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Leptin-Independent Programming of Adult Body Weight and Adiposity in Mice

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Abstract
Low birth weight and rapid postnatal weight gain are independent and additive risk factors for the subsequent development of metabolic disease. Despite an abundance of evidence for these associations, mechanistic data are lacking. The hormone leptin has received significant interest as a potential programming factor, because differences in the profile of leptin in early life have been associated with altered susceptibility to obesity. Whether leptin alone is a critical factor for programming obesity has, until now, remained unclear. Using the leptin-deficient ob/ob mouse, we show that low birth weight followed by rapid catch-up growth during lactation (recuperated offspring) leads to a persistent increase in body weight in adult life, both in wild-type and ob/ob animals. Furthermore, recuperated offspring are hyperphagic and epididymal fat pad weights are significantly increased, reflecting greater adiposity. These results show definitively that factors other than leptin are crucial in the programming of energy homeostasis in this model and are powerful enough to alter adiposity in a genetically obese strain.

Numerous epidemiological studies associate poor intrauterine growth with an increased risk of metabolic disease in adult life. In particular, infants born of low birth weight who then grow rapidly during early life are at greatest risk of developing central obesity, insulin resistance, and cardiovascular disease (1–3). These findings are recapitulated in animal models of intrauterine growth restriction (IUGR). Offspring typically have a greater degree of adiposity, exhibit increased blood pressure, and develop glucose intolerance. Furthermore, as in humans, the combination of IUGR and rapid growth in early life, so-called catch-up growth, is found to exacerbate these programming effects (4–7). Despite these robust associations, very little is known about the mechanisms that might link altered perinatal growth patterns with adult disease.

Recently, the hormone leptin has received a great deal of attention as a potential programming factor. This adipocyte-derived hormone plays a key role in the regulation of energy balance in the adult through the stimulation of energy expenditure and inhibition of food intake (8). During the perinatal period, however, the actions of leptin are quite different. In both rats and mice, there is a transient rise in circulating leptin concentrations in neonatal animals (9, 10). Despite these high concentrations, leptin does not significantly affect feeding or metabolic pathways until after the second postnatal week (11, 12).
elevated levels of leptin in early life are proposed to play a role in brain development (13–17), specifically in the development of neuronal projections from the arcuate nucleus of the hypothalamus to other regions of the hypothalamus involved in the regulation of energy balance. Studies in ob/ob mice have shown that leptin-deficient animals exhibit impaired development of these hypothalamic projections and that leptin treatment during a critical postnatal period, encompassing the normal leptin surge, can in part restore these projections and lead to a partial normalization of food intake at an early postweaning age (16).

From a programming perspective, differences in circulating leptin concentrations during the early postnatal period have been a focus of a number of studies as a potential causative factor underlying associations between early growth and later risk of disrupted energy homeostasis and metabolic disease. However, results have been conflicting. In mice, IUGR offspring of food-restricted dams exhibited an advanced and amplified leptin surge that was associated with an increased susceptibility to high-fat diet-induced obesity after weaning (18). Exogenous leptin administration, attempting to mimic this premature surge, recapitulated these outcomes in control offspring, inferring a causal link between early-life leptin and diet-induced weight gain. In contrast, a different model of IUGR in rats found that administration of leptin during the postnatal period prevented increased susceptibility to diet-induced obesity in these offspring (19). A series of studies by Pico and colleagues (20) showed that oral doses of leptin within the physiological range, given during lactation, reduces body weight gain and adiposity in adult rats. Thus, the long-term effects of alterations in postnatal leptin exposure remain controversial, and indeed, it is unclear whether changes in this hormone are key in programming offspring phenotype.

In the present study, we aimed to determine definitively whether IUGR followed by rapid postnatal growth increased body weight by leptin-dependent mechanisms. To assess this, we used our previously reported prenatal low-protein mouse model of IUGR followed by postnatal cross-fostering and litter size reduction, in which low birth weight offspring grow rapidly during the suckling period and exhibit permanent increases in body weight as adults (so-called recuperated mice) (5, 21). Using a heterozygous ob/+ mating strategy, male wild-type and ob/ob offspring from control and recuperated litters were studied from weaning until 15 wk of age. We recently determined that across a range of ages during suckling, ob/ob neonates have undetectable levels of serum leptin (22). Therefore, any programming outcomes in the ob/ob recuperated offspring must be due to leptin-independent effects.

**Materials and Methods**

**Animals and dietary manipulation**

All procedures involving animals were conducted in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act, 1986. Male and female mice heterozygous (ob+/+) for the leptin gene mutation were mated overnight and examined the following morning for the presence of a vaginal plug. Pregnant females were then transferred to fresh cages and placed on either a 20% protein diet (control diet) or an isocaloric low-protein diet (8%), as described in detail previously (23). Females were housed individually, maintained at 22°C, and had free access to water and their allocated diet for the remainder of gestation. Litter size was not altered by maternal diet (8.1 ± 0.3 vs. 7.5 ± 0.3 pups per litter for control and low-protein pregnancies, respectively; \( P > 0.05 \)). To induce catch-up growth after IUGR, pups born to low-protein dams were cross-fostered on postnatal d 3 (P3) to control-fed females and litter sizes reduced to four pups per dam (recuperated offspring). This manipulation maximizes nutritional availability, thus driving rapid catch-up growth. Control litters were those born to and suckled by dams fed the 20% protein diet, and litter size, if greater than eight pups, was reduced to eight animals at P3. Pups were weighed on the day of cross-fostering (P3) and then at P7 and P14 and at weaning.
Genotyping for the wild-type and mutant ob gene was performed by PCR, as described previously (24). At P21, male wild-type and ob/ob offspring were weaned onto a standard laboratory chow diet (23). From weaning, animals were housed individually and body weight and food intake monitored weekly before metabolic phenotyping (see below). Food was weighed at the beginning and end of the week for each animal from 4–12 wk of age to determine the number of grams of food eaten. A total of 17 control and 25 recuperated litters were generated for the present study, and heterozygous offspring and female animals were not studied after weaning.

Metabolic assessment and determination of body composition

At 14 wk of age, animals were placed in a computer-controlled, open-circuit system (Oxymax System) that was part of an integrated Comprehensive Laboratory Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH) in which oxygen consumption, carbon dioxide production, and ambulatory activity were measured over a 72-h period. Chambers had integrated photobeams to assess locomotor activity. Animals were acclimatized to monitoring cages for 72 h 1 wk before data collection (i.e. at 13 wk of age) and were weighed before and after the acclimatization and experimental periods. One week after experimental measurements were made (i.e. at 15 wk of age, 105 ± 5 d), animals were tail bled (for measurement of blood glucose with One-Touch Ultra blood glucose monitoring system; Lifescan, High Wycombe, UK) and then killed by overdose of anesthetic, blood samples were collected by cardiac puncture, body composition was measured using dual-energy x-ray absorptiometry (Lunar PIXImus; GE Medical Systems, Fitchburg, MA), and finally, tissues and organs were weighed and collected.

Measurement of serum hormones and blood lipids

Serum leptin and insulin concentrations were measured by commercial ELISA (CrystalChem, Downers Grove, IL), carried out according to the manufacturer’s instructions. For insulin determination, serum from ob/ob offspring was diluted 1:10 to obtain values within the standard curve. Serum leptin was undetectable in any ob/ob animal. In each case, all samples were measured in a single assay, and an intraassay coefficient of variance of less than 10% was achieved. Blood lipids were measured in the Mouse Biochemistry Laboratory located at Addenbrooke’s Hospital, Cambridge, UK.

Statistics

Body weight and food intake data were analyzed by repeated-measures two-way ANOVA, followed by Duncan’s post hoc testing where appropriate. Tissue and organ weights and serum hormone and metabolite data were analyzed using a generalized linear model (GLM), testing the effects of genotype and maternal diet. Where a significant interaction was detected between genotype and maternal diet by GLM, this precluded the testing of differences using the model, and data within each genotype group were therefore analyzed separately by one-way ANOVA. Nonparametric serum insulin data were log-transformed before analysis by GLM and are presented as geometric means (95% confidence intervals). All other results are presented as mean ± SEM, and P values less than 0.05 were considered significant. Metabolic data (energy expenditure and oxygen consumption) were analyzed using multiple linear regression to estimate the association between energy expenditure/ oxygen consumption and maternal diet group and genotype. The models were adjusted for body weight, fat mass, and lean mass to take into account the greatly varying body weight and composition of wild-type and ob/ob mice. All statistical analyses were performed using Stata/SE version 11.0 for Windows (StataCorp LP, College Station, TX).
Results

Postnatal growth trajectories

Offspring of ob/+ low-protein-fed dams were significantly lighter compared with offspring of ob/+ controls at P3, before cross-fostering (1.56 ± 0.04 vs. 1.69 ± 0.03 g; P < 0.01, Fig. 1A). These observations were consistent with our previously reported body weights of wild-type IUGR mice (23). After cross-fostering and litter size reductions, recuperated offspring grew more rapidly and were significantly heavier than control offspring at both P14 and P21 (P < 0.001; Fig. 1A). No differences in litter size or male to female ratios were found between diet groups (data not shown). As shown in Fig. 1B, the body weight of ob/ob offspring diverged at 7 wk of age, such that they were significantly heavier than wild-type animals from this point onward (P < 0.001). In addition, IUGR followed by rapid catch-up growth during lactation (recuperated animals) led to a permanent increase in body weight in adult life compared with control animals, and this was significant for both wild-type and ob/ob offspring (overall effect of diet, P < 0.01; Fig. 1B). Thus, body weight was increased in recuperated ob/ob offspring in the absence of any alterations in postnatal leptin profile induced by IUGR and/or rapid early growth. These differences in body weight were accompanied by increased food intake in recuperated animals. Analysis of cumulative food intake between 4 and 12 wk of age revealed that, in addition to the extreme hyperphagia of ob/ob mice, recuperated offspring ate significantly more than their control counterparts in both wild-type and ob/ob animals (Fig. 2).

Body composition and tissue weights at 15 wk of age

Analysis of body composition by dual-energy x-ray absorptiometry scan showed the expected differences between wild-type and ob/ob animals at 15 wk of age, namely an increase in percent body fat mass and a reduction in percent lean mass in ob/ob offspring. In ob/ob animals, approximately 60% of total body weight is fat mass, compared with around 20% in wild-type animals (P < 0.0001). Overall, recuperated animals had a reduction (P < 0.05) in percent lean mass (control wild-type, 79.1 ± 1.0%; recuperated wild-type, 77.1 ± 0.9%; control ob/ob, 41.0 ± 1.0%; recuperated ob/ob, 37.5 ± 1.8%) and a tendency (P = 0.09) for an increase in percent fat mass (control wild-type, 20.9 ± 1.0%; recuperated wild-type, 22.9 ± 0.9%; control ob/ob, 57.8 ± 1.4%; recuperated ob/ob, 60.0 ± 1.3%). When collecting tissues at 15 wk of age, we were unable to reliably remove all discrete adipose depots from ob/ob offspring, because the massive lipid accumulation in these animals precluded dissection of mesenteric or retroperitoneal adipose tissue. However, epididymal adipose tissue could be clearly identified and cleanly isolated from all animals, and therefore the weight of this depot was used to compare visceral adiposity between the different diet and genotype groups. Both in absolute terms (data not shown) and as a percentage of body weight (Fig. 3), ob/ob animals had a significant increase in epididymal adipose weights compared with wild-type (P < 0.0001), and in addition, there was a significant increase in epididymal adipose weight in recuperated offspring, an effect that was seen in both genotypes (P < 0.05, Fig. 3). Furthermore, in wild-type offspring, each of the epididymal, mesenteric, and perirenal adipose tissue depots were collected, and there was again a significant increase in total abdominal fat in recuperated offspring compared with control (Supplemental Fig. 1, published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org). Of other tissues collected, expected differences between wild-type and ob/ob animals were also noted for brain, kidney, liver, spleen, and thymus weights, and in line with recent data from our laboratory, we again confirmed our previous findings that recuperated animals have increased heart and spleen weights (23) (Supplemental Table 1). Analysis of blood characteristics (Table 1) revealed significant hyperglycemia, hyperinsulinemia, hypercholesterolemia, and increased free fatty acids in ob/ob offspring. There was no effect of early nutrition on any of these parameters, but in wild-type animals,
recuperated offspring had a tendency ($P = 0.07$) for increased serum leptin concentrations, consistent with their increased adiposity.

**Analysis of energy metabolism at 14 wk of age**

Both oxygen consumption and energy expenditure were greater in *ob/ob* animals compared with wild-type in absolute terms, and in addition, recuperated offspring also had a small but significant elevation in these metabolic parameters (Supplemental Table 2). In line with recent reports on appropriate analysis of metabolic data in animals with large differences in body weight and body composition (25, 26), oxygen consumption and energy expenditure data were further analyzed by regression analysis. Once adjusted for differences in body weight, fat mass, and lean mass, there was no effect of genotype on either oxygen consumption or energy expenditure. Lean mass, as would be expected, was a major determinant of both oxygen consumption and energy expenditure (full regression models in Supplemental Tables 3 and 4). Recuperated animals had very small but significantly increased oxygen consumption and energy expenditure compared with control animals ($P < 0.05$), which remained even after adjustments for the differences in body weight and composition. This suggests that the increased adiposity of recuperated animals is not due to a reduction in energy expenditure but is likely driven by differences in food intake.

**Discussion**

The most striking finding of the present study was that nutritionally induced IUGR followed by rapid growth during lactation is powerful enough to induce a permanent increase in body weight and adiposity in the genetically obese *ob/ob* mouse. These data demonstrate that the programming effects of this nutritional manipulation operate through leptin-independent pathways and thus suggest that alterations in the postnatal leptin surge are not the crucial signal in this model for long-term effects on energy homeostasis. The results also suggest that leptin-independent mechanisms invoked during rapid catch-up growth drive an increase in adipose tissue accumulation that persists into adult life.

Our findings contrast with those of Yura and colleagues (27), who reported that IUGR *ob/ob* offspring did not exhibit an increase in body weight in adult life compared with their control counterparts. This may be due to different means of inducing IUGR in the two studies (global reduction in nutrition vs. low-protein diet); however, the key difference is in the postnatal nutritional manipulations. As in human studies, it is accelerated growth in early life that is particularly detrimental in terms of increasing subsequent adiposity (2). Although it was reported that normalized nutrition of IUGR offspring during suckling resulted in catch-up growth, by weaning, body weights were equivalent to that of controls. In our model, cross-fostering and litter size reduction significantly accelerated growth during the suckling period, such that an increase in body weight compared with control offspring was evident as early as 14 d of age. We have shown previously in mice that this increased body weight is maintained in recuperated animals throughout adult life (5, 21); however, the present report is, to the best of our knowledge, the first example of a programming effect on *ob/ob* offspring body weight purely through nutritional manipulation in early life.

The increased food intake in recuperated animals observed in the present study is suggestive of altered regulation of central energy balance pathways. Importantly, this appears to be, at least in part, independent of leptin, because *ob/ob* recuperated offspring display hyperphagia compared with *ob/ob* controls. Previous studies have shown that elevated insulin during early life in rats (either through postnatal overfeeding or infusion of insulin directly to the neonate) is associated with aberrant hypothalamic morphology, perturbed levels of feeding neuropeptides, and increased body weight gain in adult life (28, 29). Given these data, and that leptin does not appear to be the critical mediator of long-term increases in adiposity, we
speculate that a similar insulin-dependent mechanism may be operational in the present model. Clearly, more studies are required to determine whether in the ob/ob recuperated animals there are alterations in central energy balance pathways.

In IUGR offspring, early postnatal catch-up growth has previously been identified as key in the subsequent programming of adult phenotype (30). In humans, although both lean and fat mass are reduced at birth in IUGR infants, during catch-up growth, fat mass is preferentially accumulated, and it is this increased adipose deposition that is predictive of later adult disease (1, 31). Despite a large body of evidence showing that rapid postnatal growth increases later adiposity, very little is known about the underlying mechanisms. It is possible that hyperinsulinemia during the postnatal period may also promote preferential accumulation of adipose tissue via increased adipocyte insulin action. In adult rodents, semistarvation followed by refeeding induces preferential adipose deposition, mediated in part through a reduction in skeletal muscle thermogenesis and accompanied by a reduction in muscle insulin sensitivity but increased insulin action in adipose tissue (32, 33). It has been suggested that a similar process that favors glucose utilization in adipose tissue and reduces growth of lean body mass might underlie early life catch-up growth (34). Consistent with this, recent studies in mice have shown that catch-up growth after IUGR results in a significant up-regulation of lipogenic gene expression in epididymal adipose tissue at 3 wk of age (6). This again could be driven by increased insulin action in adipose tissue.

In conclusion, we have demonstrated that factors induced by postnatal catch-up growth, other than leptin, are crucial in the nutritional programming of energy homeostasis and that their effects are powerful enough to alter weight gain, adiposity, and food intake in a genetically obese mouse. The identity of these factors is not known, but there is evidence from other models to suggest insulin as one candidate. Further understanding of such processes will help in the definition of how exposures during critical time windows impact on our long-term metabolic health, and this could ultimately help resolve the burden of common conditions such as type 2 diabetes and obesity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

GLM Generalized linear model
IUGR intrauterine growth restriction
P3 postnatal d 3

References


FIG. 1.
Body weights of control and recuperated offspring. A, Preweaning growth curves of control (solid line) and recuperated (dotted line) offspring. Note that because we cannot separate genotypes before 2 wk of age, average body weights within each litter were determined (n = 17–25 per group). Data are mean ± SEM. *, P < 0.05; ***, P < 0.001 recuperated vs. control offspring. B, Postweaning growth trajectory in control (solid line) and recuperated (dotted line) wild-type (∎) and ob/ob (▲) offspring from weaning at 3 wk until 15 wk of age (n = 12–19 per group). Data are mean ± SEM. **, P < 0.01; ***, P < 0.001, recuperated vs. control offspring, wild-type mice; ^, P < 0.05; ^*, P < 0.01, recuperated vs. control, ob/ob offspring.
FIG. 2.
Cumulative food intake from weaning until 12 wk of age in control (black bars) and recuperated (white bars) wild-type and ob/ob offspring (n = 12–19 per group). Data are mean ± SEM. *, P < 0.05; ***, P < 0.001 vs. same-genotype control animals.
FIG. 3.
Body composition at 15 wk of age. Epididymal adipose tissue as a percentage of body weight in control (black bars) and recuperated (white bars) at 15 wk of age (n = 12–19 per group). Data are mean ± SEM. *, P < 0.05 vs. same-genotype control animals.
TABLE 1
Serum hormone and blood lipid levels in offspring at 15 wk of age

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>ob/ob</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Recuperated</td>
<td>Control</td>
</tr>
<tr>
<td>Fed blood glucose</td>
<td>14.3 ± 0.91</td>
<td>12.2 ± 0.79</td>
<td>18.4 ± 1.45</td>
</tr>
<tr>
<td>(mmol)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum insulin</td>
<td>235 (396, 75)</td>
<td>128 (180, 76)</td>
<td>1014 (1547, 480)</td>
</tr>
<tr>
<td>(pmol)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum leptin</td>
<td>5.91 ± 1.35</td>
<td>8.07 ± 1.42</td>
<td>ND</td>
</tr>
<tr>
<td>(ng/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>2.07 ± 0.14</td>
<td>2.41 ± 0.13</td>
<td>8.08 ± 0.49</td>
</tr>
<tr>
<td>(mmol/liter)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL</td>
<td>0.19 ± 0.03</td>
<td>0.34 ± 0.05</td>
<td>3.24 ± 0.54</td>
</tr>
<tr>
<td>(mmol/liter)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL</td>
<td>1.38 ± 0.09</td>
<td>1.57 ± 0.08</td>
<td>3.40 ± 0.21</td>
</tr>
<tr>
<td>(mmol/liter)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.21 ± 0.12</td>
<td>1.09 ± 0.08</td>
<td>1.46 ± 0.15</td>
</tr>
<tr>
<td>(mmol/liter)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>0.64 ± 0.04</td>
<td>0.66 ± 0.04</td>
<td>0.95 ± 0.12</td>
</tr>
<tr>
<td>(mmol/liter)</td>
<td></td>
<td></td>
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</tbody>
</table>

Data are means ± SEM or means (95% confidence intervals) for serum insulin values (n = 8–10 per group). HDL, High-density lipoprotein; LDL, low-density lipoprotein; NS, not significant.