Role of premature leptin surge in obesity resulting from intrauterine undernutrition

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Summary

Intrauterine undernutrition is closely associated with obesity related to detrimental metabolic sequelae in adulthood. We report a mouse model in which offspring with fetal undernutrition (UN offspring), when fed a high-fat diet (HFD), develop pronounced weight gain and adiposity. In the neonatal period, UN offspring exhibited a premature onset of neonatal leptin surge compared to offspring with intrauterine normal nutrition (NN offspring). Unexpectedly, premature leptin surge generated in NN offspring by exogenous leptin administration led to accelerated weight gain with an HFD. Both UN offspring and neonatally leptin-treated NN offspring exhibited an impaired response to acute peripheral leptin administration on a regular chow diet (RCD) with impaired leptin transport to the brain as well as an increased density of hypothalamic nerve terminals. The present study suggests that the premature leptin surge alters energy regulation by the hypothalamus and contributes to "developmental origins of health and disease."

Introduction

Obesity has increased at an alarming rate in Western countries and is now a world-wide public health problem (Flier, 2004). Obesity is often associated with insulin resistance, dyslipidemia, and hypertension, thus a concept of metabolic syndrome has been proposed (Masuzaki et al., 2001; Wajchenberg, 2000). Genetic factors and/or environmental factors, such as high-calorie diet in Western life style, have been considered to attribute to the prevalence of obesity (Flier, 2004). More recently, epidemiological and experimental evidence suggest that intrauterine undernutrition is closely associated with adulthood obesity related to detrimental metabolic sequelae (Breier et al., 2001; Godfrey and Barker, 2000; Ravelli et al., 1976), giving rise to the concept of "developmental origins of health and disease" (Breier et al., 2001; Gluckman and Hanson, 2004). Involvement of perinatal exposure to glucocorticoids or pancreatic maldifferentiation in the development of impaired glucose metabolism has been demonstrated (Breier et al., 2001; Gluckman and Hanson, 2004). However, the mechanism of developmental origins of obesity is yet to be clarified.

Leptin is an adipocyte-derived satiety factor that decreases food intake and increases energy expenditure, thereby stabilizing body adiposity in many species (Friedman and Halaas, 1998). Leptin deficient *ob*/*ob* mice show marked obesity that is restored by exogenous leptin treatment (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995; Trayhurn et al., 1977). Leptin exerts its biological activities through long-form leptin receptors, expressed abundantly in the hypothalamus (Flier, 2004). However, resistance to the satiety effect of leptin is a trait of obese subjects, as circulating leptin levels are well correlated with body fat mass (Ahren and Scheurink, 1998). It remains to be elucidated whether leptin resistance at the hypothalamus is associated with the onset of obesity or metabolic disorders in offspring with intrauterine growth restriction.

In mice, plasma leptin levels rise transiently during neonatal period, termed as "neonatal leptin surge" (Ahima et al., 1998). In neonatal period, leptin alters hypothalamic neuropeptide expression and metabolic rate before exerting its anorectic effect (Ahima and Hileman, 2000; Mistry et al., 1999; Proulx et al., 2002). Moreover, neurotrophic action of leptin was recently demonstrated, which is operative only in early developmental stage, but not in adult individuals (Bouret et al., 2004a; Bouret et al., 2004b). Therefore, it is suggested that leptin surge is involved in the formation of energy-regulation circuits in the hypothalamus. However, long-term effects of physiological or pathophysiological leptin surge on hypothalamic neuronal circuits are yet to be fully clarified. Here, we demonstrate a mouse model of intrauterine undernutrition in which premature leptin surge contributes to developmental origins of obesity.

Results and discussion

Development of obesity and related metabolic disorders in offspring with intrauterine undernutrition

Maternal body weight gain during pregnancy was significantly suppressed by 30% restriction of maternal food supply (Figure

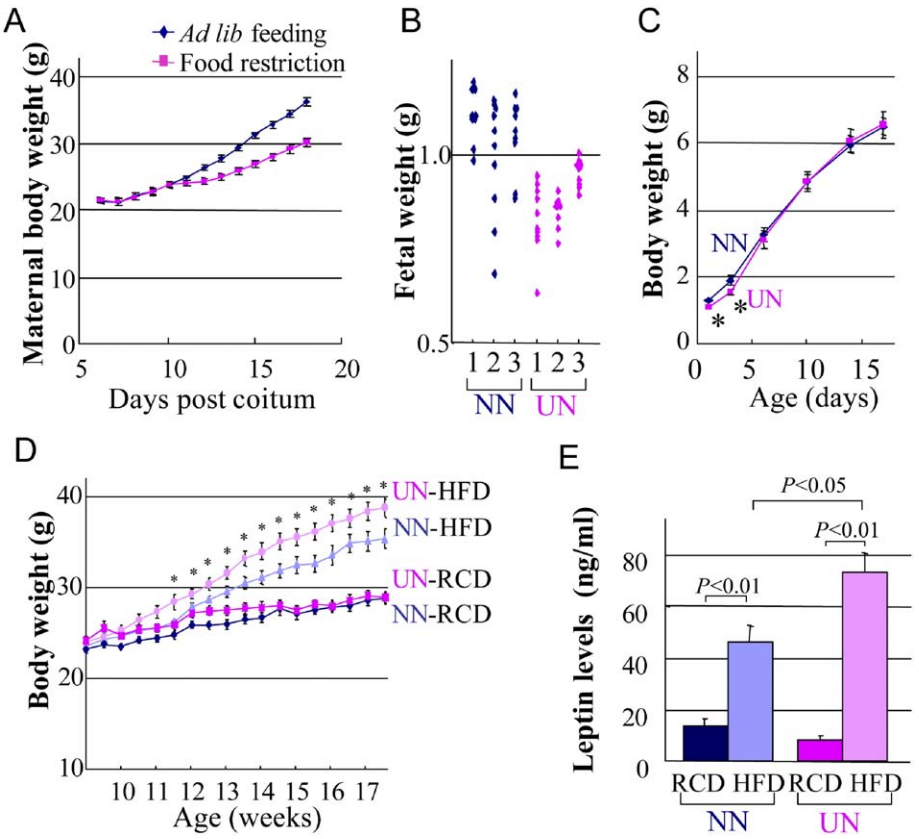

Figure 1. Exaggerated diet-induced weight gain in offspring with fetal undernutrition (UN) compared to offspring with normal fetal nutrition (NN)

A) Maternal body weight change during pregnancy. Food restriction started on day 10.5 postcoitum as described in the Experimental Procedures. Number of dams in each group is seven to eight.

B) Fetal weights at 18.5 d.p.c. in three litters in both groups. Weights of both male and female fetuses were plotted without discrimination. Litter sizes of demonstrated litters are nine to ten in both NN group and UN group.

C) Neonatal catch-up growth in UN offspring. n = 96 from 12 litters for each group. Average body weights of 96 pups including both male and female pups in each group are shown.

D) Exaggerated weight gain in UN offspring on an HFD but not on an RCD. HFD was started at 9 weeks of age.

E) Plasma leptin concentrations at 17 weeks of age. Dark and light purple lines (**A**, **C**, and **D**), dots (**B**) and columns (**E**) indicate dams or offspring with undernutrition. *p < 0.05 versus NN offspring; n.s.: not significant. In the experiment for (**D**) and (**E**), all pups were nursed at a rate of 8 pups per dam until weaning. Only male animals were used for further experiments. n = 7–15 for each group in (**D**) and (**E**). Error bars represent SEM.

1A). The UN offspring were born small (Figure 1B) with reduced lipid accumulation in the subcutaneous adipose tissue (data not shown) but caught up in body weight to NN offspring within 10 days after birth (Figure 1C). They showed no apparent difference in body weight and fat mass on an RCD thereafter (Figure 1D; Table 1). However, UN offspring developed pronounced weight gain and adiposity (40% and 30% larger in subcutaneous and epididymal fat depots, respectively) compared to NN offspring, when fed a HFD commencing at 9 weeks of age (Figure 1D; Table 1). An obesity-prone phenotype of UN offspring was confirmed in an additional three independent experiments. Serum leptin levels in UN offspring were significantly higher than those in NN offspring on an HFD ($p < 0.05$), in parallel with their adiposity (Figure 1E; Table 1). These observations are in agreement with previous reports on rodent models of fetal undernutrition and catch-up growth (Ozanne and Hales, 2004)

The UN offspring did not differ significantly from NN offspring with regard to calorie intake during the development of

Table 1. Adiposity and lipid profiles of NN and UN offspring

	NN Offspring		UN Offspring	
	RCD (n = 15)	HFD (n = 13)	RCD (n = 7)	HFD (n = 11)
Body mass index (g/cm^2)	0.33 ± 0.01	0.37 ± 0.01	0.31 ± 0.01	0.42 ± 0.01[a]
Subcutaneous adipose tissue (g)	0.52 ± 0.05	1.12 ± 0.10	0.42 ± 0.04	1.67 ± 0.14[a]
Epididymal adipose tissue (g)	0.58 ± 0.06	1.50 ± 0.15	0.48 ± 0.05	1.98 ± 0.09[b]
Leptin mRNA expression in subcutaneous adipose tissue (AU)	2.84 ± 0.69	26.26 ± 5.28	2.99 ± 0.46	21.39 ± 4.54
Serum triglyceride (mg/dl)	192.1 ± 18.2	199.6 ± 20.2	134.8 ± 9.2[a]	125.0 ± 10.9[a]
Serum total cholesterol (mg/dl)	101.3 ± 4.0	166.3 ± 16.9	92.6 ± 5.2	222.0 ± 5.8[a]
Serum HDL cholesterol (mg/dl)	47.6 ± 7.0	97.7 ± 12.7	54.0 ± 5.9	132.7 ± 6.5[b]
Serum free fatty acids (mg/dl)	0.61 ± 0.04	0.75 ± 0.06	0.69 ± 0.06	0.50 ± 0.00[b]

Samples are collected from offspring with normal intrauterine nutrition (NN) and from offspring with intrauterine undernutrition (UN) on an RCD or on an HFD at 17 weeks of age.
Differences between RCD group and HFD group were not demonstrated in both groups.
[a] $p < 0.01$ versus NN offspring.
[b] $p < 0.05$ versus NN offspring.

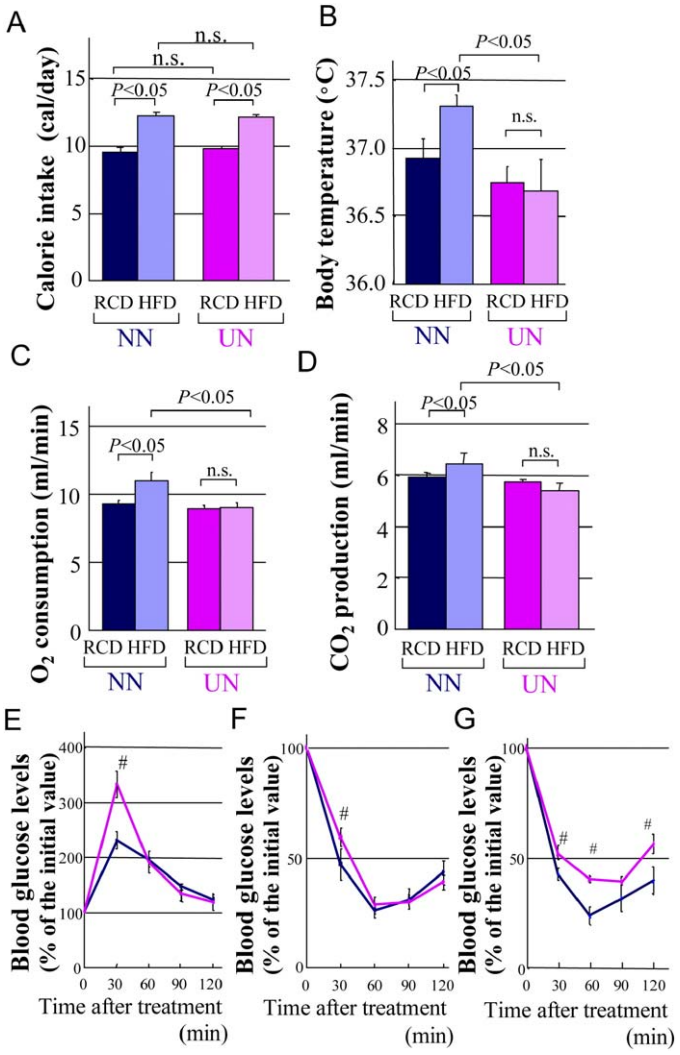

Figure 2. Blunted diet-induced thermogenesis and impaired glucose metabolism in UN offspring compared to NN offspring

A) Average daily calorie intake from 9 to 17 weeks of age on an RCD or on an HFD in NN and UN offspring. n = 7–15.

B) Resting rectal body temperature, (**C**) O_2 consumption, and (**D**) CO_2 production were measured in NN and UN offspring on an RCD or on an HFD at 13 weeks of age. n.s.: not significant. n = 7–15.

E) Glucose tolerance test was performed at 17 weeks of age on an RCD.

F and G) Insulin sensitivity was examined at 19 weeks of age on an RCD (**F**) and on an HFD (**G**). Initial glucose levels of UN and NN offspring were 100.5 ± 3.3 and 109.3 ± 5.8 mg/dl in (**E**), 95.5 ± 3.3 and 113.0 ± 4.9 mg/dl in (**F**), and 186 ± 14.8 and 157.1 ± 8.3 mg/dl in (**G**), respectively. #p < 0.05 versus NN offspring. n = 6–11 in another series of experiments from that in (**A**–**D**).

Dark and light purple columns (**A**, **B**, **C**, and **D**) and lines (**E**, **F**, and **G**) indicate UN offspring. Error bars represent SEM.

diet-induced weight gain (Figure 2A). On an HFD, NN offspring showed elevated rectal temperature and increased O_2 consumption and CO_2 production (Figures 2B–2D). On the other hand, UN offspring did not show such changes in energy metabolism, suggesting impaired diet-induced thermogenesis (DIT; Lowell and Spiegelman, 2000). These characteristics of energy metabolism in UN offspring are consistent with the "thrifty phenotype hypothesis" according to which undernutrition in utero programs offspring to conserve energy for survival under conditions of scarce food supply after birth (Hales and Barker, 2001). A significant elevation of uncoupling proteins' (UCP-1 and -2) mRNA expression in the brown adipose tissue (BAT) of both UN and NN offspring on an HFD (data not shown) could not explain clearly the mechanisms of blunted diet-induced thermogenesis in UN offspring. Nonetheless, an HFD significantly augmented free fatty acid (FFA) concentrations in NN offspring, but not in UN offspring (Table 1). Since the release of FFA from WAT stimulates mitochondrial thermogenesis in BAT (Ricquier and Bouillaud, 2000), altered lipid metabolism, such as low FFA levels in UN offspring, may partly contribute to the blunted diet-induced thermogenesis in these animals. The UN offspring also showed impaired glucose metabolism and lipid profiles compared to NN offspring (Figures 2E and 2F; Table 1). HFD deteriorated an impairment of both glucose (Figure 2G) and lipid metabolism (Table 1). These findings indicate that intrauterine undernutrition in the present study is an appropriate model for the elucidation of fetal origins of metabolic syndrome.

Neonatal premature leptin surge is associated with obesity

During the catch-up growth, the peak of leptin surge, a transient rise of serum leptin levels in mice neonates (Ahima et al., 1998) was advanced (8–10 days after birth) in UN offspring compared to that in NN offspring (16 days after birth; Figure 3A). Profiles of neonatal leptin concentrations in UN and NN offspring were fundamentally parallel with leptin mRNA expression in subcutaneous adipose tissue (Figure 3B), suggesting that altered leptin production is responsible for premature onset of leptin surge. UN offspring showed hyperglycemia and hyperinsulinemia at 8 days of age (data not shown). Increase in insulin signal augments leptin production from adipose tissue (Considine, 2001). It is likely that intrauterine undernutrition induced, at least partly through altered glucose metabolism, premature leptin surge in these offspring. According to Ahima et al. (1998), the timing for the leptin surge in mice not subjected to intrauterine undernutrition is day 10, while we observed a peak leptin surge occurring at day 16. The discrepancy might be due to differences in number of pups in each litter, i.e., only five pups per litter in the report by Ahima et al. and eight to nine pups per litter in the present study.

To examine whether premature leptin surge is associated with pronounced weight gain in UN offspring on an HFD, we induced premature leptin surge in NN offspring by exogenous administration of leptin from day 5.5 to day 10.5 of life. In addition to the significant elevation of endogenous leptin surge observed in UN offspring compared to NN offspring demonstrated in Figure 3A, serum leptin concentrations at 5.5 days of age in UN offspring were also higher than those in NN offspring in another series of experiments (data not shown). Therefore, we determined the period of exogenous leptin administration as above. Serum leptin concentration after subcutaneous leptin treatment in pups at 5.5 days of age reached its peak (3955 ± 445 ng/ml) at 2 hr after injection and returned to levels similar to vehicle-treated pups by 8 hr after injection. During the 6 days of daily leptin treatment, NN offspring treated with leptin tended to gain less weight compared to vehicle-treated groups, but the difference was not significant. This is consistent with previous findings that leptin does not exert its

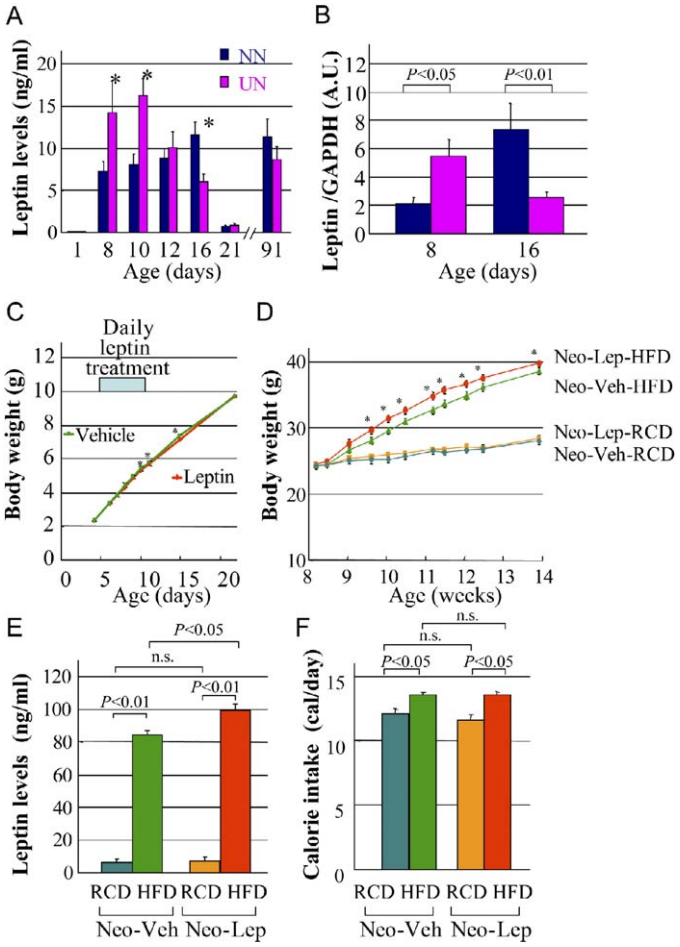

Figure 3. Premature onset of leptin surge in UN offspring as a trigger of exaggerated weight gain on an HFD

A) Serum leptin concentrations and (**B**) leptin mRNA expression in subcutaneous adipose tissue during neonatal development. Both male and female pups were assayed without discrimination. Note the earlier occurrence of leptin surge in UN offspring.

C) Body weight changes of NN offspring during neonatal leptin or vehicle treatment. Average body weights of both male and female pups are shown. n = 20–22.

D) Exaggerated weight gain on an HFD in NN offspring with early neonatal leptin treatment. HFD was started at 9 weeks of age.

E) Plasma leptin concentrations at 17 weeks of age in NN offspring with or without early neonatal leptin treatment.

F) Average daily calorie intake from 9 to 17 weeks of age on an RCD or HFD.

Dark and light orange lines (**C** and **D**) and columns (**E** and **F**) indicate offspring with neonatal leptin treatment. Neo-Lep: offspring with neonatal leptin treatment; Neo-Veh: offspring with neonatal vehicle treatment. *p < 0.05 versus control ; A.U.: arbitrary units. n = 8–10 for each column or line except for (**C**). Error bars represent SEM.

anorectic effect until the third week of life (Mistry et al., 1999; Proulx et al., 2002). They caught up in growth by the time of weaning (Figure 3C). Body weight and fat mass of leptin-treated NN offspring were similar to those of vehicle-treated NN offspring on an RCD thereafter (Figure 3D for body weight and 0.36 ± 0.05 versus 0.31 ± 0.04 g for subcutaneous adipose mass). On an HFD, however, leptin-treated NN offspring developed accelerated weight gain and adiposity compared to vehicle-treated groups (Figure 3D for body weight and 2.02 ± 0.11 versus 1.63 ± 0.17 g for subcutaneous adipose mass). An obesity-prone phenotype of offspring with neonatal leptin treatment was confirmed in an additional two independent experiments. The serum leptin levels in NN offspring with neonatal leptin treatment were significantly increased compared to vehicle-treated groups during the development of diet-induced obesity (Figure 3E). Calorie intake of leptin-treated NN offspring was similar to that of vehicle-treated groups on an HFD (Figure 3F), implying an involvement of decreased energy expenditure in the aggravation of HFD-induced obesity. Thus, as NN offspring with neonatal leptin treatment were phenotypically indistinguishable from UN offspring, premature onset of leptin surge is causally related to pronounced obesity in UN offspring on an HFD.

Leptin resistance in offspring with exposure to premature leptin surge

Resistance to the weight-reducing effect of leptin is a feature of obesity with hyperleptinemia (Flier, 2004; Friedman, 2000). It has been described that high-calorie diet induces obesity with leptin resistance by at least two different mechanisms (El-Haschimi et al., 2000), an impairment of leptin transfer through the blood-brain barrier (BBB) and/or a disturbed signal transfer in the hypothalamus. To examine the response to exogenous leptin administration in UN and NN offspring, we performed an acute intraperitoneal (i.p.) or intracerebroventricular (i.c.v.) injection of leptin in UN and NN offspring on an RCD at 8 to 10 weeks of age when they were of similar weight. Animals used in this section are a new set of mice not used in other studies. No significant difference was observed in serum leptin levels between UN and NN offspring 2 hr after i.p. injection of leptin (74.0 ± 13.9 versus 72.2 ± 6.1 ng/ml; n = 8) in a preliminary experiment. In NN offspring, body-weight increase was significantly suppressed 12 hr after i.p. leptin treatment (Figure 4A; $p < 0.05$), whereas no such effects were observed in UN offspring. In this study, i.p. injection of leptin stimulated phosphorylation of signal transducer and activator of transcription 3 (STAT3), an intracellular signaling mediator of leptin action (El-Haschimi et al., 2000), in the hypothalamus of NN offspring, but not of UN offspring (Figure 4B). Then we detected c-Fos positive cells, i.e., activated neurons (Elmquist, 2001), after i.p. injection of leptin. The number of c-Fos-positive cells was smaller in UN offspring than in NN offspring in the hypothalamic arcuate nucleus (ARH; Figure 4C) and paraventricular hypothalamus (PVH) nuclei mediating hypothalamic leptin signaling (Schwartz et al., 2000; Wilding, 2002; Figure 4D). By contrast, the response to i.c.v. injection of leptin in UN offspring was not blunted with regard to body weight (Figure 4E) and STAT3 phosphorylation in the hypothalamus (Figure 4F) compared to NN offspring. We also obtained essentially the same data in NN offspring treated neonatally with leptin (Figures 4G and 4H). These observations suggest impaired brain leptin transport (Caro et al., 1996) in UN offspring as well as in NN offspring treated neonatally with leptin. This is supported by a significant reduction in mRNA expression of the short-form leptin receptor, Ob-Ra, which is suggested to play a role in the transport of leptin across the BBB (Hileman et al., 2002) in the hypothalamus of UN offspring (1.23 ± 0.16 arbitrary units [AU]; n = 7) compared to that of NN offspring (2.09 ± 0.15 AU; n = 10; $p < 0.01$). It is likely that a premature leptin surge in UN offspring leads to resistance to peripherally administered lep-

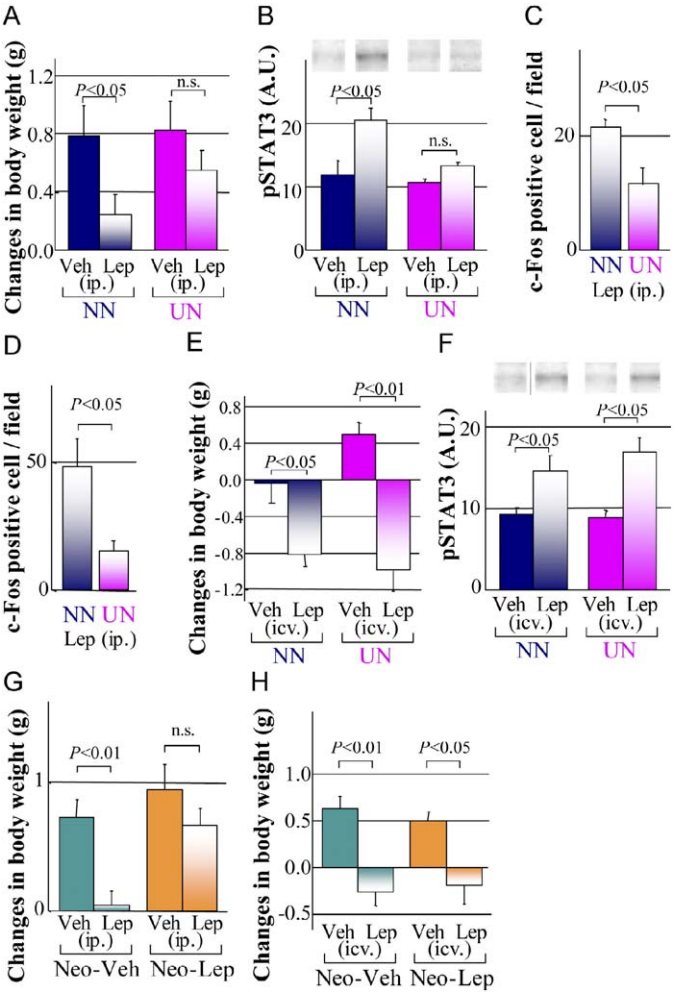

Figure 4. Resistance to peripheral leptin in adult UN and NN offspring with neonatal leptin treatment

A) Body weight changes following intraperitoneal (i.p.) leptin administration in UN and NN offspring.

B) Phosphorylated STAT3 in the entire hypothalamus after i.p. leptin administration.

C) The number of c-Fos-positive cells in arcuate nucleus of the hypothalamus after acute leptin administration at 10 weeks of age. Mean number of cells with c-Fos-like immunoreactivity after saline vehicle treatment was 9.5 ± 0.7 (n = 4) with no remarkable difference between UN and NN offspring.

D) The number of c-Fos-positive cells in paraventricular hypothalamus in the same brain tissues as in (**C**). Mean number of cells with c-Fos-like immunoreactivity after saline vehicle treatment was 8.5 ± 0.7 (n = 4) with no remarkable difference between UN and NN offspring

E) Body-weight changes following intracerebroventricular (i.c.v.) leptin administration.

F) Phosphorylated STAT3 in the entire hypothalamus after i.c.v. leptin administration.

G) Body-weight changes following i.p. leptin administration in NN offspring with neonatal leptin or vehicle treatment.

H) Body-weight changes following i.c.v. leptin administration in NN offspring with neonatal leptin or vehicle treatment.

All procedures were performed at 8–10 weeks of age. Lep: acute leptin administration; Veh: acute vehicle administration; Neo-Lep: neonatal leptin treatment; Neo-Veh: neonatal vehicle treatment; n.s.: not significant; n = 8–12; A.U.: arbitrary unit. Error bars represent SEM.

tin, accompanied by impaired leptin transport to the brain. However, neither UN offspring nor NN offspring with neonatal leptin treatment developed obesity on an RCD, suggesting that as-yet-unidentified compensatory mechanisms are operative.

On an HFD, both UN offspring and NN offspring with neonatal leptin treatment showed hyperleptinemia concomitant with pronounced obesity, indicating that they are less sensitive to circulating leptin than NN offspring without neonatal leptin treatment. It remains to be elucidated how resistance to circulating leptin might be involved in the pronounced weight gain on a high fat diet in the present study.

The hypothalamus is responsible for the development of obesity

Since the ARH plays a critical role in the central regulation of thermogenesis, we chemically injured the ARH of UN and NN offspring by neonatal administration of monosodium glutamate (MSG; Olney, 1969), which is known to induce obesity through reduced thermogenesis (Tokuyama and Himms-Hagen, 1986). No significant difference in body weight was observed between MSG-treated UN offspring and MSG-treated NN offspring on a HFD (Figure 5A), implying that the hypothalamic area damaged by MSG (i.e., ARH) is responsible for pronounced weight gain in UN offspring on a HFD. Nevertheless, the possibility cannot be fully neglected that rapid body-weight gain induced by hypothalamic ablation with MSG obscures the augmentation of diet-induced obesity after neonatal exposure to premature leptin surge. In this study, we found that in both wild-type UN offspring with endogenous premature leptin surge and NN offspring treated neonatally with exogenous leptin, the density of nerve terminals containing neuropeptide Y (NPY) or cocaine and amphetamine-regulated transcript (CART) was increased, in PVH that receives the downstream leptin signaling from ARH (Schwartz et al., 2000; Wilding, 2002; Figures 5B and 5C). These alterations were preserved even after pronounced weight gain on an HFD (data not shown). Although many animal and human studies implicate that CART is involved in thermogenesis and has an inhibitory effect against development of obesity (Hunter et al., 2004), there is some controversy about anorexigenic effect of CART neuropeptide (Kong et al., 2003). It will be interesting to elucidate the possible relationship between such hypothalamic structural changes and the development of pronounced HFD-induced obesity.

Proulx et al. (2002) revealed that leptin increased proopiomelanocortin (POMC) expression and decreased NPY and leptin-receptor expression in the hypothalamus in the developing rat. Moreover, Bouret et al. (2004a) demonstrated that leptin promotes neural projections from ARH to other hypothalamic nuclei including PVH. These findings suggest that premature onset or augmentation of neonatal leptin surge might induce organic and/or functional alterations in the developing hypothalamus.

Catch-up growth is associated with adulthood obesity in mice (Ozanne and Hales, 2004) as well as in humans (Stettler et al., 2002). The present study demonstrated that onset of leptin surge is advanced in UN offspring during the catch-up period and that this premature leptin surge contributes to a conversion to an obesity-prone phenotype in UN offspring. Impairment of leptin transfer through the BBB in the hypothalamus may be linked with this phenotypic conversion in wild-type offspring. In addition, the hypothalamic nuclei are responsible for

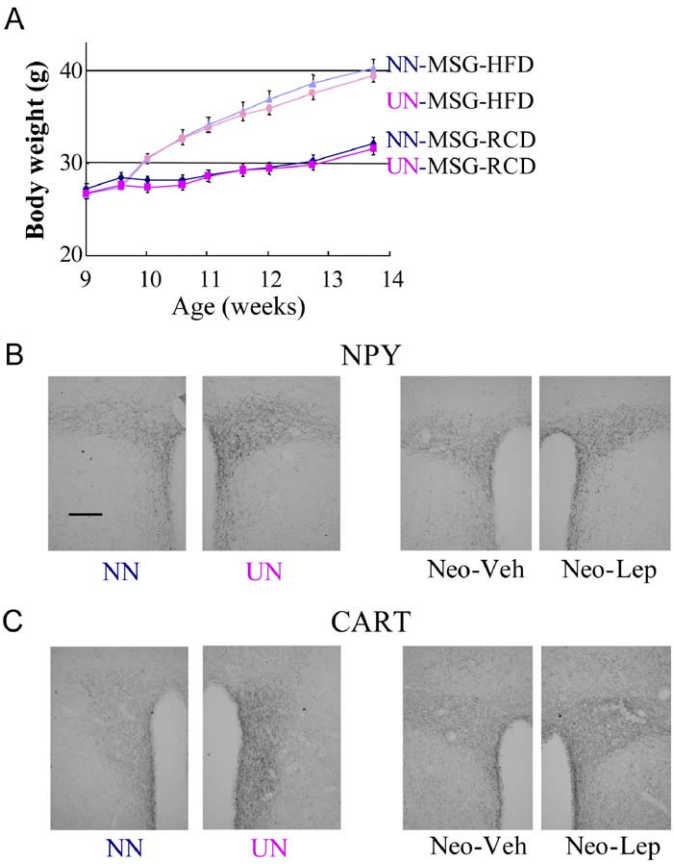

Figure 5. Involvement of the hypothalamic regulation in the exaggeration of diet-induced obesity in offspring with intrauterine undernutrition

A) Body-weight changes of UN and NN offspring with neonatal monosodium glutamate (MSG) treatment. Mice were fed an RCD or an HFD from 9 weeks of age. n = 10–12.

B) Increased nerve terminal densities of NPY in PVH of UN offspring as well as NN offspring with neonatal leptin treatment.

C) Increased nerve terminal densities of CART in PVH of UN offspring as well as NN offspring with neonatal leptin treatment.

Figures in (**B**) and (**C**) are representative of at least four mice in each study group. Neo-Lep: neonatal leptin treatment; Neo-Veh: neonatal vehicle treatment. Scale bar, 20 μm. Error bars represent SEM.

the onset of pronounced adiposity on an HFD, as demonstrated in MSG-treated UN offspring. Altered hypothalamic neural structure, including adipostatic and counter-regulatory signals, may be involved in the programming of increased adiposity by premature leptin surge. We also observed that increased adiposity on an HFD was programmed by exogenously treated premature leptin surge even in the absence of leptin signal in *ob/ob* background (S.Y., H.I., and N.S., unpublished data). This implies that altered hypothalamic neural structure in offspring with exposure to premature leptin surge might be involved in the programming of increased adiposity in addition to a decrease in sensitivity to circulating leptin.

Epidemiological evidence suggests that intrauterine undernutrition predisposes humans to obesity and metabolic syndrome later in life (Godfrey and Barker, 2000; Ravelli et al., 1976; Wajchenberg, 2000). The "fetal programming" hypothesis postulates that undernutrition in utero causes permanent changes in structure, physiology, and metabolism to adapt to restricted nutritional supply (thrifty phenotype; Godfrey and Barker, 2000). When the nutritional supply is excessive (e.g., exposure to a high-calorie diet in Western life style), such thrifty traits by fetal programming become maladaptive, that is, contribute to the development of obesity and metabolic syndrome (Wells, 2003). Currently, neonates born small for gestational age are increasing in number (Ananth et al., 2004). They show a transient overshoot in plasma leptin levels during the catch-up period (Jaquet et al., 1999). The present findings suggest the possibility that premature rise in plasma leptin levels is involved in fetal programming in these neonates and contributes to obesity and subsequent metabolic disorders after exposure to a high-calorie diet.

In conclusion, this study demonstrates that the timing of neonatal leptin surge determined by fetal nutrition contributes to the development of accelerated obesity in later life. This study also highlights the leptin surge as a target for therapeutic intervention in the developmental origins of health and disease.

Experimental procedures

Development of a mouse model of undernutrition in utero

Pregnant C57Bl/6 mice were purchased 6.5 days postcoitum (d.p.c.) from Japan Central Laboratories for Experimental Animals (Tokyo, Japan) and were divided into two groups 10.5 d.p.c. Dams were individually housed with free access to water during 14 hr/10 hr light/dark cycles (0700 hr, 2100 hr) in a specific-pathogen-free facility. The daily food supply of one group was restricted to 70% of the food consumed by the other group, fed ad libitum, based on the data of the previous day, from 10.5 d.p.c. to the day of delivery of the pups. Dams in the food-restriction group were given 2.5 g of extra food supply in the evening of 18.5 d.p.c., just before the initiation of parturition, to prevent them from eating their own pups. Litter size was examined on 1.5 days after delivery. Offspring from dam with less than 8 pups or larger than 10 pups were excluded from the study. To control litter size to 8 or 9 per dam, pups were culled or moved to other dams and were fostered until weaning, depending on the total pup numbers in every experimental course. Pups were weaned on to RCD at 21.5 days of age. Only male offspring were used in any experiments after weaning from dams. Each experimental group in all experiments consists of offspring from at least three litters. Caloric intakes by offspring after weaning were measured in groups (three to four per cage) twice a week. All experimental procedures were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University.

High fat diet (HFD)

At 9 weeks of age, HFD (containing 60% lipid by calorie mainly by lard, formula D12492, Research Diets Inc., New Brunswick, NJ, http://www.researchdiets.com/formulas/item/dio.htm) or RCD (containing 12% lipid and 20.0% protein by calorie, 3.44 kcal/g, CLEA Japan Inc., Tokyo, Japan) was supplied to NN and UN offspring.

Energy metabolism analysis

Body temperature was determined by measuring rectal core temperature 25 times with a digital thermometer (TD-320, Shibaura Electronics Co. Ltd., Tokyo, Japan). Oxygen (O_2) consumption and CO_2 production in the resting state were measured with an O_2/CO_2 metabolism measuring system (Model MK-5000, Muromachikikai, Tokyo, Japan; Nakagawa et al., 2000).

Glucose metabolism

Glucose tolerance test was performed by i.p. administration of glucose (1.5 mg/g body weight) after overnight fasting. Insulin sensitivity was examined by i.p. administration of insulin (0.75 mU/g body weight) after overnight fasting. Blood glucose concentrations were determined by Glutest Ace R (Sanwa Kagaku Kenkyusho Co. Ltd., Nagoya, Japan).

Blood sample assays
Serum concentrations of triglyceride, total cholesterol, and HDL cholesterol were determined using the Fuji DRI-CHEM system (Fuji Photo Film Co. Ltd., Tokyo, Japan). Serum leptin concentrations were determined by Mouse Leptin ELISA (R&D Systems, Inc., Minneapolis, Minnesota).

Quantitative RT-PCR analysis
Total RNA from subcutaneous adipose tissue was extracted as previously described (Masuzaki et al., 1997). Gene expression of leptin was determined by quantitative RT-PCR using TaqMan Probes (Applied Biosystems, Foster City, California), according to the manufacturer's recommendation. Primers and fluorescent probe for leptin are as follows: forward, ttcacacacg cagtcggtatc; reverse, tggtccatcttggacaaactca; TaqMan Probe, tgaagtccaag ccagtgaccctctgc. Those for short-form leptin receptor (Ob-Ra) are as follows: forward, ctgaatttccaaaagagaacgga, reverse, ggaagttggtagattgggttc atct, TaqMan Probe, tgaagtctctcatgaccact. Ribosomal RNA contents or GAPDH mRNA expression was used as an internal control.

Neonatal leptin or MSG treatment
Leptin (2.5 μg/g body weight/day) (kind donation from Amgen Inc., Thousand Oaks, California) or vehicle saline was subcutaneously administered to NN offspring daily from 5.5 to 10.5 days of age. Monosodium glutamate (MSG; 2 mg/g body weight/day) was subcutaneously administered to NN and UN offspring from 1.5 to 5.5 days of age (Olney, 1969).

Acute leptin administration to adult offspring
Leptin was administered i.p. (2.5 μg/g body weight) or i.c.v. (0.5 μg/mouse; Aizawa et al., 2000) to NN and UN offspring at 1900 hr at 8 weeks of age, and body-weight changes and food consumption were measured for the next 12 hr. The i.c.v. placement technique was confirmed by dye injection in preliminary experiment. Additionally, scanty backflow was observed after each injection.

Mice with confirmed leptin resistance or leptin sensitivity in UN offspring and NN offspring, respectively, by a single dose of i.p. leptin treatment were used for the following phosphorylated STAT3 and c-Fos immunoreactivity experiment.

Detection of phosphorylated STAT3 in the hypothalamus
Phosphorylated STAT3 protein in the entire hypothalamus of UN offspring was measured 30 min after acute i.p. (1.5 μg/g body weight) or i.c.v. (0.2 μg/mouse) administration of leptin at 10 weeks of age. Protein extraction was performed as previously described (Itoh et al., 1998). An ECL Advance kit (Amersham Bioscience Corp., Piscataway, New Jersey) was used with anti-mouse pSTAT3 antibody (1:1,000, overnight; Cell Signaling Technology, Beverly, Massachusetts) and anti-rabbit IgG antibody with HRP (1:20,000, 1 hr; Cell Signaling Technology, Beverly, Massachusetts).

Detection of activated neurons by c-Fos immunoreactivity
To prevent stress-induced c-Fos expression, intraperitoneal cannulas were placed 7 days before treatment. Leptin (0.5 μg/g body weight) or vehicle saline was gently administered through the i.p. cannulas after 2 hr of fasting at 10 weeks of age. Peak serum leptin levels were observed at 15 min after the i.p. leptin injection on preliminary examination (data not shown). The entire hypothalamus was sampled 135 min after the treatment and processed for immunohistochemistry as described previously (Yamamoto et al., 2002), using rabbit polyclonal antibody against c-Fos (1:50,000, 6 days at 4°C; Oncogen Research Products, San Diego, California). The number of c-Fos-positive cells in ARH (at –1.46 mm behind the bregma) and PVH (at –1.06 mm behind the bregma) were counted in approximately the same plane by a researcher who was blind with respect to the treatment. The distance of sections behind bregma was determined according to the atlas of mouse brain (Paxinos and Franklin, 2001).

Neuroanatomical analysis
Immunohistochemical analysis of the hypothalamus was performed as above. The antibodies used were anti-NPY antibody (1:5,000, 6 days at 4°C; Chemicon Internatinal, Temecula, California) or anti-CART antibody (1:10,000, 6 days at 4°C; Phoenix Pharmaceuticals Inc., Belmont, California). Mice were sacrificed at 12 weeks of age. The NPY and CART immunostaining was examined on the sections at –1.06 mm behind the bregma. But we compared CART immunostaining on the sections at –0.82 mm behind the bregma in NN offspring with neonatal vehicle or leptin treatment because we did not obtain exact matches at –1.06 mm behind the bregma.

Statistics
Data are expressed as mean ± SEM and the statistical significance of differences in mean values was assessed using Student's t test or analysis of variance (ANOVA) with Fisher's protected least-significant-difference test, as appropriate. Differences among means were considered significant at values of $p < 0.05$.

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