Hepatic Proliferative Response to Insulin in Severe Alloxan Diabetes

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Summary

Administration of insulin to severely diabetic rats leads to marked proliferation of liver cells. The activity of DNA polymerase and the incorporation of thymidine-3H into hepatic DNA in vivo are increased within 24 hr after insulin injections are started and reach peak levels between 48 and 72 hr. Total liver DNA increases approximately 70% during the period from 36 to 72 hr of insulin treatment. The increase in DNA is accompanied by cell division rather than being due to an increase in ploidy, and is comparable in magnitude to the proliferation of liver cells which follows surgical removal of between 43 and 68% of the liver. Hypophysectomy reduces but does not prevent cell proliferation in response to insulin. The relationship of this response to the regulation of hepatic biosynthetic activities by insulin is discussed.

Introduction

Severe insulin deficiency is characterized by impairment of somatic growth as well as by manifest abnormalities of carbohydrate and lipid metabolism. The administration of insulin to severely diabetic rats brings about dramatic alterations in the chemical and enzymatic composition of the liver and many other tissues, and restores a normal rate of growth. Some of the metabolic reorganization which follows insulin can be assumed, therefore, to be related to processes associated with growth rather than simply to alterations in the intermediary metabolism of carbohydrate and lipid. The growth-promoting action of insulin has been recognized previously (24) and is undoubtedly a very important aspect of insulin action, but for a number of reasons, it has received relatively little attention.

In an earlier study of changes in liver composition of alloxan diabetic rats brought about by insulin administration it was found that total liver DNA increased 70% or more in the 1st 3 days of insulin injections (31). During the same interval the weight of the liver increased approximately 2.5 times, whereas the weight of the carcass only increased by about 10%. Since the weight of the liver increased approximately 2.5 times, whereas the weight of the carcass only increased by about 10%. Since the earlier conclusions regarding changes in total DNA were based on chemical measurements alone, it was considered desirable to verify and extend these observations. In the experiments reported here we have measured the incorporation of labeled thymidine into hepatic DNA and the histologic localization of this process in sections of liver. We have also examined these livers for increased activity of an enzyme known to be associated with DNA replication, i.e., the soluble DNA polymerase (E.C. 2.7.7.7) described by Bollum and Potter (2). The results of these studies fully validate the earlier observations, and indicate that hepatic proliferation in response to insulin, except for a delay of about 24 hr, resembles in many respects the regenerative response which follows the removal of 43-68% of the liver from normal rats.

These findings raise several interesting questions regarding the mechanism which normally regulates liver size. First, why should this particular physiologic situation lead to massive proliferation of an organ which, in the diabetic organism, is already relatively enlarged with respect to body weight? Second, does insulin act directly upon the system of chemical signals which normally controls the rate of cell division in the liver? And, finally, is stimulation of growth throughout the organism, i.e., by the action of growth hormone and insulin in concert, a necessary prerequisite for this response on the part of the liver? Some of our experimental efforts to clarify these important questions are presented and discussed in this report.

Materials and Methods

Female Sprague-Dawley rats weighing approximately 120 gm were given a single i.v. injection of alloxan monohydrate (40 mg/kg) after a 24-hr fast. The diabetes was allowed to stabilize for approximately 1 month before experiments were performed, and only animals exhibiting marked glucosuria, decreased growth, and other diabetic stigmata were used. Insulin injections were given according to the following schedule: The initial injections consisted of 4 units of crystalline insulin (Novo Terapeutisk, Copenhagen) and 6 units of protamine-zinc insulin (Eli Lilly and Company) s.c. per 100 gm of body weight. Four units of protamine-zinc insulin were given at subsequent 24-hr intervals. In some experiments, thymidine-methyl-3H (specific activity 6.7 c/mmole, New England Nuclear Corporation) (0.8 μc gm of body weight) was given i.p. 2 hr before the animals were killed.

For some experiments severely diabetic rats were hypophysectomized (Hormone Assay Laboratories, Inc., Chicago) and maintained on a regular diet with daily injections of 0.05-0.1 mg of cortisone acetate. After 3 or 4 days, injections of insulin and growth hormone were started. The initial dose of insulin was 0.5 unit of crystalline insulin i.p. and 2.0 units of protamine-zinc insulin, s.c.; 1.0 unit of protamine-zinc insulin was given at 12-hr intervals thereafter. Growth hormone was injected s.c. at 12-hr intervals in a dose of 0.2 mg/rat. After 58 and 82 hr of treatment the rats were injected with thymidine-3H (0.8 μc/gm) and were killed 2 hr later. The liver was analyzed for thymidine incorporation and total DNA as described below. Liver glycogen concent-

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Hepatic Proliferative Response to Insulin

The concentration and blood glucose were measured as described previously (31).

After cervical transection an aliquot of liver was homogenized with 5 volumes of the buffered sucrose medium of Bollum and Potter (2). Suitable aliquots were taken into cold 10% TCA for measurement and counting of DNA. The whole homogenate was then centrifuged at 150,000 × g for 40 min in the Spinco preparative ultracentrifuge. Clear fluid was aspirated carefully from the middle region of the supernatant solution for assay of DNA polymerase activity.

For preparation of nuclei an aliquot of liver was homogenized in 10 volumes of 0.3 M sucrose, 0.001 M MgCl₂. The crude nuclear fraction obtained by centrifugation at 800 × g was then suspended in 2.4 M sucrose, 0.001 M MgCl₂ (35), and centrifuged at 150,000 × g for 40 min to sediment the nuclei which were suspended in 0.3 M sucrose, 0.001 M MgCl₂. Aliquots of this suspension were diluted in hematoxylin solution and the nuclei counted in a hemocytometer chamber. Aliquots of the suspension were taken into cold 10% TCA for determination of DNA specific activity.

DNA was determined colorimetrically as described previously (31), using the diphenylamine reagent of Dische (9). To determine acid-insoluble radioactivity, aliquots of whole homogenate were precipitated with 10% TCA, washed twice with 5% TCA, and 3 times with absolute ethanol. When most of the ethanol had evaporated the pellets were dissolved in 1–2 ml of 1 M hyamine in methanol, an aliquot was transferred to a plastic counting vial containing 15 ml of 0.03% dimethyl POPOP and 0.5% PPO in toluene and counted in a liquid scintillation spectrometer.

DNA polymerase activity was measured by a modification of methods of Bollum and Potter (2) and Bessman (1). The reaction mixture contained, in a volume of 0.3 ml, 33 μmol of glycine buffer (pH 8.0); 0.6 μmol of 2-mercaptoethanol; 3 μmol of MgCl₂; 100 μmol of DNA² (calf thymus, Type I, Sigma Chemical Company); 25 μmol each of dCTP, dTTP, and dGTP; 10 μmol of dATP; and 1 μmol of dATP-3H (Schwarz Bio Research, Inc.; specific activity 1.25 c/mmole). Two-tenths ml of enzyme preparation was added to each tube and the mixture was incubated for 10 min. At the end of the incubation period 0.3 ml of 5 nM DNA was added and the reaction was stopped by addition of 0.4 ml of cold 1 N perchloric acid. After 5 min in an ice bath, 2.5 ml of 0.001 M ATP solution was added to each tube and the tubes were sealed to stand 15 min longer. The pellets obtained after centrifugation for 10 min at 2500 rpm were dissolved in 0.1 ml of 0.2 n NaOH and the DNA was precipitated with 0.5 ml of cold 1 N perchloric acid, diluted with cold ATP solution, and centrifuged. This step was repeated once more. The precipitates were then washed twice with cold 70% ethanol, 0.03 M Tris-HCl (pH 7.0), drained, and allowed to dry. The pellets were suspended in 1.0 ml of 0.01 M Tris-HCl (pH 6.8), 0.001 M MgCl₂ and the tubes heated in a boiling bath 4 min and cooled. Twenty-five μg of crystalline DNase (Worthington Biochemical Corporation) were added to each tube and the tubes were incubated 45 min at 37°C with frequent stirring. After centrifugation, 0.7 ml of the supernatant fluid was transferred to a counting vial containing 15 ml of phosphor solution (4) and counted in a liquid scintillation spectrometer. The results were expressed as μmol of dATP incorporated into DNA/unit of DNA (or tissue weight)/min.

Results

In Chart 1 it can be seen that total liver DNA has increased about 70% by the 4th day of administration of insulin to severely diabetic rats. The chemical measurements do not show a rise in DNA until 36 hr after the 1st insulin injection, although a definite increase in both DNA polymerase activity and DNA specific...
activity can be measured by 24 hr (Chart 1). A possible explanation for this may be that during the 1st 24 hr, the liver doubles in size owing to the accumulation of large amounts of glycogen, water (31) and also, to a lesser extent, of RNA (30). These changes tend to markedly lower DNA concentration initially, and this along with other compositional changes may give rise to a small error in the chemical determination of DNA.

That the magnitude of the increase in DNA determined chemically is generally accurate is borne out by the marked rise in both the specific activity of the DNA after thymidine-3H injection and of the activity of soluble DNA polymerase. Both of these indices of DNA synthesis reach peak levels between 48 and 72 hr, at the time when total DNA is increasing most rapidly (Chart 1). Since DNA in liver cells does not turn over as do most other cellular macromolecular constituents (12), the specific activity after thymidine injection should provide an accurate estimate of the rate of synthesis. The specific activity of the DNA increased at least 20-fold at the peak, an amount comparable in magnitude to the increase following 43-68% hepatectomy of normal rats reported by Bucher and Swaffield (6). It should be mentioned that the magnitude of this increase depends to a large extent upon the measured level of thymidine incorporation in the control animals, where extraneous counts will weight the results considerably. The effectiveness of the washing procedures used to prepare the samples for counting, as well as the level of mitotic activity in nonparenchymal cells of the liver can affect this estimate. From the data in Table 1 ratios of DNA specific activity of insulin-treated to control diabetic rats were calculated. For the whole homogenate the ratio obtained was 17, whereas the ratio for purified nuclei was 32, attesting to the relatively high background level of radioactivity persisting in whole homogenates after the washing procedures. The estimates of DNA specific activity based on measurements in whole homogenates therefore should be regarded only as rough approximations of the rate of DNA synthesis in hepatocytes in vivo.

The activity of DNA polymerase in the supernatant fraction increased about 10-fold during the 1st 48 hr after insulin administration (Chart 1). This rise is comparable in magnitude to that which occurs after 68% hepatectomy (3). Peak levels of polymerase activity expressed per unit of DNA were reached at 48 hr, but the peak enzymatic activity calculated per unit of liver weight was not reached until about 72 hr, after which time the activity decreased rather abruptly.

The data in Table 1 show that a high percentage of the acid-insoluble radioactivity of liver homogenates after thymidine-3H injection was present in the nuclei. Acid hydrolysis rendered the radioactivity soluble, whereas alkaline hydrolysis did not, as would be expected if the label were present in the DNA of the nuclei. The yield of DNA in purified nuclei prepared with hypertonic sucrose amounted to about 50% of the total DNA of the homogenates and, as shown by the data in Table 1, these nuclei contained a proportionate amount of the total DNA radioactivity. This result would be expected if these nuclei truly represent a random sample of all the nuclei of the liver.

The average DNA content of nuclei isolated at various times after insulin is shown in Table 2. The values correspond well to a small error in the chemical determination of DNA.

In confirmation of these results, many mitotic figures were seen in histologic preparations from livers prepared after 60 hr of insulin treatment, while very few mitoses could be found in sections from untreated control animals.

A typical radioautograph of a tissue section prepared after a 2-hr thymidine-3H pulse from the liver of a diabetic rat 60 hr after insulin injection is shown in Fig. 1. For comparison, a radioautograph from a saline-injected control animal is shown in Fig. 2. Although the normal hepatic cellular architecture is distorted in the insulin-treated liver by the deposition of large amounts of glycogen and lipid, it can be clearly seen that the nuclei of many of the parenchymal cells are covered with grains (Fig. 1). Careful inspection of these preparations revealed uptake of thymidine into the nuclei of bile duct epithelial cells and littoral cells as well. Thymidine uptake was only rarely observed in the control sections (Fig. 2).

To assess the ability of the untreated diabetic liver to regenerate, the increase in DNA specific activity 28 hr after hepatectomy was compared in groups of untreated diabetic rats and normal rats. These results are presented in Table 3. Although the rise in DNA specific activity by 28 hr was somewhat lower in the diabetic group, it is clear that exogenous insulin is not required to support regeneration.

Experiments were carried out with hypophysectomized diabetic rats to evaluate the role of growth hormone. Hypophysectomized severely diabetic rats did not survive well without a small maintenance dose of cortisone acetate as was given in these experiments. The results are presented in Table 4. The hypophysectomized diabetic rats responded to insulin comparably to normal animals with respect to glycogen accumulation.

<table>
<thead>
<tr>
<th>ARTICLE NO.</th>
<th>TREATMENT (hr)</th>
<th>DNA CONCENTRATION/NUCLEUS OF LIVER CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Saline</td>
<td>22</td>
</tr>
<tr>
<td>2</td>
<td>Saline</td>
<td>42</td>
</tr>
<tr>
<td>3</td>
<td>Insulin, 60</td>
<td>460</td>
</tr>
<tr>
<td>4</td>
<td>Insulin, 60</td>
<td>640</td>
</tr>
</tbody>
</table>

* Units are picograms of DNA/nucleus. Each number represents the average of 2 determinations.

<table>
<thead>
<tr>
<th>EXPERIMENT</th>
<th>CONTROL</th>
<th>48</th>
<th>60</th>
<th>72</th>
<th>84</th>
<th>96</th>
<th>120</th>
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<td>10.5</td>
<td>10.7</td>
<td>11.1</td>
<td>11.2</td>
<td>10.3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>11.9±</td>
<td>13.0</td>
<td></td>
<td></td>
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In Table 1, the ratio of DNA (dpm/mg DNA X 10^9) in homogenates vs. isolated liver nuclei from alloxan diabetic rats injected for 60 hr with insulin.
Discussio

These observations confirm the earlier results which showed a rise in total liver DNA amounting to 70% or more during the 1st 3 days of insulin treatment of severely diabetic rats (31). Thymidine-3H incorporation into DNA and DNA polymerase activity provide much more sensitive tools for studying the time course of the response. The data shown in Fig. 1 indicate that DNA replication begins to increase sharply sometime between 24 and 36 hr after the 1st insulin injection. Rapid synthesis of DNA continues until about 72 hr after insulin, at which time the rate of incorporation declines. Radioautographs prepared from livers during the phase of rapid DNA synthesis (Fig. 1) show that the synthesis of DNA is occurring principally in parenchymal cells, although some incorporation is also seen in ductal and littoral cells.

It is well known that adult rat liver contains a large proportion of cells of higher ploidy (33) and that a shift to higher ploidy normally occurs during growth of the animal. Hypophysectomy and restriction of food intake have been shown to retard the development of polyplody in young rats (10). Two observations argue strongly against a change in ploidy as the basis for the burst of DNA synthesis after insulin in the diabetic animals: (a) the lack of an increase in DNA content per nucleus of magnitude sufficient to account for an increase in ploidy (Table 2), and (b) the presence of many mitotic figures in tissue sections prepared from livers during the phase of active DNA synthesis.

It is pertinent to enquire why insulin administration should produce proliferation of liver cells. The liver is capable of regulating its mass quite effectively in the face of severe insulin deficiency, as shown by the normal regenerative response of the diabetic liver to hypophysectomy (Table 3). The diabetic liver is, in fact, enlarged relative to body weight (55-65 mg/gm of body weight). This is not a consequence of cell hypertrophy but is due to an increase in the number of cells as indicated by an increase in the total DNA content of the organ relative to body weight. Why then should it rapidly enlarge further with insulin injections? The elegant studies of Leduc (20) on the effects of starvation on mitosis in liver provide some basis for understanding this phenomenon. She observed that refeeding starved mice, especially with diets rich in protein, resulted in a small wave of mitotic activity in the liver during the 1st few days of repletion. After a 28-day period of maintenance of young mice on a diet deficient in protein, the shift to a high protein diet resulted in a marked rise in mitotic activity with a peak falling between the 2nd and 3rd day of feeding of the high protein diet. Her results thus strongly implicate severe protein depletion as an important factor in setting the stage for this response.

It has been frequently observed that starvation per se does not lead to a decrease in liver DNA (13), although the volume of the liver cells is markedly decreased in starvation due to decreases in glycogen, protein, and RNA (20). The liver appears to play an important role in protein balance in the organism, since it normally contains a considerable amount of protein which can be readily utilized during fasting. The total protein content of the liver varies linearly with energy consumption when the diet contains an adequate supply of protein, and the increased liver protein on a high energy diet represents a major portion of the total increase in protein nitrogen retained in the organism (25). However, the diabetic liver, despite the increased supply of nutrients in the plasma, does not synthesize protein normally (18). It remains adjusted metabolically as though the organism were starving, and does not utilize or store the large quantities of nutrients which are available. If the liver is regarded merely as a passive metabolic arbiter, responding to variations in the supply of nutrients in the plasma as adjusted by intestinal absorption and utilization by peripheral tissues, then it is difficult to understand why the liver of the fed diabetic animal should not store carbohydrate and synthesize large amounts of protein and lipid. The fact that it resumes these activities only upon the administration of insulin strongly argues for the view that insulin is capable of acting directly upon the liver to influence its choice of biosynthetic alternatives, i.e., the synthesis of protein, lipid, and glycogen vs. the synthesis of glucose.

Although the metabolism of the liver does not appear to be directed solely by the flux of nutrients in the blood perfusing it,
it is probable that the liver normally can respond to momentary fluctuations in plasma glucose, amino acids, and lipids to a degree dependent upon the metabolic conditioning provided by various hormones, as exemplified by the insulin-dependent synthesis of hepatic glucokinase (27) and glycogen synthetase (29). Of course, it must also be considered that unknown endocrine substances released by peripheral tissues in response to insulin, could serve as signals for alterations in hepatic metabolism. In the absence of any evidence for such peripheral factors, it seems simpler to choose the alternative, allowing for a direct action of insulin upon the liver. According to this view, the proliferation of liver cells after insulin can be regarded as a consequence of the resumption of normal hepatic biosynthetic activities, perhaps particularly of increased biosynthesis of protein, under the influence of insulin. The large magnitude of the response may signify a "rebound" phenomenon analogous to that which occurs with hepatic glycogen and lipid synthesis (28, 32).

It seems unlikely that insulin directly influences liver cell proliferation for several reasons. First, of course, is the observation that the diabetic liver can regenerate normally in the almost complete absence of insulin. Second is the relatively long delay of 36 hr after insulin before the synthesis of DNA begins, in comparison with the shorter lag of only 16–20 hr after partial hepatectomy. Insulin obviously sets forces in motion which eventually lead to proliferation, but a more prompt response might be expected if insulin were itself the stimulus.

There is a considerable body of evidence which suggests that humoral factor(s) are important in mediating liver regeneration (6, 21), but nothing is known regarding their origin or chemical nature. Studies of the regulation of DNA synthesis in bacteria have led to the postulate that a specific initiator for DNA synthesis, which may be a protein, is required in addition to induction of the complement of enzymes necessary for the synthesis of the 4 major deoxyribonucleotides of DNA and their polymerization (14, 19). The possible existence of a similar initiator of DNA synthesis in mammalian cells has been inferred from observations of induction of host DNA synthesis during replication of viral DNA in mammalian cells infected with polyoma virus (11, 34). If such an initiator protein must be synthesized in order to permit DNA replication to take place in bacterial and mammalian cells, its importance as the ultimate regulator of all the highly coordinated biochemical activities involved in cell division is at present highly problematic. Moreover, the possibility that insulin influences DNA synthesis by inducing the formation of such an initiator in liver cells is rendered unlikely by the arguments mentioned in the preceding paragraph.

In bacteria the rates of change of cell mass or of synthesis of cell wall constituents may serve as signals for replication of DNA (14, 23). If a similar mechanism were to operate in the liver, then proliferation might be envisioned as a secondary consequence of cellular hypertrophy. Inasmuch as the volume of the diabetic liver doubles during the 1st 12 hr of insulin treatment (31), this possibility should perhaps receive some consideration, although it does not satisfactorily account for the relatively long delay before DNA synthesis begins.

It is particularly interesting that hepatic proliferation in response to insulin occurs in the absence of the pituitary. The slowing and reduction of the response in hypophysectomized diabetic rats is similar to the sluggish regenerative response to hepatectomy of hypophysectomized normal rats (5). An enhancing effect of growth hormone on DNA synthesis has been observed in regenerating normal liver (8). However, growth hormone does not stimulate hepatic DNA synthesis in normal or hypophysectomized rats (17). Our results with hypophysectomized diabetic rats rule out the possibility that an insulin-stimulated secretion of growth hormone is responsible for proliferation, and support the conclusion of Korner (17) that growth hormone is not itself a trigger for mitosis.

It has been observed by several investigators that insulin stimulates growth and mitosis of mammalian cells in tissue culture (7, 22). Moreover insulin has recently been shown to act synergistically with prolactin and hydrocortisone to stimulate a differentiated mammary tissue function in vitro (5). These rather diverse effects strongly suggest that the action of insulin may extend to such fundamental cellular mechanisms as the regulation of gene activity.

Increased RNA synthesis (30), glycogen synthesis (29), and protein synthesis (16, 26) take place in diabetic liver in the early period after insulin administration before DNA synthesis begins. The available experimental evidence, while far from conclusive at present, indicates that insulin may produce these early effects in liver by regulating the expression of specific regions of the genome (27, 29). The proliferation of hepatic cells appears to be somehow consequential to these earlier anabolic events, which are augmented by, but not dependent upon, growth hormone.

Acknowledgment

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References

9. Dische, Z. Über Einige Neue Charakteristische Farbreaktionen


FIG. 1. Radioautographs of diabetic rat liver after 60 hr of insulin injections. Thymidine-\(^{3}H\) was injected 2 hr prior to sacrifice. The liver tissue was fixed in Carnoy's solution, 3- to 4-\(\mu\) sections were mounted on slides and dipped in Kodak NTB-2 emulsion. After 10 days of exposure the slides were developed with Kodak D-19 developer, and fixed and stained with hematoxylin and eosin. \(\times\) 500.

FIG. 2. Radioautograph of untreated diabetic rat liver prepared as in Fig. 1. \(\times\) 500.
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