase in cAMP concentration was moderate (Table 2), as was the increase in the cytosolic free Ca^{2+} concentration (Figure 2). The increase in cytosolic Ca^{2+} produced by glucagon in hepatocytes is known to be secondary to the increase in cAMP [36]. The precise mechanisms involved are not yet completely resolved. It is worth noting that glucagon can raise cAMP concentration greatly (Figure 3, left panel). The Ca^{2+} concentration also increased with higher doses of glucagon (results not shown). Because the ED_{50} for the cAMP increase (4 nM) is higher than that for the stimulation of glycogenolysis (0.2 nM), the relationship between cAMP increase and glycogenolysis was hyperbolic rather than linear, reaching saturation at cAMP concentrations of 25 pmol per 10^6 cells (Figure 3, right panel).

Phosphorylase kinase is sensitive to both cAMP and Ca^{2+} [37]. Because EGF increased Ca^{2+} but not cAMP, and adrenaline or glucagon increased both messengers, it is understandable that the effect of EGF on phosphorylase kinase, and hence on the increase in phosphorylase *a* activity, was lower when the cells were exposed to EGF than when exposed to glucagon or adrenaline. Therefore all these effects of EGF are similar to those produced in rat hepatocytes [20]. However, if we compare the magnitudes of the responses of rat and mouse hepatocytes to EGF (see results in [20] and those described here) we observe that the response of mouse hepatocytes is stronger: EGF (always at maximal doses) increases phosphorylase *a* activity by 1.28-fold and 1.87-fold in rat and mouse hepatocytes respectively. One consequence of this is that the increase in glycogen degradation is greater in mouse (1.36-fold) than in rat (1.15-fold) hepatocytes. Whether these differences are due to the greater number of EGF receptors in mouse [3] than in rat [2] hepatocytes, or to some other cause is, however, unknown.

In isolated or cultured hepatocytes, several laboratories report a variety of effects of EGF on glycogen metabolism [17,18,20,38]. Thus, having established that mouse hepatocytes responded to EGF (at least under our conditions) in the same way as rat hepatocytes, we attempted to determine the effect in whole animals. We compared the effects of EGF and adrenaline on liver glycogen metabolism *in vivo*. Adrenaline administration produced a 5.4-fold increase in the phosphorylase *a* activity in the liver (Table 3). This resulted in a decrease in liver glycogen content of 28% at the time of animal death, and a 1.74-fold increase in plasma glucose concentration. EGF also increased phosphorylase *a* activity (Table 3), but only 2.5-fold. In agreement with this lower effect on phosphorylase, the decrease in liver glycogen was also lower. Plasma glucose concentration was moderately increased by the administration of EGF. At the time of death, the concentration of EGF in plasma was 9.2 ± 1.8 nM, which is within the range of the physiological variation in mice [5,29,32].

The decrease in liver glycogen and the increase in plasma glucose concentrations indicate that EGF causes net glycogenolysis *in vivo*. This is in agreement with the results obtained in isolated hepatocytes, discussed above, and also with those described by Peak and Agius [19]. They found that EGF decreased glycogen deposition in hepatocytes cultured in the presence of high glucose concentration. The earliest report showing an increase in glycogen synthase activity in hepatocytes exposed to EGF is not easily explained. It might indicate that EGF could increase the recycling of the glucosyl residues, provided that the stimulation of glycogen phosphorylase by EGF is well established [20,21] (Table 1). Nevertheless, our results clearly indicate that the net balance in the whole animal is glycogenolytic.

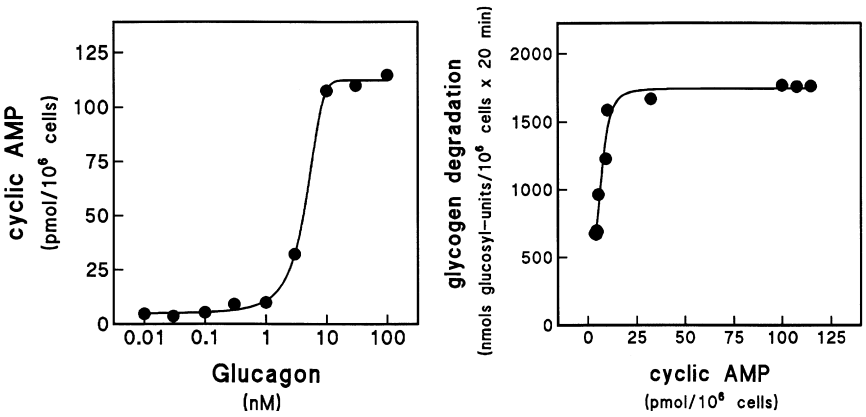

Figure 3 Relationship between cAMP and glycogen degradation in glucagon-treated hepatocytes

Left panel: isolated mouse hepatocytes were incubated in glucose-free medium in the presence of increasing concentrations of glucagon. At 5 min a sample was taken to determine cAMP concentration. Right panel: to determine glycogen degradation the incubation was continued for a further 15 min. This panel shows the relationship between the cAMP concentrations and the amount of glycogen degraded. The results are the means of duplicate values from a representative experiment. Three experiments with different cell preparations were made with similar results.

Table 3 Effects of EGF or adrenaline administration to mice on liver glycogen concentration phosphorylase *a* activity and plasma glucose

Male mice were anaesthetized (sodium pentobarbital, 60 mg/kg) before being injected with EGF (0.25 mg/kg intravenously) or adrenaline (0.37 mg/kg intravenously and 1.25 mg/kg intraperitoneally). Control animals received an identical volume of saline. The animals were killed 10 min after the administrations. Units of activity: liver phosphorylase *a*, units/g; liver glycogen, μmol glucosyl units per g; plasma glucose mM. Results are the means $\pm$ S.E.M. for 15 animals. Statistically significant differences against saline were determined by using Student's unpaired *t*-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

	Administration		
Parameter	Saline	EGF	Adrenaline
Liver phosphorylase *a*	1.0 ± 0.1	$2.5 \pm 0.3^{***}$	$5.4 \pm 0.3^{***}$
Liver glycogen	309 ± 15	$259 \pm 11^{**}$	$222 \pm 14^{***}$
Plasma glucose	9.8 ± 0.3	$11.0 \pm 0.5^{*}$	$17.0 \pm 0.8^{***}$

The whole amount of glucosyl residues released from glycogen can be calculated from data in Table 3 and the average liver weight (2.25 g). This gives a release of 112 and 196 μmol of glucosyl units on administration of EGF and adrenaline respectively. Assuming a glucose distribution space of 0.47 ml/g body weight [39], and that the increase in plasma glucose is an estimate of the increase in glucose concentration throughout the whole distribution space, we calculated the amount of free glucose that was released by the liver (26 and 158 μmol of glucose on administration of EGF and adrenaline respectively). Although we have not considered any hypothetical effect of EGF or adrenaline on glucose half-life (note that the animals were inactive because of the anaesthesia), the amount of glucose released by the liver after adrenaline injection (158 μmol) corresponds to 81% of the decrease in liver glycogen (196 μmol of glucosyl units). This is in keeping with the results obtained in isolated cells, where adrenaline not only stimulated glycogen degradation, but also inhibited glycolysis (Table 1). When mice received EGF, the amount of free glucose is only a minor proportion of the whole amount of glycogen degraded (23% according to the calculations made above). Again, this is in keeping with the results obtained in isolated cells, where EGF also stimulated glycogenolysis but did not inhibit glycolysis, it was actually increased (Table 1) [20].

Therefore, whereas adrenaline prevents the utilization by the liver itself of the glucosyl residues released from glycogen (by means of the inhibition of glycolysis), EGF does not, but it provides phosphorylated glucosyl residues that can be used by hepatocytes. Conricode and Ochs [22] have shown that EGF stimulates the pentose phosphate pathway in hepatocyte suspensions. Long exposure of cultured hepatocytes to EGF results in the induction of key enzymes of the pentose phosphate pathway [40]. Thus EGF might stimulate, by some early and some late mechanisms, the utilization of glucose by hepatocytes, which might provide pentoses for DNA synthesis, the most striking effect of this growth factor.

In conclusion, we report here the first evidence that EGF stimulates hepatic glycogen degradation *in vivo*. Our results in isolated cells and in whole animals further suggest that the glucosyl residues released might be used by the liver itself rather than released into the bloodstream.

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