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Influence of abnormally high leptin levels during pregnancy on metabolic phenotypes in progeny mice

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Makarova EN, Chepeleva EV, Panchenko PE, Bazhan NM. Influence of abnormally high leptin levels during pregnancy on metabolic phenotypes of progeny mice. Am J Physiol Regul Integr Comp Physiol 305: R1268–R1280, 2013. First published October 2, 2013; doi:10.1152/ajpregu.00162.2013.—Maternal obesity increases the risk of obesity in offspring, and obesity is accompanied by an increase in blood leptin levels. The “yellow” mutation at the mouse agouti locus (Ay) increases blood leptin levels in C57BL preobese pregnant mice without affecting other metabolic characteristics. We investigated the influence of the Ay mutation or leptin injection at the end of pregnancy in C57BL mice on metabolic phenotypes and the susceptibility to diet-induced obesity (DIO) in offspring. In both C57BL-Ay and leptin-treated mice, the maternal effect was more pronounced in male offspring. Compared with males born to control mothers, males born to Ay mothers displayed equal food intake (FI) but decreased body weight (BW) gain after weaning, equal glucose tolerance, and enhanced FI-to-BW ratios on the standard diet but the same FI and BW weight (BW) gain after refeeding (equal lean mass) compared with males born to control mothers. However, all progeny displayed equal hypothalamic expression of Agouti gene-related protein (AgRP), neuropeptide Y (NPY), and proopiomelanocortin (POMC) and equal plasma leptin and glucose levels after food deprivation. Leptin injections in C57BL mice on day 17 of pregnancy decreased BW in both male and female offspring but inhibited FI and DIO only in male offspring. Our results show that hyperleptinemia during pregnancy has sex-specific long-term effects on energy balance regulation in progeny and does not predispose offspring to developing obesity.

leptin; pregnancy; developmental programming; mice; high-fat diet

A considerable number of studies have identified leptin as a potential programming factor (48). In experimental animal models, expression of the leptin receptor has been detected in various fetal tissues, including cartilage, bone, lung, brain (16), and pancreas (18). Leptin has been shown to activate the differentiation and proliferation of fetal chondrocytes, osteoblasts (2), and islet cells (18) and to promote the migration and differentiation of hypothalamic neural progenitor cells (8). In addition, leptin may influence fetal development by affecting nutrient transport across the placenta (20, 49). Leptin administration to pregnant dams has been shown to affect mouse fetal growth (35) and rat progeny phenotypes in postnatal life (32). Taken together, these data suggest that maternal leptin levels during pregnancy may contribute to fetal developmental programming. However, whether maternal hyperleptinemia during pregnancy is a key factor involved in promoting the development of obesity in offspring remains unclear.

Both malnutrition and maternal obesity in pregnancy have been shown to predispose the offspring to becoming obese (25), although malnutrition is characterized by a decrease in blood leptin levels (21) while obesity is characterized by an increase in leptin levels. Obesity is associated with hyperglycemia, hyperinsulinemia, and hyperlipidemia, which may also have programming effects (5, 12, 15).

The results obtained in experiments administering leptin to pregnant mice or rats have been conflicting. Leptin infusion in food-restricted mice during early and midpregnancy promoted development of diet-induced obesity (DIO) in adult female offspring (36); however, the administration of leptin to both protein-restricted and adequately fed rats during the third trimester and lactation protected offspring from obesity induced by high-fat feeding (43, 44). In addition, the rats received leptin during both pregnancy and lactation. Previous reports suggest that maternal leptin may be transferred to the infant via milk (46). In neonates, leptin was shown to promote the formation of the neural network related to food intake (FI) (3), and neonatal leptin supplementation in rats prevented obesity later in life (37), whereas specific leptin blockade increased the susceptibility to DIO (1). Therefore, it remains unknown whether prenatal or postnatal maternal hyperleptinemia is responsible for the antiobesity effects of leptin administration during late pregnancy and lactation (43, 44).

The aim of this study was to investigate the influence of chronic maternal hyperleptinemia during pregnancy on meta-
iologic phenotypes and susceptibility to DIO of the progeny and to reveal the role of the late prenatal stage in causing possible programming effects of maternal leptin. To answer these questions, we used mice with inherited hyperleptinemia during pregnancy and administered leptin to the mice at the end of pregnancy. The “yellow” mutation at the mouse agouti locus (A<sup>y</sup>) causes ectopic overexpression of the agouti gene (4), which disturbs energy balance regulation via chronic blockade of melanocortin receptors by the agouti protein in the hypothalamus (28) and results in a yellow coat and the development of obesity and diabetes with age (52). Previously, we have shown that C57BL female mice with the “yellow” mutation (A<sup>y</sup>/*a) mated with males at the preobe stage display metabolic characteristics (blood levels of corticosterone, glucose, and insulin and glucose and insulin tolerance) during pregnancy and lactation that are similar to those of C57BL mice with the standard agouti genotype (a/*a). The only difference between mated A<sup>y</sup>/a and a/*a mice is that A<sup>y</sup>/a mice express blood leptin levels that are approximately two times higher during pregnancy. In suckling A<sup>y</sup>/a and a/*a mice, the leptin levels are equal (27). Therefore, this model imitates the hyperleptinemia resulting from obesity and allows us to separate the programming effects caused by elevated leptin levels from the effects of other metabolic abnormalities that are associated with obesity, and this effect is restricted to pregnancy only. We also examined whether single leptin injections in pregnant a/*a C57BL/6j mice at the end of pregnancy influence susceptibility to DIO in the offspring.

We found that female progeny were unaffected by the maternal genotype, but compared with the male progeny of the control mothers the male progeny of the A<sup>y</sup> mothers displayed a decreased growth rate after weaning, an enhanced ratio of energy intake (EI) to body weight (BW) in maturity, decreased sensitivity to exogenous leptin, decreased resistance to food deprivation, and equal EI and BW during high-fat diet feeding. Similar to offspring of the A<sup>y</sup>/a mice, female offspring were less affected by the administration of leptin to mothers than male offspring. The administration of leptin to pregnant dams at the end of pregnancy inhibited obesity in male offspring without affecting female offspring on a palatable fat and sweet diet. The data suggest that hyperleptinemia during pregnancy has sex-specific long-term effects on energy balance regulation in the progeny and does not predispose the offspring to obesity.

**METHODS**

**Ethical Approval**

All experiments were performed according to the highest standards of humane animal care with International European ethical standards (86/609-EEC) and Russian national instructions for the care and use of laboratory animals. The protocols were reviewed and approved by the Independent Ethics Committee of the Institute of Cytology and Genetics (Siberian Division, Russian Academy of Sciences).

**Diets**

The standard chow diet in pelleted form provided 3.7 kcal/g and contained 19% protein, 4% fat, and 66% carbohydrates, and the high-fat diet provided 4.8 kcal/g and contained 24% protein, 24% fat, and 42% carbohydrates. Both diets were purchased from Assortiment Agro (Moscow region, Turacovo, Russia).

Palatable food included sweet butter biscuits, lard, and sunflower seeds. The animals received palatable food in addition to standard chow. The animals received excess quantities of each foodstuff including the chow, such that their intake was ad libitum.

**Animals: Genetic Model**

C57BL/6J mice with the agouti genotypes A<sup>y</sup>/a and a/*a were bred in the vivarium of the Institute of Cytology and Genetics. The mice were housed under a 12:12-h light-dark regime (with lights switched off at 1800) at an ambient temperature of 22°C. The mice were provided access to commercial mouse chow and water ad libitum.

At 8 wk of age, females were mated with males in reciprocal crosses (A<sup>y</sup>/a x a/*a and a/*a x A<sup>y</sup>/a) and the presence of the copulatory plug was checked. Mated females were housed individually from the day of plug detection (day 1 of pregnancy). The mated females were monitored to record parturition and the number of pups, and the day of delivery was designated as postpartum day 1. To measure plasma leptin concentrations, 19 a/*a and 19 A<sup>y</sup>/a mated females were killed by rapid decapitation on day 18 of pregnancy, 5 a/*a and 5 A<sup>y</sup>/a females on postpartum day 1, and 5 a/*a and 7 A<sup>y</sup>/a females on postpartum day 10; subsequently, female trunk blood samples were collected. Fetal blood samples of the offspring of seven a/*a and seven A<sup>y</sup>/a females and newborn blood were collected. In total, 10 µl of blood from every fetus or newborn was sampled after rapid decapitation, and the samples from one litter were pooled. Blood samples from the young on postpartum day 10 were collected individually from the a/*a genotype males in the six-pup litters.

In the females killed on day 18 of pregnancy, the weights of the fetoplacental units were calculated as the weights of the uterus with fetuses divided by the numbers of fetuses. To examine maternal influence on pup growth rate during the maternal care period, BW of every pup on postpartum day 1 (when it was impossible to visually determine the agouti genotype of pups) and only a/*a pups on postpartum days 10 and 28 were measured in offspring from eight a/*a and eight A<sup>y</sup>/a mothers. All pups were born in six-pup litters. The data from male and female pups were combined because no sex differences were observed in pup BW.

The other mated females were used to investigate the maternal influences on progeny metabolic phenotypes.

**Experimental Procedures in Offspring After Weaning**

Offspring metabolism was studied in young of only the a/*a genotype (normal metabolism). To diminish the impact of litter size and maternal body conditions on offspring metabolic phenotypes, only one male and/or one female from the same mother and only those born in litters of six pups were studied. The young born to 175 dams were used in various experiments. The numbers of male and female young that were used in each experiment are indicated in Table 1. The number of young is equal to the number of mothers. For all experiments, the young born to a/*a and A<sup>y</sup>/a mothers were separated from their mothers when they were 30 days old and were then housed individually until treatment.

To investigate maternal influence on progeny growth rate and FI after weaning, BW and FI were measured in females and males once a week until the age of 10–12 wk and BW gain was calculated for every animal as the absolute change in BW during a week. FI was estimated as the difference between the initial weight of food supplied and the amount of food left on the grid. To investigate the maternal influence on progeny glucose metabolism in maturity, plasma insulin concentrations and glucose tolerance were measured in 10- to 14-wk-old females and males. For the insulin measurement, mice that had been fasted overnight (12 h) were killed by rapid decapitation and samples of trunk blood were collected. Plasma insulin concentrations were measured in mice that were used for FI and BW measurements and in the additional group of male mice (Table 1). For the glucose tolerance test (GTT), mice were fasted for 12 h and injected intraperitoneally with glucose (1 mg/g BW); blood was sampled from the...
frozen in liquid nitrogen. Collected, and entire hypothalami were excised and immediately snap-decapitated 25 h after the second injection. Trunk blood samples were collected, and entire hypothalami were excised and immediately snap-frozen in liquid nitrogen.

To examine maternal influence on sensitivity to leptin after food deprivation, we used the modified protocol published by Swart et al. (45). The authors have shown that leptin administration to 24-h food-deprived C57BL male mice did not affect 2-h FI but significantly reduced FI between 2 and 6 h of refeeding. In our experiment, male mice were food-deprived for 24 h (starting at 1800) at the age of 10–12 wk and injected with recombinant murine leptin (4.0 μg/g BW) or saline 2 h before the beginning of feeding (at 1600). FI was measured 3 h and then 24 h after the beginning of feeding.

Experiments with Leptin Injections into Dams at End of Pregnancy

To examine the influence of elevated maternal leptin levels at the end of pregnancy on progeny phenotype, 10 a/a C57BL/6J females were mated with a/a C57BL/6J males at 10 wk of age and housed individually from the day a copulatory plug was detected (pregnancy day 1). On day 17 of pregnancy, five females received intraperitoneal injections of recombinant murine leptin (4.0 μg/g BW) dissolved in saline to a final concentration of 500 μg/ml and five females were injected with saline (control). At birth the females and pups were weighed, and the litters that contained more than seven pups were reduced to seven pups. In the control group, four females had seven-pup litters and one female had a six-pup litter. In the leptin-treated group, three females had seven-pup litters and the others had eight- and nine-pup litters. On postpartum day 28 two males and two females from each litter were separated from their mothers and housed individually, and their BW and FI were measured once a week until the age of 16 wk. During this period, all animals were fed with a standard chow diet ad libitum. From the age of 16 wk, one male and one female from each litter continued to receive a standard diet and one male and one female began to receive palatable food in addition to standard chow. Their BWs and consumption of standard pellet chow were measured once a week over the course of 5 wk. The consumption of palatable food (lard, sweet butter biscuits, and sunflower seeds) was not measured. Five animals were included in every experimental group.

Materials

Murine recombinant leptin was purchased from PeproTech (Princeton, NJ).

Plasma Hormone Assays

Concentrations of leptin and insulin were measured with commercial kits (leptin: R&D Systems, Minneapolis, MN; insulin: RIA-INS-PG-125J, ChemPharmSynthesis, IBOCH of NASB, Minsk, Belarus).

Blood glucose concentrations were determined with a reflectance glucometer by the glucose oxidase method (One Touch Basic Plus, Lifescan). Plasma glucose concentrations were measured with a commercial kit (Fluittest GLU, Analyticon Biotechnologies, Lichtenfels, Germany).

Relative Quantitation Real-Time PCR

Total RNA was isolated from individual hypothalami of males with TRI Reagent (Ambion) according to the manufacturer’s instructions. The total RNA was used as a template to generate first-strand cDNA synthesis with Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI) and oligo(dT) as a primer. Applied Biosystems TaqMan gene expression assays (OhRb-LepR Mm00440181_m1, POMC Mm00435874_m1, and AgRP Mm00475829_g1) with β-actin as endogenous control [TaqMan endogenous controls with FAM dye label and MGB mouse β-actin (ACTB)] and TaqMan Gene Expression Master Mix were used for relative quantitation real-time PCR. Sequence amplification and fluorescence detection were done with the Applied Biosystems 7900HT Fast Real-Time PCR System. Reactions were performed in triplicate and the results averaged. Relative quantitation

<table>
<thead>
<tr>
<th>Treatments and Measurements</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>FI, BW, plasma insulin</td>
<td>7 9</td>
<td>12 12</td>
</tr>
<tr>
<td>Plasma insulin</td>
<td>5 3</td>
<td>4 4</td>
</tr>
<tr>
<td>GTT, blood glucose</td>
<td>6 6</td>
<td>4 4</td>
</tr>
<tr>
<td>24-h food deprivation</td>
<td>FI after FD 7 9</td>
<td>5 5</td>
</tr>
<tr>
<td>Metabolic parameters after FD</td>
<td>6 6</td>
<td></td>
</tr>
<tr>
<td>Control (FI and metabolic parameters)</td>
<td>6 6</td>
<td></td>
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</tbody>
</table>

FI, food intake; BW, body weight; GTT, glucose tolerance test; FD, food deprivation; HFD, high-fat diet. The number of young is equal to the number of mothers and the number of litters.

Table 1. Number of male and female offspring used in experimental procedures in postweaning period

To investigate maternal influence on FI regulation after food deprivation, FI, hypothalamic expression of neuropeptides and leptin receptor isoform Rb (Ob-Rb), circulating levels of leptin and glucose, and sensitivity to exogenous leptin were measured in males after 24 h of food deprivation. At the age of 12–14 wk, male mice were food deprived for 24 h (starting at 1800). Because no significant differences in daily FI were observed between males born to a/a and A/a mothers, only males born to a/a mothers were used as the control group. In some food-deprived males, FI was measured every hour for 3 h and then 24 h after the beginning of feeding. At the same time, FI and sensitivity to exogenous leptin were measured in males after 24 h of food deprivation.

Leptin sensitivity

Leptin 7 5
Saline 7 5
Leptin sensitivity after 24-h food deprivation
Leptin 7 5
Saline 6 5
HFD
HFD 7 8
Control 7 6
Total 71 72 16 16
was performed by the comparative Ct method, where Ct is threshold cycle.

Neuropeptide Y (NPY) expression was measured by semiquantitative RT-PCR with tttggtgctgctgctgctg (forward) and tctgg-

ggcttctgtcgt (reverse) primers for NPY and tttggtgctgctgctgctg (forward) and cag (reverse) primers for β-actin.

Sequence amplification was performed with a BIS thermocycler

(BIS-H, Coltsovo, Russia; www.bis.ru) with an annealing temperature of 62°C and 27 cycles for NPY cDNA amplification and an annealing temperature of 59°C and 19 cycles for β-actin cDNA amplification. PCR products were visualized on 1.5% agarose gels by ethidium bromide staining. Relative gene expression was quantified by densitometry with a Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD) and was normalized to the β-actin cDNA signals. Reactions were performed in triplicate, and the results were averaged.

Statistical Analysis

The results are presented as means ± SE from the indicated number of mice. Two-way ANOVA was used to compare BW and FI after weaning [with factors “maternal genotype” (a/a, A/a) or “treatment of mother” (saline, leptin) and “age”], blood glucose concentrations during GTT (“maternal genotype” and “time after glucose injection”), fat pad weights (“maternal genotype” and “diet”), BW changes, glucose and leptin concentrations, neuropeptide expression levels, and FI during 21 h of refeeding after food deprivation [“maternal genotype” and “treatment” (leptin, saline)]. To examine the maternal influence on the response to a leptin administration in the fed conditions, daily FI was analyzed initially by three-way ANOVA with the factors “maternal genotype,” “treatment” (leptin, saline), and “day of treatment” (day 1, 2, and 3). To examine the maternal influence on the response to a diet, BW, FI, and EI/BW were analyzed initially by three-way ANOVA with the factors “maternal genotype” or “maternal treatment,” “diet,” and “age” and then separately by two-way ANOVA in leptin-treated or control groups (“maternal genotype” and “day of treatment”) and in the males born to a/a or A/a mothers (“treatment” and “day of treatment”). To examine the maternal influence on the response to a diet, BW, FI, and EI/BW were analyzed initially by three-way ANOVA with the factors “maternal genotype” or “maternal treatment,” “diet,” and “age” and then separately by two-way ANOVA in control and HF or palatable diet animals with the factors “maternal genotype” or “maternal treatment” and “age.” In addition, multiple comparisons were performed with the post hoc Duncan test. The comparisons between single parameters were performed with a two-tailed Student’s t-test. Significance was determined as P < 0.05. The STATISTICA 6 software package (StatSoft) was used for analysis.

RESULTS

BW and Plasma Leptin Levels in Female Mice and Their Progeny

BW (without fetuses) in A/a females were slightly but significantly higher than in a/a females at the end of pregnancy and on postpartum day 1. On postpartum days 10 and 28, BWs of a/a and A/a mothers were similar (Table 2). Leptin levels were approximately twofold higher in A/a females than in a/a females at the end of pregnancy. After parturition, leptin levels decreased and remained higher in A/a females compared with a/a females on postpartum day 1 (Table 2). However, the leptin levels in a/a and A/a mothers were comparable at the peak of lactation on postpartum day 10.

Maternal genotype did not influence the weights of the fetoplacental units on day 18 of pregnancy or the pup BWs during the maternal care period. Leptin levels in the progeny were also not influenced by maternal genotype (Table 2). No significant correlations were observed between plasma leptin concentrations in the mothers and their progeny. Leptin levels were approximately threefold lower in newborns than in fetuses [0.10 ± 0.03 (n = 10), 0.38 ± 0.04 (n = 14); P < 0.0001, Student’s t-test, newborns and fetuses, respectively].

Influence of Inherited Maternal Hyperleptinemia During Pregnancy on Offspring Metabolic Phenotypes

Impact of maternal leptin levels during pregnancy on FI, BW, and glucose metabolism of male and female offspring in postweaning period. Abnormally high leptin levels during pregnancy did not influence any of the characteristics studied in female progeny. Female mice born to a/a and A/a mothers displayed the same BW (Fig. 1A) and FI (Fig. 1C) between 5 and 10 wk of age, the same BW gain after weaning (Table 3), the same fasting blood glucose levels and glucose tolerance (Fig. 1E), and the same fasting plasma insulin levels (Table 3).

Contrasting results were observed in the male progeny. Males born to a/a and A/a mothers displayed the same FI (Fig. 1D) but different growth rates during the postweaning period. At the time of separation from their mothers, the BWs of the male progeny of a/a and A/a mice were equal. Compared with males born to a/a mothers, males born to A/a mothers had lower BW gains between 5 and 7 wk of age (Table 3) and lower BWs beginning at 6 wk of age (Fig. 1B). No differences in fasting blood glucose levels or glucose tolerance (Fig. 1F) were observed between males born to a/a and A/a mothers, but fasting plasma insulin levels were significantly higher in those born to A/a mothers (Table 3). Thus prenatal exposure to high maternal leptin levels had a sex-specific effect on progeny, affecting growth rate after weaning and some aspects of glucose metabolism in male offspring.

Table 2. BW and plasma leptin concentration in female mice and their progeny on pregnancy day 18 and on postpartum days 1, 10, and 28

<table>
<thead>
<tr>
<th></th>
<th>Pregnancy day 18</th>
<th>Pp day 1</th>
<th>Pp day 10</th>
<th>Pp day 28</th>
<th>Pregnancy day 18</th>
<th>Pp day 1</th>
<th>Pp day 10</th>
<th>Pp day 28</th>
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<tbody>
<tr>
<td><strong>BW</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mother</td>
<td>26.2 ± 0.5 (19)</td>
<td>23.6 ± 0.6 (8)</td>
<td>29.9 ± 0.5 (18)</td>
<td>27.1 ± 0.9 (6)</td>
<td>27.6 ± 0.4 (19)*</td>
<td>26.9 ± 0.5 (8)**</td>
<td>29.4 ± 0.5 (13)</td>
<td>28.3 ± 0.5 (7)</td>
</tr>
<tr>
<td>Progeny</td>
<td>1.34 ± 0.03 (19)</td>
<td>1.40 ± 0.02 (48)</td>
<td>5.6 ± 0.1 (56)</td>
<td>10.3 ± 0.4 (19)</td>
<td>1.37 ± 0.03 (19)</td>
<td>1.43 ± 0.02 (48)</td>
<td>5.6 ± 0.1 (42)</td>
<td>10.7 ± 0.5 (15)</td>
</tr>
<tr>
<td>Leptin</td>
<td>22.1 ± 3.3 (19)</td>
<td>3.8 ± 1.5 (5)</td>
<td>1.1 ± 0.4 (5)</td>
<td>3.9 ± 1.2 (5)</td>
<td>48.7 ± 7.2 (19)**</td>
<td>12.6 ± 3.1 (5)*</td>
<td>1.8 ± 0.6 (7)</td>
<td>3.1 ± 1.4 (7)</td>
</tr>
<tr>
<td>Progeny</td>
<td>0.33 ± 0.06 (7)</td>
<td>0.11 ± 0.05 (5)</td>
<td>0.43 ± 0.05 (7)</td>
<td>0.10 ± 0.05 (5)</td>
<td>3.1 ± 1.4 (7)</td>
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</table>

Data (in g for BW and in ng/ml for plasma leptin concentration) are presented as means ± SE for the number of mice indicated in parentheses. The weight of progeny on pregnancy day 18 was calculated as the weight of the uterus with fetuses divided by the number of fetuses. BW of females on day 18 of pregnancy was calculated as the difference between female intravital body weight and weight of the uterus with fetuses. Pp, postpartum. *P < 0.05, **P < 0.01, ***P < 0.001, Student’s t-test, A/a vs. a/a.
Because the maternal effect on progeny development was observed only in males, only male offspring were studied in the subsequent experiments.

Impact of maternal leptin levels during pregnancy on response to HF diet in male offspring. To investigate whether enhanced maternal leptin predisposes offspring to developing DIO, adult BW-matched males born to Ay/a and control mothers were fed control (C) and HF diets. However, the HF diet did not induce pronounced obesity in males. Males that were fed either the C or HF diet showed similar BW during all periods of diet treatment that was independent of maternal genotype (3-way ANOVA with the grouping factors “age,” “diet,” and “maternal genotype”; Fig. 2A). FI and the FI-to-BW ratio were significantly lower in HF than in C males (P < 0.001, 3-way ANOVA). However, EI and EI/BW were the same in the C and HF diet groups in mice born to control mothers (Fig. 2, B and C). The genotype of the mothers influenced EI and EI/BW differently in mice fed C or HF diet. Compared with males born to a/a mothers, those born to Ay/a displayed higher EI and EI/BW in the C group (P < 0.001 for both cases, 2-way ANOVA, factors “maternal genotype” and “age”) and similar EI and EI/BW in the HF group (Fig. 2A). In contrast to mice born to a/a mothers, those born to Ay/a mothers decreased their EI and EI/BW on the HF diet compared with the C diet (Fig. 2, B and C).

Although a HF diet did not increase BW in male mice, the HF diet increased abdominal fat pad weights by approximately two-fold (Fig. 2D; P < 0.05 for diet, 2-way ANOVA with the factors “maternal genotype” and “diet”) independent of the genotype of mothers.
Thus prenatal exposure to high maternal leptin levels did not promote obesity development in adult male mice fed the HF diet and affected FI regulation only in males that were fed the standard diet and not in those fed the HF diet.

Impact of maternal leptin levels during pregnancy on FI, circulating levels of leptin and glucose, hypothalamic neuropeptide expression, and sensitivity to leptin in food-deprived male mice. To investigate whether prenatal exposure to high maternal leptin levels influences FI and BW regulation under fasting conditions, adult male offspring of A/a and control mothers were deprived of food for 24 h. Food-deprived males born to both a/a and A'/a mothers consumed more food than males fed ad libitum during the first 3 h of refeeding (Fig. 3A). FI in food-deprived males did not differ from that in males fed ad libitum during the subsequent 21 h. The cumulative 24-h FI after food deprivation in male progeny from A'/a mothers did not increase, unlike that in progeny from a/a mothers (Fig. 3A). Male progeny from A'/a mothers also displayed significantly lower BW gains during the 24 h of refeeding than males from a/a mothers (Fig. 3B).

Neither the genotype of the mother nor food restriction influenced circulating levels of glucose (Fig. 3C) or leptin (Fig. 3D) or expression of POMC in the hypothalamus (Fig. 3E). Food deprivation increased the hypothalamic expression of AgRP (Fig. 3F) and NPY (Fig. 3G) more than twofold, but maternal genotype did not influence this change in expression.

Leptin administration did not affect FI in food-deprived males from either a/a or A'/a mothers (Fig. 4A). However, leptin administration significantly reduced BW gain during 24 h of refeeding in males from a/a mothers but had no effect on BW gain in males from A'/a mothers (Fig. 4B). Independent of leptin treatment, males from A'/a mothers displayed the same FI during the first 3 h of refeeding and significantly reduced FI during the subsequent 21 h (Fig. 4A) compared with males from a/a mothers.

Thus prenatal exposure to high maternal leptin levels was associated with FI inhibition and insensitivity to exogenous leptin in food-deprived male mice.

Impact of maternal leptin levels during pregnancy on sensitivity to exogenous leptin in male offspring. To investigate whether prenatal exposure to high maternal leptin levels influences leptin signaling under normal fed conditions, leptin was administered to fed adult male offspring of A'/a and control mothers. Leptin administration affected FI in males (P < 0.01, 3-way ANOVA with the factors “maternal genotype,” “treatment,” and “day of treatment”); however, significant reduction of FI after leptin treatment was observed only in males born to a/a mothers (Fig. 5; P < 0.01, 2-way ANOVA with the factors “treatment” and “day of treatment”) and was not observed in those born to A'/a mothers. Leptin administration differentiated the males born to A'/a and a/a mothers in relation to FI: a significant influence of maternal genotype on FI was detected in the leptin-treated group (Fig. 5; P < 0.05, 2-way ANOVA with the factors “maternal genotype” and “day of treatment”) and was not detected in the control group. However, leptin administration decreased BW in males born to both a/a and A'/a mothers (Fig. 6A; P < 0.0001, 2-way ANOVA). Neither leptin treatment nor maternal genotype significantly affected hypothalamic expression of AgRP, POMC, or Ob-Rb in male progeny of a/a or A'/a mice (Fig. 6, C–E).
Plasma leptin levels at 25 h after the second injection were higher in males that received leptin than in control males \((P < 0.01, 2\text{-way ANOVA})\), but significant differences between control and leptin-treated groups were observed only in males born to \(a/a\) mothers \((P < 0.01, \text{post hoc Duncan’s test})\). Leptin-treated males born to \(Ay/a\) mothers displayed \(\sim 30\%\) lower plasma leptin levels \((P = 0.08, \text{Duncan’s test})\) than those born to \(a/a\) mothers.

Our data suggest that prenatal exposure to high maternal leptin levels decreases sensitivity to exogenous leptin in male mice.

**Influence of Leptin Administration to Dams at End of Pregnancy on Offspring Metabolic Phenotypes**

Influence of leptin administration to pregnant dams on offspring FI and BW during postweaning period. The administration of leptin to dams at the end of pregnancy did not
Influence of leptin administration to pregnant dams on offspring response to palatable food in adulthood. The HF diet did not induce obesity in the male mice; therefore, we added palatable fat and sweet food (natural lard as a source of saturated fatty acids, sunflower seeds as a source of unsaturated fatty acids, and sweet butter biscuits) to standard chow to enable the animals to freely choose the type of food. The switch to a palatable diet decreased the animal consumption of standard chow by approximately fourfold, and leptin administration to dams did not affect the amount of chow consumed. Palatable food feeding induced the development of obesity in both male and female offspring of the control mothers (Fig. 8, A and C), with a higher rate of BW gain in males than in females. During the first 2 wk of palatable food feeding, the BW of males increased by 12.0 ± 2.8% (n = 5) of the initial weight and became significantly higher than the BW of males fed standard chow (Fig. 8A); however, female BW increased only 4.1 ± 1.4% (n = 5, P < 0.05, Student’s t-test, between males and females). Leptin administration to dams at the end of pregnancy affected the response to palatable food in male and female offspring differently. No interaction was observed between “maternal treatment” and “diet” in female offspring: females born to both leptin-treated and control mothers developed obesity on the palatable diet with the same rate of BW gain (Fig. 8, C and D). In contrast, a significant interaction was detected between “maternal treatment” and “diet” in male offspring (3-way ANOVA, P < 0.01), indicating a different response to palatable food feeding in males born to control and leptin-treated mothers. Leptin administration at the end of pregnancy inhibited obesity development induced by the palatable diet in male offspring (Fig. 8B). Specifically, on the palatable diet BWs of males born to leptin-treated mothers were lower than BWs of males born to control mothers (P < 0.05, 2-way ANOVA, factors “maternal treatment” and “age”) and were the same as the BWs of control diet males that were born to leptin-treated mothers.

Thus prenatal exposure to high maternal leptin levels at the end of pregnancy decreased BW after weaning in both males and females, decreased FI on the standard diet, and inhibited obesity induced by the palatable diet only in males.

**Discussion**

In this study, we examined the influence of chronically (genetic model) and transitory (leptin injection) elevated leptin levels during pregnancy on metabolic characteristics and sus-
ceptibility to DIO in progeny mice. The advantages of using a genetic model (C57BL/6J-A^y female mice) include the ability to imitate hyperleptinemia (which is observed in obesity) and to separate the influence of elevated leptin levels from the influence of other abnormalities that accompany obesity. Furthermore, this model restricts the programming influence of maternal leptin to the prenatal stage of development.

According to our previous results (27), leptin levels in pregnant A^y mice were twofold higher than in a/a mice; however, BW was only slightly higher at the end of pregnancy. We minimized the impact of the other factors that influence the progeny phenotype, such as genetic background (26), maternal diet (7, 17), litter size, and individual maternal body conditions (38) because the dams belonged to the same line and were fed the same standard diet and because only one male and/or one female from the same mother and only from litters of six pups were studied. In addition, the use of this model allowed us to avoid the impact of stress associated with experimental manipulations (surgery, injections) on progeny development. Therefore, we assume that the influence of the A^y mutation in mothers on progeny phenotypes is related to fetal exposure to high levels of maternal leptin. To confirm this assumption and to reveal the significance of the late stage of prenatal development on the programming effect of maternal leptin, we administered leptin to pregnant a/a dams at the end of pregnancy.

The leptin injection at the end of pregnancy did not recapitulate the effects of the A^y mutation completely; however, both enhanced leptin levels during the entire pregnancy period (A^y mice) and leptin injections at the end of pregnancy had a marked effect on metabolic phenotype in the offspring. These data directly confirm the suggestion that maternal leptin is a factor involved in developmental programming (48).

Both the A^y mutation in mothers and the leptin injection in pregnant dams more strongly affected male than female offspring; therefore, the changes in maternal leptin levels during pregnancy may contribute to sex-specific programming effects of maternal diet (40), maternal obesity (33), or maternal undernutrition (34). However, the underlying mechanisms remain to be elucidated.

In the male progeny of both A^y and leptin-treated mothers, prenatal exposure to high maternal leptin levels did not predispose to the development of obesity on the standard diet, did not increase the risk of obesity on the HF diet in the offspring of A^y mothers, and protected the offspring of leptin-treated mothers on the palatable sweet and fat diet from the development of obesity. A protective effect of leptin administration to the dams at the end of pregnancy suggests that the late period of fetal development is sensitive to the programming action of leptin and the leptin effects in late pregnancy are crucial to program susceptibility to DIO.

Whether chronic hyperleptinemia in A^y mothers protects offspring from DIO remains unknown, because the HF diet used in our experiment did not induce evident obesity development during 10 wk of treatment, most likely because of diet composition. In C57BL mice, the development of obesity strongly depends on dietary fat types, and only a highly saturated fat diet is obesogenic (50). In the progeny of leptin-treated mothers, we used a palatable diet with high saturated fat (lard) and high carbohydrate contents, which is more effective to induce obesity. Therefore, further investigations with the
palatable diet are needed to determine whether prenatal exposure to chronic hyperleptinemia will protect the male progeny of Ay mothers from DIO.

Overall, our data are in accordance with results obtained in rats that have indicated that prenatal exposure to high maternal leptin levels protects male offspring from DIO (43, 44). However, a large body of evidence suggests that maternal obesity predisposes offspring to development of obesity when dietary fat and caloric density are increased (29, 39, 42, 51). Together with the data mentioned above (43, 44), our results contradict the idea that obesity in the offspring of obese mothers may be a consequence of elevated leptin levels in the blood of the mother. Maternal hyperglycemia and hyperlipidemia during pregnancy may represent the primary factors that contribute to inappropriate fetal programming in obese mothers (15), and the programming effect of hyperleptinemia possibly attenuates the negative effects of these factors for their progeny.

The phenotypic deviations observed in males that were prenatally exposed to maternal hyperleptinemia (enhanced ratio of EI to BW in the offspring of Ay mothers and resistance to DIO in the offspring of leptin-treated mothers) are in agreement with the paradigm of predictive adaptive response (14). High maternal leptin levels may be interpreted by the fetuses as a high nutritional state and result in developmental trajectories that produce phenotypes better suited for food abundance and, possibly, less suited for food deficiency. Our results confirm the last suggestion: Maternal hyperleptinemia reduced resistance to fasting in offspring. Males born to Ay mothers displayed less weight gain than males born to control mothers and did not increase their cumulative FI during 24 h of refeeding after food deprivation; however, males born to control mothers demonstrated hyperphagia. Fasting-induced hyperphagia is believed to be related to the induction of the expression of the orexigenic neuropeptides NPY and AgRP in the hypothalamus. This induction occurs quickly upon fasting and has been associated with changes in leptin and glucose levels (35). The regulation of FI at the beginning of eating was not affected by the maternal genotype: The circulating levels of glucose and leptin and the expression of AgRP and NPY were similar at the end of food deprivation, and during the first 3 h of eating the FI was also similar in males born to Ay and control mothers. FI differences between males born to Ay and control mothers were observed later when fasted animals had assuaged their hunger and ate similarly to the control animals (Fig. 4). This effect is potentially due to programming the formation of the satiety stimuli that are related to food digestion and gastric and intestinal distension.

The observed changes in regulation of energy homeostasis in the males prenatally exposed to high maternal leptin levels can be related, at least partially, to maternal influences on leptin signaling, namely, on hypothalamic sensitivity to leptin and on the regulation of leptin levels in blood. Undoubtedly, the other
aspects of energy balance regulation that were not studied in this work may be affected by maternal leptin levels, such as animal physical activity and/or the efficiency of food utilization. Leptin is known as a key regulator of energy balance (22), and it decreases EI and increases energy expenditure, inducing thermogenesis in brown adipose tissue via various pathways that may also regulate FI, while other pathways signal independently of appetite regulation (11). We discovered the influence of elevated maternal leptin on the sensitivity to leptin in male offspring; however, this effect was altered depending on the feeding conditions. When fed ad libitum, male offspring of A/α mothers had decreased sensitivity to leptin administration in relation to food consumption; however, the leptin influence on BW was unchanged. These results support data from experiments using rats reported by other authors. Leptin administration does not affect FI but reduces BW in rats treated with a HF diet and in the progeny of rats that were undernourished during pregnancy (24). The increased ratio of EI to BW in males from A/α mothers may be related to an inhibition of the anorectic effects of leptin combined with its unchanged thermogenic effects.

The maternal downregulation of leptin signaling in fed male progeny was not realized via the inhibition of hypothalamic leptin receptor expression, and we did not observe modulation of leptin-related neural peptide expression in the hypothalamus; the expression of AgRP, POMC, and Ob-Rb was similar in males born to control and A/α mothers in both the control and leptin-treated groups. However, we did not measure hypothalamic STAT3 expression and phosphorylation after leptin administration, which is a precise method for detecting changes in hypothalamic sensitivity to leptin (9). Therefore, additional experiments need to be performed to reveal whether programming effects of enhanced maternal leptin at the prenatal stage occur via the modulation of leptin action in the hypothalamus or in other targets of the offspring.

In contrast to the fed state, leptin administration did not affect FI but influenced BW gain upon refeeding after food deprivation in the offspring of A/α and control mothers: Males born to control mothers significantly reduced their BW gain, whereas males born to A/α mothers did not change their BW gain after leptin administration. Leptin plays an important role in regulating energy expenditure (11); therefore, leptin resistance may reduce the ability to adjust energy expenditure to feeding conditions.

We also found that the rate of recovery of normal blood leptin levels after the intraperitoneal injection of leptin in the fed state was faster in males born to A/α mothers compared with males born to control mothers. This finding suggests that not only leptin signaling but also regulation of leptin circulation in the blood may be affected by maternal leptin.

The mechanisms involved in the programming actions of maternal leptin remain to be elucidated. In mice, leptin has been shown to influence brain development during both the prenatal and early postnatal stages of ontogenesis (3, 47). The
modulation of leptin levels in fetuses and newborns may affect the development of neural pathways related to energy homeostasis regulation, and it has been shown in rats that maternal nutrition during pregnancy influences leptin circulation in pups (6, 31). However, we did not observe any correlation between maternal and progeny leptin levels. Irrespective of the levels of leptin in maternal blood, leptin levels in the progeny of A2 and control mothers were similar during both prenatal and postnatal stages, which suggests that maternal blood leptin may influence fetal development by affecting placenta functions. Leptin has been shown to influence placental development and global placental gene expression profiles (41), placental angiogenesis (19), and placent al nutrient transport (20, 49).

Perspectives and Significance

Our data support the hypothesis that maternal leptin is a factor involved in developmental programming. We have shown that hyperleptinemia during pregnancy has sex-specific long-term effects on the regulation of energy balance in progeny, consistent with the hypothesis of “predictive adaptive response.” Hyperleptinemia can program the male offspring to develop phenotypes that include an increased ratio of EI to BW, decreased sensitivity to leptin, and decreased resistance to food deprivation; however, hyperleptinemia does not contribute to an enhanced risk of developing obesity. More importantly, we have found that leptin administration at the end of pregnancy protects male offspring from DIO. These data demonstrate that leptin effects in late pregnancy are crucial to programming susceptibility to DIO. Therefore, the period of late fetal development may be an ontogenetic window for therapeutic interventions. A dissection of the pathways by which maternal leptin modifies offspring development is necessary to understand the mechanisms underlying developmental programming and to suggest new strategies for prenatal correction of metabolic diseases.

REFERENCES


DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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