Early Overnutrition Results in Early-Onset Arcuate Leptin Resistance and Increased Sensitivity to High-Fat Diet


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Childhood obesity increases the risk of adult obesity and diabetes, suggesting that early overnutrition permanently programs altered energy and glucose homeostasis. In the present studies, we used a mouse model to investigate whether early overnutrition increases susceptibility to obesity and insulin resistance in response to a high-fat diet (HFD). Litters from Swiss Webster dams were culled to three [chronic postnatal overnutrition (CPO)] or 10 (control) pups and then weaned onto standard chow at postnatal day (P) 23. At 6 wk of age, a subset of mice was placed on HFD, and glucose and insulin tolerance were examined at 16–17 wk of age. Leptin sensitivity was determined by hypothalamic phosphorylated signal transducer and activator of transcription-3 immuno-reactivity at P16 and adulthood after ip leptin. CPO mice exhibited accelerated body weight gain and hyperleptinemia during the preweaning period but only a slightly heavier body weight and normal glucose tolerance in adulthood on standard chow diet. Importantly, CPO mice exhibited significant leptin resistance in the arcuate nucleus, demonstrated by reduced activation of phospho-signal transducer and activator of transcription-3, as early as P16 and throughout life, despite normalized leptin levels. In response to HFD, CPO but not control mice displayed insulin resistance in response to an insulin tolerance test. In conclusion, CPO mice exhibited early and persistent leptin resistance in the arcuate nucleus and, in response to HFD, rapid development of obesity and insulin resistance. These studies suggest that early overnutrition can permanently alter energy homeostasis and significantly increase susceptibility to obesity and insulin resistance. 

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Rates of childhood obesity have risen dramatically in recent years, leading to earlier onset and increased risk for associated diseases, including type 2 diabetes and cardiovascular disease (1–3). In rodents, early overnutrition can be induced by reducing litter size, as first demonstrated by Kennedy (4). A small litter size results in increased milk availability and therefore increased consumption, which results in accelerated body weight gain during the preweaning period (5). Numerous studies using this mild manipulation, which we term chronic postnatal overnutrition (CPO), have shown a wide range of metabolic alterations, including hyperleptinemia, hyperinsulinemia, hyperphagia, and glucose intolerance in adulthood (6–14); however, the degree of these findings has varied, depending on the species and strain of rodent used.

Other models of nutritional programming, including maternal protein restriction and maternal food restriction (15, 16), exhibit hyperleptinemia during the early postnatal period, which we term chronic postnatal overnutrition (CPO), have shown a wide range of metabolic alterations, including hyperleptinemia, hyperinsulinemia, hyperphagia, and glucose intolerance in adulthood (6–14); however, the degree of these findings has varied, depending on the species and strain of rodent used.

Abbreviations: AGRP, Agouti-related peptide; ARH, arcuate nucleus of the hypothalamus; AUC, area under the curve; BAT, brown adipose tissue; CPO, chronic postnatal overnutrition; CTR, control; DIO, diet-induced obese; DMH, dorsomedial nucleus of the hypothalamus; GTT, glucose tolerance test; H&E, hematoxylin and eosin; HFD, high-fat diet; ir, immunoreactive; ITT, insulin tolerance test; NPY, neuropeptide Y; P, postnatal day; POMC, proopiomelanocortin; pSTAT3, phosphorylated signal transducer and activator of transcription-3; qPCR, quantitative RT-PCR; SOCS3, suppressor of cytokine signaling-3; UCP1, uncoupling protein 1.
natal period and some degree of leptin resistance in adulthood, suggesting that hyperleptinemia during a critical period of development may be a key signal for malprogramming of energy homeostasis. Whereas leptin is a critical trophic factor in the hypothalamus during development, inhibitory mechanisms mediating leptin effects on food intake are not fully functional until at least the fourth postnatal week (17–22). Importantly, electrophysiological studies by Davidowa and Plagemann (23) demonstrated that in 5- to 11-wk-old CPO rats, neurons in the arcuate nucleus of the hypothalamus (ARH) neurons exhibit a reduced inhibitory response to leptin, suggesting that early overnutrition may similarly result in leptin resistance. Davidowa and Plagemann (23) further suggested that early development of ARH leptin resistance may contribute to the long-term effects on body weight in the CPO model, although these studies did not examine leptin responsivity in CPO pups.

In adult models of diet-induced obesity, leptin resistance is generally localized to the ARH and results from prolonged hyperleptinemia (24–26). Because CPO pups exhibit hyperleptinemia at an age when leptin inhibitory pathways are not fully developed, we hypothesized that leptin resistance may develop rapidly during the preweaning period, resulting in malprogramming of the hypothalamic feeding neurocircuitry. In the present studies, we investigated whether CPO mice have a permanent alteration of leptin responsiveness and subsequent increased sensitivity to high-fat diet (HFD). We show that CPO mice exhibit early-onset leptin resistance that persists into adulthood, despite being maintained on a healthy standard chow diet. In response to HFD, these deficits result in the rapid development of obesity and insulin resistance, suggesting that early overnutrition can permanently alter energy and glucose homeostasis.

Materials and Methods

Animals and diets

All mice were maintained on a 12-h light, 12-h dark cycle (lights on at 0700 h) and constant temperature (23 ± 2 °C). Pregnant Swiss Webster mice (Simonsen Laboratories Inc., Gilroy, CA) were fed standard mouse chow (Purina Lab Chow no. 5001, 13.5 kcal percent fat, 58.0 kcal percent carbohydrates, 28.5 kcal percent protein; Ralston Purina Corp., St. Louis, MO), and day of birth was considered postnatal day (P) 0. Only litters that were between 7 and 12 pups at birth were used. For the control (CTR) group (13 litters total), litters were culled to 10 pups on P2. CPO litters (total of 31 litters) were initially culled to six pups on P2 and then further reduced to three pups on P5. Male offspring were weaned onto standard chow on P23 and housed three to four per cage. Pups were randomized among dams at P2 such that each litter represented pups from two to five different birth dams. This was done to ensure that body weights were matched at P2 and also increased the genetic variability within litter.

In two replicate studies, mice either remained on standard chow (25 CTR, 25 CPO mice) or were placed on a highly palatable 45% HFD (Research Diets, New Brunswick, NJ; catalog no. D12451, 45 kcal percent fat, 35 kcal percent carbohydrates, 20 kcal percent protein) at P44 and then 60% HFD (catalog no. D12492, 60 kcal percent fat, 20 kcal percent carbohydrates, 20 kcal percent protein) at P57 (38 CTR, 38 CPO mice). Body weights were measured once weekly and mice were killed at 154–174 d of age (at 1300 h) by isoflurane sedation and decapitation. Glucose and leptin responsivity were measured in adult mice as described below. Trunk blood was collected for measurement of serum leptin levels, and fat and lean mass were assessed by dual-energy x-ray absorptiometry (Lunar PIXimus II, GE Medical Systems, Madison, WI) immediately after decapitation. Percent fat and lean mass were calculated from the total carcass weight as determined by the dual-energy x-ray absorptiometry scan. The whole hypothalamus and whole interscapular brown adipose tissue (BAT) pads were removed and frozen on dry ice for measurement of RNA by real-time quantitative RT-PCR (qPCR). Heart, kidney, and liver were removed and weighed. A subset of mice was perfused for liver histology, as described below. All animal procedures were conducted in accord with accepted standards of humane animal care and approved by the Oregon National Primate Research Center Institutional Animal Care and Use Committee.

RNA isolation and qPCR analysis

RNA was analyzed by qPCR on whole hypothalamus and whole BAT as previously described (14, 27). Briefly, tissue was homogenized in 1 ml Trizol reagent (Invitrogen, Carlsbad, CA), and total cellular RNA was isolated according to the manufacturer’s specifications. Total RNA was further purified using the RNeasy minikit (QIAGEN Inc., Valencia, CA). The concentration of RNA was determined by spectrophotometry (Nanodrop ND-1000; NanoDrop Technologies, Wilmington, DE), and RNA integrity was confirmed by bioanalysis (Agilent 2100 bioanalyzer; Agilent Technologies, Inc., Santa Clara, CA). RNA samples (1 μg) were deoxyribonuclease treated and reverse transcribed using random hexamer primers (Promega, Madison, WI). Genes of interest were analyzed by individual real-time PCR assays (n = 6–9/group) using predesigned and validated real-time PCR probes and primers (Applied Biosystems, Foster City, CA) for leptin receptor (no. Mm0040186_m1) and suppressor of cytokine signaling-3 (SOCS3; no. Mm01249143_g1) in whole hypothalamus, and uncoupling protein 1 (UCP1) (no. Mm01244861_m1) for BAT, with 18S RNA used as an internal control.

Glucose (GTT) and insulin (ITT) tolerance tests

Intraperitoneal GTTs and ITTs were performed at 15 and 17 wk of age, respectively (after 9 and 11 wk on HFD), as previously described (25). For GTTs animals were fasted overnight, and glucose (1 g/kg body weight) was injected ip. Blood glucose was measured from the saphenous vein using a glucometer (Accucheck; Roche Diagnostic Corp., Indianapolis, IN) at 0, 15, 30, 60, and 120 min after injection in unanesthetized mice under manual restraint. For animals tested after 10 wk on HFD, plasma samples were collected for determination of insulin levels only at 0 and 60 min after glucose administration. For ITTs, mice were
injected with 1 U/kg human recombinant insulin (Eli Lilly, Indianapolis, IN) and blood glucose was determined at 0, 15, 30, 60, and 120 min.

**Food intake**

On P35, a subset of mice was singly housed and maintained on standard chow diet. Food hoppers were weighed at 0900 h daily, from P38 to P44 for determination of daily food intake. At P44, standard chow was replaced with 45% HFD and then again replaced with 60% HFD at P57, and 24-h food intake was determined daily.

**Temperature and activity telemetry**

Measurements of temperature and activity levels were assessed in a separate cohort of individually housed mice (10 CTR mice and five CPO mice) by implantation of G2 E-mitters (Mini-Mitter, Bend, OR) in the interscapular region, beneath the BAT pad. Activity and interscapular temperature measures were recorded automatically at 6-min intervals using ER-4000 energizer/probes and VitalView data acquisition software (Mini-Mitter), and baseline measures were averaged across 5 d (P38–42).

**Leptin responsiveness**

Central leptin responsiveness was assessed by quantifying phosphorylated signal transducer and activator of transcription-3 (pSTAT3)-immunoreactive (ir) cells in response to leptin in P16 or adult (22 wk old) mice. Adult mice were sham injected for 4 d before testing and fasted from 0800 to 1200 h on testing day, whereas pups were maintained with dams until testing. Mice were injected ip with saline or leptin (2 or 3 μg/g leptin ip for adults or pups, respectively) at 1200 h. After 45 min, mice were sedated with pentobarbital and perfused transcardially with saline followed by ice-cold, borate-buffered 4% paraformaldehyde (pH 9.5). Brains were processed as previously described (25).

To assess functional responsiveness to leptin, adult mice were injected ip with saline for 4 consecutive days. In 8-wk-old chow-fed mice (CTR vs. CPO), food was removed from hoppers at 1600 h, and then mice were injected ip with saline or leptin (2 μg/g) at 1900 h, and food was returned 15 min later. Food was reweighed at 0900 h. Injections were repeated at 1900 h on the second day and food reweighed at this time and the following morning (0900 h). At 22 wk of age, effects of injection in chow- and HFD-fed mice were assessed by measuring 48-h change in body weight after 2 consecutive days of leptin injection. A crossover design was used such that saline and leptin injections were reversed 5 d later, with each mouse serving as its own saline-injected control.

**Immunohistochemistry and histology**

Perfused brains were sectioned (30 μm) on a microtome in a one-in-six coronal series through the hypothalamus. pSTAT3 immunohistochemistry was performed as previously described (25) on every sixth section, using a rabbit anti-pSTAT3 antibody (Cell Signaling Technology, Inc., Danvers, MA; catalog no. 9145, 1:250), and staining was optimized for the ARH. pSTAT3-ir-positive cells were counted bilaterally under a light microscope by an investigator blind to the treatment group, through the rostrocaudal extent of the ARH and dorsomedial nucleus (DMH) of the hypothalamus and expressed as the total number of pSTAT3-ir-positive cells. Regions analyzed were anatomically matched across all animals with three ARH and two DMH sections being analyzed per animal (n = 3–6/group).

Perfused liver was paraffin embeded and sectioned (8 μm) on a microtome. Hematoxylin and eosin (H&E) staining was performed on two liver sections per animal and assessed under light microscopy by two independent observers blind to the treatment group. Extent of liver steatosis was graded as either mild (isolated regions of steatosis, mainly macrovesicular), moderate (scattered regions of both macrovesicular and microvesicular steatosis), or severe (extensive macrovesicular and microvesicular steatosis throughout section), using a modification of the grading system described by Brunt et al. (28). Fat content in liver was detected using an Oil Red O stain kit (American MasterTech Scientific, Inc., Lodi, CA), as per the manufacturer’s specifications, in 10 μm fresh frozen liver sections.

Images were captured under bright-field illumination using a Photometrics CoolSNAP HQ camera (Roper Scientific, Tucson, AZ) connected to a Nikon microscope (E800; Tokyo, Japan) with a Plan Apo ×10 or ×20 (NA 0.75) objective. The brightness and contrast levels of the digital images were adjusted with Adobe Photoshop (Adobe Systems, San Jose, CA).

**Statistical analyses**

For chronic HFD studies, two-way ANOVAs were conducted for the factors of treatment (CTR, CPO) and diet (CHOW, HFD). Body weights were analyzed by three-way repeated-measures ANOVAs for the factors of treatment, diet, and age, with age treated as a repeated measure. Baseline temperature measurements were calculated using averaged temperature across 5 d for each animal and two-way repeated measures ANOVAs for factors of treatment and time during either the entire lights-off or lights-on period. Baseline activity was determined by averaging measurements over 5 d for each animal, and area under the curve (AUC) measurements across 24 h were determined from this average for each animal. AUCs for GTTs and ITTs were calculated using the trapezoidal method, with baseline set as the 0 min blood glucose value for GTTs and 0 mg/dl blood glucose for ITTs. Treatment groups were compared using an independent t test for CPO vs. CTR. All significant main or interaction effects were further analyzed by Newman-Keuls post hoc analysis. Statistical analyses were conducted using Statistica software (StatSoft, Inc., Tulsa, OK). All values are represented as the mean ± SEM, and statistical significance was defined as P < 0.05. Number of animals used for all measures and the number of litters represented are provided in figure legends.

**Results**

**Characterization of young CPO mice**

As early as P9, CPO mice displayed heavier body weights compared with CTR, an effect that persisted into
adulthood (Fig. 1A). Body weight gain (Fig. 1B) during the preweaning period (P2–23) was 41% higher in CPO, and total fat mass (Fig. 1C) was 2-fold higher. We additionally observed that CPO pups exhibit significantly heavier visceral fat pads (mesenteric and omental) in addition to heavier epididymal and retroperitoneal depots (data not shown). Serum leptin levels (Fig. 1D) were 4-fold higher at P16 compared with CTR pups. This elevated leptin was not fully accounted for by level of adiposity because leptin levels were 2-fold higher in CPO mice after normalization to total fat mass (Fig. 1E). CPO pups exhibited leptin resistance in the ARH at P16, as determined by reduced pSTAT3 activation (Fig. 1, F–H), compared with CTR pups. After weaning at P23, CPO mice maintained a significant increase in caloric intake (Fig. 2A); however, intake normalized to body weight was not different. Inter-

scapular temperature (Fig. 2B) showed a modest but statistically significant reduction in CPO vs. CTR mice during the lights-off period. Conversely, young CPO mice demonstrated a slight increase in activity (Fig. 2, C and D); however, activity levels no longer differed by P60.

**Leptin and glucose responsiveness in young adult CPO mice**

In adult CTR mice, leptin reduced overnight food intake (Fig. 2E) with a greater decrease after 2 d of injections. In contrast, CPO mice showed no significant reduction in food intake after the first injection and a small but significant decrease after the second injection, indicating attenuated leptin responsiveness in CPO mice. Glucose tolerance (Fig. 2F) did not differ between CPO and CTR mice at 9 wk of age.
CPO effects on acute HFD response

Both CPO and CTR mice were hyperphagic on the first day of 45% HFD exposure (Fig. 3A). Whereas daily caloric intake returned to baseline chow levels within 2 days in CTR mice, CPO mice remained hyperphagic for 7 days. A similar response was observed when switched to 60% HFD, with a faster return to baseline. In a separate cohort, mice maintained on 45% HFD for 9 days CPO mice exhibited significantly greater cumulative caloric intake (CPO: 205.2 ± 9.1 kcal; CTR: 175.8 ± 7.2 kcal; P < 0.05), gained significantly more weight (CPO: 2.24 ± 0.88 g; CTR: −0.40 ± 0.44 g; P < 0.05), and exhibited a significantly increased feed efficiency (Fig. 3B), demonstrating that the hyperphagic response to two novel, palatable diets can rapidly increase body weight gain in CPO mice. Furthermore, during this time period, CPO mice failed to show a compensatory increase in either activity (Fig. 3C) or temperature (Fig. 3D), whereas CTR mice showed a significant increase in activity compared with CPO in response to HFD exposure.

CPO effects on chronic HFD response

In adulthood, CPO mice were only slightly heavier than CTR when maintained on standard chow (Fig. 3E), and body weight gain during this time (Fig. 3F) did not differ. Whereas both CPO and CTR demonstrated a rapid increase in body weight on a HFD, the weight gain in CPO mice was significantly greater (Fig. 3F).

By 6 months of age, body weight, fat mass, and lean mass were significantly increased by both CPO treatment and HFD (Table 1), with the greatest increase observed in CPO
HFD mice. Serum leptin levels did not differ between CTR and CPO chow mice. However, HFD increased leptin levels in all mice with a further increase in CPO HFD mice. Heart weight was significantly increased by HFD, whereas kidney weight was significantly increased by the CPO treatment alone.

Because CPO treatment affected food intake, thermogenesis, and leptin responsiveness, we assessed UCP1 mRNA as an index of thermogenesis in BAT. UCP1 mRNA was decreased in CPO chow, CTR HFD, and CPO HFD compared with CTR chow mice at 6 months of age (Table 1). At 22 wk of age, hypothalamic pSTAT3-ir was assessed in response to ip leptin injection (Fig. 4). Whereas little activation was observed in saline-injected mice, only CTR chow mice demonstrated a robust pSTAT3 induction in the ARH (Fig. 4, A and C). Remarkably, CPO
In adulthood, it is well recognized that the ARH is a primary site for central leptin actions (24) and that programmed early postnatal nutrition can permanently reprogram energy homeostasis. In contrast, leptin injection induced a marked increase in pSTAT3-ir in the DMH in all mice (Fig. 4, B and D), with no differences among treatment and diet conditions. Compared with saline-injected mice, leptin also significantly increased adiposity, eventually leading to ARH leptin resistance (25) and subsequent decreased glucose homeostasis. However, in response to a metabolic challenge (HFD), CPO mice demonstrate transient hyperphagia and rapid development of obesity, insulin resistance, and hepatosteatosis. These findings demonstrate that early postnatal nutrition can permanently reprogram energy homeostasis.

Discussion

The present studies demonstrate that early postnatal overnutrition, induced by decreasing litter size, results in a dramatic and permanent ARH leptin resistance in mice concomitant with decreased BAT thermogenesis. When maintained on standard chow diet, these abnormalities had little detrimental consequences for body weight and glucose homeostasis. However, in response to a metabolic challenge (HFD), CPO mice demonstrate transient hyperphagia and rapid development of obesity, insulin resistance, and hepatosteatosis. These findings demonstrate that early postnatal nutrition can permanently reprogram energy homeostasis.

In adulthood, it is well recognized that the ARH is a primary site for central leptin actions (24) and that prolonged increases in circulating leptin, resulting from increased adiposity, eventually lead to ARH leptin resistance...
Importantly, we demonstrate a rapid development of ARH leptin resistance in CPO pups as early as 16 d of age. In contrast to adulthood, leptin does not inhibit food intake in pups until the fourth postnatal week, indicating that downstream pathways of ARH leptin action are not fully developed (21, 22). Consequently, food intake in pups is largely regulated by availability, particularly during the first postnatal week when food intake is thought to primarily be inhibited by gastric distension (29). The absence of leptin-negative feedback in pups likely further drives the rapid increase in adiposity and leptin levels when consumption is increased, as in CPO pups. As a result, the early postnatal period in rodents is a time of increased vulnerability to excess weight gain and adiposity. Although hyperleptinemia itself may have directly induced the leptin resistance in CPO pups, it is possible that other factors are involved, including other hormones or nutrients. For instance, central administration of palmitate reduces leptin-induced STAT3 phosphorylation (30), suggesting that elevated fatty acids due to overnutrition could potentially lead to leptin resistance. Because downstream effects of leptin are not fully functional in pups, the full consequences of ARH leptin resistance in CPO pups may not be observed until later in life. However, because leptin is an essential neurotrophic factor for the development and migration of hypothalamic feeding-
related neurons (17, 18), it is possible that leptin resistance at this critical point in hypothalamic development may permanently impact the development of ARH neuron projections and that this may further impair energy homeostasis.

Surprisingly, ARH leptin resistance persisted in CPO mice maintained on standard chow diet, demonstrated by a blunted leptin-induced pSTAT3 activation, similar to that for obese mice maintained on HFD. This supports and extends previous findings from Davidowa and Plagemann (23) showing a reduced inhibitory effect of leptin on ARH neuronal firing in 5- to 11-wk-old CPO rats, and suggests that this leptin resistance may develop during the suckling period and persist throughout life. Whereas it is possible that the leptin resistance observed in adult vs. young CPO mice is mediated by different mechanisms, leptin resistance in the presence of normal circulating leptin, as in the CPO CHOW mouse, suggests that an earlier insult may have triggered this impairment. Previous studies have demonstrated that early programming, including intrauterine undernutrition (16), maternal HFD (31) and neonatal leptin administration (16) can result in long-term ARH leptin resistance. However, because these previous studies examined leptin responsivity only in adulthood, and not during the early postnatal period, it is unclear whether other prenatal models also exhibit an early onset

**FIG. 5.** GTTs and ITTs in response to chronic HFD. Glucose response in chow- or HFD-fed mice after ip administration of glucose (1 mg/g) at 15 wk age (A), corresponding AUC from 0 to 120 min, with the glucose level at 0 min considered as the baseline (B), and fasting glucose levels at 0 min (C) (CTR chow: n = 7, seven litters; CPO chow: n = 8, eight litters; CTR HFD: n = 15, eight litters; CPO HFD: n = 12, 12 litters; *, P < 0.05, main effect of diet, two way ANOVA; +, P < 0.05, vs. CTR HFD, t test). D, Plasma insulin at 0 and 60 min during the GTT (CTR CHOW: n = 5, four litters; CPO chow: n = 4, three litters; CTR HFD: n = 6, four litters; CPO HFD: n = 4, three litters; *, P < 0.05 vs. CPO chow, CTR HFD; t tests). Glucose response in 17-wk-old chow- or HFD-fed mice after ip insulin (1 mU/g) (E) and corresponding AUC from 0 to 120 min, with zero considered as the baseline (F) (CTR chow: n = 7, three litters; CPO chow: n = 7, seven litters; CTR HFD: n = 8, four litters; CPO HFD: n = 10, eight litters; *, P < 0.05 vs. all other groups; significant treatment x diet interaction, two-way ANOVA).
of leptin resistance or whether it is secondary to the onset of obesity. Because CPO chow-fed mice in the present studies did not exhibit elevated leptin levels in adulthood yet showed leptin resistance, this suggests that leptin resistance is not secondary to concurrent obesity but may be a permanent consequence of the rapid weight gain during the suckling period.

Leptin was less effective at inhibiting food intake in CPO mice, although body weight was effectively decreased. Thus, leptin-mediated effects on other pathways may remain intact in CPO mice. This is supported by the normal induction of pSTAT3 in the DMH of CPO mice. Normal DMH but not ARH leptin responsivity has previously been observed in diet-induced obese (DIO) mice (32), which also show an ARH-specific leptin resistance. The mechanisms of increased ARH susceptibility to leptin resistance are unclear but may relate to differences in access to leptin (33). This ability of other brain regions, and possibly peripheral tissues, to maintain responsivity to leptin may account for the rapid weight gain during the suckling period.

Although the general mechanisms of leptin resistance are not well understood, they likely involve reduced leptin transport into the brain, either due to reductions or impaired function in leptin transporter (Ob-Ra) and/or impaired downstream signaling (34). We observed no alterations in hypothalamic leptin receptor mRNA; however, because we did not assess individual leptin receptor subtypes, it remains possible that leptin receptor subtypes were differentially altered and/or that ARH-specific differences that were not detected by examining whole hypothalamus occurred. Previous studies examining leptin receptor subtypes in 60-d-old CPO rats found no differences in Ob-Ra, Ob-Rb, Ob-Rc, Ob-Re, or Ob-Rf in whole hypothalamus (13), although Ob-Rb was decreased in young (24 d old) CPO rats (11). This developmental difference may reflect different mechanisms of impaired leptin signaling with age or, alternatively, may relate to circulating levels of leptin, which would be elevated in younger CPO animals. Because we did not assess responsivity to central leptin administration, it remains to be determined whether leptin transport alone is impaired in CPO mice. We also found no differences in hypothalamic

**FIG. 6.** Liver histology in response to chronic HFD. H&E staining (A) and oil red O staining (B) (red, lipid staining) of liver at 6 months of age. C, Liver weight normalized to body weight (CTR CHOW: n = 8, four litters; CPO chow: n = 7, seven litters; CTR HFD: n = 14, four litters; CPO HFD: n = 14, 10 litters; significant treatment \times diet interaction: **, P < 0.005, CPO HFD vs. all other groups). D, Degree of hepatic steatosis: mild, isolated regions of steatosis, predominantly macrovesicular; moderate, scattered regions of both macrovesicular and microvesicular steatosis with regions of normal histology; severe, extensive macrovesicular and microvesicular steatosis throughout section. Scale bars, 100 μm.
SOCS3 mRNA, elevations of which have been implicated in ARH leptin resistance in response to DIO (25, 32). Again, it remains possible that regional differences that were not detected when examining whole hypothalamus occurred. Thus, the mechanisms underlying leptin resistance in CPO mice remains unclear and warrants further investigation.

CPO mice exhibited a slight reduction in temperature as well as decreased UCP1 mRNA levels. This is consistent with our previous findings of decreased sympathetic outflow to BAT, decreased UCP1 mRNA, and a reduced thermogenic response to cold in CPO rats (14). Although activity was slightly increased in juvenile CPO mice, this difference was no longer observed by 2 months of age. These modest alterations in temperature and activity did not appear to impact body weight homeostasis significantly when maintained on a standard chow diet, although reduced sympathetic tone may contribute to abnormalities under metabolic challenge. The ability to maintain normal energy homeostasis, albeit at a slightly higher body weight set point, is somewhat surprising in the face of the dramatic ARH leptin resistance. Interestingly, a recent study demonstrated that leptin receptor deletion in ARH agouti-related peptide (AGRP) and proopiomelanocortin (POMC) neurons results in rapid early growth but parallel growth rates after 9 wk of age compared with control mice (35). This normalization in adulthood suggests that leptin-independent and/or ARH leptin receptor-independent mechanisms may be able to compensate for ARH leptin resistance to some extent, perhaps when maintained on a healthy diet.

Although CPO mice maintained normal body weight homeostasis on the healthy standard chow diet, we hypothesized that the ARH leptin resistance would impair their ability to adapt to HFD. When initially placed on HFD, both CTR and CPO mice showed an initial large increase in caloric intake, presumably due to the high palatability of the diet. However, whereas the CTR mice return to baseline caloric intake within 2 d, CPO mice exhibited a prolonged hyperphagic response. This suggests that CPO mice may have an impaired ability to sense and regulate food intake according to the caloric content of the food. It is possible that this relates to the persistent ARH leptin resistance because exposure to HFD has been shown to increase leptin within 2 d, which may help normalize caloric intake (36). It is also possible that CPO mice have impairments in homeostatic feedback responsivity to other hormonal or nutritive factors. After 9 d of HFD consumption, CPO mice exhibited increased body weight gain and feed efficiency, indicating more efficient conversion of caloric intake into energy stores. Furthermore, whereas CTR mice showed a compensatory increase in activity levels, CPO mice failed to show any increases in activity or temperature to counteract the greater caloric intake. Whereas we did not measure true energy expenditure, i.e. by measuring oxygen consumption and respiratory exchange ratio, and therefore cannot conclude whether energy expenditure was truly affected, the lack of alterations in activity or temperature in CPO mice may have promoted their more rapid development of adiposity on HFD.

In response to chronic HFD exposure, both CTR and CPO mice gained considerably more weight than their chow-fed counterparts. However, HFD exposure exaggerated the body weight differences between CTR and CPO mice due to a greater rate of weight gain. This increased susceptibility to HFD-induced obesity confirms previous findings in CPO rats (10, 13). Because leptin is critical for the appropriate development of ARH neurons, ARH leptin resistance during development, such as that observed in CPO mice, might permanently alter the neuropeptide Y (NPY)/AGRP and POMC neurons of the ARH. Although two previous studies showed no alterations in NPY mRNA (10, 13), another study demonstrated elevated ARH AGRP, NPY, and cocaine- and amphetamine-regulated transcript (11) mRNA in CPO rats. Reduced pSTAT3 activation in the ARH implicates a defect at the level of the ARH neuronal cell bodies; however, because previous studies indicate that reduced ARH leptin signaling during development may reduce neuronal projection densities (17, 18), it is possible that CPO mice also exhibit alterations in downstream projections of the ARH neurons. Of particular relevance, a recent study in rats selectively bred for DIO susceptibility demonstrated ARH leptin resistance at P10 and reduced ARH projections to the paraventricular nucleus (37), with reduced AGRP fiber density persisting into adulthood. Similarly, a model of severe prenatal food restriction that results in very low postnatal leptin levels exhibited reduced ARH POMC mRNA and reduced POMC projections to the paraventricular nucleus at P21 (15). Thus, alterations in ARH leptin signaling during the early postnatal period may permanently alter ARH responsivity to leptin and the melanocortin system may be particularly impaired.

Whereas CPO treatment alone did not impair glucose tolerance, in response to HFD, CPO exacerbated the progression of glucose intolerance, leading to fasting hyperglycemia, hyperleptinemia, and insulin resistance. This prediabetic state was more severe than that observed in CTR HFD mice, which exhibited a normal response to the ITT. A more rapid advancement toward a diabetic state in CPO mice may be a consequence of the more advanced state of HFD-induced obesity. Alternatively, it is possible that CPO treatment alone increases susceptibility to insu-
lin resistance or alters glucose homeostasis but that abnormalities are only observed under metabolic challenge. The rapid development of obesity in CPO HFD mice resulted in extensive lipid accumulation in the liver. Whereas this was observed in all mice on HFD, the severity of hepatosteatosis and hepatomegaly was greater in CPO mice. Such liver abnormalities can lead to or further exacerbate hepatic insulin resistance. Because the present studies were conducted in males only, it is unknown whether female mice exhibit the same response to CPO treatment. Further investigation into possible gender differences with early overnutrition is therefore warranted.

In summary, CPO treatment resulted in a marked ARH leptin resistance throughout life, despite normal circulating leptin levels. This suggests that the ARH is highly susceptible to leptin resistance during the early postnatal period and that even a brief period of hyperleptinemia, induced by early overnutrition, can induce permanent leptin resistance. When maintained on a healthy, standard chow diet, this abnormality appears to have little detrimental consequences for body weight and glucose homeostasis. Importantly, in response to HFD, CPO mice rapidly develop obesity, insulin resistance, and hepatosteatosis. Thus, in the face of a metabolic challenge that increases leptin levels, the underlying ARH leptin resistance may impair the ability of CPO mice to regulate energy homeostasis and accelerate the detrimental effects of HFD. These studies also demonstrate how the sometimes overlooked variable of litter size can have powerful, long-term effects on body weight and metabolism and further support the importance of standardizing litter size in rodent studies. Whereas it is unknown whether nutritional programming in humans can similarly induce a permanent leptin resistance, the consequences would clearly be devastating in the presence of a HFD, leading to increased susceptibility to obesity and type 2 diabetes.

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