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Rapid and delayed effects of epidermal growth factor on gluconeogenesis

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Most reports on the effects of epidermal growth factor (EGF) on gluconeogenesis have indicated that such effects depend on the substrate used and are only observable after a lag time of 30–40 min. Recently, an immediate and transient effect of EGF on glucose synthesis was described in a perfused liver system. Here we extend the study of the effect of EGF on gluconeogenesis to isolated hepatocytes from fasted rats. The delayed effect of EGF on gluconeogenesis was studied by adding the substrate 40 min after the peptide. Under these conditions EGF increased glucose synthesis from pyruvate, decreased it when the substrate was lactate or glycerol and did not modify gluconeogensis from fructose or dihydroxyacetone. EGF did not affect the metabolic flux through glycolysis, determined as the production of lactate + pyruvate from 30 mM glucose. Furthermore, EGF did not modify the metabolic flux through pyruvate kinase, determined as the production of lactate + pyruvate from 1 mM dihydroxyacetone. The differing effects of EGF on gluconeogenesis depending on the substrate used can be explained by the effects of EGF on the cytosolic redox state (measured as the lactate/pyruvate ratio). About 20 min after the addition of EGF, the mitochondrial redox state (measured as the 3-hydroxybutyrate/acetoacetate ratio) decreased. This effect of EGF was blocked by ammonium, which also abolished the effect of the peptide on gluconeogenesis. Thus the effect of EGF at the mitochondrial level appears to be necessary for its effects on gluconeogenesis. Taken together, our results indicate that the delayed effects of EGF on gluconeogenesis are secondary to the effects of the peptide at both the mitochondrial and cytosolic levels. In addition to these delayed effects, we observed that EGF rapidly and transiently stimulated glucose synthesis from lactate, decreased the cytosolic redox state and increased oxygen consumption. All of these rapid effects required the presence of extracellular calcium and disappeared in the presence of rotenone, suggesting that this rapid effect of EGF on gluconeogenesis is secondary to the stimulation of mitochondrial respiration.

INTRODUCTION

Epidermal growth factor (EGF) is a potent mitogenic peptide for many cell types [1,2]. It binds to specific cell surface receptors which have intrinsic tyrosine kinase activity [2,3]. Upon activation, the EGF receptor undergoes autophosphorylation [4] and then phosphorylates specific substrates [5,6]. Other early events that follow EGF–receptor complex formation include phosphatidylinositol 4,5-bisphosphate hydrolysis, which generates diacylglycerol and inositol 1,4,5-trisphosphate [7]. IP_3 induces the release of stored Ca^{2+} into the cytosol. This, and the activation of plasma membrane Ca^{2+} inflow [7,8], leads to an increase in the cytosolic Ca^{2+} levels. Phosphorylation of cellular substrates and alterations of the ionic content of the cell are assumed to provide an internal stimulus for cell growth.

The liver is a target tissue for EGF action. It contains a large number of EGF receptors in both fetal and adult life [9,10]. The liver can also sequester high doses of intraportally administered EGF [11]. The clearest effect of EGF in hepatocytes is the stimulation of DNA synthesis [12], and a role for EGF in the early events of liver regeneration has been observed [13,14]. Several metabolic pathways in the liver were also found to be affected by EGF. However, published results are contradictory. Thus glucose deposition into glycogen has been shown to be increased [15], decreased [16], or not affected [17] by EGF. Furthermore, it was found that EGF counteracted [16,17] or had an additive effect [15] to the effect of insulin on glycogen synthesis. The effect of EGF on lipogenesis in rat hepatocytes has not been established unequivocally [18,19]. EGF increases glycolysis both in non-hepatic cells, such as quiescent 3T3 cells [20], and in hepatocytes isolated from fed rats [21]. In hepatocytes isolated from starved rats EGF affects gluconeogenesis, but again published results appear to be controversial. We found that EGF stimulates gluconeogenesis from pyruvate in mouse [22] and in rat [17] hepatocytes. Increased glucose synthesis from alanine, lactate/pyruvate (10:1, mol/mol) or asparagine, but not from proline or glutamine, has also been described [23]. These effects on gluconeogenesis were only observed if EGF was present 20–40 min before the addition of the gluconeogenic substrate. However, Rashed and Patel [24] recently showed that, in perfused livers, EGF rapidly and transiently increased glucose synthesis from pyruvate. In cultured hepatocytes, however, it was found that EGF did not modify gluconeogenesis from pyruvate [16]. We have reported elsewhere that EGF may increase, not affect or even decrease glucose synthesis, depending on the redox state of the substrate [25]. It was proposed that the effect of EGF on gluconeogenesis is the result of a transient [24] or stable [23] inhibition of pyruvate kinase.

We now study further the effect of EGF on gluconeogenesis in isolated hepatocytes from fasted rats. Our results indicate that the effect of EGF on gluconeogenesis may be explained by the effect on the cytosolic redox state. In addition to those delayed effects which are only observed after about 40 min, EGF rapidly and transiently stimulates gluconeogenesis from lactate.

MATERIAL AND METHODS

Chemicals

Glucagon was obtained from Novo Industri (Copenhagen, Denmark). EGF, EGTA, pyruvate, L-lactate, glycerol, fructose,

Abbreviation used: EGF, epidermal growth factor.

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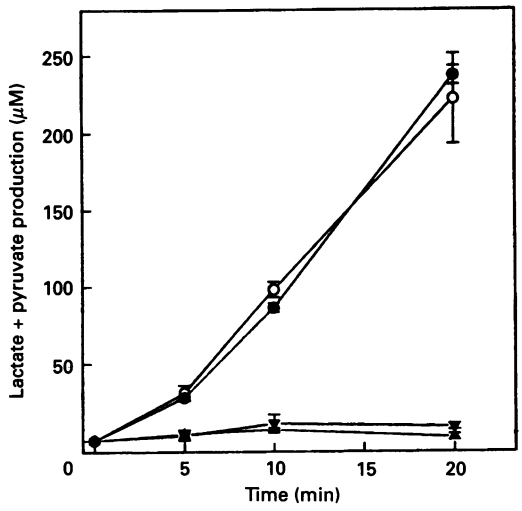

Figure 1 Effects of EGF and glucagon on metabolic flux through the pyruvate kinase reaction

Metabolic flux through the pyruvate kinase reaction was assessed in intact cells by the production of lactate + pyruvate from 1 mM dihydroxyacetone. EGF (10 nM) was added 40 min before and glucagon (10 nM) simultaneously with the substrate. ●, Control; ○, EGF; ▲, glucagon; ▼, EGF + glucagon. In all experiments each condition was performed in triplicate. Results are means ± S.E.M. of four experiments carried out with different cell preparations.

Table 1 Effect of the initial redox state of the substrate on the cytosolic redox state

Isolated hepatocytes were incubated for 40 min at 37 °C before the addition of the indicated concentrations of lactate and pyruvate; 20 min later the incubation medium was collected and processed as indicated in the Materials and methods section in order to determine lactate and pyruvate concentrations. Results are the means of two independent experiments performed in triplicate. L/P is the lactate/pyruvate ratio.

Initial			Final		
Lactate (mM)	Pyruvate (mM)	L/P	Lactate (mM)	Pyruvate (mM)	L/P
1.0	0.0	∞	0.34	0.03	11.3
2.0	0.0	∞	1.77	0.09	19.7
10.0	0.0	∞	9.68	0.20	48.4
0.9	0.09	10.0	0.47	0.03	15.7
1.8	0.18	10.0	0.97	0.08	12.1
9.0	0.9	10.0	9.75	0.57	17.1
0.5	0.5	1.0	0.57	0.06	9.5
1.0	1.0	1.0	1.21	0.21	5.8
5.0	5.0	1.0	4.0	3.93	1.0
0.09	0.9	0.1	0.34	0.22	1.5
0.18	1.8	0.1	0.47	0.77	0.6
0.9	9.0	0.1	1.5	6.00	0.3
0.0	1.0	0.0	0.25	0.23	1.1
0.0	2.0	0.0	0.31	0.88	0.4
0.0	10.0	0.0	1.07	7.5	0.1

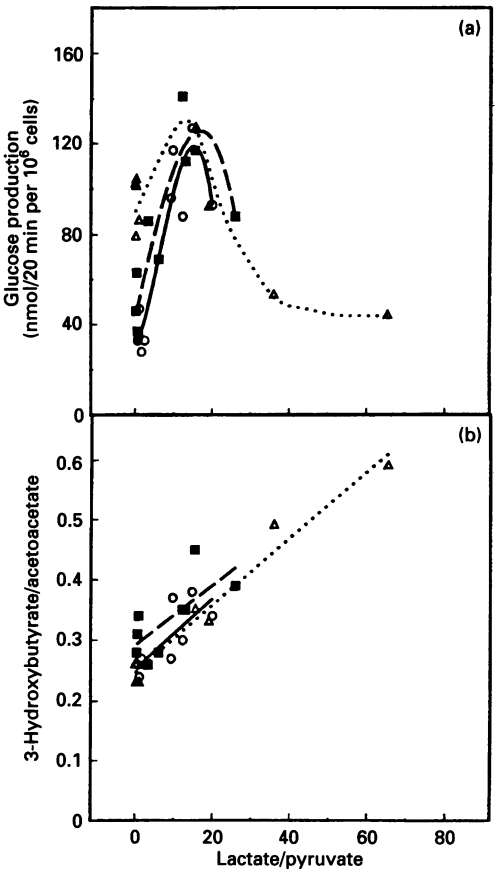

Figure 2 Relationship between the cytosolic redox state and both gluconeogenesis and the mitochondrial redox state

In the experiment shown in Table 1, glucose, acetoacetate and 3-hydroxybutyrate concentrations were also determined. The relationship between the cytosolic redox state (lactate/pyruvate ratio) and gluconeogenesis (glucose production) is shown in (**a**), and that between the cytosolic and the mitochondrial (3-hydroxybutyrate/acetoacetate ratio) redox states is shown in (**b**). Initial lactate + pyruvate concentrations: ○—○, 1 mM; ■--■, 2 mM; △----△, 10 mM.

acetoacetate, 3-hydroxybutyrate, dihydroxyacetone, glucose and rotenone were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Collagenase, Hepes, fatty acid-free albumin, NAD^+, NADH, lactate dehydrogenase, 3-hydroxybutyrate dehydrogenase and the enzymic colorimetric test for glucose were from Boehringer Mannheim (Mannheim, Germany). All other reagents were of analytical grade.

Hepatocyte isolation and incubation

Male Wistar rats (200–250 g body weight) were starved for 24 h before use. Hepatocytes were isolated by the collagenase perfusion method of Berry and Friend [26] as previously described [17], but glucose was not added to perfusion solutions. Initial cell viability measured by the Trypan Blue exclusion test was over 90 %, and decreased by about 10 % during incubation. Hormones did not affect this decrease.

Isolated hepatocytes [(1–3) × 10^6 cells/ml, final volume 2 ml] were incubated in a 20 mM Hepes (pH 7.4)-containing buffer supplemented with 1 % albumin (fatty acid-free) [17], but without glucose. Incubation was carried out with constant shaking (80 cycles/min) at 37 °C under an O_2/CO_2 (19:1) atmosphere. Under these conditions the pH was maintained at 7.4 during the whole incubation period. EGF (10 nM) was added to the cells simultaneously with or 40 min before the gluconeogenic substrate. Glucagon (10 nM) was added to the cells at the time of substrate addition. Hepatocytes after substrate addition were then further incubated for up to 20 min. To end the incubation, a sample of the suspension was placed into enough ice-cold

Table 2 Effect of EGF, added 40 min before the substrate, and glucagon on gluconeogenesis and on the cytosolic and mitochondrial redox states

Isolated hepatocytes were incubated in the absence or in the presence of EGF (10 nM); 40 min later, the gluconeogenic substrate (lactate, pyruvate, glycerol, fructose or dihydroxyacetone) was added. In cells incubated with glucagon (10 nM), the hormone was added simultaneously with the substrate. The incubation was then continued for another 20 min and processed to determine glucose, lactate (L), pyruvate (P), 3-hydroxybutyrate (3-HB) and acetoacetate (AcAc) concentrations. In all experiments each condition was performed in triplicate. Results are means ± S.E.M. of the number of independent experiments indicated. Statistical comparisons between EGF, glucagon or EGF + glucagon versus corresponding control values were by paired Student's t test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Substrate	Glucose (nmol/20 min per 10^6 cells)	Lactate (μM)	Pyruvate (μM)	3-HB (μM)	AcAc (μM)	L/P	3-HB/AcAc
Lactate (2 mM) ($n = 10$)							
Control	99 ± 6	1116 ± 56	117 ± 7	193 ± 16	457 ± 46	9.7 ± 0.5	0.44 ± 0.03
EGF	87 ± 7*	1130 ± 50	105 ± 7	182 ± 16**	455 ± 51	11.4 ± 0.7*	0.41 ± 0.03**
Glucagon	119 ± 4**	969 ± 59***	100 ± 8*	192 ± 15	445 ± 50	10.0 ± 0.7	0.44 ± 0.02
EGF + Glucagon	90 ± 5	1029 ± 51	94 ± 7*	179 ± 15*	447 ± 50	11.6 ± 0.9*	0.41 ± 0.02*
Pyruvate (1 mM) ($n = 6$)							
Control	21 ± 3	262 ± 12	312 ± 22	102 ± 5	410 ± 18	0.87 ± 0.07	0.25 ± 0.01
EGF	27 ± 3**	277 ± 18	285 ± 18	85 ± 6**	400 ± 19	1.02 ± 0.1*	0.21 ± 0.01**
Glucagon	17 ± 3*	219 ± 11**	349 ± 40	119 ± 5**	473 ± 36	0.67 ± 0.04**	0.26 ± 0.01
EGF + Glucagon	23 ± 2	226 ± 9**	279 ± 17	102 ± 5	440 ± 32	0.90 ± 0.07	0.23 ± 0.01**
Glycerol (20 mM) ($n = 4$)							
Control	64 ± 5	57 ± 3	5.0 ± 0.3	63 ± 10	461 ± 49	11.4 ± 0.6	0.14 ± 0.02
EGF	50 ± 5*	65 ± 6	3.4 ± 0.2*	40 ± 8***	479 ± 51	17.8 ± 0.6**	0.09 ± 0.02**
Glucagon	66 ± 6	57 ± 8	5.2 ± 0.2	65 ± 11	465 ± 52	10.9 ± 1.2	0.15 ± 0.03
EGF + Glucagon	48 ± 4***	64 ± 12	3.0 ± 0.8	41 ± 9	439 ± 49	21.2 ± 2.3	0.10 ± 0.02**
Fructose (5 mM) ($n = 4$)							
Control	258 ± 27	350 ± 42	159 ± 10	122 ± 8	307 ± 15	2.2 ± 0.1	0.40 ± 0.03
EGF	276 ± 23	386 ± 53	173 ± 23	109 ± 8*	308 ± 20	2.2 ± 0.1	0.35 ± 0.02*
Glucagon	290 ± 28**	258 ± 26*	125 ± 9**	88 ± 5**	301 ± 18	2.1 ± 0.1	0.30 ± 0.04*
EGF + Glucagon	315 ± 29**	279 ± 26*	137 ± 16	75 ± 6**	362 ± 13	2.0 ± 0.1	0.21 ± 0.02*
Dihydroxyacetone (1 mM) ($n = 4$)							
Control	154 ± 10	109 ± 17	24 ± 2	105 ± 6	387 ± 35	4.7 ± 0.2	0.28 ± 0.03
EGF	147 ± 11	106 ± 15	15 ± 1*	91 ± 5*	388 ± 35	7.1 ± 0.6*	0.24 ± 0.02*
Glucagon	202 ± 22*	14 ± 5**	5 ± 1**	66 ± 6**	479 ± 57*	3.2 ± 1.3	0.15 ± 0.02*
EGF + Glucagon	195 ± 23*	18 ± 6**	4 ± 1**	58 ± 4***	451 ± 41**	3.8 ± 1.2	0.13 ± 0.02**

$HClO_4$ to give a final concentration of 6%. After neutralization, glucose [27], lactate [28], pyruvate [29], acetoacetate and 3-hydroxybutyrate [30] concentrations were determined. Oxygen consumption at 37 °C was measured polarographically with a Clark electrode [31]. The cells were incubated in the absence of EGF to monitor the basal oxygen consumption. EGF (10 nM) was added 5 min later, and the oxygen consumption was recorded for the next 10 min. The experiment was performed in triplicate for each hepatocyte preparation.

RESULTS

Delayed effects of EGF

In previous studies we determined gluconeogenesis by adding either pyruvate or lactate to isolated hepatocytes and quantifying the appearance of glucose after 20 min of incubation with the substrate. Under such conditions, EGF both increased glucose synthesis from pyruvate and decreased glucose synthesis from lactate [25], but only if the peptide had been added to the cells about 40 min before substrate addition. It has been suggested that the effect of EGF on gluconeogenesis may be a consequence of an inhibition of pyruvate kinase [23,24]. In order to determine whether the observed delayed effect of EGF on gluconeogenesis can be explained by an effect on pyruvate kinase, we measured lactate and pyruvate production in cells incubated with dihydroxyacetone (Figure 1). While glucagon almost completely abolished lactate + pyruvate production by hepatocytes, EGF did not affect such production, nor did it interfere with the effect of glucagon. Furthermore, while 10 nM glucagon clearly decreased lactate + pyruvate production in hepatocytes incubated with 30 mM glucose (225 ± 13 μM and 34 ± 3 μM at 20 min for control and glucagon respectively; $P < 0.01$), 10 nM EGF did not (228 ± 20 μM lactate + pyruvate after 20 min).

Gluconeogenic flux can be modulated by other factors. It was shown that changes in the cellular redox state may alter the gluconeogenic flux [32,33]. In preliminary experiments we studied the effect of the redox state of the substrate on the redox state of the cytosol (measured by the lactate/pyruvate ratio [33]) 20 min after the addition of the substrate (Table 1). At this time, glucose production and the 3-hydroxybutyrate/acetoacetate ratio (an indicator of the mitochondrial redox state [34]) were also measured. The relationship between the lactate/pyruvate ratio (cytosolic redox state) and either gluconeogenesis or the 3-hydroxybutyrate/acetoacetate ratio (mitochondrial redox state) is shown in Figure 2. The relationship between the redox state of the cytosol and gluconeogenesis was biphasic: at a low lactate/pyruvate ratio, glucose synthesis increased as the ratio increased, while at high lactate/pyruvate ratio, glucose synthesis decreased as the ratio increased. The rate of gluconeogenesis was highest at a lactate/pyruvate ratio of 12–14. This was true for every substrate concentration used (Figure 2a) and is in agreement with previous reports [33]. The relationship between the lactate/pyruvate ratio and the 3-hydroxybutyrate/acetoacetate ratio was linear, and independent of the substrate concentration (Figure 2b).

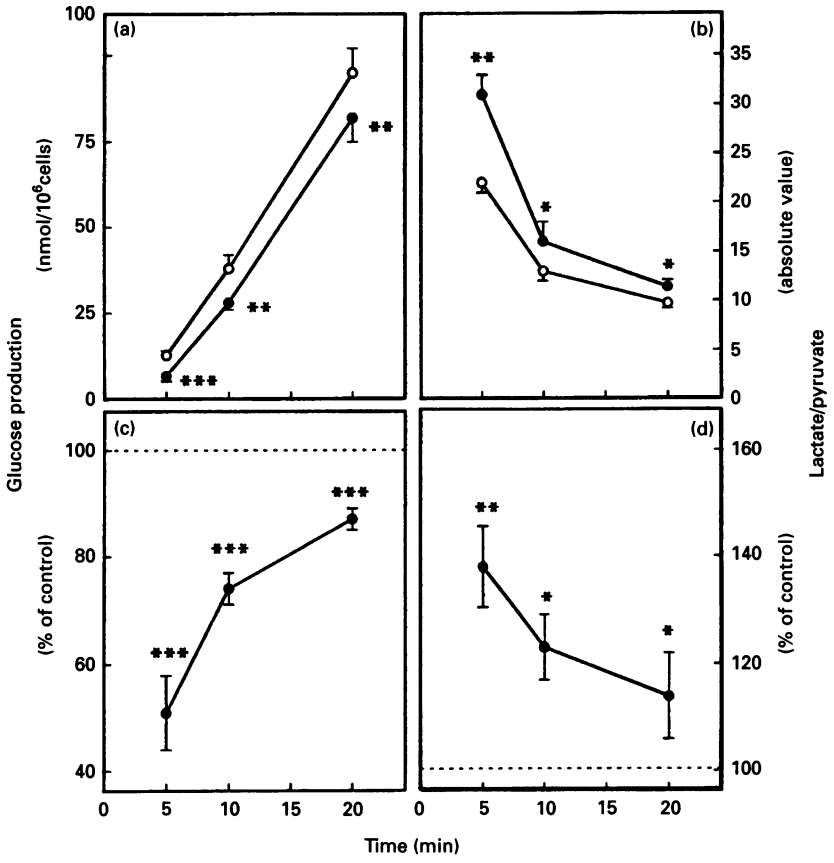

Figure 3 The delayed effects of EGF on gluconeogenesis and on the cytosolic redox state are transient

Isolated hepatocytes were incubated in the absence (○) or in the presence (●) of 10 nM EGF; 40 min later the gluconeogenic substrate (2 mM lactate) was added. After 5, 10 or 20 min of incubation in the presence of substrate, a sample was processed to determine glucose production (**a**) and the lactate/pyruvate ratio (**b**). The results shown in (**a**) and (**b**) are plotted in (**c**) and (**d**) respectively as percentages of the corresponding control value. In all experiments each condition was performed in triplicate. Results are means ± S.E.M. of six experiments carried out with different cell preparations. Statistical comparisons were by paired Student's *t* test: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Based on these results, we hypothesized that the variable effect of EGF on gluconeogenesis (increasing glucose synthesis from pyruvate, but decreasing glucose synthesis from lactate [25]) may be the consequence of an effect of EGF on the cytosolic redox state. Therefore the effects of EGF on glucose production and on the cytosolic (lactate/pyruvate ratio) and mitochondrial (3-hydroxybutyrate/acetoacetate ratio) redox states were studied using several gluconeogenic substrates (lactate, pyruvate, glycerol, fructose and dihydroxyacetone). For comparison, the effect of glucagon was also studied. The results are shown in Table 2. As others have described [35–37], glucagon increased glucose production from lactate, fructose or dihydroxyacetone, did not affect gluconeogenesis from glycerol, and decreased glucose synthesis from pyruvate. Only when the substrate was pyruvate did glucagon decrease the lactate/pyruvate ratio; when the substrate was fructose or dihydroxyacetone, glucagon decreased the 3-hydroxybutyrate/acetoacetate ratio (Table 2).

EGF increased gluconeogenesis from pyruvate, decreased this pathway when the substrate was lactate or glycerol, and did not modify gluconeogenesis from fructose or dihydroxyacetone (Table 2). When the gluconeogenic substrate was lactate, pyruvate, glycerol or dihydroxyacetone, EGF increased the lactate/pyruvate ratio, but when fructose was the substrate EGF did not alter this ratio. The relationship between the effect of EGF on the cytosolic redox state and the effect on gluconeogenesis can be better understood in a time-course experiment (Figure 3) in which the gluconeogenic substrate used was lactate. The addition of the substrate modified the cytosolic redox state, which progressively reached a new steady state (Figure 3b). EGF slowed down the rate at which this new steady state was reached. On a percentage basis, the effect was greater at 5 min than at 20 min (Figure 3d). In addition, the effect of EGF on glucose synthesis from lactate was, on a percentage

Table 3 EGF does not affect the production of total ketone bodies

Total ketone body (3-hydroxybutyrate + acetoacetate) concentrations were calculated from data presented in Table 2. Statistical comparisons between EGF and corresponding control values were by paired Student's *t* test; non-significant differences were obtained.

	3-Hydroxybutyrate + acetoacetate (μM)	
Gluconeogenic substrate	Control	EGF
Lactate (2 mM)	644 ± 61	637 ± 64
Pyruvate (1 mM)	510 ± 25	480 ± 26
Glycerol (20 mM)	523 ± 49	508 ± 49
Fructose (5 mM)	429 ± 19	417 ± 29
Dihydroxyacetone (1 mM)	429 ± 34	481 ± 54

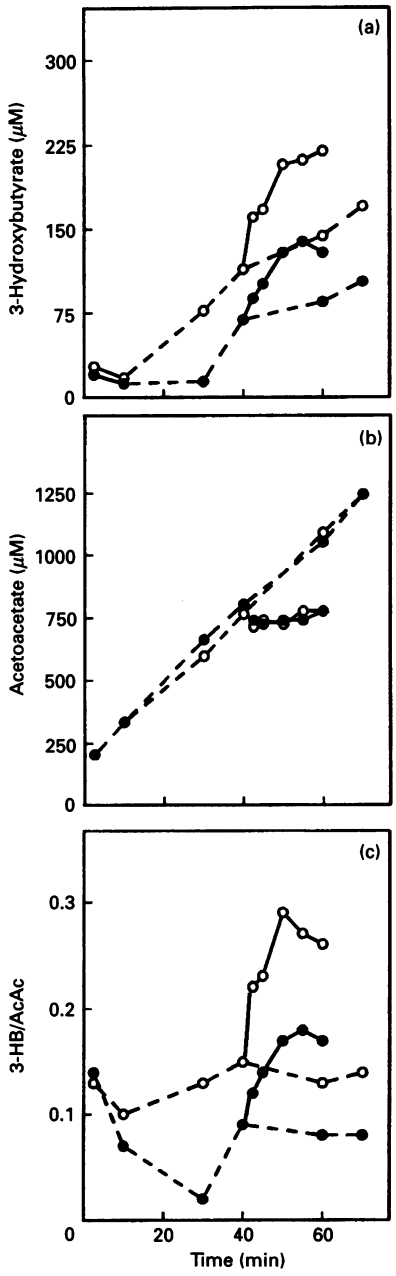

Figure 4 Effect of EGF on the mitochondrial redox state

Isolated hepatocytes were incubated at 37 °C in the absence (○) or in the presence (●) of 10 nM EGF (broken lines in both). At 40 min, 20 mM glycerol was added (continuous lines). At indicated times a sample was processed to determine the acetoacetate (AcAc) and 3-hydroxybutyrate (3-HB) concentrations. Results are means from a representative experiment performed in triplicate.

Table 4 Delayed effect of EGF on gluconeogenesis and both cytosolic and mitochondrial redox states in hepatocytes incubated in the absence of calcium or in the presence of ammonium

Isolated hepatocytes were incubated for 40 min at 37 °C with EGF (10 nM) in a medium containing no $CaCl_2$ and 0.5 mM EGTA or in a medium containing 3.2 mM NH_4Cl. Then 2 mM lactate was added and 5 min later a sample was processed to determine glucose production and the cytosolic (lactate/pyruvate; L/P) and mitochondrial (3-hydroxybutyrate/acetoacetate; 3-HB/AcAc) redox states. Three experiments with different cell preparations were performed in triplicate and the results are means ± S.E.M. Statistical comparisons versus control were by paired *t* test: $^*P < 0.05$; $^{**}P < 0.01$.

	Glucose (nmol/5 min per 10^6 cells)	L/P	3-HB/AcAc
Normal conditions (1.3 mM Ca^{2+}, no NH_4^+)			
Control	11.4 ± 1.3	37.6 ± 4.4	f0.24 ± 0.01
EGF	6.7 ± 1.2**	46.2 ± 2.9*	0.21 ± 0.01*
No Ca^{2+} + 0.5 mM EGTA (no NH_4^+)			
Control	6.6 ± 1.1	41.0 ± 0.5	0.16 ± 0.01
EGF	4.9 ± 1.0**	51.1 ± 1.4*	0.11 ± 0.01*
3.2 mM NH_4^+ (1.3 mM Ca^{2+})			
Control	3.0 ± 0.6	23.9 ± 0.7	0.05 ± 0.01
EGF	3.9 ± 0.7	23.5 ± 1.1	0.05 ± 0.01

basis, higher at 5 min than at 10 or 20 min (Figure 3c). In some experiments where the incubation with lactate was continued for up to 60 min, the effect of EGF became non-significantly different from controls at 40 min of incubation. Similar transient effects of EGF were also found using pyruvate or glycerol (results not shown).

In addition to its effects on gluconeogenesis and on the cytosolic redox state, EGF affected the mitochondrial redox state. EGF did not affect the production of total ketone bodies (3-hydroxybutyrate + acetoacetate) (Table 3), but decreased the 3-hydroxybutyrate/acetoacetate ratio in each condition studied (Table 2). To understand this effect we studied the time course of the effect of EGF on the mitochondrial redox state in the absence of gluconeogenic substrates. A gluconeogenic substrate (20 mM glycerol) was added after a 40 min incubation with EGF. Glycerol was used instead of lactate because, on a percentage basis, the effect of EGF in decreasing the 3-hydroxybutyrate/acetoacetate ratio was greater with glycerol than with lactate. Results are shown in Figure 4. In the absence of substrates, the release of both 3-hydroxybutyrate and acetoacetate was linear (Figures 4a and 4b), and EGF did not affect the total release of ketone bodies (3-hydroxybutyrate + acetoacetate). The 3-hydroxybutyrate/acetoacetate ratio was quite constant throughout the experiment in the absence of EGF, whereas EGF decreased this ratio clearly after 30 min of incubation (Figure 4c). When glycerol was added to control cells, the 3-hydroxybutyrate/acetoacetate ratio increased, indicating that a transfer of redox potential from cytosol to mitochondria took place in the cells upon the addition of this reduced substrate. This is in agreement with the results shown in Figure 2(b). In the presence of EGF, upon the addition of glycerol, the 3-hydroxybutyrate/acetoacetate ratio also increased but to a lower level than in the absence of the peptide. However, the extent of the increase was similar (2.0-fold in control cells; 2.1-fold in the presence of EGF). Thus the decrease in the 3-hydroxybutyrate/acetoacetate ratio shown in Table 2 was due to the effect of EGF, which lowered this ratio during the incubation with the peptide before the addition of the substrate.

To determine whether the effect of EGF on the mitochondrial redox state was required to produce the effect on gluconeogenesis, we studied the effect of EGF in the presence of ammonium or in the absence of extracellular calcium, since it is known that ammonium [38] and calcium [36] ions affect the 3-hydroxybutyrate/acetoacetate ratio by different mechanisms. In the absence of extracellular calcium, both the 3-hydroxybutyrate/acetoacetate ratio and gluconeogenesis decreased (Table 4), but EGF was still able to decrease both parameters and to increase the lactate/pyruvate ratio. Am-

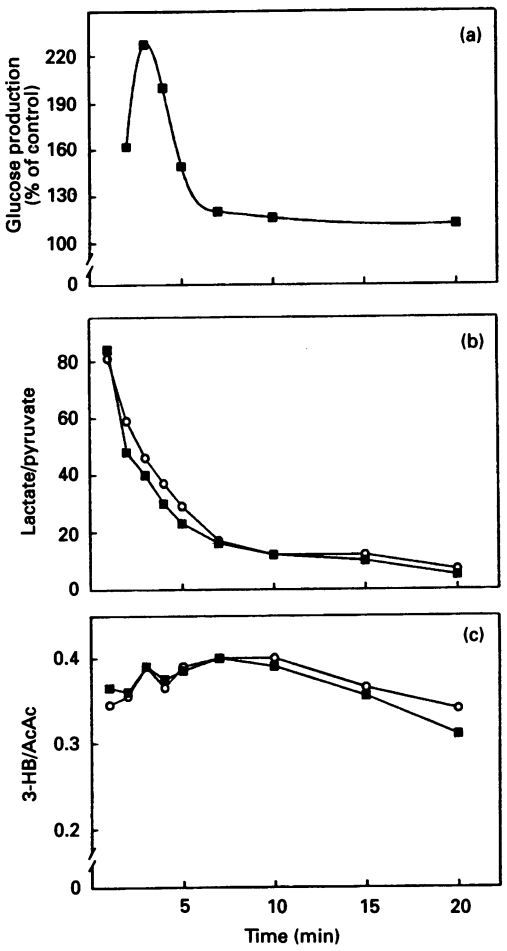

Figure 5 Rapid effects of EGF on gluconeogenesis and on the cytosolic and mitochondrial redox states

Isolated hepatocytes were incubated in the absence (○) or in the presence (■) of 10 nM EGF. Simultaneously with the peptide, the gluconeogenic substrate (2 mM lactate) was added. At indicated times, a sample was processed as indicated in the Materials and methods section to determine glucose production (expressed as a percentage of the control value) (**a**), the lactate/pyruvate ratio (**b**) and the 3-hydroxybutyrate/acetoacetate (3-HB/AcAc) ratio (**c**). Results are means of a representative experiment performed in triplicate.

monium ions produced a decrease in both the 3-hydroxybutyrate/acetoacetate and the lactate/pyruvate ratio, and in glucose synthesis from lactate. Under these conditions EGF did not affect any of these parameters (although glucagon was still able to stimulate glucose synthesis; results not shown).

Rapid effects of EGF

In a perfused liver system it has recently been shown that EGF has a rapid and transient stimulatory effect on glucose synthesis from puruvate [24]. Thus we studied the effect of EGF on glucose synthesis from pyruvate (1 mM) or lactate (2 mM) in isolated hepatocytes, adding the substrate at the same time as the peptide. EGF increased glucose synthesis from pyruvate (21 ± 3 nmol/20 min per 10^6 cells for controls and 26 ± 1 nmol/20 min per 10^6 cells for EGF; $P < 0.01$) and also from lactate (99 ± 8 mmol/20 min per 10^6 cells for controls and 109 ± 7 nmol/20 min per 10^6 cells for EGF; $P < 0.05$). Since EGF had different effects when lactate was the substrate (rapid stimulation and delayed inhibition), we further studied the earlier effects of EGF on gluconeogenesis from lactate in isolated hepatocytes.

Table 5 The rapid effects of EGF require the presence of extracellular calcium

Isolated hepatocytes were incubated in a medium containing either 1.3 mM Ca^{2+} or no Ca^{2+} plus 0.5 mM EGTA for 40 min at 37 °C in the absence of substrate and EGF. Then the substrate (2 mM lactate) and EGF (10 nM) were added. After 5 min of incubation in the presence of substrate, a sample of the suspension was processed as described in the Materials and methods section to determine glucose, lactate (L), pyruvate (P), 3-hydroxybutyrate (3-HB) and acetoacetate (AcAc) concentrations. To determine oxygen consumption, the cells were incubated as indicated in the text. In all experiments each condition was performed in triplicate. Results are means ± S.E.M. of six experiments carried out with different cell preparations. Statistical comparisons between EGF and corresponding control value were by paired Student's *t*-test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

	Glucose (nmol/5 min per 10^6 cells)	L/P	3-HB/AcAc	Oxygen consumption (nmol/min per 10^6 cells)
1.3 mM Ca^{2+}				
Control	9.5 ± 0.7	32 ± 2	0.24 ± 0.02	14.5 ± 1.3
EGF	14.1 ± 1.1***	28 ± 2*	0.24 ± 0.02	16.1 ± 1**
No Ca^{2+} + 0.5 mM EGTA				
Control	6.0 ± 0.9	35 ± 3	0.17 ± 0.02	13.8 ± 2.3
EGF	6.9 ± 1.3	34 ± 2	0.17 ± 0.02	13.8 ± 2.1

The time course of the rapid effect of EGF (adding the substrate and the peptide simultaneously) on glucose production, lactate/pyruvate and 3-hydroxybutyrate/acetoacetate ratios was next studied. The stimulatory effect of EGF on glucose synthesis was rapid and transient (Figure 5) (greatest effect at 3 min: 227% over control value). Simultaneously, EGF transiently decreased the lactate/pyruvate ratio, but it did not decrease the 3-hydroxybutyrate/acetoacetate ratio until 20 min later. Thus, while EGF (10 nM) significantly decreased the lactate/pyruvate ratio at 5 min of incubation but not at 20 min (22 ± 2 for control and 18 ± 2 for EGF at 5 min; $P < 0.01$; 8.9 ± 0.4 for control and 8.4 ± 0.3 for EGF at 20 min: not significant) (results from six independent experiments), the effect of EGF on the 3-hydroxybutyrate/acetoacetate ratio was only significant at 20 min (0.41 ± 0.03 for control and 0.42 ± 0.03 for EGF at 5 min; not significant; 0.42 ± 0.08 for control and 0.37 ± 0.07 for EGF at 20 min; $P < 0.05$) (results from 6 independent experiments). EGF did not affect the flux through the pyruvate kinase reaction, measured as lactate + pyruvate production from dihydroxyacetone (31 ± 5 and 28 ± 8 nmol/10^6 cells for control and EGF respectively at 5 min of incubation; 133 ± 18 and 121 ± 16 nmol/10^6 cells for control and EGF respectively at 20 min of incubation).

EGF increased the rate of oxygen consumption (Table 5). This effect was also transient (results not shown). The rapid effect of EGF on oxygen consumption and both the lactate/pyruvate ratio and gluconeogenesis depended on the presence of extracellular calcium, since in the absence of calcium and the presence of EGTA all these effects disappeared (Table 5). The rapid effect of EGF on glucose synthesis from lactate did not disappear in the presence of ammonium (results not shown). We also studied the effect of EGF on these parameters in cells incubated in the presence of rotenone, which inhibits electron transfer through complex I. As shown in Table 6, rotenone decreased oxygen consumption, increased the 3-hydroxybutyrate/acetoacetate ratio and decreased glucose synthesis. In the presence of rotenone, EGF did not produce any of the rapid effects described above.

Table 6 The rapid effects of EGF are abolished by rotenone

Isolated hepatocytes were incubated either in the presence or the absence of 0.5 μM rotenone for 5 min at 37 °C. Then the substrate (2 mM lactate) and EGF (10 nM) were added. After 5 min of incubation in the presence of substrate, a sample of the suspension was processed as described in the Materials and methods section to determine glucose, lactate (L), pyruvate (P), 3-hydroxybutyrate (3-HB) and acetoacetate (AcAc) concentrations. To determine oxygen consumption, the cells were incubated as indicated in the text. In all experiments each condition was performed in triplicate. Results are means ± S.E.M. of three experiments performed with different cell preparations. Statistical comparisons between EGF and corresponding control values were by paired Student's *t*-test: * $P < 0.05$; ** $P < 0.01$.

	Glucose (nmol/5 min per 10^6 cells)	L/P	3-HB/AcAc	Oxygen consumption (nmol/min per 10^6 cells)
No rotenone				
Control	14.9 ± 0.7	36 ± 1	0.42 ± 0.01	14.9 ± 0.9
EGF	20.0 ± 1.0**	30 ± 1**	0.40 ± 0.01	17.4 ± 0.6*
+ 0.5 μM rotenone				
Control	3.4 ± 0.5	44 ± 1	1.04 ± 0.02	7.9 ± 0.4
EGF	2.5 ± 0.3	46 ± 3	1.03 ± 0.02	7.9 ± 0.3

DISCUSSION

We and others have described the delayed effects of EGF on hepatic gluconeogenesis [17,22,23]. An effect of EGF on pyruvate kinase was observed [23,24], suggesting a direct modulation of gluconeogenesis by this peptide. However, reported effects of EGF on pyruvate kinase activity are contradictory: a small activation [21], a rapid and transient inhibition [24] and a slow but lasting inhibition [23] have all been described. Because pyruvate kinase is a multimodulated enzyme, we decided to determine the effect of EGF on the metabolic flux through the pyruvate kinase reaction in living hepatocytes by incubating the cells with dihydroxyacetone. The rate of lactate production from dihydroxyacetone was shown to be a good estimator *in vivo* of the flux from phosphoenolpyruvate to pyruvate in hepatocytes from fasted rats [39]. This relies on the fact that dihydroxyacetone enters the glycolytic pathway at the level of triosephosphates and thus bypasses the phosphofructokinase-1 reaction (note that this applies only to fasted rats, whose hepatocytes are depleted of glycogen). Our results indicate that, inside the hepatocytes from fasted rats, the flux through pyruvate kinase (when the cells are incubated with dihydroxyacetone) is not affected by EGF. Furthermore, measuring production of lactate + pyruvate from glucose, we did not find any effect of EGF. However, although we did not obtain any evidence for a role for pyruvate kinase or any other glycolytic enzyme in the delayed effects of EGF on gluconeogenesis, the possibility that EGF modulates pyruvate kinase in the absence of dihydroxyacetone or in the presence of other substrates cannot be excluded. The different effects of glucagon on lactate + pyruvate depending on the substrate used agrees with previous reports [37] and can be explained by the variation in the intracellular concentrations of allosteric effectors of pyruvate kinase (such as fructose 1,6-bisphosphate) produced by the different substrates used [40,41]. It is known that fructose 1,6-bisphosphate can affect the ability of physiological concentrations of glucagon to inactivate pyruvate kinase [40].

Several studies have observed that the availability of cytosolic reducing equivalents determines hormone effects on gluconeogenesis [42–44]. Results shown in Table 2 and Figure 2 and 3 suggest that the different effects of EGF on gluconeogenesis from fructose, dihydroxyacetone, lactate, pyruvate or glycerol can be explained by the effect on the cytosolic NADH/NAD^+ ratio. Thus we propose that EGF stimulates gluconeogenesis from pyruvate because it increases the cytosolic redox state, which is low when the cells are incubated with this substrate (as shown in Figure 2a, at a low cytosolic redox state, an increase leads to enhanced glucose synthesis). In contrast, EGF inhibits glucose synthesis from lactate or glycerol because the peptide also increases the cytosolic redox state, which is higher when the cells are incubated with these substrates. Note that the lactate/pyruvate ratio of about 10, found at 20 min after the addition of lactate (Table 2), is the result of a progressive reoxidation of the cells after exposure to this reduced substrate (Figure 3). EGF slows down the rate at which this reoxidation occurs (Figure 3). The lack of effect of EGF on glucose synthesis from fructose or dihydroxyacetone may be related to the lack of redox reactions in the pathway from both substrates to glucose. Furthermore, the transient nature of the delayed effect of EGF on gluconeogenesis may be the consequence of the transient nature of the effect on the cytosolic redox state (Figure 3d).

In addition to the effect on the cytosolic redox state, EGF also altered the mitochondrial redox state. The effect was only observed after about 30 min, did not require the presence of gluconeogenic substrate (Figure 4), and persisted after the addition of any of the gluconeogenic substrates studied (Table 2, Figure 4). Our results (Tables 2 and 4, and Figure 4) further suggest that the effect of EGF at the mitochondrial level is necessary for the effects on the cytosolic redox state and on gluconeogenesis, for the following reasons: (i) the delayed effect on gluconeogenesis was only observed after the effect on the mitochondrial redox state, and (ii) in the presence of ammonium, EGF did not affect glucose synthesis (note that ammonium abolished the effects of EGF on the mitochondrial redox state and on both the cytosolic redox state and gluconeogenesis).

We would emphasize that, while the effect of EGF on the mitochondrial redox state was observed under any conditions (see Table 2), this was not the case with the effect on the cytosolic redox state. It is notable also that under normal conditions the redox states in both cellular compartments were closely correlated (see Figure 2b), but EGF had an opposite effect on them. These results suggest that EGF may produce some other effect at the cytosolic level, which would be directly responsible for the effects observed on the cytosolic redox state and on gluconeogenesis. The nature of such an effect cannot be concluded from our results. Our current hypothesis is that EGF may be affecting synthesis of pyrimidines. It is known that this pathway generates NADH in the cytosol and requires aspartic acid, which can be generated from some of the gluconeogenic substrates used by us.

Taken together, our results clearly establish that the delayed effects of EGF on gluconeogenesis are secondary to some more direct effects of the peptide at both the mitochondrial and the cytosolic level, possibly related to the mitogenic action of EGF, and/or to the requirement of nitrogen sparing for cellular proliferation. This hypothesis would be in keeping with our recent observation that EGF reduces basal urea production in hepatocytes (that determined in the absence of ureogenic substrates) [45]. A hypothetical nitrogen sparing effect of EGF would also explain its effect on the mitochondrial redox state observed by us: such an effect would produce a displacement of the glutamate dehydrogenase reaction to glutamate, resulting in a decrease in the mitochondrial NADH/NAD^+ ratio.

In perfused rat liver, Rashed and Patel [24] found that EGF had a rapid and transient stimulatory effect on gluconeogenesis from pyruvate. We show here that this also occurs in isolated hepatocytes not only from pyruvate, but also from lactate. EGF

rapidly stimulated oxygen consumption, and both rapid effects (on gluconeogenesis and on mitochondrial respiration) disappeared in the absence of extracellular calcium (Table 5).

EGF is known to increase cytosolic Ca^{2+} levels in isolated hepatocytes [8,15]. Both the release of Ca^{2+} from intracellular stores [46] and the influx of extracellular Ca^{2+} [8] are stimulated by EGF. The effects of Ca^{2+} on gluconeogenesis are complex and are not yet completely understood. It is known that a rise in intracellular Ca^{2+} levels stimulates oxygen consumption [47–49], and that the mitochondrial respiratory chain is an important site for the control of the gluconeogenic flux from lactate in isolated hepatocytes from fasted rats [47,50]. We suggest that the rapid and transient effect of EGF in increasing gluconeogenesis from lactate may be the consequence of the stimulatory effect of EGF on oxygen consumption. Note that rotenone (which inhibits the mitochondrial respiratory chain at the level of NADH oxidation) abolished the rapid effects of EGF on both oxygen consumption and gluconeogenesis (Table 6). The effect of EGF on mitochondrial respiration may be the result of the Ca^{2+} signal generated by EGF.

Ca^{2+}-mobilizing hormones such as catecholamines also produce a transient stimulation of glucose synthesis that is mediated by an increase in mitochondrial respiration [43,47,48,51]. However, this effect was immediately followed by a lower but permanent stimulation of glucose synthesis which was dependent on the activation of fatty acid oxidation by these hormones [47,51,52]. Interestingly, EGF did not produce such an increase in endogenous fatty acid oxidation, as revealed by the lack of stimulation of total ketone body (3-hydroxybutyrate + acetoacetate) production under any conditions studied (Table 3, Figure 4). This may explain the differences in the rapid stimulation of gluconeogenesis between catecholamines and EGF.

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