Maternal nutritional history modulates the hepatic IGF-IGFBP axis in adult male rat offspring

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Abstract

Purpose: Alterations in early life nutrition lead to an increased risk of obesity and metabolic syndrome in offspring. We have shown that both relative maternal undernutrition and maternal obesity result in metabolic derangements in offspring, independent of the postnatal dietary environment. Since insulin-like growth factor binding protein 2 (IGFBP2) has been shown to be independently associated with obesity and diabetes risk, we examined the IGF-IGFBP axis in male rat offspring following either maternal undernutrition or maternal obesity to explain possible common pathways in the development of metabolic disorders. **Methods:** Wistar rats were time-mated and fed either a control diet (CONT), 50% of CONT (UN) or a high fat (HF) diet throughout pregnancy. Male offspring were weaned onto a standard chow diet and blood and tissues collected at postnatal day 160. Plasma and hepatic tissue samples were analysed for key players in the IGF-IGFBP system. **Results:** Both maternal UN and HF resulted in increased fat mass, hyperinsulinemia, hyperleptinemia and altered blood lipid profiles in offspring compared to CONT. Circulating IGF-1 and IGFBP3 levels and hepatic mRNA expression of IGFBP1 and IGFBP2 were significantly decreased in UN and HF offspring compared to CONT. DNA methylation of the IGFBP2 promotor region was similar between maternal dietary groups. Although chaperone gene heat-shock protein (HSP)-90 and hepatic IGFBP1 were significantly correlated in CONT offspring this effect was absent in both UN and HF offspring. **Conclusions:** This study is one of the first to directly compare two experimental models of developmental programming representing both ends of the maternal dietary spectrum. Our data suggest that two disparate nutritional models that elicit similar adverse metabolic phenotypes in offspring are characterised by common alterations in the IGF-IGFBP pathway.

Keywords: developmental programming, IGF-IGFBP axis, metabolic syndrome, rat model, gene expression

Introduction

It is well established that alterations in early life nutrition have lifelong consequences for the health and wellbeing of the individual. Clinical and animal studies have shown that both maternal undernutrition and maternal obesity lead to an increased risk of obesity and related metabolic disorders in offspring [1-4]. Despite the similarity in offspring phenotypes elicited by both maternal obesity and undernutrition, there has been limited direct investigation of these models in parallel as regards mechanisms underpinning the observed metabolic dysfunction. In particular, despite marked catch-up growth in both experimental models, little is known about the growth hormone (GH) – insulin-like growth factor (IGF) system in the setting of developmental programming.

The IGF system is integral to normal growth and development; IGF2 is known to regulate fetal and early infant growth, and IGF1 plays a dominant role in growth throughout childhood. Bioavailability of IGF1 is predominantly determined by binding to six IGF binding proteins (IGFBPs 1–6), although recent evidence indicates that IGFBPs also have direct IGF-independent metabolic effects [5]. For example, IGFBP3 has been shown to be involved in regulation of adipogenesis [6], while IGFBP2 is independently associated with obesity and diabetes risk in adults [7,8] and children [9].

IGFBP2 is the second most abundant plasma circulating IGFBP, but its physiological role remains poorly understood. Overall, there appears to be an inverse association between IGFBP2 and adiposity-related insulin resistance, but much is still unknown about the exact nature of this relationship [10]. Mice overexpressing IGFBP2 are protected from high-fat-diet-induced obesity and glucose intolerance and display increased insulin sensitivity [11]. Moreover, hyperinsulinemic clamp studies showed a 3-fold improvement in hepatic insulin sensitivity following IGFBP2 treatment of *ob/ob* mice [12]. In clinical studies, circulating IGFBP2 in adulthood has been shown to be inversely associated with current adiposity [13]

and it has been proposed as a strong marker for the metabolic syndrome in humans [8,14,15]. As IGFBP2 is the major IGFBP expressed in early life, and is also the predominant adipocyte-derived IGFBP, it is ideally positioned to act as a keystone between nutrition, growth and metabolism [16]. In addition to IGFBP2, low plasma levels of IGF-1, particularly when coupled with low levels of the potentially inhibitory IGF binding protein (IGFBP)-1 have been implicated in the pathogenesis of metabolic syndrome and cardiovascular disease [17].

Little is known about alterations in IGF-IGFBP regulation in adult offspring following early life nutritional compromise; previous studies have focussed on the immediate fetal and neonatal period following the nutritional intervention. Fetal cord blood concentrations of glucose, insulin, IGF-1, and IGF-2 are significantly reduced in pregnancies complicated by intrauterine growth restriction (IUGR) [18]. In placentas from males from preterm deliveries, there is an overexpression of both IGF-2 and IGFBP-3 genes [19]. Further, in placentas from pregnancies with IUGR, IGF-2 is also overexpressed reflecting its physiological role in optimizing energy distribution in a low-energy environment [20]. Cord serum concentrations of IGF-2 have been shown to have a significant positive effect on both birth length and weight and cord serum IGFBP-2 concentrations have been shown to correlate positively with weight gain during infancy [21,22]. Site-specific increase in IGFBP-1 phosphorylation may limit IGF-I bioavailability, which in turn could contribute to the development of fetal growth restriction [23] and IGFBP-1 protein in the placenta is negatively correlated with parameters of neonatal size [24]. There is also evidence that polymorphisms in the promoter region of IGFBP3 are associated with insulin-like growth factor binding protein (IGFBP)-3 plasma levels in cord blood and in postnatal samples [25].

The extent to which hepatic endocrine sensitivity can be programmed in utero and whether these adaptations persist into adulthood is largely unknown [26]. In addition, cytokines such as leptin may also play an important role in regulating placental formation and growth through interactions with the IGF-IGFBP axis. Relationships between cytokines and the IGF system have been shown in specific tissues [27] (for example, interleukin (IL)-6 and IGFBP-2 mRNAs in the placenta have been shown to be significantly and positively correlated)[24] but little is known about these associations in the setting of developmental programming.

The present study examined changes in the circulating/hepatic IGF-IGFBP axis in parallel models of maternal undernutrition and maternal obesity to examine whether the common phenotype of obesity and metabolic syndrome is associated with a similar dysregulation of the IGF-IGFBP axis.

Materials and Methods

The maternal high fat (HF) and undernutrition (UN) models have been described by our group previously [1,2,28]. In brief, Wistar rats (age 100±5 days) were time mated using a rat estrous cycle monitor to assess the stage of estrous before introducing the male. After confirmation of mating, rats were housed individually in standard rat cages with free access to water. All rats were kept in the same room with a constant temperature maintained at 25°C and a 12-h light, 12-h dark cycle. Animals were assigned to one of 3 nutritional groups: 1) dams fed a commercially sourced standard diet (5% fat, 18% protein, digestible energy 3.4 kcal/gm, Teklad Global Diet 2018) ad libitum throughout pregnancy and lactation (CONT group), 2) undernourished dams fed 50% of a standard diet throughout pregnancy (UN group) and 3) dams fed a high-fat diet throughout pregnancy and lactation (HF group; 24% fat, 20% protein; digestible energy 4.73 kcal/gm, Research Diets Inc. D12451). A minimum of 8 litters per maternal dietary group were used. Food intake was recorded daily until the end of pregnancy. After birth, pups were weighed and litter size adjusted to eight pups per litter (4 males and 4 females) on postnatal day 2 (P2) to ensure standardised nutrition until weaning at day 22. Plasma and livers were collected from unused male pups at P2. At weaning, all offspring were fed a commercial standard chow diet (Diet 2018) for the remainder of the study. Male offspring only were used for the present study to avoid the potential confounds of estrus dynamics on the IGF-IGFBP system [29]. At 160 days of postnatal age (P160) male offspring were DEXA scanned for body composition (Lunar Prodigy, GE, Waltham, USA), then fasted overnight and killed by injection of pentobarbitone (60 mg/kg, s.c.) anaesthesia followed by decapitation. Blood was collected into heparinised vacutainer tubes and stored on ice until centrifugation and removal of plasma for analysis. Livers were immediately dissected, weighed and snap frozen in liquid nitrogen for later analysis. All animal work was approved by the Animal Ethics Committee at the University of Auckland (Approval R652).

Molecular Analyses

RNA extraction and reverse transcription (RT)

Total RNA was extracted from hepatic tissue samples using a commercially available kit (AllPrep DNA/RNA Mini kit; Qiagen, cat 80204) as per the manufacturer's instructions. Total extracted RNA was put on ice and immediately analysed for purification and quantification or stored at -80°C for future analysis. RNA quantity and purity were analysed using the NanoDrop spectrophotometer (ND-1000 spectrophotometer; BioLab Ltd) using NanoDrop software (version 3.1.2). All samples used had a 260 nm: 230 nm ratio ≥ 1.9. RNA integrity was evaluated using gel electrophoresis. Intact RNA showed two clear bands at the 2000 bp and 5300 bp, indicating the 28S and 18S ribosomal RNA components when visualised under ultraviolet light (set to 365 nm Ethidium Bromide; UVP® BioImaging Systems).

Total RNA (5ug) was used for first strand complementary DNA (cDNA) synthesis using the Moloney Murine Leukaemia Virus Reverse Transcriptase enzyme (M-MLV-RT) (Promega Corp, Wisconsin USA) and a standard thermocycler (GeneAmp® PCR System 9700, Applied Biosystems, California, USA). A master mix was prepared containing the following: 5 µL M-MLV 5x buffer (In vitro Technologies, cat M531A), 0.5 µL M-MLV-RT (In vitro Technologies, cat M170B) and 1.25 µL of 10 µM deoxynucleosides triphosphates (dNTPs) (Global Science, cat R0181) under the following cycling conditions: an initial denaturation stage of 5 minutes at 96ºC, followed by 30 cycles of 30 seconds each of 96°C (denaturation), 60°C (annealing stage) and 72°C (extension stage). cDNA was stored at -20°C for later use in qPCR assays.

Quantitative Polymerase Chain Reaction (qPCR) Assays

For the quantification of hepatic gene expression levels and of the endogenous references rat hypoxanthine-guanine phosphoribosyltransferase (HPRT; mRNA RefSeq, NM_012583.2) and chloride channel nucleotide−sensitive 1A (CLNS1A; mRNA RefSeq, NM_031719.1), and beta actin, a quantitative PCR assay was performed using the ABI PRISMH 7900HT Sequence Detection System (Applied Biosystems; Auckland, New Zealand). HPRT and CLNS1A primers were designed using Primer 3 software (version 0.4.0, Whitehead Institute for Biomedical Research) [30] (Table 1) and manufactured by Invitrogen (Invitrogen Life Technologies; Auckland, New Zealand). Optimal primer conditions were adjusted to the following cycling conditions: Length: 20 bp (range 17–23 bp), Tm: 62°C (range 60–65°C), and amplicon length: 100–200 bp. Dissociation analyses were performed to ensure specificity and samples producing a single peak in the dissociation curves were used. Primer sequences were as follows: HPRT forward primer: TCCCAGCGTCGTGATTAGTG; HPRT reverse primer: CACACAGAGGGCCACAATGT; CLNS1A forward primer: AGTGGGGCAAAAGTGAGGTG; CLNS1A reverse primer: GATGGTAAATGAGGAGGAACAGAAG. Beta Actin and the remaining genes of interest (Hsp90, GHR, IGF–I, IGF–II, IGF–IR, IGF–IIR, IGFBP1 and IGFBP2) were purchased from commercially available sources (Qiagen, Table 1). Quantification of gene expression levels were performed under the following conditions: an initial 2 min hold period at 50ºC for normalisation (Stage 1), followed by enzyme activation at 95°C for 2 min (Stage 2); amplification of the gene product through 40 successive cycles of 95°C for 15 sec then 60°C for 1 min (Stage 3); followed by a dissociation stage of 15 sec at 95°C, 15 sec at 60°C and 2 min at 99°C (Stage 4). A standard curve was generated from the mean cycle threshold (Ct) of eight standards (1:4 serial dilution) of a known concentration in triplicate, while amplification

and dissociation curves were generated for all standards and samples (Applied Biosystems, California, USA). Each sample was run in triplicate.

qPCR normalisation

Ct values for the constitutively expressed housekeeping genes beta-actin, HPRT and CLNS1A were used as normalisation factors for the gene of interest results. Normalisation genes must be chosen specifically for each set of experimental conditions with thorough statistical analyses performed to ensure that there are no discrepancies in normalized expression levels of housekeepers in response to experimental treatment [31]. As a next level of gene expression normalization, the Quant−iT™ Oligreen® ssDNA Reagent and Kit was used (Molecular Probes, The Netherlands; cat. O1149) as an ultrasensitive fluorescent nucleic acid stain for quantifying oligonucleotides and single−stranded DNA (ssDNA) in solution. Samples were analysed in a 96-well microplate with a total reaction of 200uL in each well. Each sample was run in triplicate with 5 µL cDNA sample, 95uL TE and 100 µL of 1:200 diluted (in TE) Oligreen reagent in each well. After a 3 min incubation, fluorescence was read using a fluorescence microplate reader (Excitation/Emission 485/530 nm; BioTek® Synergy 2). Linearity was assessed by a cDNA standard curve. After background subtraction, fluorescence values were normalized in all samples and used in the calculation of overall geometric mean of the housekeeping genes.

DNA methylation

DNA methylation of the IGFBP2 gene was undertaken at the Murdoch Childrens Research Institute (Melbourne, Australia). 500ng of genomic DNA from each sample was bisulfite treated using MethylEasy Exceed kit (Human Genetics Signature, Sydney, Australia) to convert all unmethylated cytosines to uracils, which subsequently become thymines after PCR amplifications. DNA methylation levels of the Igfbp2 gene was measured using the Sequenom EpiTYPER MassArray platform (Sequenom, San Diego, USA) as previously

described [32,33]. Amplicons were designed using EpiDesigner software (http://www.epidesigner.com/) and MassArray R package [34]. Primer and target sequences, along with amplification cleavage product patterns and analyzable CpG units are provided in Figure 1. The IGFBP2 assay was designed to flank upstream region of the transcription start site of the gene. PCR amplification conditions were as follow: 95°C for 10 min; 95°C for 10 s, 56 - 60°C for 30 s, and 72°C for 1 min 30 s for 40 cycles; 72°C for 7 min. PCR amplifications and EpiTYPER reactions were performed in technical triplicates and mean methylation values were used in this analysis.

Plasma assays

Commercially available rat-specific kitsets were used for analysis of plasma insulin (CrystalChem, Downers Grove, USA Catalog #90060), leptin (CrystalChem, Catalog #90040), IGF-1 (Diagnostic Systems Laboratories, Inc; Catalog # DSL-10-29200), IGFBP2 (Mediagnost, Reutlingen, Germany, Catalog# E08), IGFBP3 (Mediagnost, Cat E031) and IGFBP1 (USCN Life Science Inc, Catalog #E90052Ra). Lipid profiles were measured using an autoanalyser (Hitachi 902 Autoanalyser, Roche Diagnostics, IN, USA). IGF and IGFBP related measurements were limited to adult P160 samples due to insufficient plasma volume from P2 offspring. Blood glucose was assayed directly at the time of cull using a Roche Accu-Chek meter.

Statistical analysis

The effect of maternal nutrition on hepatic gene expression was measured using one way analysis of variance (ANOVA) with maternal diet as a factor and where appropriate, post hoc analyses performed (Holm-Sidak) to determine which dietary treatment groups were significantly different. Liver was used as a covariate with each litter representing a single biological replicate. Data that failed to meet the criteria required for parametric analysis

(normal distribution and equal variance) were transformed where necessary. Data are presented as means ± S.E.M. unless otherwise stated and p values of less than 0.05 were considered statistically significant. All statistical analyses were performed using SigmaStat (Systat Software, San Jose, Ca, USA).

Results

Maternal weights

Maternal UN resulted in a significant reduction in gestation weights until approximately day 15 (Figure 2). There was no significant difference in maternal weight gain between the CONT and HF dams (CONT 136±5.3g, HF 142±6.1g). By the end of lactation, maternal weights were similar across all dietary groups. Gestation length (monitored every 3 hours during the daylight period) was increased by approximately 24 hours in the HF dams compared to CONT and UN groups (data not shown).

Offspring weights

Birth weights in male UN and HF offspring were reduced compared to CONT (CONT 6.3 ± 0.7g, UN 5.0 ± 0.3, HF 5.5 ± 0.4, $p<0.005$ and $p<0.01$ for UN and HF versus CONT respectively). Nose-anus length was significantly reduced at birth in UN and HF offspring compared to CONT and further reduced in UN compared to HF offspring (CONT 47.8 ± 0.3mm, UN 44.5 ± 0.3, HF 46.1 ± 0.2, $p<0.05$ for UN and HF versus CONT and $p<0.05$ for UN versus HF). HF offspring showed catch-up growth and were not different in weight to CONT at weaning (Day 22); although UN offspring demonstrated catch-up growth they remained slightly but significantly ($p<0.05$) lighter than CONT animals (CONT 61.6 ± 0.7g, UN 56.5 ± 1.04, HF 64.2± 1.2).

Adult body weights were significantly increased in HF offspring compared to CONT and were similar between CONT and UN groups (Males: CONT 562±8g, UN 594±11, HF 628±18, $p<0.005$ for maternal diet effect). Nose-anus length was significantly increased in

HF offspring compared to CONT and UN groups (CONT 261.8±1.4mm, UN 260.8±1.5, HF 268.7±1.1, $p<0.005$ for maternal HF diet effect).

Body composition

UN and HF adult male offspring had significantly increased total fat mass (%) compared to CONT offspring (Figure 3). Fat mass was further increased in HF offspring compared to UN offspring. Fat:lean ratios were significantly increased in UN and HF offspring compared to CONT and further increased in UN compared to HF offspring (CONT 0.32±0.02, UN 0.55 ± 0.03, HF 0.81 ± 0.07, $p<0.005$). Absolute liver weights were increased in UN and HF offspring compared to CONT (CONT 16.2±0.6g, UN 18.3±0.5, HF 18.1±0.6, $p<0.005$). When corrected for percent body weight, relative liver weights remained higher in UN animals compared to other groups (CONT 2.85±0.05%, UN 3.16±0.09, HF 2.83±0.04, $p<0.005$).

Plasma analysis

P2 Offspring

UN and HF offspring were hypoinsulinemic and hypoleptinemic at birth compared to CONT offspring (Insulin: CONT 2.9±0.6ng/ml, UN 1.3±0.3, HF 1.6±0.2; Leptin: CONT 5.5±1.6ng/ml, UN 0.9±0.2, HF 1.58±0.3; $p<0.05$ for effect of maternal diet).

P160 Offspring

Plasma data for adult offspring are presented in Table 2. IGF-I levels were significantly reduced in UN and HF offspring compared to CONT but were not different between UN and HF offspring. IGFBP3 levels were significantly reduced in UN and HF offspring compared to CONT but were not different between UN and HF offspring. There were no significant differences between any of the groups in fasting plasma IGFBP1, IGFBP2 or glucose

concentrations. Fasting leptin and insulin levels were significantly increased in UN and HF offspring compared to CONT. Plasma LDL, HDL and lipase levels were all significantly increased in UN and HF offspring compared to CONT.

Hepatic Gene expression

P2 offspring

There were no significant differences in hepatic gene expression at P2 with the exception of a reduction in IGFBP1 expression in HF offspring compared to CONT and UN groups (Table 3).

P160 offspring

All data are presented in Table 4 and Figure 4. Hepatic IGFBP2 and IGFBP1 mRNA levels were significantly reduced in UN and HF adult offspring compared to CONT. Hepatic HSP90 mRNA levels were reduced in UN offspring compared to CONT and there was a trend towards a reduction in HF offspring (Table 4). A strong correlation ($R^2 = 0.75$, $p<0.005$) was observed between hepatic HSP90 and IGFBP1 mRNA expression in CONT offspring which was absent in UN and HF offspring ($R^2 = 0.12$ and 0.04 respectively, Figure 5). There was a significant increase in IGF-2R mRNA levels in HF offspring compared to CONT and UN offspring. There was no difference in hepatic mRNA levels of IGF-1, GHR, IGF-2 or IGF-1R between the treatment groups.

IGFBP2 methylation

There were no significant differences in any of the CpGs analysed for IGFBP2 methylation in P2 or P160 offspring livers across the 3 maternal dietary groups (Figure 6).

Discussion

This is the first study to our knowledge to examine potential changes in the IGF-IGFBP axis in adult rat offspring using disparate models of altered maternal nutrition to induce developmental programming. It has been well established that both relative maternal undernutrition and high fat nutrition elicit a phenotype in offspring characterised by increased adiposity and altered insulin and leptin sensitivity [35-37]. In the present study, maternal UN and HF diets resulted in increased adiposity in male offspring concomitant with fasting hyperinsulinemia, hyperleptinemia and alterations in lipid profiles. We have now shown that these offspring also have reductions in circulating IGF-1 and IGFBP3 levels and significant reductions in hepatic IGFBP1 and IGFBP2 mRNA expression levels. Interestingly, the reductions in hepatic IGFBP1 and -2 mRNA expression was not paralleled by any significant changes in circulating plasma IGFBP1-2 or IGFBP2 methylation. Further, the reductions in plasma IGF-1 and IGFBP3 in programmed offspring were independent of changes in body weight/length and may therefore reflect hepatic insulin resistance, fatty liver disease or an altered body composition via increased fat:lean ratios. Both UN and HF offspring had reduced birthweights. Maternal obesity, although often associated with large offspring birthweight and macrosomia, is also characterised by fetal growth restriction, possibly due to placental insufficiency as reported by our group and others [38,39]. Of note, despite reduced birthweights and neonatal hypoleptinemia and hypoinsulinemia in UN and HF offspring, there were no marked differences in neonatal hepatic gene expression compared to controls.

We and others have shown that both maternal undernutrition and maternal obesity are associated with reduced circulating IGF-1 levels in offspring at birth [40,41]. Findings from genetic and nutrition studies suggest that fetal plasma IGF-1, potentially from a nonhepatic

source, correlates with fetal growth and that aberrant hepatic IGF-1 expression may be programmed during the fetal period but may not result in an abnormal phenotype until later adult life. In addition, we observed increased plasma cholesterol (LDL, HDL and total cholesterol) and lipase levels in UN and HF offspring compared to controls – elevations in cholesterol levels commonly parallel increased adiposity and hyperinsulinemia as shown in the current study.

Our observed reductions in IGFBP2 expression in both UN and HF offspring is in agreement with previous studies in mice where a high body weight gain was reflected by reduced hepatic IGFBP2 mRNA levels compared to control mice [42]. IGFBP2 plays a central role in the insulin-IGF system cross-talk and is closely linked to insulin resistance, thereby providing a further explanation for its association with the metabolic syndrome [43]. IGFBP2 has been reported to have marked anti-diabetic effects that are regulated in part by leptin [12] and several studies have shown that IGFBP2 overexpression can reverse diabetes in insulin-resistant *ob/ob*, Ay/a, and diet-induced obese mice, as well as insulin-deficient streptozotocin-treated mice [12]. Moreover, hyperinsulinemic clamp studies showed a 3-fold improvement in hepatic insulin sensitivity following IGFBP2 treatment of *ob/ob* mice [12]. Our data now suggest that the opposite also holds true demonstrating that reduced IGFBP2 mRNA expression is paralleled by impaired insulin sensitivity and increased adiposity in our model. These changes in IGFBP2 mRNA expression were independent of any significant difference in plasma IGFBP2 levels across the maternal dietary groups.

DNA methylation at CpG sites is an important component of epigenetic gene expression regulation, resulting in modulation of protein-DNA interactions [44]. The promoter region of the IGFBP-2 gene is rich in CpGs and lacks a TATA box and is highly conserved in

mammalian species [45]. It is therefore plausible that methylation plays an important role in regulating the expression of this gene. However, despite marked reductions in hepatic IGFBP2 mRNA expression, we observed no significant differences in IGFBP2 DNA methylation, with a generalised hypomethylation of the IGFBP2 promoter across all offspring groups. IGFBP2 hypomethylation has been reported in other model species including the zebrafish [44]. Little is known about the role of IGFBP2 methylation as relates to the metabolic syndrome although aberrant IGFBP2 methylation has been studied widely as relates to carcinomas, particularly hypermethylation in hepatic and lung cancer [46,47]. Our findings of differential expression in the absence of altered DNA methylation suggest an alternative epigenetic mechanism for regulation of the IGFBP2 gene in our rat model.

We observed reduced plasma IGF-1 levels concomitant with hyperleptinemia. The physiological relationship between leptin and IGF-I is poorly understood. Fontan *et al*. suggested that leptin could primarily regulate IGF-I secretion [48] and overexpression of leptin in transgenic mice results in a reduction in circulating IGF-1 levels [49]. However, it has also been shown that growth hormone and IGF-I can modify serum leptin levels, albeit in patients with late stage renal disease [50]. In the setting of malnutrition, IGF-I levels tend towards a decrease, while leptin levels are increased [51]. Of note, it has been reported that hyperleptinemia, low plasma IGF-I or IGFBP-3, and insulin resistance may contribute to the pathogenesis of pre-eclampsia and related fetal growth restriction [52].

Insulin acutely decreases IGF-I bioactivity through differential modulation of IGFBPs [53]. Plasma IGFBP1 levels are normally inversely regulated by insulin and glucose [54]. However, we observed no changes in fasting plasma IGFBP1 across any of the treatment groups despite marked hyperinsulinemia in UN and HF offspring with no significant

correlation between plasma insulin and plasma IGFBP1. A lack of an inverse relationship between insulin and IGFBP1 has been reported by others [55] and may relate in part to the dynamic regulation of IGFBP1 in the circulation. However, we observed marked reductions in hepatic IGFBP1 mRNA expression in UN and HF adult offspring. Hepatic production of IGFBP1 is under direct inhibitory influence of insulin and increased insulin secretion results in suppression of hepatic IGFBP1 secretion. This is in agreement with our observations of hyperinsulinemia coupled with markedly reduced hepatic IGFBP1 mRNA expression.

We report a significant positive association between hepatic HSP90 and IGFBP1 expression that was absent in UN and HF groups. HSP90 is a molecular chaperone that assists the folding or unfolding and the assembly or disassembly of other proteins and plays a well-established role in signaling protein function, trafficking and turnover [56]. Inhibition of HSP90 has been shown to induce cellular stress and pro-apoptotic pathways [57]. As such it has been proposed that stable cycling of HSP90 may serve to buffer against protein damage and therefore decreased HSP90 expression would likely lead to diminished protein functionality [56]. There are few *in vivo* data on the interaction between HSP90 and the other IGF-IGFBP gene targets apart from limited reports in the cancer literature or performed *in vitro* [58,59]. IGFBP-2 synthesis is governed by IGF receptor → phosphatidylinositol 3-kinase → Akt pathway, which is supervised by HSP90 and HSP90 inhibitors can dose-dependently decrease secretion of IGFBP-2 *in vitro* [60]. HSP90-dependent degradation of targets upstream of IGFBP-2 has therefore been proposed to result in down-regulation of IGFBP-2 in serum [60]. Blocking HSP90 may also disrupt IGF-I and IL-6-induced signaling cascades by targeting IGF-IR and STAT3, leading to significant growth-inhibitory effects [61]. It has also been shown that HSP90 interacting with IRS-2 is involved in cAMP-dependent potentiation of IGF-I signals *in vitro* [59].

One potential mechanism of IGFBP action is via altered cell-cycle dynamics. An appropriate level of IGFBP1expression is critical for proper control of the hepatocyte cell cycle [62]. We have previously reported that offspring of mothers fed a HF diet display hepatic cell cycle inhibition and associated changes in Cdkn1a (p21, cell cycle inhibitor) gene expression and DNA methylation [63]. Although cell cycle dynamics were not tested directly in this study, our previous observations of aberrant cell cycle regulation coupled with the present observation of reduced hepatic IGFBP1 expression at P2, points toward a possible mechanism for long term liver dysfunction. However, this would not account for the changes observed in UN adult offspring where although similar dysregulation of cell cycle control has been reported in a model of maternal undernutrition [64], we observed no significant changes in IGFBP1 expression in P2 livers.

Work by Zhang *et al.* has recently shown that a maternal HF diet during pregnancy and lactation altered hepatic expression of IGF-2 in adult offspring [65]. We observed no differences in hepatic IGF-2 mRNA expression in nutritionally challenged offspring at any time. These inconsistencies could relate to sex- or species-specific differences in the studies; the Zhang study utilised female mice whereas we examined male offspring to remove the potential confounds of estrous phases. Further, and in contrast to our study, in female mice offspring there was no effect of the maternal HF diet on postnatal growth and lipid profiles. We observed no changes in hepatic IGF-1 mRNA expression at birth in either UN or HF offspring, despite a reduction in birth weight compared to controls. Reduced hepatic IGF-1 mRNA expression has been reported in the late gestation fetus in the uterine ligation model [66] and these differences from the present study may simply reflect the nature of the insult or timing of sample collection (late gestation versus neonatal). Limited sample volume

precluded us from assessing circulating IGF-1 levels in neonates in the present study but we have previously reported reduced IGF-1 levels at birth in a maternal undernutrition model of growth restriction [67]. Circulating plasma IGF-1 levels were reduced in UN and HF adults compared to controls; this may be reflective of increased adiposity in these groups and the consequence of GH-resistance associated with obesity.

Both maternal undernutrition and nutritional excess resulted in obesity and metabolic dysregulation in adulthood. These data closely mimic data from clinical studies and epidemiological cohorts where both maternal under- and overnutrition result in metabolic disorders in offspring [35,36,68-70]. Although a significant increase in absolute body weight was observed only for HF offspring compared to controls, the increased adiposity observed in UN and HF offspring was paralleled by a significant increase in fat:lean ratios. This common phenotype was also characterised by marked suppression in hepatic IGFBP mRNA expression levels and is paralleled by decreased circulating levels of IGF-1 and IGFBP3. This dysregulation, particularly for IGFBPs 1 and 2, may be mediated in part by alterations in the molecular chaperone HSP90. Given the recent data on the beneficial consequences of IGFBP2 overexpression and reversal of metabolic dysfunction, the present data shows that the reverse holds true with reduced expression of IGFBP2 associated with increased adiposity and impaired insulin sensitivity. Previous work on IGFBP1 is less clear; overexpression of IGFBP1 has been shown to result in inhibition of IGF action and in profound impairment of brain development, modest inhibition of fetal and postnatal growth, and inhibition of the metabolic effects of the IGFs [71]. The observed lack of a relationship between hepatic mRNA expression for IGFBPs 1-2 and circulating plasma levels may be a consequence of other tissues contributing to the circulating IGFBP pool. For example, IGFBP2 is an adipokine secreted by white adipocytes and its regulation is known to be altered in an adipose

depot-specific manner in diet-induced obesity and insulin resistance [72]. Furthermore, both maternal under- and overnutrition are known to be associated with non-alcoholic fatty liver disease in offspring [73-75]. Circulating IGF-1 levels were reduced in UN and HF offspring compared to controls. NAFLD is known to lead to a reduction in IGF-1 and IGFBP3 with hepatic insulin resistance affecting IGF-1 levels by modulating GH-stimulated synthesis of hepatic IGF-1 [76,77]. Thus hepatic lipid accumulation as shown by us and others in these models of altered maternal nutrition may represent one mechanism underpinning the aberrant IGF-IGFBP axis.

Although not examined in the present study, animal studies suggest dysregulation of IGFBP4 is significant in the development of IUGR, although human data are lacking. It has been suggested that IGFBP4 is expressed at the maternal fetal interface and plays a role in regulating IGF bioavailability. Thus, maternal serum levels of IGFBP4 may be associated with complications of abnormal placental growth and development including IUGR [78]. In addition, obesity is characterised by a chronic state of low grade inflammation and inflammation is an important modulator of the IGF/IGFBP system leading to an overall reduction in IGF bioavailability [79]. Work by Street *et al*. showed that increases in serum concentrations of inflammatory markers in the setting of inflammatory disease were paralleled by a reduction in serum concentrations of IGF-I and IGF-II and a significant increase in insulin levels [79,80]. Future work will examine the relationship between the IGF-IGFBP system and inflammatory profiles and its association with growth patterns. We have recently published re increased inflammatory markers in offspring following either maternal undernutrition or maternal obesity [81-83]. We would therefore expect the relationship between active inflammation and modification of the IGF-IGFBP system as reported by

Street *et al*. to hold true in the setting of developmentally programmed obesity and metabolic disorders.

In summary, both maternal undernutrition and overnutrition result in obesity in adult offspring concomitant with hyperinsulinemia and hyperleptinemia. The present observations are the first to show dysregulation of a common pathway underpinning a compromised metabolic and obesogenic phenotype utilising two disparate models of altered maternal nutrition. Importantly, the data derived from our animal model closely parallel that reported in the human literature for offspring born SGA including reduced circulating plasma IGF-1 and IGFBP3 levels [84-87] and hyperinsulinemia and hyperleptinemia in early life following catch-up growth [88,89]. However, direct comparisons in many cases are difficult due to clinical intervention strategies such as GH treatment which directly impact on insulin sensitivity and components of the IGF-IGFBP axis. Further, in the present study we cannot conclude that the mechanisms leading to the dysregulation of the pathways are the same, rather the resultant changes in the IGF-IGFBP axis manifest via either under- or overnutrition are similar. However, importantly, these data further reinforce a role for IGFBP2 as a possible intervention target to ameliorate the consequences of early life nutritional compromise.

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Conflict of interest statement

The authors report no competing interests.

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Gene	Gene Symbol	Assay Name	Cat. Number
Beta Actin	ACTB	Rn_Actb_1_SG	QT00193473
HSP 90	Hsp90	Rn-Hsp90aa1_1_SG	QT00196483
GHR	GHR	Rn_Ghr_1_SG	QT00176316
IGF-1	IGF1	Rn_Igf1_2_SG	QT01745373
IGF-2	IGF2	Rn_Igf2_1_SG	QT00195594
IGF-1R	IGF1R	Rn_Igf1r_1_SG	QT00178416
IGF-2R	IGF2R	Rn_Igf2r_1_SG	QT00175490
IGFBP1	IGFBP1	Rn_Igfbp1_1_SG	QT00190841
IGFBP2	IGFBP2	Rn_Igfbp2_2_SG	QT02353113

Table 1.

Summary of Qiagen-sourced primers. HSP = heat shock protein; GHR = growth hormone receptor; IGF = insulin-like growth factor; IGFBP = insulin-like growth factor binding protein; R = receptor subtype.

Group	Con	UN	HF
Glucose (mmol/l)	5.56 ± 0.15	5.67 ± 0.36	5.72 ± 0.19
Insulin (ng/ml)	2.05 ± 0.2	3.02 ± 0.4*	4.68 ± 0.6*
Leptin (ng/ml)	10.8 ± 1.1	16.3 ± 1.8*	22.4 ± 2.4*
IGF-1 (ng/ml)	2187 ± 121	1605 ± 83*	1685 ± 168*
IGFBP1 (ng/ml)	2.69 ± 0.21	2.83 ± 0.22	2.91 ± 0.33
IGFBP2 (ng/ml)	24.4 ± 2.1	19.2 ± 1.6	20.8 ± 1.7
IGFBP3 (ng/ml)	407 ± 16.4	298 ± 20.1*	331 ± 17.9*
LDL	0.28 ± 0.02	0.45 ± 0.04*	0.39 ± 0.02*
HDL	1.06 ± 0.07	1.56±0.11*	1.29 ± 0.0*
Total cholesterol	1.88 ± 0.09	2.50 ± 0.11	2.28 ± 0.13
Lipase	9.9 ± 0.56	13.3 ± 4.4*	14.2 ± 1.1*

Table 2.

Plasma analysis in adult males at P160 for leptin, insulin, glucose, cholesterol and components of the IGF-IGFBP pathway. Data are means ± SEM; n=10 per group. * $p<0.05$ versus CONT. IGF = insulin-like growth factor; IGFBP = insulin-like growth factor binding protein; LDL = low density lipoprotein; HDL = high density lipoprotein.

Group	IGFBP1	IGF-1	GHR	HSP90	IGF-2	IGF1R	IGF2R
CONT	100 ± 6.3	100 ±6.5	100 ± 0.5	100 ±3.5	100 ±2.5	100 ± 6.5	100 ± 7.7
UN	118 ± 13.6	91 ± 8.7	102.5 ± 5.0	92 ± 10.0	117 ± 9.3	89 ± 7.2	99 ± 14.0
HF	71 ± 8.5*	89 ± 15.2	82.5 ± 5.0	97 ± 11.5	101 ± 18.3	61 ± 9.0	60 ± 14.7

Table 3.

Hepatic gene expression (expressed relative to CONT) in male offspring at postnatal day 2 (P2). Data are means ± SEM, n=10 per group. * $p<0.05$ for UN versus HF. HSP = heat shock protein; GHR = growth hormone receptor; IGF = insulin-like growth factor; IGFBP = insulin-like growth factor binding protein; R = receptor subtype.

Group	IGF-1	GHR	HSP90	IGF-2	IGF1R	IGF2R
CONT	100±12.7	100±7.9	100±7.9	100±28.9	100±23.4	100±25
UN	110±12.6	95±9.1	61±7.0*	78±13.9	77±4.9	66±10.6
HF	124±19.5	102±11	76±9.6	125±25.3	129±16.8	151±13.9**

Table 4.

Hepatic gene expression in adult male offspring at postnatal day 160 (P160). Data are means ± SEM, n=10 per group. * $p<0.05$ versus CONT, ** $p<0.05$ for HF versus UN. HSP = heat shock protein; GHR = growth hormone receptor; IGF = insulin-like growth factor; IGFBP = insulin-like growth factor binding protein; R = receptor subtype.

Figure legends

Figure 1. Primer design utilised for IGFBP2 DNA methylation analysis on the Sequenom mass array. Amplicons were designed using EpiDesigner software (http://www.epidesigner.com/) and MassArray R package.

Figure 2.

Maternal body weights during gestation and lactation. Data are means ± SEM, n=8 per group.

Figure 3.

Total body fat mass (percent) in offspring at postnatal day 160 as quantified by DEXA scan. Data are means ± SEM, n=10 per group. * $p<0.05$ for comparison between groups.

Figure 4.

(a) Hepatic IGFBP1 gene expression in offspring at postnatal day 160. Data are means ± SEM, n=10 per group. * $p<0.05$ versus CONT.

(b) Hepatic IGFBP2 gene expression in offspring at postnatal day 160. Data are means ± SEM, n=10 per group. * $p<0.05$ versus CONT.

Figure 5.

Regression analysis of hepatic IGFBP1 versus hepatic HSP90 expression. CONT: $R^2=0.75$, $p<0.005$; UN: $R^2 = 0.12$, $p=0.33$, HF: $R^2 = 0.04$, $p=0.61$. N=10 per group.

Figure 6.

Pattern of hepatic IGFBP2 DNA methylation in livers from P2 offspring (top) and adult offspring at P160 (bottom) as quantified by Sequenom MassArray platform. Data are means ± SEM, n = 10 per group.

Igfbp2

Forward Primer
(aggaagagag)GGAGTAAAGTTTTGGTTAGGGAAGA
Reverse Primer
(cagtaatacgactcactatagggagaaggct)CCAACAACAACAACAAAAACAAC
Amplicon sequence
GGAGCAAAGTCCTGGCTAGGGAAGAGTAGGGGGACCCGCCGAATCCCCTAGTAACGTAGGGTGGGCAAGTGGGCGTGTGCG
CAAGCACGTGCTCTCACGCAGGAGTGCCTTGGGGGGGAAGGGAGTGGTCTCCAAAAGGGGGAGGGGAGAAGGCAGGGGGG
GCGGGGAGAAGCAGGCTTTTTAGGACCCGGCAGCAGCGGGGGAGGAGAAAGAAGGCAAGGAGGCGTCTCCCGCGCTCGCCA
GGGCCGTGCCACCTGCCCGCTAGCTCGCCGCGCTACGGTTTCCCACTAGCCAACATGCTGCCGAGATTGGGCGGCCCCGCGCT
GCCGCTGCTCCTGCCGTCGCTGCTCTTGCTGCTGCTGTTGG
EpiTYPER cleavage pattern

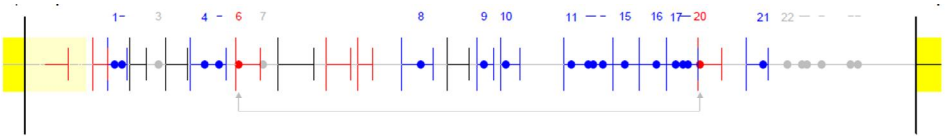


Figure 1.

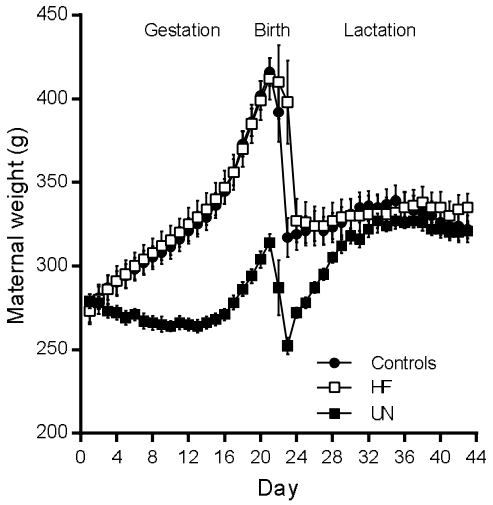

Gestation
Birth
Lactation
Maternal weight (g)
450
400
350
300
250
200
0
4
8
12
16
20
24
28
32
36
40
44
Day
Controls
HF
UN

Figure 2.

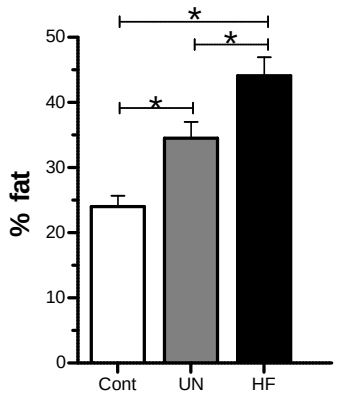


Figure 3.

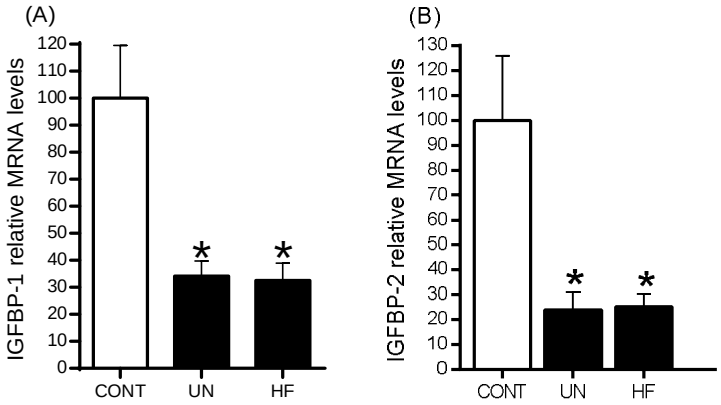

(A)
IGFBP-1 relative MRNA levels
120
110
100
90
80
70
60
50
40
30
20
10
0
CONT
UN
HF
*
*
(B)
IGFBP-2 relative MRNA levels
130
120
110
100
90
80
70
60
50
40
30
20
10
0
CONT
UN
HF
*
*

Figure 4.

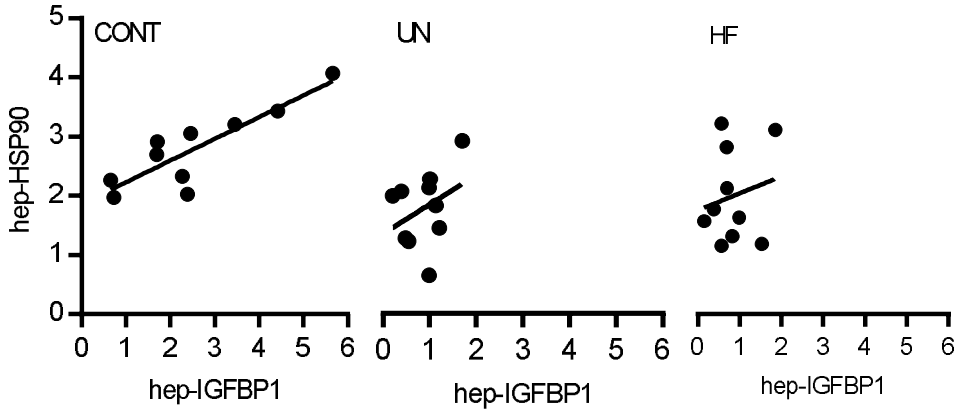
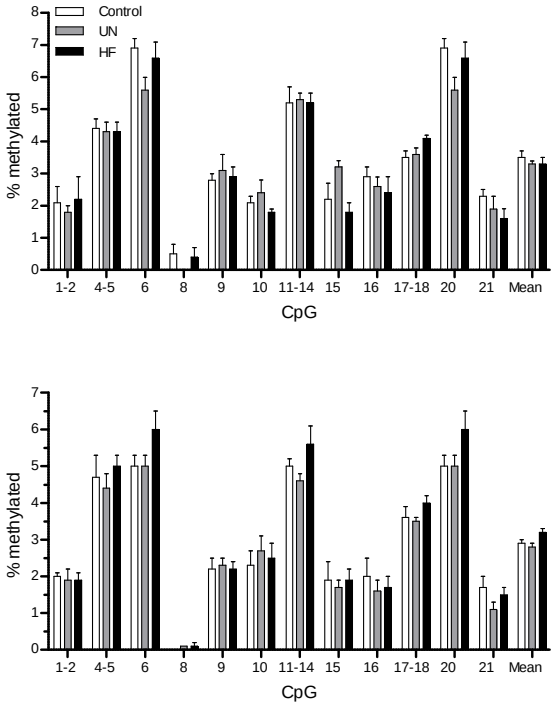

CONT
UN
HF
hep-HSP90
hep-IGFBP1
hep-IGFBP1
hep-IGFBP1
0
1
2
3
4
5
6

Figure 5.


Control
UN
HF
% methylated
0
1
2
3
4
5
6
7
8
1-2
4-5
6
8
9
10
11-14
15
16
17-18
20
21
Mean
CpG

% methylated
0
1
2
3
4
5
6
7
1-2
4-5
6
8
9
10
11-14
15
16
17-18
20
21
Mean
CpG

Figure 6.