CYCLOHEXIMIDE AND PACTAMYCIN INHIBIT THE RAPID DECREASE IN TRANSLATABLE mRNA ACTIVITY OF *P*-ENOLPYRUVATE CARBOXYKINASE (GTP)

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1. Introduction

The rate of synthesis of rat liver cytosolic *P*-enolpyruvate carboxykinase (GTP), a key gluconeogenic enzyme, rapidly decreases upon glucose feeding to fasted rats [1,2] or insulin injection to diabetic rats [2]. A corresponding decrease in translatable *P*-enolpyruvate carboxykinase mRNA occurs upon refeeding [3]. However, it appears that the decay of the polysomal mRNA for the enzyme occurs at a slower rate than that of total enzyme mRNA [3]. This suggests that during deinduction either the polysomal mRNA is selectively protected from degradation or there is a shift of the enzyme's mRNA into polysomes.

Experiments using a protein synthesis (run off) system from liver postmitochondrial supernatants show that a transient increase (30 min) in the synthesis of the enzyme occurs after glucose is tube-fed to fasted rats or insulin given to diabetic rats [4]. This results from an increase in polysomal *P*-enolpyruvate carboxykinase mRNA without a change in total translatable activity for the enzyme, constituting a transient increase in translational efficiency [4]. Thus, by being shifted into the polysomes, the mRNA for *P*-enolpyruvate carboxykinase may be relatively protected from degradation.

To examine whether non-polysomal mRNA for *P*-enolpyruvate carboxykinase is preferentially degraded, we have now used inhibitors of protein synthesis to create dissociation of polysomes (pactamycin) and polysomal aggregation (cycloheximide). However, we have found that irrespective of the polysomal state, either of these inhibitors given together with insulin abolished the hormone's effect. Further elaboration of this finding and its implication will be described.

Abbreviations: *P*-enolpyruvate carboxykinase, phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32); poly(A)$^+$ RNA, RNA containing a polyadenylic acid sequence; S-17, postmitochondrial supernatant; SDS, sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid

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2. Materials and methods

2.1. *Materials*

Alloxan monohydrate, cycloheximide, heparin, cordycepin and Hepes were purchased from Sigma Chemical Co. Pactamycin was from Upjohn Co. Insulin (glucagon-free) was a gift of Eli Lilly Co. SDS from BDH was recrystallized. 8-OH-Quinolinate was from Merck. Oligo-(dT) cellulose type III was from Collaborative Research Inc. (Waltham, MA). L-[4,5-^{3}H]-Leucine (30 Ci/mmol) was from Nuclear Research Center (Negev, Israel). NCS tissue solubilizer was from Amersham Searle Corp. The wheat germ used was a gift from General Mills, Inc.

2.2. *Treatment of animals*

Week 7 albino Sabra rats from the Hebrew University Breeding Center, were rendered diabetic by a 19 mg/100 g body wt subcutaneous injection of alloxan and used 4–7 days later based on positive glucosuria. Interperitoneal injections of insulin (5 U/100 g), cycloheximide (0.1 mg/100 g), pactamycin (0.3 mg/100 g) or cordycepin (1.5 mg/100 g) were given at the times indicated. All rats were tube fed with glucose 0.5 g/100 g body wt 90 min prior to sacrifice to ensure and standardize hyperglyceinia.

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2.3. *Preparation of polysome profiles*

This was done as in [4].

2.4. *Rate of protein synthesis*

This was determined in vivo according to [5] by injecting 12 μCi/100 g body wt [^{3}H]leucine to rats 20 min before sacrifice.

Isolation of poly(A)$^+$ RNA from postmitochondrial liver supernatants [4], translation in wheat germ lysates and determination of *P*-enolpyruvate carboxykinase mRNA translatable activity, were performed as in [3,6,7]. Preparation of wheat germ lysates was as in [8,9].

3. Results

3.1. *Effect of insulin, pactamycin and cycloheximide on ribosomal aggregation and protein synthesis*

It has been shown that short-term glucose feeding to fasted rats [4,10,11] or insulin treatment to diabetic rats [12] caused an aggregation of ribosomes with a corresponding decrease in monosome and subunit concentration [5,10,11]. Our recording of polysome profiles confirmed this finding (fig.1A), demonstrating that insulin injection to diabetic rats for 90 min caused a shift to heavier polysomes, with a corresponding decrease in small polysomes, monosomes and subunits.

Pactamycin, an inhibitor of initiation of protein synthesis [13–15]was used to degrade polysomes. When injected intraperitoneally (0.3 mg/100 g body wt) 30 min prior to insulin treatment, it caused an almost complete dissociation of polysomes (fig.1B), as evident by a great increase in monosomes and subunits with only a small fraction of polysomes consisting of 2–4 polyribosomes. At this concentration of pactamycin protein synthesis was inhibited by 89% (fig.1, legend).

Cycloheximide is known to interfere with elongation of protein synthesis [13] causing an aggregation of ribosomes [16]. A cycloheximide concentration was desired that would inhibit the elongation without interfering with initiation. As shown in fig.1C, the profile after cycloheximide treatment at 0.1 mg/100 g body wt was similar to that of insulin alone, while at 1.0 mg/100 g body wt the profile was deformed, monosome and subunit concentrations were increased as were smaller polysomes. This indicated that at this concentration, cycloheximide was probably interfering with initiation of protein synthesis as well. Therefore, at 0.1 mg/100 g body wt cycloheximide was chosen and at this concentration protein synthesis was inhibited by 76% (fig.1, legend).

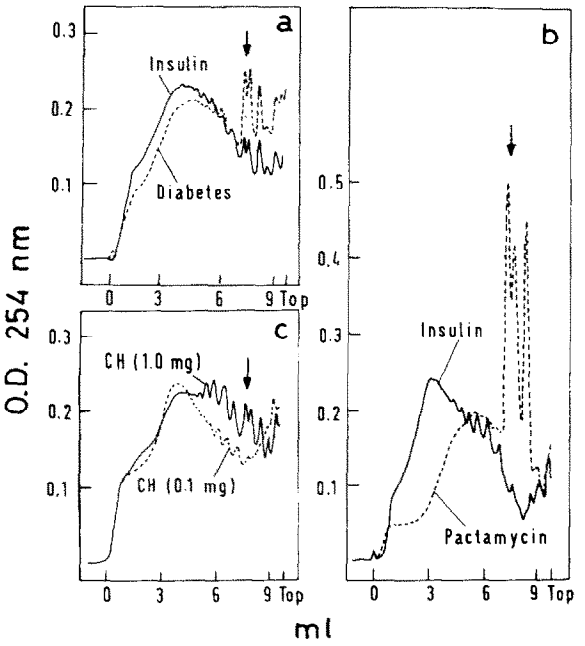

Fig.1. Sucrose gradients. Preparations of gradients and S17 samples followed by scanning of profiles were as in section 2. (A) Polysomal profiles of diabetic rat and diabetic rat injected with insulin (5 U/100 g) 90 min before sacrifice. (B) Polysomal profiles of diabetic rats injected with insulin (5 U/100 g) for 90 min with or without pactamycin (0.3 mg/100 g) 30 min before insulin. At this pactamycin concentration pactamycin inhibited protein synthesis by 89%. (C) Polysomal profiles of diabetic rats treated with insulin (5 U/100 g) for 60 min and cycloheximide at 1.0 mg/100 g or 0.1 mg/100 g before sacrifice. At 0.1 mg/100 g protein synthesis was inhibited by 76%. All rats were given 5 g glucose/kg for 90 min before sacrifice. Arrows indicate position of monosomes.

3.2. *Translatable activity of poly(A)$^+$ RNA*

Template activity of poly(A)$^+$ RNA for synthesis of total and released proteins as well as *P*-enolypruvate carboxykinase was quantitated in the wheat germ translation system and was proportionately linear with increasing amounts of poly(A)$^+$ RNA (results not shown). As shown in table 1, insulin injected to diabetic rats for 60 and 90 min, affected a marked decrease of the *P*-enolpyruvate carboxykinase mRNA, while the template activity for the synthesis of total

Table 1
Translatable activity of poly(A)$^+$ RNA from diabetic rats treated with insulin, cycloheximide and pactamycin

Treatment	Time (min) after insulin injection	No. of prep.	[^{3}H]Leucine incorporation (× 10^{-3}) into		
			Released Protein (dpm)	Enzyme (dpm)	% dpm in enzyme
None	–	10	3570 ± 200	6.55 ± 0.67	0.185 ± 0.017
Insulin	60	5	3660 ± 300	3.32 ± 0.61	0.090 ± 0.015
Insulin	90	6	4360 ± 540	2.94 ± 0.36	0.069 ± 0.009
Insulin and cycloheximide	60	4	4510 ± 190	10.71 ± 1.32	0.239 ± 0.022
Insulin and cycloheximide	90	7	4160 ± 500	7.39 ± 0.81	0.182 ± 0.020
Insulin and pactamycin	90	4	3750 ± 400	6.13 ± 0.34	0.170 ± 0.016

Diabetic rats were used as such or treated with insulin (5 U/100 g) for the times indicated. Cycloheximide (0.1 mg/100 g body wt) and pactamycin (0.3 mg/100 g body wt) were given 30 min prior to insulin. All rats were given 5 g glucose/kg at 90 min before sacrifice. Poly(A)$^+$ RNA was isolated from the S17 fraction and translated in the wheat germ system as in section 2. The [^{3}H]leucine incorporation into released proteins and *P*-enolpyruvate carboxykinase, and % incorporation in the enzyme, are expressed as dpm/3.6 μg poly(A)$^+$ RNA, and are the means of the number of preparations ± SEM

proteins remained essentially the same. When expressed as % incorporation it was evident that the insulin treatment for 60 and 90 min reduced translatable enzyme mRNA by ~1/2 and 2/3rds, respectively. A $t_{1/2}$ of 55 min. was obtained for this decay which is in close agreement with previous calculations of 45 min in glucose-fed fasted rats [2,3], 40 min in cordycepin treated fasted rats [2], or 40 min in dBcAMP pretreated Reuber H-35 cells when dBcAMP is removed [17].

Pactamycin, (0.3 mg/100 g body wt) given 30 min prior to insulin treatment of diabetic rats, inhibited the decay of *P*-enolpyruvate carboxykinase mRNA activity. Both [^{3}H]leucine incorporation into released proteins and into the enzyme after pactamycin and insulin treatment were identical to that of untreated diabetic rats (table 1).

Cycloheximide (an inhibitor of elongation) had a similar effect. Given at the times indicated in table 1, cycloheximide inhibited the insulin-mediated decay of *P*-enolpyruvate carboxykinase mRNA activity.

These results suggested that either the decay of *P*-enolpyruvate carboxykinase mRNA activity was protein synthesis-dependent, or that the specific effect of insulin on that decay required protein synthesis. To distinguish between these two possibilities, we used cordycepin, an inhibitor of poly(A)$^+$ RNA appearance in the cytoplasm [18]. As seen in table 2, cordycepin caused a rapid decline in the translatable mRNA for the enzyme when given to diabetic rats. By 90 min the template activity for the synthesis of the enzyme was 1/3rd that of untreated diabetic animals (table 1), while template activity for total protein synthesis was unaffected. This effect of cordycepin was inhibited by either pactamycin or cycloheximide (given 30 min before cordycepin) with the mRNA activity for the synthesis of *P*-enolpyruvate carboxykinase remaining at the diabetic levels (or slightly above) (table 2).

Cycloheximide and pactamycin, injected alone into diabetic rats for 2 h had little or no effect. When percent incorporations into enzyme were compared with those of the diabetic controls (table 1), a slight increase was noted. In order to determine if this increase was due to an accumulation of *P*-enolpyruvate carboxykinase mRNA, cycloheximide was injected for 4 h and the translatable activity of total poly(A)$^+$ RNA was determined. The results (not shown) indicated no additional increase in % incorporation into the enzyme. Thus the possibility that cycloheximide (or pactamycin) induced an increase in translatable mRNA for the enzyme was unlikely.

Table 2
Translatable activity of poly(A)$^+$ RNA from diabetic rats treated with cordycepin, cycloheximide and pactamycin

Treatment	Time (min) injected before sacrifice	No. of prep.	[^{3}H]Leucine incorporation (× 10^{-3}) into:		
			Released protein (dpm)	Enzyme (dpm)	% dpm in enzyme
Cordycepin	90	6	3970 ± 220	2.78 ± 0.48	0.072 ± 0.008
Cycloheximide	120	4	3360 ± 290	8.18 ± 0.98	0.251 ± 0.041
Cordycepin and cycloheximide	90	3	4430 ± 630	9.93 ± 2.70	0.224 ± 0.046
Pactamycin	120	2	4200	11.79	0.281
Cordycepin and pactamycin	90	3	4330 ± 430	8.88 ± 0.84	0.205 ± 0.006

All rats were diabetic. Cycloheximide and pactamycin were injected alone or 30 min prior to cordycepin at concentrations as in table 1. Cordycepin was given at 1.5 mg/100 g body wt. Isolation of poly(A)$^+$ RNA and translation in the wheat germ system were as in section 2. Results are the mean dpm ± SEM of [^{3}H]leucine incorporation/3.6 μg poly(A)$^+$ RNA

4. Discussion and conclusion

This evidence clearly demonstrates a rapid decay of translatable *P*-enolpyruvate carboxykinase mRNA initiated by insulin or cordycepin treatment of diabetic rats. The rate at which the mRNA activity decreases, corresponds to reported rates at which the synthesis of the enzyme declines by similar treatment [1–3] indicating that the decrease in the rate of synthesis of *P*-enolpyruvate carboxykinase reflects predominantly the decrease in its functional mRNA.

The effects of both insulin and cordycepin were completely inhibited by either cycloheximide or pactamycin, irrespective of the polysomal state or aggregation. While it is not known whether insulin accelerates the degradation or stops the synthesis of the mRNA, cordycepin is known for its inhibitory effect on the appearance of poly(A)$^+$ RNA in the cytoplasm [18]. Thus, with cordycepin treatment, the observed decay of *P*-enolpyruvate carboxykinase mRNA represents the rate of inactivation in the absence of its replenishment. The inhibition of this decay by either pactamycin or cycloheximide, assuming no additional effects, indicates that the system for mRNA inactivation requires protein synthesis. Conceivably, this is because the turnover rate of this assumed inactivating system is very rapid.

This system is evidently not unique for *P*-enolpyruvate carboxykinase mRNA. Recent evidence [19] suggests that tyrosine amino transferase and tryptophan oxygenase mRNAs (known for short half-lives) act similarly to *P*-enolpyruvate carboxykinase mRNA. Cycloheximide injected to rats preinduced with hydrocortisone caused increased translatable activity of both these mRNA whether free or bound to polysomes [19]. Furthermore, α-amanitin decreased these two mRNAs activities and its effect was prevented by cycloheximide, again implying that cycloheximide stabilized the mRNAs in the absence of their synthesis. On the other hand no effect of cycloheximide was noticable on albumin mRNA [19] which has been shown to be of a longer turnover rate [20] than *P*-enolpyruvate carboxykinase mRNA. That this effect of cycloheximide is a more general phenomenon is supported by the report that it affected an increase in the amount of poly(A)$^+$ RNA in 3T6 cells in vitro [21].

From these findings the possible existence in rat liver of a general rapidly turning over inactivating system of mRNA is raised. Since various inhibitors of protein synthesis block the effect of this system, a peptide factor may be involved. Regulation of this system or factor would therefore be of great significance in regulating (cellular) translatable mRNAs.

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