Effects of maternal immobilization stress on birth weight and glucose homeostasis in the offspring

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KEYWORDS
Prenatal stress; Corticosterone; Glucose tolerance; Birth weight; Fructose feeding; Triglycerides

Abstract
Recent epidemiological studies have shown strong associations between low birth weight and the incidence of diabetes in the adult offspring. It has been hypothesized that exposure to maternal glucocorticoids programs cellular changes in the fetus which increases the susceptibility of the offspring to diabetes. Stressors produces large increases in maternal glucocorticoids. The present study determined the effects of immobilization stress during weeks one, two or three of pregnancy on offspring birth weight, glucose homeostasis, and the ability of the offspring to cope with metabolic stress. Immobilization stress produced large increases in maternal levels of ACTH and corticosterone, but did not affect birth weight of the pups. Chronic administration of high fructose diet, a metabolic stressor, to 60 days old control and prenatally stressed offspring produced large increases in plasma levels of triglyceride and insulin. However, there were no differences between the groups either in peak levels, or in the rates of increase and decrease (upon discontinuation of the diet) of plasma triglyceride and insulin concentrations. Basal levels of glucose and insulin, and areas under the glucose and insulin plasma concentration–time curves after an i.p. glucose dose were similar between 120 days old control and prenatally stressed offspring. These results suggest that in young adult rats prenatal immobilization stress did not affect glucose homeostasis or the ability of these rats to cope with chronic metabolic stress.

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1. Introduction
Non-insulin dependant diabetes (type 2) is a chronic disease that exerts an enormous economic and social cost in modern society (Seidell, 2000). It accounts for 90% of all cases of diabetes in the United States (Skyler and Oddo, 2002). Research examining the mechanism(s) of the etiology of diabetes has focused on genetic issues and factors like diet, lack of exercise, and obesity. Recent epidemiological studies have shown strong associations between low birth weight and diabetes in adulthood (Eriksson et al., 2003; Barker, 2003; Ozanne et al., 2004; Hales and Ozanne, 2003). The associations are independent of factors like smoking, alcohol consumption, obesity and social class,
although these factors certainly add to the effects of early life (Phipps et al., 1993). The authors of the studies have made the provocative suggestion that the intra-uterine environment is a major determinant of diabetes. They have hypothesized that increased exposure of the fetus to glucocorticoids programs long term cellular changes, which produces low birth weight and predisposes the organism to diabetes in adult life.

A variety of synthetic glucocorticoids are administered to pregnant women in preterm labor to promote lung maturation in the fetus and reduce mortality in preterm infants. Some of these clinical studies have demonstrated that in utero exposure to glucocorticoids reduces birth weight in infants (Gur et al., 2004; Murphy et al., 2002; Bloom et al., 2001). Interestingly, recent longitudinal studies have demonstrated that prenatal exposure to glucocorticoids is also associated with elevated blood pressure and increased behavioral disturbances in adolescence (Doyle et al., 2000; Trautman et al., 1995). However, to the best of our knowledge, clinical studies have not systematically examined the effects of prenatal glucocorticoid exposure on glucose and insulin homeostasis in adolescence or adulthood. In contrast, a number of animals studies have shown that prenatal exposure to glucocorticoids decreases birth weight (Reininisch et al., 1978; Ain et al., 2005; Jobe et al., 1998), and is associated with insulin resistance, hyperglycemia (Lindsay et al., 1996; Nyirenda et al., 1998), and hypertension (Benediktsson et al., 1993; Gatford et al., 2000) in the adult offspring. These changes occur long after the last exposure to glucocorticoids, and the mechanisms mediating these effects are not clearly understood. Unfortunately, most of these studies have used synthetic glucocorticoids like dexamethasone, betamethasone, and prednisone. The tissue distribution, receptor affinities, metabolism routes, and pharmacokinetics of these steroids are markedly different from the physiologically relevant glucocorticoid (Reul et al., 1987; Diederich et al., 1997). Consequently, their administration results in non-physiological exposure of the fetus to glucocorticoids. Also, many of these studies have used techniques, and doses that produce glucocorticoid levels that are much higher than those encountered physiologically.

One of the largest physiological increases in glucocorticoids is produced by stressors. In humans, low birth weight has been associated with elevated placental levels of corticotropin releasing hormone (a secretagogue of cortisol) and higher fetal cord blood cortisol levels suggesting a role for endogenous glucocorticoids in producing fetal growth retardation (Goland et al., 1993; Wadhwa et al., 2004). However, to the best of our knowledge, clinical studies have not examined the association between maternal stress and glucose and insulin homeostasis in the offspring. Numerous animals studies have examined the effects of maternal stress on different development end points in the offspring. A variety of prenatal stress paradigms decrease birth weight of rodents and non-human primates (Pollard, 1984; Cabrera et al., 1999; Drago et al., 1999; Schneider et al., 1999; Lesage et al., 2004). However, and much to our surprise, there is limited information on the role of maternal stress and resultant glucocorticoid production in the etiology of altered glucose metabolism and diabetes in the offspring. We are interested in determining, in rats, if fetal exposure to glucocorticoids via prenatal stress will produce irreversible alterations in glucose metabolism in the exposed animals. The primary hypothesis guiding these studies is that prenatal stress will increase the cumulative exposure of the fetus to maternal corticosterone (B), the primary glucocorticoid in rats. This exposure will ‘program’ irreversible cellular changes in the fetus, and lead to reduced birth weight, and long term alterations in glucose metabolism in the adult offspring.

The specific aim of these studies was to determine the effects of immobilization stress administered during the first, second, and third week of pregnancy on birth weight and glucose homeostasis in the offspring. Glucose homeostasis was evaluated by measuring basal fasting plasma glucose, and insulin levels and by the conduct of i.p. glucose tolerance tests in the adult offspring. Also, we evaluated the ability of prenatally stressed rats to cope with chronic metabolic stress administered during adulthood. Adult rats prenatally stressed during weeks one, two, or three of gestation were exposed to the chronic metabolic stress of a high fructose diet. In normal rats, a high fructose diet causes hyperglycemia, hyperinsulinemia, and hypertriglyceridemia, and has been used as a model for Syndrome X (Reed et al., 1994; Tobey et al., 1982; Bhanot et al., 1994). In control and prenatally stressed rats, we measured plasma triglyceride, insulin, and glucose levels before, during, and after discontinuation of a 3 week regimen of high fructose diet.

2. Methods

2.1. Materials

D- (+) glucose was purchased from Sigma Co. (St. Louis, MO), and bovine aprotinin was obtained.
from Bayer (Kankakee, IL). The fructose diet containing 60% fructose, 21% protein, and 5.2% fat was purchased from Harlan Teklad (Madison, WI). The diet derived 66% of its calories from carbohydrate (fructose), 20% from protein, and 13% from fat.

2.2. Animal experiments

All animal experiments were approved by the University of the Sciences in Philadelphia Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications no. 80–23, revised 1978).

2.3. Experiments with pregnant rats

All animals were allowed to acclimatize to the vivarium for 2 weeks prior to the start of the experiments. Virgin female Sprague–Dawley rats (225–250 g, Charles-River, MA) were mated by housing one male rat with two female rats. Day one of pregnancy was assigned upon observation of sperm in the daily morning vaginal smears. Rats were then singly housed throughout the pregnancy. Pregnant rats were divided into four groups of 5–7 rats per group. Group 1 was subject to immobilization stress on days 1–7 of pregnancy while groups 2 and 3 received immobilization stress on days 8–14 and 15–21 of pregnancy, respectively. Group 4 consisted of control rats that were left undisturbed throughout their pregnancy. Stress was administered in a room adjacent to the room in which the rats were housed. Immobilization stress was administered in three, 45 min sessions in the morning, afternoon, and evening. Stress was administered by enclosing rats in flexible plastic cones (Braintree Scientific Inc., Braintree, MA). The wide end of the cone was securely taped such that it conformed to the individual body size of the animals. 0.25 mL tail tip blood samples were collected from mothers in groups one, two, or three before (basal) and immediately after the 45 min morning stress session (stress) on the first and last day of the seven day immobilization stress regimen. Basal samples taken before the administration of stressor were obtained within two minutes of moving the animal from its home cage. Blood samples were collected in cold EDTA and aprotinin lined polypropylene tubes, centrifuged at 4 °C, and plasma harvested and stored at −70 °C pending analyses for corticosterone and ACTH.

2.4. Experiments with offspring

Upon birth offspring from litters in all four groups were weighed. Offspring were weaned on day 21 post-birth and housed in isosexual groups according to prenatal treatment. Only male offspring from each litter were used in all subsequent experiments.

2.5. Fructose feeding experiment

On day 60, 1–2 male offspring were randomly chosen from each litter in each of the four groups. A 0.3 mL tail tip blood sample was taken from all animals (basal). Rats were then transferred onto a fructose diet for the next 21 days. Tail tip blood samples (0.3 mL) were taken from rats in all groups on day 7, 14, and day 21 of the fructose diet treatment To assess the rate of recovery from the effects of chronic ingestion of fructose, the fructose diet was discontinued on day 21, and rats in all groups were transferred to a diet of regular lab chow. Three days later, a final 0.3 mL tail tip blood sample was obtained from all rats (day 24). On the days of blood sampling, food was withdrawn at 8:00 am and blood collected at 1:00 pm after a 5 h fast. Blood was collected in cold EDTA and aprotinin lined polypropylene tubes, centrifuged at 4 °C, plasma collected, and stored at −70 °C pending analyses of triglyceride, insulin, and glucose levels.

To determine if alterations in biochemical parameters were due only to the fructose diet, and to account for possible (but unlikely) changes in biochemical parameters due to the passage of time (24 days), male offspring from litters in each of the four groups (4-6 rats per group) were housed alongside the fructose fed animals, and fed a lab chow diet for 24 days. Tail tip blood samples (0.3 mL) were obtained from these animals, processed and stored as described above, and used for the measurement of triglyceride, insulin, and glucose levels.

2.6. Glucose tolerance test

On day 120, two randomly chosen male offspring from each litter in all four groups were administered an i.p. glucose tolerance test. Animals were fasted overnight, and the following morning glucose tolerance tests were started and completed between 8:30 AM–11:30 AM. Animals were administered a 2 gm/kg i.p. glucose dose, and 0.3 mL tail tip blood samples were taken in EDTA and aprotinin lined polypropylene tubes just before the dose and at 15, 30, and 60 min after the dose. Samples were
centrifuged and plasma harvested and stored at −70 °C pending analyses for glucose and insulin.

2.7. Biochemical methods

Plasma corticosterone concentrations were determined using a RIA kit supplied by ICN Biomedicals, Inc. (Costa Mesa, CA). The inter-day coefficient of variation of the assay was 7.1%. ACTH plasma concentrations were determined using a RIA kit supplied by DiaSorin, Inc (Stillwater, MN). The inter-day coefficient of variation of the assay was 9.4% at a concentration of 20 pg/mL and 7.2% at a concentration of 500 pg/mL.

Plasma triglyceride concentrations were measured using the GPO–Trinder reagents kit supplied by Sigma Diagnostics (St. Louis, MO). The inter-day coefficient of variation of the assay was 3.5%.

Plasma insulin concentrations were measured by RIA using a kit supplied by LINCO Research (St. Charles, Missouri). The kit uses an antibody made specifically against rat insulin. The inter-day coefficient of variation of the assay was 4.1%

2.8. Data analyses

All data are expressed as mean ± SEM. In order to compute mean birth weight of a group, all pups in a litter from the group were weighed and the average pup weight of the litter computed. This was repeated for all litters in the group, and the mean of these values is reported as the mean birth weight of the group. Similarly, whenever two rats from a litter were used, the values of biochemical parameters obtained from the two rats were averaged and reported as a single point rather than treat each offspring as a separate sample. Consequently, in experiments with the offspring the sample size in each of the four groups never exceeded the number of treated dams in each group. Such a conservative method of data analyses is recommended for the analyses of offspring data from multiparous species as it minimizes litter effects (Holson and Pearce, 1992; Zorilla, 1997).

In the fructose experiment, biochemical parameters were repeatedly measured from the same set of rats over the 24 day course of the experiment. Therefore, plasma triglyceride, insulin, and glucose data were analyzed with a repeated measure 2-way ANOVA followed, where appropriate, by a Newman-Keuls post-hoc multiple comparison test.

In the glucose tolerance test experiment, the total area under the plasma concentration–time curves for glucose and insulin were determined using the linear trapezoidal rule. The basal areas were then subtracted, and the net area under the plasma concentration–time curves reported. Basal glucose and insulin levels (prior to the i.p. glucose injection), and net area under the plasma concentration–time curve data between groups were compared with a one way ANOVA followed, where appropriate, by a Newman-Keuls post-hoc multiple comparisons test.

All statistical tests were conducted at a 0.05 level of significance.

3. Results

As shown in Table 1, immobilization stress during weeks 1, 2, or 3 of pregnancy had no effect on the gestation length, litter size, or birth weight of the

<table>
<thead>
<tr>
<th>Parameter (units)</th>
<th>Treatment</th>
<th>Control</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestation length (days)</td>
<td></td>
<td>22.6 ± 0.2 b</td>
<td>22.3 ± 0.2</td>
<td>22.2 ± 0.2</td>
<td>22.3 ± 0.2</td>
</tr>
<tr>
<td>Percent maternal weight gain (%)</td>
<td></td>
<td>64.5 ± 2</td>
<td>56.2 ± 2*</td>
<td>60.6 ± 3</td>
<td>54.8 ± 2*</td>
</tr>
<tr>
<td>Litter size</td>
<td></td>
<td>14.1 ± 1</td>
<td>13.7 ± 0.6</td>
<td>14.8 ± 0.7</td>
<td>14.0 ± 1</td>
</tr>
<tr>
<td>Birth weight (gms)d</td>
<td></td>
<td>6.4 ± 0.2</td>
<td>6.3 ± 0.2</td>
<td>5.9 ± 0.2</td>
<td>6.0 ± 0.2</td>
</tr>
</tbody>
</table>

a Immobilization stress administered during weeks 1, 2, or 3 of pregnancy or animals left undisturbed throughout the pregnancy (control).

b Mean ± SEM n = 6–7.

c Percent weight gain relative to the weight on day 1 of pregnancy. Day 1 weight in all groups ranged from 231–278 g.

d All pups in a litter were weighed and the average pup weight of a litter computed and reported as a single point. This was done for all litters in a group and the mean of these points is reported as the group mean in the table.

* Significantly different from control, p < 0.05, one way ANOVA with Newman-Keuls post hoc test.
pups. However, stress during weeks 1 and 3 of pregnancy reduced the magnitude of pregnancy induced increase in maternal body weight.

Table 2 shows that immobilization stress administered during weeks 1, 2, or 3 of pregnancy produced large increases in the plasma concentrations of ACTH. In all three groups, the increase in stress induced plasma ACTH levels were observed both on the first and last day of the stress regimen. This suggest that there was no acclimatization to the chronic (7 days) stress regimen. Basal ACTH levels were increased over the course of the 7 days immobilization stress regimen probably reflecting a chronic stress induced increase in basal hypothalamus—pituitary activity.

Plasma B levels showed a pattern of changes similar to that of its secretagogue, ACTH. Stress produced marked increases in plasma B levels both on the first and last day of the chronic stress regimen (Table 3). However, in contrast to the ACTH data, there were no increases in basal B levels over the course of the seven day immobilization stress regimen, except in animals stressed during week three of pregnancy. In this group, basal B levels on day seven were significantly higher than the corresponding B levels on day one.

Table 4 shows the body weight profile of control male offspring, and male offspring prenatally stressed during weeks one, two, or three of gestation. No statistically significant changes were observed in the body weight of control male offspring or male offspring prenatally stressed during weeks one, two, or three of gestation. However, stress during weeks 1 and 3 of pregnancy reduced the magnitude of pregnancy induced increase in maternal body weight.

### Table 2: Basal and immobilization stress induced ACTH levels in pregnant rats

<table>
<thead>
<tr>
<th>Pregnancy week during which stress was administered</th>
<th>Plasma ACTH concentration (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td></td>
<td>Basal</td>
</tr>
<tr>
<td>Week 1</td>
<td>46 ± 4a</td>
</tr>
<tr>
<td>Week 2</td>
<td>45 ± 5</td>
</tr>
<tr>
<td>Week 3</td>
<td>43 ± 4</td>
</tr>
</tbody>
</table>

++ *p* < 0.01, +++ *p* < 0.001 significantly different from basal concentration on the same day; 1-way ANOVA, Newman-Keuls post-hoc test. ** *p* < 0.01, *** *p* < 0.001 significantly different from day 1 basal concentration; 1-way ANOVA, Newman-Keuls post-hoc test. Pregnant rats were subject to 7 days of an immobilization stress regimen during weeks 1, 2, or 3 of pregnancy. Plasma ACTH levels were measured before (basal) and immediately after (stress) the morning stress session on day 1 and 7 of the stress regimen.

### Table 3: Basal and immobilization stress induced corticosterone levels in pregnant rats

<table>
<thead>
<tr>
<th>Pregnancy week during which stress was administered</th>
<th>Plasma corticosterone concentration (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td></td>
<td>Basal</td>
</tr>
<tr>
<td>Week 1</td>
<td>44 ± 15a</td>
</tr>
<tr>
<td>Week 2</td>
<td>31 ± 12</td>
</tr>
<tr>
<td>Week 3</td>
<td>17 ± 2</td>
</tr>
</tbody>
</table>

+++ *p* < 0.001, significantly different from basal concentration on the same day; 1-way ANOVA, Newman-Keuls post-hoc test. ** *p* < 0.01, *** *p* < 0.001 significantly different from day 1 basal concentration; 1-way ANOVA, Newman-Keuls post-hoc test. Pregnant rats were subject to 7 days of an immobilization stress regimen during weeks 1, 2, or 3 of pregnancy. Plasma corticosterone levels were measured before (basal) and immediately after (stress) the morning stress session on day 1 and 7 of the stress regimen.

### Table 4: Body weight profile of male control rats and male rats prenatally stressed during weeks 1, 2, or 3 of gestation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body Weight (gms)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Birtha</td>
</tr>
<tr>
<td>Control</td>
<td>6.4 ± 0.2b</td>
</tr>
<tr>
<td>Week 1</td>
<td>6.3 ± 0.2</td>
</tr>
<tr>
<td>Week 2</td>
<td>5.9 ± 0.2</td>
</tr>
<tr>
<td>Week 3</td>
<td>6.0 ± 0.2</td>
</tr>
</tbody>
</table>

a Includes both male and female offspring.
b Mean ± SEM; n = 5–7.
differences were observed between the groups in either birth weight or in body weight at any of the three ages examined. As shown in Fig. 1(A), administration of a 60% fructose diet caused marked increases in plasma triglyceride concentrations in control and all three groups of prenatally stressed offspring. These results show that we were able to reproduce literature reports which demonstrate high fructose diet induced increases in plasma triglyceride concentrations. The increase persists throughout the 21 day period of fructose diet administration (2-way repeated

Figure 1  Plasma triglyceride (A), insulin (B), and glucose (C) concentrations in control and prenatally stressed rats before, during, and after discontinuation of a 21 days high fructose diet regimen. Rats prenatally stressed during weeks 1 (WK 1), 2 (WK 2) or 3 (WK 3) of gestation or left undisturbed throughout gestation (C) were administered a 60% fructose diet for 21 days. Blood was sampled from rats in all groups before starting the fructose diet (BASAL), on days seven (DAY 7), 14 (DAY 14), and 21 (DAY 21) after starting the diet, and three days after discontinuation of the diet (DAY 24). See the Methods section for more details on the experimental protocol. Bars represent mean ± SEM of 5–7 rats per group.
measures ANOVA, significant effect of duration, \( P < 0.001 \). Three days after discontinuation of the diet, triglyceride levels returned to their basal values (day 24 = basal, Newman–Keuls post-hoc test). However, there were no statistically significant differences in plasma triglyceride concentrations between the four groups at any of the time periods. Similarly, there were no significant differences between the groups either in the rates of increase of triglyceride concentrations or in their rates of recovery subsequent to stopping the fructose diet on day 21 (2-way repeated measures ANOVA, main effect of prenatal stress, \( P = 0.18 \); prenatal stress \( \times \) duration interaction, \( P = 0.425 \)).

As previously reported in the literature, our results (Fig. 1(B)) demonstrate that the 60% fructose diet increased plasma insulin concentrations by day 7 which persisted throughout the 21 day diet regimen (2-way repeated measures ANOVA, significant duration effect, \( P < 0.001 \)). Three days after discontinuation of the diet, insulin levels decreased but remained higher than the basal levels (day 24 > basal, Newman–Keuls post-hoc test). As previously reported for triglyceride levels, there were no differences in plasma insulin levels between the four groups in the rats, or in the rates of increase or decrease of insulin levels (2-way repeated measures ANOVA, main effect of prenatal stress, \( P = 0.628 \), prenatal stress \( \times \) duration interaction, \( P = 0.067 \)).

Fig. 1(C) shows the effect of chronic fructose diet administration on plasma glucose levels. Surprisingly, a 2-way repeated measure ANOVA revealed a significant duration effect (\( P < 0.001 \)). More detailed 1–way repeated measure ANOVA revealed that statistical significance was achieved due to small (7%) decrease in glucose levels between discrete periods in the WK 2 and WK 3 groups. Results from the literature suggest that chronic administration of a high fructose diet does not affect plasma glucose levels. In the present study, the relevance of the small changes in glucose levels in two of the four groups is unclear. There were no statistically significant differences between the four groups in glucose levels (2-way repeated measure ANOVA, main effect of prenatal stress, \( P = 0.681 \), prenatal stress \( \times \) duration interaction, \( P = 0.671 \)).

Additional animals from the four groups were housed alongside the fructose fed animals, and fed lab chow for the entire 24 day duration of the experiment. These rats served as negative controls for the above mentioned experiment and plasma triglyceride, insulin, and glucose levels were measured in these animals at the same sampling times. Analyses of the triglyceride and insulin data demonstrated no duration effect (2-way repeated measure ANOVA), which suggest that changes in these biochemical parameters observed in the fructose fed animals were due to the diet and not the passage of time (data not shown). Surprisingly, plasma glucose levels in these negative controls showed a small but significant decrease over the 24 day period (data not shown). The very small magnitude of these changes is of questionable relevance.

Analyses of body weights during the fructose diet experiment demonstrated no statistical differences in body weights between the control and prenatally stressed rats either prior to, during, or three days after discontinuation of a high fructose diet (Table 5). Also, comparison of body weights during the five measurement periods between control offspring fed a high fructose diet, and control offspring fed lab chow (negative controls) during the same period revealed no significant differences in body weight (data not shown). This confirms literature reports that the high fructose diet mediated increases in plasma triglyceride and insulin concentrations are not accompanied by disproportionate increases in body weight.

Table 5  Body weight profile of control and prenatally stressed rats before, during, and after a 21 day regimen of high fructose diet

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body weight (gms)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
</tr>
<tr>
<td>Control</td>
<td>365 ±18*</td>
</tr>
<tr>
<td>Week 1</td>
<td>351 ± 7</td>
</tr>
<tr>
<td>Week 2</td>
<td>351 ± 4</td>
</tr>
<tr>
<td>Week 3</td>
<td>360 ± 7</td>
</tr>
</tbody>
</table>

* Mean ± SEM; \( n = 5–7 \).
while Table 6 summarizes the results of the i.p. glucose tolerance test. Basal plasma glucose and insulin levels in the control group were 103 mg/dL and 0.39 ng/mL, respectively. Prenatal stress during weeks 1, 2, or 3 of gestation did not affect basal glucose or insulin plasma concentrations. Administration of a 2 gm/kg i.p. glucose dose caused increases in glucose and insulin levels. In the control group, the net area under the glucose and insulin plasma concentration time curves were 10,071 (mg/dL)×min and 35.9 (ng/mL)×min, respectively. Prenatal stress during weeks one, two, or three of gestation did not affect the net area under the plasma glucose and insulin concentration—time curves.

4. Discussion

Our studies were designed to examine if maternal stress during discrete periods of gestation and the consequent exposure of the developing fetuses to B, would affect glucose and insulin homeostasis in the adult offspring. In our study, basal B levels on day one in all three groups were similar (Table 3). These basal levels were obtained between the first (in group week 1) and 15 (in group week 3) day of pregnancy. The results support reports which demonstrate that basal B levels during pregnancy are not significantly affected until day 14 of pregnancy and begin to rise about day 18 of pregnancy (Waddell and Atkinson, 1994; Atkinson and Waddell, 1995). In the group that received stress during the last week of pregnancy (week 3), basal B levels on day seven were higher than the corresponding levels on day one. This is probably not a stress related phenomenon, and reflects the dam’s preparation for impending parturition (Waddell and Atkinson, 1994). The immobilization stress paradigm used in our studies caused massive increases in plasma levels of B and in the levels of

<table>
<thead>
<tr>
<th>Parameter (units)</th>
<th>Treatment</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal plasma glucose (mg/dL)</td>
<td>Control</td>
<td>103 ± 3³</td>
<td>105 ± 4</td>
<td>107 ± 4</td>
</tr>
<tr>
<td></td>
<td>Week 1</td>
<td>105 ± 4</td>
<td>107 ± 4</td>
<td>104 ± 4</td>
</tr>
<tr>
<td>Net glucose AUC (mg/dL)×min</td>
<td>Week 2</td>
<td>9876 ± 490</td>
<td>7814 ± 1432</td>
<td>10037 ± 1274</td>
</tr>
<tr>
<td>Basal plasma insulin (ng/mL)</td>
<td>Week 3</td>
<td>0.39 ± 0.1</td>
<td>0.67 ± 0.2</td>
<td>0.58 ± 0.1</td>
</tr>
<tr>
<td>Net insulin AUC (ng/mL)×min</td>
<td></td>
<td>35.9 ± 10</td>
<td>35.3 ± 7</td>
<td>30.6 ± 7</td>
</tr>
</tbody>
</table>

One hundred and twenty day old male control rats or rats subject to prenatal immobilization stress during weeks 1, 2 or 3 of gestation were administered an i.p. glucose tolerance test as described in the Methods section. Blood samples were taken before (basal) and at varying times after the glucose dose, and net area under the plasma glucose and insulin concentration—time curves (AUC) were computed as described in the Methods section.

³ Mean ± SEM; n = 6–7.
its secretagogue ACTH. Despite the chronic seven day stress regimen, stress induced increases in B and ACTH levels were similar on day one and seven of this regimen suggesting no biochemical acclimatization to the stressor. Some studies have shown that a chronic stress regimen induces activation of the basal activity of the HPA axis and increases in basal levels of B (Vernikos et al., 1982). However, in the present study, such increases were observed in the basal levels of ACTH but not in the levels of B probably due to the episodic nature of the chronic stress regimen.

Maternal stress during weeks 1, 2, or 3 of pregnancy did not adversely affect reproductive performance as assessed by gestation length and litter size. However, immobilization stress during weeks one and three reduced maternal weight gain during pregnancy; further evidence of the efficacy of the stress regimen employed in these studies. Prenatal immobilization stress did not affect birth weight of the offspring. A review of the literature shows inconsistency in the effects of prenatal stress on birth weight with reports of both decreases (Pollard, 1984; Cabrera et al., 1999; Drago et al., 1999; Patin et al., 2002; Lesage et al., 2004) and no change (Von Hoersten et al., 1995; Guo et al., 1993; Holson et al., 1995; Gerardin et al., 2005). In most of these studies, the weight of each individual pup in all litters of a group was treated as an independent observation in the statistical analyses. Such an inflation of sample size can produce spurious statistical significance and might account for the reported inconsistency in the effects of prenatal stress on birth weight. In our studies, the average pup weight of each litter in a group was computed and the values used to compute the group mean. Consequently the sample size for birth weight analyses in each group was equal to the number of dams in the group. It must be noted that if we had incorrectly treated individual pups in a litter as independent samples we would have a much larger sample size, and would have obtained statistical significance for the effects of prenatal stress on birth weight.

Our next study determined if prenatal stress affected the ability of the adult offspring to cope with metabolic stress. A high fructose diet is a well established paradigm of a metabolic stressor. Chronic administration of a high fructose diet is known to increase triglyceride and insulin plasma concentrations along with concomitant loss of glycemic control. Therefore, it has often been used as an animal model of the metabolic syndrome. The exact molecular mechanisms mediating the effects of a high fructose diet are presently unclear. Results of recent studies suggest that a high fructose diet alters protein levels involved in stress pathways, and acts by inducing hepatic stress (Kelley et al., 2004).

Consistent with literature reports, our studies demonstrate that a high fructose diet caused increased increases in plasma levels of triglyceride and insulin within 1 week of the start of diet administration (Reed et al., 1994; Tobey et al., 1982; Bhanot et al., 1994). Continued diet administration did not increase the magnitude of changes in these parameters. Three days after discontinuation of the high fructose diet, triglyceride levels decreased to their basal values and insulin levels markedly decreased but continued to be slightly higher than basal values. These results corroborate literature reports, and demonstrate rapid recovery of biochemical parameters from the effects of chronic high fructose diet (Dai and McNeill, 1995). Furthermore, as reported in the literature, measurement of body weight during the experiment demonstrated that the fructose diet mediated increases in plasma insulin and triglyceride concentrations were not accompanied by a disproportionate increase in body weight (Reed et al., 1994; Bhanot et al., 1994; Dai and McNeill, 1995). Prenatal stress during weeks, one, two, or three of gestation did not affect the rate of increase, peak levels, or the rate of recovery of plasma triglyceride and insulin concentrations. These results suggest that prenatal stress did not affect the ability of 60 day old offspring to cope with the hepatic stress induced by administration of a chronic high fructose diet.

Results from our final studies demonstrated that prenatal stress did not affect basal glucose or insulin levels in 120 day old rats and did not affect glucose tolerance as measured by glucose and insulin area under the plasma concentration—time curves after an i.p. glucose load. In contrast to these results, subcutaneous administration of dexamethasone, a synthetic glucocorticoid, to dams during the third week of gestation, decreased offspring birth weight, and produced glucose intolerance in the 6 month old offspring (Nyirenda et al., 1998). The fetus is normally protected from exposure to the much higher levels of maternal glucocorticoid by the placental enzyme 11β-hydroxysteroid dehydrogenase, type 2 (11β-HSD2). This enzyme converts physiologically active glucocorticoid (corticosterone in rats) to the inactive 11-keto products (11-dehydrocorticosterone in rats) (Monder and White, 1993; Heller et al., 1988; Burton and Waddell, 1994). Dexamethasone is a poor substrate for 11β-HSD2 (Siebe et al., 1993). Consequently, its administration to pregnant dams probably results in high, non-physiological exposure...
of the fetus to glucocorticoids, and could account for its ability to decrease birth weight and alter glucose tolerance in the offspring. Few studies have systematically explored fetal exposure to B after maternal stress. One of the few studies that measured maternal and fetal B levels after immobilization stress showed surprisingly small increases in fetal B plasma levels despite huge increases in maternal B—a testimony to the protective efficacy of placental 11\(^\beta\)-HSD 2 (Ward and Weisz, 1984). Maternal stress probably results in a much more modest exposure of the fetus to maternal B which might account for its inability to affect glucose tolerance in the offspring.

Prenatal restraint stress in an illuminated environment has been demonstrated to cause modest (18%) increases in basal glucose levels in five months old male rats (Vallee et al., 1996). However, the number of pups examined in this study was greater than the number of treated dams and the results might be partially confounded by litter effects. Interestingly, a recent study using an experimental design similar to our study subjected pregnant rats to restraint stress in an illuminated environment during the last week of gestation. The authors demonstrated prenatal stress mediated reduction in fetal weight at term (Lesage et al., 2004). Additionally, the authors showed that 24 months old prenatally stressed rats had elevated basal glucose levels and higher glucose levels in response to a oral glucose load as compared to offspring of control rats. However, consistent with our results, these authors did not observe differences between the control and prenatally stressed groups either in basal insulin levels or in insulin levels in response to the glucose load (Lesage et al., 2004). The originators of the ‘restraint stress in an illuminated environment’ paradigm created the illuminated environment with the use of high wattage bulbs with the accompanying generation of significant amounts of heat (Ward and Weisz, 1984). Such a restraint paradigm is different from the immobilization paradigm used in our studies which is conducted at ambient temperature. The possible use of such a paradigm by the aforementioned investigators could account for the differences in findings between the studies. Also, it is conceivable that the relatively small and selective effects of prenatal stress on glucose levels are manifested only in aged animals. Due to experimental constraints, we evaluated glucose tolerance in four months old animals, in contrast to the 24 months old animals used by the earlier investigators. This could also account for our inability to observe prenatal stress induced alterations in glucose tolerance. Age specific effects of prenatal insults on glucose tolerance have been previously reported. A review of the literature showed that maternal low protein diets, a widely used paradigm of prenatal insult, which is hypothesized to act by increasing fetal exposure to glucocorticoids actually improved glucose tolerance in younger animals (Langley SC et al., 1994; Shepherd et al., 1997), and deterioration of glucose tolerance was only observed in aged offspring (Hales et al., 1996; Petry et al., 2001).

Finally, a review of the literature demonstrates that many studies which examine the effects of maternal treatments on development in the offspring of multiparous species like rats are confounded by litter effects. The investigators treat a relatively small number of dams, then proceed to use multiple offspring from a litter in their experiments, and treat each offspring in a litter as an independent observation. This dramatically inflates the sample size of the study, which in turn inflates the alpha level (normally set at 0.05), and leads to spurious statistical significance (Holson and Pearce, 1992; Zorilla, 1997). In all our experiments, whenever more than one pup in a litter was used to measure a biological parameter, the values obtained were averaged and reported as a single point. Consequently in the analyses of offspring data, the sample size of the group never exceeded the number of treated dams in that group. Such a conservative method of data reporting and statistical analyses is more accurate and is highly recommended in the evaluation of maternal treatments on the offspring of multiparous species (Holson and Pearce, 1992; Zorilla, 1997). In our studies, it minimized the risk of obtaining spurious statistical significance, and consequently improved the reliability of our conclusions.

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References

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Lindsay, R.S., Lindsay, R.M., Waddell, B.J., Seckl, J.R., 1996. Prenatal glucocorticoid exposure leads to offspring hyperglycaemia in the rat: studies with the 11 beta-hydroxysteroid dehydrogenase inhibitor carbexonolone. Diabetologia 39, 1299-1305.


