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CYCLOHEXIMIDE INHIBITION OF INSULIN CONTROL OF LIVER GLYCOGEN SYNTHASE b INTO a CONVERSION*

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SUMMARY

Cycloheximide given to insulin-treated alloxan diabetic rats results in the inhibition of insulin-induced liver glycogen synthase b into a conversion without affecting the level of synthase b. The effect of cycloheximide, believed to elevate cAMP in liver of normal rats, is independent of cAMP levels of the insulin-treated diabetic rat. The inhibition of insulin-mediated synthase b to a conversion by cycloheximide does not appear to be the result of a cycloheximide-induced cAMP-dependent phosphorylation of synthase a to b and suggests that insulin control of synthase b and a interconversions is dependent upon cycloheximide-sensitive protein synthesis.

INTRODUCTION

The regulation of phosphorylation-dephosphorylation interconversions of metabolic control enzymes (e.g. glycogen synthase and phosphorylase) has been defined in terms of an "on-off" system. The "on-off" switching mechanism (rapid phase) is believed controlled by fluctuations of small molecular weight metabolites (e.g. cAMP) sensitive to hormonal stimuli (glucagon, catecholamines) or by large molecular weight molecules (heat-stable inhibitor of phosphoprotein phosphatase) which have not, as yet, been directly associated with any particular class of stimuli (1-3).

The times required for either glucocorticoid or insulin restoration of liver glycogen synthase b into a conversion activity, which is lost in adrenalectomized (4) as well as diabetic rats (5-7), are such to suggest the hormones act in a sequential manner. The hormonal actions which are believed to be operative (5) are that the glucocorticoid is initially concerned with synthesis (slow phase) of the protein(s) required for synthase b to a transformation and insulin is subsequently needed to activate (rapid phase) the synthase transformation system.

The metabolic manifestation of glucocorticoid and insulin effects on liver glycogen synthesis is the same (deposition) and it might be expected that a concerted mechanism of hormone action does exist, the nature of which could

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be experimentally differentiated. The present study was designed to present evidence that the insulin effect on liver glycogen synthase b into a conversion can be inhibited in vivo by cycloheximide which, in the diabetic rat, is independent of the cAMP levels in liver (8,9) suggesting that insulin action, like glucocorticoid, is mediated via the regulation of the synthesis of synthase phosphatase (slow phase).

MATERIALS AND METHODS

Male, Sprague-Dawley rats (200-250 g) were injected with 150 mg alloxan/kg body weight and used 5-6 days later if judged "diabetic" (5) 48 hours after treatment by the glucose in tail-vein blood using Dextrostix (Ames). Blood glucose and liver glycogen were determined when animals were killed and livers removed (5).

Liver glycogen synthase was determined as the amount of glucose (glc) transferred from UDP-glc to primer glycogen using the method described by Thomas et al (10). The assays for synthase and endogenous capability for b into a conversion have been described (5-7). The enzyme preparation is an 8000 x g (10 min) extract obtained from livers blended for 30 sec in 3 volumes of 0.1 M glycylglycine, pH 7.4, containing 0.02 M 2-mercaptoethanol and 20% (v/v) glycerol. The extent of conversion of endogenous synthase b into a, upon incubation of the extract at 20° (5), is measured from catalytic criteria representative of physiological "activation" of synthase believed associated with liver glycogen synthesis in vivo and is estimated from the increased activity in the absence of glc-6-P using a -/+glc-6-P assay (5-7). Synthase was assayed in 0.25 ml (final volume after initiating the reaction upon addition of enzyme) containing 13.75 μmole glycylglycine, pH 7.4, 0.5 μmole UDP-glc (with 14,000 cpm UDP-[^{14}C]glc), 0.5 μmole Mg^{2+}(acetate salt), 1.25 mg rabbit liver glycogen, and 2.5 μmole of either glc-6-P, for b plus a activity, or Na_2SO_4, for a activity. After incubation at 37° for 4 min, 0.1 ml was spotted on Whatman ET-31 filter paper and processed for counting as described (10). Synthase is expressed as nmole glc incorporated into glycogen.

Rats were treated by intramuscular injection of insulin, cycloheximide, actinomycin D and [^{3}H]leucine as described for each experiment. [^{3}H]leucine incorporation into trichloroacetic acid (TCA)-precipitable protein was determined by homogenizing an aliquot of whole liver with 6% TCA. The acid precipitate was washed two times by resuspending the pellet in 10% TCA followed by centrifugation. The pellet was dissolved in 1.0 ml of 1.0 N NaOH, containing 0.4% deoxycholate, and 0.1 ml of the solution was counted in Aquasol (45% efficiency). The dose of [^{3}H]leucine was 5 μCi (sp. ac. 140-160 Ci/mmole) given one hour before sacrifice. cAMP was determined from whole liver aliquots (rapidly frozen in 10% TCA) by the method of Gilman (11) using the kit supplied by Amersham. UDP-[^{14}C]glc and [^{3}H]leucine were from New England Nuclear Corp., Boston. All other reagents, the sources of which have not been designated, were obtained from Sigma or Fisher Scientific and were the best quality available.

RESULTS

Fig. 1 shows the effect of 5 mg cycloheximide/kg body weight on synthase b into a conversion from normal, diabetic and insulin-treated diabetic rats. It can be seen that cycloheximide treatment completely inhibits the insulin-induced appearance of liver capability for synthase b to a conversion lost on alloxan treatment. Cycloheximide has no effect on the endogenous synthase b

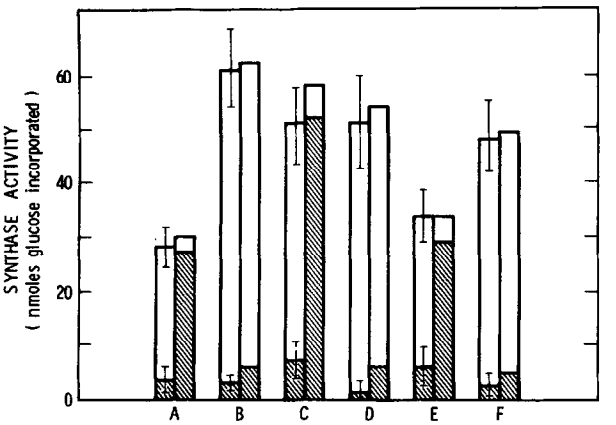

Fig. 1: The effect of cycloheximide on liver glycogen synthase b into a conversion. Synthase b to a conversion was measured by preincubation of the enzyme preparation for 60 min at 20° and assaying for synthase b and a. Open bars, synthase b determined in the presence of 10 mM glc-6-P and crossed bars, synthase a determined in the presence of 10 mM Na_2SO_4. For each pair of bars, the one on the left represents synthase b and a activities at 0 min of preincubation and the bar on the right, the activities at 60 min of preincubation. The increased activity of synthase a at 60 min is the extent of b to a conversion, as described (5,7). The vertical lines at 0 min represent the range of values of at least 6 animals done for each experiment. A, normal; B, alloxan diabetic; C, diabetic treated with 4 units of insulin 3 hr before sacrifice; D, insulin-treated diabetic given 5 mg cycloheximide/kg body wt 4 hr before sacrifice; E, normal rat treated with cycloheximide only; F, diabetic rat treated with cycloheximide only.

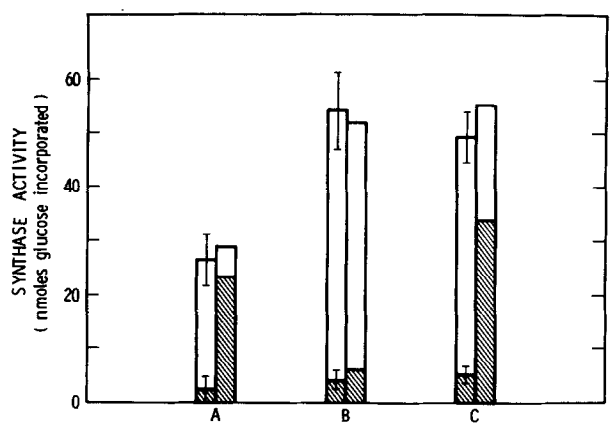

Fig. 2: The effect of actinomycin D on liver glycogen synthase b into a conversion. Assay methods and bar descriptions are the same as Fig. 1. The vertical lines represent the range of values of at least 3 animals done for each experiment. All animals were treated with 1 mg actinomycin D/kg body wt 4 hr before sacrifice. A, normal; B, alloxan diabetic; C, diabetic treated with 4 units insulin 3 hr before sacrifice.

TABLE 1: Effect of Cycloheximide Treatment on cAMP in Liver

Treatment	cAMP (pmoles)	[^{3}H]leu (cpm)	Protein (mg)	Glycogen (mg)	Glucose** (mg)
Normal (5)*	1112 ± 122	8904 ± 980	131 ± 36	47.7 ± 7.0	1.13 ± 0.29
+ cycloheximide (3)	1520 ± 210	1685 ± 200	129 ± 25	54.2 ± 9.0	1.20 ± 0.23
+ insulin (3)	1086 ± 96	13840 ± 700	188 ± 12	--	--
+ insulin and cycloheximide (5)	1238 ± 147	1944 ± 116	151 ± 40	--	--
Diabetic (5)	1244 ± 150	15419 ± 780	170 ± 46	5.2 ± 0.6	4.16 ± 0.57
+ cycloheximide (7)	1276 ± 191	1288 ± 130	140 ± 46	6.4 ± 0.8	4.80 ± 0.64
+ insulin (7)	1131 ± 158	12548 ± 750	176 ± 55	68.4 ± 14.0	2.30 ± 0.46
+ insulin and cycloheximide (7)	1131 ± 180	1763 ± 106	142 ± 45	5.7 ± 1.4	2.22 ± 0.33

*Numbers in parentheses are the number of animals done.

**Values for glucose are per ml blood; the other determinations (cAMP, [^{3}H]leu, Protein, Glycogen) are per g liver wet weight.

into a conversion capability of normal liver nor does it affect the absence of the conversion reaction in the non-treated diabetic rat. In addition, the increased synthase activity of the diabetic animals is not reversed by insulin or cycloheximide alone or in combination except at much higher doses of cycloheximide (TABLE 2). The animals appear well fed and although severely diabetic, the near doubling of the synthase activity, which has been observed by others (6) as well, can not be accounted for by a great change in liver weight (normal, 8-9 g livers average; diabetic, 7-8 g livers average). In view of the time-period of cycloheximide treatment (4 hr), the data of Fig. 1 suggest that compared to the level of synthase in the normal, the half-time of decay of synthase b activity of the diabetic is in excess of 4 hr.

Fig. 2 shows the effect of actinomycin D (1 mg/kg body weight) on normal, diabetic and insulin-treated diabetic liver capability for endogenous synthase b into a conversion activity. It can be seen that the drug had no effect on the synthase conversion capability of the normal, non-treated or hormone-treated diabetic animal. As with cycloheximide, actinomycin D had no effect on the elevated synthase activity of the diabetic suggesting the change in total assayable activity (presumably all in the b form of enzyme) is the result of extra-nuclear events.

It has been reported that cycloheximide can elevate the cAMP in whole animal liver as well as isolated liver cells (8). In view of this observation, cycloheximide inhibition of the insulin-induced synthase b to a conversion in the diabetic (Fig. 1) might result from the elevated cAMP and consequent in-

TABLE 2: Effect of Cycloheximide on Total Synthase Activity

Cycloheximide* (mg/kg)	Normal	Diabetic	Diabetic + Insulin
0	30 ± 3 (10)**	68 ± 9 (10)	66 ± 10 (10)
5	28 ± 5 (4)	57 ± 8 (4)	54 ± 11 (4)
10	31 ± 7 (4)	58 ± 8 (10)	61 ± 8 (10)
45	30 ± 7 (8)	36 ± 9 (4)	30 ± 3 (6)

*The 5 mg/kg doses of cycloheximide were given in equally divided amounts at two-hour intervals over the four-hour period. The other doses were given as four equally divided amounts over the four-hour treatment.

**The activity of glycogen synthase (+glc-6-P) is expressed as the nmoles glucose incorporated into glycogen. The numbers in parentheses are the number of separate determinations made.

creased activity of cAMP-dependent protein kinase (2). The increased cAMP-dependent protein kinase activity under these conditions would result in an apparent cycloheximide inhibition in that synthase a formed consequent to synthase phosphatase would rapidly be converted, via phosphorylation, to synthase b. TABLE 1 shows the results of cAMP determinations from livers of treated and non-treated normal and diabetic rats. In addition to the cAMP levels (pmole/g liver), data are shown for [^{3}H]leucine incorporation into TCA-precipitable protein (cpm/g liver) as well as for the protein and glycogen levels (mg/g liver) and blood glucose (mg/ml blood). The [^{3}H]leucine incorporation data was used as a marker to monitor the effectiveness of cycloheximide inhibition of protein synthesis.

The results of TABLE 1 show cycloheximide treatment of the normal rat elevates somewhat the mean level of cAMP; however, the range of values are well within the range for the non-treated normal. Since cycloheximide treatment did not alter synthase b to a conversion in the normal (Fig. 1,E), it does not seem likely that the slightly higher cAMP level would inhibit the formation of synthase a. The cAMP level of the non-treated normal agree with values reported by others (12). TABLE 1 shows as well that cycloheximide does not significantly elevate cAMP of the insulin-treated diabetic nor does insulin lower cAMP levels, as has been reported previously (9). In this study, cycloheximide does inhibit [^{3}H]leucine incorporation into acid insoluble protein (90%) in the insulin-treated diabetic suggesting that cycloheximide does effectively inhibit protein synthesis as well as the liver capability for the transformation of synthase b into a (Fig. 1).

The data of TABLE 1 show that the protein levels of liver are not significantly altered so as to account for the near doubling of the synthase b activity noted in the diabetic. The slight increase in the mean level of liver

protein in the diabetic is similar to that recently reported by Miller (13). However, the range of protein values in the diabetic is similar to that of the normal.

Of interest is the observed increase of [^{3}H]leucine incorporation into acid-insoluble protein of the insulin-treated normal as well as the untreated and insulin-treated diabetic. This might result from a combination of increased re-utilization of [^{3}H]leucine as well as an increased turnover rate of large molecular weight proteins (greater than 37,000 daltons; Ref. 14).

In TABLE 1, blood glucose and liver glycogen values show the expected changes associated with diabetes, as well as the changes upon insulin treatment, relative to normal values (5).

Increasing the dosage of cycloheximide given to the diabetic rats in the absence or presence of insulin, TABLE 2, did not affect synthase activity until the dose was quite large (45 mg/kg body weight). Protein synthesis, measured by [^{3}H]leucine incorporation, was inhibited to the same extent as with the lower dose used for the experiments of Fig. 1 and TABLE 1 (data not shown).

The experiments of TABLE 2 were designed to show that synthase b of the diabetic was not, as judged by the catalytic criteria of enzyme activity, altered by a significant inhibition upon treatment with the lower cycloheximide dose in the presence or absence of the hormone. A large effect of cycloheximide on synthase b activity might suggest that the enzyme molecule has been altered so as not to be capable of undergoing b into a conversion, if phosphatase were present, thereby suggesting cycloheximide to have an apparent inhibitory effect on synthase phosphatase.

DISCUSSION

The results of this study suggest that, similar to the effect of adrenal glucocorticoid (4), insulin is concerned with the synthesis of the synthase phosphatase (slow phase) rather than an activation mechanism (rapid phase) as a component of a concerted action with steroid-induced synthesis of the phosphatase protein. It can not be ruled out that a large molecular weight modulator of synthase phosphatase activity is dependent on insulin-induced protein synthesis, analagous to the heat-stable inhibitor of phosphorylase phosphatase (3); however, the modulator of synthase phosphatase might act as either an activator or de-inhibitor of the enzyme (15).

Our results show as well that cycloheximide does not significantly change the cAMP levels in the liver of the diabetic rat in contrast to the response of normal liver cells to the inhibitor (8). In the absence of a cycloheximide-induced elevated liver cAMP, cycloheximide inhibition of insulin-induced synthase b to a conversion does not appear to be the result of a cycloheximide-

activated cAMP-dependent protein kinase actively recycling synthase a into b when the diabetic animal is treated with the hormone and cycloheximide.

The studies in this report provide tentative evidence that the turnover time of synthase phosphatase is significantly less than of the synthase participating in the b to a conversion reaction. Such an effect appears independent of insulin control of the level of synthase b as has been reported earlier (16).

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