ABSTRACT

Maternal undernutrition in pregnancy is associated with intrauterine growth restriction (IUGR). With enhanced nutrient support, IUGR newborns may demonstrate a rapid catch-up growth and, paradoxically, the development of obesity in adult offspring. As hypothalamic control of appetite is likely set during the fetal or neonatal period, nutrient stress and perhaps nutrient enhancement during these periods may alter appetite setpoints and contribute to programming of adult obesity. We postulated that the degree of nutrient enhancement during the newborn period may modulate the programming of appetite regulating hormones, body composition and the propensity to adult obesity in IUGR newborns. Pregnant rats received from day 10 to term gestation and throughout 21 days of lactation either ad libitum (AdLib) food (control, N=12) or 50% food-restriction (FR, N=12) to produce IUGR newborns. The two groups of offspring were studied at day 1 (AdLib vs FR), and to create two distinct model groups of newborn catch-up growth (immediate, delayed) among the IUGR newborns, half of the IUGR pups born to FR mothers were cross-fostered to AdLib dams whereas the other half were nursed by their own FR dams. Similarly, half of the pups born to AdLib mothers were nursed by AdLib dams, and half cross fostered to FR dams. Thus there were 4 groups of pups at 3 weeks: the IUGR immediate catch-up growth (FR/AdLib); IUGR delayed catch-up growth (FR/FR); Control (AdLib/AdLib), and a lactation FR control group (AdLib/FR). From 3 weeks to 9 months of age, all offspring were provided ad libitum rat chow. Daily body weights and food intake from weaning to 9 months was recorded. Body composition was determined by DEXA at 3 weeks and 9 months. Plasma leptin and ghrelin levels were analyzed at ages 1 day, 3 week and 9 month. Maternal FR during pregnancy resulted in IUGR pups (6.0±0.3 vs 7.1±0.3 g, p<0.01) with decreased leptin (0.66±0.03 vs 1.63±0.12 ng/ml, p<0.001) and increased ghrelin levels (0.43±0.03 vs 0.26±0.02 ng/ml, p<0.01). Maternal FR during lactation (FR/FR)
further impaired the IUGR offspring growth at 3 weeks. However, by 9 months of age these pups had attained normal body weight, % body fat and plasma leptin levels. Conversely, IUGR offspring nursed by AdLib dams (FR/AdLib) exhibited rapid catch-up growth at 3 weeks, and continued accelerated growth resulting in increased weight, % body fat, and plasma leptin levels. These results suggest that the degree of newborn nutrient enhancement and timing of IUGR newborn catch-up growth, may determine the programming of orexigenic hormones and offspring obesity.

**Key Words:** Appetite regulating hormones, leptin, ghrelin, catch-up growth, maternal food-restriction.
INTRODUCTION

There is a well recognized epidemic of adult obesity in western society. In the United States current data indicate that more than 20% of adults are clinically obese (BMI \( \geq 30 \text{ kg/m}^2 \)) and an additional 30% are overweight (BMI \( \geq 25 \text{ kg/m}^2 \)) (15, 29). The rates of obesity have increased markedly from 1986 to the present time and give no indication of abating (24). In conjunction with the prevalence of adult obesity, childhood and adolescent obesity has increased at an alarming rate with approximately 20% of children now classified as obese (6, 17). There have been numerous postulates as to the etiology of the obesity "epidemic", with the majority attributing to the availability of a high fat, high caloric diet. Barker et al hypothesize a mechanism of developmental programming of adult offspring, such that small for gestational age newborns developed a "thrifty phenotype", which predisposed them to increased weight gain as offspring (1, 12). Paradoxically, small for gestational age offspring have not only an increased risk of obesity, but a notable risk of hypertension, diabetes, and cardiovascular disease as adults (2, 9, 11, 20). The potential for these adult onset diseases has been demonstrated in humans and a variety of animal models including sheep and rats (1, 4, 7, 13, 27).

Maternal rats have been widely used as a model for creation of intrauterine growth restricted (IUGR) newborns. Undernutrition or malnutrition of maternal rats during select periods of pregnancy has been demonstrated to result in newborn pups that are growth restricted (8, 28). When provided appropriate newborn nutrition, these growth restricted newborn pups may demonstrate rapid catch-up growth, such that their body weights exceed that of controls by 12 weeks of postnatal life (8, 16, 26). The mechanism for the rapid catch-up growth and the eventual development of obesity is unclear. However, it may include aspects of enhanced orexigenic mechanisms (appetite), reduced anorexic mechanisms, highly efficient metabolism of substrates and/or reduced energy expenditure.
As hypothalamic control of appetite is likely set during the fetal or neonatal period, nutrient stress and perhaps nutrient enhancement during these periods may alter appetite setpoints and contribute to programming of adult obesity. We postulated that the degree of newborn nutrient enhancement and timing of catch-up growth may modulate the programming of appetite regulating hormones, body composition and the propensity to adult obesity in IUGR newborns.

MATERIAL AND METHODS

Maternal Rat and Diet: A model of rat dams 50% food restricted during pregnancy was utilized. Studies were approved by the Animal Research Committee of Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center and were in accordance with the American Association for Accreditation of Laboratory Care and National Institutes of Health guidelines. First-time-pregnant Sprague Dawley rats (Charles River Laboratories, Inc, Hollister, CA) were housed in a facility with constant temperature and humidity and on a controlled 12 h light-12 h dark cycle. At 10 days of gestation, rats were provided either an ad libitum diet of standard laboratory chow (LabDiet 5001, Brentwood, MO, USA: protein 23%, fat 4.5%, metabolizable energy 3030 kcal/kg) or a 50% food restricted diet determined by quantification of normal intake in the ad libitum fed rats. The respective diets were given from 10 day of pregnancy to term and throughout the 21 day lactation period.

Maternal body weights and the food intake were recorded daily. Rat dams gave birth normally and at day 1 after birth, pups were limited to eight (4 males and 4 females) per litter to normalize rearing. The study offspring were categorized as an immediate catch-up growth and a delayed catch-up growth group.

The Offspring: As stated above, control dams (n=12) received ad libitum (AdLib) food, whereas study dams were 50% food-restricted (FR; n=12) to produce IUGR newborns. Thus, 2 groups of
offspring were studied at day 1 (AdLib vs FR). To create two groups of newborn catch-up growth (immediate, delayed) among the IUGR newborns, half of the IUGR pups born to FR mothers (n=6) were nursed after cross-fostering to AdLib dams (FR/AdLib) whereas the other half were nursed by their own FR dams (FR/FR; n=6). As the control group, half of the pups born to AdLib mothers were nursed by AdLib dams (AdLib/AdLib). To control for nutrient restriction only during lactation, half of the pups born to AdLib dams were cross fostered to FR dams (AdLib/FR). Thus there were 4 groups of pups at 3 weeks: the IUGR immediate catch-up growth (FR/AdLib); IUGR delayed catch-up growth (FR/FR); Control (AdLib/AdLib), and a lactation FR control group (AdLib/FR). At 3 weeks of age offspring in all four groups were housed individually and weaned to *ad libitum* feed.

**Body Weights and Food Intake**: Each litter from the four groups was weighed weekly, and the weight of an individual pup was calculated from it (i.e., litter weight/number of pups). The first weight was recorded at 1 d of age and subsequent weights were taken at 7, 14 and 21 days of age. Thereafter, the body weight and food intake were monitored weekly on individual basis.

**Body composition**: At 3 weeks and 9 months of age, offspring of both sexes underwent a non-invasive dual-energy x-ray absorptiometry (DEXA) scanning using DXA system with software program for small animal (QDR 4500A, Hologic, Bedford, MA, USA). In vivo scan of whole body composition was obtained, including lean and fat tissue mass, total mass and percent body fat determinations.

**Plasma Hormones**: Pups at 1 day of age were decapitated and blood collected in capillary tubes. One male and one female from each litter at ages three weeks and nine months were fasted overnight, and blood was collected via cardiac puncture in heparinized and EDTA/aprotinin (780 KIU/ml of blood) tubes for determination of plasma leptin and plasma ghrelin levels, respectively. Leptin and ghrelin concentrations were determined by a RIA using a commercial Kit (Rat Leptin...
RIA Kit, LINCO Research, Inc., St. Charles, MO, USA; and, Rat Ghrelin RIA Kit, Phoenix Pharmaceuticals, Inc., Belmont, CA, USA).

Statistical Analysis: Differences between control and the experimental groups were compared using either unpaired t-test (1 day old neonate), repeated measures of ANOVA (body weight and food intake), or ANOVA with Dunnett’s post hoc tests (body composition and plasma hormones). At ages 1 day and 3 weeks, combined data for males and females are shown since no sex differences were evident. However, at the age of 9 months, sex differences justified analyzing the data according to sex. Values are expressed as means ± SE.

RESULTS

Growth

At 1 day after birth, pups of food restricted dams weighed significantly less than pups of ad libitum fed dams consistent with IUGR (6.0 ± 0.3 vs 7.1 ± 0.3 g, \( P < 0.01 \)). The subsequent growth pattern showed either an immediate or a delayed catch-up growth, dependent upon the postnatal maternal nutrition. Thus, despite the reduced body weight at birth, by 3 weeks of age IUGR offspring exposed to dams provided ad libitum food during lactation (FR/AdLib) demonstrated markedly increased weight gain such that they exceeded the body weight of control pups. In contrast, IUGR pups that were exposed to maternal food restriction during lactation (FR/FR) remained significantly below the body weight of control pups at 3 weeks. Similar growth patterns were demonstrated by AdLib/FR pups at 3 weeks. By 9 months of age, FR/AdLib offspring were markedly heavier whereas the FR/FR and Ad Lib/FR offspring had attained body weights comparable to the controls (Figure 1).
In all groups, the male offspring had significantly higher body weights than females from the age of 4 weeks and this difference became perceptibly accentuated with advancing age (Figure 1).

**Food Intake**

In the period following weaning, the FR/AdLib offspring increased food intake compared to controls, and this trend of hyperphagia persisted throughout adult life. Conversely, the AdLib/FR and FR/FR offspring showed similar consumption of food as the control offspring (Figure 2). However, when the food intake was adjusted for body weight, all three experimental groups consumed more food than controls, which was apparent from the age of 4 to 8 weeks, after which no differences were discernable between the groups. In general, the male offspring consumed more food than females (Figure 2).

**Body composition**

At 3 weeks of age, the FR/AdLib offspring exhibited catch-up growth with differential impact on specific tissue mass. They had significantly higher body and percentage lean body mass with comparable percentage body fat to the controls. Conversely, the FR/FR and AdLib/FR offspring showed significantly reduced body mass and percentage body fat with increased percentage lean body mass (Figure 3).

Nonetheless by 9 months of age, the FR/AdLib offspring (male and female) were markedly heavier with excess percentage body fat, though, the percentage lean body mass was now significantly reduced. In contrast, the AdLib/FR and FR/FR had attained a comparable body composition as the control offspring, illustrating a catch-up growth albeit a delayed one (Figure 4). In all groups, the adult male offspring had higher body mass than female offspring, though the lean body mass and fat when adjusted for body mass was analogous. The exception was the FR/AdLib female adult offspring, which had even higher percentage body fat at 9 months of age (Figure 4).
Plasma Hormones

The influence of maternal food restriction on the appetite regulating hormones in the offspring was investigated. 1 day old neonates of food restricted dams had significantly reduced plasma leptin (0.66 ± 0.03 vs 1.63 ± 0.12 ng/ml, \(P < 0.001\)) and increased plasma ghrelin levels (0.43 ± 0.03 vs 0.26 ± 0.02 ng/ml, \(P < 0.001\)) than controls.

However, the plasma leptin levels in the FR/AdLib offspring, despite being reduced at birth, were significantly greater at 3 weeks. Furthermore, this increment was also evident at 9 months of age. Conversely, the ghrelin levels were similar to the control offspring at both 3 weeks and 9 months of age. (Figure 5).

In contrast, the FR/FR and AdLib/FR offspring had plasma leptin levels that were similar to the controls whereas the plasma ghrelin levels were significantly elevated at 3 weeks of age. This trend was still evident at 9 months of age (Figure 5).

In general, the male adult offspring had significantly higher plasma leptin and lower plasma ghrelin levels compared to female offspring.

DISCUSSION

The three novel findings of this study are of primary importance in understanding the development of obesity in intrauterine growth restricted babies. First, the rate and timing of postnatal catch-up growth plays a critical and significant role. Second, this appears to be associated with altered orexigenic and anorexigenic regulatory mechanisms. Third, the resulting phenotype has markedly different muscle versus fat mass. These results further emphasize both the plasticity and the potential of the critical appetite regulating hormones in the pathogenesis of programming-induced obesity.
In the present study, exposure to maternal food restriction *in utero* resulted in offspring with reduced body weight, decreased plasma leptin, and increased plasma ghrelin at one day of age. It appears that the appetite regulating hormones in the growth restricted pups are altered in a direction expected under conditions of “starvation”. The phenomena of low birth weight as a result of nutritional perturbations *in utero* have been established in numerous studies (1, 7, 28), and its potential risk for adult diseases has been extensively demonstrated in humans and animal models (1, 3, 11, 13). Leptin and ghrelin are involved in the hypothalamic regulation of energy homeostasis. The decreased plasma leptin among growth restricted newborns is consistent with previously reported studies (5, 10). However, this represents the first report of increased plasma ghrelin among growth restricted pups. Ghrelin is a significant appetite stimulator secreted by the stomach. These results suggest that elevated plasma ghrelin may potentiate or drive the appetite so as to facilitate rapid weight gain, and indeed these offspring exhibit hyperphagia in the immediate post-weaning period.

Whether this nutritionally compromised environment persisted or was alleviated in the postnatal period ultimately determined the phenotype of the offspring. For instance, if the growth restricted newborns were subjected to further insult, where maternal food restriction persisted through the nursing period as well (i.e., FR/FR), it resulted in a delayed catch-up growth in presence of continued elevated ghrelin levels. This reduction in the rate of postnatal catch-up growth was also observed in the control offspring reared by mothers 50% food restricted during lactation (i.e., AdLib/FR). The rate of catch-up growth was significantly reduced as these offspring remained significantly smaller than controls at three weeks of age. However, by nine months of age, the catch-up growth had reached levels of the controls. In addition, these offspring consumed more food per body weight during the early post-weaning period. As shown in Figure 1, the delayed catch-up growth pups demonstrated a similar rate of growth, though its onset shifted by
approximately three weeks. These results indicate that the mechanism of programming is less likely to be dependent upon the rate of catch-up growth and more likely involves the period of time at which the catch-up growth occurs. At nine months of age, the delayed catch-up growth group had comparable body mass, lean body mass and percent body fat. These offspring showed evidence of elevated plasma ghrelin levels, suggesting that a continued appetite orexigenic drive may potentiate further catch-up growth. Whether this growth rate would ultimately exceed that of controls at more advanced adult ages is unknown. However, these offspring exhibited normal levels of plasma leptin indicating a markedly different phenotype from those offspring that experienced only prenatal exposure to maternal food restriction (i.e., FR/AdLib).

The FR/AdLib offspring demonstrated a rapid catch-up growth and increased food intake during the early post-weaning period. More importantly, these offspring at 9 months of age had higher body mass with increased body fat and reduced percentage lean body mass. Whereas they demonstrated reduced plasma leptin at birth, the plasma leptin levels were markedly increased at three weeks and nine months, suggesting a leptin resistance was contributing towards an inhibition of anorexia. Similar leptin resistance has been demonstrated in obese humans (23, 25) as well as in offspring of rat dams undernourished in pregnancy (26). These results suggest that rapid catch-up growth may in part be related to elevated ghrelin levels, which may result in leptin resistance with increased body fat and reduced lean mass potentially leading to obesity and other metabolic disorder in later life.

Several maternal conditions have been linked to obesity in both human and rodent offspring. Paradoxically, both maternal nutrition deprivation (2, 16, 21, 26) and maternal obesity (19) produce obese offspring. The association of impaired fetal development on obesity has been clearly demonstrated in the Dutch Famine Study where poor maternal nutrition resulted in increased rates of obesity in adult males (20). In the present study, however, maternal food restriction during the
second half of gestation was associated with both male and female offspring obesity, though the impact was more profound among females. As expected, males were heavier than females, with similar percentage lean body and fat mass. In contrast to humans (14, 22), but consistent with studies on rat (18), leptin concentrations were generally higher in males than female offspring. In addition, this is the first study that notes sex difference in the levels of plasma ghrelin with males exhibiting significantly lower levels.

As rats represent an altricial species, the period of lactation has been correlated with a third trimester of human gestation. Thus, these results would suggest that nutritional interventions may be of importance during the last trimester of human pregnancy. Although one may not be able to extrapolate from rats to humans, these results suggest the potential to modify rates of catch-up growth, so as to reduce the programming of orexigenic and anorexic mechanisms.

In conclusion, these results demonstrate that offspring obesity may result from prenatal and/or newborn programming of orexigenic and anorexigenic regulatory mechanisms, and that this programming may be dependent upon the timing of nutrient reduction and nutrient availability. These results suggest that the prevention of immediate rapid newborn catch-up growth (e.g. nutrient manipulation) may reduce the risk of childhood and/or adult onset obesity.
ACKNOWLEDGMENTS

The authors acknowledge Linda Day, Stacy Behare and Glenda Calvario for technical assistance.

GRANTS

This work was supported by the National Institutes of Health K01 DK 063994, the American Heart Association 0455117Y, and the March of Dimes.
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FIGURE LEGENDS

**Figure 1**  Mean body weights of male (upper panel) and female (lower panel) offspring from 1 day to 9 months of age in controls (○), FR/AdLib (●), AdLib/FR (▼) and FR/FR (▼). The inset figures show the body weights of males and females from 1 day to 3 weeks of age. Number of animals studied per group was 24 males and 24 females (from 6 litters) till 3 weeks of age, after which all offspring were weaned onto ad libitum diet. Thereafter data is shown from 18 males and 18 females (from 6 litters) till 9 months of age. For clarity, the significance symbols at different time points are omitted from the graph. Significantly different from control: FR/AdLib, at day 1, and from 3 to 36 weeks; AdLib/FR, from 1 to 16 weeks; and FR/FR, from 1 day to 30 weeks.

**Figure 2**  Post-weaning food intake of male (upper panel) and female (lower panel) offspring from 4 to 5 months in controls (○), FR/AdLib (●), AdLib/FR (▼) and FR/FR (▼). Number of animals studied per group was 18 males and 18 females (from 6 litters). *P* < 0.01, FR/AdLib vs control offspring.

**Figure 3**  Body mass (upper panel), percentage lean body mass (middle panel) and percentage body fat (lower panel) in 3 week old offspring. Since no sex differences were evident, combined data of males (24) and females (24) from 6 litters in the 4 groups is shown. *P* < 0.001 vs control offspring.

**Figure 4**  Body mass (upper panel), percentage lean body mass (middle panel) and percentage body fat (lower panel) in 9 month old offspring. Since sex differences were evident, data for male (■) and female (□) are shown separately. Number of animals studied per group was 18 males and 18 females (from 6 litters) at 9 months of age. *P* < 0.001 vs control offspring.
**Figure 5**  Plasma leptin and ghrelin levels. At 3 weeks (upper panels), combined data for males (n=6) and females (n=6) is shown. At 9 months (lower panels), data is shown separately for 6 males (■) and 6 females (□) from 6 litters. *P < 0.001 vs control offspring.*