Regulation of Hepatic Hydrogen Synthesis During Fetal Development: Roles of Hydrocortisone, Insulin, and Insulin Receptors (glycogen synthetase/organ culture/glucocorticoids)

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ABSTRACT In fetal rat liver in utero, an increase in glycogen and the glycogen synthetic enzyme glycogen synthetase (EC 2.4.1.11) occurs between gestational days 17 and 19. We used an organ culture system for finding the stimulus for increased enzyme activity and defining the relationship of this increase to glycogen synthesis. In fetal-liver explants from an earlier period (gestational day 16), hydrocortisone causes an elevation in total glycogen synthetase activity. This effect, which can be blocked by actinomycin D, is strikingly similar in time course and magnitude to the normal increase in utero. However, in order for glycogen synthesis to proceed after hydrocortisone increases glycogen synthetase levels, insulin is required. Unlike hydrocortisone, insulin does not increase total glycogen synthetase activity; it appears to act by converting the b or phospho form of synthetase to the a or dephospho form. Insulin alone does not stimulate glycogen synthesis in explants from 16-day fetal liver, although no defect in insulin binding was demonstrable. These findings support the hypothesis that the increase in liver/glycogen synthesis during the last trimester requires glucocorticoids for the developmental induction of glycogen synthetase and insulin for activation of the enzyme.

The glycogen content of fetal rat liver increases greatly during the last few days of gestation (1). Experiments in utero have shown that adrenal corticosteroids can increase glycogen in fetal liver as early as the 15th day of gestation (2). Experiments in vitro with term (gestational day 21) fetal-liver explants in organ culture have shown that insulin, but not hydrocortisone, stimulates glycogen synthesis at the end of gestation (3, 4). We postulated that this difference in the action of hydrocortisone in utero and in organ culture may be explained on the basis of differences in gestational age. The major developmental increase in glycogen and the glycogen synthetic enzyme glycogen synthetase (EC 2.4.1.11) occurs between the 17th and 19th days of fetal life in the rat (5). It is possible that the effect of hydrocortisone may be fully expressed in term tissue. We therefore studied the action of hydrocortisone and insulin on glycogen metabolism in fetal rat liver explants from day 16 of gestation. At this stage of gestation, hydrocortisone caused an increase in total glycogen synthetase (a+b) that was strikingly similar to the normal induction of the enzyme in utero. Glycogen synthesis, however, required insulin. Unlike glucocorticoids, insulin did not increase total glycogen synthetase (a+b), but appeared to convert the b or phospho form of synthetase to the a or dephospho form. In term fetal liver, total glycogen synthetase had already reached adult levels and was not further increased by hydrocortisone, whereas insulin increased synthetase a and stimulated glycogen synthesis.

The two hormones had distinctly different effects on glycogen synthetase. Glucocorticoids stimulated the developmental induction of total glycogen synthetase (a+b), while insulin activated the enzyme. This interaction of insulin and glucocorticoids may account for the appearance of glycogen in developing rat liver.

MATERIALS AND METHODS

Pregnant Osborne–Mendel rats (date of conception known) were obtained from the NIH pathogen-free colony. The methods used for preparation of fetal-liver explants and maintaining them in organ culture have been described (3). A synthetic medium, BGJb (Fitzon–Jackson modification), was used for these experiments; the medium was supplemented with 1 mg/ml of crystalline bovine albumin (Armour Pharmaceutical, Chicago, Ill.) and was at pH 7.4 when fully equilibrated with the gas phase of 95% O2-5% CO2. Hydrocortisone acetate (Calbiochem, Los Angeles, Calif.), 10 mM in ethanol; dexamethasone, pregnenolone, and progesterone (Sigma, St. Louis, Mo.), 10 mM in ethanol; insulin (E. Lilly, Indianapolis, Ind.), 2 mg/ml in mM HCl; or actinomycin D (Merck, Sharp and Dohme, Rahway, N.J.), 2 mg/ml in BGJb medium, were added alone or in combination at appropriate dilutions to the medium in individual culture dishes. At the end of the experimental periods, explants with supporting screens were frozen in liquid nitrogen.

Glycogen synthetase a and total synthetase (a+b) were measured by the incorporation of [14C]glucose from UDP-[14C]glucose into glycogen. Tissue (approximately 2 mg) was removed from liquid nitrogen and dispersed immediately by sonication in 200 μl of a solution (pH 7.5) containing 20 mM glycyglycine, 100 mM NaF, 3 mM dithiothreitol, and 5 mM EDTA at pH 7.5. A 25-μl sample of homogenate was added to 25 μl of reaction mixture for assay of synthetase. The final concentrations of components in the 50-μl assay for synthetase a were: 2.5 mM UDPglucose (0.8 Ci/mmol), 0.5% glycogen, and 200 mM β-glycerophosphate at pH 8.0. Final concentrations of components in the 50-μl assay for synthetase a+b were 10 mM UDPglucose (0.1 Ci/mmol), 10 mM glucose-6-phosphate, 0.5% glycogen, and 100 mM Tris-HCl at pH 9.0. The reactions were incubated at 30° for 10 min. Glycogen labeled with [14C]glucose was isolated by the filter-paper method of Thomas et al. (6). In both assays accumulation of 3454
TABLE 1. Specificity of glucocorticoids for induction of glycogen synthetase in explants from gestational day 16

<table>
<thead>
<tr>
<th>Additions</th>
<th>Hr</th>
<th>Synthetase a+b (units/g of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. None</td>
<td>20</td>
<td>3.5 ± 0.5</td>
</tr>
<tr>
<td>2. Insulin (0.1 U/ml)</td>
<td>20</td>
<td>4.1 ± 0.4</td>
</tr>
<tr>
<td>3. Hydrocortisone (10 μM)</td>
<td>20</td>
<td>7.5 ± 0.5</td>
</tr>
<tr>
<td>4. Actinomycin D (20 μg/ml)</td>
<td>22</td>
<td>3.7 ± 0.4</td>
</tr>
<tr>
<td>5. Actinomycin D (20 μg/ml)</td>
<td>22</td>
<td>3.7 ± 0.4</td>
</tr>
<tr>
<td>+ Hydrocortisone (10 μM)</td>
<td>20</td>
<td>3.7 ± 0.4</td>
</tr>
</tbody>
</table>

1. None                    | 60 | 3.5 ± 0.4                           |
2. Hydrocortisone (0.1 μM) | 60 | 15.0 ± 1.3                          |
3. Dexamethasone (0.1 μM)  | 60 | 16.2 ± 1.5                          |
4. Pregnenolone (0.1 μM)   | 60 | 3.5 ± 0.06                          |
5. Progesterone (0.1 μM)   | 60 | 2.9 ± 0.02                          |

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Fig. 1. Development of glycogen synthetase a+b in utero and in vitro. In utero: Glycogen synthetase a+b was measured in fetal liver at the indicated gestational ages (●). Each point represents the mean ± SEM for three litters. In vitro: Explants from fetuses (gestational day 16) were cultured in control medium (A) or in medium containing 10 μM hydrocortisone (Δ) for the indicated times, and glycogen synthetase a+b was measured. Each point represents the mean ± SEM of explants from 4 separate culture dishes.

Since we previously showed that insulin caused a rapid and linear increase in glycogen content in term fetal liver (5), we studied the effect of insulin in 16-day explants that had first been exposed to hydrocortisone for 40 hr (Table 2). Hydrocortisone increased total synthetase (a+b) over 40 hr, but did not increase synthetase a significantly above control levels and did not increase the glycogen content. Insulin, however, increased synthetase a and increased glycogen content in the explants that had been exposed to hydrocortisone. In the explants that had not been exposed to hydrocortisone, insulin did not increase synthetase a and did not stimulate glycogen synthesis.

In term explants, total glycogen synthetase (a+b) levels remained high over 40 hr in culture and were not affected by hydrocortisone. Insulin, but not hydrocortisone, increased synthetase a and stimulated glycogen synthesis (Table 3). The glycogen content of term fetal liver is initially 700–1000

Table 2. Effect of insulin and hydrocortisone on glycogen synthetase and glycogen levels in fetal-liver explants from gestational day 16

<table>
<thead>
<tr>
<th>Hydrocortisone (10 μM)</th>
<th>Insulin (0.1 U/ml)</th>
<th>Glycogen* (mg/g of protein)</th>
<th>Synthetase a+b (units/g of protein)</th>
<th>Synthetase a+b (units/g of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>4 ± 1</td>
<td>0.10 ± 0.01</td>
<td>3.3 ± 0.6</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>4 ± 1</td>
<td>0.10 ± 0.02</td>
<td>3.0 ± 0.5</td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>5 ± 1</td>
<td>0.16 ± 0.04</td>
<td>14.9 ± 1.3</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>11 ± 1</td>
<td>0.48 ± 0.06</td>
<td>11.7 ± 1.3</td>
</tr>
</tbody>
</table>

Explant from 16-day fetuses were incubated for 40 hr in BGJ medium (55 mM glucose) with and without hydrocortisone (10 μM). Explants were then exposed to insulin (0.1 U/ml): *glycogen was measured 5 hr after exposure to insulin; b glycogen synthetase a and glycogen synthetase a+b were measured 2 hr after exposure to insulin. Each entry is the mean ± SEM of explants from separate culture dishes: *b = 5; n = 4.

Biologically active [3H]insulin was prepared as described (8). Explants were cultured in BGJ medium for 18 hr and then transferred to a Tris-buffered Ringer's solution (9) with 2% albumin at pH 7.5, containing 1 μg/ml of [3H]insulin plus various concentrations of unlabeled insulin or other additions. At the end of the experiment, explants were rinsed in the buffer (4°) and the [3H]bound was measured.

RESULTS

In utero, the induction of glycogen synthetase occurs between gestational days 17 and 19. When explants from day 16 of gestation were placed in organ culture, the developmental induction of total glycogen synthetase did not occur spontaneously. The addition of hydrocortisone (0.01–10 μM) to the medium caused an increase in total glycogen synthetase (a+b); the time course and magnitude of the increase were strikingly similar to the normal induction in utero (Fig. 1). The synthetic glucocorticoid, dexamethasone, produced a similar increase in enzyme level whereas pregnenolone or progesterone did not have this effect. The action of hydrocortisone was inhibited by actinomycin D (20 μg/ml) (Table 1).

Although hydrocortisone stimulated the developmental induction of total glycogen synthetase (a+b) in vitro, the attendant net synthesis of glycogen did not occur. For this, the addition of insulin was required. The glycogen content was not increased in 16-day explants exposed to either hydrocortisone or insulin for 40 hr; however, glycogen content was increased 4-fold in explants exposed to the combination of both hormones for 40 hr (from 4 ± 1 to 16 ± 2 mg/g of protein).
studies did not seem to account for the failure of insulin to act on 16-day explants.

**DISCUSSION**

The in vitro studies with explants in organ culture suggest that both glucocorticoids and insulin have important roles in the development of glycogen synthesis in fetal liver. Hydrocortisone appears to induce total glycogen synthetase (a+b) in vitro; this action is inhibited by actinomycin D, suggesting that DNA-dependent RNA synthesis is involved. However, the actual net synthesis of glycogen requires insulin. Unlike glucocorticoids, insulin does not change total glycogen synthetase (a+b), but appears to act by converting synthetase b to synthetase a.

The action of hydrocortisone on explants from gestational day 16 can be related quite convincingly to the development of glycogen synthetase in utero. The time course and magnitude of the increase in vitro is strikingly similar to the induction in utero. The fetal adrenal cortex begins to secrete glucocorticoids on gestational day 16 or 17 and could therefore provide the natural stimulus for the development of the enzyme (5). Furthermore, Jacquot and Kretchmer (5) showed that fetal hypophysectomy by decapitation early in gestation prevented the normal developmental induction of glycogen synthetase in fetal liver. Hence the induction of glycogen synthetase in utero correlates with the onset of fetal glucocorticoid secretion, and glucocorticoid deprivation inhibits the induction.

With respect to a possible role for insulin in utero, it should be noted that the fetal-rat pancreas contains immunoreactive insulin by day 14 of gestation (13) and that insulin concentrations at term are quite high (50-200 μU/ml) (14). Thus, plasma insulin concentrations are high at an appropriate time in gestation.

Insulin stimulates glycogen synthesis in term fetal liver, but not in liver from an earlier gestational period (15-16 days). This action correlates with the ability of insulin to increase synthetase a in the absence of any change in total synthetase content. Insulin therefore appears to act by converting the β or phospho-form of synthetase to the α or dephospho form. The regulation of interconversion of enzyme forms by phos-

**Table 3. Effect of insulin and hydrocortisone on glycogen synthetase and glycogen levels in utero (gestational day 16) explants.**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Glycogen (mg/g of protein)</th>
<th>Synthetase a (units/g of protein)</th>
<th>Synthetase a + b (units/g of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>8 ± 1</td>
<td>0.34 ± 0.09</td>
<td>29 ± 3</td>
</tr>
<tr>
<td></td>
<td>Hydrocortisone</td>
<td>7 ± 1</td>
<td>0.29 ± 0.03</td>
</tr>
<tr>
<td>B</td>
<td>0</td>
<td>8 ± 1</td>
<td>0.37 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>Insulin</td>
<td>46 ± 2</td>
<td>1.26 ± 0.10</td>
</tr>
</tbody>
</table>

(4) Explants from term fetuses were incubated in B16G5 medium for 40 hr with and without hydrocortisone (10 μM). Glycogen was then extracted and measured (n = 5), and glycogen synthetase a and a + b were measured (n = 4); values are mean ± SEM.

(8) Explants from term fetuses were incubated in B16G5 medium for 40 hr and then exposed to insulin (0.1 U/ml). Synthetase a and synthetase a + b were measured 2 hr after exposure to insulin (n = 4). Glycogen was extracted and measured 5 hr after the addition of insulin (n = 5); values are mean ± SEM.
phorylation and dephosphorylation is obviously complex. Since insulin receptors are present early in gestation, the failure of insulin to act at this stage may be due to lack of development of some critical component of the interconversion process. Since glucocorticoids affect the ability of 16-day explants to respond to insulin, glucocorticoids may also be involved in the development of the interconversion system.

Another consideration, which adds to the complexity of the system, is that the action of insulin on glycogen synthesis in term tissue is partially inhibited by cycloheximide (3). Thus, the action of insulin may involve protein synthesis, although de novo synthesis of glycogen synthetase does not seem to be involved.

In conclusion, the organ culture system has provided insight into the roles that hydrocortisone and insulin have in the developmental appearance of glycogen in fetal liver.

**NOTE ADDED IN PROOF**

Plas et al. have recently shown that hydrocortisone increases glycogen synthetase \(a+b\) in primary cultures of fetal-rat hepatocytes [Plas, C., Chapeville, F., and Jacquot, R. (1973) *Develop. Biol.* 32, 82–91]. Hydrocortisone also stimulates glycogen synthesis in their system. The culture medium used by Plas et al. contains 10% fetal-calf serum. Insulin contained in the fetal-calf serum could account for the stimulation of glycogen synthesis in their system.

We thank Dr. R. Kahn for communicating data on insulin binding before publication.