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Intrauterine growth restriction combined with a maternal high-fat diet increased adiposity and serum corticosterone levels in adult rat offspring

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Original Article

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Abstract

Intrauterine growth restriction (IUGR) and fetal exposure to a maternal high-fat diet (HFD) independently increase the risk of developing obesity in adulthood. Excess glucocorticoids increase obesity. We hypothesized that surgically induced IUGR combined with an HFD would increase adiposity and glucocorticoids more than in non-IUGR offspring combined with the same HFD, findings that would persist despite weaning to a regular diet. Non-IUGR (N) and IUGR (I) rat offspring from dams fed either regular rat chow (R) or an HFD (H) were weaned to either a regular rat chow or an HFD. For non-IUGR and IUGR rats, this study design resulted in three diet groups: offspring from dams fed a regular diet and weaned to a regular diet (NRR and IRR), offspring rats from dams fed an HFD and weaned to a regular diet (NHR and IHR) and offspring from dams fed an HFD and weaned to an HFD (NHH and IHH). Magnetic resonance imaging or fasting visceral and subcutaneous adipose tissue collection occurred at postnatal day 60. IHH male rats had greater adiposity than NHH males, findings that were only partly normalized by weaning to a regular chow. IHH male rats had a 10-fold increase in serum corticosterone levels. IHH female rats had increased adiposity and serum triglycerides. We conclude that IUGR combined with an HFD throughout life increased adiposity, glucocorticoids and triglycerides in a sex-specific manner. Our data suggest that one mechanism through which the perinatal environment programs increased adiposity in IHH male rats may be via increased systemic glucocorticoids.

Introduction

Intrauterine growth restriction (IUGR) impacts up to 15% of pregnancies and increases the risk of obesity, thus contributing to the global obesity epidemic.[1,2] With the frequent consumption of a high-fat diet (HFD) worldwide and the association of obesity with hypertensive disorders of pregnancy, increasingly IUGR infants are born to mothers that consume an HFD or are obese.[3–8] In certain populations, even up to half of infants born to obese mothers are IUGR.[6] Both fetal exposure to a maternal HFD and IUGR independently predispose the individual to adult-onset obesity.[9–12] Importantly, fetal exposure to a maternal HFD and IUGR independently increase the risk of developing obesity even when dietary and lifestyle choices are taken into account, suggesting a role for fetal programming of increased obesity risk.[12–18] Obesity that develops secondary to an adverse *in utero* environment is often depot-specific, with the visceral adipose tissue (VAT) increasing in excess to the subcutaneous adipose tissue (SAT). Furthermore, in children increased visceral adipose that develops secondary to an adverse *in utero* environment occurs without changes to the body mass index (BMI), thus making the clinical determination of increased visceral adipose challenging.[19] Importantly, increased visceral adiposity in childhood increases the risk of developing metabolic syndrome, which increases the risk of cardiovascular disease and early mortality.[20–26] Multiple studies demonstrate the persistence of epigenetic and physiological changes resulting from an adverse perinatal environment both into adulthood and to subsequent generations despite consumption of a healthy diet in the offspring.[27,28] Despite the clinical importance of the perinatal environmental impact on obesity risk, it is unknown whether IUGR that occurs in the setting of a maternal HFD enhances the maternal HFD-induced offspring obesity risk.

Understanding how the *in utero* environment impacts future adiposity is critical, as current clinical recommendations primarily emphasize maternal weight gain and caloric intake, not diet composition, during pregnancy.[29–31] Yet, it is not known if there are overlapping mechanisms or cumulative effects of IUGR and a maternal HFD on developing increased visceral adiposity. One potential mechanism includes disruption to glucocorticoid-mediated fat deposition. Increased adiposity secondary to an increased glucocorticoid effect can be the result of excess secretion of circulating glucocorticoids by the hypothalamic–pituitary–adrenal axis or liver, increased intracellular glucocorticoid receptor (*GR*) density, or dysregulated

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intracellular glucocorticoid metabolism via the enzyme 11-β hydroxysteroid dehydrogenase 1 (*11-β HSD 1*) (as reviewed by Oakley and Cidlowski[32]). Conditions that increase circulating glucocorticoid levels increase visceral adiposity,[33] and increased glucocorticoids modulate adipogenesis and increase the risk of developing metabolic syndrome.[34–39] Glucocorticoid signaling induces transcription of proadipogenic factors through activation of *GR* isoforms, including active GR isoform (*GR-α*).[40,41] Binding of glucocorticoid to *GR-α* induces transcription of proadipogenic and lipogenic factors, such as ATP-binding cassette sub-family A member 1 (*Abca1*).[42] The adipocyte enzyme *11-β HSD 1* catalyzes the conversion of inactive glucocorticoid (11-dihydrocorticosterone in rodents, cortisone in humans) to active glucocorticoid (corticosterone in rodents, cortisol in humans).[43] Via generation of active glucocorticoids, overexpression of *11-β HSD 1* induces visceral adipose accumulation in rodents, while *11-β HSD 1* knock-out mice show reduced visceral adiposity.[34,44]

Increased glucocorticoids also induce the adipocyte differentiation and lipid metabolism regulator, peroxisome proliferator-activated receptor gamma (*Pparγ*), via increased expression of transcription factors CCAAT/enhancer-binding proteins β and Δ.[45] Isoforms of *Pparγ* include *Pparγ1* and *Pparγ2*; both *Pparγ1* and *Pparγ2* are expressed in adipose tissue.[46] Activation of either isoform of *Pparγ* induces differentiation of preadipocytes and increases adipocyte storage capacity for circulating lipids, potentially through regulation of adipose triglyceride lipase in adipocytes.[47,48]

The etiology of increased adiposity has not been studied in an animal model that combines IUGR and a maternal HFD. Thus measurement of circulating corticosterone and triglyceride levels and adipose tissue levels adipogenic and lipogenic proteins may provide insight into the mechanism through which IUGR and a maternal HFD increase visceral adiposity both independently and in combination. We hypothesized that the combined *in utero* insults of IUGR and a maternal HFD would enhance the programmed adipose tissue dysregulation and increase visceral adiposity in adult rat offspring more than either *in utero* insult alone. Specifically, IUGR combined with a maternal HFD would have greater visceral adiposity, circulating corticosterone and triglyceride levels, and visceral adipose protein levels of *GR-α* and *11-β HSD 1* than either IUGR rats or rats exposed to a maternal HFD. Because an adverse *in utero* environment programs obesity independent of lifestyle choices, we also hypothesized that weaning IUGR offspring exposed to a maternal HFD to a standard, regular rat chow would not fully reverse the combined IUGR- and maternal HFD-induced adipose tissue dysregulation.

Methods

Animals husbandry and study design

The University of Utah Animal Care Committee approved all procedures. Rat husbandry and the rat model were as previously described.[49] In brief, 45-day-old male and non-pregnant female Sprague Dawley rats were obtained from Charles River Laboratories, Inc. (Wilmington, MA, USA). Rats were exposed to 12 h light/dark cycles. Male rats used for breeding were kept on a regular rat chow (TD.8640; Reg, Harlan-Teklad, Madison, WI, USA) throughout the study. Female rats used for mating were fed one of two diets, either a regular rat chow (TD.8640) or a HFD rat chow (TD.110526; Harlan-Teklad). The regular chow had a caloric density of 3 kcal/g and contained 17% kcals from fat, consisting of 60 g/kg soybean oil and 0.03 wt/wt cholesterol, 54% kcals from carbohydrate, and 29% kcals from protein. The HFD had a caloric density of 4.3 kcal/g and contained 44% kcals from fat, consisting of milkfat and 10 g/kg soybean oil, and 1% wt/wt cholesterol, 40% kcals from carbohydrate, and 16% kcals from protein. The fat in the HFD was 65% saturated fat. The HFD also contained 0.5% cholic acid to aid in fat absorption as rats do not have gall bladders. Protein content in the HFD was lower than the regular diet. The protein content was chosen to mimic current protein consumption in the United States,[50] is sufficient for normal mammalian growth,[51] and balances dietary decreases in carbohydrates and protein while increasing fat intake. Further, protein content was significantly higher than levels used in low-protein dietary studies.[52]

After 5 weeks on either the regular diet or HFD, at 80–90 days of life, non-pregnant female rats were mated by placing the male rats into the female rat cages. Pregnancy day 0.5 was determined by the presence of sperm on a vaginal swab the following morning. Bilateral uterine artery ligation was performed on day 19 of a 21-day gestation using anesthesia alone as a control, as described previously.[53] We previously reported that maternal caloric intake did not vary due to diet or surgical intervention, with all dams consuming an average of 63 kcal/day.[49] Dams were allowed to deliver vaginally and litters were culled to six for rearing consistency, with three males and three females per litter. Only one rat per sex per litter was used in the experiments outlined in this manuscript, with the exception that body weights from birth (P0) were averaged per sex from each litter, and the average body weight per sex from each litter was then averaged across the litters in each group and analyzed between groups. At the age of weaning (P21), offspring from dams fed an HFD were either continued on the HFD or changed to a regular rat chow (TD.8640). For each of the normal non-IUGR (N) and IUGR (I) rats, our study design resulted in three experimental groups: offspring exposed to a maternal regular diet and weaned to a regular diet (RR); offspring exposed to a maternal HFD and weaned to a regular diet (HR); offspring exposed to a maternal HFD and weaned to an HFD (HH) (Fig. 1). All offspring were killed at postnatal day (P) 60 after an overnight fast and anesthetized with isoflurane before decapitation. Tissues were flash frozen in liquid nitrogen and stored at −80°C or fixed in formalin

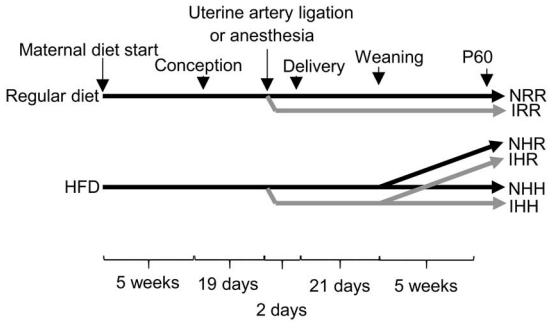

Fig. 1. Schematic of the timeline of dietary and surgical interventions in the rat model. Either a maternal regular diet or a maternal high-fat diet (HFD) were started 5 weeks before conception and continued through gestation and lactation. Nineteen days after conception, either bilateral uterine artery ligation to induce intrauterine growth restriction (IUGR) (I) or anesthesia alone for non-IUGR (N) rats was performed 2 days before delivery. At P21, the normal time of weaning, regular diet rats were weaned to a regular diet (RR), and maternal HFD fed rats were weaned to either a regular diet (HR) or to an HFD (HH). Post-weaning diets were continued for an additional 5 weeks, until the offspring were 60 days old. In the figure, the normal, non-IUGR rat timeline is shown in black lines, and IUGR rats shown in gray lines.

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for further analysis. Female offspring were harvested only in estrus to minimize confounders of the estrus cycle.[54]

Volumetric assessment of adipose depots

Magnetic resonance imaging (MRI) images of the abdomen and pelvis were obtained and data analyzed as previously described.[55] In brief, MRI was performed at P60 at the University of Utah small animal imaging core facility. Paired images of fat plus water and water alone were obtained from diaphragm to pelvis. Image analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). MRI images were analyzed using retroperitoneal fat as a representative visceral adipose depot. MRI image analysis was undertaken on four rats from different litters per group. These rats were not used for other analyses in this study.

Analysis of adipocyte size

The left retroperitoneal adipose tissue was used as a representative visceral adipose depot, and the left flank adipose tissue was used as a representative subcutaneous adipose depot. Adipose tissue was fixed in 10% formalin, embedded in paraffin, sliced to 5 µm thickness and stained with hematoxylin and eosin at the University of Utah Dermatopathology laboratory. Adipocyte diameter was quantified using Bioquant True Color Windows Image Analysis System (R&M Biometrics, Nashville, TN, USA). Diameter was measured as the longest distance from one end of the adipocyte to the other. All adipocytes where the entire adipocyte appeared in the field were measured per field from 15 high powered fields taken from six rats for each adipose depot. These rat tissues were not used for other analyses in this study.

Analysis of serum corticosterone, triglycerides, leptin and adiponectin

After an overnight fast, mixed arterial and venous blood was collected in serum separator tubes (BD Vacutainer; BD, Franklin Lakes, NJ, USA) after decapitation and placed on ice. Serum was separated from cellular components within 30 min of collection, flash frozen in liquid nitrogen and stored at −80°C. Serum corticosterone was measured using an ELISA kit (Cayman Chemical Company, Ann Arbor, MI, USA), with a detection limit of 30 ng/ml. Triglyceride levels were measured by ARUP Laboratories (Salt Lake City, UT, USA). Serum leptin and adiponectin were quantified using an ELISA kit (Millipore, St. Charles, MI, USA), with kit detection limits of 0.08 ng/ml for leptin and 0.155 ng/ml for adiponectin. Serum from six rats from different litters per group was analyzed. All six rats used in serum analyses were also used in adipocyte protein and messenger RNA (mRNA) analyses.

Protein analysis

VAT and SAT protein was isolated using whole cell lysates in a buffer containing 150 mM NaCl, 50 mM Tris at pH 7.4, 1 mM EDTA, 0.25% Na-deoxycholate, 1% Igepal CA-630. Total protein was quantified using a BSA standard curve measured by a bicinchoninic assay (Pierce, Rockford, IL, USA); 50 µg protein was run on a 10% SDS-PAGE gel (Bio-Rad, Hercules, CA, USA) and transferred to a polyvinylidene difluoride membrane. Membranes were blocked with 5% milk. Antibodies used to identify and quantify specific protein content included *GR-α* (Abcam, Cambridge, UK), *11-β HSD 1* (Sigma-Aldrich, St. Louis, MO, USA), leptin (Millipore, Billerica, MA, USA), adiponectin (Cell Signaling Technology, Danvers, MA, USA) and *Pparγ* (Santa Cruz Biotechnology, Dallas, TX, USA), which detects both isoforms of *Pparγ*. Hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) (Proteintech, Rosemont, IL, USA) was used as an internal control as *Hprt1* did not vary by intrauterine condition or diet. Protein was detected with Western Lightning-enhanced chemiluminescence (PerkinElmer Life Sciences, Waltham, MA, USA) using goat anti-rabbit horseradish peroxidase secondary antibody from Cell Signaling Technology (Beverly, MA, USA). Blots were quantified using a Kodak Image Station 2000R (Eastman Kodak/SIS, Rochester, NY, USA).

Due to the large number of rat diet and intrauterine condition groups, not all samples were able to be run on a single western blot gel. Therefore, protein samples from NRR and IRR rats were run on every gel along with either samples from both NHR and IHR or both NHH and IHH rats. Each target protein was first compared with the loading control (*Hprt1*), then protein levels from the NRR rat group were compared between the two western blot gels (one containing NRR, IRR, NHR and IHR protein samples, and other containing NRR, IRR, NHH and IHH protein samples). Target protein levels from the NHR and IHR rats were normalized for differences between the NRR data between the two sets of gels to account for differences in baseline loading and exposure of the gel. Sexes were run on separate gels and analyzed separately. Given the large number of samples, representative western blot images were cropped from the gels in the figures.

Adipocyte mRNA analysis

Total adipocyte mRNA was isolated as described previously using the RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA, USA).[55] Total RNA was quantified spectrophotometrically. The complementary DNA (cDNA) was synthesized from 1 µg RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) per manufacturer's protocol.

Real-time reverse transcriptase polymerase chain reaction (RT-PCR)

Semi-quantitative real-time RT-PCR quantification was performed using *Hprt1* as an internal control, as Ct values of *Hprt1* did not differ between any of the groups. Relative quantification of PCR products was based on differences between *Hprt1* and the target using the comparative Ct method (TaqMan Gold RT-PCR manual; PE Biosystems, Foster City, CA, USA).[56] Assays were performed with Taqman Gene Expression Assays (Applied Biosystems) for target gene *Abca1*.

Statistics

Data were expressed as mean ± S.E.M., except for adipocyte diameter, for which data were expressed as median and interquartile range in a box and whisker plot. Two-way analysis of variance with Fisher's protected least-significant difference was used for data analysis, using variables of diet and intrauterine environment. A P value $\leqslant 0.05$ was considered statistically significant. Results of statistical analyses are shown for data from IUGR relative to non-IUGR rats of the same maternal and offspring diet, and all groups relative to NRR. Male and female data were analyzed separately (Fig. 1).

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Results

Weight gain and food intake

Male NHH rats consumed less kilocalories per day than NRR male rats, but otherwise there were no differences in food or caloric intake in this study. There was no difference between maternal diet groups in the time to achieve pregnancy (regular diet females were pregnant at 1.7 ± 0.2 days, HFD females were pregnant at 2.7 ± 0.5 days), and >95% of female rats with a vaginal swab containing sperm became pregnant in all groups. Maternal diet also did not impact the number of pups per litter (regular diet litter size 10.6 ± 0.8 pups per litter; HFD litter size 12.3 ± 0.8 pups per litter, $P > 0.05$). Maternal HFD decreased the male to female ratio (regular diet male:female ratio 2.3 ± 0.4, HFD male:female ratio 1.2 ± 0.2, $P = 0.019$). While we previously reported that maternal HFD intake induced mild growth restriction of about 5% in both male and female offspring at birth, with a larger number of rats per group in this study, the decreased body weight in the maternal HFD fed rats at P0 no longer reached statistical significance ($P = 0.1$ for both males and females).[57] While only female IUGR rats from maternal HFD fed dams weighed less than regular diet fed dams by P21, by P60, all rats exposed to the maternal HFD weighed less compared with sex-matched maternal regular diet fed rats, regardless of post-weaning diet (Table 1). Decreased rat body weight occurred despite the same total caloric intake, with the exception of decreased caloric intake in NHH male rats. None of the rats in our study ate more than the normal non-IUGR regular diet group (NRR). Both male and female NHH and IHH rats consumed more fat and cholesterol than the other four groups of rats, with no difference between IUGR and non-IUGR within any diet group (Table 1).

Volumetric assessment of adipose depots and adipocyte size

The greatest visceral and subcutaneous adiposity was seen in male offspring in the combined *in utero* insults of IUGR and a maternal HFD when weaned to an HFD (IHH males) (Fig. 2a and 2b). Compared with NRR males, all other male rat groups had greater visceral adiposity (Fig. 2a and 2b). Changing the post-weaning diet to a regular diet decreased visceral adiposity by 35% but did not fully reverse the impact of maternal HFD exposure and/or IUGR on visceral adipose volume in NHR and IHR males (Fig. 2b). IUGR increased visceral adiposity in males compared with respective diet non-IUGR rats (Fig. 2b). The greatest subcutaneous adiposity in female rats was seen in the combined *in utero* insults of IUGR and a maternal HFD when weaned to an HFD (IHH females), findings which were normalized by weaning to a regular diet (Fig. 2c and 2d).

The greatest visceral and subcutaneous adipocyte size was seen in the combined *in utero* insults of IUGR and a maternal HFD

Table 1. Growth and food intake at P60

	NRR	IRR	NHR	IHR	NHH	IHH
Males						
Body weight P0 (g)	4.3 ± 0.1	3.3 ± 0.1*	na	na	3.9 ± 0.2	3.3 ± 0.1*†
Body weight P21 (g)	60 ± 1	55 ± 3	na	na	59 ± 2	54 ± 3
Body weight P60 (g)	349 ± 6	346 ± 7	324 ± 13*	310 ± 8*	289 ± 6*	294 ± 12*
Food intake (g/day)	32 ± 1	31 ± 1	28 ± 1	31 ± 0	18 ± 1*	20 ± 1*
Food intake (kcal/day)	95 ± 4	93 ± 4	84 ± 3	93 ± 1	77 ± 5*	88 ± 3
Fat intake (kcal/day)	16 ± 1	16 ± 1	14 ± 1	16 ± 1	34 ± 2*	39 ± 1*
Cholesterol intake (mg/day)	0.8 ± 0	0.8 ± 0	0.8 ± 0	0.9 ± 0	195 ± 10*	223 ± 6*
Females						
Body weight P0 (g)	4.1 ± 0.1	3 ± 0.2*	na	na	3.6 ± 0.2	2.9 ± 0.1*†
Body weight P21 (g)	56 ± 2	52 ± 4	na	na	55 ± 2	47 ± 2*†
Body weight P60 (g)	222 ± 3	211 ± 4	202 ± 4*	192 ± 5*	203 ± 6*	212 ± 4
Food intake (g/day)	20 ± 0	20 ± 1	19 ± 1	20 ± 1	13 ± 1*	13 ± 0*
Food intake (kcal/day)	60 ± 1	61 ± 3	58 ± 4	60 ± 3	58 ± 4	58 ± 2
Fat intake (kcal/day)	10 ± 0	10 ± 1	10 ± 1	10 ± 0	26 ± 2*	25 ± 1*
Cholesterol intake (mg/day)	0.5 ± 0	0.5 ± 0	0.5 ± 0	0.5 ± 0	135 ± 8*	146 ± 8*

Groups: Body weights from rats at P0 were averaged for all live offspring rats per sex in each litter, and the average body weight per sex from each litter was averaged for 10 litters per group. Body weights were obtained from the following numbers of offspring rats each from a different litter at P21 and P60.
Normal non-intrauterine growth restriction (IUGR), maternal regular diet, regular diet at weaning (NRR): at P21, $n = 27$ male and $n = 22$ female; at P60, $n = 24$ male and $n = 22$ female.
IUGR, maternal regular diet, regular diet at weaning (IRR): at P21, $n = 10$ male and $n = 8$ female; at P60, $n = 20$ male and $n = 20$ female.
Normal non-IUGR, maternal high-fat diet (HFD), regular diet at weaning (NHR): at P60, $n = 14$ male and $n = 14$ female.
IUGR, maternal HFD, regular diet at weaning (IHR): at P60, $n = 24$ male and $n = 21$ female.
Normal non-IUGR, maternal HFD, HFD at weaning (NHH): at P21, $n = 10$ male and $n = 13$ female; at P60, $n = 18$ male and $n = 25$ female.
IUGR, maternal HFD, HFD at weaning (IHH): at P21, $n = 7$ male and $n = 9$ female; at P60, $n = 20$ male and $n = 35$ female.
P0 and P21 maternal HFD offspring growth data are shown under the NHH and IHH columns, and not-applicable (na) is instead written into the columns under NHR and IHR for this data.
Data presented as mean ± S.E.M. for rats each from different litters.
*Significant difference between the control group and NRR, $P \leqslant 0.05$; †significant difference between IHH and NHH, $P \leqslant 0.05$. No difference was found comparing IHR with NHR.

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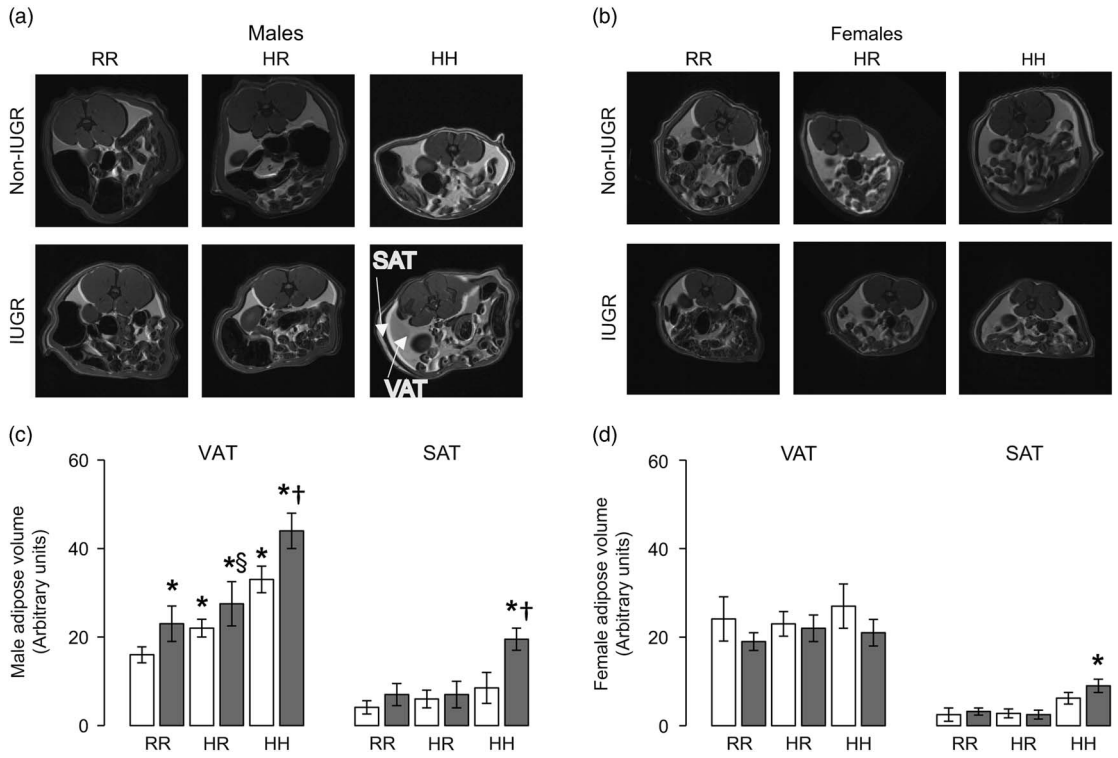

Fig. 2. The combined *in utero* insults of intrauterine growth restriction (IUGR) and a maternal high-fat diet (HFD) increased visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) in male rats (*a*,*b*) and SAT in female rats (*c*,*d*). Weaning to a regular rat chow did not normalize IUGR or maternal HFD-induced increased adiposity in male rats. White bars represent normal, non-IUGR (N) rats and gray bars represent IUGR (I) rats. Maternal and offspring diets are denoted below the graphs [regular diet rats were weaned to a regular diet (RR), and maternal HFD fed rats were weaned to either a regular diet (HR) or to an HFD (HH)]. A total of four rats each from different litters per group were used in these analyses. Statistical significance is denoted as follows: $^{*}P \leqslant 0.05$ for NHR, NHH, IRR, IHR and IHH rats compared with sex-matched NRR rats; $^{\S}P \leqslant 0.05$ for IHR rats compared with NHR sex-matched rats; $^{\dagger}P \leqslant 0.05$ for IHH rats compared with sex-matched NHH rats.

when weaned to an HFD in both sexes (IHH males and females) (Fig. 3a–3d). Weaning to a regular diet decreased visceral adipocyte size by 38% but did not fully reverse the impact of maternal HFD exposure in NHR and IHR males (Fig. 3a and 3b). IUGR increased visceral adipocyte size in males compared with respective diet non-IUGR rats (Fig. 3b). Weaning maternal HFD fed rats to a regular diet normalized adipocyte size in the subcutaneous adipose but not the visceral adipose in both sexes.

Analysis of serum corticosterone, triglycerides, leptin and adiponectin

The greatest serum corticosterone was seen in males rats in the combined *in utero* insults of IUGR and a maternal HFD when weaned to an HFD (IHH males), findings which were normalized by weaning to a regular diet (Fig. 4a). The greatest serum triglyceride level was seen in female rats in the combined *in utero* insults of IUGR and a maternal HFD when weaned to an HFD (IHH females), findings which were normalized by weaning to a regular diet (Fig. 4d). The greatest serum adiponectin was seen in female rats in the combined *in utero* insults of IUGR and a maternal HFD when weaned to an HFD (IHH females), findings which were normalized by weaning to a regular diet (Supplemental Fig. 1).

Adipose depot protein quantification

The greatest amount of leptin protein was found in male rats in the combined *in utero* insult of IUGR and a maternal HFD when weaned to an HFD (IHH males) in both VAT and SAT, findings which were normalized by weaning to a regular diet (Fig. 5a). The lowest amount of adiponectin protein was found in non-IUGR and IUGR female rats exposed to a maternal HFD when weaned to an HFD (NHH and IHH females) in both VAT and SAT, with no difference between NHH and IHH female adiponectin protein levels (Fig. 5d). This increased adiponectin protein in was normalized by weaning to a regular diet (Fig. 5d).

The greatest amount of *Pparγ2* protein in male rats was found in the combined *in utero* insults of IUGR and a maternal HFD when weaned to an HFD (IHH males), findings which were normalized by weaning to a regular diet (Fig. 6c). The lowest *Pparγ1* and *Pparγ2* protein levels in female rats were seen in the combined *in utero* insults of IUGR and a maternal HFD when weaned to an HFD (IHH females), findings which were normalized by weaning to a regular diet (Fig. 6b and 6d). Normalizing maternal HFD fed rats to a regular diet decreased *Pparγ1* protein levels in male subcutaneous adipose, regardless of *in utero* IUGR insult (Fig. 6a).

The combined *in utero* insults of IUGR and a maternal HFD did not change *11-β HSD 1* protein levels in any male rat groups in the visceral adipose and in any female rat groups in VAT and SAT (Fig. 7a and 7b). Combined maternal and offspring HFD decreased *11-β HSD 1* protein levels in male rat subcutaneous adipose, regardless of *in utero* IUGR insult (Fig. 7a). The combined *in utero* insults of IUGR and a maternal HFD did not change *GR-α* protein levels in male or female rat VAT and SAT (Fig. 7c and 7d). Normalizing maternal HFD fed rats to a regular

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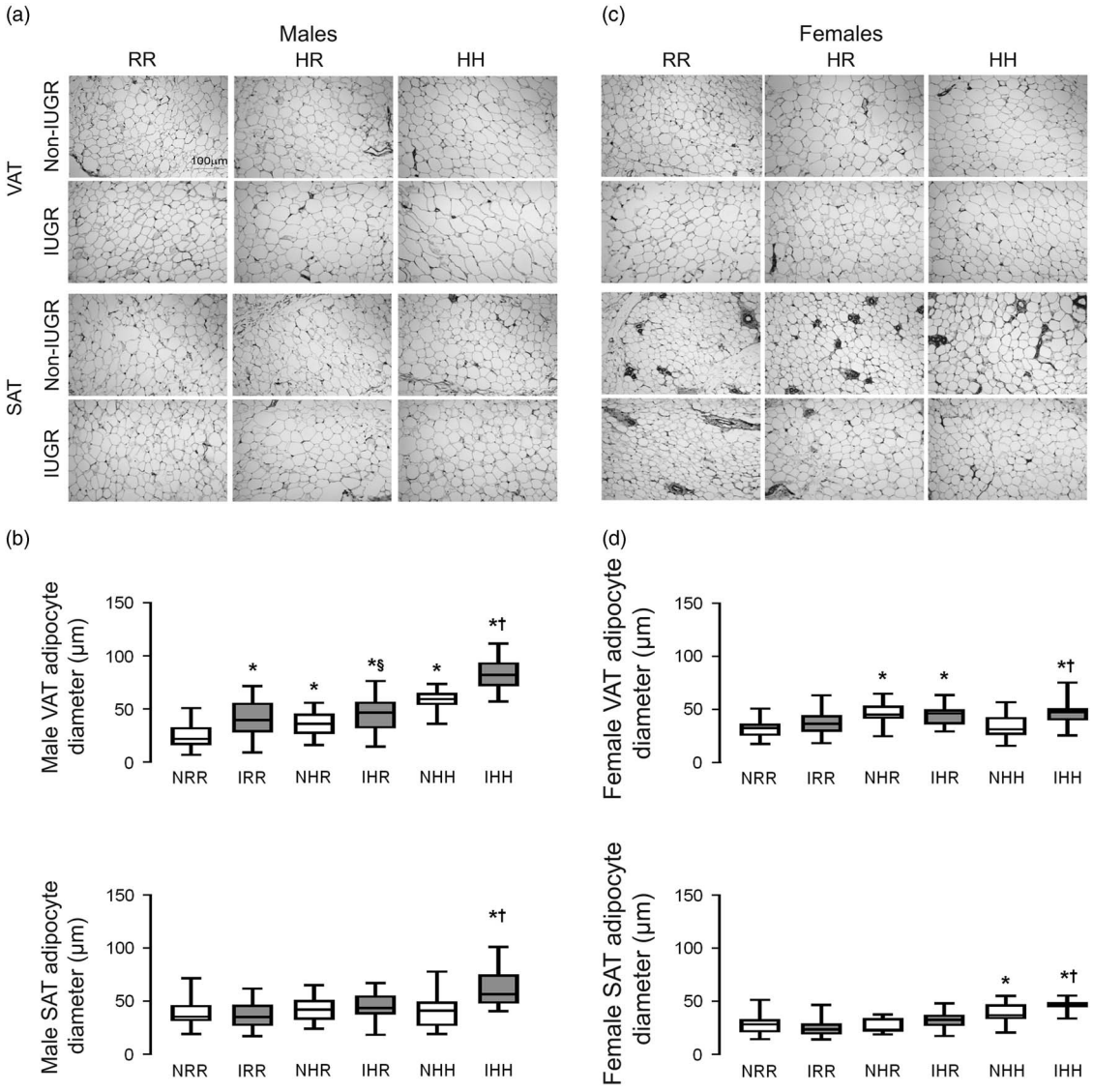

Fig. 3. The greatest adipocyte size was found in the combined *in utero* insult of intrauterine growth restriction (IUGR) and a maternal high-fat diet (HFD), which does not normalize by weaning to a regular diet in the visceral adipose tissue (VAT). Representative hematoxylin and eosin stained adipocyte images of VAT and subcutaneous adipose tissue (SAT) for normal non-IUGR (N) and IUGR (I) male (*a*) and female (*c*) rats; and quantification of adipocyte size is shown below the representative images for male (*b*) and female (*d*) VAT and SAT. Scale bar in the upper left panel represents 100 μm. White bars represent normal non-IUGR rats and gray bars represent IUGR rats. Quantification of adipocyte diameter data shown as box and whisker plots, with median and 25–75% interquartile range. Maternal and offspring diets are denoted below the graphs [regular diet rats were weaned to a regular diet (RR), and maternal HFD fed rats were weaned to either a regular diet (HR) or to an HFD (HH)]. A total of six rats from different litters per group were used in these analyses. Statistical significance is denoted as follows: $^{*}P \leqslant 0.05$ for NHR, NHH, IRR, IHR and IHH rats compared with sex-matched NRR rats; $^{\S}P \leqslant 0.05$ for IHR rats compared with NHR sex-matched rats; $^{\dagger}P \leqslant 0.05$ for IHH rats compared with sex-matched NHH rats.

diet decreased *GR-α* protein levels in male visceral and female subcutaneous adipose, and increased *GR-α* protein levels in male subcutaneous and female visceral adipose, regardless of *in utero* IUGR insult (Fig. 7c and 7d).

Adipose depot mRNA quantification

The greatest *Abca1* mRNA levels were seen in male rats in the combined *in utero* insults of IUGR and a maternal HFD when weaned to an HFD (IHH males), findings which were normalized by weaning to a regular diet (Fig. 8a). *Abca1* mRNA levels were decreased in all other male rat subcutaneous adipose (Fig. 8a). The least *Abca1* mRNA levels were seen in female rats exposed to a maternal HFD and weaned to an HFD (NHH and IHH females), findings which were normalized by weaning to a regular diet (Fig. 8b).

Discussion

In our study, the combined *in utero* insults of a maternal HFD and IUGR increased visceral and subcutaneous adiposity, serum corticosterone and serum triglyceride levels in a sex-dependent manner. Weaning offspring to a regular diet did not fully normalize the increased adiposity secondary to the combined *in utero* insults. Increased circulating corticosterone levels and increased levels of downstream *GR* transcription targets suggests that increased adiposity may be in part secondary to increased circulating corticosterone in IHH male rats. Increased adiposity

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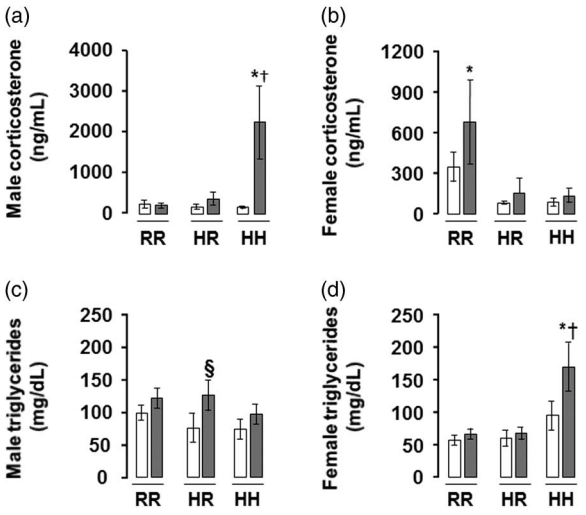

Fig. 4. The greatest corticosterone level was in the combined *in utero* insult of intrauterine growth restriction (IUGR) and a maternal high-fat diet (HFD) in male rats, and the greatest triglyceride level was in the combined *in utero* insult of IUGR and a maternal HFD in female rats, findings which were normalized by weaning to a regular diet. Fasting serum corticosterone levels shown in (*a*,*b*) and triglycerides in (*c*,*d*). White bars represent normal non-IUGR (N) rats and gray bars represent IUGR (I) rats. Maternal and offspring diets are denoted below the graphs [regular diet rats were weaned to a regular diet (RR), and maternal HFD fed rats were weaned to either a regular diet (HR) or to an HFD (HH)]. A total of six rats from different litters per group were used in these analyses. Statistical significance is denoted as follows: $^{*}P \leqslant 0.05$ for NHR, NHH, IRR, IHR and IHH rats compared with sex-matched NRR rats; $^{\S}P \leqslant 0.05$ for IHR rats compared with NHR sex-matched rats; $^{\dagger}P \leqslant 0.05$ for IHH rats compared with sex-matched NHH rats.

secondary to the combined *in utero* insults likely occurs through multiple mechanisms and in a sex-specific manner.

Ample evidence now exists demonstrating that maternal obesity or HFD intake programs obesity in her children. When adjusted for sex, age and socio-economic factors, a study of 504 children in India born to mothers with normal glucose tolerance demonstrated that increased maternal adiposity predicted increased adiposity in her children.[58] When controlling for lifestyle factors, maternal obesity more than doubled the risk of obesity in 2–4-year-old children as found in a study of 8494 children.[18] Further, a longitudinal study of 179 children ages 6–11 years old showed increased metabolic syndrome in children exposed to maternal obesity compared with those born to women with a normal pre-pregnancy body weight.[12] Similar to epidemiological studies, rodent studies of maternal HFD intake show increased offspring adipose tissue depot volume. A maternal HFD in non-IUGR mice induced increased adiposity, which was not reversed by a standard chow postnatally and further exacerbated by HFD feeding.[59] Maternal HFD feeding in rats also induced increased adiposity in offspring, which was not improved by fish oil supplementation.[60] Offspring from non-obese, maternal HFD fed dams had increased adiposity, indicating that in the absence of maternal obesity, an HFD alone can increase offspring adiposity.[61] Our study also showed that a maternal HFD alone increased visceral adipose depot volume and adipocyte size in male rats. We then showed that these results are further exacerbated by weaning to an HFD and are not normalized by weaning to a regular chow. Increased caloric consumption, particularly of fat, increases body weight and fat deposition.[62] Unlike in humans, it is common for rodents to decrease or maintain their food intake when fed an HFD, although this finding is not universal.[63,64] The reason behind the discrepancy in HFD food intake between studies is unknown, but may be secondary to the species studied or to the specific components of the diet and palatability. Diet composition of HFDs with or without additional sucrose enhanced weight gain and adiposity differentially depending on the age of the rat.[65] The male rats in our study consumed the same total calories as the NRR males, except for decreased caloric intake of the NHH males, a finding that was associated with decreased body weight but not decreased visceral adiposity. Following the rats in our study to an older age may show increased body weight in addition to increased adiposity.

IUGR alone impacts the development of adult-onset obesity. At the age of 50 years, women exposed during fetal life to the Dutch famine of 1944–1945 had increased BMI and waist circumference.[13] Further, without exposure to famine, low birth weight was associated with increased waist to hip ratios independent of lifestyle in a study of 1084 adult men born between 1920 and 1943.[17] Studies in rodents also indicate that adverse *in utero* environments resulting in IUGR increase adipose deposition in the offspring.[55,66] Spontaneous IUGR occurs in piglets and is associated with increased body fat at 12 months of age.[67] Decreased placental nutrient transfer in sheep leads to increased visceral adiposity in adolescence.[68] Our data presented here supports the growing body of literature that IUGR even without a maternal HFD increases visceral adiposity, with all male IUGR rat groups having increased adiposity compared with diet-matched non-IUGR male rats.

The role that catch-up growth, or the rapidity with which a growth-restricted individual's weight matches that of non-growth-restricted peers, impacts the development of obesity. Rapid catch-up growth increases the risk of developing obesity in both humans and animal models.[69–72] We previously reported that at postnatal day 21, male IUGR rats from either dams on an HFD or dams on a regular diet had caught up in body weight to the non-IUGR male rats, while IUGR female rats weighed less than non-IUGR females.[57] In our current study, regardless of post-weaning diet and despite having increased visceral adiposity, maternal HFD exposed rats weighed less than regular diet non-IUGR rats at P60. Similar results are found in the literature, where maternal HFD fed offspring rats showed normal body weight early in life but increased adiposity with aging.[61] In another study, adolescent rat offspring of HFD fed dams weighed the same as offspring from normal diet fed dams, but by adulthood the maternal HFD fed offspring weighed more.[73] Taken together, these data suggest that a latent predisposition to increased adiposity may exist even if offspring do not weigh more in early life. Following our IUGR and HFD fed rats to an older age may reveal eventual weight gain in addition to increased adiposity. Further, IUGR has been shown to increase the preference for palatable foods, particularly sweet-tasting food, which is thought to play a role in catch-up growth and eventual formation of obesity.[74] In our study, IUGR rats fed an HFD did not consume more calories than the non-IUGR regular diet fed rats. Discrepancy in food intake and weight gain in IUGR individuals from the literature and from our study may be due to the lower carbohydrate content in the HFD compared with the regular diet, suggesting that the HFD was not more palatable than the regular diet.

The mechanisms for increased adiposity depend on sex, type of *in utero* environment, and post-weaning diet. One mechanism for increased adiposity despite similar caloric intake is exposure to excess endogenous or exogenous glucocorticoids. In humans with cortisol excess, visceral adiposity is increased more than

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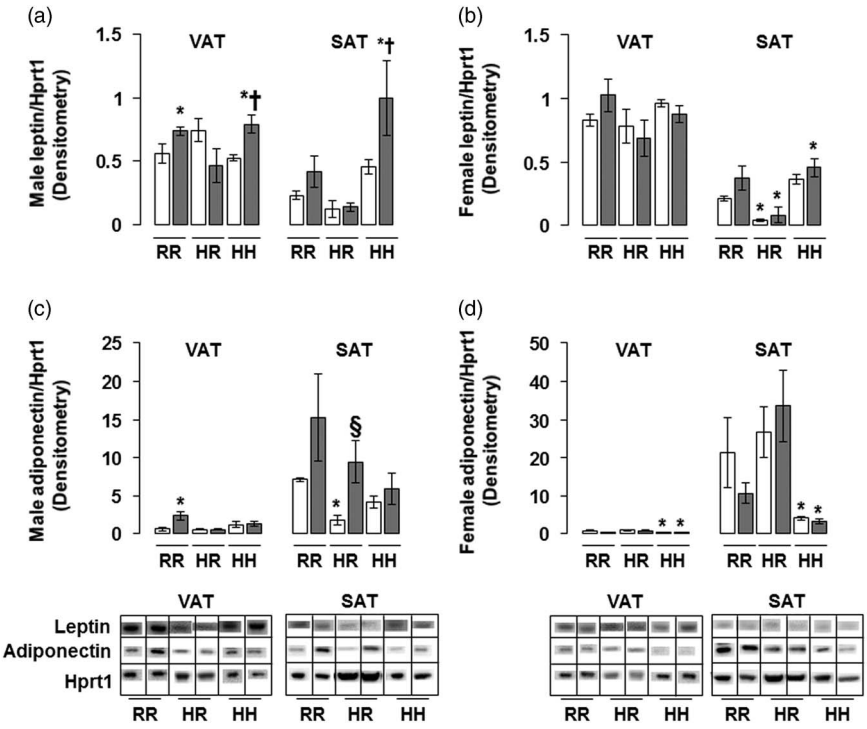

Fig. 5. The greatest leptin protein was found in the combined *in utero* insult of intrauterine growth restriction (IUGR) and a maternal high-fat diet (HFD) in male visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT); and the least adiponectin protein was found in the combined *in utero* insult of IUGR and a maternal HFD in female VAT and SAT. These findings were normalized by weaning to a regular diet. Western blot protein quantification of VAT and SAT leptin (*a*,*b*) and adiponectin (*c*,*d*). Due to the large number of samples, western blots were run as follows per sex: western blots with NHH and IHH samples were run on one gel, and NHR and IHR were run on a second gel. NRR and IRR samples were run on both western blots. All samples were first normalized to hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) control protein levels, then controlled for variation in NRR protein levels between blots. Representative western blot images are shown below the graphs, with one representative lane shown per rat group. White bars represent normal non-IUGR (N) rats and gray bars represent IUGR (I) rats. Maternal and offspring diets are denoted below the graphs [regular diet rats were weaned to a regular diet (RR), and maternal HFD fed rats were weaned to either a regular diet (HR) or to an HFD (HH)]. A total of six rats from different litters per group were used in these analyses. Statistical significance is denoted as follows: $^{*}P \leqslant 0.05$ for NHR, NHH, IRR, IHR and IHH rats compared with sex-matched NRR rats; $^{\S}P \leqslant 0.05$ for IHR rats compared with NHR sex-matched rats; $^{\dagger}P \leqslant 0.05$ for IHH rats compared with sex-matched NHH rats.

subcutaneous adiposity.[75] Similarly in animal models, excess corticosterone administration increases visceral adiposity in rats.[76,77] The impact of the *in utero* environment on circulating glucocorticoids can be seen in IUGR rodent studies. Maternal food restriction in rats followed by HFD feeding of the offspring impacts *GR* and *11-β HSD 1* levels, while increasing white adipose tissue volume and leptin levels.[78] Similar to our current results, IUGR male rats exposed *in utero* to maternal tobacco smoke also have increased visceral adiposity in association with increased circulating corticosterone without changing *GR-α* protein levels.[66] Offspring HFD feeding alone did not change circulating corticosterone levels or *11-β HSD 1* or *GR-α* mRNA levels in adult rats.[79] In *GR* knock-out mice, obesity resulting from HFD feeding does not require the *GR*, whereas obesity resulting from exogenous glucocorticoid administration requires an intact *GR*, emphasizing the importance of multiple pathways to increased adiposity.[80] In our current study, a maternal HFD with or without a post-weaning HFD in non-IUGR male rats (NHR and NHH) increased visceral adiposity and adipocyte size, without changing circulating corticosterone or visceral adipose *11-β HSD 1* protein. Despite increased circulating corticosterone in female IUGR rats on a regular diet (IRR females), visceral and subcutaneous adiposity, *GR-α* protein and *Abca1* mRNA levels are unchanged, suggesting that mildly increased circulating corticosterone alone does not necessarily increase adiposity or glucocorticoid signaling. Conversely, female non-IUGR and IUGR rats exposed to a maternal HFD and weaned to a regular diet (NHR and IHR) did not have increased circulating corticosterone, but had increased *GR-α* protein in VAT and increased visceral adipocyte diameter. NHR and IHR female rats did not have increased downstream transcription target *Abca1* mRNA levels in VAT, suggesting that the increased adipocyte diameter may be due alterations in non-glucocorticoid pathways. *GR-α* protein was decreased in NHR males, which may indicate an appropriately decreased response to normal levels of circulating glucocorticoids as a result of increased visceral adiposity. Male IHH *GR-α* protein is unchanged from NHH in both visceral and subcutaneous adipose depots, which combined with the significantly increased circulating glucocorticoids may in part explain the etiology of increased visceral and subcutaneous adipose depot volume and adipocyte size. Increased circulating corticosterone and stable *GR-α* protein may allow for increased *GR* signaling and thus increased visceral adipose in the IHH male rats, as suggested by increased GR transcription target *Pparγ2* protein and *Abca1* mRNA levels, and similar to results found in our previous study of maternal tobacco smoke exposure on offspring adiposity.[66]

The perinatal environment also impacts adipocyte expression of *Pparγ*. Increased adiposity and adipocyte size is associated with increased *Pparγ* levels in HFD fed rats, particularly when fed a HFD from the time of weaning.[65] Further, adipose tissue *Pparγ* protein levels are increased by both maternal HFD feeding and IUGR throughout life.[81] In our study, maternal HFD feeding

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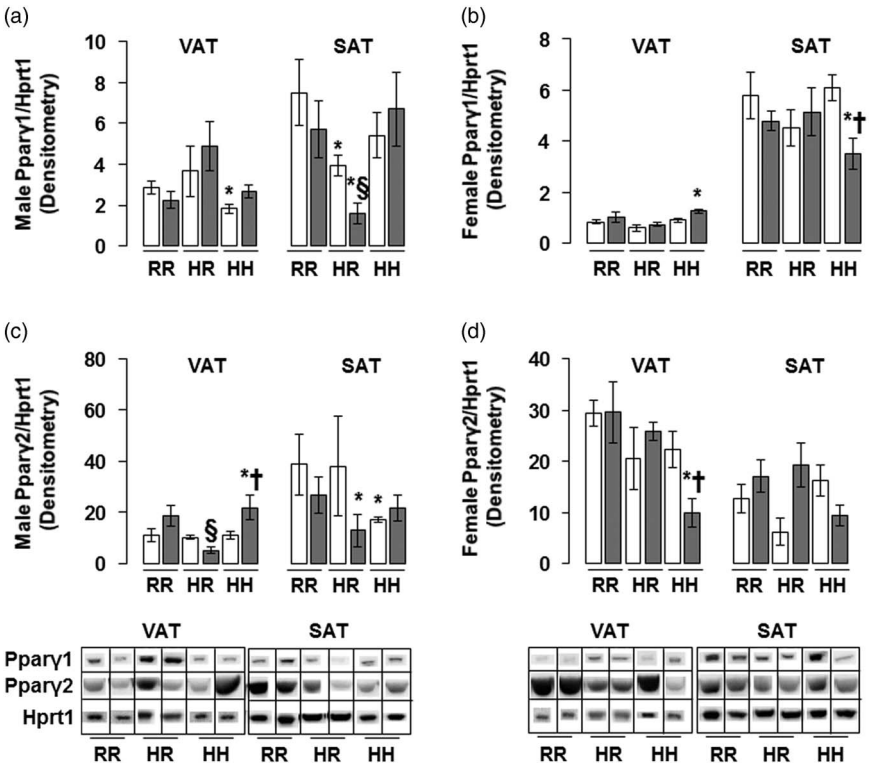

Fig. 6. The greatest peroxisome proliferator-activated receptor gamma 2 (*Pparγ2*) protein was found in the combined *in utero* insult of intrauterine growth restriction (IUGR) and a maternal high-fat diet (HFD) in male visceral adipose tissue (VAT), while the least *Pparγ1* protein was found in the combined *in utero* insult of IUGR and a maternal HFD in female subcutaneous adipose tissue (SAT) and *Pparγ2* protein was found in the combined *in utero* insult of IUGR and a maternal HFD in female VAT. These findings were normalized by weaning to a regular diet. Western blot protein quantification of VAT and SAT *Pparγ1* (*a*,*b*) and *Pparγ2* (*c*,*d*). Due to the large number of samples, western blots were run as follows per sex: western blots with NHH and IHH samples were run on one gel, and NHR and IHR were run on a second gel. NRR and IRR samples were run on both western blots. All samples were first normalized to hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) control protein levels, then controlled for variation in NRR protein levels between blots. Representative western blot images are shown below the graphs, with one representative lane shown per rat group. White bars represent normal non-IUGR (N) rats and gray bars represent IUGR rats (I). Maternal and offspring diets are denoted below the graphs [regular diet rats were weaned to a regular diet (RR), and maternal HFD fed rats were weaned to either a regular diet (HR) or to an HFD (HH)]. A total of six rats from different litters per group were used in these analyses. Statistical significance is denoted as follows: $^{*}P \leqslant 0.05$ for NHR, NHH, IRR, IHR and IHH rats compared with sex-matched NRR rats; $^{\S}P \leqslant 0.05$ for IHR rats compared with NHR sex-matched rats; $^{\dagger}P \leqslant 0.05$ for IHH rats compared with sex-matched NHH rats.

alone and IUGR alone did not increase adipose tissue *Pparγ* protein levels. However, we found that the combination of a maternal HFD and IUGR increased *Pparγ2* protein and visceral adiposity in male rats without increasing serum triglycerides. In males, maternal HFD or IUGR rats may compensate for increased serum fatty acids by appropriately depositing the fat into adipocytes, a physiologic storage depot for circulating fatty acids, thus increasing adipocyte hypertrophy. Increased visceral adipose *Pparγ2* protein in IHH males may have contributed to increased circulating lipid deposition into the visceral adipocytes. Conversely, IHH female rats had increased SAT volume, adipocyte size and serum triglycerides, and decreased visceral and subcutaneous adipose *Pparγ* isoforms. Translational repression of *Pparγ* via administration of microRNA miR-130b in HFD fed obese mice reduced fat deposition, a process suggested to be mediated by increased lipolysis.[82] Taken together, our findings suggest that IHH females have either decreased uptake of serum lipids into this physiologic storage depot or enhanced lipolysis.

Many of the findings of our study were sex-specific. Sex-specific differences in outcomes were also seen with a sex-bias toward female offspring secondary to a maternal HFD intake during pregnancy in our model. Sex-bias during pregnancy can be due to multiple factors including maternal diet, fat source, stress and animal genetics, though the mechanisms through which this occurs remain unclear.[83–88] Often these stressful events result in a greater proportion of female offspring at birth. While we do not have data on maternal stress in our rat model to determine whether stress may have played a role in sex-bias at birth, placing the female rats on an HFD before conception could have contributed to the decreased male:female sex ratio. Long-term metabolic outcomes are also impacted after exposure to a HFD or IUGR. Male mice exposed to a maternal HFD had higher corticosterone levels than male mice exposed to a maternal control diet, while female mice remained unaffected.[89] Further, despite higher corticosterone levels, male mice exposed to a maternal HFD did not have changes in *11-β HSD 1* protein levels.[89] Increased adipose depot volume and adipocyte size often occur with decreased circulating or local production of adiponectin or increased leptin due to leptin resistance.[90,91] Consistent with increased adipose depot volume and adipocyte size, increased leptin protein was found in the visceral and subcutaneous adipose of IHH male rats while decreased circulating adiponectin was found in the IHH female rats. The reason for the differential metabolic effects between male and female rats remain unclear, but may be secondary to differential hormonal regulation of the hypothalamic–pituitary–adrenal axis.[92,93] While we have not examined the potential role of sex-specific neuroendocrine

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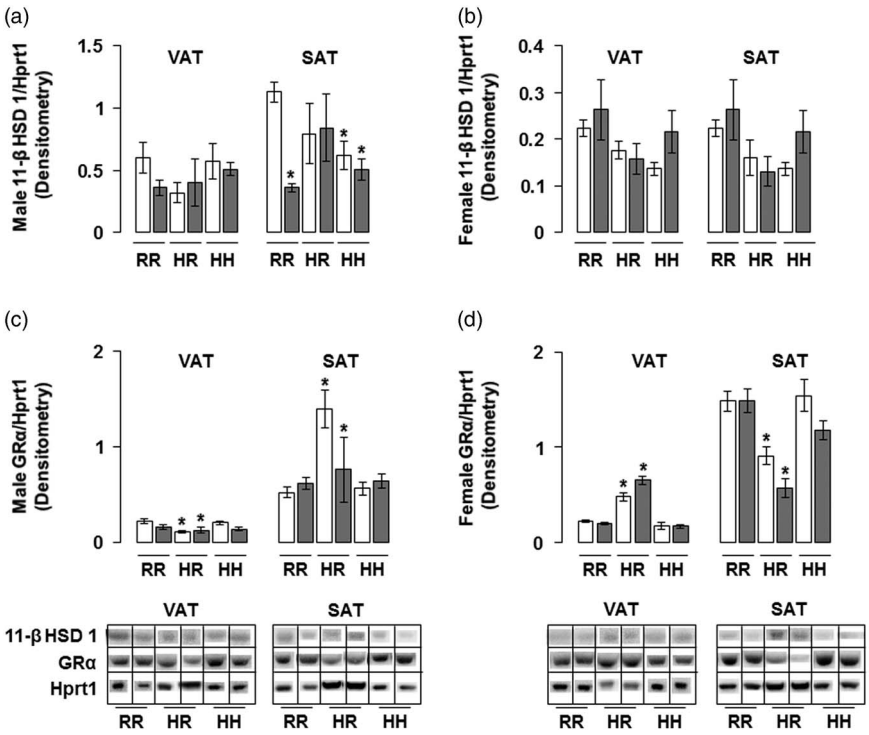

Fig. 7. The combined *in utero* insults of intrauterine growth restriction (IUGR) and a maternal high-fat diet (HFD) did not change 11-β hydroxysteroid dehydrogenase 1 (*11-β HSD 1*) protein levels in any male rat groups in the visceral adipose tissue (VAT) and in any female rat groups in VAT or subcutaneous adipose tissue (SAT) (*a*,*b*). The combined *in utero* insults of IUGR and a maternal HFD did not change active glucocorticoid receptor (*GR-α*) protein levels in male or female rat VAT or SAT (*c*,*d*). Western blot protein quantification of VAT and SAT *11-β HSD 1* in (*a*,*b*) and *GR-α* in (*c*,*d*). Due to the large number of samples, western blots were run as follows per sex: western blots with NHH and IHH samples were run on one gel, and NHR and IHR were run on a second gel. NRR and IRR samples were run on both western blots. All samples were normalized to hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) control protein levels, then controlled for variation in NHR protein levels between blots. Representative western blot images are shown below the graphs, with one representative lane shown per rat group. White bars represent normal non-IUGR (N) rats and gray bars represent IUGR (I) rats. Maternal and offspring diets are denoted below the graphs [regular diet rats were weaned to a regular diet (RR), and maternal HFD fed rats were weaned to either a regular diet (HR) or to an HFD (HH)]. A total of six rats from different litters per group were used in these analyses. Statistical significance is denoted as follows: $^{*}P \leqslant 0.05$ for NHR, NHH, IRR, IHR and IHH rats compared with sex-matched NRR rats. No difference was found between IHR and NHR sex-matched rat data, or between IHH and NHH sex-matched rat data.

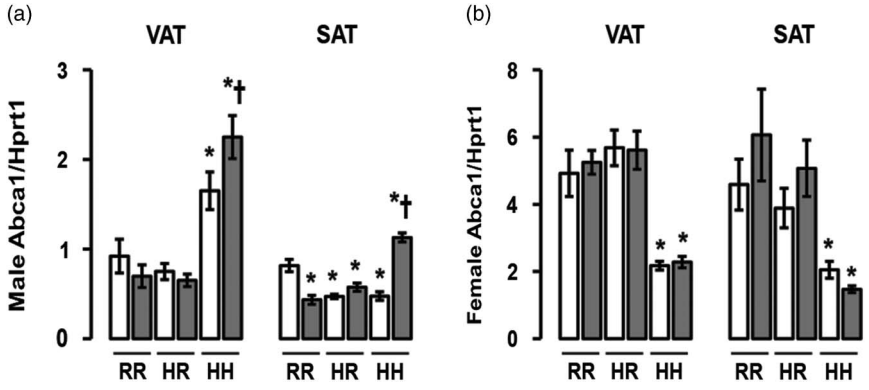

Fig. 8. The greatest ATP-binding cassette sub-family A member 1 (*Abca1*) messenger RNA (mRNA) was found in the combined *in utero* insult of intrauterine growth restriction (IUGR) and a maternal high-fat diet (HFD) in male visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT), and the least *Abca1* mRNA was found in IUGR (I) and non-IUGR (N) rats from a maternal HFD in female VAT and SAT. These findings were normalized by weaning to a regular diet. mRNA quantification of VAT and SAT *Abca1* mRNA (*a*,*b*). White bars represent normal non-IUGR rats and gray bars represent IUGR rats. Maternal and offspring diets are denoted below the graphs [regular diet rats were weaned to a regular diet (RR), and maternal HFD fed rats were weaned to either a regular diet (HR) or to an HFD (HH)]. A total of six rats from different litters per group were used in these analyses. Statistical significance is denoted as follows: $^{*}P \leqslant 0.05$ for NHR, NHH, IRR, IHR and IHH rats compared with sex-matched NRR rats; $^{\dagger}P \leqslant 0.05$ for IHH rats compared with sex-matched NHH rats. No difference was found between IHR and NHR sex-matched rat data.

alteration in our rat model, other authors have shown that increased sex steroids can induce systemic cortisol release.[94]

Differential effects between VAT and SAT were seen in our study, including increased visceral adiposity outpacing that of the subcutaneous adiposity in male rats. VAT remains more strongly correlated with metabolic risk. Increased VAT was more highly correlated with metabolic syndrome than increased SAT in 3001 healthy men and women from the Framingham Heart Study.[95] The mechanism through which visceral adipose excess may confer a greater metabolic risk is not fully defined and likely

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multifactorial, possibly including increased metabolic activity with secretion of vasoactive substances, inflammatory markers, adipocytokines, growth factors and markers of fibrinolysis.[96–102] Further, the protein and mRNA expression profiles between VAT and SAT vary.[103–105] The inherent differences between VAT and SAT and sex-specific responses of these tissues to perinatal insults likely contributed to the increased depot-specific adiposity seen in this rat model.

Several potential limitations of this study must be addressed. First, the HFD rat food contains less protein than the regular diet food. The HFD protein content is sufficient for normal mammalian growth, and was selected to be similar to the typical American diet and to prevent severe decreases in carbohydrate intake in this model.[50,51] Dietary changes in carbohydrate, fat and protein intake have been shown to be associated with persistent metabolic changes in the offspring, therefore we anticipate that the alteration of carbohydrate, fat and protein each contributed to the metabolic changes in our HFD fed rats.[28,106,107] However, despite decreased protein and carbohydrate intake in the dam, offspring from dams fed a HFD weighed the same at birth as offspring from dams fed a regular diet, suggesting that the maternal HFD was sufficient for normal growth. Second, we used an anesthesia control group to generate our non-IUGR rats because sham surgery induces mild growth restriction,[49] therefore we surmise that data obtained from a sham surgery group would be partway between that of the bilateral uterine artery ligation and current anesthesia control. Thus, inclusion of a separate sham surgery group may not further enrich our knowledge of how IUGR and a maternal HFD impact development of obesity. Third, we have not manipulated glucocorticoid signaling in this model to demonstrate a more definitive mechanism through which IUGR and a maternal HFD increase adiposity. Lastly, our data only focuses on one time-point (P60) to measure early adulthood onset of obesity. Studies are underway to determine when adiposity increases in this rat model and if increased adiposity secondary to an adverse in utero environment persists.

In conclusion, the combined *in utero* insults of IUGR and a maternal HFD increased visceral and subcutaneous adiposity and serum corticosterone and triglycerides in adult rat offspring more than either *in utero* insult alone and in a sex-specific manner. Weaning offspring from the combined *in utero* insults to a regular rat chow did not fully normalize the increased visceral adiposity seen in male rats. Our findings suggest that the increased systemic but not locally produced corticosterone in IHH male rats may have increased glucocorticoid signaling, leading to increased visceral and subcutaneous adiposity despite stable caloric intake. Further, increased visceral and subcutaneous adiposity occurred without increasing body weight, making body weight gain a poor indicator of adiposity in our rat model. Implications of this work are two-fold; first, body weight remains a poor indicator of visceral adipose accumulation resulting from perinatal insults, and second, even when consuming a low-fat diet, the impact of an adverse perinatal environment on increased visceral adiposity persists into early adulthood.

Supplementary materials. To view supplementary material for this article, please visit https://doi.org/10.1017/S2040174418000016

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Conflicts of Interest. None.

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