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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ABSTRACT

The World Health Organization has identified hypercholesterolemia to be one of the major symptoms encompassing the metabolic syndrome. Moreover, epidemiologic 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 catalyzes 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, we maintained Wistar rats 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_{max}/K_{m}) was significantly increased in LP2 offspring at postnatal day 130 compared with 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 with 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 (Barker et al., 1989; Curhan et al., 1996a,b; Leon et al., 1996; Nilsson et al., 1997). 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; Martin et al., 2003; Finken et al., 2006; Eriksson, 2011). 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, 1992, 2001; 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, 2002, 2003, 2004; Singhal and Lucas, 2004). However, very little is known about drug disposition in adult life of low-birth-weight 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 the postnatal life of low-birth-weight infants (Boehm et al., 1990; Ozanne et al., 1996; Burns et al., 1997; 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. Among 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 and 15 days (Thummel et al., 1996; Thummel and Wilkinson, 1998; Lee et al., 1999). Interestingly, although the CYP3A4 isozyme is responsible for the metabolism of approximately half of all marketed drugs (Wrighton et al., 1996), it remains to be investigated whether the 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 the pharmacokinetics of a drug to changes in hepatic P450 enzyme activity.

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ABBREVIATIONS: ATF, activating transcription factor; β-HSD1, 11β-hydroxysteroid dehydrogenase-1; CAR, constitutive androstane receptor; Ct, cycle threshold; CYP7A1, cholesterol 7α-hydroxylase; ER, endoplasmic reticulum; IUGR, intrauterine growth restriction; LP, low protein; MPR, maternal protein restriction; P450, cytochrome p450; PXR, pregnane X receptor; RT-PCR, reverse-transcription polymerase chain reaction; UPLC-PDA, ultraperformance liquid chromatography with photodiode array detection.
The long-term programming of Cyp3a activity was recently demonstrated by Tajima et al. (2012) in mice offspring born of mothers receiving a high-fat diet during pregnancy. Specifically, they correlated a decrease in hepatic Cyp3a activity in 6-week-old offspring with reduced triazolam substrate clearance (Tajima et al., 2012). Although that was 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 regimen is a relevant animal model to study the developmental origins of adult diseases because MPR shares features common with placental insufficiency—induced intrauterine growth restriction (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 (Ozanne et al., 1996; Burns et al., 1997; 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 after 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 associated with catch-up growth (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 liver were noted at 4 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 I of drug metabolism (Avadhani et al., 2011), we hypothesized that MPR-derived low-birth-weight 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, control).

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-hour light/dark cycle. For 3 weeks, these rats were left to acclimatize to the animal care facility, and their reproductive cycles were observed. 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). 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 with the control offspring, which received a control diet throughout prenatal and postnatal life. Food and water was provided at libitum. The food intake of these offspring was monitored by measuring their food consumption every third day, as previously published elsewhere (Sohi et al., 2011).

At postnatal day 21 (d21), a subset of the offspring were sacrificed, and the medial lobe liver tissue was excised and snap frozen for quantitative reverse-transcription polymerase chain reaction (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 ultraperformance liquid chromatography (UPLC). 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 (Guan et al., 2005; Chanson-Reig et al., 2009; Sohi et al., 2011)

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 (1987) (TRizol; Invitrogen, Carlsbad, CA). The RNA was treated with deoxyribonuclease to remove any contaminating DNA, then 4 μg of the total RNA was reverse transcribed to cDNA using random primers and SuperScript II RNase H–reverse transcriptase (Invitrogen). Primer sets directed against rat CYP2B1, CYP3A23, CYP3A2, and CYP2C11, constitutive androstane receptor (Car), pregnane X receptor (Pxr), and β-actin were generated via Primer Express software (PE Applied Biosystems, Boston, MA) 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 CFX384 Real Time System was employed using the DNA binding dye IQTM SYBR green supermix (Bio-Rad Laboratories, Mississauga, Ontario, Canada). The cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. The cycle threshold (Ct) was set at a level where the exponential increase in PCR amplification was approximately parallel between all samples. All primer sets produced amplification 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−ΔΔCt, in which ΔΔCt 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. (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 minutes at 4°C. The subsequent supernatant was centrifuged at 105,000g for 60 minutes at 4°C. The microsomal pellet was resuspended in 100 mM potassium phosphate buffer containing 20% glycerol at pH 7.4, and the protein concentration was determined by colorimetric BCA Protein Assay (Pierce Corporation, Madison, WI). 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
Enzymes in the liver of MPR-derived low-birth-weight offspring over the long term. Quantitative 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, and CYP2B1, respectively, was observed in the liver of MPR offspring who were placed on a normal protein diet after weaning (LP2) compared with the control group (Fig. 1, A, C, and D). Interestingly, in MPR offspring subjected to protein restriction throughout pregnancy and postnatal life (LP1), there was no difference in hepatic CYP3A23, CYP3A2, CYP2C11, or CYP2B1 mRNA expression (Fig. 1, A–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 (Fig. 1, A, C, and D). There was also a noticeable trend toward an increase in CYP3A2 mRNA expression in the LP2 offspring compared with the control and LP1 offspring ($P < 0.06$) (Fig. 1B). The average steady-state mRNA levels for the control group were 25.03, 24.85, 31.96, 20.81, and 20.72 Ct for CYP3A23, CYP3A2, CYP2B1, CYP2C11, and $\beta$-actin, respectively.

We also evaluated whether the two LP dietary regimes differentially affected 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 Fig. 2, there was no difference in Car mRNA levels between the LP1 and LP2 offspring. However, there was a slight increase in the hepatic Car mRNA level in the LP2 offspring when compared with the controls.

### 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, a testosterone metabolism assay was performed using rat liver microsomes. This assay is used to determine CYP3A and CYP2C11 enzyme activities, as testosterone metabolism to $6\beta$-OH testosterone and $16\alpha$-OH testosterone has been previously observed to be mediated by CYP3A and CYP2C11 enzymes, respectively. $16\alpha$-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 $2\alpha$-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 were determined for this study. $V_{\text{max}}/K_{\text{m}}$, a measure of intrinsic clearance for $6\beta$-OH testosterone, $16\alpha$-OH testosterone, and $2\alpha$-OH testosterone, was significantly elevated by 1.9-, 3.25-, and 2.14-fold,

### Results

A Switch to a Normal Protein Diet in Postnatal Life of MPR-Derived Low-Birth-Weight Offspring Leads to Increases in the Level of Steady-State Hepatic CYP3A23, CYP2C11, and CYP2B1 mRNA at Postnatal Day 130. In this study, we wanted to investigate the expression and function of major P450 drug metabolizing cytochrome P450 enzymes previously described by 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 (Souidi et al., 2005; Velenosi et al., 2012). We used 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 250 $\mu$l for timed enzymatic reactions. The linear rate of production of metabolites was determined by varying the time, protein, and relevant substrate concentrations before conducting enzymatic reactions. Formation of testosterone metabolites ($6\beta$-OH testosterone and $16\alpha$-OH testosterone) was determined to be linear at 10 minutes. The reactions were initiated by the addition of 1 mM NADPH to microsomal samples containing varying concentrations of testosterone. The reaction was terminated by the addition of 50 $\mu$l of ice-cold acetonitrile followed by a 15-minute incubation on ice and centrifugation to pellet the precipitated protein (Velenosi et al., 2012).

#### Testosterone Metabolite Analysis by UPLC with Photodiode Array Detection

Testosterone metabolite analysis was performed by solid-phase extraction followed by UPLC with photodiode array (PDA), using methods previously described elsewhere (Velenosi et al., 2012). The solid-phase extraction cartridge (C18, Strata-X Polymeric Reverse Phase 33 mm, Phenomenex, Torrance, CA) was conditioned according to the manufacturer’s specifications. 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. We then used 1 ml of methanol containing 0.1% triethylamine and 0.1% trifluoroacetic acid to elute the analytes into clean glass test tubes. The eluent was dried, reconstituted in mobile phase, and injected on a Kinetex C18 column (1.7 mm; Phenomenex) for testosterone and metabolite separation. The column was maintained at 40°C in a Waters Acquity UPLC H-Class System (Waters Corporation, Milford, MA). The mobile phase flow and gradient used for each assay was the same as previously described elsewhere (Velenosi et al., 2012). An 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 were <10%.

#### Statistics

All results were expressed as the mean of arbitrary values ± the standard error of the mean (S.E.M.). $P < 0.05$ was considered statistically significant for differences between the mean values, evaluated using the unpaired Student’s t test for RT-PCR and testosterone enzyme kinetics in liver microsomes via UPLC. One-way analysis of variance (ANOVA) followed by a Bonferroni’s multiple post hoc test was used to evaluate the statistical significance of the differences for the results when comparing the effect of all the dietary regimes for quantitative RT-PCR analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (5’-3’)</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A23</td>
<td>FWD ACC AGT GGA AGA CTC AAC AGT GTG ATG T</td>
<td>XM 00375127</td>
</tr>
<tr>
<td></td>
<td>REV TCA CAG GGA CAG GTT TGC CTT TCT</td>
<td>XM 00375127</td>
</tr>
<tr>
<td>CYP3A2</td>
<td>FWD CTC GCT GTT TGA TTA AAG ATT TG</td>
<td>BC 089765</td>
</tr>
<tr>
<td></td>
<td>REV TTC AGA CAC GGT TGA CTT TCT</td>
<td>BC 089765</td>
</tr>
<tr>
<td>CYP2C11</td>
<td>FWD CCC TGA GGT CTG TGT GGA TGG GC</td>
<td>BC 088146</td>
</tr>
<tr>
<td></td>
<td>REV GGG GCA CTT TGC TCT TCT</td>
<td>BC 088146</td>
</tr>
<tr>
<td>CYP2B1</td>
<td>FWD GCT GTA CTT CCC CCA TGT CG</td>
<td>XM 00375035</td>
</tr>
<tr>
<td></td>
<td>REV ATC AGT GTG TGT CAT TTT ACT GCG G</td>
<td>XM 00375035</td>
</tr>
<tr>
<td>Car</td>
<td>FWD GGT GTC TGT GTC CTT GAT GC</td>
<td>AB 204900</td>
</tr>
<tr>
<td></td>
<td>REV AGG CAC AAC GTA GTG TTG AGT</td>
<td>AB 204900</td>
</tr>
<tr>
<td>Pxr</td>
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<tr>
<td>$\beta$-actin</td>
<td>FWD AGG AGG CCC AGA GCA AGA</td>
<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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respectively, in the LP2 offspring compared with the control offspring and by 1.8-, 4.33-, and 2.5-fold, respectively, in the LP2 offspring compared with the LP1 offspring at postnatal day 130 (Fig. 3, A–C). There was no difference in V_{max}/K_{m} for 6β-OH testosterone, 16α-OH testosterone, or 2α-OH testosterone between the LP1 and control offspring (Fig. 3, A–C). The increases in intrinsic clearance of testosterone metabolites corresponded with the increases in CYP2B1, CYP2C11, and CYP3A23 mRNA expression, respectively (Fig. 1, A, D, and C). The Michaelis-Menten kinetic parameters for 6β-OH testosterone, 16α-OH testosterone, and 2α-OH testosterone are presented in Table 2.

Expression of the Major Hepatic P450 Drug Metabolizing Enzymes Is Unaltered by MPR at Postnatal Day 21. Because an increase in CYP3A, CYP2B, and CYP2C11 expression and activity was observed by adulthood in the MPR offspring that received a normal (20%) protein diet after weaning (LP2), we further pursued whether this increase was exclusively due to a switch in diet after weaning or was persistent before the normal protein was restored at postnatal day 21. Interestingly, the MPR offspring displayed no statistically significant difference in CYP3A23, CYP3A2, CYP2B1, or CYP2C11 mRNA levels compared with the controls at postnatal day 21 (Fig. 4). The average steady-state mRNA levels for the control group were 24.85, 23.68, 26.45, 33.57, and 20.89 Ct 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 after lactation, they exhibited no differences in expression of these P450 enzymes. Collectively, this study suggests that an inappropriate...
Low-Birth-Weight Rats Have Elevated Hepatic Drug Metabolism

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 after 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, there is a possibility that 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 toward 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 contributes to a suggestion 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 action of the low-protein diet itself during pregnancy and lactation.

Because 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 with 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 (Honkakoski et al., 1998; Yoshinari et al., 2001). However, it is unlikely that the modest increase in CAR expression is completely responsible for the observed changes in CYP3A23 and CYP2C11 expression.

TABLE 2

<table>
<thead>
<tr>
<th>Microsome</th>
<th>6β-OH Testosterone</th>
<th>16α-OH Testosterone</th>
<th>2α-OH Testosterone</th>
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<tbody>
<tr>
<td></td>
<td>$K_m$ μM</td>
<td>$V_{max}$ pmol/min/mg protein</td>
<td>$V_{max}/K_m$ mg/mg</td>
</tr>
<tr>
<td>Control</td>
<td>172 ± 18</td>
<td>182 ± 27</td>
<td>0.97 ± 0.16</td>
</tr>
<tr>
<td>LP1</td>
<td>185 ± 47</td>
<td>155 ± 16</td>
<td>1.03 ± 0.17</td>
</tr>
<tr>
<td>LP2</td>
<td>123 ± 19</td>
<td>214 ± 21</td>
<td>1.85 ± 0.22 $^{bc}$</td>
</tr>
</tbody>
</table>

$^a$ P < 0.05 LP1 compared with control.
$^b$ P < 0.001 LP2 compared with control.
$^c$ P < 0.05 LP2 compared with LP1.
$^d$ P < 0.05 LP2 compared with control.
$^e$ P < 0.001 LP2 compared with LP1.

Fig. 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 1 mM 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 was expressed as the mean ± S.E.M.; n = 5–6/group, where each n represents an offspring derived from a different mother.
Moreover, the role of endocrine factors, which are key regulators of these P450 enzymes, cannot be ruled out and deserves further consideration. For instance, corticosteroids have been previously demonstrated to upregulate CYP3A23 and CYP2C11 (Huss et al., 1996; Daskalopoulos et al., 2012). Interestingly, our laboratory has recently demonstrated that the LP dietary regimen leads to an upregulation of hepatic 11β-hydroxysteroid dehydrogenase-1 (11β-HSD1) (Vo et al., 2013). An increase in 11β-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 life span. 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. (2008) demonstrated that tunicamycin–induced ER stress in HepG2 cells induced the expression of CYP2B6, the human ortholog of CYP2B1. 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 from 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 our study. In addition, ATF5 has been observed to interact with the C/EBPα family of transcription factors as well as to synergistically potentiate the actions of nuclear receptor Car, both of which are 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 as observed in these offspring. For instance, despite strong clinical and animal evidence linking IUGR to hepatic dysfunction and elevated cholesterol levels in adult life, little is 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). Because the rat ortholog of CYP2C9 (i.e., CYP2C11) activities were 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 toward reducing circulating cholesterol levels, a hypothesis that remains to be tested. Moreover, to completely understand the impact of low birth weight on alterations in the 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 in pregnancy, 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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Authorship Contributions

Participated in research design: Sohi, Urquhart, Hardy. Conducted experiments: Sohi. Contributed new reagents or analytic tools: Urquhart, Hardy. Performed data analysis: Sohi, Barry, Veloseni. Wrote or contributed to the writing of the manuscript: Sohi, Barry, Veloseni, Urquhart, Hardy.

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