

Prenatal and Postnatal Pathways to Obesity: Different Underlying Mechanisms, Different Metabolic Outcomes

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Obesity and type 2 diabetes are worldwide health issues. The present paper investigates prenatal and postnatal pathways to obesity, identifying different metabolic outcomes with different effects on insulin sensitivity and different underlying mechanisms involving key components of insulin receptor signaling pathways. Pregnant Wistar rats either were fed chow *ad libitum* or were undernourished throughout pregnancy, generating either control or intrauterine growth restricted (IUGR) offspring. Male offspring were fed either standard chow or a high-fat diet from weaning. At 260 d of age, whole-body insulin sensitivity was assessed by hyperinsulinemic-euglycemic clamp, and other metabolic parameters were measured. As expected, high-fat feeding caused diet-induced obesity (DIO) and insulin resistance. Importantly, the insulin sensitivity of IUGR offspring was similar to that of control offspring, despite fasting insulin hypersecretion and in-

creased adiposity, irrespective of postnatal nutrition. Real-time PCR and Western blot analyses of key markers of insulin sensitivity and metabolic regulation showed that IUGR offspring had increased hepatic levels of atypical protein kinase C ζ (PKC ζ) and increased expression of fatty acid synthase mRNA. In contrast, DIO led to decreased expression of fatty acid synthase mRNA and hepatic steatosis. The decrease in hepatic PKC ζ with DIO may explain, at least in part, the insulin resistance. Our data suggest that the mechanisms of obesity induced by prenatal events are fundamentally different from those of obesity induced by postnatal high-fat nutrition. The origin of insulin hypersecretion in IUGR offspring may be independent of the mechanistic events that trigger the insulin resistance commonly observed in DIO. (*Endocrinology* 148: 2345–2354, 2007)

THE GROWING INCIDENCE of obesity has a profound impact on global health, including risk of cardiovascular diseases, stroke, and type 2 diabetes. Over recent years, a number of interrelated environmental factors have been identified that lead to obesity development. First, it is now widely accepted that environmental changes during fetal development play a key role in determining susceptibility to obesity and metabolic disease in adult life (1–5). Adverse prenatal environmental influences are believed to induce permanent adaptive changes in the developing fetus that act to promote survival in the short term but may increase vulnerability to later obesogenic environmental stimuli (1–4, 6). Second, there is increasing evidence that a range of postnatal environmental conditions amplify the risk of metabolic disorders in adult life (7, 8). Such factors in rodent studies include neonatal overnutrition through either reduced litter size or overnutrition from lactating obese dams (7, 9–11) and postweaning diet-induced obesity (12, 13). However, comparative studies between prenatally and postnatally induced

obesity have yet to be performed. Such investigations are required to establish appropriate clinical strategies for prevention and management of the disease process.

Our previous research in rats has shown that maternal undernutrition during pregnancy leads to intrauterine growth restriction (IUGR) and has long-term metabolic consequences in offspring. These IUGR offspring show catch-up growth after weaning (14–17) and develop metabolic abnormalities in adult life that include obesity, hyperinsulinemia, hyperleptinemia, and hypertension (15–18). When these offspring are exposed to a high-fat (HF) diet during postnatal life, the obesity development and resulting hyperleptinemia and leptin resistance are further amplified (18).

The aims of the present study were first to investigate the effects of IUGR in rats (by maternal undernutrition during pregnancy) on obesity development, insulin sensitivity, and regulation of energy metabolism in adult offspring. Second, we sought to compare these changes in metabolic regulation and obesity development in IUGR adult offspring with those resulting from a long-term postnatal HF diet. Third, we investigated the effect of calorie restriction on metabolic health of IUGR rats, because it has been shown that long-term calorie restriction lowers the risk of metabolic disorders (19, 20). We hypothesized that maternal undernutrition during pregnancy leads to the development of obesity in offspring through metabolic changes independent of those induced by a postnatal HF diet. We further hypothesized that in adult IUGR offspring, these metabolic perturbations are amelio-

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Abbreviations: AD, Maternal *ad libitum* food intake during pregnancy; BMI, body mass index; CR, calorie restricted; FAS, fatty acid synthase; GLUT4, glucose transporter 4; HF, high fat; IUGR, intrauterine growth restriction; PI, phosphatidylinositol; PKC, protein kinase C; PLSD, projected least significant difference; SREBP-1c, sterol regulatory element-binding protein 1c.

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rated by calorie restriction throughout postnatal life. We explored these hypotheses by *in vivo* studies in IUGR offspring of rats undernourished during pregnancy, using the hyperinsulinemic-euglycemic clamp and a range of key molecular and biochemical markers of insulin sensitivity and lipid regulation.

Materials and Methods

Experimental design

Virgin Wistar rats (age, 100 ± 5 d; $n = 15$ per group) were time mated. A rat estrous cycle monitor (Fine Science Tools, Foster City, CA) was used to quantify vaginal impedance as a marker of the stage of estrus of the female rats before introducing the male (21). After confirmation of mating, rats were housed individually in standard rat cages containing wood shavings as bedding and free access to water. All rats were kept in the same room with a constant temperature (25 C) and a 12-h light, 12-h dark cycle. Mothers were assigned to one of two nutritional groups: group 1, standard diet *ad libitum* (Teklad 2018; Harlan, Oxfordshire, UK) throughout pregnancy, and group 2, undernutrition (30% of *ad libitum* intake) of a standard diet throughout gestation. Food intake and maternal weights were recorded daily until birth. After birth, pups were weighed and litter size recorded. Pups from undernourished mothers were cross-fostered onto dams that were fed *ad libitum* throughout pregnancy (AD). In both groups, litter size was adjusted to eight pups per litter to assure adequate and standardized nutrition until weaning. After weaning, male offspring from the two groups of dams 1) AD offspring and 2) IUGR offspring from undernourished mothers were divided into three balanced postnatal nutritional groups to be fed either a standard rat chow (C) (Teklad 2018), a HF diet, or a calorie-restricted (CR) diet (70% of the daily intake of the *ad libitum* standard chow-fed rats). The standard chow diet provided 3.4 kcal/g (dry weight) and contained 18.9% protein, 5.7% fat, and 57.3% carbohydrate. The HF diet comprised Teklad diet 2018 supplemented with high-quality beef fat, clarified butter, corn oil, molasses sugar, casein, and Teklad vitamin (40060) and mineral (AIN-36, 170915) mixes. The HF diet provided 5 kcal/g (dry weight) and contained 28.7% protein, 30% fat, and 31.1% carbohydrate. The protein/energy ratio and vitamin and mineral content in the two diets were identical and in accordance with the requirements for standard rat diets. Body weights and food intake of all offspring were measured daily throughout the study. All procedures involving animals were carried out with the prior approval of the Animal Ethics Committee of the University of Auckland.

Hyperinsulinemic-euglycemic clamp

At 263 ± 2 d of age, a hyperinsulinemic-euglycemic clamp was performed as described previously after an overnight fast ($n = 6$ per group) (22–24). Briefly, rats were anesthetized using halothane, and polyethylene catheters were inserted into the right carotid artery for blood sampling and the right jugular vein for infusion of insulin and glucose. The level of anesthesia was monitored regularly and body temperature maintained with a heated mat (37 C). Human insulin (Actrapid; Novo Nordisk, Bagsværd, Denmark) in saline and BSA (1 mg/ml) was infused at a rate of 20 mU/kg·min to induce hyperinsulinemia. The infusion rate of glucose [20% D-(+)-glucose in sterile saline] using a Harvard digital syringe pump was adjusted to reach and maintain euglycemia (100–120 mg/ml). Arterial blood samples were taken at baseline and every 10 min for measurement of circulating glucose levels. Patency of the carotid catheter was maintained by flushing with heparinized saline (20 mU/ml) after each blood sample. When euglycemia had been maintained at steady state for 30 min, a final arterial blood sample was collected and the rats were killed by decapitation.

Other physiological measures

A parallel cohort of male offspring was maintained until 270 d for assessment of endocrine and metabolic parameters uncompromised by the hyperinsulinemic-euglycemic clamp ($n = 6$ per group). After an overnight fast, rats were killed by decapitation under halothane anesthesia. Blood was collected into heparinized tubes and stored on ice (4

C) until centrifugation and removal of plasma for analysis. Body length (nose to anus) and suprarenal fat pad and liver weights were recorded. Liver and quadriceps muscle were snap frozen in liquid nitrogen and stored at -80 C for analysis.

Materials

Analytical grade biochemicals were obtained from BDH Laboratory Supplies (Poole, UK) or Sigma-Aldrich Inc. (St. Louis, MO) unless otherwise specified; reagents and apparatus for SDS-PAGE and immunoblotting were from Bio-Rad (Hercules, CA).

Antibodies

Antibodies for insulin receptor β (sc-711) and protein kinase C (PKC) ζ (sc-216) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), glucose transporter 4 (GLUT4) antibody (AB1346) was from Chemicon International (Temecula, CA), and phosphatidylinositol (PI) 3-kinase p85 antibody (06–195) was from Upstate Biotechnology (Lake Placid, NY). Antirabbit secondary antibody (raised in mouse; A2074) was from Sigma-Aldrich, and antimouse secondary antibody (raised in donkey; KO504) was from Santa Cruz Biotechnology.

Plasma assays

Fasting plasma insulin was measured by ELISA (Mercodia AB, Uppsala, Sweden). Plasma C-peptide, leptin, and ghrelin were measured with commercially available RIA kits (LINCO Research, St. Charles, MO). Plasma glucose and free fatty acids were measured by enzyme colorimetric assay using an automated bioanalyzer (Roche/Hitachi GOD-PAP; Roche Diagnostics, Penzberg, Germany). All samples were analyzed in a single assay.

Tissue triglyceride storage

Triglycerides were extracted from 100 mg muscle and liver samples with chloroform (25) and were quantified by enzyme colorimetric assay in an automated bioanalyzer (Roche/Hitachi GOD-PAP).

Glycogen storage

Glycogen was measured with an assay based on that of Roehrig and Allred (26). A total of 0.05–0.1 g liver or muscle was weighed into polypropylene tubes and combined with 19 vol (0.45–0.9 ml) of 10 mmol/liter sodium acetate buffer (pH 4.6). Samples were homogenized on ice using an Ultra Turrax homogenizer, and 0.5 ml homogenate was transferred to a 2-ml vessel containing 0.1 ml amyloglucosidase (60 U/ml). The mixture was then incubated at 37 C in a water bath for 2 h to digest the glycogen to free glucose. Samples were centrifuged for 5 min and analyzed for glucose concentration in an automated bioanalyzer (Roche/Hitachi GOD-PAP). Glycogen concentrations were expressed relative to tissue weight. All samples were analyzed on a single assay.

Real time-PCR analysis of phosphoenolpyruvate carboxykinase (PEPCK), fatty acid synthase (FAS), and sterol regulatory element-binding protein 1c (SREBP-1c)

Total RNA was isolated with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA), treated with DNase I, and further purified with the RNeasy Mini Kit (QIAGEN, Valencia, CA). RNA was dissolved in nuclease-free water and stored at -80 C. The concentration of the resulting RNA was measured using a NanoDrop spectrophotometer, and integrity was assessed by Agilent Bioanalyzer RNA Lab-on-a-Chip. An aliquot of RNA (4 μ g) was subjected to RT by the SuperScript III First-Strand Synthesis System (Invitrogen). TaqMan probes and primers for PEPCK, FAS, and sSREBP-1c were designed by and obtained from Applied Biosystems as Assay-on-Demand Kits using a two-step PCR procedure. For real-time PCR analysis, 1 μ l RT product was combined with 10 μ l Universal PCR mix (2 \times), 1 μ l Assay-on-Demand mix (20 \times), and 8 μ l nuclease-free water. TaqMan PCR amplification and detection was performed using an ABI Prism 7900 HT (Applied Biosystems) in 384-well plates. Samples and controls were analyzed in triplicate. Levels

of cDNA were quantified relative to the standard curve generated from a reference sample and normalized to glyceraldehyde-3-phosphate dehydrogenase expression.

Analysis of liver and muscle proteins

Key insulin-signaling protein levels were quantified in the liver and quadriceps muscle by Western blotting. Protein was extracted from 50–100 mg tissue sample in ice-cold RIPA lysis buffer (which contains Triton X-100, sodium dodecyl sulfate, sodium chloride, Tris-HCl, deoxycholic acid, sodium orthovanadate, and Complete Mini EDTA-free protease inhibitors from Roche Diagnostics) with a homogenizer probe. The resulting supernatant was removed and centrifuged at 10,000 rpm for 15 min at 4 C. The clear lysates were collected and centrifuged at 10,000 rpm for an additional 15 min and stored at –80 C. Protein levels were then quantified in the lysates using the Lowry method (27). Equal amounts of protein (20 μ g per 15 μ l) were dissolved using Laemmli's sample loading buffer, at 10%, and milliQ water to make the volume up to 15 μ l. Proteins were separated by SDS-PAGE using 0.75-mm-thick 8% acrylamide running gels. The proteins were then transferred to nitrocellulose membranes with a Criterion blotter (Bio-Rad), blocked for nonspecific binding, exposed to primary antibodies, and visualized using horseradish-peroxidase-coupled secondary antibodies and enhanced chemiluminescence detection substrate (SuperSignal West Dura Extended Duration Substrate Kit). Immunoreactive proteins were quantified via exposure to autoradiographic film (Agfa-Gevaert, Mortsel, Belgium), which was developed/fixated in a Kodak Xomatic \times 1000 processor, and specific bands were quantified on a GS800 densitometer (Bio-Rad) using Quantity One quantification software (Bio-Rad).

Statistical analysis

All measures were analyzed by ANOVA using StatView for Windows, version 5.0 (SAS Institute Inc., Cary, NC) statistical software. Differences between the groups were compared by Fisher's projected least significant difference (PLSD) *post hoc* analysis. Data are presented as means \pm SEM unless otherwise stated. $P < 0.05$ was considered significant.

Results

Postnatal growth and development of obesity

As expected (14–18), maternal undernutrition during pregnancy caused intrauterine growth restriction and reduced birth weight but did not change litter size. Male IUGR offspring were shorter and weighed significantly less at birth than AD offspring (body length: AD 49.3 ± 0.18 mm, IUGR 44.9 ± 0.22 mm, $P < 0.001$; body weight AD 6.21 ± 0.05 g, IUGR 4.41 ± 0.05 g, $P < 0.001$). At weaning, IUGR offspring remained significantly lighter than AD offspring. Subsequently, IUGR offspring displayed catch-up growth and by 260 d of age had attained body weights similar to those of AD offspring (Table 1) but remained shorter than AD offspring

(Table 1). Physiological and metabolic parameters were similar in the parallel cohorts of rats used for the hyperinsulinemic-euglycemic clamp and for the basal metabolic investigations.

Also as expected, introduction of a HF diet at weaning accelerated growth in both the IUGR and AD offspring. At 260 d of age, HF-fed animals were significantly heavier ($P < 0.0001$) and longer ($P < 0.01$) than chow-fed animals (Table 1). In contrast, rats fed a CR diet from weaning grew more slowly than the chow-fed offspring, remaining lighter and significantly shorter than the AD offspring (Table 1). Body mass index [BMI = nose-to-tail length (cm)/body weight (g)] (2) was calculated at 260 d as a marker of obesity (Table 1). In comparison to chow-fed rats, HF diet led to an increased BMI and CR diet to a reduced BMI. For all three postweaning diet groups, IUGR offspring had a significantly higher BMI than did AD offspring.

Markers of obesity were assessed at 260 d of age (Table 2). Fat deposition in suprarenal fat pads was highest in HF-fed rats, with a 100% increase in fat pad weight relative to body weight compared with chow-fed animals ($P < 0.001$). Prenatal undernutrition caused an increase in suprarenal fat pad weight in all three postnatal diet groups relative to the corresponding normally nourished groups ($P < 0.05$), with the prenatally and postnatally undernourished (IUGR-CR) group showing 50% heavier fat pads compared with the AD-CR group. Plasma leptin concentration, a marker of whole-body adiposity, was higher in IUGR offspring than in AD offspring ($P < 0.05$) and was further increased by postnatal HF diet. A postweaning CR diet reduced plasma leptin concentrations relative to chow-fed offspring. IUGR offspring showed a reduction in fasting total ghrelin levels ($P < 0.01$), reflecting increased fat deposition in these animals. As expected, CR-fed rats showed markedly elevated total ghrelin levels compared with both chow-fed and HF-fed animals.

Insulin secretion and action

Circulating plasma insulin, C-peptide, and glucose levels. Consistent with previous studies from our laboratory (16–18), adult IUGR offspring showed significantly elevated fasting plasma insulin levels (Fig. 1A). Interestingly, this was also observed in the IUGR-CR group relative to the AD-CR group despite the overall reduction of plasma insulin by postweaning calorie restriction. However, a postnatal HF diet did not

TABLE 1. Body weight, length, and BMI

Prenatal nutrition	Postnatal nutrition	Weaning weight (g)	Δ BW at 260 d (g)	Body length nose to anus at 260 d (mm)	BMI [(cm/g) ²]
AD	C	48.42 \pm 1.48	661.83 \pm 16.75	256.5 \pm 6.6	1.81 \pm 0.10
AD	CR	52.33 \pm 1.17	445.00 \pm 9.52 ^f	245.7 \pm 6.4 ^d	0.89 \pm 0.03 ^f
AD	HF	50.33 \pm 1.05	808.00 \pm 17.66 ⁱ	266.8 \pm 6.2 ^h	2.71 \pm 0.15 ^t
IUGR	C	46.00 \pm 0.44 ^c	696.67 \pm 31.14	246.5 \pm 5.0 ^b	2.22 \pm 0.21 ^a
IUGR	CR	44.29 \pm 1.10 ^c	453.36 \pm 7.13 ^f	239.6 \pm 5.3 ^{b,d}	0.98 \pm 0.03 ^{a,f}
IUGR	HF	43.92 \pm 1.89 ^c	853.67 \pm 37.11 ⁱ	258.2 \pm 5.0 ^{b,h}	3.22 \pm 0.25 ^{a,i}

Rats were weaned at 22 d of age. Change in body weight (Δ BW), nose-to-anus body length, and BMI (nose-to-tail length/body weight) were calculated at 270 d of age. C, Chow postnatal diet; CR, postnatal CR diet; HF, postnatal HF diet. Values are mean \pm SEM (n = 12 in all groups) and calculated using a two-way ANOVA and *post hoc* Fisher's PLSD test.

^{a-c} AD vs. IUGR: ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$.

^{d-f} C vs. CR: ^d $P < 0.05$, ^e $P < 0.01$, ^f $P < 0.001$.

^{g-i} C vs. HF: ^g $P < 0.05$, ^h $P < 0.01$, ⁱ $P < 0.001$.

TABLE 2. Markers of obesity

Prenatal nutrition	Postnatal nutrition	Suprarenal fat (% BW)	Leptin (ng/ml)	Ghrelin (pg/ml)	Glucose (mmol/liter)
AD	C	2.72 ± 0.22	47.45 ± 9.91	2345 ± 149.4	8.62 ± 0.31
AD	CR	1.11 ± 0.14 ^f	11.95 ± 1.70 ^f	3290 ± 275.8 ^f	6.90 ± 0.28 ^f
AD	HF	5.72 ± 0.30 ⁱ	66.50 ± 2.24 ^g	2315 ± 207.9	7.63 ± 0.17 ^h
IUGR	C	3.48 ± 0.39 ^a	77.75 ± 18.46 ^a	2025 ± 182.7 ^a	9.67 ± 0.31 ^c
IUGR	CR	1.55 ± 0.08 ^{a,f}	39.47 ± 25.17 ^{a,f}	3083 ± 290.0 ^{a,f}	7.67 ± 0.22 ^{c,f}
IUGR	HF	6.14 ± 0.28 ^{a,i}	99.00 ± 15.57 ^{a,g}	1655 ± 46.02 ^a	9.01 ± 0.27 ^{c,h}

All results are from male Wistar rats at 270 d of age. Endocrine data were measured in heparinized plasma from fasting trunk blood samples. C, Chow postnatal diet; CR, postnatal CR diet; HF, postnatal HF diet. Values are mean ± SEM (n = 6 in all groups) and calculated using a two-way ANOVA and *post hoc* Fisher's PLSD test.

^{a-c} AD vs. IUGR: ^a *P* < 0.05, ^b *P* < 0.01, ^c *P* < 0.001.

^{d-f} C vs. CR: ^d *P* < 0.05, ^e *P* < 0.01, ^f *P* < 0.001.

^{g-i} C vs. HF: ^g *P* < 0.05, ^h *P* < 0.01, ⁱ *P* < 0.001.

affect plasma insulin levels. Fasting plasma C-peptide levels, a marker of insulin secretion from the pancreas, varied in parallel with plasma insulin levels (Fig. 1B), being increased in the IUGR groups relative to the AD groups, decreased by postweaning calorie restriction, and unaffected by a postnatal HF diet. Fasting plasma glucose levels were slightly but significantly increased in IUGR offspring relative to AD offspring in all nutritional subgroups and were unaffected by a postnatal HF diet but decreased by a postnatal CR diet (Table 2).

Whole-body insulin sensitivity assessment by hyperinsulinemic-euglycemic clamp. Because elevated plasma insulin levels are commonly viewed to reflect the presence of insulin resistance, we used the hyperinsulinemic-euglycemic clamp method to investigate whole-body insulin sensitivity at 260 d of age. This is the gold-standard methodology for the *in vivo* assessment of insulin sensitivity (23, 24, 28). Despite elevation of fasting plasma insulin and glucose levels in IUGR offspring, maternal undernutrition did not lead to a change in glucose disposal rate at steady state when normalized for insulin levels (glucose infusion rate per kilogram per minute normalized for achieved insulin level) (Fig. 1C). Thus, adult IUGR offspring showed a similar level of insulin sensitivity

to AD offspring. In contrast, a significant reduction in insulin sensitivity was observed in HF-fed rats compared with chow-fed rats (*P* < 0.05). Furthermore, postweaning calorie restriction caused an increase in glucose disposal rate (*i.e.* increased insulin sensitivity) in both IUGR and AD offspring (*P* < 0.01).

Glucose metabolism

Glycogen storage in liver and muscle. Intriguingly, prenatal undernutrition had a marked effect on insulin-sensitive glycogen storage in liver and muscle (quadriceps) at maturity (Fig. 2, A and B). In liver, glycogen was markedly elevated in IUGR offspring (*P* < 0.001) in all postnatal diet groups. The CR diet reduced liver glycogen content in both IUGR and AD offspring (*P* < 0.001), as expected, but the HF diet had no effect. Similar effects were observed in muscle of all postnatal diet groups: an increase in glycogen storage in IUGR offspring relative to AD offspring (*P* < 0.05), a reduction by CR diet (*P* < 0.05), and no effect of an HF diet.

Hepatic PEPCK. Hepatic expression of PEPCK is an indicator of insulin-dependent gluconeogenic capacity. Hepatic PEPCK mRNA expression, measured by real-time PCR and

FIG. 1. The effect of maternal undernutrition and postnatal HF diet or calorie restriction in male Wistar rats on plasma insulin concentration (A), plasma C-peptide concentration measured in fasting trunk plasma at 270 d of age in an independent cohort (B), and *in vivo* insulin sensitivity as assessed by the hyperinsulinemic-euglycemic clamp at 260 d of age (C). C, Chow postnatal diet; CR, postnatal CR diet; HF, postnatal HF diet. Values are mean ± SEM (n = 6 in all groups) and calculated using a two-way ANOVA and *post hoc* Fisher's PLSD test. AD vs. IUGR: *, *P* < 0.05; **, *P* < 0.001; ***, *P* < 0.001; C vs. CR: †, *P* < 0.05; ††, *P* < 0.01; †††, *P* < 0.001; C vs. HF: #, *P* < 0.05; ##, *P* < 0.01; ###, *P* < 0.001.

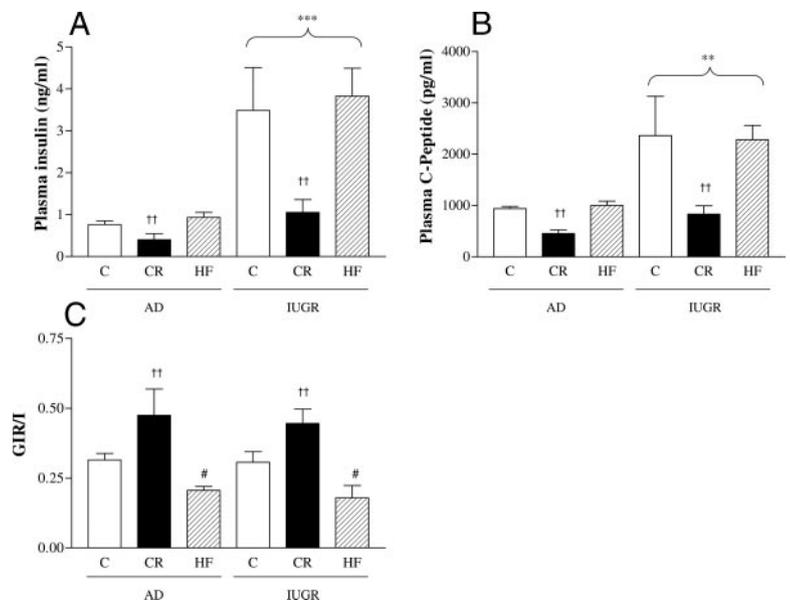
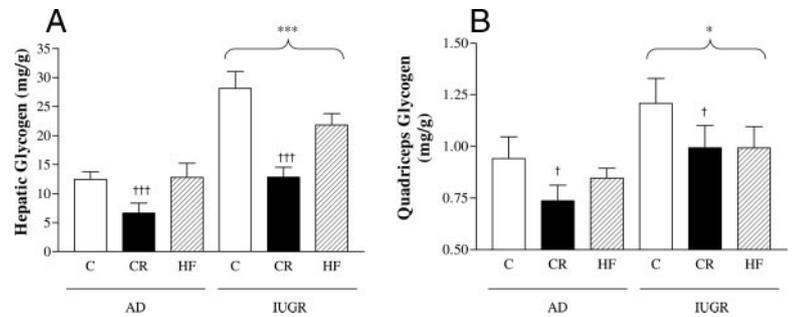


FIG. 2. The effect of maternal undernutrition and postnatal HF diet or calorie restriction on hepatic glycogen content (A) and quadriceps muscle glycogen content (B) in male Wistar rats from samples at 270 d of age. Glycogen is expressed as milligrams glycogen per gram of wet tissue. C, Chow postnatal diet; CR, postnatal CR diet; HF, postnatal HF diet. Values are mean \pm SEM ($n = 6$ in all groups) and calculated using a two-way ANOVA and *post hoc* Fisher's PLSD test. AD vs. IUGR: *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.001$; C vs. CR: †, $P < 0.05$; ††, $P < 0.01$; †††, $P < 0.001$; C vs. HF: #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$.



expressed relative to the internal control glyceraldehyde-3-phosphate dehydrogenase, was not altered by a postnatal HF diet but was increased ($P < 0.01$) by a postnatal CR diet compared with animals on standard chow (Table 3). Prenatal nutritional status did not affect hepatic PEPCK mRNA expression.

Insulin signaling proteins in liver and muscle. Western blot analysis revealed that postweaning calorie restriction significantly ($P < 0.05$) increased levels of insulin receptor β -subunit protein and the p85 regulatory subunit of PI 3-kinase in both liver and quadriceps muscle, regardless of prenatal nutritional status (Table 4 and Fig. 3, A and B). This observation is in agreement with the elevated insulin sensitivity observed in these animals. Neither IUGR nor a postnatal HF diet affected levels of these proteins.

Hepatic expression of PKC ζ was significantly elevated in IUGR offspring compared with AD offspring ($P < 0.05$) (Fig. 3C). Importantly, postnatal HF nutrition had the opposite effect of prenatal undernutrition, causing a marked decrease in PKC ζ levels in liver that is consistent with the decrease in insulin sensitivity measured by hyperinsulinemic-euglycemic clamp. In muscle, prenatal or postnatal nutritional status had no effect on levels of PKC ζ or on the insulin-sensitive GLUT4 (Table 4).

Lipid metabolism

In accordance with the hyperinsulinemic-euglycemic clamp data, the triglyceride content of both liver and muscle was similar in IUGR and AD offspring (Table 5). However, the triglyceride content of liver was more than doubled by postnatal HF nutrition ($P < 0.001$), whereas postnatal CR nutrition reduced triglyceride levels in both liver ($P < 0.05$) and quadriceps muscle ($P < 0.01$) compared with animals on

a chow diet. In parallel with triglyceride storage, circulating triglyceride levels were similar in IUGR and AD offspring, whereas CR nutrition reduced plasma triglyceride levels (Table 5). Fasting plasma free fatty acid concentrations were significantly increased in IUGR offspring ($P < 0.05$), whereas postnatal nutritional status had no significant effect (Table 5).

Hepatic mRNA levels of FAS, a key regulator of hepatic fatty acid synthesis, and of SREBP-1c were quantified by real-time PCR (Table 3). FAS mRNA expression was significantly increased in IUGR offspring ($P < 0.01$) and was significantly decreased by postnatal HF feeding ($P < 0.001$) (Table 3). SREBP-1c mRNA expression was markedly decreased by postnatal CR nutrition ($P < 0.01$) but was unaffected by IUGR (Table 3).

Discussion

The present study investigated the metabolic effects of, and interactions between, various prenatal and postnatal nutritional regimens. IUGR was achieved by restricting maternal nutrition to 30% of *ad libitum* intake. Groups of IUGR offspring and offspring of normally nourished mothers were subjected to three different nutritional regimens from weaning until adulthood: *ad libitum* feeding of standard chow to serve as a reference level of nutrition, *ad libitum* feeding of a HF diet to generate diet-induced obesity, and calorie restriction by feeding standard chow at 70% of *ad libitum* intake. Both IUGR and a postweaning HF diet led to adult obesity, but by different underlying mechanisms. We have summarized these differences schematically in Fig. 4.

Insulin secretion and insulin action clearly differed between the two groups. IUGR offspring showed increased fasting plasma insulin levels, and the parallel increase in C-peptide secretion indicates that this results from enhanced

TABLE 3. Expression of hepatic regulators of glucose and adipose tissue measured by real-time PCR

Prenatal nutrition	Postnatal nutrition	Hepatic PEPCK mRNA	Hepatic FAS mRNA	Hepatic SREBP-1c mRNA
AD	C	1.21 \pm 0.10	0.87 \pm 0.13	1.35 \pm 0.13
AD	CR	2.16 \pm 0.27 ^f	1.24 \pm 0.34	1.03 \pm 0.12 ^e
AD	HF	1.14 \pm 0.15	0.44 \pm 0.08 ^h	1.40 \pm 0.30
IUGR	C	1.03 \pm 0.12	2.15 \pm 0.36 ^b	1.93 \pm 0.28
IUGR	CR	1.77 \pm 0.13 ^f	1.07 \pm 0.18 ^b	1.73 \pm 0.02 ^e
IUGR	HF	1.02 \pm 0.12	0.80 \pm 0.15 ^{b,h}	1.04 \pm 0.05

All results were taken from liver samples of male Wistar rats at 270 d of age and quantified by real-time PCR. C, Chow postnatal diet; CR, postnatal CR diet; HF, postnatal HF diet. Values are mean \pm SEM ($n = 6$ in all groups) and calculated using a two-way ANOVA and *post hoc* Fisher's PLSD test.

^{a-c} AD vs. IUGR: ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$.

^{d-f} C vs. CR: ^d $P < 0.05$, ^e $P < 0.01$, ^f $P < 0.001$.

^{g-i} C vs. HF: ^g $P < 0.05$, ^h $P < 0.01$, ⁱ $P < 0.001$.

TABLE 4. Insulin-signaling proteins in muscle

Prenatal nutrition	Postnatal nutrition	Insulin receptor β	PI 3-kinase p85	PKC ζ	GLUT-4
AD	C	100.00 \pm 16.36	100.00 \pm 14.85	100.00 \pm 31.72	100.00 \pm 38.09
AD	CR	133.86 \pm 14.31 ^d	186.95 \pm 29.25 ^d	129.74 \pm 48.99	83.348 \pm 41.73
AD	HF	114.17 \pm 14.82	159.46 \pm 33.87	142.10 \pm 60.98	53.56 \pm 25.36
IUGR	C	101.62 \pm 11.27	157.20 \pm 41.09	134.97 \pm 33.07	109.82 \pm 36.11
IUGR	CR	146.28 \pm 21.26 ^d	224.17 \pm 58.94 ^d	139.98 \pm 53.63	74.14 \pm 21.25
IUGR	HF	123.28 \pm 15.00	62.426 \pm 13.64	68.07 \pm 31.83	108.93 \pm 28.74

All results are from quadriceps muscle samples of male Wistar rats at 270 d of age. C, Chow postnatal diet; CR, postnatal CR diet; HF, postnatal HF diet. Protein samples were quantified using Western blotting and densitometric analysis of protein bands of interest. Values are expressed as percentage of AD chow-fed animals. Values are mean \pm SEM (n = 6 in all groups) and calculated using a two-way ANOVA and *post hoc* Fisher's PLSD test.

^{a-c} AD vs. IUGR: ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$.

^{d-f} C vs. CR: ^d $P < 0.05$, ^e $P < 0.01$, ^f $P < 0.001$.

^{g-i} C vs. HF: ^g $P < 0.05$, ^h $P < 0.01$, ⁱ $P < 0.001$.

insulin secretion rather than the impaired insulin clearance that has been observed in the obese insulin-resistant state (29). Conversely, elevated plasma insulin and C-peptide levels were not observed in diet-induced obesity. Importantly, despite showing increased fasting plasma insulin levels, IUGR offspring did not show any change in insulin sensitivity compared with AD offspring, whereas in agreement with previous findings (23, 30, 31), rats fed a HF diet developed insulin resistance regardless of prenatal nutritional history. These observations, together with previous studies (30–34), suggest that, in rats, an obesogenic HF diet does not lead to insulin hypersecretion, as confirmed by unchanged C-peptide levels in the present study, but does lead to decreases in glucose metabolism in peripheral tissues in association with reduced insulin sensitivity. The obesity and insulin resistance observed in HF-fed offspring and the obesity and insulin hypersecretion observed in IUGR offspring are based on different metabolic mechanisms.

We used the gold-standard method for direct *in vivo* assessment of whole-body insulin sensitivity, the hyperinsulinemic-euglycemic clamp (23, 24, 28). Due to the extent of obesity and age of our rats, it was necessary to perform these studies under halothane anesthesia. It is possible that the use

of anesthesia in our protocol may have masked small differences in insulin sensitivity between AD and IUGR rats. Although there are some reports of relatively minor metabolic changes induced by halothane anesthesia (35, 36), many researchers have successfully performed glucose clamps under anesthesia (22, 24, 37). Insulin doses were carefully adapted in accordance with the experimental conditions, and all animals were exposed to the same carefully regulated levels of anesthesia. Furthermore, the experimental design included both positive and negative controls (the postnatal HF and CR diets, respectively) for the assessment of insulin sensitivity. The results were fully consistent with the molecular and protein analysis data, thereby confirming the validity and biological relevance of our data set.

Transport of glucose across the cell membrane is considered to be the rate-controlling step of glucose metabolism in muscle and liver (38). Impaired insulin-stimulated glucose transport rather than impaired insulin-signaling protein phosphorylation is believed to be responsible for insulin resistance in type 2 diabetes (38, 39). Insulin regulates glucose transport in muscle and hepatic tissue through activation of insulin receptor substrate-dependent PI 3-kinase. Downstream effectors of PI 3-kinase are postulated to mediate

FIG. 3. The effect of maternal undernutrition and postnatal HF diet or calorie restriction on hepatic insulin receptor β (A), p85 subunit of PI 3-kinase (B), and PKC ζ (C) in male Wistar rats at 270 d of age with representative Western blots at the top. Graphs show corresponding densitometric analysis of protein bands of interest as percentage of the AD chow-fed group. C, Chow postnatal diet; CR, postnatal CR diet; HF, postnatal HF diet. Values are mean \pm SEM (n = 6 in all groups) and calculated using a two-way ANOVA and *post hoc* Fisher's PLSD test. AD vs. IUGR: *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.001$; C vs. CR: †, $P < 0.05$; ††, $P < 0.01$; †††, $P < 0.001$; C vs. HF: #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$.

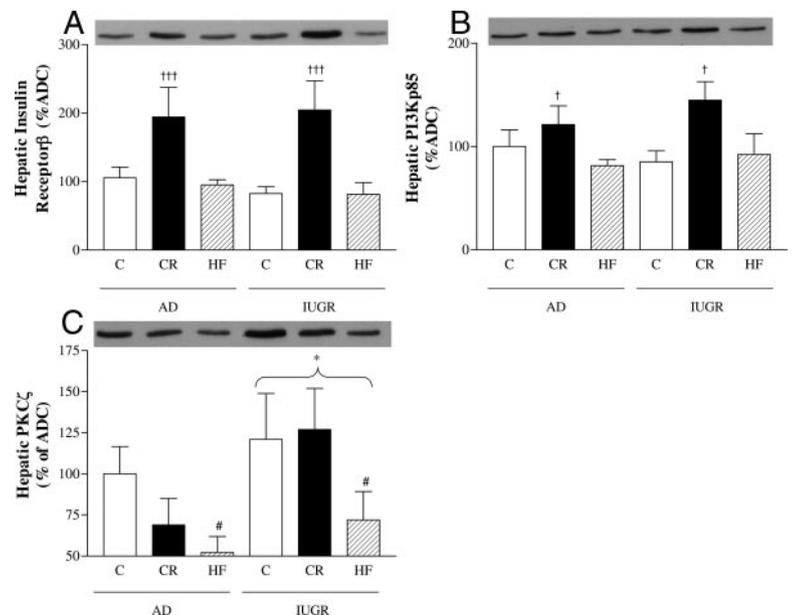


TABLE 5. Plasma lipids and triglyceride deposition in liver and muscle

Prenatal nutrition	Postnatal nutrition	Plasma free fatty acids (mmol/liter)	Plasma triglycerides (mmol/liter)	Liver triglycerides ($\mu\text{mol/g}$)	Quadriceps triglycerides ($\mu\text{mol/g}$)
AD	C	0.70 \pm 0.09	1.90 \pm 0.36	22.90 \pm 3.07	11.90 \pm 2.46
AD	CR	0.67 \pm 0.08	0.62 \pm 0.04 ^f	5.86 \pm 0.61 ^d	6.78 \pm 0.73 ^f
AD	HF	0.47 \pm 0.10	1.26 \pm 0.22 ^h	58.27 \pm 8.58 ⁱ	10.76 \pm 1.44
IUGR	C	0.67 \pm 0.07 ^a	2.50 \pm 0.36	20.33 \pm 4.44	17.29 \pm 3.03
IUGR	CR	0.90 \pm 0.11 ^a	0.67 \pm 0.04 ^f	17.09 \pm 12.29 ^d	8.97 \pm 1.38 ^f
IUGR	HF	0.73 \pm 0.08 ^a	1.68 \pm 0.22 ^h	45.53 \pm 16.43 ⁱ	11.14 \pm 1.18

All results were taken from plasma or tissue samples of male Wistar rats at 270 d of age. C, Chow postnatal diet; CR, postnatal CR diet; HF, postnatal HF diet. Plasma samples were quantified by colorimetric assay. Liver and quadriceps muscle triglycerides are expressed as micromoles per gram of wet tissue. Values are mean \pm SEM (n = 6 in all groups) and calculated using a two-way ANOVA and *post hoc* Fisher's PLSD test.

^{a-c} AD vs. IUGR: ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$.

^{d-f} C vs. CR: ^d $P < 0.05$, ^e $P < 0.01$, ^f $P < 0.001$.

^{g-i} C vs. HF: ^g $P < 0.05$, ^h $P < 0.01$, ⁱ $P < 0.001$.

changes in levels of atypical PKC ζ , which in turn stimulates GLUT4 translocation to the cell membrane and subsequent glucose transport (39, 40) and also increases insulin internalization *in vitro* and thus insulin action within the liver (41).

We observed increased hepatic PKC ζ expression in IUGR offspring but decreased hepatic PKC ζ expression in insulin-resistant HF-fed animals. The important role of PKC ζ in enhancing insulin action has been reported through investigations into exercise in high-performance athletes (42, 43). We speculate that increased PKC ζ expression in IUGR offspring may drive increased insulin receptor internalization, enhanced clearance of insulin from the circulation, and increased glucose transport across the cell membrane. Conversely, in an obesogenic environment resulting from HF nutrition, diminished hepatic PKC ζ may reduce glucose transport across the cell membrane and may represent a key cellular defect in the chronic insulin-resistant state, as observed in type 2 diabetes (44). Standaert *et al.* (45) reported no change of PKC ζ action in the livers of nondiabetic mice fed on a HF diet. However, the mice in these studies were subjected to relatively short periods of HF nutrition compared with the long-term exposure to HF nutrition used in our present study.

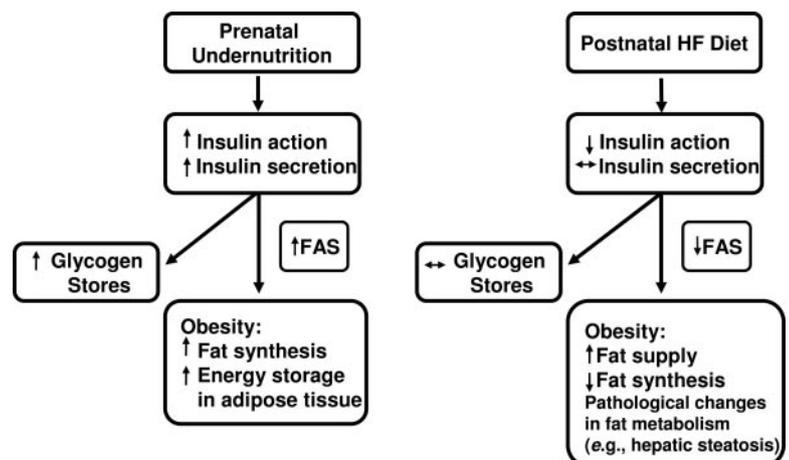
Glucose metabolism

Our observations of differential changes in plasma insulin levels and hepatic PKC ζ expression in obese IUGR and

HF-fed rats are consistent with differences in glycogen storage in these animals. We observed elevated storage of glycogen in both liver and quadriceps muscle in IUGR offspring but not in HF-fed animals. The increased fasting plasma insulin levels and increased PKC ζ expression in IUGR offspring may facilitate insulin-stimulated glucose uptake and subsequent glycogenesis. Additionally, the activity of glycogen synthase is impaired in animals with insulin resistance, and patients with type 2 diabetes show low glycogen synthase activity and decreased glycogen storage (38, 46). Nevertheless, changes in glycogen synthase or glycogen phosphorylase gene expression do not consistently account for increased glycogen storage (47), and insulin and leptin, acting in concert, may have a stimulatory effect on glycogen deposition and glucose incorporation into glycogen (47). Importantly, the IUGR offspring in the present study displayed hyperleptinemia, and animals from a parallel cohort have been shown to develop leptin resistance (18). Hyperleptinemia induces sparing of glycogen stores rather than diminished glycogenolysis during the transition from a fed to a fasted state without change in either glycogen synthase or phosphorylase activities (47). Thus, peripheral leptin resistance in conjunction with insulin hypersecretion, under conditions of maintained sensitivity to elevated insulin, may account in part for the increased glycogen storage in IUGR offspring seen in the present study.

In skeletal muscle, GLUT4 is translocated to the cell mem-

FIG. 4. Schematic representation of the different mechanisms of obesity development in prenatally undernourished and diet-induced obese rats. Prenatal influences (*i.e.* maternal undernutrition) lead to an increase in insulin action and secretion. Prenatal influences also cause a related elevation in hepatic and muscle glycogen stores. In prenatally undernourished rats, obesity develops that is associated with an increase in fat synthesis through elevations in hepatic FAS and storage of fatty acids for energy in peripheral adipose tissue sites. In contrast, diet-induced obesity through HF nutrition leads to decreased insulin action and insulin resistance. Diet-induced obesity is associated with reduced hepatic fat synthesis. The increased fat supply through diet leads to lipid accumulation in organs (*e.g.* hepatic steatosis). Diet-induced obesity does not result in changes of insulin secretion or glycogen storage.



brane in response to insulin, facilitating glucose uptake for storage as glycogen (42). Although we did not see changes in total GLUT4 protein content in muscle tissue of IUGR rats, it is feasible that there is a higher level of insertion of GLUT4 into the plasma membrane, resulting in increased glucose uptake and glycogen storage. This interpretation is supported by the observation of increased GLUT4 translocation into the plasma membrane of skeletal muscle in the offspring of mice undernourished during pregnancy (43).

The source of substrate for the higher plasma glucose levels and glycogen storage in IUGR rats is unclear. Increased hepatic gluconeogenesis appears to be unlikely; PEPCK is the key rate-controlling enzyme in gluconeogenesis, and hepatic PEPCK expression is normally negatively regulated by insulin (48). In many animal models of obesity and type 2 diabetes, insulin resistance leads to a 2- to 3-fold increase in gluconeogenesis and PEPCK mRNA (49, 50) despite elevated insulin levels, resulting in hyperglycemia (51). In contrast, IUGR rats from the present study showed higher plasma insulin levels, slightly higher plasma glucose levels, and unchanged expression of hepatic PEPCK compared with prenatally normally nourished animals, suggesting that the obesity of IUGR offspring in our study was independent of the insulin-resistant state commonly observed in diet-induced obesity.

Lipid metabolism

Under normal physiological conditions, fatty acids and triglycerides are exported from the liver and stored in adipose tissue. The obese IUGR offspring in this study showed elevated hepatic FAS mRNA, a marker of increased fat synthesis, and elevated plasma free fatty acids. This is consistent with the increased expression of hepatic PKC ζ in IUGR offspring, because hepatic atypical PKCs are regulators of lipid-synthesizing enzymes such as FAS (52). In contrast, HF feeding suppressed hepatic expression of PKC ζ and FAS, indicating a reduced requirement for endogenous fat synthesis, but increased hepatic triglyceride deposition. The lipostatic theory of obesity postulates that decreased insulin action in adipose tissue causes pathophysiological triglyceride accumulation in nonadipose tissues such as the liver, leading to whole-body insulin resistance (30, 53–56), and our observation of elevated hepatic triglyceride storage and insulin resistance in HF-fed rats is consistent with this proposal.

Nevertheless, not all obese individuals are insulin resistant, and fat distribution rather than total-body fat mass may be critical for the development of insulin resistance. Relative to sc adipocytes, visceral adipocytes are more sensitive to the lipolytic effects of catecholamines and less sensitive to the lipogenic effects of insulin (57, 58). In our IUGR offspring, the markedly elevated plasma leptin levels could not be explained by the modest increase in visceral (suprarenal) fat deposition, suggesting a relative increase in sc fat deposition. Indeed, we have recently observed that the elevation in total body fat in IUGR offspring, measured by dual-energy x-ray absorptiometry, is not reflective of visceral fat pad size alone (59). Because these animals maintained physiological whole-body sensitivity to insulin in the face of insulin hypersecretion,

we speculate that their obesity may be the result of increased insulin-driven fat deposition in the more insulin-sensitive sc adipose tissue. This contrasts with the insulin-resistant HF-fed rats, where increased dietary lipid supply leads to hepatic steatosis and massively increased visceral fat accumulation.

Implications of this study

The developmental origin of disease paradigm suggests that the fetus adjusts its metabolic set points in response to early-life cues that forecast the postnatal nutritional environment (2–5). This hypothesis predicts that animals undernourished *in utero* should anticipate a nutritionally sparse postnatal environment and display increased efficiency of energy storage and use. Our observations support this concept. When IUGR offspring of dams undernourished throughout pregnancy were exposed to postweaning calorie restriction, they showed evidence of improved energy storage and use when compared with similarly fed offspring of normally nourished dams. This was seen as increased fat storage (higher suprarenal fat content and elevated plasma leptin levels) and more effective glucose use (higher plasma insulin and glucose levels and increased glycogen stores).

In this study, we assessed insulin sensitivity by the hyperinsulinemic-euglycemic clamp method and by measuring a number of key cellular markers of insulin action. We conclude that the intrauterine growth restriction caused by maternal undernutrition, although leading to insulin hypersecretion and obesity, does not itself cause decreased insulin sensitivity (60). This is an important finding, because it is common, although not universal (61, 62), in the experimental or clinical literature to equate findings of hyperinsulinemia or reduced glucose tolerance with insulin resistance (8, 63–67). Particularly in a clinical setting, the need for rapid and minimally invasive assessment of insulin sensitivity has led to methodological approaches such as single measurements of fasting insulin or glucose tolerance tests. However, it is important to distinguish between hyperinsulinemia resulting from lack of insulin uptake and from insulin hypersecretion. Glucose tolerance tests represent pancreatic β -cell insulin secretion rather than tissue sensitivity to insulin (61). Of particular concern, small for gestational age children who have experienced intrauterine growth restriction are often classified as insulin resistant when assessed by the homeostasis model assessment method (68, 69).

Interestingly, the mechanisms responsible for catch-up growth during the early postnatal period are thought to influence associations between IUGR and risks for subsequent obesity (70). We have been able to show a clear distinction between the effects of IUGR (in this case via maternal undernutrition), which leads to insulin hypersecretion with maintained insulin sensitivity, and of postnatal hypercaloric nutrition, which leads to insulin resistance. Therefore, it is feasible that an increase in insulin secretion and enhanced insulin action in IUGR offspring, which would support catch-up growth, could be masked, under obesogenic conditions when insulin resistance develops as shown in the present study with a HF diet. The nature of this paradox, based on postnatal hypercaloric nutrition, was clearly iden-

tified in our present study and may be a reason, at least in part, why a number of studies of IUGR offspring, in both clinical and animal settings, have been classified as insulin resistant. Therefore, the present study suggests that careful assessment of postnatal nutrition is critical in the interpretation of the metabolic consequences of IUGR and that caution is required in designing therapeutic strategies.

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