Hypertrophic fat cells in hypophagic intrauterine growth restricted rats without catch-up growth

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Background: Intrauterine growth restriction (IUGR) with catch-up growth increases the risk of obesity, coronary events, and insulin resistance. Our previous study showed that IUGR rats which exhibited catch-up growth had increased food intake due to altered expression of the genes encoding orexigenic (ghrelin) and anorexigenic (peptide YY, or PYY, and cholecystokinin, or CCK) peptides in the gastrointestinal tract. However, it remains unclear whether IUGR without catch-up growth leads to a decrease in food intake mediated by gut hormones.

Methods: We measured food intake, fat mass, lipids profile and evaluated glucose metabolism in IUGR rat offspring which did not show catch-up growth.

Results: The IUGR offspring ate significantly less food than control offspring because of the decrease in ghrelin. These IUGR offspring had reduced visceral fat mass but bigger lipid droplets. Serum adipocytokine levels also indicated hypertrophy of fat cells with increased leptin and decreased adiponectin.

Conclusions: These results indicate that intrauterine undernutrition changes food intake after birth that are mediated by ghrelin. These observations suggest a catch-up growth mechanism due to hyperphagia and provide insight into the risk of metabolic disorders in IUGR with and without catch-up growth.

Key words: maternal food restriction, intrauterine growth restriction, adipocytokine, ghrelin, rat

Introduction

Fetal growth is dependent on many factors including genetic, placental, and maternal factors. Maternal undernutrition during gestation reduces placental weight and nutrition supply to fetus, in particular key nutrients such as amino acids and glucose. This can lead to a disruption of normal fetal development and intrauterine growth restriction (IUGR).1-3 Extensive data have shown that IUGR offspring have increased hypertension and decreased glucose uptake in response to insulin in later life.4,5 IUGR with catch-up growth also leads to an increase in obesity, insulin resistance and plasma leptin levels, as well as increased risk of coronary events.6-7 Furthermore, it has been observed that the amount of visceral fat in catch-up IUGR children is already excessive by 4 years of age.8 Catch-up growth in weight is excessive weight gain, and catch-up growth is a known metabolic risk factor in IUGR.9-11 Catch-up growth in most IUGR children occurs in early postnatal life, while approximately 15% of IUGR children remain small in terms of weight through to adult life.12 A previous report has shown that the absence of catch-up growth in IUGR programs improves insulin sensitivity.13 To date there are few reports on the metabolic status of this IUGR subgroup that do not exhibit catch-up growth. Marcio et al. reported that a low plasma level of adiponectin is one of several independent predictors of glucose intolerance.14 Recent evidence implicates leptin in the regulation of glucose homeostasis. Additional data is required about not only glucose tolerance but also adipocytokines regarding IUGR without catch up growth.15
the mechanism of catch-up growth is still unclear, although it has been attributed to hyperphagia in some studies. Ghrelin is an orexigenic peptide produced by the stomach. Cholecystokinin (CCK) is an anorexigenic peptide synthesized in the duodenum and jejunum, and peptide YY (PYY) also suppresses appetite and is secreted from ileum and colon. Ghrelin stimulates appetite and increases food intake, while PYY and CCK reduce food intake. Our previous study showed the IUGR adult rats had increased food intake due to the altered expression of the genes encoding orexigenic (ghrelin) and anorexigenic (PYY and CCK) peptides in the gastrointestinal tract and also exhibited obesity. However, it remains unclear whether IUGR without catch-up growth leads to a decrease in food intake mediated by gut hormones. In the present study, we utilized an IUGR rat model by restricting maternal diet (50% of ad libitum) during gestation. We investigated the impact of IUGR without catch-up growth on food intake, to see whether or not this is influenced by gut hormones, and to find out whether or not this influences risk factors associated with IUGR. Therefore, we measured food intake, gut hormones, fat volume, glucose tolerance, and adipocytokine profiles.

Materials and Methods

Animals and experimental procedures

All experiments were conducted in accordance with the Rules of Animal Experimentation and the Guide for the Care and Use of Laboratory Animals published by Hamamatsu University School of Medicine. This research program was approved by the Animal Experimental Committee at Hamamatsu University School of Medicine.

Male and female Sprague-Dawley rats, 8 weeks of age, were purchased from Nippon SLC (Hamamatsu) and were housed in a facility with temperature maintained at a consistent 22°C and a 12/12 hour light/dark cycle. All rats were acclimatized to this housing for 1 week before the beginning of the study. The rats were allowed to mate, and day 0 of pregnancy was defined as the day a positive smear was found. On day 7 of gestation, females were assigned to 1 of 2 dietary groups for the duration of gestation: (a) standard diet fed ad libitum, the Control group (C offspring) (Rodent Lab Diet EQ 5L37, containing 3.12 kcal/g with 10.5% calories from fat, 25% from protein, and 64.5% from carbohydrates; PMI Nutrition International, Brentwood, MO, USA); or (b) 50% of the ad libitum diet, the Restriction group (R offspring). The amount of R diet was determined daily by quantifying the food intake among the pregnant control rats, and providing 50% of this amount. The food intake of females in both groups was recorded daily until delivery.

There was no significant difference in the number of pups between the two groups. Pups from undernourished mothers were fostered by control mothers. Litter size was adjusted to 8 pups per litter (4 males and 4 females) to ensure adequate and standardized nutrition until weaning. Both groups were fed a standard diet. Food intake (daily) and body weight (weekly) were measured until the rats were 8 months old. We took 6 male samples for each of the following measurements from each group (C and R), and excluded the R offspring with catch-up growth, which were defined as those offspring with body weight within 2 SD below the mean of the C offspring (Figure 1).

Intraperitoneal glucose tolerance test (IPGTT)

At 30 weeks of age, 1 male pup from every litter was selected for an intraperitoneal glucose tolerance test (IPGTT) after an overnight fasting period. A baseline blood sample was collected from the tail vein at 0 minutes, after which 50% D-glucose solution (2.5 g/kg body weight) was injected intraperitoneally into the animal. Blood samples were obtained from the tail vein at 15, 30, 60, and 120 minutes after the injection. Blood glucose concentration was immediately determined using an enzyme electrode method and the plasma rapidly frozen for later measurement of plasma insulin using an enzyme linked immunosorbent assay (ELISA) kit (Morinaga Institute of Biological Science, Kanagawa).

Plasma and tissue sampling

At 3, 8, and 33 weeks, 1 male pup from each litter in both dietary groups was selected and decapitated after overnight fasting. Blood samples were collected by cardiac puncture. Samples were centrifuged and the plasma was stored at -70°C for subsequent analysis. Plasma levels of total cholesterol (TC) and triglycerides (TG) were measured using an enzymatic assay and the GPO-HDAOs glycerol blanking method (SRL, Tokyo).

The fat tissue was evaluated using the Adiposity Index, an index of visceral adiposity. The wet weights of the retroperitoneal, mesenteric, and epididymal fat pads were determined, and the Adiposity Index was computed using the following formula: Adiposity Index = (sum of the weights of the three deposits/body weight) × 100.

ELISA

Blood from each rat was collected into a tube containing
Figure 1. On day 7 of gestation, females were assigned to 1 of 2 dietary groups for the duration of gestation: (A) standard diet fed ad libitum (the Control group, C offspring) or (B) 50% of the ad libitum diet (the Restriction group, R offspring). Both groups were fed a standard diet after weaning.

Figure 2. Changes in (A) body weight and (B) food intake in group R (open squares) and group C (closed circles). Values are expressed as means ± SD. *P < 0.05, **P < 0.01
EDTA aprotinin (500 kallikrein inhibiter units/ml) (Roche Diagnostics GmbH, Mannheim, Germany) and centrifuged. Plasma was separated and stored at -80°C for later analysis. Ghrelin concentrations were determined using a Desacyl-Ghrelin ELISA kit (Mitsubishi Chemical Medicine Corporation, Tokyo). Leptin levels were analyzed using a leptin ELISA kit (Morinaga Institute of Biological Science, Kanagawa). Adiponectin levels were analyzed using an adiponectin ELISA kit (Circulex, Nagano).

RNA isolation and real-time polymerase chain reaction (PCR)
Total RNA was extracted from tissues using RNeasy Mini Kits (Qiagen Science, Germantown, MD, USA). The RNA preparations had A260/280 ratios of approximately 1.8. The quality of RNA was checked visually after electrophoresis and staining with ethidium bromide. The expression levels of specific mRNAs were quantified using real-time polymerase chain reaction (PCR). First, RNA was reverse transcribed using an Advantage RT-for-PCR Kit (Clontech Laboratories, Mountain View, CA, USA) followed by amplification of the target cDNAs using QuantiTect SYBR Green PCR Kits (QIAGEN Sciences), according to the manufacturer’s protocol. The primer sequences were:
- 5’-AGAGGCCGCACTAAATAAGTA-3’ (forward) and 5’-GCAGGAGTGCTGGAGTTT-3’ (reverse) for ghrelin;
- 5’-AGCGGTATGGGAAAGAGAATCA-3’ (forward) and 5’-ACCACCTGGCACCACCTCTTG-3’ (reverse) for PYY;
- 5’-AGCCTGGCAAATGAGAAAACA-3’ (forward) and 5’-TGCAATTCACACTCTGAAACA-3’ (reverse) for CCK;
- 5’-AGTCCCTGCCCCTTGACACA-3’ (forward) and 5’-GATCAGGCGCTCCTTAACACTAA-3’ (reverse) for 18S rRNA.

The PCR cycling included 15 minutes of heating at 95°C, followed by 40 cycles of 94°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds in a StepOne-Plus Real-Time PCR System (Applied Biosystems Inc., Foster City, CA, USA). The specificity of each PCR product was verified by both melting curve analysis (one peak) and agarose gel electrophoresis (determination of expected band size). All mRNA levels were normalized

Figure 3. A: Evaluation of fat mass groups C and R at 33 weeks. B: Fat cell size in the epididymal fat of both groups at 33 weeks (solid and open bars, respectively).
to indicate statistical significance.

Results

Body weight and food intake

At birth, R offspring weighed significantly less than C offspring (5.8 ± 0.14 vs. 7.0 ± 0.09 g, P < 0.01), and this difference continued to the end of the present study. The weight of R offspring remained 2 SD less than that of C offspring throughout the study. R offspring ate significantly less food than did C offspring from weaning until the end of the experiment (Figure 2A,B).

Fat mass weight and morphological examination

Total visceral fat mass was compared using an adiposity index, which was significantly smaller in the R offspring (5.3 ± 0.7%) than that in the C offspring (6.5 ± 0.7%) (Figure 3A). Lipid droplets in the epididymal fat were compared between the C and R offspring. Figure 2B shows that small fat cells in the R were bigger than those in the C offspring, and the median area square measurement of the adipocytes from the R offspring (5,876 μm²/cell) was significantly larger compared with
that of the C offspring (5,041 μm²/cell) (Figure 3B).

**IPGTT**

Fasting glucose was not significantly different between C offspring (70 ± 7 mg/dl) and that of the R offspring (75 ± 6 mg/dl). After intraperitoneal injection, blood glucose of the R offspring was significantly lower than that of the C offspring. At 120 minutes after injection, the glucose level of the R offspring decreased compared with that of the C offspring, but this was not significant (Figure 4A). The average blood insulin level of the R offspring was significantly lower than that of the C offspring at 120 minutes (Figure 4B). The HOMA-R of the R offspring (0.21 ± 0.11) was significantly lower than that of the C offspring (0.37 ± 0.05) (Figure 4C).

**Plasma concentrations of TG, TC, ghrelin, leptin, and adiponectin**

At 3, 8, and 33 weeks, the R offspring had significantly lower plasma ghrelin (Figure 5A), PYY (Figure 5B), and CCK (Figure 5C), and significantly higher plasma leptin, than did the C offspring (Table 1). These hormones affected their appetite. There was no significant difference in plasma TG or TC between the C and R offspring (data not shown). At 3, 8, and 33 weeks, the R offspring had significantly lower plasma adiponectin than did the C offspring (Table 1).

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**Figure 5.** Plasma gut hormone levels in groups C and R (solid and open bars, respectively) at 3, 8, and 33 weeks. Values are expressed as mean ± SD. *P < 0.05, **P < 0.01. A: Ghlerin. B: PYY. C: CCK. Gene expression levels of ghrelin, PYY, and CCK in groups C and R (solid and open bars, respectively) at 3, 8, and 33 weeks. D: Ghrelin in the stomach. E: PYY in the ileum. F: CCK in the duodenum.

**Table 1.** Plasma leptin and adiponectin levels

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<td>Leptin</td>
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<td>0.8 ± 0.25*</td>
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<td>Adiponectin (ng/ml)</td>
<td>2.0 ± 0.2</td>
<td>1.5 ± 0.3*</td>
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Gene expression of Ghrelin, PYY, and CCK
At 3 and 8 weeks, mRNA levels of ghrelin in the stomach (Figure 5D), PYY in the ileum (Figure 5E), and CCK in the duodenum (Figure 5F) were significantly decreased in the R offspring compared with those in the C offspring. These relationships mirrored the results for plasma concentrations.

Discussion
Previous reports regarding IUGR without weight catch-up growth have shown that glucose tolerance was not impaired. This is consistent with our results in the present study, with these data suggesting that glucose tolerance and hyperinsulinemia were improved in the R group, as was the insulin response to the IPGGT. We did, however, observe some unusual changes in expression of adipocytokines that are derived from adipose tissue. Reported data have shown that serum leptin concentration is positively correlated with the percentage of body fat and that serum adiponectin concentrations in obese subjects were significantly lower than those in non-obese subjects. In our study, R offspring had less fat volume than did C offspring. However, the R offspring displayed increased leptin and decreased adiponectin compared with that in the C offspring. In another report, Jaquet et al. reported BMI in IUGR did not have a positive correlation with serum leptin. Adipocyte volume determines leptin levels, and there is also a negative correlation between adipocyte size and adiponectin levels. We, therefore, considered that the adipocytokine levels correlated with the fat cell size irrespective of the fat mass volume in IUGR. The fat cells in the R group rats readily developed hypertrophy. As mice gain weight, enlargement of adipocytes occurs as the number of adipocyte cells increases. Spalding et al. reported that the number of adipocytes is set during childhood and the adipocyte number in lean individuals is less than that in obese ones. We anticipated that R group offspring would have a lower number of fat cells than did control mice. Based on these results, we consider that decreased PYY and CCK have less of an effect on food intake than does increased ghrelin.

Our study has some limitations. First, a female rat can have multiple babies with each pregnancy, and maternal nutrition may not uniformly affect each fetus. Furthermore, we selected 4 male and 4 female rats from each of the litters, which may result in selection bias. Second, PYY and CCK increase after feeding and inhibit food intake. Therefore, a decrease of these gut hormones during the fasting state would have less effect on food intake. Third, the ability to overcome starvation in the IUGR rats may be weaker than that in the controls due to their smaller body size, resulting in decreased PYY and CCK.

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It is unclear what factors influenced the observed change in these hormones. Several gastrointestinal hormones can contribute to food intake by modulating the activity of the gut-brain axis.27 It will be necessary to measure any changes of brain factors that might influence the appetite in IUGR. There is also an unknown mechanism associated with the fat cells in the R group rats that readily developed hypertrophy. A hypothesis that would merit consideration for future studies would, therefore, be that fat cells make up are determined during fetal growth. As a result of this, if proven, both cell growth and adipocytokine secretion would be changed.

These results indicate that intrauterine undernutrition alters food intake through changes in orexigenic (ghrelin) and anorexigenic (PYY and CCK) peptides and of visceral fat cell characteristics. This is consistent with a catch-up growth mechanism due to hyperphagia and suggests that there may be a risk of metabolic disorders in IUGR both with and without catch-up growth.

References


