Plasma Luteinizing Hormone Levels in Normal and Prenatally Stressed Male and Female Rat Fetuses and Their Mothers

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ABSTRACT

Concentrations of luteinizing hormone (LH) were measured in plasma of fetal and neonatal rats obtained from control mothers and from mothers exposed to stress from Days 14 to 21 of gestation. The regimen of stress used is known to be associated with an abnormal ontogenetic pattern of testosterone secretion from the fetal testes. The overall ontogenetic pattern of immunoreactive LH levels in plasma was similar in male and female rats, and was unaffected by stress. In all groups, LH was low from Days 16 to 20 of gestation, and then rose progressively through birth, i.e. Day 23. However, stress in the mother significantly decreased the already low levels of LH between Days 16 and 20, as indicated by a larger percentage of samples from stressed fetuses of both sexes with LH levels below the limit of sensitivity of the assay. Sex differences in both the control and stressed group became evident only after Day 20 of gestation, with plasma concentrations of females exceeding those of males from Day 21 to 23 post-conception.

INTRODUCTION

The extent to which the sex behavioral potential of male rodents is masculinized and feminized depends on the amount of testosterone available to the developing nervous system during critical stages of perinatal ontogeny. In rats, stress experienced during fetal development increases the males' potential for female sexual behavior, and impairs their ability to display male patterns (see review Ward, 1984). We have shown that male fetuses from mothers stressed during pregnancy have higher than normal titers of plasma testosterone and testicular A4-A3-3B-ol steroid dehydrogenase (3B-HSD) activity on Day 17 of gestation, but lack the surge in both testicular androgenogenic enzyme activity and blood testosterone levels found in normal males on Days 18 and 19 (Orth and Weisz, 1980; Ward and Weisz, 1980, 1984; Weisz and Ward, 1980; Orth et al., 1983). These findings suggest that the incomplete masculinization and feminization of sexual behavioral patterns of prenatally stressed males result from changes in the ontogenetic pattern of androgenogenic activity of the fetal testes which, in turn, lead to an abnormal pattern of plasma testosterone.

Testosterone secretion of Leydig cells in rats is thought to come under the control of pituitary luteinizing hormone (LH) during the last week of gestation. Therefore, the present study was undertaken to assess whether the changes in plasma testosterone levels and testicular androgenogenic enzyme activity that characterize stressed rat fetuses are associated with altered levels of plasma LH. In addition, we measured LH levels in control male and female rats since there are discrepancies in the literature regarding the ontogenetic pattern of plasma LH in rat fetuses. Males have been reported to have levels of LH
higher than females on Days 16–20 of gestation (Chowdhury and Steinberger, 1976), equivalent to females on Days 17.5–19.5 (Chapman et al., 1982; Salisbury et al., 1982), or lower than females on Days 19–21 (Slob et al., 1980) or 20.5–21.5 (Habert and Piccon, 1982). In the present study, plasma samples were taken from pregnant and female fetuses of stressed and control mothers killed on Days 16–22 of pregnancy and from their newborn pups on Day 23 post-conception (pc).

MATERIALS AND METHODS

Animals

The litters of 121 nulliparous female rats (Harlan Sprague-Dawley, Madison, WI), mated between 70 and 90 days of age, were used. All animals were housed in a temperature controlled vivarium (23°C), maintained on a reversed day-night cycle (lights off, 0800–2000 h). Purina rat Chow (Ralston-Purina, St. Louis, MO) and water were available ad libitum.

Procedure

Estrous females were mated by being placed with males in the middle of the dark period (1100–1300 h) until two ejaculations were observed. The afternoon of mating was considered Day 0 of gestation; Half of the animals were assigned randomly to the control group and half to the stress group. The animals were stressed by being placed into 13 x 5 x 8 cm Plexiglas holders (A. H. Thomas, Philadelphia, PA), illuminated by two 150-W floodlights (2150 lm/m²) for 45 min at 0900, 1300, and 1700 h (Ward, 1972). This treatment was given daily beginning on Day 14 of gestation and, unless a group was killed at an earlier stage, continued through Day 21 of gestation. Control animals were not handled until they were killed.

Mothers from each treatment group were killed on Days 16 through 22 of gestation, between 1300 and 1700 h. The stressed mothers were killed at 45 min after the second stress session of the day. The pregnant animals were decapitated, after being stunned by a blow to the head, and the trunk blood was collected in a test tube that was centrifuged for 5 min. The serum was flash-frozen in a bath of methanol and dry ice. Plasma was collected from fetuses and neonates as described previously (Ward and Weise, 1984). The sex of each fetus was determined by visualizing the gonads. The plasma from animals of the same sex, age, and treatment was pooled to a volume of 0.3 ml per sample. On Days 22 and 23, the blood from a single pup provided a 0.3 ml sample. On Days 16 and 17, in most cases, the plasma from all littermates of the same sex was needed to complete a sample. Plasma from fetuses of different litters was not pooled. The samples were flash-frozen and stored at −70°C until the time of assay.

Plasma samples were obtained also from groups of neonatal animals. Only pups born on Day 23 pc were used. They were killed within 12 h of birth. To minimize stress to the neonates, the mother was anesthetized by an injection of Chloropent (Fort Dodge Laboratories, Inc., Ft. Dodge, IA) and kept with the litter. The pups were removed individually from the nest and killed. Plasma samples were collected as for the fetuses. Neonatal samples were not collected.

The titer of LH were measured using radioimmunoassay with kits supplied by the Rat Pituitary Distribution Program, NIAMDD. The microassay method of Coquelin and Bronson (1980) was used with slight modification. For assay of fetal and neonatal samples, LH (NIAMDK LH-1-5) was iodinated to approximately 100 µCi/lug using chloramine T (Greenwood et al., 1963). The following reagents were added to 10 x 75 mm borosilicate test tubes in two consecutive overnight incubations at room temperature: (1) 50 µl of LH (NIAMDK RP-1) in 1% bovine serum albumin (BSA)-phosphate-buffered saline (PBS) buffer, or 20 µl of plasma in 30 µl of buffer, plus 50 µl of NIADD anti-rat LH 5.4 at 1:15,000 dilution in 0.1 M ethylene diamine tetraacetic acid (EDTA)-PBS without normal rabbit serum; and (2) 50 µl [125I]-LH (NIAMDK LH-1-5) in 1% BSA-PBS at 20,000 dpm. On the third day, 100 µl of trichloroacetic acid (TCA) and rabbit immunoglobulin (BioRad Laboratories, Richmond, CA) were added and the tubes were incubated for 6 h at room temperature. After the addition of 2 ml 0.1 M PBS and centrifugation, the radioactivity in each tube was counted in a gamma counter (Beckman Instruments). The LH concentrations were expressed as nanogram equivalents of NIAMDK LH RP-1.

The assay procedure for the maternal samples was identical to that used for the fetal and neonatal samples with the exception of the reagents used. In the assays of the maternal samples, NIADD rLH-I-6 was used as the iodination standard, NIADD anti-rat LH-S-7 (1-7000) as the first antibody, and NIADD rLH-RP-2 as the reference standard. The values
obtained for the maternal samples were mathematically corrected so that they could be expressed in terms of NIAIDK LHRP-1. The intrasay coefficient of variation for the assays of the maternal and fetal samples was 2.9 and 3.3%, respectively. LH was measured in all of the fetal and neonatal samples in a single assay, as were those from the mothers. The sensitivity of the assay was 0.16 ng LH-RP/1/tube (8 ng/ml).

RESULTS

The number of fetal/neonatal samples assayed for each of the 32 sex-age-treatment combinations and the number of litters contributing samples to each group are shown in Table 1. Of the 67 samples from 16-day-old fetuses, 50% were assayed in duplicate, as were all but 27 of the 205 samples from the remaining groups. Only measurements for which the values for duplicate samples differed by less than 20% were included in the data analyses. For purposes of statistical evaluation, fetal samples containing levels of LH below the level of detectability of the assay were assigned a value of 8 ng/ml. The ratio of samples containing detectable levels of LH to total number of samples assayed are summarized in Table 1.

Because LH titers in many of the samples from the Day 16–20 groups fell below the level of detectability of the assay (see Table 1), these data were analyzed using nonparametric statistics, i.e. Kruskal-Wallis

![Image]

**TABLE 1.** The ratio of number of samples from male and female fetuses of control and stressed litters that contained detectable amounts of luteinizing hormone (8 ng/ml or above) to the total number of samples in each day, sex, and treatment group. Samples were obtained on Days 16–22 of gestation and on the day of birth (Day 23).*

<table>
<thead>
<tr>
<th>Control</th>
<th>Stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Day</td>
<td>Ratio</td>
</tr>
<tr>
<td>16</td>
<td>2/18</td>
</tr>
<tr>
<td>17</td>
<td>1/14</td>
</tr>
<tr>
<td>18</td>
<td>4/14</td>
</tr>
<tr>
<td>19</td>
<td>6/15</td>
</tr>
<tr>
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</tr>
<tr>
<td>21</td>
<td>2/23</td>
</tr>
<tr>
<td>22</td>
<td>3/21</td>
</tr>
<tr>
<td>23</td>
<td>1/17</td>
</tr>
</tbody>
</table>

The number of litters contributing samples to each group were: Day 14, control = 20, stress = 18; Day 17, control = 9, stress = 20; Day 18, control = 4, stress = 4; Day 19, control = 6, stress = 6; Day 20, control = 4, stress = 4; Day 21, control = 2, stress = 5; Day 22, control = 8, stress = 8; Day 23, control = 3, stress = 3.

**FIG. 1.** The percentage of plasma samples containing levels of luteinizing hormone (LH) above the level of sensitivity of the assay (8 ng/ml) in groups of male and female rat fetuses derived from control mothers or mothers exposed daily beginning on Day 14 of pregnancy and continuing until killed.
Circulating levels of LH in control and stressed mothers are summarized in Table 2. A two-way analysis of variance revealed no treatment effect, but the changes over days were significant (p<0.001). Maternal LH rose between Days 16 and 19 of gestation (t-tests, p<0.01), and again between Days 20 and 22 (p<0.02). However, since the focus of the study was on the effects of stress on fetal rats, the number of maternal samples measured on each day of gestation was small.

**DISCUSSION**

Testosterone secretion by fetal testes, like that of adult testes, has been shown to depend on LH (Picon and Kruszka, 1976; Sanyal and Villee, 1977; Feldman and Bloch, 1978; Picon and Gangnerau, 1980). Several lines of evidence indicate that in the rat the dependence of the testes on pituitary LH begins around Day 18 of gestation (Naessany et al., 1981; Habert and Picon, 1982; Salisbury et al., 1982). Consequently, it is reasonable to propose that circulating LH is one of the determinants of the surge of testosterone secreted by the normal testes on Days 18 and 19 pc and that the abnormal pattern of steroidogenic activity in fetal Leydig cells of stressed females (Orth et al., 1983) could be due to alterations in circulating LH levels.

As shown in the present study, while stressing the mother did not induce major changes in the developmental pattern of circulating LH in her fetuses, it did affect the absolute level of LH at certain stages of development. Specifically, maternal stress was associated with a significant reduction of plasma LH titers in both male and female fetuses before Day 20 of gestation. This effect was seen at a time when LH levels in control fetuses were generally low, or at below the limit of detectability of the assay, and before there were any sex differences in LH.

<p>| TABLE 2. Mean ± SEM luteinizing hormone titers (ng/ml serum) in pregnant rats on Days 16-22 of gestation. |
|---|---|---|</p>
<table>
<thead>
<tr>
<th>Day</th>
<th>N</th>
<th>Control</th>
<th>Stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>17</td>
<td>40.9 ± 3.13</td>
<td>14</td>
</tr>
<tr>
<td>17</td>
<td>7</td>
<td>41.1 ± 3.99</td>
<td>4</td>
</tr>
<tr>
<td>18</td>
<td>6</td>
<td>37.7 ± 3.93</td>
<td>7</td>
</tr>
<tr>
<td>19</td>
<td>5</td>
<td>61.8 ± 13.04</td>
<td>2</td>
</tr>
<tr>
<td>20</td>
<td>7</td>
<td>45.1 ± 6.62</td>
<td>2</td>
</tr>
<tr>
<td>21</td>
<td>4</td>
<td>63.0 ± 14.60</td>
<td>4</td>
</tr>
<tr>
<td>22</td>
<td>5</td>
<td>38.5 ± 9.72</td>
<td>2</td>
</tr>
</tbody>
</table>

[p<0.0005], Day 17 [p<0.005], Day 18 [p<0.03], Day 19 [p<0.0007], Day 20 [p<0.03].

The data from days 21, 22, and 23 were submitted to a 3-way analysis of variance (sex X days X treatment). Sex, days, and the interaction between these were significant (p<0.001). As Figure 2 illustrates, on each of the 3 days, females had higher LH levels than the corresponding male group (t-test, p<0.002). LH increased in every group between Days 21 and 22 (p<0.002). In addition, in stressed males, there was a decrease between the last day of gestation and birth, i.e. Days 22 and 23 (p<0.003).
It is unfortunate that it is exactly during this period, before Day 20 of gestation, that the sensitivity of currently available techniques for measuring LH, whether by bioassay (e.g., Picon and Habert, 1981; Habert and Picon, 1982) or immunoassay, precludes assigning precise values to plasma LH levels. Nevertheless, it is clear that a dramatic surge in testosterone secretion occurs in unstressed fetuses (Weisz and Ward, 1980) when the level of this gonadotropin is low. However, a number of factors have been identified that could act in concert during this phase of Leydig cell development to ensure increased testosterone production in the presence of low levels of LH in the circulation. These include increases in the responsiveness of the fetal testes to LH (Warren et al., 1975; Picon and Ktorza, 1976; Sanyal and Viller, 1977; Feldman and Bloch, 1978; Picon and Gangneau, 1980), in the number of Leydig cells (Roosen-Runge and Anderson, 1959), in LH receptors (Warren et al., 1984), and in the activity of steroidogenic enzymes (Orth and Weisz, 1980; Orth et al., 1983).

The period before Day 20 of pregnancy, when stressing the mother alters plasma LH levels in her fetuses, coincides with the time when this treatment has a profound effect on the ontogenetic pattern of plasma testosterone titers (Ward and Weisz, 1980, 1984) and of steroidogenic enzyme activity in the testes (Orth et al., 1983) of her male offspring. The mechanism by which stressing the mother further decreases the already low levels of LH found in rat fetuses between Days 16 and 20 and the role of this change in the altered secretory activity of fetal Leydig cells remains to be determined. In adults, stress can decrease the levels of circulating LH (Gray et al., 1978; Tachè et al., 1978; Collu et al., 1979) and testosterone (Repčeková and Mikulaj, 1977; Gray et al., 1978). Opioid peptides, which are released during stress (Guillemín et al., 1977), could be involved in mediating this effect. These peptides can reduce blood levels of both testosterone and LH (Bruni et al., 1977; Cicero et al., 1979), and may lower the responsiveness of the testes to LH (Charpentet et al., 1982). The hypothesis that opioid peptides are involved is the effect of stress on testicular function in rat fetuses and, therefore, in the loss of the prenatal testosterone surge is supported by several observations made in our laboratories. Administration of the opioid receptor blocker naloxone to stressed mothers restored to normal the levels of 3β-HSD in Leydig cells of their male fetuses on Day 19 of gestation (Ward et al., 1983) and prevented the increase in lordosis behavior typically seen in their male offspring in adulthood (Ward et al., 1986).

The importance of the timing of the prenatal surge of testosterone on Days 18–19 pc for normal differentiation of behavioral potentials in male rats is evident from our previous work (Ward and Weisz, 1980, 1984; Ward, 1984). To determine whether the decrease in concentrations of LH between Days 16 and 20 pc plays a role in suppressing the surge of testosterone in the stressed fetuses will require a detailed examination of other factors that may be involved in the timing of this surge. These include the development of LH receptors and the responsiveness of the fetal Leydig cells to LH.

After Day 20, plasma LH concentrations rose in both sexes, and differences between the sexes became evident. Paradoxically, the increase in LH after Day 20 of gestation occurs at a time when plasma testosterone levels (Weisz and Ward, 1980) and the activity of steroidogenic enzymes in Leydig cells (Orth and Weisz, 1980) are actually falling in control males. The basis for the decline in Leydig cell function remains to be defined. It is unlikely to be due to downregulation of LH receptors, since the responsiveness of fetal Leydig cells to LH, unlike those of adults, increases rather than decreases following exposure to gonadotropins (Warren et al., 1982). Similarly, the loss in number of fetal Leydig cells after Day 19 of gestation (Roosen-Runge and Anderson, 1959) cannot account for the decrease in testosterone biosynthesis, since the activity of steroidogenic enzymes has been shown to decrease within individual Leydig cells (Orth and Weisz, 1980). One contributing factor could be the marked decline in circulating levels of progesterone that occurs in fetal rats during the last days of gestation (Weisz and Ward, 1980), since addition of progesterone to fetal testes cultured in vitro results in a marked increase in testosterone synthesis (Sanyal and Viller, 1977).

Before Day 20, there appears to be an absence of negative feedback to testosterone since plasma LH titers are not different in males and females, despite large sex differences in plasma testosterone levels on Days 18 and 19 of gestation. The marked sex difference in circulating LH titers after Day 20 pc may reflect maturation of the negative feedback system to testosterone secreted by the gonads of the males. This conclusion is supported by several lines of evidence (Naesens et al., 1981; Picon and Habert, 1981;
Habert and Picon, 1982; Naessutty and Picon, 1982; Salisbury et al., 1982).

It has been shown that sexual differentiation in the rat is influenced not only by the prenatal surge in testosterone but also by a second surge of testosterone that occurs during the first few hours after birth (Corbier et al., 1978; Corbier, 1983). This rise in plasma testosterone levels, unlike the prenatal one, is accompanied by a concomitant rise in plasma LH (Corbier et al., 1978). Whether exposure has an effect on this postnatal rise in testosterone and LH remains to be determined. In this context it is interesting to note that plasma LH titers decrease between the last day of gestation and the day of birth in stressed males, but remain constant in control males.

The ontogenetic pattern of circulating LH levels in fetal rats obtained in the present study is in general agreement with that reported by others (Slob et al., 1980; Habert and Picon, 1982; Salisbury et al., 1987). The sole exception to this are the findings of Chowdhury and Steinberger (1976). These investigators described high levels of LH in male fetuses on Day 16 of gestation, followed by a rapid decline over the next 3 days. No LH was detectable in the plasma of females until the day of birth, when titers in females were higher than in males. The basis for this marked discrepancy in findings in this one study remains obscure.

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