Glucose- and insulin-dependent induction of ATP citrate lyase in primary cultures of rat hepatocytes

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ATP citrate lyase, which is involved in the translocation of the lipogenic precursor acetyl-CoA from mitochondria to cytosol, was studied in primary cultures of hepatocytes from adult rats. After an initial decrease at the first day of culture the enzyme activity was nearly constant during the following days. It could be enhanced between 24 h and 48 h in culture about 1.5-fold by elevation of the insulin concentration to 10^{-7} mol/l, 2.2-fold by elevation of the glucose concentration from 5 to 25 mmol/l and 3.5-fold by simultaneous elevation of insulin and glucose. The increase of activity was about linear with time for 24 h and could be blocked by cycloheximide, which inhibits protein synthesis at the translational level. Both observations suggest that the enhancement of activity was due to induction rather than to activation by interconversion. The glucose-dependent induction was furthermore evidenced by immunotitration which indicated a parallel increase of activity and enzyme protein.

Since acetyl-CoA formed in the mitochondria cannot penetrate the inner mitochondrial membrane, it is transported into the cytosol after condensation with oxaloacetate as citrate. Cytosolic acetyl-CoA is then supplied by ATP-dependent cleavage of citrate which is catalysed by ATP: citrate oxaloacetate-lyase (EC 4.1.3.8) and which can be regarded as the first enzyme of the carbohydrate-dependent synthesis of fatty acids and cholesterol (Kornacker & Lowenstein, 1965a). Correspondingly, ATP citrate lyase is important, beside acetyl-CoA carboxylase, for the regulation of the glucose-dependent formation of lipids in liver and adipose tissue. Compared with the short-term regulation of the enzyme by phosphorylation and dephosphorylation (Janski *et al.*, 1979; Ramakrishna & Benjamin, 1981) the long-term adaptation based on hormone-dependent (Nepokroeff *et al.*, 1974; Spence *et al.*, 1979) and diet-dependent (Takeda *et al.*, 1967; Zakim *et al.*, 1967; Gibson *et al.*, 1972) induction and degradation seem to be more important. The ATP citrate lyase level is decreased by diabetes mellitus and starvation, while it can be enhanced drastically by insulin and refeeding of a fat-free, carbohydrate-rich diet. Whereas it is obvious that insulin increases the enzyme level, no unequivocal information seems to be available whether carbohydrate enhances the

ATP citrate lyase level by itself or via an elevation of the insulin concentration. Since it is difficult to differentiate primary and secondary effects of an inducing agent in the intact animal due to endogenous compensatory regulation, studies on isolated organ preparations are necessary in order to answer this question. Primary cultures of isolated hepatocytes are useful for the study of the long-term regulation of enzymes, since they can be maintained for several days preserving their liver-specific functions (Bonney *et al.*, 1974; Savage & Bonney, 1978; Oliver *et al.*, 1978; Katz & Ick, 1981).

In the present investigation the influence of glucose on the long-term regulation of ATP citrate lyase was studied in primary cultures of hepatocytes with and without addition of insulin.

The enzyme activity was enhanced significantly by elevation of the glucose concentration in the absence of insulin. The glucose-mediated enhancement could be further increased by the presence of insulin, depending on the hormone concentration. The inhibition of this enhancement by inhibitors of protein synthesis, as well as immunotitration of enzyme activity, indicated that the metabolite-dependent increase of ATP citrate lyase was due to induction rather than to activation. Some of the results have been documented in a preliminary abstract (Katz, 1980).

Experimental

Materials

Malate dehydrogenase, coenzymes, culture medium and foetal calf serum were supplied by Boehringer Mannheim. Collagenase type II, penicillin and streptomycin sulphate were from Sigma. L-[U-¹⁴C]Leucine was purchased from Amersham-Buchler. Bovine insulin, dexamethasone, 3,3',5-triiodo-L-thyronine, cycloheximide, oleic acid, glucose and bovine serum albumin were obtained from Serva. All other chemicals were reagent grade from Merck. Albumin was defatted according to Chen (1967); oleic acid was bound to albumin as described by Garland *et al.* (1964).

Cell culture

Hepatocytes were isolated from male Wistar rats (150–200g, Winkelmann, Paderborn, Germany) as described earlier (Katz *et al.*, 1979). The cells were cultured in medium 199 with Earle's salts (Morgan *et al.*, 1950) containing glucose (5.5 mmol/l), defatted serum albumin (2g/l), NaHCO₃ (18 mmol/l), Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] (15 mmol/l), streptomycin sulphate (117mg/l) and penicillin (60mg/l). The osmolarity was adjusted to 285 mosmol/l, and the pH of the medium gassed with air/CO₂ (19:1) was 7.40. The standard medium contained insulin (5×10^{-10} mol/l) and dexamethasone (10^{-9} mol/l). Hepatocytes [$(2-2.5) \times 10^6$] suspended in 3 ml of standard medium containing 5% (v/v) foetal calf serum were inoculated into 60mm Falcon dishes for tissue culture (Becton-Dickinson, Heidelberg, Germany). The incubation was performed at 37°C in an incubator gassed with humidified O₂/CO₂/N₂ (14:5:81) mimicking arterial O₂ tensions (Nauck *et al.*, 1981). After 4 h and 24 h the standard medium was changed; foetal calf serum was then omitted. The induction of ATP citrate lyase was initiated in 24 h cultures by increasing the glucose, insulin or glucose-plus-insulin level as indicated. General protein synthesis was measured by incorporation of L-[U-¹⁴C]leucine (0.6 μ Ci/ μ mol) into secreted and cellular protein according to Mans & Novelli (1960) under culture conditions which led to an induction of ATP citrate lyase. In some experiments cycloheximide (30 μ mol/l) was added to inhibit protein synthesis.

Enzyme assay

After washing with a solution containing NaCl (130 mmol/l), KCl (30 mmol/l) and Tris/HCl (5 mmol/l), pH 7.4, the cell monolayers were scraped off and homogenized at 2°C in a glass homogenizer with a solution containing NaCl (100 mmol/l), KCl (70 mmol/l), dithioerythritol (2 mmol/l), MgCl₂ (1 mmol/l), EDTA (0.2 mmol/l) and Tris/HCl

(20 mmol/l), pH 8.0. ATP citrate lyase activity was determined by a photometric assay modified according to Srere (1959) in a 100 000g supernatant using the following test conditions: potassium citrate (20 mmol/l), MgCl₂ (10 mmol/l), NADH (0.3 mmol/l), ATP (7 mmol/l), 2-mercaptoethanol (10 mmol/l) and malate dehydrogenase (1.5 units/l) in Tris/HCl (150 mmol/l), pH 8.0. After a short preincubation the citrate cleavage was started by addition of coenzyme A to a final concentration of 0.2 mmol/l.

Acetyl-CoA carboxylase was measured radiochemically as described earlier (Katz & Ick, 1981). Lactate dehydrogenase was determined photometrically according to Bergmeyer *et al.* (1974) in the 100 000g supernatant.

The immunotitration of ATP citrate lyase activity was performed by preincubation of aliquots of the 100 000g supernatant with increasing amounts of a specific antibody to rat liver ATP citrate lyase. The enzyme, isolated according to Linn & Srere (1979), was used for immunization of rabbits. The antibody was partially purified by precipitation of the γ -globulin fraction in the presence of Na₂SO₄ (1.2 mol/l) in phosphate buffer (50 mmol/l), pH 7.4. After preincubation for 30 min at 30°C and then for 4 h at 2°C the immunoprecipitate was removed by centrifugation at 100 000g for 20 min. The enzyme activity was determined in the supernatant. DNA was measured as described by Oliver *et al.* (1978). The statistical evaluation was performed using Student's *t*-test.

Results

The ATP citrate lyase activity decreased rapidly during the first 24 h of culture under standard conditions. After this initial decrease, however, only a slight further loss of enzyme activity was observed during the following days (Fig. 1), which indicates that a new steady state was reached in the cultures. The long-term influence of glucose and insulin on the enzyme activity was studied therefore during the second day of culture.

In the presence of low glucose concentrations (5.5 mmol/l), increasing insulin to 10^{-7} mol/l resulted in a moderate but significant increase of ATP citrate lyase activity to 150% during 24 h (Fig. 2a), while the enzyme activity was only slightly decreased without insulin. The most distinct alteration of the citrate lyase activity dependent on the insulin concentration was observed at hormone concentrations between 10^{-9} and 10^{-8} mol/l. In the presence of high glucose concentrations (20 mmol/l) the elevation of insulin led to a more pronounced increase of ATP citrate lyase activity to 220% (Fig. 2a).

Fig. 1. ATP citrate lyase activity in primary cultures of male rat hepatocytes under basic glucose (5.5 mmol/l) and insulin (5×10^{-10} mol/l) concentrations
Cells were cultured in medium 199 containing defatted bovine serum albumin (2 g/l) and dexamethasone (10^{-9} mol/l). Foetal calf serum (5%, v/v) was present during the first 4 h only. The medium was changed after 4, 24 and 48 h. Values are means $\pm$ S.E.M. for eight cultures.

Increasing the basic glucose concentration to 20 mmol/l in the presence of a low level of insulin (5×10^{-10} mol/l), led to a significant increase of ATP citrate lyase to 230%, which was about linear with time during 24 h. The increase in activity was linear up to a concentration of 20 mmol/l and declined slightly at higher glucose concentrations (Fig. 2b). The glucose-dependent enhancement of the citrate lyase activity could be increased further by simultaneous elevation of the insulin concentration to 10^{-7} mol/l. The enhancement of enzyme activity observed under this condition was equal or even slightly greater than the sum of the isolated effects of glucose or insulin alone. On the other hand, the glucose-dependent increase of ATP citrate lyase was diminished but not abolished in the absence of insulin (Fig. 2b).

Table 1. Inhibition of glucose- and glucose-plus-insulin-dependent enhancement of the ATP citrate lyase level by cycloheximide

Hepatocytes (24 h cultures) were incubated for 8 h rather than for 24 h with or without addition of glucose (15 mmol/l), insulin (10^{-7} mol/l) and cycloheximide (30 μ mol/l). The relatively short incubation period was necessary, since after longer incubation in the presence of cycloheximide morphological alterations of the hepatocytes occurred. Values are means $\pm$ S.E.M. for three to six determinations of a representative experiment. Significant ($P < 0.005$) differences from the corresponding cultures without cycloheximide are indicated by *.

Addition	...	ATP citrate lyase [μ mol $\cdot$ min ⁻¹ $\cdot$ (g of DNA) ⁻¹]		
		None	Glucose	Glucose plus insulin
Control		169 $\pm$ 8	237 $\pm$ 9	260 $\pm$ 8
+ Cycloheximide		174 $\pm$ 7	165 $\pm$ 3*	182 $\pm$ 9*

The addition of tri-iodothyronine at 10^{-5} mol/l, as well as an increase of the glucocorticoid dexamethasone to 10^{-7} mol/l, did not significantly alter the glucose- or the insulin-dependent alteration of ATP citrate lyase in hepatocyte cultures prepared from normal adult rats. Since in presence of high glucose concentrations the formation of lactate led to a slight acidification of the culture medium, the influence of pH on the ATP citrate lyase level was determined using different bicarbonate concentrations in the medium. No significant influence of the pH was observed between pH values 7.20 and 7.55.

The long-term enhancement of the ATP citrate lyase activity by glucose as well as by insulin could be inhibited by cycloheximide in a concentration sufficient to block protein synthesis at the translational level (Tables 1 and 2). The inhibition suggests that not only the insulin-dependent but also the glucose-dependent enhancement of enzyme activity was due to induction rather than to activation of the enzyme. In view of the relatively slow enhancement of the ATP citrate lyase activity, it would be desirable to study the induction and its inhibition over a longer time period. However, an exposure of hepatocyte cultures to inhibitors of protein synthesis for more than 12 h results in morphological alterations of the cells indicating a decrease in viability.

In order to correlate ATP citrate lyase activity with the amount of enzyme protein, a relative quantification of the enzyme protein was performed by immunotitration with increasing amounts of a specific antibody, according to Maurizi *et al.* (1978). Determination of soluble ATP citrate lyase activity

Fig. 2. Insulin-dependent (a) and glucose-dependent (b) enhancement of ATP citrate lyase activity

Hepatocytes of male rats were cultured under standard conditions. The induction period between 24 and 48 h in culture was initiated by medium change and addition of insulin in the presence of low ($\bullet$, 5.5 mol/l) or high ($\circ$, 20 mmol/l) glucose concentrations (a) or by addition of glucose in the absence (Δ) or the presence of low ($\bullet$, $5 \times 10^{-10} \text{ mol/l}$) and high ($\circ$, 10^{-7} mol/l) insulin concentrations (b). Values are means $\pm$ S.E.M. for six to twelve cultures.

Table 2. General protein synthesis in hepatocyte cultures during glucose-dependent induction of ATP citrate lyase

The incorporation of [^{14}C]leucine ($0.6 \mu\text{Ci}/\mu\text{mol}$) into trichloroacetic acid-precipitable secreted and cellular protein was determined in hepatocyte cultures during induction of ATP citrate lyase. It was linear at least for 12 h. Values are means $\pm$ S.E.M. for three experiments with duplicates.

Addition	Incorporation [$\mu\text{mol} \cdot \text{h}^{-1} \cdot (\text{g of DNA})^{-1}$] of [^{14}C]leucine into:		
	Secreted protein	Cellular protein	Total protein
None	181 ± 6	504 ± 18	685 ± 17
Glucose (15 mM)	208 ± 6	535 ± 46	743 ± 43
Glucose (15 mM) + insulin (10^{-7} M)	189 ± 4	536 ± 26	725 ± 21
Cycloheximide ($30 \mu\text{M}$)	8 ± 2	18 ± 5	26 ± 6

in the supernatant after immunoprecipitation revealed that a significantly higher amount of antibody was necessary for precipitation of the enzyme activity after glucose-dependent enhance-

ment compared with controls before induction (Fig. 3). The correlation between the increase in enzyme activity and enzyme protein also demonstrates the existence of a metabolite-dependent induction.

The specificity of this effect was examined by measuring general protein synthesis in the presence of different glucose concentrations (Table 2). The elevation of glucose to 20 mmol/l with or without simultaneous addition of insulin did not alter significantly the incorporation of [^{14}C]leucine into secreted or cellular protein. Protein synthesis was essentially blocked by cycloheximide ($30 \mu\text{mol/l}$), which was sufficient to prevent the glucose-dependent induction of ATP citrate lyase (Table 1). Furthermore, the specificity of the glucose-mediated long-term regulation of lipogenic enzymes is demonstrated by comparison of ATP citrate lyase, acetyl-CoA carboxylase and the non-inducible cytosolic enzyme lactate dehydrogenase (Table 3). The two lipogenic enzymes were enhanced in the presence of high glucose and glucose-plus-insulin concentrations, while lactate dehydrogenase was not altered. The addition of a high concentration of oleate as an alternative energy substrate did not affect any of the enzyme activities.

Table 3. *Effect of glucose and oleate on the lipogenic enzymes ATP citrate lyase and acetyl-CoA carboxylase as well as on lactate dehydrogenase*

Hepatocytes were cultured under standard conditions except that the concentration of albumin in the medium was increased to 8 g/l. Glucose, insulin and albumin-bound oleate were added with the medium change to 24 h cultures. The enzyme activities were determined 24 h later. Values are means $\pm$ S.E.M. for three to six experiments.

Addition	ATP citrate lyase [$\mu\text{mol} \cdot \text{min}^{-1} \cdot (\text{g of DNA})^{-1}$]	Acetyl-CoA carboxylase [$\mu\text{mol} \cdot \text{min}^{-1} \cdot (\text{g of DNA})^{-1}$]	Lactate dehydrogenase [$\mu\text{mol} \cdot \text{min}^{-1} \cdot (\text{mg of DNA})^{-1}$]
None	149 $\pm$ 7	75 $\pm$ 5	168 $\pm$ 5
Glucose (15 mM)	310 $\pm$ 21	144 $\pm$ 10	176 $\pm$ 7
Glucose (15 mM) + insulin (10^{-7} M)	370 $\pm$ 14	201 $\pm$ 31	198 $\pm$ 7
Oleate (2 mM)	162 $\pm$ 8	69 $\pm$ 5	174 $\pm$ 7

Fig. 3. *Immunotitration of ATP citrate lyase activity in hepatocyte cultures before (●) and after (○) glucose-dependent induction for 24 h*

Aliquots (100 μl) of cell extract were incubated with increasing amounts of an immunoglobulin G preparation of a specific antiserum (1.5 mg of protein/ml of homogenization buffer) for 30 min at 30°C and then for 4 h at 2°C. The volume was adjusted to 150 μl by addition of homogenization buffer. The enzyme activity was determined in the supernatant after sedimentation of the immunoprecipitate at 100 000 g for 20 min.

Discussion

It is shown by the present investigation that ATP citrate lyase could be maintained in primary hepatocyte cultures at a nearly constant level, which is

reached after a rapid decrease during the first day of culture. The elevation of insulin or glucose concentrations in the medium during the second day of culture led to a significant increase of the enzyme activity, which was due to induction.

This observation is in line with studies *in vivo* that demonstrated an insulin-dependent (Lakshmanan *et al.*, 1972; Kumar, 1977) and a diet-dependent (Kornacker & Lowenstein, 1965a; Zakim *et al.*, 1967) regulation of lipogenic key enzymes. Both the carbohydrate-dependent (Gibson *et al.*, 1972; Finkelstein *et al.*, 1979) as well as the insulin-dependent (Spence *et al.*, 1979) long term regulation of the ATP citrate lyase are caused by enzyme induction as evidenced by radioimmunochemical studies. However, endogenous regulatory mechanisms may superimpose primary hormonal- or especially metabolite-dependent effects in the intact animal. Thus, no unequivocal information could be obtained from these studies *in vivo* whether the carbohydrate-mediated enhancement of ATP citrate lyase is effected by metabolite-dependent induction (Kornacker & Lowenstein, 1965b; Zakim *et al.*, 1967) or by a secondary elevation of the insulin concentration (Nepokroeff *et al.*, 1974).

Using the less complex system of primary hepatocyte cultures, a direct glucose-dependent induction of the ATP citrate lyase activity could be demonstrated in the present investigation, which was not mediated by, but modulated by, insulin. The mean glucose and insulin concentrations responsible for induction were in the physiological range observed in the portal vein (Berger *et al.*, 1975; Strubbe & Steffens, 1977), since the initial glucose and insulin levels at the beginning of the induction period were decreased to about 75% and 20% respectively by degradation within 24 h of culture (Katz & Ick, 1981). Thus both effects may be of physiological significance for the regulation of carbohydrate-dependent lipogenesis in liver. The enhancement of ATP citrate lyase seems to be due to a specific induction rather than to a general metabolic effect, as general protein synthesis was not affected by high levels of glucose and no change in

ATP citrate lyase activity was observed with oleate as energy substrate instead of high glucose concentration. Moreover, a selective glucose- and insulin-dependent long-term regulation was seen with the lipogenic enzymes ATP citrate lyase and acetyl-CoA carboxylase, but not with lactate dehydrogenase. However, it was not possible to decide whether the carbohydrate-dependent induction was caused by glucose or by a glycolytic metabolite, as was proposed by Kornacker & Lowenstein (1965b). During the preparation of the present paper, Spence & Pitot (1982) demonstrated that ATP citrate lyase was induced by low concentrations of fructose and glycerol in the absence of insulin, while low glucose concentrations were effective only in the presence of insulin. That may indicate an induction by a glycolytic metabolite rather than by glucose itself. Nevertheless, further studies using cell-free protein synthesis will be necessary to answer this question conclusively. It can be excluded that the induction was due to acidification by glucose-dependent lactate production, as experimental changes of the pH in the culture medium within the physiological range did not influence the ATP citrate lyase level.

In contrast with the hormone-mediated induction of glucokinase (Katz *et al.*, 1979; Schudt, 1979) and of tyrosine aminotransferase (Michalopoulos *et al.*, 1978), the elevation of the basic glucocorticoid concentration did not enhance significantly the glucose-dependent or the insulin-dependent induction of acetyl-CoA carboxylase (Katz & Ick, 1981) and ATP citrate lyase. Due to the long-term effect of thyroid hormones (Spence *et al.*, 1979), no addition of tri-iodothyronine was necessary in order to obtain glucose-dependent or insulin-dependent induction in hepatocyte cultures prepared from normal, not thyroidectomized, rats.

Although the existence of metabolite-dependent induction and repression of enzymes in mammalian systems has been postulated on the basis of studies *in vivo* of ATP citrate lyase (Kornacker & Lowenstein, 1965b), δ -aminolaevulinate synthase (Hickman *et al.*, 1968), ornithine transaminase and threonine dehydratase (Pitot & Peraino, 1963), it has been difficult to substantiate this hypothesis in studies on whole animals. Conclusive results on metabolite-dependent regulation of enzyme levels have been presented, however, by studies using liver cell cultures, which demonstrate the glucose repression of glucagon-mediated induction of tyrosine aminotransferase (Michalopoulos *et al.*, 1978) as well as the carbohydrate-dependent induction of glucokinase (Spence *et al.*, 1981) and acetyl-CoA carboxylase (Katz & Ick, 1981).

As with the latter two enzymes ATP citrate lyase is involved in the transformation of glucose to lipids during the absorptive phase. Therefore a co-ordinate regulation not only of ATP citrate lyase and

acetyl-CoA carboxylase (Gibson *et al.*, 1972; Finkelstein *et al.*, 1979) but also of other enzymes involved in this metabolic pathway seems to be reasonable. The described metabolite-dependent induction ensures that even in the absence of insulin a basic adaptation to an enhanced supply of carbohydrates is present.

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