Department of Pharmaceutical Analysis and Drug Metabolism[1], College of Pharmaceutical Sciences, Zhejiang University; The Children Hospital of Zhejiang University School of Medicine[2], Hangzhou, Zhejiang, People's Republic of China

Expression of CYP3A23/1, CYP3A2, PXR, CAR and HNF4α in large-for-gestational-age neonatal rats

Shaoqing Ni[1,2], Xiumin Wang[2], Jue Wang[2], Su Zeng[1], Zhengyan Zhao[2]

Received October 15, 2008, accepted November 14, 2008

Su Zeng, Department of Pharmaceutical Analysis and Drug Metabolism, College of Pharmaceutical Sciences, Zhejiang University, No. 388 Yu Hang Tang Road, Hangzhou, Zhejiang 310058, P.R. China
nsqshc@163.com

Zhenyan Zhao, The Children Hospital of Zhejiang University School of Medicine, No. 57 Zhu Gan Xiang, Hangzhou, Zhejiang 310003, P.R. China
zhaozy@zju.edu.cn

Pharmazie 64: 252–257 (2009) *doi: 10.1691/ph.2009.8769*

Background: The available information about hepatic drug-metabolizing enzymes in the large-for-gestational-age (LGA) neonate is limited. *Objective:* To determine whether LGA status alters expression of the cytochrome p450 enzymes, CYP3A23/1 and CYP3A2, and nuclear receptors PXR, CAR and HNF4α. *Methods:* Appropriate-for-gestational-age (AGA) and LGA pups from 10 litters of rats ($n = 15$ pups/group) were used. Hepatic expression levels of CYP3A23/1, CYP3A2, pregnane X receptor (PXR), constitutive androstane receptor (CAR), and HNF4α mRNA were detected by quantitative real-time RT-PCR. Protein expression was determined immunohistochemically and expression levels were evaluated using staining intensity scores and percentage of positive hepatocytes. *Results:* The mean body weights of AGA and LGA rat pups were 6.20 ± 0.49 and 7.77 ± 0.70 g (mean ± SD), respectively ($P < 0.001$). Liver weight/body weight ratios trended higher in the LGA group ($5.18 \pm 0.50\%$, mean ± SD) than in the AGA group ($4.90 \pm 0.01\%$, mean ± SD), but the difference was not significant. CYP3A2 mRNA levels were higher in LGA than in AGA pups ($P < 0.05$), but there were no group differences in CYP3A23/1 mRNA levels. HNF4α levels were also higher in the LGA group ($P < 0.05$), but PXR and CAR mRNA levels did not differ between the groups. The staining intensity and frequency of CYP3A23/1-positive and HNF4α-positive hepatocytes differed between LGA and AGA pups, whereas no significant differences in CYP3A2 or CAR protein expression were observed between groups. *Conclusions:* LGA status affects the hepatic expression of CYP3A23/1, CYP3A2, and HNF4α, suggesting that further research on this issue is warranted.

1. Introduction

Large-for-gestational-age (LGA) infants, which account for approximately 1%–10% of all deliveries, are those newborns whose weight exceeds the 90th percentile for their gestational age, whether delivered preterm, term or post-term. Appropriate-for-gestational-age (AGA) infants are those with birth weights between the 10th and 89th percentile (Wang et al. 1994). LGA infants show a propensity for decreased insulin sensitivity at birth and are at elevated risk for developing obesity, cardiovascular disease and diabetes later in life (Ahlsson et al. 2007). It has also been shown that as children, LGA infants born of mothers without confirmed impaired glucose tolerance during pregnancy show higher insulin concentrations than children who were AGA at birth (Evagelidou et al. 2006). Insulin is an important regulator of inducible and liver-specific gene expression (Sidhu and Omiecinski 1999). Collectively, these observations suggest that the expression and/or regulation of a subset of drug-metabolizing enzymes might be altered in LGA infants, and could lead to clinically relevant drug-drug interactions. However, the available information on hepatic cytochrome P-450 CYP3A enzymes and their related nuclear receptors in LGA infants is very limited.

The CYP3A subfamily is the most abundant and clinically important of the CYP enzymes in the liver. The CYP3A enzymes are responsible for the maintenance of steroid homeostasis as well as the metabolism of approximately 50% of the most commonly used drugs (Johnson 2003; Yu et al. 2005; Pikuleva 2006). Among the major functional CYP3A isoforms, CYP3A4 is the predominant form in humans, and is the primary enzyme involved in catalyzing the metabolism and clearance of a large proportion of clinical medications, including many pediatric drugs. It has been suggested that alterations in the expression or activity of these CYP3A isoforms is a key predictor of drug responsiveness and toxicity (Thummel and Wilkinson 1998).

Studies indicate that the ligand-activated nuclear receptors, pregnane X receptor (PXR; *NR1I2*) and constitutive an-

drostane receptor (CAR; *NR1I3*), regulate the expression of genes such as CYP3A4, orphan nuclear receptor, hepatocyte nuclear factor-4alpha (HNF4α; *HNF4A*), and that this genetic regulation is critically involved in PXR- and CAR-mediated transcriptional activation of CYP3A4 (Tirona et al. 2003). Given these regulatory relationships, PXR, CAR and HNF4α can be expected to be important factors in the interacting network of CYP3A4-mediated drug metabolism with potentially significant impacts on clinical responses to drugs. To the best of our knowledge, however, there have been no reports on the expression of these nuclear receptors and their relation to CYP3A expression in LGA newborns either in human or rodents.

CYP3A23/1 and CYP3A2 are rat main CYP3A. (Cooper et al. 1993; Mahnke et al. 1997), and rodents are often used as a model for human drug/toxicology studies, whereas no references offer effective method of establishing LGA model as to our knowledge. Therefore, we have opted instead to obtain high-birth-weight pups from pregnant rats fed an ordinary diet, which can represent a portion of the LGA maternal nutritional status. The present study employed this high-birth-weight model to determine the expression of CYP3A23/1, CYP3A2, PXR, CAR and HNF4α in LGA and AGA neonatal rats using real-time reverse-transcription polymerase chain reaction (RT-PCR) and immunohistochemistry. The goal of this work was to improve our understanding of changes in CYP3As and nuclear receptors in the LGA condition.

2. Investigations and results

2.1. Body and liver weight of LGA and AGA neonatal rats

Body weights of AGA and LGA pups were 6.20 ± 0.49 and 7.77 ± 0.70 g (mean ± SD), respectively ($P < 0.001$) and the corresponding liver weights were 0.31 ± 0.06 and 0.40 ± 0.04 g (mean ± SD) ($P < 0.001$). Body and liver weights were weakly correlated in the LGA group ($r = 0.465$, $P = 0.05$), and strongly correlated in the AGA group ($r = 0.750$, $P = 0.001$). Liver weight was slightly higher as a percentage of body weight in the LGA group ($5.18 \pm 0.50\%$, mean ± SD) than in the AGA group ($4.90 \pm 0.01\%$, mean ± SD); however, this apparent difference did not reach statistical significance.

2.2. mRNA expression of CYP3A23/1, CYP3A2 isoforms and nuclear receptors

CYP3A23/1, CYP3A2, PXR, CAR and HNF4α mRNA expression was detected in all samples examined (Fig. 1). There were no significant differences in the level of CYP3A23/1, mRNA expression (calculated as $2^{CT[GAPDH] - CT[target]}$) between LGA and AGA pups, but CYP3A2 mRNA levels were higher in LGA pups than in AGA pups ($P < 0.05$). HNF4α mRNA levels were also higher in LGA pups than in AGA pups ($P < 0.05$), while PXR and CAR expression levels were not significantly lower.

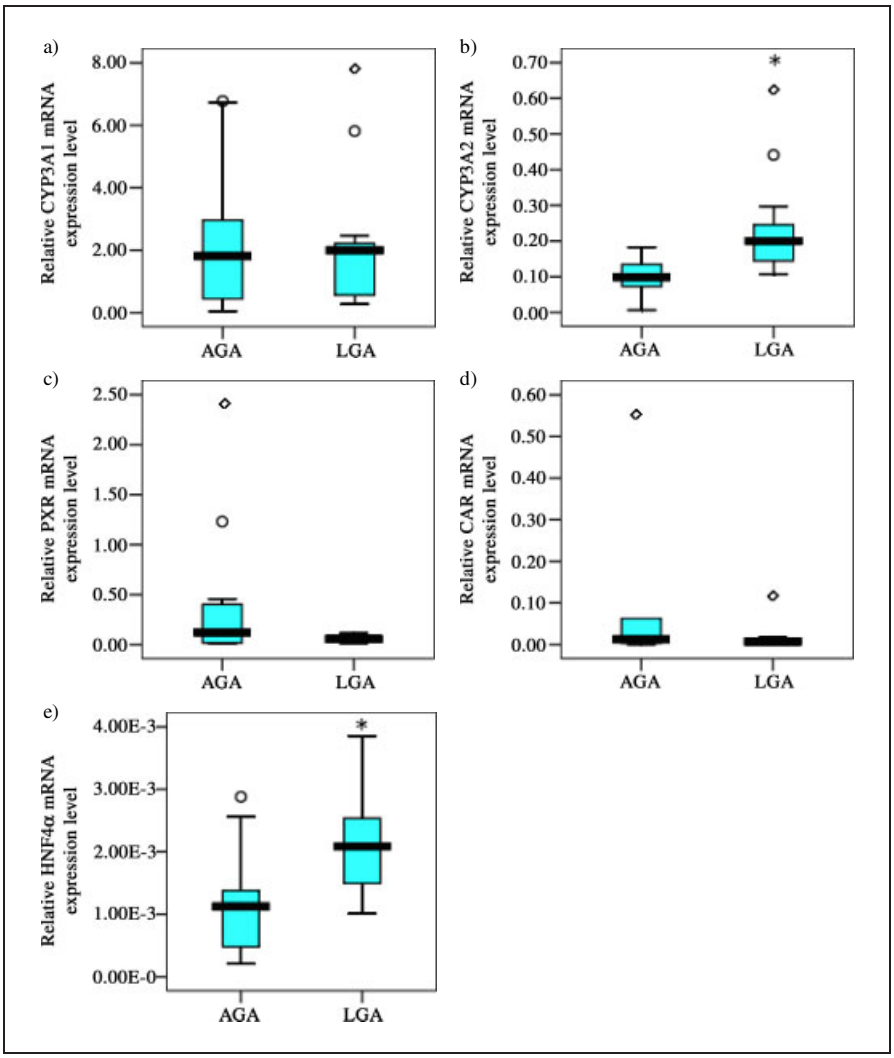

Fig. 1:
Relative levels of CYP3A23/1, CYP3A2, PXR, CAR and HNF4α mRNA expression in AGA and LGA groups ($n = 15$ pups/group) as assessed by real-time RT-PCR. Values were normalized against GAPDH. Data are presented as box-and-whisker plots: boxes represents the 25th and 75th percentiles and the heavy bar represents the median. Whiskers delineate the 10th and 90th percentile levels. ○ denotes each data point that is more than 1.5 times the interquartile range; ◇ denotes outliers that are more than 3 times the interquartile range; * represents a significant difference compared to the AGA group ($P < 0.05$)

2.3. Correlations between mRNA expression of CYP3A23/1, CYP3A2 and their receptors

The level of CYP3A1 message correlated strongly with that of CYP3A2 in both the LGA ($r = 0.839$, $P < 0.001$) and AGA ($r = 0.718$, $P < 0.001$) groups. There was a positive correlation between the levels of CYP3A2 and HNF4α mRNA ($r = 0.562$, $P = 0.029$) in the AGA group. A statistically significant correlation was also observed between PXR and CAR expression in the AGA group ($r = 0.869$, $P < 0.000$), and between PXR and HNF4α in the LGA group ($r = 0.799$, $P < 0.000$). However, we did not observe positive correlation between other pairs in both groups.

2.4. Protein levels of CYP3A23/1, CYP3A2 and nuclear receptors

The protein expression scores of CYP3A23/1, CYP3A2, CAR and HNF4α in the liver of LGA and AGA pups are summarized in Table 1. All proteins examined showed a widely dispersed staining pattern in the hepatic lobule with no evidence for zonally restricted expression (Fig. 2). CYP3A23/1, CYP3A2 and CAR immunolabeling were evident in the cytoplasm of hepatocytes from both groups. There was positive staining for HNF4α protein in the nucleus and cytoplasm of some hepatocytes in LGA pups, while little HNF4α immunoreactivity was seen in the AGA group tissue. PXR protein staining was largely absent in both LGA and AGA groups. The staining intensity and frequency of CYP3A23/1-positive and HNF4α-posi-

Table 1: CYP3A23/1, CYP3A2, CAR and HNF4α protein expression in neonatal AGA and LGA rat hepatic sections by immunohistochemistry

Protein	Group	No.	Score[1]				χ^2	P value
			–	+	++	+++		
CYP3A23/1	AGA	9	0	3	6	0	7.86	0.005*
	LGA	9	0	0	4	5		
CYP3A2	AGA	9	0	1	4	4	1.14	0.291
	LGA	9	0	0	3	6		
CAR	AGA	9	0	0	5	4	0.21	0.647
	LGA	9	0	0	4	5		
HNF4α	AGA	9	9	0	0	0	4.8	0.028*
	LGA	9	5	1	3	0		

[1] Protein expression score corresponds to the sum of both staining intensity (0, negative; 1, weak; 2, intermediate; 3, strong) and percentage of positive hepatocytes (0, 0% positive; 1, < 25% positive; 2, ≥ 25 and ≤ 50% positive; 3, > 50% positive). Sums were classified as "–" (0), "+" (1–2), "++" (3–4) and "+++" (5–6); a score greater than 2 was regarded as positive (see Material and Methods). * represents a significant difference compared to the AGA group

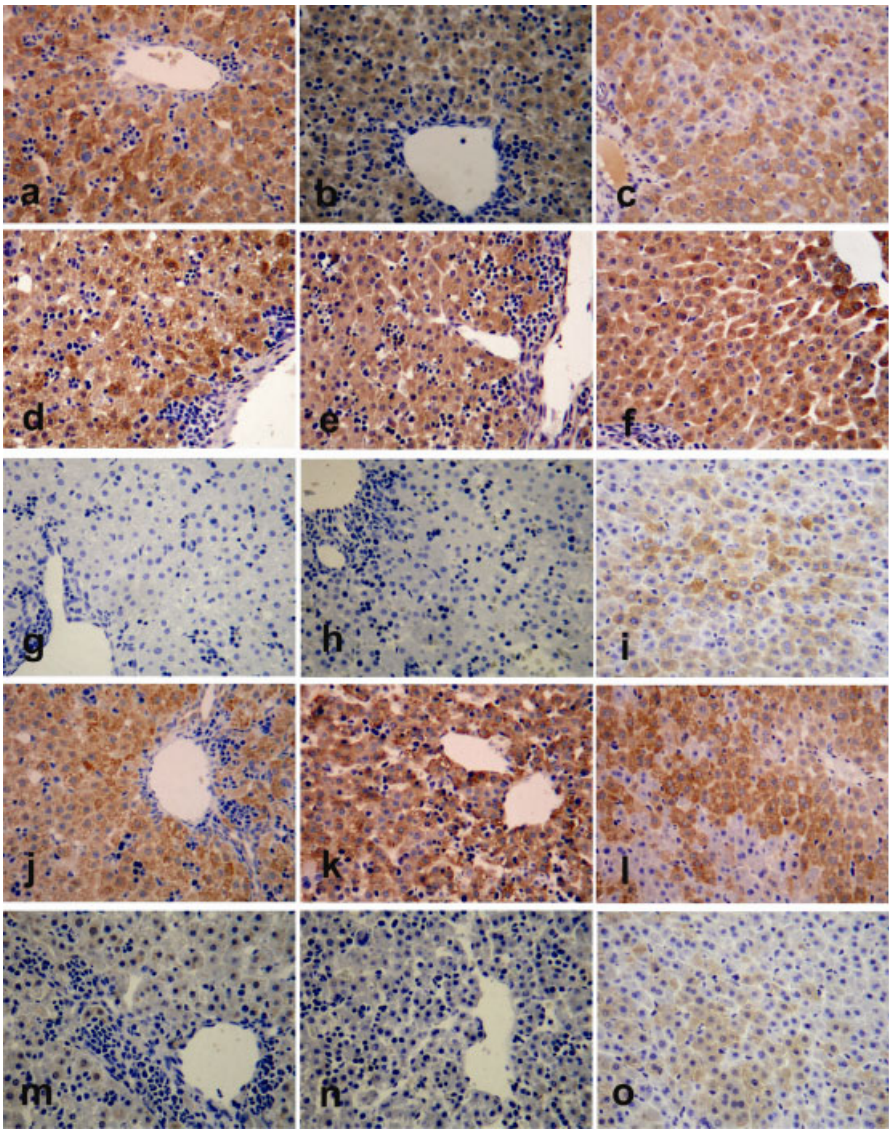

Fig. 2:
Photomicrographs of hepatic sections from rat pups in LGA and AGA groups immunostained for CYP3A23/1, CYP3A2, PXR, CAR and HNF4α. CYP3A23/1 (top row: a–c), CYP3A2 (d–f), PXR (g–i), CAR (j–l) and HNF4α (bottom row: m–o) in the LGA group (left column), AGA group (middle column) and in a 21-day-old male rat (positive control; right column). Protein staining is brown; hematoxylin counterstain is blue; original image 200× magnification

tive hepatocytes were greater in LGA pups than in AGA pups ($P < 0.05$), while no significant group differences in CYP3A2 and CAR expression levels were observed.

3. Discussion

Our study suggested that LGA status had significant effects on hepatic mRNA expression of CYP3A2 and HNF4α, and protein expression of CYP3A23/1 and HNF4α. Since CYP3A and HNF4α are critically involved in drug metabolism, these results may have clinical implications for optimal medication in LGA children. However, a specific LGA model is difficult to establish. To our knowledge, existing references do not offer an effective method of establishing LGA model. De Assis et al. (2006) reported that a high-fat diet during the extent of gestation could increase the birth weight of Sprague Dawley rats, (about 6.1 g vs. 7.1 g in control and high-fat diet groups). However, our prior efforts to develop such models using high-fat diets for pregnant rats were unsuccessful. Other studies also indicated that maternal high fat feeding and maternal obesity had no effect on the birth weight of their offspring (Buckley et al. 2005; Shankar et al. 2008). Furthermore, mothers of LGA infants are not always obese women. As result, we have opted instead to obtain high-birth-weight pups from pregnant rats fed an ordinary diet. The birth weight classification and birth weight of LGA neonates in our study was comparable with the reference (Gelardi et al. 1990; Wang et al. 1994; Merzouk et al. 2000; de Assis et al. 2006). For example, Merzouk et al. (2000) reported that rat pups whose birth weights above the 90th percentile greater were 6.8 g. Accordingly, LGA neonate rats obtained in our model can represent a portion of the LGA maternal nutritional status.

In our high-birth-weight model, the weight of LGA pups was significantly higher than that of AGA pups. Although our study revealed a tendency toward a higher liver/body weight ratio in LGA pups than in AGA pups, these differences did not reach statistical significance. Because the liver is the major organ for drug biotransformation and elimination, any relative decrease in its size could have functional implications for the metabolism and elimination of drugs in newborns (Frattarelli et al. 2005; Allegaert et al. 2007). Furthermore, the relative low correlation between LGA hepatic and body weight also implied that the developmental pattern of liver weight in LGA neonates could be different from that of AGA pups. Additional studies using larger numbers of animals will be needed to confirm whether this trend has physiological significance.

Although CYP3A23/1 mRNA expression did not differ between the LGA and AGA pups, we did find that the staining intensity and frequency of CYP3A23/1-positive hepatocytes were higher in LGA pups than in AGA pups ($P < 0.05$). On the contrary, we found that CYP3A2 mRNA levels were higher in LGA pups than in AGA pups ($P < 0.05$). Meanwhile, staining intensity and frequency of CYP3A2-positive hepatocytes did not differ between LGA and AGA groups. These discrepancies imply that CYP3A23/1 and CYP3A2 are under different transcriptional and/or post-transcriptional regulation in the LGA condition. Consistent with this, CYP3A23/1 and CYP3A2 are known to be differentially regulated during development (Mahnke et al. 1997) and the CYP3A2 promoter binds a different set of transcription factors than does that of CYP3A23/1 (Huss et al. 1999). In addition, although rat CYP3A23/1 and CYP3A2 share 89% amino acid sequence similarity, three of five of their C-terminal residues are different, suggesting possible differences in post-translational modifications. Moreover, the close relationship between CYP3A23/1 and CYP3A2 mRNA suggests that there may be an interaction among isoforms that could also contribute to the discrepancy between mRNA and protein expression of CYP3A23/1 and CYP3A2.

The absence of PXR staining in both LGA and AGA tissues cannot be attributed to technical problems since staining was clearly evident in a positive control 21-day-old male rat. However, the possibility that the sensitivity of the antibody is limited cannot be ruled out. Although CAR and PXR share some common ligands and regulate overlapping sets of target genes (Roth et al. 2008), their modes of activation are distinct. PXR is located in the nucleus, has low basal activity and is highly activated upon ligand binding. In contrast, non-induced CAR resides in the cytoplasm. Activated CAR translocates to the nucleus to activate its target genes and CAR localization and activity are regulated by various protein phosphorylation events (Moreau et al. 2008). In our studies, CAR was investigated in its basal state and, accordingly, was observed primarily in the cytoplasm of hepatocytes.

Hepatic expression of HNF4α at the mRNA and protein levels was significantly higher in LGA than in AGA neonatal rats. HNF4α is critical for regulating glucose transport and glycolysis and is crucial for maintaining glucose homeostasis (Stoffel and Duncan 1997). It is a key transcription factor regulating responses to xenobiotics through activation of the PXR gene during fetal liver development and it is essential for morphological and functional differentiation of hepatocytes (Kamiya et al. 2003; Parviz et al. 2003). Our study revealed that PXR mRNA expression was strongly associated with HNF4α expression. These findings indicate that elevated HNF4α expression could alter PXR-mediated drug metabolism. The elevated expression of HNF4α in LGA pups could be a response to maternal nutrition status.

Our study did not reveal statistically significant correlations between CYP3A23/1, CYP3A2 and HNF4α, PXR and CAR mRNA levels in the LGA group. This suggests that the altered expression of CYP3A23/1 and CYP3A2 was probably due to other signaling pathways and may involved combined effects of HNF4α, PXR, and CAR in LGA rat pups.

Insulin concentrations may be higher in LGA than in AGA conditions (Evagelidou et al. 2006). Previous studies indicate that hepatic CYP3A levels are increased in experimentally generated diabetic rats and reduced to normal levels by insulin treatment (Yamazoe et al. 1989). A dose-dependent decrease in the relative abundance of hepatic CYP3A mRNA with increasing dosage of insulin has been reported in the cow (Lemley et al. 2008). However, in the present study, we did not observe suppression of CYP3A expression in LGA pups. Hence there may be another signaling pathway or a different modulation system in LGA pups.

The prevalence of LGA infants has increased over the last ten years, along with the increase in overweight adolescents (Surkan et al. 2004). LGA infants remain heavier and taller and show higher insulin concentrations than their AGA counterparts (Evagelidou et al. 2006). Children who are LGA at birth and are exposed to an intrauterine environment of diabetes or maternal obesity are prone to accumulate fat in early childhood and are at an increased risk of developing metabolic syndrome, characterized by hypertension, insulin resistance and hypertriglyceridemia

(Hediger et al. 1999; Boney et al. 2005). Childhood and adult obesity are among the cardiovascular risk factors now considered to be "programmed" by early life experience. Perhaps counter-intuitively, babies subjected to either an early life environment of nutritional deprivation or one over-rich in nutrients appear to be at risk. Indeed there is a 'U-shaped' relationship between birth weight and risk of adult obesity (Curhan et al. 1996). Changes in physiological, pathophysiological, and/or nutritional conditions can alter the expression of drug-metabolizing enzymes (Yoshinari et al. 2006; Hirunpanich et al. 2007). To date, however, the impact of maternal hyper-nutritional status/high infant birth weight on neonatal hepatic CYP3A expression has remained unknown. To the best of our knowledge, ours is the first study to describe the molecular changes in hepatic CYP3A and nuclear receptors, PXR, CAR and HNF4α that occur in LGA neonatal rats.

In conclusion, LGA affects hepatic expression of CYP3A23/1, CYP3A2 and HNF4α. These observations have potential implications for optimizing medication therapy in human LGA infants. Furthermore, these kinds of alterations could be fetally programmed and might lead to permanent ontogenic changes in drug response. Our findings provide a basis for additional studies on appropriate medication regimens in children born LGA.

4. Experimental

Experimental methods were based on our previous work with minor modifications (Ni et al., 2008).

4.1. Animal model

All procedures used in this study were approved and performed in accordance with the guidelines established by the Animal Ethics Committee of Zhejiang University School of Medicine. Pregnant, 12-week-old Sprague–Dawley (SD) rats (250–300 g) obtained at the 2nd day of pregnancy from Zhejiang Medical Science Academy (Hangzhou, China) were used to generate the offspring used in this study. The rats were housed in a 22 ± 1 °C environment at 60% humidity and were maintained on a 12 h light/12 h dark cycle.

4.2. Feeding protocol

The rats were housed individually in standard cages, and water and food were provided *ad libitum*. The rats were fed a standard commercial rat diet, provided fresh daily, containing (per 100 g) 22.5 g protein, 57.0 g carbohydrates, 3.9 g fat, 8.0 g cellulose, 1.0 g minerals, 5.0 g mixed vitamins and 2.5 g water. The body weight of pups was determined at birth. All the pups had not suckled and were sacrificed within 2 h of being born.

4.3. Sample treatment

Rat pups were categorized as AGA or LGA based on birth weight data obtained from the previous 1200 rats (100 broods) fed a standard commercial rat diet in our laboratory. AGA was defined as having a birth weight between the 10th and 89th percentiles, while LGA was defined as having a birth weight at or above the 90th percentile. The AGA birth-weight range was 5.2 to 7.2 g, and the LGA group included pups with a birth weight above 7.2 g. AGA (n = 15) and LGA (n = 15) pups were collected from 10 litters of rats. All animals were sacrificed by decapitation and exsanguination. Body and liver weights of pups were recorded. One portion of the liver was snap-frozen in liquid nitrogen and stored at −80 °C until ready for RNA extraction. The remainder of the liver was fixed in 10% neutral-buffered formalin for immunohistochemical examination.

4.4. RNA extraction and real-time PCR

Total RNA was extracted from approximately 20–30 mg liver tissue using the AxyPrep Multisource Total RNA Miniprep Kit (Axygen biosciences, CA 94587, USA) according to the manufacturer's protocol. The quality of the RNA was assessed by agarose gel electrophoresis and quantity was determined spectrophotometrically. cDNA was synthesized using oligo $(dT)_{18}$ (TaKaRa Biotechnology), dNTPs (TaKaRa Biotechnology), ribonuclease inhibitor (Bio Basic Inc) and M-MuLv reverse transcriptase (RevertAid™, Fermentas life sciences) as recommended by the supplied protocol.

Gene-specific primers (Table 2) were designed based on sequences deposited in GenBank. The PCR mixture included 10 μL SYBR Green PCR Master Mix reagent (SYBR Premix Ex Taq™, TaKaRa biotechnology), 0.4 μL 10 μM each primer, and 2 μL cDNA in a total volume of 20 μL. PCR reactions were performed twice in duplicate using a LightCycler System (Roche Applied Sciences). Thermocycling was performed using an initial ramp to 95 °C followed after 10 s by 40 cycles of 95 °C for 5 s (denature) and 60 °C for 20 s (anneal). After PCR, dissociation curves were established using the built-in melting curve program of the LightCycler to confirm the presence of a single PCR product. Standard curves were established by diluting a known concentration of cDNA from a 21-day-old male rat liver (10-fold dilutions over 5 logs) to evaluate the efficiency of PCR and determine R^2 values. The slope should be between −3.2 and −3.7 and $R^2 > 0.99$. To analyze results after real-time RT-PCR experiments, we quantified the target gene transcript relative to that of the references gene transcript, GAPDH, using the ΔC_T method ($2^{CT[GAPDH] - CT[target]}$), as described by the Bio-Rad real-time PCR protocol.

4.5. Immunohistochemistry

CYP3A23/1, CYP3A2, PXR and CAR proteins were detected using a two-step immunohistochemical technique with the DAKO Envision system. All formalin-fixed, paraffin-embedded liver tissues from the two groups were examined (n = 9 for each group). In each case, 4 μm-thick serial paraffin sections were transferred to silanized slides. After deparaffinization and hydration with xylene and graded alcohols, the antigens were unmasked by heating under pressure for 100 s in 0.01 mol/L citrate buffer, pH 6.0. Endogenous peroxidase activity was quenched by placing slides in 3% hydrogen peroxide for 10 min. After washing with phosphate-buffered saline (PBS), the slides were incubated overnight at 4 °C in a humidified atmosphere with rabbit anti-rat primary antibodies against CYP3A23/1 (AB1253, Chemicon, CA, USA; 1 : 150 dilution), CYP3A2 (AB1276, Chemicon, CA, USA; 1 : 200 dilution), PXR (sc-25381, Santa Cruz, CA, USA; 1 : 50 dilution) and CAR (sc-13065, Santa Cruz, CA, USA; 1 : 50 dilution). After incubating with primary antibodies, cells were labeled with a goat anti rabbit peroxidase (HRP)-labeled polymer IHC kit (DAKO, Glostrup, Denmark) as described by the manufacturer. The sections were visualized by peroxidase-diaminobenzidine staining and then counterstained with hematoxylin for 1 min.

HNF4α was detected immunohistochemically using a goat polyclonal primary antibody against HNF4α (sc-6556, Santa Cruz) and a biotinylated secondary antibody followed by streptavidin-peroxidase detection of an HRP–Streptavidin complex (immunoCruz™ staining system SC-2053, Santa Cruz). The staining procedure was different from that described above as follows: 1) the unmasking antigen buffer was 0.01 mol/L EDTA, pH 9.0; 2) the blocking solution contained goat serum; and 3) the primary antibody dilution was 1 : 75.

As blank controls, sections were incubated with PBS instead of the primary antibody. For negative controls, appropriate volumes of normal rabbit or goat serum were substituted for the primary antibody to rule out the possibility of a false-positive response by the secondary antibody. A liver from a 21-day-old male rat was used as a positive control. Images were captured with a digital camera mounted to an Olympus microscope (BX 60 Olympus Optical Co., Japan).

Table 2: Quantitative RT-PCR primers and their products

Gene	Sense	Antisense	Product Length (bp)
CYP3A23/1	GATGTTGAAATCAATGGTGTGT	TTTCAGAGGTATCTGTGTTT	290
CYP3A2	AGTAGTGACGATTCCAACATAT	TCAGAGGTATCTGTGTTTCTT	251
PXR	GAGCTCTGGGCAGAAACATCATC	GGCCAACATGGTTCCACCTC	146
CAR	CATTTCCATGCCCTGACTTGTG	AGGCTGGACAATGGCGTCTC	133
HNF4α	CGGGCTGGCATGAAGAAAG	AGCGCATTAATGGAGGGTAGG	101
GAPDH	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA	143

Samples were analyzed by calculating the percentage of positive cells in five fields after viewing at 200× magnification with an Olympus microscope (BX 60 Olympus Optical Co., Japan). Protein expression was evaluated using a score corresponding to the sum of both (a) staining intensity (0, negative; 1, weak; 2, intermediate; 3, strong) and (b) percentage of positive hepatocytes (0, 0% positive; 1, $< 25\%$ positive; 2, ≥ 25 and $\leq 50\%$ positive; 3, $> 50\%$ positive). Using summed score values (maximum possible = 6), samples were classified as "−" (0), "+" (1–2), "++" (3–4) and "+ + +" (5–6). A score greater than 2 was regarded as positive (Lee et al. 2003). Each sample was scored in duplicate by two blinded observers.

4.6. Statistical analysis

Statistical analyses were performed with R software 2.6.0. Group differences in mRNA and protein expression levels were detected by an independent-sample t-test or Mann-whitney U tests as appropriate. Pearson or spearman's rho (data points with skewed distributions) correlation coefficients were used to compare correlations between groups as appropriate. A P value less than 0.05 was considered significant in all cases.

Acknowledgement: The authors would like to thank technician Weizhong Gu and Yidong Wu for technical assistance. This work was supported by a grant from the natural science foundation of Zhejiang province (Y2080183).

References

Ahlsson FS, Diderholm B, Ewald U, Gustafsson J (2007) Lipolysis and insulin sensitivity at birth in infants who are large for gestational age. Pediatrics 120: 958–965.

Allegaert K, Anderson BJ, van den Anker JN, Vanhaesebrouck S, de Zegher F (2007) Renal drug clearance in preterm neonates: relation to prenatal growth. Ther Drug Monit 29: 284–291.

Boney CM, Verma A, Tucker R, Vohr BR (2005) Metabolic syndrome in childhood: association with birth weight, maternal obesity, and gestational diabetes mellitus. Pediatrics 115: e290–296.

Buckley AJ, Keseru B, Briody J, Thompson M, Ozanne SE, Thompson CH (2005) Altered body composition and metabolism in the male offspring of high fat-fed rats. Metabolism 54: 500–507.

Cooper KO, Reik LM, Jayyosi Z, Bandiera S, Kelley M, Ryan DE, Daniel R, McCluskey SA, Levin W, Thomas PE (1993) Regulation of two members of the steroid-inducible cytochrome P450 subfamily (3A) in rats. Arch Biochem Biophys 301: 345–354.

Curhan GC, Chertow GM, Willett WC, Spiegelman D, Colditz GA, Manson JE, Speizer FE, Stampfer MJ (1996) Birth weight and adult hypertension and obesity in women. Circulation 94: 1310–1315.

de Assis S, Khan G, Hilakivi-Clarke L (2006) High birth weight increases mammary tumorigenesis in rats. Int J Cancer 119: 1537–1546.

Evagelidou EN, Kiortsis DN, Bairaktari ET, Giapros VI, Cholevas VK, Tzallas CS. Andronikou SK (2006) Lipid profile, glucose homeostasis, blood pressure, and obesity-anthropometric markers in macrosomic offspring of nondiabetic mothers. Diabetes Care 29: 1197–1201.

Frattarelli DA, Ergun H, Lulic-Botica M, Lehr VT, Aranda JV (2005) Vancomycin elimination in human infants with intrauterine growth retardation. Pediatr Infect Dis J 24: 979–983.

Gelardi NL, Cha CJ, Oh W (1990) Glucose metabolism in adipocytes of obese offspring of mild hyperglycemic rats. Pediatr Res 28: 641–645.

Hediger ML, Overpeck MD, McGlynn A, Kuczmarski RJ, Maurer KR, Davis WW (1999) Growth and fatness at three to six years of age of children born small- or large-for-gestational age. Pediatrics 104: e33.

Hirunpanich V, Sethabouppha B, Sato H (2007) Inhibitory effects of saturated and polyunsaturated fatty acids on the cytochrome P450 3A activity in rat liver microsomes. Biol Pharm Bull 30: 1586–1588.

Huss JM, Wang SI, Kasper CB (1999) Differential glucocorticoid responses of CYP3A23 and CYP3A2 are mediated by selective binding of orphan nuclear receptors. Arch Biochem Biophys 372: 321–332.

Johnson TN (2003) The development of drug metabolising enzymes and their influence on the susceptibility to adverse drug reactions in children. Toxicology 192: 37–48.

Kamiya A, Inoue Y, Gonzalez FJ (2003) Role of the hepatocyte nuclear factor 4alpha in control of the pregnane X receptor during fetal liver development. Hepatology 37: 1375–1384.

Lee CH, Lee MK, Kang CD, Kim YD, Park DY, Kim JY, Sol MY, Suh KS (2003) Differential expression of hypoxia inducible factor-1 alpha and tumor cell proliferation between squamous cell carcinomas and adenocarcinomas among operable non-small cell lung carcinomas. J Korean Med Sci 18: 196–203.

Lemley CO, Butler ST, Butler WR, Wilson ME (2008) Short communication: insulin alters hepatic progesterone catabolic enzymes cytochrome P450 2C and 3A in dairy cows. J Dairy Sci 91: 641–645.

Mahnke A, Strotkamp D, Roos PH, Hanstein WG, Chabot GG, Nef P (1997) Expression and inducibility of cytochrome P450 3A9 (CYP3A9) and other members of the CYP3A subfamily in rat liver. Arch Biochem Biophys 337: 62–68.

Merzouk H, Madani S, Chabane Sari D, Prost J, Bouchenak M, Belleville J (2000) Time course of changes in serum glucose, insulin, lipids and tissue lipase activities in macrosomic offspring of rats with streptozotocin-induced diabetes. Clin Sci (Lond) 98: 21–30.

Moreau A, Vilarem MJ, Maurel P, Pascussi JM (2008) Xenoreceptors CAR and PXR activation and consequences on lipid metabolism, glucose homeostasis, and inflammatory response. Mol Pharm 5: 35–41.

Ni S, Wang X, Wang J, Lu S, Zeng S, Zhao Z, Yu L, Chen S (2008) Effects of interauterine undernutrition on the expression of CYP3A23/3AI, PXR, CAR and HNF4alpha in neonate rats. Biopharm Drug Dispos 29: 501–510.

Parviz F, Matullo C, Garrison WD, Savatski L, Adamson JW, Ning G, Kaestner KH, Rossi JM, Zaret KS, Duncan SA (2003) Hepatocyte nuclear factor 4alpha controls the development of a hepatic epithelium and liver morphogenesis. Nat Genet 34: 292–296.

Pikuleva IA (2006) Cytochrome P450s and cholesterol homeostasis. Pharmacol Ther 112: 761–773.

Roth A, Looser R, Kaufmann M, Blattler SM, Rencurel F, Huang W, Moore DD, Meyer UA (2008) Regulatory cross-talk between drug metabolism and lipid homeostasis: constitutive androstane receptor and pregnane X receptor increase Insig-1 expression. Mol Pharmacol 73: 1282–1289.

Shankar K, Harrell A, Liu X, Gilchrist JM, Ronis MJ, Badger TM (2008) Maternal obesity at conception programs obesity in the offspring. Am J Physiol Regul Integr Comp Physiol 294: R528–538.

Sidhu JS, Omiecinski CJ (1999) Insulin-mediated modulation of cytochrome P450 gene induction profiles in primary rat hepatocyte cultures. J Biochem Mol Toxicol 13: 1–9.

Stoffel M, Duncan SA (1997) The maturity-onset diabetes of the young (MODY1) transcription factor HNF4alpha regulates expression of genes required for glucose transport and metabolism. Proc Natl Acad Sci USA 94: 13209–13214.

Surkan PJ, Hsieh CC, Johansson AL, Dickman PW, Cnattingius S (2004) Reasons for increasing trends in large for gestational age births. Obstet Gynecol 104: 720–726.

Thummel KE, Wilkinson GR (1998) In vitro and in vivo drug interactions involving human CYP3A. Annu Rev Pharmacol Toxicol 38: 389–430.

Tirona RG, Lee W, Leake BF, Lan LB, Cline CB, Lamba V, Parviz F, Duncan SA, Inoue Y, Gonzalez FJ, Schuetz EG, Kim RB (2003) The orphan nuclear receptor HNF4alpha determines PXR- and CAR-mediated xenobiotic induction of CYP3A4. Nat Med 9: 220–224.

Wang X, Guyer B, Paige DM (1994) Differences in gestational age-specific birthweight among Chinese, Japanese and white Americans. Int J Epidemiol 23: 119–128.

Yamazoe Y, Murayama N, Shimada M, Yamauchi K, Kato R (1989) Cytochrome P450 in livers of diabetic rats: regulation by growth hormone and insulin. Arch Biochem Biophys 268: 567–575.

Yoshinari K, Takagi S, Yoshimasa T, Sugatani J, Miwa M (2006) Hepatic CYP3A expression is attenuated in obese mice fed a high-fat diet. Pharm Res 23: 1188–1200.

Yu AM, Fukamachi K, Krausz KW, Cheung C, Gonzalez FJ (2005) Potential role for human cytochrome P450 3A4 in estradiol homeostasis. Endocrinology 146: 2911–2919.