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.

A difference in sensitivity of the reaction to glycogen inhibition was present between fed and fasted animals. Increasing concentrations of glycogen had only a slight inhibitory effect in extracts from fed animals but considerably reduced activity in extracts from fasted animals. Insulin administration reduced the sensitivity of the phosphatase reaction to glycogen inhibition. This could explain, at least in part, the increased phosphatase activity noted in the insulin-treated, fasted rats since glycogen was routinely added to the homogenizing buffer.

Glycogen synthase D phosphatase catalyzes the hydrolytic cleavage of phosphoryl groups from glycogen synthase D to form synthase I. Conversion of synthase I back to synthase D is catalyzed by a cAMP-dependent protein kinase which has broad substrate specificity (1-3). It also may be catalyzed by a non-cAMP-dependent protein kinase (4). Synthase D phosphatase may have a rather broad substrate specificity (5-7), but has not been as well studied as the kinase.

The I form of synthase is active in vivo but it is generally accepted that the D form has little or no activity except under special circumstances (8). Since the glycogen synthase catalyzed reaction is rate-limiting (9), regulation of the proportion (per cent) of total synthase in the I or active form will regulate the overall rate of glycogen synthesis.

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.

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.

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-[14C]glucose and [14C]glucose-1-P were obtained from New England...
The pulverized tissue was homogenized in 60% glycerol, 10 mM EDTA, nitrogen-cooled stainless steel percussion mortar. For synthase assay the same day. The frozen tissue was crushed to a fine powder in a liquid nitrogen-cooled aluminum clamps (~196°C). The atria and aorta were removed and the tissue was stored at -20°C until assay later.

Blood was collected in heparinized tubes and placed on ice. The atria and aorta were collected from the heart and the ventricles homogenized in 50 mM imidazole, 0.5% glycogen, pH 7.0 (1/5, w/v), using Potter-Elvehjem homogenizing tubes and motor-driven Tetlon pestles. The homogenate was centrifuged at 4°C for 10 min. The supernatant was treated twice with Dowex 1-X4 resin to remove anionic inhibitors of the phosphatase reaction (15). Total synthase I activities are not significantly affected by this treatment. In most studies 13.3 mM EDTA (final concentration 10 mM) also was present in the homogenizing solution in order to inhibit protein kinase activity.

Synthase phosphatase was assayed by incubating the supernatant at 30°C using endogenous substrate. The reaction was stopped by adding an aliquot of the supernatant to cold 200 mM KF, 7.5 mM EDTA, 10 mM potassium phosphate, pH 7.8, in a ratio of 1/8, v/v. The diluted sample was immediately placed on ice and used for synthase and phosphatase assays. No phosphatase activity could be demonstrated in the pellet. Eighty-five to ninety percent of the synthase was present in the supernatant and there was no difference in the per cent of enzyme in the supernatant of fed, fasted, or insulin-treated animals. In diabetic animals 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.

In some studies proteins in the 7700 x g supernatant were digested in 500 ~1 of 30% KOH. Extracts for glucose-6-P and ATP determinations were prepared by placing 100 ~1 of the 1/4 supernatant into glass tubes containing 10% KOH. ATP and glucose-6-P were determined by the fluorometric method of Lowry (20), plasma glucose by the Nelson method (21), proteins by the method of Zak and Cohen (22), and cAMP by radioimmunoassay using a commercial kit (Schwarz/Mann). In the column chromatography studies, 500 ~1 of tissue supernatant was digested in 500 ~1 of 30% KOH. Extracts for glucose-6-P and ATP determinations were prepared by placing 100 ~1 of the 1/4 supernatant on 300 ~1 of frozen 3 M perchloric acid and thawing slowly.

Glycogen synthase was assayed using the method of Thomas et al. (16). Units are micromoles of UDP-[14C]glucose incorporated into glycogen per min. Glycogen phosphorylase was assayed by the method of Gilboe et al. (14, 15). Units are micromoles of [14C]glucose-1-P incorporated into glycogen per min. Synthase I kinase was assayed as previously described (18). Glycogen was determined by a phenol/sulfuric acid method (19), and ATP and glucose-6-P by the fluorometric method of Lowry (20), plasma glucose by the Nelson method (21), proteins by the method of Zak and Cohen (22), and cAMP by radioimmunoassay using a commercial kit (Schwarz/Mann). In the column chromatography studies, 500 ~1 of tissue supernatant were applied to a Sephadex G-25 column (1 x 15 cm) which previously had been equilibrated with homogenizing buffer at 4°C. The protein fraction was eluted with the same buffer and collected visually. This sample was used for phosphatase assay.

In some studies proteins in the 7700 x g supernatant were precipitated using ammonium sulfate, 38% final concentration, at 0°C. After mixing for 10 min the precipitate was collected by centrifugation and resuspended in 1 ml of homogenizing buffer. All of the phosphatase activity was present in this fraction. This sample was collected as described above before phosphatase assay.

In the experiments the heart was quickly removed and immediately frozen using liquid nitrogen-cooled aluminum clamps (~196°C). The atria and aorta were removed and the tissue was stored at -20°C until assay later.

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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 percent 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 the phosphatase activity could be demonstrated. In fasted animals a small increase after insulin administration.

![Fig. 1. Increase in percent of synthase in the I form in rat heart after insulin administration. The number of animals studied at each time point appears in parentheses at the 15-min time point. Controls were grouped and homogenization time. * indicates a statistically significant response to insulin. Total synthase activity was similar for each group (~1 unit/g wet weight).](http://www.jbc.org/doi/fig/1.jpg)
but statistically significant stimulation of phosphatase was present as early as 5 min after insulin administration (Δ%I = 2.5/min after insulin, 1.9/min for controls p < 0.5).

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 μM 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 activity.

Fig. 2. Synthase D phosphatase activity in heart extract. The number of animals studied appears in parentheses. Total synthase activity was similar for all groups (~2 units/g wet weight) and remained stable during the incubation. Phosphatase assays are done using fresh tissue. Freezing reduces synthase activity approximately 50%. Blood sugars were 113 ± 5, 89 ± 6, and 440 ± 58 mg/dl (± S.E.) for fed, fasted, and diabetic animals, respectively.

Fig. 3. Activation of synthase D phosphatase activity by insulin. Animals were killed 16 min after insulin. * indicates the increased activity after insulin is statistically significant. The number of animals studied is indicated in parentheses. Total activity was similar for all groups (~2 units/g wet weight) and remained stable during the incubation. Blood sugars were 113 ± 5, 89 ± 6, and 50 ± 6 mg/dl for fed, fasted, and insulin-treated animals, respectively.

Fig. 4. Synthase D phosphatase activity in alloxan diabetic rats. Insulin was given 30 min before the rats were killed. The rate was statistically significantly greater (p < 0.05) in the insulin-treated animals. Total synthase activity was ~2 units/g wet weight and remained stable during the incubation. The mean initial per cent of synthase I for normal, diabetic, and insulin-treated animals was 12%, 6% and 11%, respectively. Blood sugars were 113 ± 5, 590 ± 51, and 480 ± 20 mg/dl for the normal, diabetic, and insulin-treated diabetic animals, respectively. n = 13 for controls and 6 for each diabetic group.

Fig. 5. Activation of synthase D phosphatase by glucose. Glucose was given 15 min before the rats were killed. The increase in rate was statistically significant (p < 0.05). The mean initial per cent of synthase I for fed, fasted, and glucose-treated rats was 15%, 7% and 6%, respectively. Blood sugars were 88 ± 12, 79 ± 4, and 101 ± 13 mg/dl for the fed, fasted, and glucose-treated animals, respectively. n = 6 for each group.
Insulin Effects on Heart Glycogen Synthase Phosphatase

Table I

Metabolite concentration in extracts from normal (non-diabetic) rat heart

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Glycogen in Fed</th>
<th>Glycogen in Fasted</th>
<th>Glycogen in Fasted + insulin</th>
<th>Glucose-6-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>mg/ml</td>
<td>mg/ml</td>
<td>mg/ml</td>
<td>µM</td>
</tr>
<tr>
<td>Fed</td>
<td>0 0.8 ± 0.1 (10)</td>
<td>0.5 ± 0.1 (9)</td>
<td>1.2 ± 0.2 (13)</td>
<td>10 ± 1</td>
</tr>
<tr>
<td></td>
<td>10 0.5 ± 0.1</td>
<td>4.6 ± 0.2</td>
<td>6.4 ± 0.5</td>
<td>120 ± 10</td>
</tr>
<tr>
<td>Fasted</td>
<td>0 1.3 ± 0.1 (13)</td>
<td>6.2 ± 0.3 (12)</td>
<td>1.0 ± 0.1 (12)</td>
<td>10 ± 1</td>
</tr>
<tr>
<td></td>
<td>10 1.2 ± 0.2</td>
<td>6.4 ± 0.5</td>
<td>6.2 ± 0.3</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>Fasted + insulin</td>
<td>0 1.3 ± 0.2 (6)</td>
<td>6.4 ± 0.5 (6)</td>
<td>5.8 ± 0.3 (6)</td>
<td>140 ± 24</td>
</tr>
<tr>
<td></td>
<td>10 1.0 ± 0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Tissue was homogenized in the absence of glycogen.

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

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.

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.

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 x 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, and 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 cated in the physiological regulation of the synthase system in activity (33) in a noncompetitive manner and has been impli-

tions present. This suggested that one effect of insulin was to 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-

crived in rat diaphragm muscle (36), also could play a role.

Glycogen has only a slight effect on synthase phosphatase activity in extracts from fed animals but in fasted animals produced a considerable concentration-dependent inhibition. 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-
gen concentration and the fraction of synthase in the I form in an isolated diaphragm muscle preparation. Insulin increased the fraction of synthase in the I form for all glycogen concentra-
tions present. This suggested that one effect of insulin was to desensitize the net synthase D to synthase I conversion reaction to glycogen inhibition. The present results in heart from fasted animals are in agreement with this and localize the insulin effect to the phosphatase reaction.

Das (38) has reported a higher per cent of synthase I in normal fed compared to diabetic animals. These results are similar to those in the present study. He also concluded that there was reduced synthase phosphatase activity in heart extracts from diabetic animals which was restored to normal 1 h after insulin administration; apparently shorter time inter-

vals were not studied. Calculation of the phosphatase rates in the extracts from fed normal, diabetic, and insulin-treated animals from the data presented, however, reveals essentially

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 activity (33) in a noncompetitive manner and has been implicated in the physiological regulation of the synthase system in that organ. 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 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.

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-

un published observations.
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 is known to stimulate 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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