Ontogeny of the Response of the Hypothalamo-Pituitary-Adrenal Axis to Maternal Immobilization Stress in Rats

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Abstract. In this study we investigated the response of the rat fetal hypothalamo-pituitary-adrenal (HPA) axis to an acute maternal stress in late gestation.

On day 20 of gestation, pregnant rats were exposed to forced immobilization stress for up to 60 min. In mothers, a significant increase in plasma ACTH and corticosterone (B) was observed at 20 and 60 min. The ACTH content in the maternal pituitary decreased significantly at 60 min. Fetal blood pH was decreased by the maternal stress, showing a hypoxic condition of the fetus. Fetal plasma ACTH increased transiently at 20 min. Fetal plasma B increased at 20 and 60 min. ACTH in the fetal pituitary and the placenta did not show marked changes due to the maternal stress.

Pregnant rats on day 18–21 of gestation were subjected to a 20 min maternal stress. In the basal condition without stress, fetal plasma ACTH and B showed parallel ontogenic patterns, having a peak value on day 19 of gestation. Fetal plasma ACTH as well as plasma B were increased significantly by the maternal stress at all points evaluated. These results indicate that fetal hypoxia is important in stress transmission to the fetal HPA axis in this type of maternal stress, and the fetal HPA axis responds to the stress as early as day 18 of gestation.

(IN RATS, chronic exposure to stress in the last gestational week induces permanent impairment of reproductive functions [1–3] and of response to stress in the hypothalamo-pituitary-adrenal (HPA) axis [4] of the offspring in later life. These permanent effects of prenatal stress are considered to be derived from alterations in fetal neuroendocrine activity during the critical period of functional brain differentiation. It is postulated that maternal adrenal function is one of the most important factors affecting the fetal endocrine system under maternal stress [5–6] and it is well known that hyperproduction of maternal corticosteroids suppresses fetal HPA activity [7–9].

Meanwhile recent reports [10–11] demonstrate that fetal neurotransmitters, hypothalamic hormones and systemic hormones respond to acute maternal stress on day 20 or 21 of gestation as they do in adults under stress. However, little information is available to indicate whether or not the fetal HPA axis is concomitantly activated by this type of maternal stress. Our recent study demonstrates an acute change in fetal hypothalamic corticotropin releasing hormone (CRH) as well as in fetal plasma ACTH in response to maternal stress [12].

The following study was carried out to investigate further the acute response of the fetal HPA axis to maternal stress on day 20 of gestation, and to examine the ontogeny of stress response in fetuses at day 18–21 of gestation.

Materials and methods

Animals

Pregnant rats of the Wistar-Imamichi strain were used in this study. The animals were kept individually in cages under a constant lighting schedule (light: 0800 h-2200 h) with free access to food and water. All experiments were performed...
in the morning (sacrificing between 0900 h-1200 h) considering the diurnal variation in HPA activity.

**Experiment 1**

On day 20 of gestation, the rats were decapitated without stress or after exposure to 20 and 60 minutes' forced immobilization. Two fetuses were rapidly dissected out and their trunk blood was collected immediately in a heparinized glass capillary. The blood sample was applied to the IL1304 Blood Gas System (Instrumentation Laboratory Co., USA) and blood pH was measured. The measurement was done within 2 min of sacrificing the mother animal.

**Experiment 2**

On day 20 of gestation, the rats were subjected to an acute forced immobilization stress. They were decapitated before, 20 and 60 min after starting the stress (5 mothers in each group). The trunk blood samples from mothers and fetuses of each sex were collected in the plastic tubes containing EDTA, and centrifuged immediately to obtain the plasma. The blood from 3-4 fetuses of the same sex was pooled. The maternal and fetal pituitaries, the placentae and plasma samples were placed on dry ice immediately, and stored at -80°C until assayed. Tissue ACTH was extracted from the tissue homogenate in 0.1N HCl after 10 minutes’ incubation at 96°C. The acidic supernatants were subjected to assay immediately after neutralization with 1N NaOH. Plasma levels of ACTH and corticosterone and pituitary and placental ACTH were measured by specific radioimmunoassays.

**Experiment 3**

Pregnant rats on day 18 to 21 of gestation were subjected to a single 20 min immobilization stress. Stressed rats and untreated controls were decapitated and plasma samples were collected from mothers and fetuses in the manner described above. The number of mothers used for this experiment was 12-16 in each group. Plasma levels of ACTH and corticosterone were measured by radioimmunoassays. Because of the limited volume of fetal plasma, corticosterone levels were determined in randomly selected samples.

**RIA of ACTH**

A double-antibody RIA kit was purchased from the Diagnostic Products Co., USA. The sensitivity of this assay was around 0.8 pg/tube. Intra- and interassay variations in this system were 4.7 and 7.2%, respectively. The values were expressed as pg human ACTH (1-39), which was approximately equal to μ IU medical research council (MRC) reference preparation of human ACTH 74/555.

**RIA of corticosterone**

The antibody raised against corticosterone-3-CMO-BSA (diluted 1:2000) was used for this assay. [1, 2-3H(N)]-corticosterone (2042.4 GBq/mmol) was obtained from the NEN Research Products Co. and standard corticosterone was purchased from the Sigma Co. The sensitivity of the assay was about 0.04 ng/tube, and the coefficients of variation for intra- and interassay were 5.2 and 5.6%, respectively.

**Statistical analysis**

All data were expressed as the mean ± SEM. One- and two-way analysis of variance (ANOVA) were used for statistical analysis. Duncan’s multiple range test was performed to compare the paired data, and a P level lower than 0.05 was considered significant, unless otherwise stated.

**Results**

**Fetal blood pH under stress on day 20 of gestation (Table 1)**

Fetal peripheral blood pH was evidently decreased after 20 and 60 min of the maternal immobilization stress. Rather severe hypoxemia was observed in the early stage of stress exposure.

<table>
<thead>
<tr>
<th>stress (min)</th>
<th>pH</th>
<th>significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.205±0.017 (6)</td>
<td>p&lt;0.01a</td>
</tr>
<tr>
<td>20</td>
<td>7.078±0.031 (5)</td>
<td>p&lt;0.01a</td>
</tr>
<tr>
<td>60</td>
<td>7.106±0.040 (4)</td>
<td>p&lt;0.05a</td>
</tr>
</tbody>
</table>

+: mean±SE (n).
a: As compared to the values at 0 min, by Duncan’s multiple range test.
ACTH and corticosterone under stress on day 20 of gestation

The dilution curves of maternal and fetal plasma, as well as of the tissue extract from the pituitary and placenta were parallel to the standard curve (Fig. 1).

In mothers subjected to the acute forced immobilization (Fig. 2), the maximal response of the HPA axis was found, being accompanied with a sustained increase in plasma ACTH and corticosterone, and a gradual decrease in ACTH contents in the pituitary. Also in the fetuses of both sexes, there was a transient but significant increase in plasma ACTH at 20 min (Fig. 2A). However, no statistically significant change was observed in the ACTH level of the fetal pituitary and placenta (Fig. 2B). Fetal plasma corticosterone increased from 20 min and remained high up to 60 min in both sexes (Fig. 2C).

Stress response in mothers in late pregnancy

A marked and constant response of plasma ACTH was observed all through the days evaluated (Fig. 3A). Basal plasma corticosterone (Fig. 3B) showed a significant increase as pregnancy progressed. The increase in plasma corticosterone due to stress was significant on all days of gestation examined.

Stress response in fetuses in late gestation

The male and female fetuses in the control group showed a characteristic ontogenic pattern of plasma ACTH and corticosterone (Figs. 4, 5). The maximal level was observed on day 19 of gestation, which was significantly higher than the values on all other days, except male fetuses on day 18. The ACTH levels of the control fetuses were significantly higher than those of mothers throughout gestation, and the corticosterone levels of the fetuses were significantly higher than those of mothers except on day 21 of gestation. A good correlation was found between plasma ACTH and
Under the maternal stress, fetal plasma ACTH and corticosterone increased significantly on all days examined. These two parameters also correlated well.

In both basal and stressed conditions, no apparent sex difference was observed in fetal plasma levels of ACTH and corticosterone.

**General analysis by ANOVA (Table 2)**

The results showed that the maternal stress clearly activated the HPA axis not only in mothers but also in fetuses. Ontogenic change in the HPA activity was evident in fetuses. No interaction of the 2 factors was detected in ACTH, but it was significant in corticosterone.

**Discussion**

Immunohistochemical studies in rat fetuses demonstrate that hypothalamic neurons containing CRH are detected in the paraventricular nucleus and in the median eminence on day 17.5–18 of gestation [13–14], and that ACTH stained cells
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Fig. 5. Plasma levels of ACTH (A) and corticosterone (B) in female fetuses under maternal forced immobilization for 20 min. Blank columns represent the control groups without stress, and dotted columns represent the stress groups. Number of samples for A and B ranged from 10-21 and 3-7, respectively. *, **, ***: As compared to the date-matched controls; p<0.05, p<0.01 and p<0.001 by one-way ANOVA. A (control): p<0.05: day 19 vs day 18, 20; day 21; days 21 vs days 18, 19 and 20. A (stress): p<0.05: day 19 vs days 18 and 21. B (control): p<0.05: all pairs. B (stress): p<0.05: day 21 vs days 18 and 20; day 19 vs days 18, 20 and 21.

Table 2. Influence of stress and gestational age on plasma levels of ACTH and corticosterone, evaluated by ANOVA

<table>
<thead>
<tr>
<th>parameter</th>
<th>factor</th>
<th>mother</th>
<th>male fetus</th>
<th>female fetus</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>d.f. F value</td>
<td>d.f. F value</td>
<td>d.f. F value</td>
</tr>
<tr>
<td>ACTH</td>
<td>main effects</td>
<td>4,34 271.0 p&lt;0.001</td>
<td>4,116 49.4 p&lt;0.001</td>
<td>4,114 63.5 p&lt;0.001</td>
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<tr>
<td></td>
<td>stress</td>
<td>1,34 1070.7 p&lt;0.001</td>
<td>1,116 47.1 p&lt;0.001</td>
<td>1,114 45.0 p&lt;0.001</td>
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<tr>
<td></td>
<td>date of gestation</td>
<td>3,34 0.6 ns</td>
<td>3,116 47.9 p&lt;0.001</td>
<td>3,114 62.9 p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>2-way interaction</td>
<td>3,34 0.2 ns</td>
<td>3,116 1.0 ns</td>
<td>3,114 1.4 ns</td>
</tr>
<tr>
<td>corticosterone</td>
<td>main effects</td>
<td>4,31 303.0 p&lt;0.001</td>
<td>4,29 90.6 p&lt;0.001</td>
<td>4,23 73.1 p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>stress</td>
<td>1,31 1077.8 p&lt;0.001</td>
<td>1,29 155.9 p&lt;0.001</td>
<td>1,23 121.0 p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>date of gestation</td>
<td>3,31 56.0 p&lt;0.001</td>
<td>3,29 72.3 p&lt;0.001</td>
<td>3,23 53.8 p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>2-way interaction</td>
<td>3,31 5.3 p&lt;0.01</td>
<td>3,29 11.7 p&lt;0.001</td>
<td>3,23 3.8 p&lt;0.05</td>
</tr>
</tbody>
</table>

It is also established that the negative feedback mechanism of corticosterone in the fetus begins to operate in the last week of gestation [8, 19, 23] and that maternal corticosterone is transferred in part through the placenta [24-25]. In regard to the latter finding a question arises: Does the fetal HPA axis respond to the maternal stress despite increased maternal corticosterone? The present data on day 20 of gestation clearly demonstrated a transient increase in ACTH in fetal plasma, suggesting an active response of the fetal pituitary under the maternal immobilization stress. This observation confirmed our previous report [12], and the results by Cohen et al. [26] that ether inhalation causes an increase in plasma ACTH in the fetus on day 20. Since no transplacental passage of ACTH takes place [27-28], the source of ACTH in fetal circulation is considered to be the fetal pituitary. Though corticotropin-like activity is found in the rat placenta [29], the content is too small to contribute to the increase in fetal plasma ACTH under the maternal stress.

The mechanism of stress-transmission from the mother to the fetus is still unclear. Morishima et al. reported that the maternal psychological stress by
illumination and restraint reduces uterine blood flow [30], and decreases fetal pO₂ [31] in the rhesus monkey. The decrease in fetal blood pH under the maternal stress is in the same line as those findings. The temporal change in pH correlates with the variation in fetal hypothalamic CRH, and it shows an inverse relationship to plasma ACTH [12]. Hence the insufficient oxygen supply to the fetus could be a direct trigger to the fetal CNS to activate the fetal HPA axis.

The ontogenic pattern of fetal plasma corticosterone and ACTH in the present study was generally in good agreement with previous findings [32-33]. We observed that fetal plasma ACTH and corticosterone varied in parallel with each other and the highest values were found on day 19 of gestation. Chatelain et al. [34] reported that maximal fetal plasma ACTH was found on day 18 of gestation, and Boudouresque et al. [33] on day 19. Our results were similar to those of the latter group. The discrepancy in absolute ACTH values seems to be due to the different measurement systems used in these studies.

The ontogenic development of a stress response before birth was our main subject of interest. The results of early studies are controversial: no considerable increase in fetal plasma corticosterone was detected 20 min after the ether-laparotomy stress [35], while a significant increase was found 20 min after laparotomy and histamine injection on day 21 of gestation [9]. Recent reports indicate, however, an evident increase in fetal plasma corticosterone and/or ACTH 20 min after ether inhalation [26] or maternal immobilization [32, 11-12]. Our present results demonstrated that there was a significant response of the fetal pituitary-adrenal axis as early as day 18 of gestation, namely, in the very early developmental stage of the HPA axis. In view of this fact, it is conceivable that the HPA axis starts its function in the prenatal period, as already mentioned.

It is of interest that the stress response of plasma corticosterone in mothers and fetuses was dependent on gestational age, as confirmed in the present study by the significant 2-way interactions in the analysis of variance. Meanwhile the response of ACTH seemed to be independent of gestational days. The sensitivity of the adrenal gland to ACTH might be altered in the course of pregnancy. The increase in fetal corticosterone due to maternal stress was very limited on day 21, probably followed by the well-known stress non-responsive period after birth. The maternal corticosterone increase in late pregnancy could be a factor in suppressing fetal adrenal function, while transfer of corticosterone through the placenta was reported to decrease significantly on the last day of pregnancy [25]. Further studies are needed to elucidate the regulation of fetal adrenal function.

In conclusion, the present data indicate that the maternal stress due to acute immobilization causes hypoxia in the fetus, and the stress-induced response of the fetal HPA axis occurs from as early as day 18 of gestation. Our results support the hypothesis that fetal HPA activity is influenced by maternal stress in late gestation, which would be important in developing to the so-called “prenatal stress syndrome” in later life.

Acknowledgements

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References

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