Insulin Stimulation of Heart Glycogen Synthase D Phosphatase (Protein Phosphatase)*

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Insulin rapidly produced an increase in per cent of total heart glycogen synthase in the I form in fed rats. In fasted rats the response was diminished and delayed. In diabetic animals there was no response over the 15-min time period studied. Since synthase phosphatase activity is necessary for synthase D to I conversion, the phosphatase activity was determined in extracts from these groups of animals. In the fasted and diabetic rats phosphatase activity was less than one-half of that in fed animals. Administration of insulin to fasting animals increased synthase phosphatase activity to a level approaching that of fed animals by 15 min. In diabetic animals insulin also stimulated an increase in synthase phosphatase activity but 30 min were required for full activation. Insulin had no effect in normal fed animals.

Insulin activation of synthase phosphatase activity in heart extracts from fasted animals was still present after Sephadex G-25 chromatography and ammonium sulfate precipitation. Thus insulin had induced a stable modification of the phosphatase itself or of its substrate synthase D rendering the latter a more favorable substrate for the reaction.

In the present study we have determined the effect of insulin administration on the synthase D/I ratio and on the synthase D phosphatase activity in heart extracts from fed, fasted, and alloxan diabetic rats. These are physiological states where circulating insulin concentrations are expected to be relatively high, low, and very low, respectively. Insulin clearly stimulated phosphatase activity in fasted and diabetic rats and induced a stable enzyme protein modification either of synthase phosphatase itself or of synthase D rendering it a more favorable substrate for the reaction.

It has been known for several years that insulin administration results in a rapid increase in the per cent of synthase in the I form and stimulates glycogen synthesis in many tissues (10) including heart (11, 12). Similar results were obtained following the physiological rise in circulating insulin which occurs with glucose feeding (13).

The mechanism by which insulin stimulates a conversion of synthase D to synthase I is as yet unclear. It could result from a decrease in protein kinase activity, an increase in synthase D phosphatase activity or both.

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Part of this data has been presented previously in abstract form (14).

MATERIALS AND METHODS

Glucose-6-P, glucose-2-P, ATP, EDTA, UDP-glucose, rabbit liver glycogen, imidazole, and alloxan were purchased from Sigma Chemical Co. Glycogen was passed over a mixed bed, ion exchange resin (Amberlite MB-3, Mallinckrodt) before use. Glucose-labeled UDP-\[^{14}C\]glucose and \[^{14}C\]glucose-1-P were obtained from New England
Insulin administration to fed rats resulted in rapid increase in the per cent of heart glycogen synthase in the I form as wc (11) and others (12) have shown previously. In rats fasted 19 to 21 h the initial per cent of synthase I was lower and there was no increase after insulin administration. In diabetic animals the initial per cent of synthase I was similar to that in the normal fasting animals and changed little over the time course studied (Fig. 1). In none of these experiments was there a significant change in total synthase activity.

Since an active synthase D phosphatase is necessary for synthase D to synthase I conversion, the activity of this enzyme was studied in extracts of heart from fed, fasted, and diabetic animals. In extracts from fed rats the phosphatase reaction was essentially linear for 10 min and the per cent of synthase I increased from 13 to 46% of the total synthase activity present (Fig. 2). With prolonged incubation over 90% of the synthase D is converted to synthase I under these assay conditions. In extracts from fasted rats the phosphatase activity was only 40% of that seen in fed animals. In extracts from diabetic rats the phosphatase activity was even further reduced being only about 30% of that in the fed animals. In all of these experiments there was little change in total synthase activity.

Potassium fluoride, which is a known inhibitor of the phosphatase, completely inhibited the reaction in extracts from fed and fasted rats, and no competing protein kinase activity was present. After allowing the phosphatase reaction to go essentially to completion, addition of ATP, MgCl₂, and cAMP resulted in a rapid decrease in per cent of synthase I to initial values (data not shown). This further validated that we were measuring synthase phosphatase activity in the assay. Diabetic animals were not studied.

When insulin was administered intraperitoneally to fasted rats and the rats killed 15 min later synthase phosphatase activity clearly had been stimulated and the velocity of the phosphatase reaction approached that seen in normal fed animals (Fig. 3). In fed animals only a slight stimulation of phosphatase activity was produced. In fasted animals a small
but statistically significant stimulation of phosphatase was present as early as 5 min after insulin administration ($\Delta \% I = 2.5/\text{min after insulin}$, $1.9/\text{min for controls}$ $p < 0.05$).

In alloxan diabetic animals insulin stimulated phosphatase activity but the response was delayed. A full activation did not occur until 30 min after insulin (Fig. 4).

Since the dose of insulin used in the above studies was in the pharmacological rather than physiological range, normal fasted rats were given glucose intravenously in order to increase circulating insulin. A rapid stimulation of synthase phosphatase activity occurred (Fig. 5) suggesting an increase in circulating insulin concentration within the normal range also is effective.

In order to determine whether insulin given for a longer period of time than necessary to fully activate the phosphatase (15 min) could cause a larger increase in per cent of synthase I, fasted rats were killed 30 min after insulin was given. Glucose (2.8 mmol/kg IV) was given 15 min after insulin to prevent severe hypoglycemia. The per cent of synthase I increased from a control value of 6.6 to 10.4% 30 min after insulin ($p < 0.01$). The increment rise was similar to that in fed rats.

ATP strongly inhibits synthase phosphatase in other tissues (23-27) and has been reported to inhibit a protein phosphatase partially purified from bovine heart (28). In the phosphatase reaction mixture used in the present studies the ATP concentration was less than 40 $\mu$M and was essentially the same in all animal groups. Thus a difference in ATP concentration could not have influenced the results obtained. Also hydrolytic products of ATP are unlikely to be important.

Glucose-6-P stimulates synthase phosphatase activity (29-31) and reverses ATP inhibition of synthase phosphatase...
**Table I**

| Incubation | Glycogen | Glycogen | Glucose-6-P |
|------------|----------|----------|-------------|
| min        | mg/ml    | mg/ml    | µM          |
| Fed        | 0        | 0.8 ± 0.1| 5.8 ± 0.2   |
|            | (10)     | (9)      | (5)         |
| 10         | 0.5 ± 0.1| 4.6 ± 0.2| 90 ± 10     |
| Fasted     | 0        | 1.3 ± 0.1| 6.2 ± 0.3   |
|            | (13)     | (12)     | (5)         |
| 10         | 1.2 ± 0.2| 6.4 ± 0.5| 130 ± 18    |
| Fasted + insulin | 0    | 1.3 ± 0.2| 6.4 ± 0.5   |
|            | (12)     | (6)      | (5)         |
| 10         | 1.0 ± 0.1| 5.8 ± 0.3| 140 ± 24    |

* Tissue was homogenized in the absence of glycogen.

* Tissue was homogenized in the presence of 5 mg/ml of glycogen.

In skeletal muscle (23). The initial glucose-6-P concentration in the phosphatase assay mixtures was very low but increased considerably during the incubation. However, the concentrations were similar in extracts from fed and fasted animals (Table I). The phosphatase rate was essentially linear with time in spite of the glucose-6-P concentration change. Thus under the conditions used glucose-6-P did not detectably affect the results.

**Fig. 6.** Sephadex G-25 column chromatography (left) of extracts from fasted and insulin-treated rats. Total synthase activity was similar for both types of animal (~0.22 unit/ml) and remained stable throughout the incubation. The initial per cent of synthase I for the fasted and insulin-treated rats was 8% and 11%, respectively. n = 4 for each group. Ammonium sulfate precipitation of supernatant proteins, followed by Sephadex G-25 chromatography of the resuspended pellet (right). Total synthase activity was ~0.12 unit/ml and remained stable throughout the incubation. Initial mean per cent of synthase I was 10.9% (Fed), 5.5% (Fasted), and 4.8% (Insulin). n = 3 for each group.

**Fig. 7.** Glycogen inhibition of heart synthase D phosphatase. Hearts from fed, fasted, and insulin-treated, fasted rats were homogenized in 50 mM imidazole, pH 7.0, with varying concentrations of glycogen. Glycogen concentration in the homogenizing solution was (O) 0 mg/ml (0.5 for fed), (●) 2.5 mg/ml, (△) 5.0 mg/ml, (▲) 7.5 mg/ml, and (x) 10 mg/ml. Total synthase activity, initial per cent of synthase I and blood sugars were similar to those reported in Fig. 4. n = 7 for each line.

In order to demonstrate further that the insulin effect was due to a change in the phosphatase, or its protein substrate, synthase D, and was not due to a small molecular weight effector, Sephadex G-25 chromatography of the heart extracts was done prior to assay. Insulin activation of the phosphatase reaction was still present (Fig. 6). In other experiments proteins in the 7700 × g supernatant were precipitated using ammonium sulfate, resuspended in homogenizing solution, and passed through a Sephadex G-25 column equilibrated with the same solution. Insulin activation of the phosphatase was still demonstrable. These studies indicate that insulin has induced a stable modification of the phosphatase, synthase D its substrate, or both. If small molecular weight activators are important in the mechanism they must be tightly bound to the proteins or have induced a stable change in the protein.

Although glycogen has been reported to inhibit synthase phosphatase activity (32), it was routinely added (5 mg/ml) in our studies in order to assure stability of synthase D; this also reduced any variability in phosphatase activity due to variation in endogenous glycogen concentration. In order to investigate the mechanism of insulin activation of synthase phosphatase the assay was done using variable concentrations of glycogen in the homogenizing buffer. In extracts from fed animals glycogen had little effect on the reaction velocity (Fig. 7) but in extracts from fasted animals considerable inhibition was produced. Insulin administration to the fasted animals
from fed, fasted, and insulin-treated, fasted rats the initial synthase phosphatase activity was considered. In extracts synthase phosphatase activity (6). Therefore, the possibility substrate for a partially purified protein phosphatase having that the phosphorylase a concentration could be regulating that organ. In heart, phosphorylase a has been reported to be a tivity (33) in a noncompetitive manner' and has been impli- activity of the synthase activity.

resulted in a reduction in glycogen inhibitability. When the reaction velocity versus glycogen concentration was plotted and the data extrapolated to zero glycogen concentration, the effect of insulin essentially disappeared (Fig. 8). The assays could not be done in the absence of glycogen due to instability of the synthase activity.

In liver, phosphorylase a inhibits synthase phosphatase ac-

DISCUSSION

Insulin stimulated a rapid increase in per cent of synthase I in fed but not normal fasted or fed diabetic animals. Synthase phosphatase activity was considerably diminished in the fasted and fed diabetic animals and this could account for the reduced ability of insulin to promote a synthase D to synthase I conversion over the relatively short time periods studied.

Insulin administration to fasted or alloxan diabetic rats stimulated synthase phosphatase. Some increase was seen by 5 min but by 15 min phosphatase activity in fasted animals was stimulated to levels approaching those in fed animals. In diabetic animals (Fig. 4) the increase in activity was slower but still reached a normal fed value by 30 min after insulin was given. The insulin-induced change in synthase phosphatase activity of fasted animals was clearly due to a stable modification of the phosphatase protein or its substrate, synthase D, and was not due to a small molecular weight modifier.

Little activation of synthase phosphatase could be shown in heart extracts from fed animals after insulin administration in spite of stimulation of an increase in per cent of synthase I. This suggests the presence of an additional mechanism which further alters the synthase kinase-synthase phosphatase activity ratio in favor of the latter enzyme. The nature of this remains unknown. Tissue cAMP concentration was deter-

Fig. 8. Glycogen inhibition of heart synthase D phosphatase. The data from Fig. 8 were used to calculate phosphatase rate in the various groups of rate (0 to 7 min). Glycogen concentration is that measured in the incubation mixture during assay. n = 7 for each point.

Fig. 9. Per cent phosphorylase a activity in extracts from fed, fasted, and insulin-treated rats. Total phosphorylase activity was similar in all animals (~40 units/g wet weight) and remained stable throughout the incubation. n = 13 for each group.

The insulin-induced change in synthase phosphatase in the a form decreased rapidly and by 5 min of incubation had reached a low stable level which was similar in the three groups (Fig. 9). In spite of the dramatic decrease in phosphorylase a concentration during the incubation there was little change in the velocity of the synthase phosphatase reaction. Thus under the conditions used, phosphorylase a did not significantly influence the results.

In the latter group insulin reduced the sensitivity of the reaction to glycogen inhibition and could largely explain the increased phosphatase activity observed. Several years ago Danforth (37) reported an inverse relationship between glyco-

In liver, phosphorylase a inhibits synthase phosphatase ac-

In heart, phosphorylase a has been reported to be a substrate for a partially purified protein phosphatase having synthase phosphatase activity (6). Therefore, the possibility that the phosphorylase a concentration could be regulating synthase phosphatase activity was considered. In extracts from fed, fasted, and insulin-treated, fasted rats the initial phosphorylase a activity was similar (Fig. 9). The per cent of phosphorylase in the a form decreased rapidly and by 5 min of incubation.

1 Unpublished observations.
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no difference between the three groups. The different assay conditions and long incubation period used (1 h) make comparison with the present study difficult.

There are several reports of reduced synthase phosphatase activity in liver extracts from diabetic animals (39-41) but the response to insulin administration has been variable. In alloxan diabetic rats, Gold (39) found that 1 h was required before an insulin effect on liver synthase D to synthase I converting activity could be demonstrated. Nichols and Goldberg (40) reported a significant increase in the diminished activity within 10 min after insulin administration. In pancreatectomized dogs that had not received maintenance insulin for 2 to 4 days, an infusion of insulin with glucose for 30 to 120 min had no effect on liver synthase phosphatase activity. In animals controlled with daily insulin injections, however, an insulin and glucose infusion resulted in an increased synthase phosphatase activity within 5 to 15 min (41). In some animals glucose infusion alone increased the activity and since glucose stimulates synthase phosphatase activity directly in liver (42) the contribution of insulin to the rapid activation of synthase phosphatase in these animals is difficult to determine.

It is generally accepted that insulin does not enter cells but rather interacts with cell surface receptors (43). The intracellular mediator or mediators of insulin are uncertain. Insulin has been reported to increase cGMP concentration in certain cells (44) and it has been suggested to be an intermediate in the action of insulin. In the present study direct addition of insulin to the homogenates had no effect on synthase phosphatase activity nor did addition of cGMP to a final concentration of 1.0 mM. Thus, the mediator through which insulin activates heart synthase phosphatase remains unknown.

Since this enzyme appears to be a rather broad specificity protein phosphatase (5-7), activation of the enzyme or alterations in its substrates could produce other intracellular effects on insulin. In addition, activation by insulin of similar but distinctly different phosphoprotein phosphatases may be important both intracellularly and in the mediation of insulin-stimulated transmembrane transport.

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