Original article

MOLECULAR METABOLISM

Neonatal overnutrition causes early alterations in the central response to peripheral ghrelin

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ABSTRACT

Objective: Excess nutrient supply and rapid weight gain during early life are risk factors for the development of obesity during adulthood. This metabolic malprogramming may be mediated by endocrine disturbances during critical periods of development. Ghrelin is a metabolic hormone secreted from the stomach that acts centrally to promote feeding behavior by binding to growth hormone secretagogue receptors in the arcuate nucleus of the hypothalamus. Here, we examined whether neonatal overnutrition causes changes in the ghrelin system.

Methods: We used a well-described mouse model of divergent litter sizes to study the effects of postnatal overfeeding on the central and peripheral ghrelin systems during postnatal development.

Results: Mice raised in small litters became overweight during lactation and remained overweight with increased adiposity as adults. Neonatally overnourished mice showed attenuated levels of total and acyl ghrelin in serum and decreased levels of *Ghrelin* mRNA expression in the stomach during the third week of postnatal life. Normalization of hypoghrelinemia in overnourished pups was relatively ineffective at ameliorating metabolic outcomes, suggesting that small litter pups may present ghrelin resistance. Consistent with this idea, neonatally overnourished pups displayed an impaired central response to peripheral ghrelin. The mechanisms underlying this ghrelin resistance appear to include diminished ghrelin transport into the hypothalamus.

Conclusions: Early postnatal overnutrition results in central resistance to peripheral ghrelin during important periods of hypothalamic development. Because ghrelin signaling has recently been implicated in the neonatal programming of metabolism, these alterations in the ghrelin system may contribute to the metabolic defects observed in postnatally overnourished mice.

Keywords Ghrelin; Hypothalamus; Nutrition; Programming; Hormone; Tanycytes

1. INTRODUCTION

Over the past three decades, the prevalence of obesity and type II diabetes has increased at an alarming rate, including among children and adolescents [1,2]. Epidemiological data have suggested that excess nutrition and growth during pre- and/or post-natal life may contribute to the etiology of obesity and related diseases in later life, particularly in an environment with a wide availability of calorie-dense foods [3,4]. The results of experiments in a variety of animal models also support a link between the perinatal nutritional environment and the programming of energy balance "set points" [5–8]. Because of the importance of postnatal organ development, including that of the brain, animal models of postnatal metabolic programming have been developed to specifically target this developmental period. An animal model that has proven very useful for the study of postnatal overfeeding is a reduction of litter size. Pups raised in small litters (SL) display accelerated growth during the pre-weaning period, and these animals remain overweight throughout life [9–12]. In addition, postnatally overfed animals show accelerated and exacerbated weight gain and altered glucose tolerance when fed an obesogenic diet [10,12].

The precise biological substrates that mediate the effects of early postnatal overfeeding on later metabolic health are not fully understood. However, there is a growing appreciation that the developmental programming of hypothalamic systems involved in energy balance regulation by the perinatal environment represents a possible cause of obesity and related diseases. The rodent hypothalamus develops primarily during the postnatal period under the influence of intrinsic and environmental cues (see [13] for a review). Perturbations

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Abbreviations: AgRP, agouti-related peptide; ARH, arcuate nucleus; DMH, dorsomedial nucleus; GOAT, ghrelin O-acyltransferase; GHSR, growth hormone secretagogue receptor; HFHS, high-fat/high-sucrose diet; LHA, lateral hypothalamic area; MBH, mediobasal hypothalamus; ME, median eminence; NL, normal litters; NPY, neuropeptide Y; P, postnatal day; POMC, pro-opiomelanocortin; PVH, paraventricular nucleus; SL, small litter

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in the development of projections from the arcuate nucleus of the hypothalamus (ARH) are a common feature of animals subjected to nutritional insults during perinatal life, including in postnatally overfed mice [14–19]. Changes in the circulating levels of metabolic hormones, such as the adipocyte hormone leptin, in response to nutritional challenges that occur during early life represent a likely cause for the nutrition-induced alterations in hypothalamic development. For example, maternal obesity and postnatal overnutrition increase leptin levels throughout postnatal life and cause central leptin resistance during critical periods of hypothalamic development [10,16,20]. In contrast, maternal malnutrition during pregnancy and/or lactation blunts the naturally occurring postnatal leptin surge [14,15,21]. Remarkably, daily leptin treatment during early postnatal life in pups born to malnourished dams normalizes their metabolic abnormalities [22], indicating that the developmental actions of leptin contribute to the adult metabolic phenotype.

The gut-derived hormone ghrelin is also particularly well suited to transmit signals to the developing brain in response to alterations in the nutritional environment. In adults, circulating ghrelin levels are influenced by nutritional status [23–26], and neonatal ghrelin plays an important role in hypothalamic development and lifelong metabolic regulation [27]. However, whether ghrelin levels are regulated in response to nutritional challenges during early life and whether neonatal nutrition influences the hypothalamic reponse to ghrelin remain unknown. In the present study, we used the small litter model to determine whether overnutrition during early postnatal life influences the development of the ghrelin system and the sensitivity of hypothalamic neurons to ghrelin.

2. MATERIAL AND METHODS

2.1. Animals

Offspring of C57BL6 mice (Charles River Laboratory) produced in our mouse colony were used in these studies. Litters were normalized to 7 pups per litter on postnatal day 1 (P1), with 4 male and 3 female pups per litter. On P3, some litters were culled to 3 pups per litter (2 male + 1 female pups, SL = small litter) to induce postnatal overnutrition, whereas the control litters were maintained with 7 pups/litter (NL = normal litter). Animals were fed standard chow following weaning unless otherwise specified. Only male pups were used for the studies. Each experimental group in all experiments consisted of offspring from at least 3 litters. The animal usage was in compliance with and approved by the Institutional Animal Care and Use Committee of the University of Lille and the Saban Research Institute of the Children's Hospital of Los Angeles.

2.2. Physiological measurements

Pups were weighed once every two days from P4 to P22 and once weekly after weaning using an analytical balance. Body composition analysis (fat/lean mass) was performed at P120 using a LaTheta 100 X-ray Computed Tomography scanner. Food intake and respiratory exchange ratio (RER) were monitored at P90 using a combined indirect calorimetry system (TSE systems). Briefly, after adaptation for 3 days, O_2 and CO_2 production were measured for 3 days to determine the respiratory exchange ratio. In addition, food intake was determined continuously by the integration of weighing sensors fixed at the top of the cage from which the food containers were suspended into the sealed cage environment. Blood glucose levels were measured on P180 after overnight fasting using a glucometer (OneTouch Ultra, Johnson & Johnson). For the high-fat/high-sucrose (HFHS) challenge, mice were divided into two groups on P120: a first group was fed a standard chow diet for 8 weeks (also referred as "control" mice). A second group was fed for 8 weeks with a high-fat diet (60% fat) plus sucrose in the drinking water (20% wt/vol) (also referred as "HFHS" mice).

2.3. Ghrelin assays

Pups were decapitated on P8, P12, P14, P16, P22 and P60 and trunk blood was collected in a chilled tube containing Pefabloc (AEBSF, Roche Diagnostics). The collected serum was also acidified with 1 N HCl to achieve a final concentration of 0.05 N and then stored at −20 C until use. Total and acyl ghrelin levels in the serum were assayed using ELISA kits (Millipore). Acyl ghrelin levels were also characterized in mouse neonates injected with ghrelin. For this, P16 mice were injected with various doses of ghrelin (5, 10, and 50 ug/kg) or saline, and 15 min after injection the trunk blood was collected as described above.

2.4. Real-time qPCR analysis

The ARH (including the median eminence), DMH, and stomach of P14, P16, P22, and P60 mice ($n = 4–6$ per group) were dissected. For the ghrelin-induced changes in neuropeptide expression, P14 mice were given an intraperitoneal injection of ghrelin (Phoenix Pharmaceuticals, 2 mg/kg) or vehicle alone (0.9% NaCl) ($n = 3–5$) and were sacrificed 2 h later. Total RNA from the ARH and DMH was isolated using the Arcturus PicoPure RNA Isolation Kit (Life Technologies). Total RNA from the stomach was isolated using the RNeasy Lipid Tissue kit (Qiagen). cDNA was generated using the high-capacity cDNA Reverse Transcription Kit (Life Technologies). Quantitative real-time PCR analysis was performed using TaqMan Fast Universal PCR MasterMix. The mRNA expression was calculated using the 2^{-ddCt} method after normalization to *gapdh* (Mm99999915_g1) as a housekeeping gene. The inventoried TaqMan Gene expression assays for *Ghsr* (Mm00616415_m1), *Ghrelin* (Mm00445450_m1), *Goat* (Mm01200389_m1), *Pomc* (Mm00435874_m1), *Agrp* (Mm00475829_g1), *Npy* (Mm03048253_m1) were used. All assays were performed using an Applied Biosystems StepOnePlus real-time PCR system.

2.5. Chronic neonatal injection of ghrelin

Starting at P12, pups were treated twice daily with intraperitoneal injections of ghrelin (Phoenix Pharmaceuticals, 10 ug/kg) for a total of 10 days. Controls received equivolume injections of vehicle (0.9% NaCl).

2.6. cFos analysis

On P14, P16, P22, and P60 mice were given an intraperitoneal injection of ghrelin (Phoenix Pharmaceuticals, 2 mg/kg) ($n = 4–7$) or vehicle alone (0.9% NaCl) ($n = 3–9$) and were then perfused 2 h later with a solution of 4% paraformaldehyde. A separate cohort of mice received an intracerebroventricular injection of ghrelin at P14. For this purpose, 1 ul of ghrelin (240 ug/ml) or vehicle (0.9% NaCl) was stereotactically infused over 3 min into the lateral ventricle (1 mm lateral to Bregma 0, depth of 3 mm) under isoflurane anesthesia. Pups were perfused 90 min later with a solution of 4% paraformaldehyde. The brains were then frozen, cut into 30-um thick sections, and processed for cFos immunostaining using standard procedures [28]. Briefly, after pretreatment overnight in a mixture of 0.3% Triton X-100 and 2% normal goat serum, sections were incubated for 48 h at 4 C in a rabbit primary antiserum directed against the N-terminal domain of Fos (Ab-5, Oncogene; 1:2,000). The primary antibody was localized with Alexa Fluor 488 Goat anti-Rabbit IgG (Invitrogen; 1:200). The sections were counterstained using bis-benzamide (Invitrogen; 1:3,000) to visualize the cell nuclei and coverslipped with buffered glycerol (pH 8.5).

