PROTEIN RESTORATION IN LOW BIRTH WEIGHT RAT OFFSPRING DERIVED FROM MATERNAL LOW PROTEIN DIET LEADS TO ELEVATED HEPATIC CYP3A AND CYP2C11 ACTIVITY IN ADULTHOOD

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LOW BIRTH WEIGHT RATS HAVE ELEVATED HEPATIC DRUG METABOLISM

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Abbreviations: 11-HSD1, 11-hydroxysteroid dehydrogenase-1; CAR, constitutive androstane receptor; CVD, cardiovascular disease; cDNA, complementary DNA; P450, cytochrome p450; ER, endoplasmic reticulum; IUGR, intrauterine growth restriction; LP, low protein; MPR, maternal protein restriction; PXR, pregnane x receptor, RT-PCR, reverse transcription-polymerase chain reaction; UPLC-PDA, ultraperformance liquid chromatography with photodiode array detection
Abstract

The World Health Organization has identified hypercholesterolemia to be one of the major symptoms encompassing the metabolic syndrome. Moreover, epidemiological evidence indicates that low birth weight offspring are at greater risk of developing the metabolic syndrome. Previous work in our laboratory demonstrated that maternal protein restriction (MPR) results in impaired fetal growth and hypercholesterolemia in adulthood. This was attributed to repression of hepatic CYP7A1, a rate limiting enzyme that catabolizes cholesterol to bile acids. Another important function of hepatic cytochrome P450 enzymes is the phase I oxidative metabolism of drugs (i.e. statins for hypercholesterolemia), which can significantly impact pharmacokinetics. We hypothesized that MPR offspring may have altered ability to metabolize drugs in adulthood. To address this hypothesis, Wistar rats were maintained on a 20% protein diet (Control) or a low 8% protein diet throughout prenatal and postnatal life (LP1) or exclusively during prenatal life and weaning (LP2). Intriguingly CYP3A and CYP2C11 intrinsic clearance ($V_{\text{max}}/K_m$) was significantly increased exclusively in LP2 offspring at postnatal day 130 compared to control or LP1 offspring, as evaluated by testosterone enzyme kinetics in liver microsomes. The increase in activity was secondary to an increase in CYP3A23 and CYP2C11 mRNA. Collectively, these findings suggest that a low birth weight offspring followed by postnatal catch-up growth may have a diminished response to xenobiotics metabolized by CYP3A and CYP2C11 enzymes.
Introduction

Clinical studies have reported a strong inverse correlation between birth weight and metabolic risk factors associated with cardiovascular disease (CVD) (Barker, et al., 1989; Nilsson, et al., 1997; Curhan, et al., 1996; Curhan, et al., 1996; Leon, et al., 1996). Therefore, the likelihood of prescribing medication for the management of these metabolic symptoms (i.e. statins for hypercholesterolemia) can be considered to be greater in these offspring. This may be particularly relevant in cases of low birth weight offspring, which undergo nutrition-induced accelerated growth in neonatal life and display an earlier onset of these symptoms (Straka, et al., 1990; Crowther, et al., 1998; Yajnik, 2000; Eriksson, 2011; Finken, et al., 2006; Martin, et al., 2003). The underlying reason behind this phenomenon can be explained by the “Predictive Adaptive Response” hypothesis, which suggests that adverse events during development induce adaptations suited for survival in a similar predictive environment but can become maladaptive if a ‘mismatch’ to the predictive environment occurs, leading to a “thrifty phenotype” (Hales and Barker, 2001; Hales and Barker, 1992; Rickard and Lummaa, 2007). Clinically, this hypothesis has been supported by evidence where accelerated growth due to higher nutrient exposure in preterm infants results in an increase in markers of the metabolic syndrome by adolescence (Singhal, et al., 2001; Singhal, et al., 2002; Singhal, et al., 2003; Singhal and Lucas, 2004; Singhal, et al., 2004). However, very little is known about drug disposition in adult life of low birth offspring. Moreover, the role of accelerated growth as result of a nutrition mismatch in postnatal life has not been examined.
Several clinical and animal studies have observed liver dysfunction in postnatal life of low birth weight infants (Boehm, et al., 1990; Burns, et al., 1997; Ozanne, et al., 1996; Rees, et al., 2000; Lillycrop, et al., 2008). Given that approximately 75% of prescribed drugs are metabolized in the liver (Wienkers and Heath, 2005), liver dysfunction may alter the pharmacokinetic parameters of several drugs in the postnatal life of these infants. Amongst the prescribed drugs eliminated by the liver, three fourths of these are metabolized by the cytochrome P450 (P450) family of enzymes (Wienkers and Heath, 2005). In humans, reduced intravenous midazolam clearance, a measure of hepatic CYP3A4 activity, has been observed in preterm infants aged between 2 to 15 days (Lee, et al., 1999; Thummel and Wilkinson, 1998; Thummel, et al., 1996). Interestingly, the CYP3A4 isozyme is responsible for the metabolism of approximately half of all marketed drugs (Wrighton, et al., 1996). Yet, it remains to be investigated whether pharmacokinetics of drugs in the liver are altered in adult life of low birth weight offspring.

Animal models make it feasible to investigate alterations in pharmacokinetics of a drug to changes in hepatic P450 enzyme activity. The long-term programming of Cyp3a activity was recently demonstrated by Tajima et al. in mice offspring born of mothers receiving a high fat diet during pregnancy (Tajima, et al., 2012). Specifically, they correlated a decrease in hepatic Cyp3a activity in six week old offspring with reduced triazolam substrate clearance (Tajima, et al., 2012). Although an important finding, it remains to be established whether similar long-term programming of P450 enzyme activities occur when in utero insults result in low birth weight offspring. In rodent models, low birth
weight offspring undergoing accelerated growth rates due to a postnatal nutrition mismatch display hypertension (Boubred, et al., 2009), obesity (Desai, et al., 2007), hypercholesterolemia (Nusken, et al., 2008), insulin resistance (Desai, et al., 2007) and reduced longevity (Jennings, et al., 1999). Therefore, it would be imperative to also determine the adverse consequences of altering the postnatal nutrition environment of low birth weight offspring on long-term programming of hepatic drug metabolizing P450 enzymes.

Maternal protein restriction (MPR) dietary regime is a relevant animal model to study the developmental origins of adult diseases, since MPR shares features common with placental insufficiency induced intrauterine growth restriction (PI-IUGR), which occurs in 8% of pregnancies and produces protein deficiency in the developing fetus (Crosby, 1991; Lamarche, et al., 1998; Ross and Beall, 2008). We and others have previously demonstrated that MPR (8% protein) during pregnancy and lactation in wistar rats leads to asymmetric IUGR offspring (Desai and Hales, 1997, Sohi, et al., 2011), which exhibit impaired liver function in adulthood (Burns, et al., 1997; Ozanne, et al., 1996; Rees, et al., 2000; Lillycrop, et al., 2005). In addition, we have observed that these offspring display high circulating and hepatic cholesterol levels exclusively when they are faced with a nutritional mismatch of a normal (20%) protein diet post-weaning. This was attributed to the long-term repression of cholesterol 7α-hydroxylase (CYP7A1), which is rate-limiting for the catabolism of cholesterol to bile acids (Sohi, et al., 2011). It is noteworthy that decreased CYP7A1 expression is also associated with Endoplasmic Reticulum (ER) stress in a rat model of hypothyroidism (Zhou, et al., 2009). This is of
great interest considering that we have recently demonstrated that these MPR offspring exhibit elevated markers of ER stress (Sohi, et al., 2013). Specifically, higher steady-state levels of phosphorylated eukaryotic initiation factor (eIF)-2α at Serine 51 and glucose regulated protein Grp78 in the livers were noted at four months of age (Sohi, et al., 2013). In contrast, MPR derived offspring that were maintained on a low protein diet throughout life did not display hypercholesterolemia or hepatic ER stress. As P450 enzymes (i.e. CYP3A23, CYP3A2 and CYP2C11) reside in the ER of the liver and are involved with Phase 1 of drug metabolism (Avadhani, et al., 2011), we hypothesized that MPR derived low birth offspring may have an impaired activity of P450 drug-metabolizing enzymes in adulthood when faced with a nutritional mismatch of a normal (20%) protein diet in postnatal life. To address this, the effects of a low protein diet throughout life (LP1) or until lactation (LP2) were compared relative to a normal protein diet throughout life of the offspring (C).
Materials and Methods

Animals and Dietary Regimes

All procedures were performed in accordance with the guidelines set by the Canadian Council of Animal Care and upon approval of the Animal Care Committee of the University of Western Ontario. Female and male Wistar rats at breeding age (250 g) were purchased from Charles River (La Salle, St.Constant, Quebec, Canada). These rats were housed in individual cages and maintained at room temperature on a 12-h light, 12-h dark cycle. For 3 weeks, these rats were left to acclimatize to the animal care facility and their reproductive cycles were followed. At the onset of proestrus, these rats were mated. Impregnation was confirmed by the presence of sperm in the vaginal smear the next morning. Upon confirmation of impregnation (gestation day 1), the rats were fed either a control diet containing 20% protein or a LP diet containing 8% protein. The LP diet contained similar fat content and was made isocaloric by a 14% increase in carbohydrates (Bio-Serv, Frenchtown, NJ, USA). At birth, the litter size was reduced to eight animals (four females and four males), with weights closest to the litter mean. This ensured a standard litter size for all mothers. Three different dietary regimes were administered to these offspring. Offspring derived from a maternal LP diet were either administered the LP diet throughout postnatal life (LP1) or until the end of weaning (LP2). The LP1 and LP2 offspring were compared to the Control offspring which received a control diet throughout prenatal and postnatal life. Food and water was provided ad libitum. The food intake of these offspring were monitored by measuring their food consumption every third day, and have been previously published (Sohi, et al., 2011).
At postnatal day 21 (d21), a subset of the offspring were sacrificed and the medial lobe liver tissue were excised and snap frozen for quantitative RT-PCR. At postnatal day 130 (d130), the pups were also sacrificed and the medial lobe liver tissue was excised and frozen for quantitative RT-PCR and for testosterone enzyme kinetics in liver microsomes via ultra performance liquid chromatography. We did not examine the female offspring to prevent confounding factors related to their estrous cycle and their hormonal profile. Moreover, the maternal low protein model has been demonstrated to exhibit early life programming effects in a sexually dimorphic manner, which was not the focus of this study (Sohi, et al., 2011; Chamson-Reig, et al., 2009; Guan, et al., 2005)

**Real Time PCR Analysis**

Total RNA from Wistar rat medial lobe liver tissue was extracted at d21 and d130 by the one-step method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987) (TRIzol, Invitrogen, Carlsbad, CA, USA). RNA was treated with deoxyribonuclease to remove any contaminating DNA. 4 µg of the total RNA was reverse transcribed to cDNA using random primers and Superscript II RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Primer sets directed against rat CYP2B1, CYP3A23, CYP3A2, CYP2C11, Car, Pxr, and β-actin were generated via Primer Express software (PE Applied Biosystems, Boston, MA, USA) based on published sequences (Table 1). The relative abundance of each transcript was determined by real-time quantitative PCR as previously published (Hardy, et al., 2006). For the quantitative analysis of mRNA expression, the Bio-Rad CFX384 Real Time System was employed using the DNA binding dye IQTM SYBER green supermix (Bio-Rad, Mississauga, Ontario, Canada). The cycling
conditions were 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min. The cycle threshold was set at a level where the exponential increase in PCR amplification was approximately parallel between all samples. All primer sets produced amplicons of the expected size and sequence. We calculated the relative fold changes using the comparative cycle times (Ct) method with β-actin as the reference guide. Over a wide range of known cDNA concentrations, all primer sets were demonstrated to have good linear correlation (slope=-3.4) and equal priming efficiency for the different dilutions compared with their Ct values (data not shown). Melt curve analysis was conducted at the end of the PCR reaction to ensure a single peak for all primers. Moreover, all primer sets were validated by amplifying cDNA followed by running the product on an agarose gel to confirm a single band at the expected amplicon size. Given that all primer sets had equal priming efficiency, the ΔCt values (primer internal control) for each primer set were calibrated to the experimental samples with the lowest transcript abundance (highest Ct value), and the relative abundance of each primer set compared with calibrator was determined by the formula, $2^{-\Delta\Delta Ct}$, in which $\Delta\Delta Ct$ is the calibrated Ct value.

**Hepatic Microsome Isolation**

Wistar rat liver microsomes were isolated by differential centrifugation using methods described previously by Velenosi et al. (Velenosi, et al., 2012). Briefly, 0.9% NaCl solution was used to rinse liver tissue. The rinsed tissue was homogenized in 1.15% KCl solution containing 1 mM EDTA and was centrifuged at 9000g for 20 min at 4°C. The subsequent supernatant was centrifuged at 105,000g for 60 min at 4°C. The microsomal
pellet was resuspended in 100 mM potassium phosphate buffer containing 20% glycerol at pH 7.4 and protein concentration was determined by colorimetric BCA Protein Assay (Pierce Corp., Madison, WI, USA). Microsomal protein extract was stored at -80°C for further analysis.

**Hepatic Metabolism of Testosterone by CYP3A and CYP2C11 Enzymes**

Metabolic activity of CYP3A and CYP2C11 in hepatic microsomes was determined using methods previously described by Velenosi et al. (2012) (Velenosi, et al., 2012). Testosterone was selected as a probe for CYP3A and CYP2C11 enzyme activities based on previously documented selective metabolism by specific rat P450 isozymes (Velenosi, et al., 2012; Souidi, et al., 2005). 50 mM potassium phosphate buffer and 2 mM MgCl₂ (pH 7.4) with 1 mg/ml hepatic microsomal protein equating to a final volume of 250μl was used for timed enzymatic reactions. Linear rate of production of metabolites was determined by varying time, protein and relevant substrate concentrations prior to conducting enzymatic reactions. Formation of testosterone metabolites (6β-OH testosterone and 16α-OH testosterone) was determined to be linear at 10 minutes. The reactions were initiated by addition of 1mM NADPH to microsomal samples containing varying concentrations of testosterone. The reaction was terminated by addition of 50 μl of ice-cold acetonitrile followed by 15-minute incubation on ice and centrifugation to pellet precipitated protein (Velenosi, et al., 2012)
Testosterone Metabolite Analysis by Ultra Performance Liquid Chromatography with Photodiode Array (UPLC-PDA) Detection

Testosterone metabolite analysis was performed by solid-phase extraction followed by UPLC-PDA using methods previously described (Velenosi, et al., 2012). The solid-phase extraction cartridge (C18, Strata-X Polymeric Reverse Phase 33 μm; Phenomenex, Torrance, CA) was conditioned according to manufacturer’s specification. Carbamazepine was used as an internal standard for testosterone quantification. The analytes and internal standard were passed across the packing of the cartridge by gravity. The cartridges were then washed with 1 ml of Milli-Q water followed by 1 ml of 50:50 methanol/water. 1 ml of methanol containing 0.1% triethylamine and 0.1% trifluoroacetic acid was used to elute the analytes into clean glass test tubes. The eluent was dried, reconstituted in mobile phase and injected on a Phenomenex Kinetex C18 column (1.7 μm particle size, 50 x 2.1 mm; Torrence, CA) for testosterone and metabolite separation. The column was maintained at 40°C in a Waters ACQUITY UPLC H-Class System. The mobile phase flow and gradient used for each assay was the same as previously described (Velenosi, et al., 2012). ACQUITY UPLC PDA detector (Waters) was used to detect testosterone (245 nm) and carbamazepine at (290 nm) for quantification. The interassay and intraassay coefficients of variation of the analytes was <10%. 

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Statistics

All results were expressed as the mean of arbitrary values ± the standard error of the mean (SEM). The significance of differences (p<0.05) between mean values were evaluated using the unpaired Student’s t-test for RT-PCR and testosterone enzyme kinetics in liver microsomes via ultra performance liquid chromatography. One-way analysis of variance (ANOVA) followed by a Bonferroni’s Multiple Comparison post hoc test, was used to evaluate significance of differences for results comparing the effect of all the dietary regimes for Q-RT-PCR analysis.
Results

A switch to a normal protein diet in postnatal life of MPR derived low birth weight offspring leads to increases in the levels of steady-state hepatic CYP3A23, CYP2C11 and CYP2B1 mRNA at postnatal day 130

In this study, we wanted to investigate the long-term effects of MPR derived low birth weight offspring on expression and function of major P450 drug metabolizing cytochrome enzymes in the liver. Q-RT-PCR analysis was conducted to examine the steady-state mRNA expression of hepatic CYP3A23, CYP3A2, CYP2C11 and CYP2B1 at postnatal day 130. A 1.79, 1.45 and 1.94 fold increase in CYP3A23, CYP2C11, CYP2B1 respectively was observed in the livers of MPR offspring who were placed on a normal protein diet post-weaning (LP2) compared to the Control (Figure 1A, C, D). Interestingly, in MPR offspring subjected to protein restriction throughout pregnancy and postnatal life (LP1), there was no difference in hepatic CYP3A23, CYP3A2, CYP2C11 and CYP2B1 mRNA expression (Figure 1A, B, C, D). Upon comparing the two MPR dietary regimes, the LP2 offspring displayed a 1.93, 1.27 and 3.04 fold elevation in hepatic CYP3A23, CYP2C11 and CYP2B1 mRNA expression, respectively, relative to LP1 (Figure 1A, C, D). There was also a noticeable trend to an increase in CYP3A2 mRNA expression in LP2 offspring compared to Control and LP1 offspring (P<0.06) (Figure 1B). The average steady-state mRNA levels for the Control group were 25.03, 24.85, 31.96, 20.81, 20.72 cycles threshold for CYP3A23, CYP3A2, CYP2B1, CYP2C11 and β-actin, respectively.

We also evaluated whether the two LP dietary regimes differentially affect the steady state mRNA levels of the nuclear receptors Car and Pxr, considering that they
serve as master transcription factors regulating the transcription of several xenobiotic detoxification enzymes (Wang, et al., 2012). As depicted in Figure 2, there was no difference in Car mRNA levels between the LP1 and LP2 offspring. However, there was a slight increase in hepatic Car mRNA level in the LP2 offspring when compared to Control.

**Elevated expression of hepatic CYP3A23 and CYP2C11 correlates with increases in their drug metabolizing activity in MPR offspring.**

To evaluate whether postnatal 20% protein dietary restoration in MPR derived low birth weight offspring also impacted long-term function of hepatic CYP3A and CYP2C11 enzymes, testosterone metabolism assay was performed using rat liver microsomes. This assay is used to determine CYP3A and CYP2C11 enzyme activities, as testosterone metabolism to 6β-OH testosterone and 16α-OH testosterone has been previously observed to be mediated by CYP3A and CYP2C11 enzymes, respectively. 16α-OH testosterone is also an indicator of CYP2B1 under conditions when CYP2B1 is induced (Williams and Borghoff, 2000; Chovan, et al., 2007). Therefore, we also measured 2α-OH testosterone, given that CYP2C11 also metabolizes testosterone to this metabolite while CYP2B1 does not (Chovan, et al., 2007). Full enzyme kinetics of these metabolites was determined for this study. Vmax/Km, a measure of intrinsic clearance for 6β-OH testosterone, 16α-OH testosterone and 2α-OH testosterone, was significantly elevated by 1.9, 3.25 and 2.14 fold respectively in LP2 offspring compared to the Control offspring and by 1.8, 4.33 and 2.5 fold respectively in LP2 offspring compared to the LP1 offspring at postnatal day 130 (Figure 3 A, B, C). There was no difference in Vmax/Km for 6β-OH
testosterone, 16α-OH testosterone and 2α-OH testosterone between LP1 and Control offspring (Figure 3 A, B, C). The increases in intrinsic clearance of testosterone metabolites corresponded with the increases in CYP2B1, CYP2C11 and CYP3A23 mRNA expression, respectively (Figure 1D, C, A). The Michaelis-Menten kinetic parameters for 6β-OH testosterone, 16α-OH testosterone and 2α-OH testosterone are presented in Table 2.

**The expression of the major hepatic P450 drug metabolizing enzymes were unaltered by MPR at postnatal day 21.**

Since an increase in CYP3A, CYP2B and CYP2C11 expression and activity was observed by adulthood in MPR offspring that received a control protein diet post-weaning (LP2), we further pursued whether this increase was exclusively due to a switch in diet post-weaning or was persistent before the normal protein was restored at postnatal day 21. Interestingly, MPR offspring displayed no significant difference in CYP3A23, CYP3A2, CYP2B1 and CYP2C11 mRNA levels compared to Control at postnatal day 21 (Figure 4). The average steady-state mRNA levels for the Control group were 24.85, 23.68, 26.45, 33.57, 20.89 cycles threshold for CYP3A23, CYP3A2, CYP2B1, CYP2C11 and β-actin, respectively.
Discussion

In this study we present the novel finding that MPR derived low birth weight rat offspring have elevated CYP3A and CYP2C11 activity in adulthood, exclusively when faced with a nutritional mismatch of a normal (20%) protein diet in postnatal life. This was found to coincide with increases in their steady-state mRNA levels. Interestingly, when low birth weight offspring were maintained on a low protein diet post-lactation, they exhibited no differences in expression of these P450 enzymes. Collectively, this study suggests that an inappropriate dietary intervention strategy in IUGR offspring may augment important hepatic drug-metabolizing enzymes in adult life.

Contrary to what we had initially hypothesized, administering a normal protein diet post-weaning to maternal low protein diet derived low birth weight offspring (LP2) resulted in elevated expression of drug metabolizing enzymes CYP3A23 and CYP2C11 in adulthood. The LP2 offspring displayed corresponding increases in intrinsic enzymatic activity of these P450 enzymes. Given that testosterone is also a substrate for these P450 enzymes, it is noteworthy that circulating testosterone has been previously reported to be decreased in these LP2 offspring (Chamson-Reig, et al., 2009). Our data provide evidence for a potential mechanism behind this observation. However, a previous study has reported a decrease in testis weight and function in offspring derived from a low protein diet during pregnancy and lactation (Zambrano, et al., 2005). Therefore, the possibility of decreases in testosterone synthesis may also contribute to a reduced circulating testosterone level in the LP2 offspring. Conversely, no differences in the steady-state mRNA expression and activity of these P450 enzymes were observed when
the IUGR offspring were maintained on a low protein diet (LP1). Moreover, other than a trend to a decrease in CYP2C11, no significant changes in expression of any of these P450 enzymes were observed at postnatal day 21, which represents the end of lactation, a time point where normal protein was restored for LP2 offspring. This goes to suggest that the postnatal nutritional mismatch of a normal (20%) protein diet is likely the more significant contributing factor to elevated CYP3A23 and CYP2C11 expression in adult life, as opposed to the direct actions of the low protein diet itself during pregnancy and lactation. Since, increases in CYP3A and CYP2C11 activity were consistent with CYP3A23 and CYP2C11 steady-state mRNA expression in LP2 offspring, we postulated transcriptional mechanisms to underlie the increases in activity. Specifically, we attempted to investigate the role of xenobiotic sensing nuclear receptors Pxr and Car which represent major transcription factors involved with the transcriptional induction of P450 drug-metabolizing enzymes in humans (Willson and Kliewer, 2002). In this study we observed a slight increase in Car mRNA levels in LP2 offspring when compared to the Control group. However, there was no difference in Car levels between LP1 and LP2 offspring. We also observed a noticeable increase in the well characterized increases in expression of the Car target gene CYP2B1 in the LP2 offspring (Yoshinari, et al., 2001; Honkakoski, et al., 1998). However, it is unlikely that the modest increase in CAR expression is completely responsible for the observed changes in CYP3A23 and CYP2C11 expression. Moreover, the role of endocrine factors, which are key regulators of these P450 enzymes, cannot be ruled out and deserve further consideration. For instance, corticosteroids have been previously demonstrated to upregulate CYP3A23 and CYP2C11 (Daskalopoulos, et al., 2012; Huss, et al., 1996). Interestingly, our laboratory
has recently demonstrated that LP2 dietary regime leads to an upregulation of hepatic 11-hydroxysteroid dehydrogenase-1 (11-HSD1) (Vo, et al., 2013). An increase in 11-HSD1 would be indicative of an increased conversion of inactive corticosteroids to their active form. Therefore, it is possible that a potential increase in active corticosteroids may underlie the upregulation of these P450 enzymes in the livers of LP2 offspring in adulthood.

In support of the main tenet of the “predictive adaptive response” hypothesis, MPR derived low birth weight rat offspring when faced with a nutritional mismatch in postnatal life, have been previously reported to display hypercholesterolemia (Nusken, et al., 2008), visceral obesity (Desai, et al., 2007), hypertension (Boubred, et al., 2009), type 2 diabetes (Desai, et al., 2007) and reduced longevity (Jennings, et al., 1999). Conversely, in the absence of protein restoration, rat IUGR offspring have been observed to live longer (Sasaki, et al., 1982). Given that the liver plays a key role in metabolism, any alterations in its function can lead to the development of the metabolic syndrome and reduced lifespan. We recently observed indices of hepatic ER stress exclusively in MPR derived IUGR offspring, which received restored maternal protein in postnatal life (Sohi, et al., 2013). Interestingly, an elegant study by Pascual et al. in 2008 demonstrated that tunicamycin-induced ER stress in HepG2 cells induced the expression of CYP2B6, the human ortholog of CYP2B1 (Pascual, et al., 2008). The molecular mechanism behind this induction was attributed to ER stress activation of liver enriched activating transcription factor 5 (ATF5), which shares close sequence homology to the more ubiquitously expressed ATF4. ATF4 activation is known to occur via its selective translation during a period of global protein translation attenuation due to elevated phosphorylation of eukaryotic initiation factor 2α. Therefore, it is possible that activation of ATF5 due to the
previously observed hepatic increases in phosphorylation of eukaryotic initiation factor 2α (Pascual, et al., 2008), may also cause the induction in CYP2B1 expression observed in the study. Interestingly, ATF5 has been observed to interact with C/EBPα family of transcription factors, as well as synergistically potentiate the actions of nuclear receptor Car, which are both established regulators of the CYP2B6 gene (Neuvonen, et al., 2006). With the use of Alggen PROMO™ software, we have identified multiple putative C/EBPα binding sites at the promoter of all the P450 enzymes examined in this study. Therefore, it is conceivable that under conditions of stress (i.e. ER stress), the liver of LP2 low birth weight offspring responds by elevating hepatic xenobiotic metabolizing enzymes in an attempt to increase their detoxification capacity.

To date there have been no clinical studies conducted to evaluate the effects of low birth weight on the pharmacokinetics of drugs in adulthood. This is particularly relevant to drugs that would likely be used to manage the symptoms of the metabolic syndrome that are observed in these offspring. For instance, despite strong clinical and animal evidence linking IUGR to hepatic dysfunction and elevated cholesterol levels in adult life, there is little known about whether statin pharmacokinetic or pharmacodynamic parameters are altered. Out of all the statins that target the liver, simvastatin, lovastatin and atorvastatin are metabolized primarily through CYP3A4, and fluvastatin is metabolized through CYP2C9 (Neuvonen, et al., 2006). Since, the rat ortholog of CYP2C9 (i.e. CYP2C11) activities was elevated in adulthood, it is likely that low birth weight rat offspring would metabolize fluvastatin in the liver at a faster rate. Our data suggest that low birth weight rat offspring may require larger doses of fluvastatin to maintain efficacy towards
reducing circulating cholesterol levels, a hypothesis which still remains to be tested. Moreover, in order to completely understand the impact of low birth weight on alterations in pharmacokinetics of drugs, several additional factors need to be evaluated, mainly changes in plasma binding protein levels and drug transporter function. It is also important to determine whether different insults, including infection, stress, placental dysfunction and inflammation, leading to low birth weight would similarly impact drug pharmacokinetic measures in adult life.

In summary, this study highlights that low birth weight offspring faced with a nutritional mismatch of a normal (20%) protein diet in postnatal life have elevated activity of hepatic phase I drug metabolizing enzymes CYP3A and CYP2C11 in adulthood. Moreover, maintaining these low birth weight offspring on a low protein diet prevented the increases in expression of these enzymes. It is plausible that these offspring sense the nutritional mismatch as an unanticipated insult and consequently respond by increasing their detoxification capacity. In light of this study, future studies examining a need for optimizing drug dosing to ameliorate symptoms of metabolic syndrome in IUGR offspring that display nutritional mismatch induced accelerated growth would be useful.
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Authors Contributions

Participated in Research Design Study: Sohi, Urquhart, Hardy

Conducted Experiments: Sohi

Contributed new reagents or analytic tools: Urquhart, Hardy

Performed data analysis: Sohi, Barry, Velenosi

Wrote or contributed to the writing of the manuscript: Sohi, Barry, Velenosi, Urquhart, Hardy

Hardy
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Footnotes

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure Legends

Figure 1: Quantitative RT-PCR mRNA level analysis of A. CYP3A23, B. CYP3A2, C. CYP2C11, D. CYP2B1 in the livers of rat offspring derived at postnatal d 130. RNA was extracted and reverse transcribed for quantitative RT-PCR. mRNA level expression was assessed via Q-RT-PCR using primers specific for CYP3A23, CYP3A2, CYP2C11, CYP2B1 and β-actin. The relative levels of each mRNA transcript were normalized to that of the levels of each β-actin mRNA transcript. Results were expressed as the mean ± SEM. * Control vs LP2 Significant difference (P < 0.05), # LP1 vs LP2 Significant difference (P < 0.05); n = 7–8/group, where each n represents an offspring derived from a different mother.

Figure 2: Quantitative RT-PCR mRNA level analysis of A. Pxr and B. Car in the livers of rat offspring derived at postnatal d 130. RNA was extracted and reverse transcribed for quantitative RT-PCR. mRNA level expression was assessed via Q-RT-PCR using primers specific for Pxr, Car and β-actin. The relative levels of each mRNA transcript were normalized to that of the levels of each β-actin mRNA transcript. Results were expressed as the mean ± SEM. * Control vs LP2 Significant difference (P < 0.05); n = 7–8/group, where each n represents an offspring derived from a different mother.

Figure 3: Michaelis-Menten plots of A. 6β-OH testosterone, B. 16α-OH testosterone and C. 2α-OH testosterone after incubation of rat liver microsomes with 1mM NADPH and various concentrations of testosterone. Liver microsomes were extracted and timed enzyme reaction was performed for testosterone metabolite analysis via solid-phase extraction followed by UPLC-PDA detection. Each data point on the curves were
expressed as the mean ± SEM. n = 5–6/group, where each n represents an offspring derived from a different mother.

**Figure 4**: Quantitative RT-PCR mRNA level analysis of **A. CYP3A23**, **B. CYP3A2**, **C. CYP2C11**, **D. CYP2B1** in the livers of rat offspring derived at postnatal day 21. RNA was extracted and reverse transcribed for quantitative RT-PCR. mRNA level expression was assessed via Q-RT-PCR using primers specific for CYP3A23, CYP3A2, CYP2C11, CYP2B1 and β-actin. The relative levels of each mRNA transcript were normalized to that of the levels of each β-actin mRNA transcript. Results were expressed as the mean ± SEM. n = 7–8/group, where each n represents an offspring derived from a different mother.
**Table 1:** Real Time PCR Primers

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<td>REV ATC CAC AGA CCT TGC CAA CTC CTT</td>
<td>BC 089765</td>
</tr>
<tr>
<td>CYP2C11</td>
<td>FWD CCC TGA GGA CTT TTG GGA TGG GC</td>
<td>BC 088146</td>
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<td>CYP2B1</td>
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<td>β-actin</td>
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<td>NM 031144</td>
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<tr>
<td></td>
<td>REV TTG GTT ACA ATG CCG TGT TCA</td>
<td>NM 031144</td>
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</table>
Table 2: Michaelis-Menten kinetic values for P450 probe substrates in Control and LP2 offspring rat liver microsomes at Day 130.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Control</th>
<th>LP1</th>
<th>LP2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Km (µM)</td>
<td>Vmax (pmol/min/mg protein)</td>
<td>Vmax/Km (µl/min/mg protein)</td>
</tr>
<tr>
<td>6β-OH Testosterone</td>
<td>172 ± 18</td>
<td>182 ± 27</td>
<td>0.97 ± 0.16</td>
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<tr>
<td>16α-OH Testosterone</td>
<td>185 ± 47</td>
<td>155 ± 16</td>
<td>1.03 ± 0.17</td>
</tr>
<tr>
<td>2α-OH Testosterone</td>
<td>123 ± 19</td>
<td>214 ± 21</td>
<td>1.85 ± 0.22**#</td>
</tr>
</tbody>
</table>

Data are presented as means ± SEM.

*P < 0.05 LP2 compared with Control.

**P < 0.001 LP2 compared with Control.

#P < 0.05 LP2 compared with LP1.

**P < 0.001 LP2 compared with LP1.

†P < 0.05 LP1 compared with Control.
Fig 1. Bar graphs showing the ratio of CYP3A1, CYP3A2, CYP2C11, and CYP2B1 mRNA expression compared to control, LP1, and LP2 groups. Significant differences are indicated by asterisks (*) and hash marks (#).
Fig 4