Neonatal overnutrition in mice exacerbates high-fat diet-induced metabolic perturbations

Zhiguo Liu¹, Chun Yan Lim², Michelle Yu-Fah Su², Stephanie Li Ying Soh², Guanghou Shui⁴, Markus R Wenk⁴, Kevin L Grove³, George K Radda², Weiping Han^{2,4} and Xiaoqiu Xiao^{1,2}

¹Laboratory of Lipid and Glucose Metabolism, The First Affiliated Hospital of Chongqing Medical University, 1 Youyi Road, Yuzhong District, Chongqing 400016, People's Republic of China

²Laboratory of Metabolic Medicine, Singapore Bioimaging Consortium, Agency for Science Technology and Research (A*STAR), #02-02 Helios, 11 Biopolis Way, Singapore 138667, Singapore

³Division of Neuroscience, Oregon National Primate Research Center, Oregon Health and Science University, 505 NW 185th Avenue, Beaverton, Oregon 97006, USA

⁴Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 119275, Singapore

Correspondence
should be addressed
to X Xiao or W Han

Email
xiaoxq@cqmu.edu.cn or
weiping_han@sbic.a-star.edu.sg

Abstract

Neonatal overnutrition results in accelerated development of high-fat diet (HFD)-induced metabolic defects in adulthood. To understand whether the increased susceptibility was associated with aggravated inflammation and dysregulated lipid metabolism, we studied metabolic changes and insulin signaling in a chronic postnatal overnutrition (CPO) mouse model. Male Swiss Webster pups were raised with either three pups per litter to induce CPO or ten pups per litter as control (CTR) and weaned to either low-fat diet (LFD) or HFD. All animals were killed on the postnatal day 150 (P150) except for a subset of mice killed on P15 for the measurement of stomach weight and milk composition. CPO mice exhibited accelerated body weight gain and increased body fat mass prior to weaning and the difference persisted into adulthood under conditions of both LFD and HFD. As adults, insulin signaling was more severely impaired in epididymal white adipose tissue (WAT) from HFD-fed CPO (CPO-HFD) mice. In addition, HFD-induced upregulation of pro-inflammatory cytokines was exaggerated in CPO-HFD mice. Consistent with greater inflammation, CPO-HFD mice showed more severe macrophage infiltration than HFD-fed CTR (CTR-HFD) mice. Furthermore, when compared with CTR-HFD mice, CPO-HFD mice exhibited reduced levels of several lipogenic enzymes in WAT and excess intramyocellular lipid accumulation. These data indicate that neonatal overnutrition accelerates the development of insulin resistance and exacerbates HFD-induced metabolic defects, possibly by worsening HFD-induced inflammatory response and impaired lipid metabolism.

Key Words

- ▶ adipose tissue
- ▶ fatty acids
- ▶ glucose transport
- ▶ insulin resistance
- ▶ obesity

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Introduction

The prevalence of obesity among adults has increased significantly since the 1980s (Flegal *et al.* 2012). The WHO

estimates that globally there are more than one billion overweight adults, including 400 million that are

clinically obese. As it is a major contributor to the increased occurrence of life-threatening chronic diseases such as coronary heart disease and type 2 diabetes mellitus (T2DM), obesity has been considered a worldwide health concern. In addition to excessive energy consumption in adult life, a significant contributor to the dramatic increase in obesity is imbalanced nutrition at early stage of life. This is reflected by a 'U'-shaped relationship between birth weight and adult BMI, suggesting that both low- and high-birth weights are risk factors for future obesity (Demmelmair *et al.* 2006, Martin-Gronert & Ozanne 2010). In addition, weight gain from the first week to 2 years of life may be a critical determinant for the development of obesity and metabolic syndrome several decades later (Armitage *et al.* 2004, Toshke *et al.* 2004, Patel & Srinivasan 2010). Therefore, both pre- and postnatal nutrition have significant effects on the long-term regulation of body weight and energy homeostasis in adulthood.

A number of rodent models have been established and characterized to mimic early imbalanced nutrition in humans, such as by altering uterine blood flow or changing maternal dietary intake during fetal or immediate postnatal stages (Patel & Srinivasan 2002, Armitage *et al.* 2004, Langley-Evans *et al.* 2005, Plagemann 2005, Patel & Srinivasan 2010) and controlling food availability through litter size manipulation (Plagemann *et al.* 1999a,b, Velkoska *et al.* 2005, Xiao *et al.* 2007, Boullu-Ciocca *et al.* 2008, Glavas *et al.* 2010, Habbout *et al.* 2012). In the latter model, when pups are raised in small litter, e.g. three pups per litter, presumably milk intake of each individual pup is greater than that of its control (CTR) that is raised in a normal-sized litter with around ten pups per litter. These chronic postnatal overnutrition (CPO) animals are characterized by persistent overweight and early onset of obesity, hyperleptinemia, hyperinsulinemia, glucose intolerance, impaired hypothalamic feeding circuitry, impaired norepinephrine turnover, and brown adipose tissue thermogenesis (Plagemann *et al.* 1999a,b, Xiao *et al.* 2007, Boullu-Ciocca *et al.* 2008, Glavas *et al.* 2010, Habbout *et al.* 2012). CPO manipulation results in leptin resistance specifically in the arcuate nucleus of hypothalamus during the early postnatal period that persists into adulthood. When fed with a high-fat diet (HFD), CPO mice exhibit hyperphagia and accelerated development of obesity and insulin resistance (Glavas *et al.* 2010). These studies indicate that neonatal overnutrition may permanently alter energy homeostasis and significantly increase susceptibility to obesity and insulin resistance in the CPO mice, especially when they are faced with high-energy availability as adults.

Excess lipid supply is a major contributing factor to the development of insulin resistance. Obese animals and humans are characterized by the presence of chronic low-grade inflammation, which may causally contribute to the onset of insulin resistance, cardiovascular diseases, and T2DM (Hotamisligil *et al.* 1993, Xu *et al.* 2003, Iyer *et al.* 2010). Chronic lipid surplus leads to adipocyte hypertrophy and apoptosis and local increases in the production of non-esterified fatty acids (NEFA). Through increased expression of pro-inflammatory cytokines and chemokines, and infiltration of macrophages and other immune cells into adipose tissue, obesity-induced inflammation is potentiated and could directly inhibit insulin pathways (Cinti *et al.* 2005, Strissel *et al.* 2007). In support of this concept, anti-inflammatory drugs have been shown to improve obesity-related hyperglycemia (Goldfine *et al.* 2008). Furthermore, mice with genetically disrupted inflammatory pathways are protected from HFD-induced insulin resistance (Klover *et al.* 2005, Weisberg *et al.* 2006). To investigate whether the metabolic perturbations induced by neonatal overnutrition are associated with inflammation and abnormalities in nutrient metabolism, in particular lipid metabolism, and whether neonatal overnutrition pre-conditions mice to be more susceptible to metabolic defects induced by energy oversupply in adulthood, we examined alterations of insulin signaling pathways in white adipose tissue (WAT) and skeletal muscle (SKM) in CTR and CPO mice under low-fat diet (LFD) and HFD.

Materials and methods

Animals and diets

Male offspring of Swiss Webster mice were used in this study. All animals were maintained under a 12 h light:12 h darkness (lights on at 0700 h) cycle and constant temperature ($23 \pm 2^\circ\text{C}$). Pregnant Swiss Webster mice (Simonsen Laboratories, Inc., Gilroy, CA, USA) were maintained on standard mouse chow, housed individually, and monitored closely for the day of birth, which was considered as postnatal day 0 (P0). Litters were culled to ten pups on P2 for the CTR group, while for the CPO group, to six pups on P2 and then to three pups on P5. A subset of mice was used for the measurement of stomach weight and milk composition on P15 (see below) and the rest of the animals were weaned onto either a LFD (Research Diets, New Brunswick, NJ, USA, cat #D12450B) or a 60% HFD (Research Diets, cat #D12492) on P21 and housed three to four per cage. The LFD provided

3.84 kcal/g of energy (70.0% carbohydrate, 20.0% protein, and 10.0% fat) whereas the 60% HFD provided 5.24 kcal/g of energy (20.0% carbohydrate, 20.0% protein, and 60.0% fat). Food and water were available *ad libitum* unless fasting was required for an experiment, in which case all animals were fasted for the same length of time. To assess insulin sensitivity, a subset of mice from each treatment received i.p. injections of saline or insulin (1 U/kg of body weight) 20 min before killing. These adult mice were killed by decapitation at about 150 days of age between 1000 and 1200 h. Epididymal WAT and hind limb gastrocnemius SKM were quickly removed and dissected free of connective tissues and used for RNA and protein extraction, or for histological study. All animal studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Agency for Science, Technology and Research (A*STAR) and Chongqing Medical University.

Analysis of lipids from gastric contents using HPLC and mass spectrometry

CTR and CPO pups were separated from their dams, weighed to determine body weight, and fat mass and killed on P15. Stomachs were quickly removed and weighed as a measure of food intake. Extraction and analysis of lipids from the gastric contents were performed as previously described (Fei *et al.* 2008, Low *et al.* 2008). Lipids from gastric contents were extracted with hexane and blown dry and samples were dissolved in 100 μ l chloroform/methanol (2:1; vol/vol). An Agilent HPLC system (Agilent Technologies, Inc., Palo Alto, CA, USA) coupled with a triple quadrupole/ion trap mass spectrometer (4000Qtrap; Applied Biosystems) was used for quantitation of individual lipids. Based on the product ion and precursor ion analysis of head groups, multiple reaction monitoring transitions were set up for quantitative analysis of various polar lipids (Fei *et al.* 2008), and results were expressed as normalized intensities to corresponding internal standards. Neutral lipids were analyzed using a sensitive HPLC/ESI/MS method (Low *et al.* 2008). Briefly, separation of triacylglycerol (TAG) from polar lipids was carried out on an Agilent Zorbax Eclipse XDB-C18 column (i.d. 4.6 $\times$ 150 mm). Selective ion monitoring was used to record major phospholipids, sterol, TAG, and diacylglycerol (DAG) species. DAG and TAG were calculated as relative contents to the spiked

d5-TAG 48:0 internal standard, while sterols were normalized to corresponding deuterated standards.

Body composition measurement

Body compositions of CTR and CPO were measured with an EchoMRI 100 (Echo Medical Systems, Houston, TX, USA) as previously described (Gustavsson *et al.* 2008). Briefly, unanesthetized mice were weighed first before they were put in a mouse holder and inserted in the MR analyzer. Readings of body fat mass and body lean mass were given within 1 min.

Indirect calorimetry by Comprehensive Lab Animal Monitoring System

Oxymax/Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH, USA) was used to measure individual mice on their oxygen consumption (VO_2), carbon dioxide production (VCO_2), activity, and food intake as described previously (Lou *et al.* 2010). Mice were weighed and individually housed in chambers maintained at $24 \pm 1^\circ\text{C}$ and given free access to LFD or HFD and water. All the measurements were taken every 15 min for 6 days after the mice were acclimatized for 1 day. All data collected were averaged from 6 days of monitoring. VO_2 and VCO_2 were normalized to body weight and expressed as milliliters per kilogram per hour. Food intake was expressed as kilocalories per 12 h using consumed food at light or dark phase multiplying by 3.84 for LFD and 5.24 for HFD.

Serum hormone and biochemical assays

Trunk blood samples, obtained from mice of around 5 months of age, were allowed to clot on ice and were centrifuged for 20 min at 2000 *g*, and serum was stored at -80°C until use. Serum leptin levels were measured using commercial RIA Kits (Linco Research, Inc., St Charles, MO, USA). Serum NEFA and TAG levels were quantified using colorimetric NEFA Kits (Wako Chemicals, Richmond, VA, USA), TAG reagent, and standard (Sigma) respectively.

Adipose tissue and SKM histology

Fresh WAT and SKM were post-fixed in 4% paraformaldehyde for 48 h, paraffin-embedded, and then sectioned on a microtome at 8 μm . WAT inflammation was assayed by macrophage-specific marker F4/80 using anti-mouse

F4/80 antibody (1:100; Serotec, Raleigh, NC, USA; Cinti *et al.* 2005). Oil Red O staining was performed on unembedded sections, which were counterstained with hematoxylin.

RNA isolation and real-time RT-PCR

WAT and SKM were homogenized in TRIzol Reagent (Invitrogen) and total RNA was isolated. The quality and concentration of the RNA were determined by measuring the absorbance at 260 and 280 nm, and RNA integrity was confirmed using an Agilent 2100 Bioanalyzer. For mRNA quantification, both TaqMan and SYBR Green methods were used. Inventoried TaqMan primers and probes for adiponectin, DAG acyltransferase-2 (DGAT2), fatty acid synthase (FAS), glucose transporter 4 (GLUT4), insulin receptor substrate 1 (IRS-1), lipoprotein lipase (LPL), and fatty acid translocase (FAT/CD36) were obtained from Applied Biosystems, and mouse GAPDH was used as an endogenous control. SYBR Green primers were designed using Primer Express Software from Applied Biosystems and the sequences are available upon request. Quantitative real-time PCR was performed as described previously, but with an additional dissociation step for SYBR Green analysis (Xiao *et al.* 2004). All transcriptional levels were normalized to GAPDH and expressed as the fold change compared with CTR-LFD group.

Protein preparation and western blotting

Frozen WAT was sliced and thawed in ice-cold lysis buffer (containing in mM: 20 Tris-Cl, pH 7.5, 150 NaCl, 1% Triton X-100, 10 NaF, and 1 EDTA) supplemented with 1 mM Na_3VO_4 , 1 mM phenylmethylsulphonyl fluoride, and protease inhibitor cocktail (Santa Cruz Biotechnology). Tissues were further disrupted and homogenized and then subjected to constant agitation on an orbital shaker for 1 h at 4 °C. The tissue lysate was then centrifuged at 10 000 *g* for 10 min at 4 °C to remove insoluble materials. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories). Samples containing 20 µg total proteins were resolved by SDS-PAGE, transferred onto PVDF membranes (Bio-Rad), and then subjected to immunodetection. Antibodies against acetyl CoA carboxylase (ACC), FAS, phospho-Akt-S473, phospho-Akt-T308, total Akt, and IRS-1 were from Cell Signaling Technology, Inc. (Danvers, MA, USA). Tubulin was used as loading control. For the analysis of phosphorylation of IRS-1 at tyrosine residues, anti-IRS-1 antibodies were bound to protein A-coupled

Sepharose beads and incubated with proteins lysates overnight at 4 °C with agitation. Immunocomplexes were detected by anti-phospho-tyrosine antibody (Abcam, Inc., Cambridge, MA, USA).

Statistical analysis

Data are expressed as the mean $\pm$ S.E.M. One-way ANOVA, followed by Newman-Keuls's multiple range test and Student's *t*-tests, were used to determine significant differences among groups. In some experiments, when both litter size (CTR and CPO) and food type (LFD and HFD) were considered, two-way ANOVA and Bonferroni post-tests were used to determine significant differences. Statistical analyses were conducted using GraphPad Prism Software (GraphPad Prism, San Diego, CA, USA), and statistical significance was defined as $P < 0.05$.

Results

Altered milk composition and intake by adjusting postnatal litter size

In agreement with previous reports (Plagemann *et al.* 1999a,b, Xiao *et al.* 2007, Boullu-Ciocca *et al.* 2008, Glavas *et al.* 2010), a significant increase in body weight and fat mass was observed in CPO compared with CTR mice (Fig. 1A and B). Stomach weight of CPO pups was significantly increased compared with CTR and the difference remained significant after normalization to body weight (Fig. 1C and D). For polar lipid species (phospholipids and sphingolipids) in gastric contents, no significant difference was found between CPO and CTR pups (data not shown). For neutral lipids, TAG was significantly increased in gastric contents from CPO pups while DAG, free cholesterol (FC), and cholesterol ester (CE) were not different between the two groups (Fig. 1E).

Reduced metabolic rate and locomotor activity in CPO mice

On LFD, adult CPO mice displayed higher body weight, fat mass, and serum leptin and TAG levels than age-matched CTR mice (Table 1). When compared with CTR mice on HFD (CTR-HFD), body weight and fat mass were significantly higher in CPO mice fed HFD (CPO-HFD), while serum levels of glucose, NEFA, and TAG did not differ between CTR-HFD and CPO-HFD (Table 1). We then examined the metabolic characteristics of adult CPO and CTR mice after fed with LFD or HFD for 18 weeks using the Oxymax/CLAMS system.

Figure 1

Increased milk intake in CPO mice. Body weight (A), body composition (B), gastric weight (C and D), and lipid profiles from gastric contents (E) were analyzed in CTR and CPO mice at postnatal day 15. Data are expressed as

means $\pm$ s.e.m. For the analysis of body weight, body composition, and gastric weight (A, B, C and D), $n=16$ for each group. For lipid analysis (E), $n=5-6$. * $P<0.05$ vs CTR.

Compared with CTR, CPO mice showed lower VO_2 during both light and dark phases of a 24-h monitoring period on LFD. The decreasing trend was also found during the dark phase on mice that were fed with HFD (Fig. 2A and B). Similar change pattern was observed for VCO_2 (Fig. 2C). Furthermore, we examined locomotor activity to explore whether reduced VO_2 in CPO mice could be accounted for by decreased physical activity. As shown in Fig. 2D, CPO mice exhibited significant reduction in locomotor activity on either LFD or HFD during the dark phase. Although CPO mice fed with LFD displayed higher energy intake during the dark phase, total daily food intake (combined amount of light phase and dark phase) did not differ between CTR and CPO on either LFD or HFD (Fig. 2E).

Altered HFD-induced insulin resistance in WATs but not in SKM in CPO mice

To gain insight into cellular and molecular mechanisms underlying systemic insulin resistance, we investigated alterations in the insulin-signaling cascade and insulin response in CTR and CPO mice that were fed on LFD or HFD. Compared with CTR-LFD mice, expression of IRS-1 and insulin-sensitive GLUT4 in WAT was downregulated by 60% in CTR-HFD mice, and even more pronounced reduction (70% for IRS-1 and 80% for GLUT4) was observed in CPO-HFD (Fig. 3A and B). As shown in Fig. 3C and D, insulin-stimulated IRS-1 phosphorylation at tyrosine residues was significantly reduced in CPO mice either

Table 1 Physiological and biochemical measurements in male CTR and CPO mice. Swiss Webster pups were raised with standard litter size (ten pups per litter, CTR) and small litter size (three pups per litter, CPO) during lactation and weaned to low- and high-fat diets (LFD and HFD) on *postpartum* day 21 (P21). Mice were killed at the age of 150 days. All data are presented as mean $\pm$ s.e.m.

	CTR-LFD	CPO-LFD	CTR-HFD	CPO-HFD
Body weight (g)	45.8 $\pm$ 1.3 ^a	55.0 $\pm$ 1.0 ^b	57.3 $\pm$ 1.6 ^b	65.9 $\pm$ 1.4 ^c
Fat mass (g)	11.8 $\pm$ 0.9 ^a	16.1 $\pm$ 0.9 ^b	20.7 $\pm$ 1.1 ^c	24.1 $\pm$ 1.4 ^d
Glucose (mM)	8.8 $\pm$ 0.5 ^a	11.8 $\pm$ 1.3 ^{a,b}	15.8 $\pm$ 1.8 ^{b,c}	19.4 $\pm$ 2.5 ^c
Leptin (ng/ml)	2.79 $\pm$ 0.5 ^a	6.76 $\pm$ 1.1 ^b	10.8 $\pm$ 1.0 ^{b,c}	13.5 $\pm$ 1.4 ^c
NEFA (mEq/l)	0.46 $\pm$ 0.05 ^a	0.58 $\pm$ 0.03 ^{a,b}	0.61 $\pm$ 0.05 ^{a,b}	0.71 $\pm$ 0.07 ^b
Triacylglycerol (mg/dl)	105 $\pm$ 6.2 ^a	139 $\pm$ 6.5 ^b	152 $\pm$ 8.9 ^b	170 $\pm$ 12.1 ^b

Columns with differing superscripts (a, b, c or d) indicate values that are significantly different from each other (two-way ANOVA and Bonferroni post-tests were used to determine significant differences, $n=18-24$ for the measurement of body weight and fat mass, and 6-12 for glucose, leptin, NEFA, and triacylglycerol measurements).

Figure 2

Decreased energy expenditure and locomotor activity in CPO mice. CPO and CTR mice on LFD or HFD were analyzed by indirect calorimetry using the CLAMS. (A) Circadian rhythm of oxygen consumption in CTR on LFD (CTR-LFD, black line), CPO on LFD (CPO-LFD, red line), CTR on HFD (CTR-HFD, blue line), and CPO on HFD (CPO-HFD, dark red line). Data are presented as an average of 6 days of measurement over a 24-h monitoring

period. Rate of daily oxygen consumption (B), carbon dioxide production (C), locomotor activity (D), and food intake (E) were analyzed for CTR and CPO mice on each diet during light and dark periods averaged from 6 days of measurement over a 24-h monitoring period, $n=3$ per group. * $P<0.05$ vs CTR on the same diet.

under LFD or HFD diets. The same change was observed for Akt phosphorylation at Serine 473 residue in CTR and CPO mice with LFD. But pAkt was almost completely blocked upon insulin stimulation in these animals under HFD and no significant difference was found (Fig. 3C and D). Compared with changes in WAT, the effect of HFD on SKM IRS-1 and GluT4 was minor (Fig. 4A and B).

Aggravated macrophage infiltration and inflammation in adipose tissue of HFD-CPO mice

We assessed the degree of macrophage infiltration in WAT by labeling adipose tissue sections with F4/80,

a macrophage marker. As shown in Fig. 5A, almost no immunoreactivity to F4/80 was observed in CTR-LFD mice, while macrophage infiltration was readily observed in the CPO-LFD group. Moreover, extensive signs of macrophage infiltration and inflammation were observed in both HFD treatment groups. We further examined the expression of pro- and anti-inflammatory cytokines in adipose tissues. As illustrated in Fig. 5B, there is no significant change in adiponectin mRNA level between CTR and CPO on LFD. However, HFD induced a significant reduction in adiponectin in CTR and CPO mice with a more pronounced reduction in CPO mice. In contrast, with a HFD, TNF α was increased by 125 and 200% in CTR

Figure 3

Impaired insulin signaling in WAT of CPO mice. Expression levels of IRS-1 (A) and GLUT4 (B) in WAT were evaluated by quantitative PCR in CTR and CPO on LFD or HFD. The expression levels are expressed as fold change of CTR-LFD. Columns with differing superscripts (a, b or c) indicate values that are significantly different from each other, $n=6-7$ for each group. (C) Total protein from WAT lysates was analyzed by western blot using antibodies against phospho-Akt-S473, total Akt, and IRS-1, or phospho-tyrosine

antibody on immunoprecipitated samples with IRS-1 antibody. Representative immunoblots were obtained from four independent experiments. The phosphorylated levels of IRS-1 (pIRS-1, $n=4$ (D)) and Akt (pAkt, $n=4$ (E)) on the bands were quantified with ImageJ Software, normalized to the quantified IRS-1 and Akt bands, and were expressed as the fold change of CTR-LFD with saline injection. * $P<0.05$.

and CPO respectively (Fig. 5C). In addition, HFD induced a significant increase in interleukin 1 β (IL1 β) and monocyte chemoattractant protein 1 (MCP1) and induction of IL1 β and MCP1 was exaggerated in CPO-HFD mice when compared with CTR-HFD (Fig. 5D and E).

Increased sensitivity to HFD-induced dysfunction in WAT lipid metabolism in CPO mice

We further tested the levels of several key enzymes in *de novo* fatty acid synthesis, lipid transport, and storage. No difference was observed in the levels of these enzymes between CTR-LFD and CPO-LFD mice (Fig. 6). However, HFD significantly decreased ACC1 by 60 and 72%, DGAT2

by 33 and 60%, and LPL by 30 and 37% in CTR and CPO respectively. The similar trend was also observed for FAS, and stearoyl-coenzyme A desaturase 1 (SCD1). Notably, a more profound reduction in FAS, SCD1, and DGAT2 was observed in CPO-HFD mice when compared with CTR-HFD (Fig. 6). HFD-induced reduction in ACC and FAS was confirmed by immunoblots (Fig. 6C).

Increased HFD-induced fat accumulation in SKM of CPO mice

Ectopic fat accumulation is a critical contributing factor to insulin resistance and to the development of T2DM. We determined whether CPO led to enhanced fat

Figure 4
Altered insulin signaling in SKM of CPO mice. Expression levels of IRS-1 (A) and GLUT4 (B) in SKM were evaluated by quantitative PCR in CTR and CPO on LFD or HFD. The expression levels are expressed as fold change of CTR-LFD. Columns with differing superscripts (a or b) indicate values that are significantly different from each other, $n=6-7$ for each group.

accumulation in the SKM. Gene expression analysis for *de novo* fatty acid synthesis, lipid transport, and storage of SKM showed the opposite changes compared with WAT. Under LFD, SKM mRNA levels of ACC2 and LPL from CPO mice were increased by 130 and 80% respectively, and the effect was similar to the HFD-induced change in CTR mice (Fig. 7B and C). When animals were maintained on HFD, mRNA levels of FAS, LPL, and FAT/CD36 in CPO mice were significantly higher than their CTR counterparts (Fig. 7A, B, C and D). Consistent with increased lipid synthesis, transport, and storage, CPO-HFD mice exhibited profound intramyocellular lipid (IMCL) deposition in SKM sections, while CTR mice revealed lower lipid accumulation as indicated by Oil Red O staining (Fig. 7E).

Discussion

Altered nutritional experiences, either *in utero* or in early postnatal periods critical for development and maturation of organogenesis, can give rise to long-term consequences for developing organisms and have significant effects in the context of the current obesity epidemic (Plagemann 2005, Patel & Srinivasan 2010). Mice with early postnatal overnutrition had an impaired ability to sense and regulate feeding according to the caloric content of the food and thereby exhibited increased sensitivity to HFD and accelerated development of adiposity. These abnormalities may be attributable to leptin resistance in the arcuate nucleus of hypothalamus (Glavas *et al.* 2010). However, molecular mechanisms underlying the increased susceptibility to insulin resistance induced by postnatal overnutrition in peripheral metabolic organs are poorly appreciated. In the current studies, we provided direct evidence that at the early postnatal stage, decreasing litter size induced dramatic overnutrition due to increased milk intake and increased fat contents of the milk, resulting in rapid growth in body weight. In addition, mice with postnatal exposure to overnutrition displayed an impaired insulin-signaling cascade in WAT as adults. When challenged with HFD, these mice exhibited more pronounced inflammatory responses, impairment of the insulin signaling cascade, and dysregulated gene expression of lipid metabolism in WAT. Our data suggested that the abnormalities observed in CPO mice increased susceptibility to insulin resistance and exacerbated the detrimental outcome in response to HFD in adulthood.

Rodent pups suckled in litters of varying sizes have been extensively used as experimental models in studying metabolic and behavior development. Animals raised in small litters show an accelerated body weight gain prior to weaning (Plagemann *et al.* 1999a,b, Xiao *et al.* 2007, Boullu-Ciocca *et al.* 2008, Glavas *et al.* 2010). However, it is still unclear how litter size reduction results in accelerated growth rates. Using stomach weight as an index of energy intake in pups, the current study showed that pups from small litters (CPO) had a significant increase in milk intake, and the change is consistent with the increase in body weight and fat mass. In addition, we observed higher TAG levels in gastric contents from CPO pups, indicating increased fat content of the milk. This is in agreement with an earlier report showing that pups suckled in small litters had increased ability of nutrient extraction (Fiorotto *et al.* 1991). These results suggest that in addition to alterations in maternal behavior, locomotor activity, and absorption of small intestine (Fiorotto *et al.* 1991,

Figure 5

Macrophage infiltration and inflammation in WAT of CPO mice. (A) Micrographs of representative F4/80 immunohistochemical staining of WAT from CTR and CPO mice that were fed on LFD or HFD. Expression levels of adiponectin (B), TNFα (C), IL1β (D), and MCP1 (E) were measured

by quantitative PCR and expressed as fold change of CTR-LFD. Columns with differing superscripts (a, b or c) indicate values that are significantly different from each other, $n=6-7$ for each group.

Mozes *et al.* 2007), increased energy intake, via increasing amount of milk and TAG intake, contributes to the alteration in metabolic phenotype in CPO pups.

One critical contributor to obesity-related insulin resistance and T2DM is impaired adipose function (Rondinone *et al.* 1997, Waki & Tontonoz 2007, Guilherme *et al.* 2008). Insulin signaling, including activation of IRS-1 and Akt, is essential in glucose uptake and nutrient substrate metabolism and is also involved in insulin-mediated secretion of adiponectin from adipocytes (Bogan & Lodish 1999). Upon insulin stimulation, IRS-1 undergoes phosphorylation at multiple tyrosine residues to enable insulin-signaling transmission. In this study, when fed with a HFD, CPO mice displayed more pronounced reduction of WAT *Glut4* mRNA. More importantly, insulin stimulated IRS-1 and Akt phosphorylation was significantly attenuated in CPO mice under conditions of both LFD and HFD in contrast to CTR mice,

suggesting a greater insulin resistance in CPO. These data confirm and extend previous findings using the same CPO model (Glavas *et al.* 2010) and suggest that severe abnormalities in the WAT insulin-signaling cascade play a crucial role in increased susceptibility to neonatal overnutrition-induced hyperglycemia, insulin insensitivity, and glucose intolerance in the presence of HFD.

Chronic inflammation is a key component in the pathogenesis of insulin resistance and metabolic syndrome, and WAT is a central player as both a source and site of inflammation (Hotamisligil *et al.* 1993, Xu *et al.* 2003, Iyer *et al.* 2010). This prompted us to investigate whether impaired WAT insulin signaling is associated with chronic inflammation in WAT. Adiponectin is an adipokine with potent anti-inflammatory and insulin-sensitizing properties (Hung *et al.* 2008). Under our experimental conditions, adiponectin levels in CPO mice showed a decreasing tendency on LFD and

Figure 6

Altered expression of key enzymes in lipid metabolism in WAT of CPO mice. Expression levels of FAS (A), ACC (B), SCD1 (D), LPL (E), and DGAT2 (F) were measured by quantitative PCR and expressed as fold change of CTR–LFD. Columns with differing superscripts (a, b or c) indicate values that are

significantly different from each other, $n=6-7$ for each group. (C) Protein levels of FAS and ACC were analyzed by western blot. FAS and ACC protein levels were quantified with ImageJ Software with tubulin as a loading control and were presented under each band, $n=4$. * $P<0.05$ vs CPO–LFD.

significant reduction on HFD, while IL1 β displayed increased expression. These findings are generally similar to those described in a previous report (Boullu-Ciocca *et al.* 2008). F4/80 is a specific marker for mature macrophages (Weisberg *et al.* 2003) and MCP1 is a pro-inflammatory

cytokine produced by macrophages and a potent chemotactic factor for monocytes (Kanda *et al.* 2006). The extent of WAT macrophage infiltration shown in current studies was most severe in CPO–HFD based on F4/80 immunohistochemical staining. In further support

Figure 7

Altered expression of key enzymes in SKM lipid metabolism and intramyocellular lipid accumulation in CPO mice. Expression levels of FAS (A), ACC (B), LPL (C), and FAT/CD36 (D) were measured by quantitative PCR and expressed as fold change of CTR–LFD. Columns with differing

superscripts (a, b or c) indicate values that are significantly different from each other, $n=6-7$ for each group. (E) Micrographs of representative Oil Red O staining of SKM from CTR and CPO mice that were fed on LFD or HFD. Arrows indicated intramyocellular lipid accumulation.

of this result, MCP1 expression in CPO-HFD was significantly higher than that in CTR-HFD. Thus, based on the fact that CPO on LFD exhibited a modest WAT dysfunction, such as adipocyte hypertrophy, macrophage infiltration, and abnormal production of adipokines, and that CPO, in response to HFD, developed more severe WAT insulin resistance and inflammation, we suggest that CPO pre-conditions the mice for increased susceptibility to insulin resistance by enhancing inflammation in visceral WAT in response to HFD.

In addition to impairing insulin-dependent GluT4 translocation and subsequent glucose utilization, WAT insulin resistance results in reduced lipogenesis, enhanced lipolysis, and abnormal adipokine secretion. A variety of key components of WAT *de novo* fatty acid synthesis (ACC and FAS), desaturation (SCD1), and esterification (DGAT) are coordinately downregulated at the transcriptional level in this study, accompanied by elevated circulating NEFA levels in both CTR and CPO mice under HFD condition, with greater changes in CPO-HFD mice. These findings suggest that the capacity of WAT to store lipids is possibly suppressed due to impairment in insulin signaling, consistent with previous reports that link adipocyte insulin signaling and lipogenic functions (Blüher *et al.* 2002, Poulain-Godefroy *et al.* 2008). Accordingly, the selective downregulation of adipocyte lipogenic genes in the insulin-resistant state might reduce lipid storage in WAT and promote accumulation of TAG and other lipids in non-adipose tissues such as muscle and, therefore, lead to peripheral insulin resistance.

SKM is another major site in glucose metabolism. The impaired SKM insulin signaling is crucial for systemic insulin resistance and precedes the development of diabetes (DeFronzo & Tripathy 2009, Pagel-Langenickel *et al.* 2010). However, our study showed that HFD exerts minor effects on *Irs1* and *Glut4* mRNA in SKM compared with the change in WAT, agreeing with a previous report (Anai *et al.* 1999). NEFA uptake by SKM is controlled by both circulating lipid levels and specific transporters, particularly LPL and FAT/CD36 (Koonen *et al.* 2005, Goldberg *et al.* 2009). Here, we found upregulated LPL and FAT/CD36 expression in SKM and IMCL deposition in HFD-fed CTR and CPO mice, with stronger effects in CPO-HFD mice. This is consistent with the notion that NEFA uptake into the muscle is significantly elevated in insulin-resistant muscle (Bonen *et al.* 2004). Furthermore, we observed increased SKM lipogenesis as evidenced by upregulation of FAS and ACC expression, suggesting that SKM *de novo* fatty acid synthesis might also contribute to IMCL deposition on HFD. IMCL directly inhibits insulin-

stimulated IRS-1 tyrosine phosphorylation (Kim *et al.* 2002), which subsequently attenuates GLUT4 translocation (Yuan *et al.* 2001) and glucose conversion into glycogen for storage (Kim *et al.* 2002). These abnormalities are involved in the development of hyperglycemia.

In summary, this study provides evidence that exposure to overnutrition during early postnatal periods can result in long-term abnormalities in metabolic homeostasis. Postnatal overnutrition causes WAT dysfunction in adulthood, either through impairment of lipid metabolism or disruption of production and secretion of adipokines, and thereby inhibits the role of WAT in regulating energy homeostasis. In addition, excess supply of NEFA and inflammatory mediators and reduced adiponectin expression may contribute to the impaired function of SKM. The findings that CPO may precondition mice to be more sensitive to developing metabolic abnormalities and insulin resistance in response to energy overload in adulthood highlight the importance of a balanced energy intake during early development. However, it should be noted that there are shortcomings in current studies using adipose tissue in evaluation of mRNA levels of *Glut4* and lipogenic genes. The profound changes in adipose tissue cell population, increased infiltration of macrophages for instance, would not accurately reflect adipocyte-specific changes in the adipose tissue preparation. Future studies will address these issues using isolated adipocytes and will further examine functional changes in activities of enzymes related to lipid metabolism and determine whether epigenetic modification is involved in the metabolic malprogramming caused by CPO.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Z L, C Y L and M Y-F S contributed equally to this work.

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