IMPAIRMENT OF INDUCTION OF
GLYCOLYTIC ENZYMES AND
DEVELOPMENT OF INSULIN RESISTANCE IN RATS
AS A RESULT OF
CONTINUOUS INSULIN TREATMENT

By
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ABSTRACT

It has been established that the administration of insulin to rats for 10 days results in a considerable increase of RNA synthesis as well as in the activity of hexokinase and pyruvate kinase in liver cells. Electron microscopic studies have shown that on the 6th day of insulin treatment the size of hepatocytes nucleoli and the number of free ribosomes increase. Moreover on the 10th day of treatment the glycogen content is changed in the liver cells. Following long-term insulin treatment (16–20 days) the liver cells lose their capacity to respond by enhanced RNA synthesis and by the induction of hexokinase and pyruvate kinase.

It has also been demonstrated that insulin administration for 10–15 days significantly decreases blood sugar. However, continuous insulin treatment (up to 25–27 days) elevates blood sugar to values surpassing even the normal value on day 27 of treatment.

The administration of insulin to rats for 25–27 days leads to a decreased tolerance to glucose load. The content of immunoreactive insulin in the blood plasma increases considerably on day 10 of insulin injection and continues to rise more sharply on subsequent days of treatment.
A number of hormones act by inducing in target cells the DNA dependent synthesis of RNA molecules programming enzymes which provide hormonal physiological effects (Salganik 1968; Oravec & Körner 1972). It has been shown that in hepatocytes insulin induces a set of enzymes responsible for glycolysis and glycogen storage: hexokinase (ATP: D-hexose-6-phosphotransferase, EC 2.7.1.1), phosphofructokinase (ATP: D-fructose-6-phosphate-1-phosphotransferase, EC 2.7.1.11), pyruvate kinase (ATP: pyruvate-phosphotransferase, EC 2.7.1.40), glycogen synthetase (UDP glucose: glycogen-α-4-glycosil transferase. EC 2.4.1.11) (Weber et al. 1965, 1966; Ilyin 1969). In insulin deficiency the induction of these enzymes is diminished resulting in impaired utilization of glucose, hyperglycaemia and some other symptoms characteristic of diabetes (Ilyin 1971).

Recently, it has been demonstrated in this laboratory that continuous administration of inductors, i.e. of hormones or other compounds to animals leads to the failure of target cells to react by the synthesis of corresponding inductive enzymes. Thus, if rats are given cortisol daily for 20–25 days, liver cells lose their capacity to respond to the administered hormones by enhanced synthesis of RNA and glycogenic enzymes (Salganik et al. 1968). In rats fed a galactose-rich diet the induction of liver enzymes which transform galactose into glucose is impaired and symptoms of galactosaemia develop (cataract, liver lesions, growth retardation) (Salganik et al. 1971a).

It thus appeared possible that the insulin effect could exhibit a similar pattern and that continuous hormone administration might lead to the loss of the capacity of the liver cells to respond to insulin treatment by the induction of glycolytic enzymes. In turn, this might result in the impairment of glucose uptake, in hyperglycaemia (in spite of high concentration of blood insulin) and to other symptoms characteristic of insulin resistant diabetes.

The aim of the study was to investigate these ideas.

**MATERIALS AND METHODS**

The compounds used in the experiments were: protamine-zinc-insulin ("Orfas", USSR). lactate dehydrogenase (LDH), ATP, ADP, NAD-H, NADP ("Reanal", Hungary), glucose-6-phosphate dehydrogenase ("Serva", FRG), cortisol-acetate ("Richter", Hungary), 14C-adenine, specific activity 28 mCi/mg ("Isotope", USSR). The reagents for radioimmunological assays of insulin content (Insulin Radioimmunoassay KIT) containing 125I-insulin (specific activity 100 mCi/mg) and the double antibodies were purchased from CEA-CEN-SORIN (France-Belgium-Italy).

**Methods**

Male Wistar-strain rats, weighing 130–150 g, were injected daily with 2 IU protamine-zinc-insulin given subcutaneously for 1–27 days. A group of rats given insulin for 21 days subsequently received 5 mg cortisol-acetate per 100 g body weight intra-
peritoneally for 3 days. Glucose was added to the animals' ration and they were allowed water ad libitum. On different days of the period of continuous hormone treatment, 12 h after the last insulin injection or 5 h after cortisol administration, the animals were sacrificed. One hour before sacrifice the rats were injected with a dose of 30 μCi/100 g body weight 14C-adenine (specific activity 28 mCi/mg) intraperitoneally.

D-RNA and r-RNA were extracted from the nuclei of liver cells according to the procedure of Georgiev & Mantyeva (1962a). D-RNA is a fraction of nuclear RNA abundant in m-RNA (Georgiev & Mantyeva 1962b). The efficiency of measurement of radioactivity in RNA samples was 2.7%o. Radioactivity was expressed as cpm/mg of RNA. The method of Spirin (1958) was used to determine the RNA content in the samples.

In order to assay the activity of liver glycolytic enzymes the liver was removed at low temperature and washed with 0.14 M KCl. Two g of liver were homogenized in 25 ml 0.14 M KCl, using a Potter-Elvehjem homogenizer, filtered through nylon and centrifuged at 90 000 x g for 1 h.

The supernatant was used as the source of glycolytic enzymes. Protein concentration was determined according to Lowry et al. (1951). Hexokinase activity was measured using the methods of Di Pietro & Weinhouse (1960) and expressed as ΔE340/10 min/mg of supernatant protein. A somewhat modified procedure of Weber et al. (1965) was used to assess the activity of pyruvate kinase. Three ml of the reaction mixture (final volume) contained 125 μmoles Tris-HCl (pH 7.4), 18.8 μmoles MgSO₄, 4 μmoles ADP, 42 μmoles KCl, 36.8 μmoles PEP, 1.3 μmoles NADH. The reaction was started by adding 0.5 ml of supernatant and 10 μg LDH. The activity of pyruvate kinase was expressed as ΔE340 for 5 min of incubation of the mixture per 1 mg of supernatant protein at room temperature.

![Graph showing hexokinase activity](image)

**Fig. 1.**

Hexokinase activity in the liver of rats under prolonged insulin (PZI) administration (ΔE₃₄⁰ per 10 min of incubation at 22°C).
The content of blood sugar and the tolerance to glucose load were determined 12 h after the last insulin injection. Blood glucose was measured by the orthotoluindine method with the modifications introduced by Golikov (1970) and expressed as mg/100 ml. In order to test tolerance to glucose loads, glucose (250 mg per 100 g body weight in 1 ml of water) was administered through a tube inserted into the oesophagus; blood sugar was measured every hour during 4 h. Blood samples were taken from the tail vein.

The content of immunoreactive insulin in the blood was established according to the method of Morgan & Lazarow (1963) using antibodies to insulin and 125I-insulin. The determinations were performed 12 h after the last injection of the hormone. The insulin estimates were expressed in ng per 1 ml of plasma.

For electron microscopy the liver of 20 rats was used. Of these, 5 rats were used as controls and the remainder were killed 6, 10 and 16 days after insulin injection was started. 5 rats at each time. Pieces of the right liver lobe were fixed in paraformaldehyde and OsO4 according to Fahimi & Karnovsky (1966), embedded with a styrol-metacrylate mixture using the method of Stockem & Komnick (1970). Thin sections were made on a Tesla BS 490 A ultratome. They were stained with uranylacetate and lead citrate and observed with a Tesla B 3-413 electron microscope with an accelerating voltage of 80 kV.

RESULTS

Changes in RNA synthesis and activity of glycolytic enzymes of rat liver under continuous insulin treatment

Fig. 1 shows that as a result of the daily administration of insulin for 5 days the activity of liver hexokinase increases approximately by 70%. On day 10 the hexokinase activity exceeds twice its basic level. However, if animals continue to receive insulin, the hexokinase activity decreases, in spite of the treatment, and by day 20 it parallels the basic activity.

The activity of rat liver pyruvate kinase under the effect on insulin exhibits a comparable pattern: it markedly increases during the 5 days of daily insulin injections, so that on day 10 of treatment pyruvate kinase activity surpasses that of the control about 2 times. Prolonged administration of insulin (days 16 and 21) results in the loss of the capacity of liver cells to respond to the regular hormone injection, as expected, by elevated pyruvate kinase activity, which at this time does not exceed the control level (Fig. 2).

The increase in the activity of glycolytic enzymes under the effect of insulin is the result of induction mediated by DNA-dependent RNA synthesis (Weber et al. 1965). Bearing this in mind, we have investigated RNA synthesis during continuous administration of insulin. The data presented in Table 1 and Fig. 3 show that the incorporation of 14C-adenine into D-RNA and r-RNA is enhanced in the first few days of insulin treatment. at the time when the activity of glycolytic enzymes is also raised. Maximum D-RNA synthesis in the liver occurred 10 days after insulin treatment was commenced. However, after
Pyruvate kinase activity in rat liver under continuous PZI administration (ΔE_{340} per mg of protein for 5 min at 22°C).

Table 1.
Changes in D-RNA synthesis of rat liver under continuous insulin administration and subsequent cortisol treatment (cpM/mg RNA).

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Control</th>
<th>Treatment</th>
<th>Insulin</th>
<th>Insulin for 21 days + subsequent cortisol administration for 3 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Single injection</td>
<td>10 days</td>
<td>21 days</td>
</tr>
<tr>
<td>1</td>
<td>752</td>
<td>1187</td>
<td>-</td>
<td>775</td>
</tr>
<tr>
<td>2</td>
<td>1032</td>
<td>1260</td>
<td>-</td>
<td>1219</td>
</tr>
<tr>
<td>3</td>
<td>1034</td>
<td>1547</td>
<td>-</td>
<td>926</td>
</tr>
<tr>
<td>4</td>
<td>1076</td>
<td>1239</td>
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<td>-</td>
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<td>1026</td>
<td>1645</td>
<td>2447</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>3670</td>
<td>5568</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>2213</td>
<td>-</td>
<td>1001</td>
</tr>
<tr>
<td>8</td>
<td>1887</td>
<td>-</td>
<td>-</td>
<td>1242</td>
</tr>
</tbody>
</table>

Each experiment was designed in such a way that animals in all the experimental groups were sacrificed simultaneously and studied under similar conditions.
Changes in r-RNA synthesis in the liver of rats under continuous insulin administration; o—o subsequent cortisol administration for 3 days.

20 daily injections of the hormone the rate of RNA synthesis is decreased to the control values.

The results obtained indicate that under continuous insulin administration the cells of rat liver, lose the ability to react to the administration of insulin by promoting the synthesis of RNA and glycolytic enzymes.

Changes in the content of blood insulin under continuous administration of the hormone

As shown in Table 2, the insulin content in the blood of intact rats takes up on an average 0.82 ng/ml. Insulin administration for 10 days results in the increase of blood insulin by about 70 %. Subsequent treatment with insulin elicits a sharp increase in the quantity of immunoreactive plasma insulin. Thus,

<table>
<thead>
<tr>
<th>Days of insulin treatment</th>
<th>0</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Content of insulin in blood</td>
<td>0.82 ± 0.15</td>
<td>0.79 ± 0.09</td>
<td>1.03 ± 0.13</td>
<td>1.40 ± 0.20</td>
<td>&gt; 8.00</td>
</tr>
</tbody>
</table>

The number of animals in the experiment is indicated in brackets. Blood insulin was determined 12 h after the last hormone injection.
Table 3.
Changes in blood sugar in rats under continuous insulin administration.

<table>
<thead>
<tr>
<th>Days of insulin treatment</th>
<th>0</th>
<th>6</th>
<th>10</th>
<th>16</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood sugar (mg/100 ml)</td>
<td>85 ± 4.8</td>
<td>74 ± 7.6</td>
<td>62 ± 3.9</td>
<td>73.8 ± 2.1</td>
<td>94 ± 1.9</td>
</tr>
</tbody>
</table>

The number of animals in experiment is indicated in brackets. Blood sugar was determined 12 h after the last hormone injection.

on day 20 of treatment the content of plasma insulin was almost ten-fold that of the control animals (Table 2).

Impairment of glucose tolerance in rats as a result of continuous insulin administration

As a result of continuous insulin administration, the blood sugar decreased on day 10 of treatment and falls to approximately 70% of the initial level.

Fig. 4.
Changes in blood sugar content under glucose load in rats treated with insulin for 25 days. 1: intact rats; 2: rats induced with insulin for 25 days; the glucose load was given 12 h after the last insulin injection.
Fig. 5.
Impairment of glucose tolerance in rats continuously treated with insulin for 27 days. 1: intact rats; 2: rats induced with PZI for 27 days; glucose load was given 1 week after the last insulin injection.

(Table 3). However, on day 16 the blood sugar rises slightly, despite continuous insulin injection, and on day 25 its level is even somewhat greater than the initial values (Table 3).

One hour after glucose administration (200 mg/100 g body weight) to intact rats a 10 mg/100 ml elevation of blood sugar occurs. But in 3–4 h the blood sugar falls to the initial values or even lower (Fig. 4).

A different pattern is observed in animals treated with insulin for 25 days. In such animals 1 h after glucose administration the blood sugar content exceeds the initial level by 15 mg/100 ml. In 3–4 h this does not fall as in

Fig. 6.
Ultrastructure of hepatocyte of control rat. × 18 000.

Fig. 7.
Hepatocyte of rat 6 days treated with insulin. The increase in nucleolus size and the number of free ribosomes in cytoplasm is seen. ×18 000.

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the controls and still surpasses on an average the initial value by 10 mg/100 ml. This gives experimental evidence for a peculiar impairment of glucose tolerance developing in rats subjected to long-term insulin treatment. This impairment was made particularly clear in experiments in which rats were treated with insulin for 25–27 days and in which glucose tolerance was investigated one week after hormone administration was stopped. In such animals, 1 h after glucose load the blood sugar surpassed the initial level by 40 mg/100 ml and even more after 2 h; 4 h later it was still higher than the initial level by 30 mg/100 ml (Fig. 5).

Changes in hepatocyte ultrastructure under continuous insulin treatment

As a result of daily insulin injection the nucleoli size and content of free ribosomes increased in the rat hepatocytes on day 6 (Figs. 6 and 7). By day 10 the glycogen content was sharply augmented in the hepatocytes (Fig. 8). A number of vacuoles were identified in the regions of glycogen deposition. In spite of daily insulin administration the glycogen content considerably decreased in rat hepatocytes by day 16 (Fig. 9). On day 20 most of the cells were hardly distinguishable from the controls.

DISCUSSION

A number of experiments have shown that the increase in the activity of glycolytic enzymes under insulin treatment is accompanied by RNA synthesis and inhibited by actinomycin D and ethionine (Weber et al. 1966). These data and some direct evidence (Pilkis 1970) suggest that insulin induces de novo synthesis of glycolytic enzymes. Obviously, it is not the activation of pre-existing enzymes but rather the increase in their amount, is responsible for the effect of insulin on glycolysis. On the basis of the experimental results presented in this work, we conclude that as a result of prolonged insulin treatment the liver cells lose their ability to react to the administered hormone by enhanced DNA-dependent RNA synthesis and by the induction of glycolytic enzymes responsible for the utilization of glucose. In spite of the sharply increased blood content of immunoreactive insulin, the liver cells of such animals are resistant to the inductive action of very large doses of the hormone.

Fig. 8.
Hepatocyte of rat treated 10 days with insulin. Note abundant accumulation of glycogen and vacuoles in cytoplasm. × 18 000.

Fig. 9.
Hepatocyte of rat treated for 16 days with insulin Glycogen storage is evident. × 18 000.
Obviously, owing to this resistance the glucose load in such animals leads to prolonged hyperglycaemia.

In normal animals glucose load stimulates the release of insulin from the pancreatic cells into the blood stream, thereby promoting glucose utilization by the cells of the liver and muscles (Matschinsky et al. 1971). In animals deprived of the ability to react to insulin injection by the induction of glycolytic enzymes, glucose load results in a rather stable condition of hyperglycaemia (Figs. 4 and 5). The blood content of immunoreactive insulin estimated at the time of glucose load, was still very high. This obviously means, that the impairment of glucose tolerance is not concerned with the inhibition of insulin release by the pancreatic β-cells which could occur as a result of continuous insulin treatment.

Electron microscopy showed an increased size of nucleoli on day 6 after insulin treatment was started. This is, obviously, related to an enhanced r-RNA synthesis occurring at this time. On day 10 the glycogen deposition increases in the cells, presumably in consequence of induction hexokinase and glycogen synthetase.

Abolition of the effect of insulin on hepatocytes on day 16 and 20 is possibly associated with the loss of cell capacity to react to the hormone by the induction of the synthesis of RNA and the corresponding enzymes.

What are the causes of the development of the resistance of target cells to the inductive action of insulin? Studies of the mechanisms interfering with the induction of glycogenic enzymes under long-term cortisol treatment, have shown that an impaired induction is associated with changes in the properties and composition of liver chromatin associated with a rise in histones content and a fall in chromatin template activity (Argutinskaya et al. 1973).

An enhanced RNA synthesis under the effect of cortisol in vitro has been demonstrated in isolated liver cells nuclei of intact rats (Lukacs & Sekeris 1967). Nuclei isolated from the liver of animals subjected to long-term cortisol treatment fail to respond to incubation with cortisol by augmented RNA synthesis (Salganik et al. 1971b). It thus seems probable that during prolonged insulin administration changes may also occur in the properties of liver cell chromatin that prevent induction.

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