Inhibition of Rat Liver Glucose-6-Phosphate Dehydrogenase Synthesis by Glucagon*

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In fasting-refeeding experiments glucagon was found to inhibit the induction of rat liver glucose-6-P dehydrogenase. The kinetics of induction in the presence of glucagon indicated that the hormone decreased the rate of enzyme synthesis without altering the rate of enzyme degradation. Immunological titration of the amount of glucose-6-P dehydrogenase protein present in control and glucagon treated rats suggests that glucagon does not produce an inhibited species of the enzyme. Isolation of radioactive glucose-6-P dehydrogenase by precipitation with a specific antiserum provided direct evidence that glucagon inhibited the synthesis of the enzyme. Under identical conditions glucagon had no effect on the levels of 6-phosphogluconate dehydrogenase.

For the past several years we have been using the rat liver hexose monophosphate shunt dehydrogenases, glucose-6-P dehydrogenase (n-glucose-6-phosphate:NADP oxireductase, EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6-phospho-D-glucose:NADP oxireductase (decarboxylating), EC 1.1.1.44) as models to study the nutritional and hormonal regulation of enzyme levels in a mammalian system. These dehydrogenase are lipogenic enzymes regulated by dietary and hormonal stimuli in a fashion coordinate with other lipogenic enzymes (1-3). Using kinetic methods of analysis developed by Berlin and Schimke (4) we have shown that changes in levels of the hexose monophosphate shunt dehydrogenases in response to carbohydrate feeding are due to a change in rates of synthesis for both glucose-6-P dehydrogenase (5) and 6-phosphogluconate dehydrogenase (6). The rate of synthesis of these enzymes is proportional to the dietary intake of carbohydrate. Fat in the diet or insulin affect the rate of synthesis of glucose-6-P dehydrogenase (5, 7) and 6-phosphogluconate dehydrogenase indirectly (6) by affecting the dietary consumption of carbohydrate which in turn alters the rate of enzyme synthesis.

In a preliminary report (8) cyclic adenosine 3':5'-monophosphate or glucagon were shown to inhibit the induction of glucose-6-P dehydrogenase during fasting-refeeding experiments. This was the first report that cyclic AMP could inhibit the induction of a lipogenic enzyme.

The studies here were designed to investigate the mechanism by which hormone-mediated alterations in the level of cyclic AMP affect the level of glucose-6-P dehydrogenase. Our results demonstrate that cyclic AMP decreases the rate of synthesis of glucose-6-P dehydrogenase without altering the rate of enzyme degradation or producing an inhibited species of the enzyme. They also demonstrate that 6-phosphogluconate dehydrogenase levels are not affected by cyclic AMP.

EXPERIMENTAL PROCEDURE

Materials—Glucagon and DEAE-cellulose were purchased from Sigma; Diastrol ultrafiltration membranes from Amicon Corporation; Freund’s complete adjuvant and agarose from Difco; and C-labeled amino acid mixture (52 mCi/matom) from a protein hydrolysate of an algal suspension was purchased from Amersham/Searle.

Treatment of Animals—Young, male Sprague-Dawley rats obtained from local suppliers were fed rat chow or a 60% glucose diet as previously described (5). Daily weights were recorded and food consumption was measured with the use of nonspill metabolism feeders.

Animals were trained in the use of these feeders prior to the start of the experiment. Glucagon injections were given subcutaneously at a dose of 0.2 mg/100 g rat every 6 hours.

Preparation and Assay of Hexose Monophosphate Shunt Dehydrogenases—Methods for the preparation of rat liver supernatant fractions, the assay of glucose-6-P dehydrogenase activity, and the assay for protein were previously described (5). The activity of 6-phosphogluconate dehydrogenase was assayed as before (6).

Kinetic Analysis of Rates of Enzyme Synthesis and Degradation in Vivo—Rates of enzyme degradation were obtained as previously described (5) by kinetic analysis of the time courses for the changes in enzyme specific activities from one steady state level to another steady state level. Since the final steady state enzyme level is a result of the ratio of the rate of enzyme synthesis to the rate of enzyme degradation, the rate of synthesis can be calculated from the observed value for the final enzyme level and the value for the rate of degradation.

Immunological Procedures—Antiserum was prepared against glucose-6-P dehydrogenase which was purified as described previously (9). An equal volume of complete Freund’s adjuvant was emulsified with 11 mg of purified glucose-6-P dehydrogenase and injected subcutaneously at two sites on the back of a goat. An additional 5 mg of purified glucose-6-P dehydrogenase in phosphate buffer, pH 7.0, were injected subcutaneously 8 weeks later. The goat was bled 1 week later and the serum obtained was stored at -20°C.

Ouchterlony double diffusion patterns and quantitative precipitin

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1 The abbreviation used is: cyclic AMP, cyclic adenosine 3':5'-monophosphate.
tests were performed as outlined by Ouchterlony (10) and Williams and Chase (11), respectively. Immunochemical titrations were done according to the method of Kabat and Mayer (12).

Preparation of Rat Liver Supernatant Fractions for Immunochemical Analysis—Glucose-6-P dehydrogenase was partially purified by DEAE-cellulose column chromatography. A DEAE-cellulose column was equilibrated with 50 ml of 0.01 M potassium phosphate buffer, pH 7.0, for each ml of column bed volume. The column was eluted with 0.05 M potassium phosphate buffer, pH 7.0, (speed 2 ml/min) until the optical density at 280 nm was less than 0.05 A units. Glucose-6-P dehydrogenase was eluted with 0.2 M potassium phosphate buffer, pH 7.0, at a slow speed (0.25 ml/min). Fractions were collected in 1.3-ml volumes, assayed, and fractions with greater than 1.5 units of enzyme per ml were pooled.

Seventy to eighty per cent of the original glucose-6-P dehydrogenase activity was recovered from the column, and the per cent recovery was corrected for in calculating the radioactivity in that amount of enzyme present in 2 g of liver.

Isotopic Determination of Rate of Synthesis of Glucose 6-P Dehydrogenase—The rate of synthesis of glucose-6-P dehydrogenase was measured through isolation of the pulse-labeled enzyme by immunoprecipitation. Rats were injected intraperitoneally with 50 μCi of 14C-amino-acids and killed after 30 min. Rat liver supernatant fractions were prepared and glucose-6-P dehydrogenase was partially purified using DEAE-cellulose chromatography as described under "Experimental Procedure." Because immunoprecipitation involved a heat step, heat unstable radioactivity-labeled protein was removed prior to immunoprecipitation by heating the DEAE-cellulose eluate at 30° for 30 min, refrigeration at 4° overnight, and freezing at −20° for at least 1 day. The thawed eluate was centrifuged at 8000 × g and 4° for 1 hour and the supernatant fraction was filtered through glass wool. This procedure removed nonspecific precipitates. The antiserum was also heated at 100° for 30 min to remove precipitates that might nonspecifically absorb radioactivity and then centrifuged at 12,100 × g for 15 min at 4°.

Approximately 40 units of glucose-6-P dehydrogenase were then precipitated from a 5.0-ml volume of the heat treated DEAE-eluate by the addition of antiserum sufficient to precipitate 1.25 times that amount of glucose-6-P dehydrogenase. The immunoprecipitate formed after incubation at 30° for 15 min and at 4° for 24 hours. The immunoprecipitate was collected by centrifugation at 4° for 1 hour at 8000 × g. The resulting supernatant fraction was then used to obtain a second, control immunoprecipitate to correct for the amount of radioactivity nonspecifically absorbed to the first immunoprecipitate. Thus, 40 units of nonradioactive glucose-6-P dehydrogenase, purified by chromatography on DEAE-cellulose, was added to the supernatant fraction remaining after removal of the first immunoprecipitate. Glucose-6-P dehydrogenase antiserum was then added to precipitate this nonradioactive glucose 6-P dehydrogenase. This second, control immunoprecipitate was then collected in the same manner as the first immunoprecipitate.

Immunoprecipitates were drained for 2 min and suspended in a few drops of a wash solution of 0.15 M sodium chloride in 0.01 M potassium phosphate buffer, pH 7.0, (11) and transferred quantitatively with the aid of 1.5 ml of wash solution to a clean centrifuge tube. After 20 min in an ice bath the precipitates were centrifuged at 8000 × g for 1 hour. After three washes the precipitates were dissolved in a few drops of 0.5 M ammonium hydroxide and transferred quantitatively to a Whatman No. 3MM paper disc. The paper discs were then placed in a few drops of 0.5 M trichloroacetic acid 80° for 20 min, 95% ethanol for 15 min, and washed in 5% trichloroacetic acid at room temperature for 15 min, 5% ethanol-ether (1:1) for 15 min. The discs were air dried before counting. The radioactivity in the immune precipitates on the discs was counted as described for the immunoprecipitates.

We have previously reported that cyclic AMP or glucagon were able to prevent the induction of glucose-6-P dehydrogenase which normally occurs when fasted rats are fed a high carbohydrate diet (8). The cyclic AMP could have decreased the amount of the enzyme either by decreasing the rate at which it was synthesized or by increasing the rate at which it was degraded. A second possibility would be that cyclic AMP stimulated the phosphorylation of glucose-6-P dehydrogenase resulting in the formation of an inhibited form of the enzyme. The possibility that glucagon (via cyclic AMP) altered the rate of glucose-6-P dehydrogenase synthesis or degradation was checked by following the time course for the dietary induction of glucose 6-P dehydrogenase in the presence and absence of glucagon (Fig. 1).

Rats were fasted for 2 days and then fed a 60% glucose diet for 4 days. Those animals receiving glucagon were injected every 6 hours while the control rats were injected with saline. If glucagon had lowered the activity of glucose-6-P dehydrogenase by increasing the rate of enzyme degradation, induction in the presence of glucagon would have followed the time course represented by the dashed line (half-life equals 7.8 hours). The induction actually observed either in the presence or absence of glucagon (solid lines) followed a time course with a half-life of 15 hours which agrees with estimates made previously (5). Thus glucagon does not appear to alter the rate of the enzyme's degradation. Glucagon could either cause a decrease in the rate at which the enzyme is synthesized or the formation of an inhibited species of the enzyme. This latter possibility was tested by the experiment reported in Fig. 2.
The rat liver supernatant fractions were concentrated 2- or 3-fold by ultrafiltration under nitrogen pressure using a Diaflo ultrafilter PM 30 membrane. Glucose-6-P dehydrogenase did not pass through the ultrafiltration. The enzyme activity was measured in the supernatant fraction. Glucose-6-P dehydrogenase activity was found to be 0.69 units per milliliter of supernatant fraction. The open circles (○) represent the specific activity of glucose-6-P dehydrogenase, while the closed circles (●) represent the specific activity of the enzyme from the glucagon-treated rats. Since this was not the case, the enzyme was degraded or by producing an inhibited enzyme. Since the glucagon-treated rats were fasted for 2 days and refed a 60% glucose diet for 4 days. Glucagon was injected from the start of refeeding. The dietary consumption of carbohydrate in the control animals was 21.7 ± 0.8 kcal per 100 g rat per day and that of the glucagon-treated rats was 22.3 ± 0.5 kcal per 100 g rat per day. The closed circles (●) represent a control animal with a glucose-6-P dehydrogenase specific activity of 0.69. The open circles (○) represent two pooled glucagon-treated animals with a specific activity of 0.36. The rat liver supernatant fractions were concentrated 2- or 3-fold by ultrafiltration under nitrogen pressure using a Diaflo ultrafilter PM 30 membrane. Glucose-6-P dehydrogenase did not pass through the membrane and was not inactivated. The concentrated supernatant fractions were diluted with 0.15 M potassium chloride until the control and experimental supernatant fractions were of equal glucose-6-P dehydrogenase activity. Variable amounts of glucose-6-P dehydrogenase were then incubated with 0.2 ml of antiserum in a volume of 1.9 ml at 30°C for 15 min and at 4°C for 36 hours. The immunoprecipitate was collected by centrifugation in a desk top centrifuge at 8000 × g for 15 min and aliquots of the supernatant fraction were assayed for glucose-6-P dehydrogenase activity.

In this experiment an antiserum against rat liver glucose-6-P dehydrogenase was used to titrate the amount of glucose-6-P dehydrogenase protein present in the livers from rats treated with and without glucagon. Since the antiserum will precipitate all of the glucose-6-P dehydrogenase activity from rats treated with glucagon, the glucagon does not produce a partially inhibited glucose-6-P dehydrogenase which is incapable of reacting with the antiserum. Furthermore, 0.2 ml of antiserum will precipitate approximately 12 units of glucose-6-P dehydrogenase whether or not the enzyme came from rats injected with glucagon. If glucagon had produced a species of glucose-6-P dehydrogenase which had a lower catalytic activity per mol of enzyme, it would have taken a correspondingly larger amount of antiserum to precipitate one unit of enzyme from the glucagon-treated rats. Since this was not the case, we have concluded that glucagon does not produce an inhibited species of glucose-6-P dehydrogenase. Since the injection of glucagon does not lower the specific activity of glucose-6-P dehydrogenase by altering the rate at which the enzyme is degraded or by producing an inhibited enzyme, it must act by decreasing the rate of enzyme synthesis.

The most direct method to demonstrate the ability of glucagon to decrease the rate of glucose-6-P dehydrogenase synthesis would be to show a decreased incorporation of radioactive amino acids into the enzyme in the presence of the hormone. This requires the ability to isolate radioactive glucose-6-P dehydrogenase in high yield from liver. The most convenient method to do this is by precipitating the enzyme with a specific antiserum. Accordingly, rat liver glucose-6-P dehydrogenase was purified to homogeneity by the criteria of disc gel electrophoresis and used to produce an antiserum in a goat as described under "Experimental Procedure." During the course of these experiments we found it necessary to purify radioactive glucose-6-P dehydrogenase by a batchwise elution from DEAE-cellulose prior to immunoprecipitation in order to reduce background radioactivity nonspecifically absorbed to the antigen-antibody precipitate. Therefore, glucose-6-P dehydrogenase from a DEAE-cellulose eluate was used as the source of antigen for characterization of the specificity of the goat antiserum.

The results of a quantitative precipitin reaction between antibody and glucose-6-P dehydrogenase are shown in Fig. 3. Variable amounts of glucose 6-P dehydrogenase from a DEAE-cellulose eluate were added to constant amounts of goat antiserum. The smoothness of the immunoprecipitin curve indicated the formation of one antibody-antigen complex (14). If the antiserum contained more than one antibody, the curve would have had more than one plateau region. From the equivalence point, it was determined that 60 units of glucose-6-P dehydrogenase were precipitated by 1 ml of antiserum. In the zone of antibody excess, less than 1% of the original activity remained in the supernatant fraction. Similar results (not shown) were obtained for a quantitative precipitin reaction between antibody and glucose-6-P dehydrogenase from a crude rat liver supernatant fraction.

The Ouchterlony double diffusion patterns in Fig. 4 show that formation of one antigen-antibody complex from the diffusion of antiserum against glucose-6-P dehydrogenase after purification on DEAE-cellulose. Several concentrations of antiserum were used to ascertain that there was no additional band formation near the wells. The single band of precipitation was due to the formation of an immunoprecipitate containing glucose-6-P dehydrogenase as the band stained for glucose-6-P dehydrogenase activity. Together these results establish that the antiserum is specific for glucose-6-P dehydrogenase.

Table I illustrates two separate experiments in which this antiserum was used to isolate glucose-6-P dehydrogenase from the livers of rats injected with 14C-amino acids for 90 min prior to sacrifice. In preliminary experiments we found that only low levels of radioactivity are incorporated into rat liver glucose-6-P dehydrogenase in vivo. This necessitated a preliminary purification of the radioactive glucose-6-P dehydrogenase on DEAE-cellulose in order to lower the amount of radioactivity nonspecifically absorbed to the immunoprecipitate. After this purification step the amount of radioactivity absorbed to the second, control immunoprecipitate was 80 to 90 cpm and these have been subtracted from the values listed under Column A in Table I. In a second preliminary experiment, we found that 2 days after initiating refeding there was a maximum increase in the rate of glucose-6-P dehydrogenase synthesis so this time period was selected for the experiments reported here.

Rats were fasted for 2 days and refed the 60% glucose diet for 2 days. Glucagon was injected every 6 hours during the 2 days of refeeding. One hour after the last injection of glucagon each rat was injected with 50 μCi of 14C-labeled amino acid mixture and killed 30 min later. The glucose-6-P dehydrogenase was
In Experiment I glucagon decreased the specific activity by the relative rate of synthesis of the enzyme (A/B). The result is the relative rate of synthesis of glucose-6-P dehydrogenase as described under “Experimental Procedure.” Open circles represent the amount of protein in the immunoprecipitate and closed circles the unprecipitated glucose-6-P dehydrogenase activity.

then isolated as described under “Experimental Procedure.” The radioactivity in that amount of enzyme present in 2 g of liver is reported in Column A. This value is divided by the radioactivity in total soluble protein (Column B) in order to correct for alterations in the size of amino acid pools or differences in the amount of radioactivity actually injected. The result is the relative rate of synthesis of the enzyme (A/B). In Experiment I glucagon decreased the specific activity by 40% and there was a corresponding 40% decrease in the relative rate of enzyme synthesis. In Experiment II, glucagon produced a 52% decrease in specific activity and this was accompanied by a 38% decrease in the relative rate of glucose-6-P dehydrogenase synthesis. Thus, in both experiments the decrease in specific activity was accompanied by a decrease in the rate of enzyme synthesis. Considering the difficulty of these experiments and the low amount of radioactivity incorporated into the enzyme, we believe this provides direct evidence that glucagon acts by decreasing the rate of glucose-6-P dehydrogenase synthesis.

6-Phosphogluconate dehydrogenase synthesis, like glucose-6-P dehydrogenase synthesis, is increased during fasting-refeeding experiments (6). However, 6-phosphogluconate dehydrogenase specific activity only increases about 5-fold under conditions where glucose-6-P dehydrogenase increases in excess of 20-fold (Fig. 1). The reasons for these differences are not clear. However, during the course of the experiment presented in Fig. 1 we measured the activity of 6-phosphogluconate dehydrogenase in the control and glucagon-treated animals and observed that glucagon did not decrease the activity of this enzyme even though glucose-6-P dehydrogenase activity decreased by 48% in the same animals. These results are illustrated in Fig. 5 where the 6-phosphogluconate dehydrogenase activity from the glucagon-treated rats in Fig. 1 are superimposed over similar data for control rats taken from data published previously (see Fig. 1 of Ref. 6). Glucagon did alter the time course or the extent of the dietary induction of 6-phosphogluconate dehydrogenase. More recently we have used 3H-amino-acids to estimate the relative rate of 6-phosphogluconate dehydrogenase synthesis in rats treated with or without glucagon.2 These data also indicate that glucagon has no effect on the rate of 6-phosphogluconate dehydrogenase synthesis.

**DISCUSSION**

The purpose of this research was to gain insight into the mechanism by which glucagon inhibited the dietary induction of glucose-6-P dehydrogenase. Previous experiments had dem-

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shown that cyclic AMP could duplicate the effect of glucagon and that neither agent affected the appetite of the animals (8). This is particularly important since we have shown that the rate of synthesis of both glucose-6-P dehydrogenase (3, 7) and 6-phosphogluconate dehydrogenase (6) increases in proportion to increases in the intake of dietary carbohydrate. Glucagon had no effect on the intake of dietary carbohydrate so glucagon must act by some mechanism other than by decreasing the appetite. Fig. 1 illustrates that glucagon does not alter the rate at which the enzyme is degraded in vivo. In addition, determination of the amount of glucose-6-P dehydrogenase protein present by titration with a specific antiserum (Fig. 2) shows that a 48% decrease in enzyme activity is accompanied by a corresponding decrease in the amount of immunoreactive glucose-6-P dehydrogenase protein. This demonstrates that glucagon did not produce a partially inhibited species of the enzyme. The possibility remains, however, that glucagon could favor the formation of a species of glucose-6-P dehydrogenase which was completely inactive and also unable to react with the anti-glucose-6-P dehydrogenase serum.

These data suggest that glucagon prevents the dietary induction of glucose-6-P dehydrogenase by decreasing the rate at which the enzyme is synthesized. Fig. 1 supports this conclusion. In this kinetic method for calculating rates of enzyme synthesis and degradation, the rate of enzyme synthesis is equal to Ph where P is the steady state specific activity of the enzyme and h is the first order rate constant for enzyme synthesis; the rate of enzyme degradation, the rate of enzyme synthesis so they could be used for gluconeogenesis and would also decrease the levels of this class of enzymes which use blood glucose for fatty acid synthesis. This suggests that glucagon acts at both the level of enzyme activation and the level of enzyme synthesis in order to achieve an orderly regulation of metabolism.

The hexose monophosphate shunt dehydrogenases should be particularly useful in gaining insight into the relationships between the dietary and hormonal regulation of enzyme synthesis. The rate of synthesis of both glucose-6-P dehydrogenase and 6-phosphogluconate dehydrogenase is correlated with the intake of dietary carbohydrate while only glucose-6-P dehydrogenase synthesis is decreased by glucagon.

Thus, 6-phosphogluconate dehydrogenase should represent a model for an enzyme whose rate of synthesis is regulated by dietary factors while glucose-6-P dehydrogenase would represent a model for enzymes whose rate of synthesis is regulated by both dietary and hormonal factors. Together these two models should provide some unique insights into the mechanisms by which dietary and hormonal factors regulate enzyme synthesis.

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