Maternal prenatal undernutrition alters the response of POMC neurons to energy status variation in adult male rat offspring

Christophe Breton, Marie-Amélie Lukaszewski, Pierre-Yves Risold, Mihaela Enache, Johann Guillemot, Guillaume Rivière, Fabien Delahaye, Jean Lesage, Isabelle Dutriez-Casteloot, Christine Laborie, and Didier Vieu

1Neurosciences et Physiologie Adaptatives, UPRES EA 4052, Equipe Dénutrition Maternelles Périnatales, SN4, Université de Lille I, Villeneuve d’Ascq; and 2Oestrogènes, Expression Génique et Pathologies du Système Nerveux Central, UPRES EA 3182, Besançon, France

Submitted 3 September 2008; accepted in final form 10 December 2008

Breton C, Lukaszewski M, Risold PY, Enache M, Guillemot J, Rivière G, Delahaye F, Lesage J, Dutriez-Casteloot I, Laborie C, Vieu D. Maternal prenatal undernutrition alters the response of POMC neurons to energy status variation in adult male rat offspring. Am J Physiol Endocrinol Metab 296:E462–E472, 2009. First published December 16, 2008; doi:10.1152/ajpendo.90740.2008.—Epidemiological studies suggest that maternal undernutrition predisposes the offspring to development of energy balance metabolic pathologies in adulthood. Using a model of a prenatal maternal 70% food-restricted diet (FR30) in rats, we evaluated peripheral parameters involved in nutritional regulation, as well as the hypothalamic appetite-regulatory system, in nonfasted and 48-h-fasted adult offspring. Despite comparable glycemia in both groups, mild glucose intolerance, with a defect in glucose-induced insulin secretion, was observed in FR30 animals. These animals also exhibited hyperleptinemia, despite similar visible fat deposits. Using semiquantitative RT-PCR, we observed no basal difference of hypothalamic proopiomelanocortin (POMC) and neuropeptide Y (NPY) gene expression, but a decrease of the OB-Rb and an increase of insulin receptor mRNA levels, in FR30 animals. These animals also exhibited basal hypercorticosteronemia and a blunted increase of corticosterone in fasted compared with control animals. After fasting, FR30 animals showed no marked reduction of POMC mRNA levels or intensity of β-endorphin-immunoreactive fiber projections. By contrast, NPY gene expression and immunoreactive fiber intensity increased. FR30 rats also displayed subtle alterations of food intake: body weight-related food intake was higher and light-dark phase rhythm and refeeding time course were modified. A defect in glucose-induced insulin secretion together with a blunted increase of corticosterone in FR30 animals may contribute to basal hypercorticosteronemia.

Address for reprint requests and other correspondence: C. Breton, Neurosciences et Physiologie Adaptatives, UPRES EA 4052, Equipe Dénutrition Maternelles Périnatales, SN4, Université de Lille I, F-59655 Villeneuve d’Ascq, France (e-mail: christophe.breton@univ-lille1.fr).

IN ADDITION TO LIFESTYLE and dietary factors, increasing evidence suggests that the origin of some metabolic disorders that manifest in adult life may be traced to development. Indeed, epidemiological studies have shown that adverse environmental factors leading to intrauterine growth retardation (IUGR) and low birth weight may predispose individuals to later onset of energy balance metabolic pathology development (9, 17, 18, 20, 29). This has led to the concept of the developmental origin of adult diseases, also called “fetal programming,” or the Barker hypothesis (4). As illustrated by the Dutch Famine Study, offspring of women exposed to famine during early pregnancy displayed an increased risk of adiposity and glucose intolerance, as well as hypertension, later in life (41).

To obtain insights into the underlying mechanisms, numerous animal models, including maternal undernutrition, have been developed to promote intrauterine fetal programming (47, 56). These studies confirmed that impaired fetal development has long-term metabolic consequences, sensitizing the offspring to hyperphagia and obesity, particularly when they are fed a hypercaloric diet (22, 26, 53), reduced leptin and insulin sensitivity (10, 26, 50, 51), type 2 diabetes (43), and elevated blood pressure (32, 44). In adults, the pivotal role of the hypothalamus, especially the arcuate nucleus (Arc), in the maintenance of energy homeostasis controlling the nutritional status and energy storage level is well established (45). Peripheral hormones and energetic substrates act on feeding centers by modulating the expression and release of hypothalamic orexigenic and anorexigenic peptides, such as neuropeptide Y (NPY) and α-melanocyte-stimulating hormone [a neuropeptide derived from proopiomelanocortin (POMC) processing in the hypothalamus], respectively (2). Moreover, the hypothalamus plays an essential role in programming of fetal and neonatal development. In rodents, several studies suggest that hypothalamic “malprogramming” begins in utero but continues in early postnatal life during the suckling period, leading to a disturbed organization and, consequently, long-lasting dysfunction in adulthood. Indeed, perinatal (i.e., fetal or neonatal) modification of energy status, such as in uterine artery-ligated rat dams (40), perinatal low-protein-fed rat dams (37, 39), gestational diabetic rat dams (35, 36), and perinatal overfeeding (30, 38) and underfeeding (13, 30, 42) of neonatal rats, may permanently alter the structure of hypothalamic nuclei and the appetite-programming system in later life. These studies have mostly described alterations in NPY neurons, as well as increased gene expression, in fetal and postnatal offspring, supporting the notion that the NPY system is a key target of perinatal developmental programming, possibly disturbing body weight set point (13, 30, 38, 39, 40, 42). By contrast, very few experimental data are available from adult offspring (22, 35).
Among hypothalamic programming factors, a central role for leptin has been suggested by a study in which subcutaneous administration of leptin on postnatal days 3–13 reversed the hyperphagia and obesity in adult offspring of rats subjected to prenatal undernutrition (54). As a potential mechanism, leptin was found to promote neuronal outgrowth from the Arc to the paraventricular nucleus of the hypothalamus (PVN) during postnatal development and, thus, is highly involved in the plasticity and hardwiring of the hypothalamic appetite-regulatory circuits (5). In particular, we showed that maternal undernutrition prevents the postnatal surge of plasma leptin, disturbing preferentially the hypothalamic wiring, as well as the gene expression of the anorexigenic POMC, in male rat pups (11). Recently, it was shown that early postnatal blockade of leptin leads to long-term leptin resistance and increased susceptibility to diet-induced obesity in rats (3).

Using a model of prenatal maternal 70% food-restricted diet (FR30) in rats throughout gestation, we previously showed that FR30 induces IUGR and programs some metabolic syndrome (FR30) in rats throughout gestation, we previously showed that FR30 induces IUGR and programs some metabolic syndrome (9). We hypothesized that prenatal maternal FR30 induces developmental programming that durably modifies levels of key circulating factors acting on the hypothalamus, as well as hypothalamic gene expression, in adult offspring. The programmed modifications may result in alterations of long-term energy homeostasis and food intake in adulthood. To test our hypothesis, we investigated endocrine and metabolic parameters, hypothalamic appetite-regulatory factor gene expression, and related peptide levels in nonfasted and 48-h-fasted FR30 and control 4-mo-old rats. This study sheds new light on the manner in which maternal undernutrition disturbs the hypothalamic POMC anorexigenic circuit over the long term, as well as on the peripheral controlling factors that are critically involved in the lifelong regulation of food intake, body weight, and energy metabolism.

MATERIALS AND METHODS

Animal Model of Programming

Wistar rats (300 g body wt; Charles River Laboratories, L’Arbresle, France) were housed six per cage in a room maintained on a 12:12-h light-dark schedule (lights on at 0700) and controlled temperature (22–23°C). After 8 days of acclimation, females were mated with a male for 1 night. If spermatozoa were found in vaginal smears, the next day was considered day 0 of pregnancy. Pregnant females were transferred to individual cages, where they had free access to commercial food (regular rat chow: total digestible energy 2,900 cal/g (16% protein, 3% fat, and 60% carbohydrate); SAFE D04, UAR, Augy, France). Tap water was available ad libitum. Each pregnant dam was randomly assigned to the control (CT, n = 12) or food-restricted (FR30, n = 12) group. CT pregnant dams were fed ad libitum; pregnant dams from the FR30 group were fed 30% (7.2 g) of the daily intake of CT pregnant dams from day 1 of pregnancy until delivery (day 21). At parturition, each litter usually contained 7–13 fetuses. Pups were weighed, and litter size was adjusted to eight pups per dam (males preferred) for CT and FR30 mothers. Feed-restricted pups were nursed by FR30 dams fed ad libitum during lactation. To obviate any litter effects, animals used for each experiment were randomly chosen from different litters, and only a limited number of animals (n = 1–2) was used from each litter. After they were weaned, all animals were caged individually and fed ad libitum. Body weight and food intake of the offspring were measured weekly until adulthood. All parameters of adult male offspring from the CT and FR30 groups were measured at 4 mo of age. Animal use accreditation by the French Ministry of Agriculture (no. 04860) has been granted to our laboratory for experimentation with rats. Experiments were conducted in accordance with the principles of laboratory animal care of the European Communities (European Communities Council Directive of 1986, 86/609/EEC).

Decapitation and Plasma and Tissue Collection

At 2 days before they were killed, animals were randomly divided into two groups (n = 6): one group was fasted for 48 h, and the other was fed ad libitum. Nonfasted and 48-h-fasted animals were rapidly weighed and killed by decapitation between 9 and 10 AM. Trunk blood samples were collected into prechilled tubes containing EDTA (20 μl of a 5% solution), gently shaken, and centrifuged at 4,000 g for 10 min at 4°C. Aliquots of the supernatants were stored at −20°C. Hypothalami, liver, and white adipose tissue (WAT) and interscapular brown adipose tissue (BAT) fat pads were rapidly removed, weighed, frozen in liquid nitrogen, and stored at −80°C. For immunohistochemistry experiments, brains were fixed by intracardiac perfusion using buffered 4% paraformaldehyde solution. For double-label (POMC-c-Fos) in situ hybridization experiments, brains were removed and frozen on dry ice and stored at −80°C.

Food Intake

Food consumption was recorded weekly from weaning to adulthood in both groups. Four-month-old CT and FR30 animals (n = 6 per group) were housed individually in metabolic cages. After 8 days of acclimation, food intake of rats that remained in their home cages was measured twice a day at the beginning and end of the light phase (9 AM and 6 PM, respectively). All animals were presented with the same amount of food. Intake was measured for 3 consecutive wk by subtraction of the uneaten food and is presented as mean of food intake in grams. After these same animals were fasted for 48 h, we investigated the time course of refeeding between 9 AM and noon in both groups by measuring food intake at regular intervals 1, 2, and 3 h later.

Metabolic Parameters

For intraperitoneal glucose tolerance tests (IPGTT), rats were fasted overnight (16 h). Basal blood glucose level was determined using an automatic glucometer (Glucotrend 2, Roche Diagnostics) before the glucose injection (2 g/kg body wt, n = 12 per group). Tail vein blood glucose was then measured at 0, 15, 30, 60, and 120 min after injection. For insulin secretion assays, plasma insulin was measured to determine glucose-stimulated secretion at the same time. Plasma insulin concentrations were measured by ELISA (DRG International).

Endocrine Parameters

All plasma endocrine parameters under resting conditions and after 48 h of fasting were investigated using commercially available kits. Blood glucose and plasma insulin levels were determined as described above. Plasma leptin concentrations were measured with an active murine leptin ELISA kit (Diagnostic Systems Laboratories). Plasma corticosterone levels were determined by a competitive enzyme immunoassay (Immunodiagnostic Systems, Boldon, UK). Assay kits were used to determine the contents of plasma triglycerides and total cholesterol (61238 Triglyceride Enzymatique PAP100, 61218 Cholesterol Liquide, BioMérieux, France), as well as free cholesterol and free fatty acid (catalog nos. 279-47106 and 999-75406, Wako Chemicals, Neuss, Germany). Plasma C-reactive protein (CRP) levels were determined using a CRP ELISA kit (Millipore, Chemicon Products). Each point was measured in duplicate. Sensitivity of the assay was 0.07 ng/ml for insulin, 0.04 ng/ml for leptin, 0.55 ng/ml for corticosterone, and 2.5 ng/ml for CRP, and the intra- and interassay coeffi-
Semiquantitative RT-PCR

Hypothalamic RNA was extracted and purified (n = 6 per group) using the TRIzol reagent (GIBCO BRL, Strasbourg, France). The quality of the total RNA was assessed by determination of the ratio of absorbance at 260 nm to absorbance at 280 nm and by gel electrophoresis in agarose. To ensure appropriate amplification in the exponential phase for each target, we carried out PCR amplification in separate reactions with different numbers of cycles, but with similar amounts of the corresponding cDNA templates generated in single RT reactions. These preliminary experiments allowed us to determine the optimal cycle number for each primer pair for linear semiquantitative amplification, as described and validated elsewhere (6).

Briefly, 3 μg of total DNase-treated RNA were reverse transcribed into cdNA using 3 μg of random hexamers and 200 U of Moloney’s murine leukemia virus RT (GIBCO BRL). One-thirtieth of the first-strand synthesis reaction was amplified using 1 U of Taq polymerase (Qiogen, Illkirch, France) and forward and reverse primers at 2 μM each. The cycling parameters were as follows: 94°C for 90 s, 60°C for 90 s, and 72°C for 120 s. Negative control RT-PCR were performed by omission of RT from the reaction mixture or addition of H2O, instead of cdNA template, to the reaction mixture. The position of the primers and the predicted size of the amplification products are summarized in Table 1. Moreover, the priming sites were separated by an intron, which prevented amplification of any contaminating genomic DNA. Cyclophilin B was used as an internal standard. Each sample was denatured, separated on a 1% agarose gel, visualized by ethidium bromide, and quantified by Multi-Analyst software. The experiment was performed in triplicate and gave similar results. After amplification, the samples were separated on a 1% agarose gel, visualized by ethidium bromide, and quantified by Multi-Analyst software. The relative levels of expression are presented as the densitometric ratio of the test gene to the housekeeping gene (cyclophilin B).

Table 1. Primers used for semiquantitative RT-PCR analysis

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Accession No.</th>
<th>PCR Product, bp</th>
<th>Forward Position</th>
<th>Reverse Position</th>
<th>No. of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclophilin B</td>
<td>AF071225</td>
<td>456</td>
<td>155–179</td>
<td>586–610</td>
<td>23</td>
</tr>
<tr>
<td>POMC</td>
<td>AF510391</td>
<td>379</td>
<td>79–103</td>
<td>434–458</td>
<td>25</td>
</tr>
<tr>
<td>NPY</td>
<td>M20373</td>
<td>354</td>
<td>63–87</td>
<td>393–417</td>
<td>30</td>
</tr>
<tr>
<td>Leptin receptor (OB-Rb)</td>
<td>NM_012596</td>
<td>544</td>
<td>2265–2285</td>
<td>2788–2808</td>
<td>34</td>
</tr>
<tr>
<td>Insulin receptor</td>
<td>NM_017071</td>
<td>332</td>
<td>4110–4134</td>
<td>4417–4441</td>
<td>33</td>
</tr>
<tr>
<td>c-Fos</td>
<td>X06769</td>
<td>546</td>
<td>434–458</td>
<td>955–979</td>
<td>35</td>
</tr>
</tbody>
</table>

Accession numbers correspond to mRNA sequences. POMC, proopiomelanocortin; NPY, neuropeptide Y.

NPY, β-Endorphin, and c-Fos Immunohistochemistry

As previously described (11), brains were postfixed for 24 h in 4% paraformaldehyde in PBS, cryoprotected by incubation for 24 h in 0.05 M PBS containing 20% sucrose, and frozen in liquid nitrogen. The hypothalami were cut into serial 12-μm sections on a cryostat, mounted on gelatin-coated slides, and stored at −80°C. Sections from FR30 and CT brains (n = 6 rats per group) were incubated for 48 h at 4°C in the primary antibodies at the appropriate dilutions in PBS containing 0.3% Triton X-100 and 10% lactoprotein. The rabbit NPY antiserum (generously provided by Dr. H. Vaudry, University of Rouen, Rouen, France) was used at a dilution of 1:500. The rabbit antiserum to β-endorphin (prepared in our laboratory) was used at a dilution of 1:200. The rabbit anti-c-Fos (Santa Cruz Biotechnology, Santa Cruz, CA) was used at a dilution of 1:500. Labeling was revealed with secondary antibodies conjugated to Cy3TM (1:400 dilution; Jackson Immunoresearch Laboratories, Interchim) or Alexa Fluor (1:400 dilution; Molecular Probes, Interchim) for 2 h at room temperature. c-Fos immunoreactivity was revealed with a peroxidase-labeled goat anti-rabbit IgG (1:500 dilution; Vector Laboratories, Burlingame, CA). c-Fos protein was visualized using a nickel-intensified diaminobenzidine reaction to produce a black precipitate in cell nuclei. Semiquantitative analysis of the immunoreactive fibers projecting to different nuclei was based on averages of 20–50 coronal sections from 6 rats, depending on the area investigated. Images were observed on a fluorescence microscope (Olympus). Fluorescent images of coronal sections were captured spanning most of the nucleus investigated. Fibers were considered labeled when their respective staining was clearly above background. Semiquantification of fluorescence intensity was performed using image analysis software (analySIS 3.0, Soft Imaging System, Olympus), as previously described (11), and the relative density of NPY- and β-endorphin-immunoreactive fibers was assigned a score ranging from + to ++++ (see Table 3; also see Figs. 5 and 6).

Double-Label In Situ Hybridization

Coronal sections (12 μm thick) throughout the Arc were cut with a cryostat, mounted onto gelatin-coated slides, and air-dried. POMC and c-Fos RT-PCR fragments (Table 1) were subcloned into pGEM-T Easy, linearized, and used as riboprobes. The 35S-labeled c-Fos cRNA probe was mixed with the digoxigenin-labeled POMC cRNA probe in hybridization buffer (35S-labeled probe at 1.5 × 106 disintegrations/min and 50 ng of digoxigenin-labeled probe in 50 μl of buffer). Coverslips were applied, and the sections were hybridized at 55°C overnight. The coverslips were removed, and the slides were rinsed three times for 10 min each in 2× saline-sodium citrate (SSC) at room temperature. The slides were digested with RNase A (20 μg/ml) for 45 min, washed twice for 5 min each in 2× SSC, once for 10 min in 1× SSC, and once for 10 min in 0.5× SSC at room temperature and then washed twice for 30 min each in 0.1× SSC at 55°C. The slides were then washed briefly in buffer A [100 mM Tris·HCl (pH 7.5) and 150 mM NaCl] and incubated for 30 min at room temperature in blocking reagent (Boehringer Mannheim). Then the slides were washed briefly in buffer A and incubated for 3 h at 37°C in anti-digoxigenin alkaline phosphatase-conjugated antibody (Boehringer Mannheim) diluted 1:1,000 in buffer A. The slides were rinsed twice for 15 min each in buffer A and once for 10 min in buffer B [100 mM Tris·HCl (pH 9.5), 100 mM NaCl, and 50 mM MgCl2] at room temperature. Then the slides were incubated for 3 h at 37°C in buffer B with 0.34 mg/ml nitro blue tetrazolium salt and 0.18 mg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (Boehringer Mannheim). The slides were washed in 10 mM Tris·HCl (pH 8.0) and 1.0 mM EDTA to stop the chromogen reaction. Then the slides were placed in 70% ethanol for 15 s and air-dried. The slides were then dipped in 60% collodion (Wako Chemical) dissolved in isoamyl acetate, air-dried, and dipped in autoradiography emulsion. After 20 days of exposure, the slides were developed and mounted in an aqueous mounting medium. To assess the nonspecific labeling with digoxigenin-labeled probe and 35S-labeled probe, we used sense RNA.
probes on some adjacent sections from experimental animals, but we detected no specific signal. To estimate the percentage of POMC cells coexpressing the c-Fos gene, we examined six sections per animal, including the ventromedial part of the hypothalamus, at high magnification under bright-field illumination. Under these conditions, POMC mRNA-containing cells appear as dark-colored cell bodies and c-Fos mRNA-containing cells as autoradiographic clusters.

Statistical Analysis

Values are means ± SE. Statistical analysis was performed by two-way ANOVA (with prenatal undernutrition and diet as factors) followed by Newman-Keuls post hoc analysis. P < 0.05 was considered statistically significant.

RESULTS

Dam and Litters

There were no significant differences in the number of progeny per dam (9.8 ± 1.5 and 7.8 ± 0.8 pups/litter from CT and FR30 dams (n = 12 each), respectively, P = not significant (NS)) or the ratio of male births to total births in each litter (0.59 ± 0.09 and 0.46 ± 0.06 for CT and FR30 dams, respectively, P = NS). Maternal undernutrition resulted in fetal growth retardation, as reflected by decreased body weight at birth. As shown in Table 2, the body weights of male newborn pups differed between the CT and FR30 groups: 6.29 ± 0.11 and 4.45 ± 0.13 g (n > 30 each), respectively (P < 0.001). Body weight of FR30 rats born at full term was reduced by ~30%. From birth until adulthood, body weight of FR30 rats remained lower until 4 mo of age.

Adult Male Rats

Body composition and plasma hormone levels. As reported in Table 2, body weight of 4-mo-old FR30 rats remained reduced ~11% compared with CT rats (473.2 ± 7.2 vs. 422.5 ± 6.8 g, n > 30 each, P < 0.001), reflecting a partial postnatal catch-up growth. There were no significant absolute weight differences in the liver or the WAT (perirenal, epididymal, and subcutaneous) and interscapular BAT fat pads in 4-mo-old CT rats compared with maternal undernourished animals. Although their body weight was reduced, no change was observed in fat pads of FR30 rat adult offspring. When expressed relative to body weight, the weights of the perirenal, epididymal, and subcutaneous WAT fat pads were increased by 23%, 21%, and 12%, respectively, whereas the weight of the interscapular BAT fat pad was decreased by 12%.

Plasma triglyceride contents significantly decreased, whereas free fatty acid levels increased, after fasting in both groups. However, at 4 mo of age, regardless of the feeding conditions (fasted or nonfasted), no significant difference was noted in plasma lipid parameters between FR30 and CT rats. Nonfasting and fasting leptin concentrations were ~1.5-fold higher in FR30 than CT rats: 11.82 ± 1.71 vs. 6.28 ± 0.99 ng/ml (n = 6 each, P < 0.05) and 4.45 ± 0.49 vs. 2.63 ± 0.56 ng/ml (n = 6 each, P < 0.05) in nonfasted and fasted animals, respectively. In the nonfasted condition, corticosterone concentrations were about fourfold higher in FR30 than CT animals: 4.60 ± 1.86 vs. 1.24 ± 0.25 μg/dl (n = 6 each, P < 0.05). In CT animals, serum corticosterone concentration was ~25-fold higher in the fasted than nonfasted condition: 31.21 ± 1.82 vs. 1.24 ± 0.25 μg/dl (n = 6 each, P < 0.01). By contrast, 48-h-fasted FR30 rats showed no significant increase of corticosteronemia compared with nonfasted FR30 animals. Plasma CRP was increased in nonfasted FR30 compared with CT animals (43.81 ± 4.11 vs. 30.56 ± 3.06 μg/ml, n = 6 each, P < 0.05), whereas no difference was observed between fasted FR30 and CT animals.

Glucose tolerance test and insulin response. The glucose and insulin curves from the IPGTT are shown in Fig. 1A and B. CT and FR30 rats had comparable nonfasted, as well as fasted, blood glucose concentrations (Table 2). In the nonfasted state, insulin concentrations were higher in the FR30 than CT animals (4.34 ± 0.59 vs. 2.71 ± 0.32 μg/l, n = 12 each, P < 0.01), whereas in the fasted state, insulin levels of FR30 animals were not different from those of CT animals (Table 2). However, as depicted in Fig. 1A, plasma glucose was significantly higher in FR30 than CT rats (n = 12 per group) during the IPGTT at 30 min (P < 0.05) and 60 min (P < 0.01). This partial glucose intolerance of FR30 animals might be explained

Table 2. Effects of maternal undernutrition (FR30) on birth weight and morphometric and biological parameters of adult rats at rest and after 48 h of fasting

<table>
<thead>
<tr>
<th></th>
<th>CT Nonfasted</th>
<th>CT Fasted</th>
<th>FR30 Nonfasted</th>
<th>FR30 Fasted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth wt, g</td>
<td>6.29 ± 0.11</td>
<td>450.8 ± 5.6*</td>
<td>4.45 ± 0.13*</td>
<td>398.6 ± 9.5*</td>
</tr>
<tr>
<td>Adult body wt, g</td>
<td>473.2 ± 7.2</td>
<td>450.8 ± 5.6*</td>
<td>4.45 ± 0.13*</td>
<td>398.6 ± 9.5*</td>
</tr>
<tr>
<td>Liver wt, g</td>
<td>11.88 ± 1.75</td>
<td>11.68 ± 1.02</td>
<td>10.95 ± 3.98</td>
<td>10.46 ± 3.98</td>
</tr>
<tr>
<td>Perirenal WAT wt, g</td>
<td>9.97 ± 3.4</td>
<td>10.55 ± 4.11</td>
<td>10.55 ± 4.11</td>
<td>10.55 ± 4.11</td>
</tr>
<tr>
<td>Epididymal WAT wt, g</td>
<td>10.66 ± 4.23</td>
<td>10.55 ± 4.11</td>
<td>10.55 ± 4.11</td>
<td>10.55 ± 4.11</td>
</tr>
<tr>
<td>Subcutaneous WAT wt, g</td>
<td>0.59 ± 0.17</td>
<td>0.59 ± 0.32</td>
<td>0.26 ± 0.10</td>
<td>0.26 ± 0.10</td>
</tr>
<tr>
<td>Interscapular BAT wt, g</td>
<td>0.33 ± 0.13</td>
<td>0.33 ± 0.13</td>
<td>0.33 ± 0.13</td>
<td>0.33 ± 0.13</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>128.16 ± 41</td>
<td>34.02 ± 12.5*</td>
<td>146.37 ± 25</td>
<td>39.38 ± 6.7*</td>
</tr>
<tr>
<td>FFA, mmol/l</td>
<td>0.34 ± 0.19</td>
<td>0.63 ± 0.08</td>
<td>0.42 ± 0.07</td>
<td>0.82 ± 0.12*</td>
</tr>
<tr>
<td>Glucose, g/l</td>
<td>0.96 ± 0.03</td>
<td>0.73 ± 0.03*</td>
<td>0.95 ± 0.03</td>
<td>0.79 ± 0.04</td>
</tr>
<tr>
<td>Insulin, μg/l</td>
<td>2.71 ± 0.32</td>
<td>0.63 ± 0.13</td>
<td>4.34 ± 0.59*</td>
<td>0.62 ± 0.04*</td>
</tr>
<tr>
<td>Leptin, ng/ml</td>
<td>6.28 ± 0.99</td>
<td>2.63 ± 0.56</td>
<td>11.82 ± 1.71*</td>
<td>4.45 ± 0.49*</td>
</tr>
<tr>
<td>Corticosterone, μg/dl</td>
<td>1.24 ± 0.25</td>
<td>31.21 ± 1.82*</td>
<td>4.6 ± 1.86*</td>
<td>6.53 ± 2.76*</td>
</tr>
<tr>
<td>CRP, μg/ml</td>
<td>30.56 ± 3.06</td>
<td>361.14 ± 1.75</td>
<td>43.81 ± 4.11*</td>
<td>35.9 ± 3.73</td>
</tr>
</tbody>
</table>

Values are means ± SE [n > 30 rats/group (for weight values) and n = 6 rats/group (for fasted vs. nonfasted)]. WAT, white adipose tissue; BAT, brown adipose tissue; FFA, free fatty acid; CRP, C-reactive protein. *P < 0.05; **P < 0.01; ***P < 0.001 vs. CT in the same condition (i.e., fasted or nonfasted). dP < 0.05; eP < 0.01; fP < 0.001 vs. nonfasted.

AJP-Endocrinol Metab • VOL 296 • MARCH 2009 • www.ajpendo.org
and FR30 rats (137.44 and 136.05 ± 9 cal/g, respectively), FR30 rats displayed much higher weight-related food energy intake during the light phase (35.32 ± 0.43 vs. 17.66 ± 0.63 cal/g, \( P < 0.001 \)). As shown in Fig. 2D, after 48 h of starvation, absolute food intake during the 1st h of refeeding was reduced in FR30 compared with CT animals (5.47 ± 0.47 vs. 6.7 ± 0.13 g, \( n = 6 \) each, \( P < 0.05 \)), was increased during the 2nd h (2.67 ± 0.48 vs. 1.19 ± 0.41 g, \( n = 6 \) each, \( P < 0.05 \)), and returned to equal levels during the 3rd h. Measurement of weight-related food intake confirmed lower food energy intake during the 1st h of refeeding (37.59 ± 0.51 vs. 41.08 ± 0.79 cal/g, \( P < 0.05 \)) and much higher food energy intake during the 2nd h of refeeding (18.35 ± 0.63 vs. 7.29 ± 0.21 cal/g, \( P < 0.001 \)) in FR30 than CT rats.

**Hypothalamic mRNA contents of NPY, POMC, and leptin and insulin receptors.** Using RT-PCR analysis, we were unable to demonstrate any significant difference in hypothalamic NPY and POMC mRNA expression levels between nonfasted FR30 and CT animals (Fig. 3, A and B). However, as expected, fasting state led to expression differences for both neuropeptides. The mRNA levels of hypothalamic NPY increased significantly in fasted compared with nonfasted CT animals \( (P < 0.01) \), whereas only a tendency to increased expression was observed in fasted FR30 rats (Fig. 3A). We demonstrated a significant decrease in hypothalamic POMC mRNA levels in fasted compared with nonfasted CT animals \( (P < 0.05) \). However, fasted FR30 animals showed no significant decrease of POMC mRNA levels compared with nonfasted FR30 animals, but POMC mRNA levels were higher in fasted FR30 than fasted CT animals \( (P < 0.05; \ Fig. 3B) \). In addition, we detected a significant decrease in the mRNA content of the long isoform of the leptin receptor, OB-Rb \( (P < 0.001) \), as well as an increase of insulin receptor mRNA level \( (P < 0.001) \), in the hypothalamus of nonfasted FR30 compared with CT animals. After 48 h of fasting, no variation of OB-Rb mRNA level was observed in CT animals, whereas OB-Rb mRNA levels increased in FR30 rats, returning to a level comparable to that observed in CT animals \( (P < 0.001; \ Fig. 3C) \). Insulin receptor mRNA content increased significantly in fasted compared with nonfasted CT animals \( (P < 0.001) \), whereas insulin receptor mRNA levels did not vary between fasted and nonfasted FR30 animals (Fig. 3D).

**Distribution of c-Fos-expressing cells.** Animals were killed between 9 and 10 AM. At this stage, under resting conditions, a subtle distribution difference was observed in both groups throughout the ventromedial part of the hypothalamus, where only a few isolated c-Fos-expressing cells were evident. However, FR30 rats exhibited a striking increase in the number of c-Fos-containing cells in the ventral part of the Arc (Fig. 4B) compared with CT animals, which showed far fewer labeled nuclei in this hypothalamic area (Fig. 4A). The pattern of c-Fos induction observed in FR30 animals closely resembles the distribution of POMC-immunoreactive perikarya (data not shown). In addition, CT animals displayed a slight increase in the number of c-Fos-containing cells in the hypothalamic ventromedial nucleus (VMH), whereas no c-Fos labeling was visible in the VMH in FR30 animals (Fig. 4A). At 48 h of fasting, a large decrease was observed in the intensity of c-Fos-immunoreactive nuclei in the ventral Arc of FR30 rats at the limit of detection, whereas a few c-Fos-expressing cells

**Food intake.** As described in Fig. 2C, absolute total daily food consumption over 24 h was similar in CT and FR30 animals in adulthood: 25.11 ± 0.74 and 25.14 ± 0.81 g, respectively \( (n = 6, P = NS) \). However, weight-related food intake, calculated as energy in food ingested, was higher in FR30 than CT rats: 173 ± 17 vs. 154 ± 12 cal·g body wt·J−1·J−1 \( (n = 6 \) each, \( P < 0.05) \). Although rats are nocturnal animals, absolute food intake was lower during the dark phase (Fig. 2B; 20 ± 0.77 vs. 22.19 ± 0.71 g, \( n = 6 \) each, \( P < 0.05 \)) and higher during the light phase (Fig. 2A; 5.14 ± 0.42 vs. 2.88 ± 0.24 g, \( n = 6 \) each, \( P < 0.001 \)) in the FR30 than CT group, resulting in modified light-dark phase food intake rhythm. Although we observed no significant difference of weight-related food intake during the dark phase between CT by a defect in glucose-induced insulin secretion and/or a decrease in insulin sensitivity. To test the first hypothesis, we examined potential changes in insulin levels after an intraperitoneal bolus of glucose (Fig. 1B). The increase over basal insulin levels was transiently lower in FR30 than CT rats after the glucose bolus at 15 min \( (P < 0.05) \) but no difference was observed from 30 to 120 min in B.
were detectable in CT animals. c-Fos labeling was undetectable in the VMH in fasted CT animals (data not shown).

Colocalization of c-Fos and POMC mRNA. To identify the cells expressing the c-Fos gene in the Arc in FR30 rats, we performed double-label in situ hybridization using a digoxigenin-labeled POMC cRNA and a 35S-labeled c-Fos cRNA probe. Figure 4D shows a representative coronal section of the ventral part of the Arc processed via double-label in situ hybridization. The distribution of cells expressing the c-Fos gene overlapped the distribution of POMC neurons. Approximately 30% of the c-Fos mRNA-containing cells also expressed the POMC gene.

Immunocytochemistry of NPY- and β-endorphin-containing neurons and fibers. In the fed state, NPY and β-endorphin antisera displayed intensely labeled cell bodies, with the expected pattern in the Arc of CT and FR30 animals. As mentioned above, POMC-containing neurons were distributed throughout the medioventral part of the Arc more ventrally than the NPY-containing perikarya. The distribution and number of NPY- and POMC-immunoreactive perikarya showed no visible difference between CT and FR30 rats. Labeled axons were observed in many brain regions with both antisera. No obvious distribution and intensity differences in the innervation of the brain areas by NPY were observed between FR30 and CT rats (Table 3). Also, there was no visible difference in POMC-immunoreactive fiber distribution and intensity between the two groups in brain regions such as the PVN (Fig. 5, A and B), the dorsomedial hypothalamic nucleus (DMH), and the paraventricular nucleus of the thalamus (PVT; Fig. 5, A and B; Table 3). As expected, after 48 h of fasting, a similar marked increase in the fluorescence intensity and number of NPY-labeled axons was observed in all brain areas of CT as well as FR30 animals (Table 3). By contrast, no decrease in the fluorescence intensity and number of β-endorphin-labeled axons was observed in fasted FR30 compared with fasted CT animals in the PVN (Fig. 5, C and D), DMH, and PVT (Fig. 5, C and D; Table 3).

DISCUSSION

The principal finding of this study is that maternal prenatal undernutrition leads to alterations of the hypothalamic POMC system in the adult male FR30 rat. Under resting conditions, we observed no obvious differences in the distribution and number of NPY and POMC neurons, the NPY and β-endorphin density of Arc projections, and hypothalamic NPY and POMC gene expression between CT and FR30 rats. However, after fasting, FR30 animals displayed no marked fall of POMC mRNA or β-endorphin-immunoreactive fiber intensity throughout innervated brain areas, whereas NPY mRNA and NPY-immunoreactive fiber intensity were increased. Accordingly, after starvation, food intake was reduced in FR30 rats during the 1st h of refeeding, suggesting an adaptation to fasting. Recent studies pointed out the
importance of melanocortin in refeeding-induced PVN neuronal activation and satiety (48).

We confirmed that POMC neurons are a hypothalamic target of maternal undernutrition. Indeed, we recently demonstrated a dramatic reduction of the postnatal leptin surge affecting preferentially the anorexigenic POMC system in weanling male rats exposed to a 50% maternal food restriction in late gestation and lactation (11). We hypothesized that lactation may have been compromised in the FR30 dams during the early postpartum period until the increase of food intake could compensate for the deficient state. This might, at least partly, account for subtle perturbations of the POMC system, which continues to develop during the first 2 wk of lactation.

These findings are in agreement with data that showed short- and long-term consequences of manipulation of maternal nutrition and hormone level during the perinatal period on hypothalamic nuclei structure, as well as neuropeptide expression levels, of the offspring (13, 22, 30, 35–39, 40, 42). In contrast to related models that have mostly reported disorganization of the NPY system in offspring (13, 30, 35, 36, 38, 39, 40, 42), alterations of the NPY system in adult FR30 rats are weak, if not negligible. An explanation for such a discrepancy might be that, apart from leptin, mechanisms beyond maternal undernutrition that are thought to influence hypothalamic appetite programming during the perinatal period are largely unknown and might differ depending on the models used.

Despite showing increased food intake relative to body weight, FR30 adult offspring showed reduced body weight and no change in visible fat pads. Some hypotheses may explain this apparent discrepancy. On the one hand, it has been shown that IUGR might be responsible for somatic growth alterations leading to shorter (body length) and lighter (body weight) rat offspring (21, 25, 53, 55). Thus, in addition to skeletal growth modifications (16, 21), we cannot rule out the possibility that a different lean-to-fat mass ratio due to a deficit in muscle mass and/or different body fat pad distribution occurs in adult FR30 rats (25). To address this question, further experiments, such as absorptiometric studies, are needed to measure the total body composition of FR30 adult rats. On the other hand, additional mechanisms, such as modified spontaneous motor activity and adipose tissue and/or muscle thermogenesis, may also account for increased energy expenditure in FR30 adult rats (12).

FR30 animals exhibited hyperleptinemia, as well as similar visible fat deposits. When expressed relative to body weight, the weights of the white fat pads were increased by 20% in FR30 compared with CT adult rats. These findings are in agreement with a recent report of hyperleptinemia without an obese phenotype in mice with adipose androgen receptor deficiency (57). As supported by others (26, 51, 53), hyperleptinemia may be interpreted as a leptin-resistant state.

At rest, we found no difference in expression of NPY and POMC mRNA levels between FR30 and CT groups. After 48 h of fasting, fasted FR30 rats showed no marked decrease of POMC mRNA levels compared with nonfasted FR30 animals.
negative feedback to the hypothalamic-pituitary-adrenal (HPA) axis by reducing hypothalamic corticotropin-releasing hormone expression (19), whereas FR30 rats continued to display a high basal level of circulating corticosterone. At rest, we also observed a decrease in hypothalamic OB-Rb mRNA levels in FR30 rats. Leptin resistance based on a specific decrease in hypothalamic OB-Rb mRNA has been reported in hyperleptinemic rats (27). In addition, we observed an increase of hypothalamic insulin receptor mRNA levels in FR30 rats. Concurrently, increased hypothalamic insulin signaling, as well as hyperinsulinemia, may also influence leptin sensitivity (33). However, in view of the widespread hypothalamic distribution of leptin and insulin receptors, further studies are required to more precisely localize their expression in hypothalamic nuclei of FR30 compared with CT adult rats. Interestingly, we observed that plasma CRP, a liver-derived protein defined as a low-grade inflammatory marker, was increased in nonfasted FR30 rats. Recent data suggest that leptin resistance may be mediated by circulating CRP, which directly binds leptin and attenuates its physiological functions by inhibiting the binding of leptin to its receptor (7).

Alternatively, hyperleptinemia might normally stimulate the appetite-programming system. Indeed, in contrast to hyperleptinemia resulting from increased fat mass, enhanced plasma leptin in mice with adipose androgen receptor deficiency did not cause leptin resistance (57). As suggested by others (22, 42), FR30 rats might have developed an adaptive resetting of the neuronal activation threshold, which could explain normalized POMC and NPY gene expression levels. Leptin is known to increase c-Fos expression only in POMC neurons (15). Thus the increase of the number of c-Fos-expressing POMC neurons in the ventral Arc might result from chronic leptin activation in FR30 rats. In addition, we cannot rule out the possibility that hyperleptinemia in FR30 rats might impede the decrease of POMC gene expression after fasting (1). Additional experiments are needed to investigate the leptin-induced activation of the signal transducer and activator of transcription 3 or suppressor of cytokine signaling 3 (33) gene expression levels in distinctive populations of Arc neurons.

Taken together, our data suggest that the appetite-regulatory mechanisms would be differently programmed in FR30 rats. The fact that FR30 adult rats displayed higher body weight–related food intake, despite no change in basal POMC and NPY, raises the question about the mechanisms underlying long-term hypothalamic energy regulation. In addition to the Arc, it would be important to determine the role of other hypothalamic nuclei, such as the VMH, DMH, PVN, and lateral area, which also contain numerous orexigenic and anorexigenic factors that play an important role in food intake regulation.

We previously showed that maternal undernutrition programs the HPA axis of the offspring throughout its lifespan (28). In particular, perinatal maternal food restriction led to

Table 3. Estimation of the labeling intensity of β-endorphin- and NPY-immunoreactive fibers in paraventricular and dorsomedial hypothalamic nuclei, as well as paraventricular nucleus of thalamus of adult rats

<table>
<thead>
<tr>
<th>β-Endorphin</th>
<th>NPY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonfasted</td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>+++</td>
</tr>
<tr>
<td>FR30</td>
<td>+++</td>
</tr>
<tr>
<td>Fasted</td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>+</td>
</tr>
<tr>
<td>FR30</td>
<td>+++</td>
</tr>
</tbody>
</table>

Labeling intensity of fibers provided by β-endorphin and NPY antisera was scored as follows: ++++, intense; ++, moderate; +, low. Similar variations were observed in the different areas investigated.
hypercorticosteronemia accompanied by disturbed glucocorticoid feedback, leading to 8-mo-old male rats that were unable to cope with severe stress (14, 46). Our data demonstrated hypercorticosteronemia in FR30 rats as early as 4 mo of age and a blunted increase of corticosterone after fasting. Hypothalamic POMC system perturbations might, at least partly, be responsible for altered HPA activity (19, 52). Indeed, fasted FR30 rats displayed modifications of POMC-derived peptide-labeled axons in brain areas involved in HPA axis regulation, such as the PVN (19, 52) and PVT (23). Accordingly, a recent study showed elevated basal plasma corticosterone, elevated basal but attenuated stress-induced ACTH secretion, and inappropriately elevated corticotropin-releasing hormone expression in the PVN in POMC-deficient (POMC<sup>−/−</sup>) mice with the selective restoration of pituitary POMC (49).

Chronic increased circulating glucocorticoids are known to raise glycemia by promoting gluconeogenesis, insulin resistance, and reduced insulin secretion (8). However, glucose and insulin concentrations were similar in fasted FR30 and CT animals, whereas mild glucose intolerance accompanied by a
defect in glucose-induced insulin secretion was observed in fasted FR30 animals. Contrary to the rat FR30 model described by Vickers and others (26, 53–55), we observed no difference in the plasma insulin levels between the fasted CT and FR30 groups. This discrepancy is probably due to the fact that, in their FR30 model, pups from undernourished mothers were cross-fostered onto dams that had been fed ad libitum. This marked difference between both models emphasizes the importance of normal lactation and catch-up growth during early lactation of undernourished pups (10, 26, 53).

In our model, contrary to the Barker hypothesis, impaired glucose tolerance was not caused by insulin resistance but, at least in part, by decreased insulin secretion. These findings are consistent with data demonstrating that the effect of maternal undernutrition on offspring blood glucose and insulin levels largely depends on the species, as well as the protocols, used. Thus the different outcome of maternal FR30 for regulation of glycemia in offspring may reflect fetal and/or postnatal programming differences, such as the timing of sensitive developmental windows, the nature of the malnutrition, or the genetic background of the species (29, 43).

Finally, FR30 rats exhibited dysregulated day-night food intake rhythm. In rats, recent studies reported that prenatal maternal feeding modifications may program the biological clock and circadian rhythm of the offspring (34). In addition, exposure of the fetal developing brain to an excess of circulating glucocorticoids, which occurred during gestation in undernourished FR30 dams, might result in resetting of circadian rhythms of the HPA axis in the offspring (31). These factors might, at least in part, account for impaired central circadian clock activity and sleep-arousal balance perturbation in FR30 rats. This hypothesis is supported by additional observations. On the one hand, at rest, the morning increase in plasma insulin level observed in FR30 rats might reflect post-prandial insulinemia as a result of modified light-dark food intake rhythm. Feeding and increased insulin levels induce c-Fos expression in POMC neurons (48). Accordingly, in the morning, FR30 rats exhibited a striking increase in the number of c-Fos-containing cells in the ventral part of the Arc. About 30% of the c-Fos-expressing cells were POMC neurons. On the other hand, during the dark period of the cycle, the ventromedial Arc spontaneously exhibited increased c-Fos immunoreactivity, whereas very low c-Fos labeling was detectable during the light period. This nocturnal induction occurred at the nuclear level of POMC-producing neurons (24). Thus activation of the Arc melanocortin-signaling system might contribute to the termination of food intake and satiety, a period that might be delayed in FR30 rats (24, 48).

In conclusion, we have demonstrated that maternal undernutrition differently programs the long-term appetite-regulatory system in their offspring, especially the response of POMC neurons to energy status and food intake rhythm.

ACKNOWLEDGMENTS

The authors thank V. Montel and A. Dickes-Coopman for technical assistance.

GRANTS

This study was supported by grants from the French Ministry of Education and grants from the Conseil Régional du Nord-Pas de Calais.

REFERENCES

7. Chen K, Li F, Li J, Cai H, Strom S, Bisello A, Kelley DE, Friedman-Engelbregt MJT, van Weissenbruch MM, Lips P, van Lingen A, Roos Houdijk ECAM, Engelbregt MJT, Popp-Snijders C, Delemarre-van de Waal HA. Distinct physiologic and circadian rhythm of the offspring may reflect fetal and/or postnatal programming differences, such as the timing of sensitive developmental windows, the nature of the malnutrition, or the genetic background of the species (29, 43).

23. Jaferi A, Bhatnagar S. Corticosterone can act at the posterior paraventricular nucleus to inhibit hypothalamic-pituitary-adrenal activity in an-