Mechanism of Control of Hepatic Glycogenesis by Insulin*

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SUMMARY

Insulin produces an increase in the conversion of the D form (glucose-6-P dependent) of liver glycogen synthase to the I form (glucose-6-P independent) when injected intravenously into fed alloxan diabetic rats. Insulin activates glycogen synthase previously inactivated by glucagon in isolated perfused livers from normal rats, while increasing [14C]glucose incorporation into glycogen and decreasing glycogen-stimulated glucose production. This change in synthase activity is associated with a decrease in synthase (protein) kinase activity and tissue adenosine 3',5'-monophosphate levels when insulin is used to antagonize the effects of glucagon. Finally, insulin alone has a direct effect on the activation of glycogen synthase (D to I shift) in perfused livers from normal rats if insulin infusion is begun after an initial 30-min perfusion period. This effect is maximal between 6 and 15 min and is no longer present after 30 min. Associated changes include an increase in [14C]glucose incorporation into glycogen at 10 min and a decrease in synthase kinase activity at 6 and 15 min. This decrease in kinase activity is not associated with a decrease in apparent hepatic concentrations of adenosine 3',5'-monophosphate.

The enzymatic pathway involved in glycogen synthesis has been characterized in most mammalian tissues. Glycogen synthesis in liver is at least partially controlled by the activity of glycogen synthase (1), the enzyme responsible for catalyzing the incorporation of glucose from uridine diphosphoglucose into glycogen (2). The active form of synthase (I or glucose-6-P independent) is converted to the inactive form of synthase (D or glucose-6-P dependent) by an ATP requiring phosphorylation reaction catalyzed by active synthase (protein) kinase (3). The activity of kinase is in turn regulated at least in part by alterations in tissue levels of cyclic AMP (4). Therefore, any hormone or factor affecting cyclic AMP concentration can alter synthase activity through an effect on the kinase. On the other hand, the inactive form of synthase is converted to the active form by a dephosphorylation reaction catalyzed by synthase phosphatase (5). Current evidence suggests that the phosphatase may also exist in two forms, thus providing an additional site for control of glycogen synthesis (5).

The effect of insulin to promote the activation of glycogen synthase (D to I shift) in the liver in vivo has been well documented (6–9). Many workers have reported interactions of insulin with glucagon, epinephrine, and cyclic AMP in the perfused rat liver (10–15). The action of insulin to promote glycogen synthase activation has been well characterized in diaphragm (16–18), skeletal muscle in vivo (19, 20), heart in vivo (20, 21) and in vitro (22), fat pad in vitro (23), human placenta in vitro (24), and tumor cells in vitro (25). From these reports it could be inferred that glycogen synthesis in the liver is at least partially dependent on the action of insulin to activate glycogen synthase. However, the question of whether or not insulin actually enhances hepatic glycogenesis has remained controversial due to an absence of evidence obtained in vitro.

The purpose of the present study was to explore the extent to which insulin mediates hepatic glycogen synthesis. We have studied the action of insulin to alter glycogen synthase from a basal state in diabetic rat liver in vivo and the antagonism between the effects of glucagon and insulin on glycogen synthase in livers of normal rats in vitro. Finally, we describe a set of conditions whereby insulin promotes glycogen synthase activation from a basal state (absence of glucagon) in livers of normal rats perfused in vitro.

MATERIALS AND METHODS

Male Wistar rats fed ad libitum with Purina laboratory chow and weighing from 100 to 150 g were used after anesthetization with sodium pentobarbital (50 mg per kg). Experimental diabetes was induced where indicated by intravenous injection of alloxan (60 mg per kg) and was diagnosed 2 days later by a maximal positive urine sugar test using "Tes-Tape." For experiments in vivo, 48-hour alloxan diabetic rats were anesthetized as previously described and both femoral triangles were exposed. The animals' tongues were secured to facilitate breathing and body temperature was maintained with heat from 100-watt light bulbs. Blood samples were taken from the femoral veins prior to the injection of either insulin or 0.9% NaCl solution-albumin. The livers were removed at the indicated times by abdominal incision. Livers were analyzed immediately after surgical excision.
The technique of liver perfusion (26) and the apparatus used for the procedure have been described in detail by Exton and Park (27). Livers were perfused at 37° with oxygenated (95% O2-5% CO2) Krebs-Henseleit bicarbonate buffer, pH 7.4, containing 3% Fraction V bovine serum albumin and 25% washed bovine red blood cells at a constant flow of 7 ml per min.

Liver perfusions were terminated by rapidly freezing livers with Wollenberger clamps (28) cooled in liquid nitrogen. Frozen livers were prepared for analyses by powdering in a percussion mortar at liquid nitrogen temperature (27) and were stored at -70°.

Aliquots of frozen liver powder were analyzed for glycogen (29) and the hydrolyzed glucose determined by the alkaline ferricyanide method using the Technicon Auto-Analyzer. [14C]Glycogen was determined as previously described (30).

Glycogen synthase was extracted from fresh liver or frozen liver powder with 100 mm potassium fluoride-10 mm EDTA, pH 7.8 (w/v, 1:5) and assayed without added sulfate using the method of Thomas et al. (31). Data were expressed as per cent I (active) form. Blood glucose was determined as described above, and tissue protein was determined by the Lowry procedure using the Technicon Auto-Analyzer.

Glycogen synthase kinase I was extracted from frozen liver powder with 5 mm phosphate-2 mm EDTA (w/v, 1:3) at pH 7. After centrifugation at 12,000 x g for 15 min, the supernatants were passed rapidly (10 min total time) through 2 Sephadex G-50 columns (1 cm x 15 cm), all at 4°. The first column was equilibrated with the extraction buffer and the second with 50 mm Tris-5 mm EDTA at pH 7.8. Aliquots of the protein eluate from the second column were assayed for synthase kinase activity using added synthase I as substrate for the kinase in the absence and presence of cyclic AMP as described by Shen et al. (32). Kinase activity determined in the absence of cyclic AMP was expressed as the active or cyclic AMP independent form (I form), and that determined in the presence of added cyclic AMP was expressed as total kinase or cyclic AMP independent plus dependent forms (I plus D forms).

The phosphate-EDTA buffer consistently increases apparent synthase 1 activity in liver homogenates since there is no fluoride to inhibit synthase phosphatase. Table I thus demonstrates that insulin does activate hepatic glycogen synthase in alloxan diabetic rats. This agrees with previous data obtained in vivo (8-9, 34).

The activation of hepatic glycogen synthase by insulin in vivo does not show whether or not this is a direct action of the hormone on the liver or merely an indirect effect mediated by another effector system. Physiologically, insulin appears to act to antagonize the effects of glucagon since it has been well documented that insulin counteracts some of the actions of glucagon in the perfused rat liver (10-15). The next series of experiments

**RESULTS**

Optimal conditions for demonstrating an effect of insulin on hepatic glycogen synthesis appear to require a slight insulin deficiency. Since an acute insulin deficiency develops 48 hours after a single injection of alloxan, fed alloxan diabetic animals were used. After 15 min, an initial blood sample was withdrawn from the femoral vein and the animals were then injected with 0.2 ml of either insulin (2 units) or 0.9% NaCl solution. In the first series of animals, the livers were excised 5 min after insulin injection, and in the second series after 15 min. Final blood samples were taken 15 min after injection.

After 5 min of insulin exposure, glycogen synthase was increased from a control value of 21% I synthase to an insulin treated value of 38% (Table I). The same effect was observed after the 15-min period where a control value of 3% I synthase was increased to 14% by insulin. As seen in this table, insulin also produced a decrease in blood glucose of 72 mg/100 ml, while the control showed no significant change. The difference in absolute levels of glycogen synthase activity between the 5- and 15-min experiments was due to a difference in extraction procedures. The first series (5 min) was extracted in phosphate-EDTA buffer rather than the usual potassium fluoride-EDTA. The phosphate-EDTA buffer consistently increases apparent synthase I activity in liver homogenates since there is no fluoride to inhibit synthase phosphatase. Table I thus demonstrates that insulin does activate hepatic glycogen synthase in alloxan diabetic rats. This agrees with previous data obtained in vivo (8-9, 34).

**TABLE I**

**Effect of insulin on liver glycogen synthase in alloxan diabetic rats**

The in vivo technique and experimental conditions are described in the text and under “Materials and Methods.” The 5-min series was extracted using phosphate-EDTA buffer and the 15-min series was extracted in KF-EDTA buffer. The concentration of insulin employed was 2 units.

| Insulin | Exposure time | Glycogen synthase | Blood glucose change |
|---------|---------------|--------------------|----------------------|
| 0 (6)   | 5             | 21 ± 2             | -14 ± 7              |
| + (6)   | 5             | 38 ± 7             | -72 ± 21             |
| 0 (4)   | 15            | 3 ± 2              | 0                    |
| 1 (1)   | 15            | 11 ± 4             | 0                    |

*p is less than 0.025 versus control.
was designed to determine if the antagonism extended to control of synthase activity.

Livers from normal fed rats were perfused for 2 hours in a recirculating system. The 1st hour of perfusion was carried out without substrate or hormone to produce a state of relative insulin deficiency. At the beginning of the 2nd hour, 1.0 μCi of [14C]glucose was injected into both recirculation reservoirs. Glucagon was infused at 0.25 pmoles per min into one reservoir and glucagon plus insulin (insulin at 5 munits per min) into the other reservoir. Infusions were continued through the 2nd hour. Blood perfusate samples were taken every 20 min throughout the perfusion.

Glucagon produced an increase in the rate of perfusate glucose accumulation. The least number of observations and the experimental procedure are described in the text and under "Materials and Methods." The final concentration of glucagon was 3.75 X 10^-8 M and of insulin was 4 X 10^-8 M.

Table II shows the effects of glucagon plus and minus insulin on [14C]glucose incorporation into glycogen in the same livers. Approximately 800 cpm per g of liver were incorporated into glycogen with glucagon alone, whereas insulin plus glucagon produced a 2-fold increase in radioactive glycogen deposition. It could be argued however, that the increase in [14C]glycogen deposition with insulin plus glucagon was due to higher specific activity of [14C]glucose since insulin decreased total perfusate glucose values from 17 mM with glucagon alone to 11 mM with glucagon plus insulin. This would have produced final calculated specific activities of 2720 cpm per pmole with glucagon and 3860 cpm per pmole with glucagon plus insulin in a final volume of 50 ml. The 40% increase in specific activity could not satisfactorily account for the 100% difference in glycogen deposition. This possibility will be experimentally circumvented in the next series. Also shown in Table II, insulin caused a trend toward a sparing effect on total liver glycogen levels although not statistically significant.

Liver glycogen synthase activity was determined in the same series (Table II). With glucagon alone, synthase I activity was 14%, whereas with insulin plus glucagon, synthase I activity increased to 21%. Therefore, these data demonstrate that insulin activates glycogen synthase in the presence of very low concentrations of glucagon in the isolated perfused rat liver. Insulin caused a 38% decrease in tissue cyclic AMP from a glucagon-stimulated level of 0.68 pmoles to 0.42 pmoles per mg of liver, a figure which is close to basal levels of the nucleotide (14). These data are consistent with an activation of synthase due to a decrease in synthase inactivation by cyclic AMP. This effect of insulin to lower tissue cyclic AMP in the presence of glucagon agrees with the original report of the same effect by Jefferson et al. (11).

Since glucose alone has been shown to activate glycogen synthase activity in the perfused rat liver (37, 38), the inactivation of synthase by low levels of glucagon in the last series of experiments was complicated by the higher final glucose concentrations with glucagon as opposed to the lower glucose concentrations in livers treated with glucagon plus glucagon. This would, in effect, make it more difficult to demonstrate the synthase activating capability of insulin. Also, the incorporation of [14C]glucose into glycogen was complicated by a difference in final specific activities of [14C]glucose. Therefore, the next series of experiments was designed to circumvent these two complicating factors.

Livers were perfused for 2 hours as previously described, except that supplemental glucose infusion was begun the 2nd hour in control (0.7 pmoles of glucose per min) and glucagon plus insulin (4.2 pmoles of glucose per min) livers in order to equalize glucose accumulation with that previously observed with glucagon alone. All other additions and infusions were the same as those described for Fig. 1. Fig. 2 shows glucose accumulation in this series, and, as can be seen, glucose accumulation is essentially the same for the three different sets of conditions. Therefore, the problems of specific activities and glucose effects from the last series should be minimized.

Table III demonstrates that the effect of insulin on [14C]glycogen...
Effects of glucagon plus and minus insulin on perfused liver glycogen deposition. The experimental procedure is described in the text. The least number of observations for each point was six. The final concentrations of glucagon and insulin were the same as those described for Fig. 1.

**Table III**

**Effects of glucagon plus and minus insulin on perfused liver glycogen deposition.**

| Hormone(s) infused | Glucose infused | Glycogen concentration (cpm/g wet liver) |
|--------------------|----------------|----------------------------------------|
| Control (8)        | +             | 43620 ± 4330                           |
| Glucagon (6)       | 0             | 1959 ± 510                              |
| Glucagon + insulin (6) | +        | 4768 ± 1246b                           |

*p is less than 0.01 versus control.
*b p is less than 0.05 versus glucagon.

glycogen deposition as seen in Table II was not due to different specific activities, but rather to a direct effect of insulin to activate glycogen synthase. The sparing effect of insulin on tissue glycogen is also significant under these conditions. This table also emphasizes that very small concentrations of glucagon can reduce [14C]glycogen deposition by about 95%.

Table IV shows the effects of glucagon plus and minus insulin on synthase and synthase kinase activities and on cyclic AMP levels in perfused livers under the conditions described for Fig. 2. The mean control value of 70% I synthase indicates that glucagon caused a 65% decrease in synthase activity, whereas insulin partially reversed this effect of glucagon. Synthase (protein) kinase activity expressed as per cent I kinase (per cent active form) was increased by approximately 60% with glucagon, and as shown in the next column, the effect was probably exerted through the glucagon-stimulated increase in tissue cyclic AMP. Insulin, on the other hand, reversed the effect of glucagon on per cent I synthase kinase probably through its effect to lower glucagon-elevated tissue cyclic AMP levels. Therefore, it appears that the action of insulin to activate glucagon-inactivated synthase is at least partially due to a decrease in the hepatic cyclic AMP level which is responsible for a decrease in the activity of the synthase inactivating enzyme, glycogen synthase kinase.

The next series of experiments was designed to answer the basic question of whether or not insulin directly regulates glycogen synthase under basal metabolic conditions. Livers from fed rats were perfused by recirculation of substrate-free blood buffer mixture for 30 min to reach basal metabolic conditions and to achieve a relatively ahormonal state. After 30 min, insulin infusion was begun on the experimental side at a rate of 10 milliunits per ml of perfusate and continued for 6, 15, or 30 min. The experiments were terminated by freezing the livers as previously described. Blood samples were taken from the recirculation reservoirs at 0, 15, 30, 45, and 60 min.

Glucose production from 30 to 60 min was unaffected by this concentration of insulin (Fig. 3). After 30 min of equilibration perfusion, the control and experimental livers had the same per cent I synthase activities (Table V). At 6 min, the control livers showed 29% synthase I, whereas the insulin treated livers had a mean synthase activity of 38%. The same relative effect of insulin was seen at 15 min. At 30 min, the insulin effect was no longer statistically significant. The increases in synthase activity observed with time may be due to the increasing glucose concentration in the perfusate. The effect of insulin on glycogen synthase under basal metabolic conditions was rapidly manifest (6 to 15 min) and short in duration (30 min) in the perfused liver. This is similar to the effect of insulin on heart glycogen synthase activation, *in vitro* (22). Glycogen synthase kinase activity was determined at 6 and 15 min to elucidate the mechanism of insulin.
Effects of insulin on glycogen synthase, synthase kinase, and cyclic AMP in perfused liver

Experimental details are the same as those described for Fig. 3. The least number of observations for each mean was seven.

| Insulin | Exposure time | Glycogen synthase | Synthase kinase | Cyclic AMP per mg liver |
|---------|---------------|-------------------|-----------------|------------------------|
| min     | % I           | nmoles/g protein/5 min | %               |                        |
| 0       | 22 ± 1        | 104 ± 7           | 46 ± 2          | 0.37 ± 0.02            |
| +       | 22 ± 1        | 104 ± 7           | 46 ± 2          | 0.35 ± 0.03            |
| 0       | 38 ± 2        | 104 ± 3           | 36 ± 3A         | 0.30 ± 0.02            |
| +       | 41 ± 3        | 135 ± 9           | 40 ± 5          | 0.28 ± 0.01A           |
| 0       | 46 ± 4        |                   |                 |                        |
| +       | 52 ± 6        |                   |                 |                        |

Not significantly different from control.

*p is less than 0.01 versus control.

**p is less than 0.025 versus control.

Discussion

Control of mammalian hepatic glycogen synthesis by insulin has remained a controversial topic primarily due to the lack of evidence obtained in vivo and to several negative reports, in vivo (39) and in vitro (12, 38). Another factor contributing to this controversy has been the demonstration of the control of hepatic glycogen synthesis by glucagon in the perfused rat liver (37, 38), a system that is apparently hormonally deficient. Since it has been well established that mammalian liver has an intrinsic ability to regulate circulating glucose concentrations (40-43) and that circulating glucose concentrations can regulate glycogen synthesis, most research has been directed at the mechanism of the glucose control of glycogen synthesis. Regardless of the undisputed importance of glucose in controlling hepatic glycogenesis, it would seem an error to ignore previous reports of insulin activation of glycogen synthase in vivo preparations (6-9, 34). Data presented in this paper confirm the reports previously cited regarding insulin activation of glycogen synthase in vivo, and in addition, clearly demonstrate for the first time that insulin increases glycogen synthase activity in the isolated perfused rat liver under at least three different sets of experimental conditions. The insulin-mediated change in glycogen synthase activity can be correlated with a decrease in synthase I kinase activity, also demonstrated in liver for the first time. This stable enzyme effect can be readily explained by the decrease in tissue levels of cyclic AMP when insulin is used to antagonize the effect of glucagon. However, this explanation does not necessarily appear to be the answer for the activation of hepatic glycogen synthase by insulin alone. As shown in Table V, synthase I kinase activity is decreased by insulin under basal metabolic conditions, while the tissue levels of the cyclic nucleotide remain apparently unchanged. These data are similar to studies of Villar-Palasi and Wenger (44) where they found synthase I kinase activity reduced in skeletal muscle extracts of rats that had been treated with insulin in vivo. This decrease in activity was not correlated with a similar decrease in cyclic AMP. This was later confirmed and extended by Shen et al. (52) with diaphragm in vivo. Thus, it appears that insulin alters the synthase I kinase activity without apparently altering the level of cyclic AMP.

There are two very obvious and perhaps not opposing views regarding this action of insulin. First, since the free metabolically active pool of cyclic AMP may be very small compared to the bound pool found under basal metabolic conditions, a small decrease in cyclic AMP below base line would be difficult to detect using current techniques. Second, the action of insulin to inactivate glycogen synthase kinase under basal metabolic conditions may be due to an insulin-mediated alteration in the sensitivity of the cyclic AMP-dependent kinase. This would mean that the same concentration of cyclic AMP would be less effective in the presence of insulin.

The present data with perfused liver could be interpreted to include both mechanisms. In certain physiological situations, insulin acts in direct opposition to the effects of glucagon. Here, insulin could act first by lowering glucagon-stimulated levels of cyclic AMP. March 23, 2020

There are several negative reports regarding insulin activation of hepatic synthase in vivo (12, 38). Undoubtedly, there are additional negative findings which have not been reported. This is probably due to several important experimental complications. First, Glinzmann and Mortimore (12) reported that they were unable to show any effect on glycogen synthase using glucagon plus and minus insulin. However, it appears that the concentration of glucagon that they used was about 4 × 10^-11 M. Using this higher concentration of glucagon, the present investigators were also unable to obtain the synthase effect. Therefore, the difference was probably due to the higher glucagon concentrations employed. This may be explained by the fact that inactivation of glycogen synthase by protein kinase is more sensitive to cyclic AMP than is activation of phosphorylase kinase and subsequently phosphorylase activation by protein kinase (39). This would mean that synthase would be maximally inactivated while phosphorylase activation was being initiated. Second, the effect of insulin to activate synthase from basal metabolic conditions seemed to require a short equilibration period (ap-
looked for effects of insulin on glycogen synthase activation. Insulin effects on glucose accumulation in the perfusate are not readily discernible (19). Since there are no clear-cut effects of insulin on glycogen accumulation, many investigators have not looked for effects of insulin on glycogen synthase activation during this time period. Lastly, the effect of insulin on synthase activation under basal conditions appears to be of rapid onset and of short duration, at least under conditions studied by the present investigators. With longer exposure times, this effect of insulin could have been missed. Therefore, it would appear that most of the negative data can be readily explained by one or more of these complicating factors.

Since the present study has not yet included determination of tissue synthase phosphatase activity, no mention has been made of this enzyme. This should not be taken as a suggestion that the importance of this enzyme is doubtful in insulin control of glycogenesis. Bishop (5) has shown that infusion of insulin into dogs results in an activation of hepatic synthase phosphatase between 5 and 15 min, and that glucagon promptly reduces this activity back to control levels. Evidence was also presented that synthase phosphatase exists in forms of different activity. It has also been shown that the synthase-activating enzyme (phosphatase) is almost absent in alloxan-diabetic rat liver and that administration of insulin results in restoration of the enzyme to normal levels (45). Therefore, it would appear that insulin may act to activate hepatic synthase phosphatase, and at the same time, maintain its integrity. With insulin involved in the control of glycogenesis by way of synthase phosphatase, a third possible mechanism could be included in control of hepatic glycogenesis by insulin.

In conclusion, the present data complete the hypothesis that mammalian hepatic glycogen synthase is directly controlled by insulin in vivo and in vitro. Further, it completes the experimental evidence for a general action of insulin to control glycogenesis by controlling glycogen synthase interconversion in diaphragm (16-18), skeletal muscle (19, 20), heart (20-22), fat (23), human placenta (24), tumor cells (25), and finally in liver (6-9, present paper).

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