Histochemical and Cytogenetical Effects of Insulin on Mouse Liver in vivo and in vitro**

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Histochemically detectable changes in the liver of the mouse after subcutaneous injection of a single sublethal dose of insulin and the effect of insulin on mitotic rate and chromosome changes in cultured mouse liver cells have been studied.

No insulin-induced necrosis or hydropic degeneration of perportal cells was observed. The most marked changes found were a diminution of glycogen and an accumulation of sudanophilic lipid, first in the perportal cells, then throughout the lobule, followed by a rapid restoration to normal. There were no changes in the mitochondria with the sublethal dose.

Mitotic rates were increased with 0.5mg% for 10 to 20 hours treatment but no chromosome changes were observed.

These observations indicate that insulin causes disturbance of metabolic processes in the liver, which might be interpreted as signs of incipient injury, but insulin does not give any damage at the chromosomal level.

It is well known from the work of Cori(1950) and others that a number of hormones interfere at some point or other in the course of carbohydrate metabolism. The most important stimulator is generally recognised to be insulin. Insulin is well known to stimulate the rate of glucose uptake in rat diaphragm muscle and to increase the energy output (Krahl, 1956). Engel and Scott(1950) described a linear relationship between the increase of glycogen and the log dose of insulin injected in the rat pancreas. Also, Bullough (1954) reported that with glucose as substrate, insulin increase the number of mitosis in mouse epidermis. The mitotic rate is primarily determined by the supply of intra-cellular glucose which is in turn apparently a function of the degree of activity of the glucokinase reaction.

Also, that a single toxic dose of insulin produces hypoglycemia and causes severe periportal liver injury is a well known fact. When synthalin, which also reduces the amount of glucose in the blood, had been considered for use as a substitute of insulin, many investigators reported the effect of synthalin on the liver(Herbertson, 1958; Davis, 1958) and pancreas (Davis, 1952). Davis (19

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* Received November 24, 1978
** This study was supported by a Faculty Research Grant (1977) of Yonsei University College of Medicine.
58) has shown that the acute liver lesions of synthalin poisoning in the rabbit consist of periportal hydropic degeneration, loss of glycogen, and a form of necrosis often characterized by the presence of intracellular polymorphonuclear leukocytes. According to Herbertson (1958), the administration of synthalin to guinea pigs causes necrosis, hydropic swelling and fatty degeneration of the parenchymal cells in the peripheral parts of the liver lobules. Therefore, according to both Davis and Herbertson, one of the most striking features of the liver damage is the localization in the periportal zone in contrast to the centrolobular hepatotoxic agents.

The present experiments were designed to examine the histochemical effect of a single sublethal dose of insulin in mouse liver and further the effect of insulin on mitotic rate and the chromosome changes in vitro on mouse liver cells.

**MATERIALS AND METHODS**

1. **Animals and Insulin**

The mice used in these experiments were both male and female albino mice. In all of the experiments the animals were 2 to 3 months old and weighed 26 to 32 gm. Insulin (Isophane insulin, U.S.P.N.P.H., produced by E.R.Squibb and Sons, Inc., New York,) was diluted to 1 mg in 1 cc of Hanks' solution.

2. **Histochemical changes in vivo**

Insulin was administered by subcutaneous injection and always given at 9 a.m. except to some animals that were to be sacrificed 18 hours after injection. The doses tested were 0.1 mg, 0.2 mg, 0.3 mg, 0.5 mg, all per 30 gm body weight, and 4-10 male and female mice were used for each dose. The surviving mice were sacrificed 24 hours after injection. A dose of 0.3 mg/30 gm body weight was finally selected because mice that received 0.5 mg/30 gm body weight died within 24 hours, whereas mice that received 0.2 mg/30 gm body weight did not appear ill and no changes were observed in the liver. Three males and three females were sacrificed 2, 4, 6, 8, 12, 18, 24 and 48 hours after injection.

Routine histological changes were observed in livers fixed in formalin and stained with Delafield's hematoxylin and eosin. Glycogen was demonstrated by the periodic acid-Schiff technique. Natural fat was preserved by frozen sections of 6 microns, stained with oil red 0. Mitochondria were fixed and stained by the Regaud method.

3. **Cell culture**

The normal mouse livers were rinsed three times with Hanks' solution. Tissues were prepared by trypsinizing with 0.25% trypsin (GIBCO, 1:300) at room temperature for 20 minutes. Cells were seeded in small glass petri dishes (50mm) with McCoy's media supplemented with 20% fetal calf serum and antibiotics. Cells were subcultured once before starting the experiments.

4. **Mitotic index and chromosome changes**

The cells were trypsinized and seeded in petri dishes and incubated for 24 hours. Media were changed and incubation was resumed for another 16 hours. Insulin was diluted in media and added for final concentrations of 0.125mg%, 0.25mg% and 0.5mg% in 4, 10 and 20 hours. About 4 hours before harvesting the cells, colcemid (0.5ug.ml) was added. The cells were trypsinized and treated with hypotonic solution (0.075 M KCl) for minutes
and fixed with 3:1 ethanol: acetic acid, air dried and stained with Giemsa.

RESULTS

None of the mice that received either 0.1 mg or 0.2mg per 30gm body weight exhibited any ill effects and the liver cells appeared entirely normal. Among those that received 0.3mg per 30gm body weight and subsequently were sacrificed at 2, 4, 6, 8, 12, 18, 24 and 48 hours after injection, changes were observed in the livers as follows.

GLYCOGEN—In untreated control mice, all cells throughout the lobule of the liver were heavily laden with glycogen (Fig. 1). Two to 4 hours after insulin injection, the livers were still full of the glycogen throughout the lobules. Eight hours after injection, there was a sharp line of demarcation between the glycogen-poor periportal cells and the glycogen-rich central cells. At this time (5 p.m.) the glycogen content of the liver was low in control animals. Twelve hours after injection treatment, liver glycogen was very variable. Among the mice injection at 9:00 p.m. and sacrificed at 9:00 a.m. the following day, one exhibited glycogen diminution throughout the lobule and the other two, again showed very clear lines of demarcation between glycogen-poor periportal cells and the glycogen-rich central cells (Fig. 2). In 3 mice injected at 9:00 a.m. and sacrificed 9:00 p.m. the liver was entirely devoid of histochemically demonstrable glycogen, whereas in control animals, glycogen was abundant at this hour (9:00 p.m.). Eighteen hours after insulin treatment, in mice injected at 3:00 p.m. and sacrificed at 9:00 a.m. the following day, there was a complete loss of glycogen from the entire lobule (Fig. 3). Twenty-four hours after insulin injection, in mice injected and sacrificed at 9:00 a.m., one of the three mice had a moderate amount of glycogen evenly distributed throughout the liver lobule, but in the other two mice glycogen was beginning to reappear in the central cells. After 48 hours, glycogen was abundant throughout the lobules as in the control untreated, mice.

NEUTRAL FAT—In untreated control mice there was only a trace amount or none of demonstrable sudanophilic fat at 9:00 a.m. and 9:00 p.m. After 2 to 6 hours, only trace amounts were present, without lobular zonation, and the livers appeared essentially normal. After 8 hours, there were tiny to medium sized fat droplets in the periportal and midzonal cells but none in the centrolobular cells. After 12 hours, in those injected at 9:00 a.m. and sacrificed at 9:00 p.m., there was no fat accumulation in the liver, whereas in those injected at 9:00 p.m. and sacrificed at 9:00 a.m. the following day, there was a pronounced perportal fat infiltration (Fig. 4), with even more fat present than in similar mice sacrificed 8 hours after injection. After 18 hours, in those injected at 3:00 p.m. and sacrificed at 9:00 a.m., the following day, there was a large number of small to medium sized droplets throughout the lobule. After 24 hours, the droplets in the periportal cells were large (Fig. 5), and they decreased in size toward the central vein. Forty-eight hours after insulin injection, there was only a trace amount of lipid in an occasional, isolated cell. The liver was essentially normal.

MITOCHONDRIA—In the normal mouse liver, mitochondria exhibit varying forms in different parts of the lobule. There is a transition from long slender filaments in the centrolobular cells to short thick rods in the periportal cells. The liver mitochondria of all mice treated with a single sublethal dose of insulin were normal in appearance.
Table 1. Mitotic Index in % after insulin

<table>
<thead>
<tr>
<th>mg% Insulin</th>
<th>0</th>
<th>4</th>
<th>10</th>
<th>20</th>
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<tbody>
<tr>
<td>0.5</td>
<td></td>
<td>1.4</td>
<td>2.2</td>
<td>1.9</td>
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<tr>
<td>0.25</td>
<td></td>
<td>1.2</td>
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<td>0.125</td>
<td></td>
<td>1.2</td>
<td>1.4</td>
<td>1.8</td>
</tr>
<tr>
<td>Control</td>
<td>1.2</td>
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MITOTIC RATE and CHROMOSOME CHANGES—The mitotic index observed after different concentrations and different hours of insulin treatments are shown in Table 1. The control culture had a mitotic index of 1.2%. There were no significant effect effects on mitotic rate with all concentrations with 4 hours treatment. However, with all concentrations with 10 to 20 hours exposure, increases in mitotic rate were observed. Insulin does not cause any chromosome changes in vitro. The normal mouse karyotypes are shown in Figure 6.

DISCUSSION

In mice, sublethal doses of insulin produced only transient changes in glycogen and accumulation of fat droplets in the perportal cells 12 hours after insulin injection, in all cells of the lobule after 18 hours, and after 24 to 48 hours the liver had returned to its normal appearance. These changes in glycogen and lipid probably represent a disturbance of metabolic processes in the liver and might be interpreted as signs of incipient injury.

While the changes in lipids were very consistent, there was some individual variation in the extent of glycogen loss and in its lobular distribution, even in the relatively small number of mice used in these experiments. Controlled feeding of the animals might reduce the variation. Furthermore, there is a distinct difference in effect on the glycogen content when insulin is administered at different times of the day, as demonstrated when insulin was injected at 3:00 p.m. and 9:00 p.m. instead of at 9:00 a.m. These variations suggest that the response to insulin may vary with a diurnal cycle of activity in the liver, i.e., the maximum glycogen deposits are during the night, when assimilatory processes predominate, and minimum deposits are during the day, when liberation is at its height.

The study of the incipient damage to centrolobular cells by carbon tetrachloride in sulfaguanidine-protected mice, by Wilson and Leduc (1957), has shown that, in the centrolobular cells of both protected and unprotected liver, changes occur in cytoplasmic ribonucleoprotein, glycogen, and netural fat, but changes in mitochondria and in the nuclear deoxyribonucleoprotein occur only in an unprotected liver and are indications of irreversible damage. The present study shows a sublethal dose of insulin affected only the glycogen and fat of the liver, and the mitochondria and the nuclei were not damaged. Therefore the slight damage produced by insulin on the liver is even less severe than that procued by carbon tetrachloride in sulfaguanidine-protected mice.

In Davis’s (1958) experiment, he gave glucose, either by intraperitoneal injection or by an intravenous drip, to prevent death from hypoglycemia. He indicated that this procedure did not seem to affect the liver lesion. The reason the mice die in this study is presumably a large body surface/body weight ratio. Mice use a large amount of glycogen from the body in order to maintain their temperature. After synthalin (insulin substitute) treatment hypoglycemia follows, so that available glucose is inadequate. Possibly death might be prevented after the lethal dose of
synthalin by injection of glucose and by keeping the mice warm. However, Herbertson (1958) stated that most of his animals (guinea pigs) survived even without treatment of glucose after injection of a single toxic dose of synthalin. He suggested that those which did not survive apparently died from renal failure, 4 to 6 days after the injection. Preliminary study of the mice kidney indicated that there was severe injury to the kidney. These observations suggest an approach to the problem presented by the mouse which might be worthy of further study.

Insulin has been said, first by Gey and Thalheimer (1924), to affect cells directly in tissue culture, and Waymouth (1954) concluded that the any true hormonal effect in tissue cultures is uncertain. Prop and Hendrix (1965) reported that the mitotic rate increases with the dose of insulin in organ cultures of total mammary gland of the mouse. He suggested that the mechanism of action of insulin is more directly connected with the action of other hormones in serum which is used in media rather than by only influencing the glucose metabolism, as mentioned by Bern and Rivera (1960) with other hormones. The absence of changes in the chromosome structure confirms that the insulin probably effects only the cell surface in vitro as suggested by Bullough (1954) in epidermal mitosis of the adult male mouse.

REFERENCES

Corm CF: Rep 1st Inter Cong Biochem Cambridge, 1950
Davis JC: Hydopic degeneration of the cells of the pancreatic islets produced by synthalin A. J Path & Bact 64:575, 1952
Davis JC: Lesions in the rabbit liver produced by synthalin. J Path & Bact 76:97, 1958
Gey GO, Thalheimer W: Observations on the effects of insulin introduced into the medium of tissue cultures. J Am Med Assn 82:1609, 1924
Fig. 1. Liver section of untreated, control mice. All cells throughout the lobules of the liver are heavily laden with glycogen at 9:00 a.m. (PAS, ×450)

Fig. 2. Liver section of 12 hours after the insulin treatment. Very sharp lines of demarcation between the glycogen-poor periportal cells and the glycogen-rich central cells. (PAS, ×450)
Fig. 3. Liver section of 18 hours after the insulin treatment. A complete lose of glycogen from the entire lobule. (PAS, ×100)

Fig. 4. Liver section of 12 hours after the insulin treatment. A pronounced fat infiltration in periportal region. (Oil red O, ×450)
Fig. 5. Liver section of 24 hours after the insulin treatment. Still heavily laden with fat and droplets in the periportal cells are large. (Oil red O, ×450)

Fig. 6. The karyotype of normal mouse liver cells.