Table 5
Changes in nuclear receptors genes expression in dam's liver, fetal liver, and placenta of pregnant rats treated with PCN

Gene	Accession no.		Fold change	*t* Test
Nuclear receptor subfamily 1, group 1, member 2 (Nr1i2)	NM_052980	Dam's liver	1.336*	0.000
		Fetal liver	0.963	0.622
		Placenta	1.093	0.753
Nuclear receptor (CAR) (Nr1i3)	NM_022941	Dam's liver	1.102	0.567
		Fetal liver	1.050	0.787
		Placenta	1.109	0.785

* $P < 0.05$.

normalization was performed in each experimental datum, fold changes (average of signals of treated groups/average of signals of control groups) were calculated. Students *t* test or Welchs *t* test was done. In this study, we picked up the probes focusing on drug metabolizing CYPs isozymes (Table 1) and two orphan nuclear receptors for CYP inducers, Nr1i2 and Nr1i3. Among CYP isozymes examined, probes of which significance level was $P < 0.05$ and Absolute Call was present, were picked up.

Reverse transcriptase polymerase chain reaction (RT–PCR) for CYP3A1 mRNA

Total RNA was prepared as described above. For RT–PCR analysis, we selected CYP3A1 gene because it was the only gene which was commonly up-regulated in dam's liver, placenta, and fetal liver. PCR was performed with pairs of oligonucleotide primers corresponding to the cDNA sequences of the rat mRNA. PCR was carried out with 1 μl of cDNA sample in a 100-μl reaction mixture containing 50 pM of sense and antisense primer, 1.25 U of rTaq, 10 × PCR buffer, and dNTP mixture (Takara, Ohtsu, Japan). This was immediately followed by preheating at 95°C for 7 min, denaturation at 95°C for 1 min, annealing for 1 min, and extension at 72°C for 1 min using Takara PCR Thermal Cycler SP (Takara). Annealing temperatures and cycle numbers are shown in Table 2. Optimal cycle numbers were determined in a preliminary experiment to ensure that the amplification was in the linear range and not during the plateau phase. PCR products were identified by electrophoresis on 2% agarose gel (Nippon Gene Co. Ltd.) followed by ethidium bromide (Invitrogen) staining. Fluorescent-gel imaging was carried out using an ultraviolet-CCD video system Fas-III (Toyobo, Tokyo, Japan). The relative band density against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was represented as the mean ± standard deviation (SD) for three dams, and statistical analysis was carried out using Students *t* test or Welchs *t* test.

Results

Histopathological findings

In the dam's liver, there were no histopathological changes in both CO and Sa groups (Figs. 1a, c). In PCN group, slight swelling of hepatocytes was observed (Fig. 1b). After the PB treatment, moderate vacuolization of hepatocytes was observed (Fig. 1d).

In the fetal liver, slight swelling of hepatocytes was observed in PCN group (Fig. 1f). There were no histopathological changes in CO (Fig. 1e), Sa, and PB groups. In the placenta, there were no histopathological changes in all groups.

Findings of microarray analysis

The selected results of microarray analysis on CYPs in the dam's liver, placenta and fetal liver are shown in Tables 3 and 4. Among 40 probes for drug metabolizing CYPs isozymes, probes showing significant changes were picked up. In Tables 5 and 6, the results of microarray analysis on two orphan nuclear receptors are shown.

In dam's liver, the gene expression of CYP3a18, Cyp3A1 (CYP3A subfamily), and cytochrome P-450e (CYP2B subfamily) prominently increased in PCN group (Table 3). The gene expression of Cyp2a1 (CYP2A subfamily) increased moderately, and that of Cyp2f1 (CYP2F subfamily), CYP4F4 (CYP4F subfamily), p450Md mRNA for cytochrome P-450 (CYP2C subfamily), CYP3A9 (CYP3A subfamily), and Cyp2b3 (CYP2B subfamily) also showed a

Table 6
Changes in nuclear receptors genes expression in dam's liver, fetal liver, and placenta of pregnant rats treated with PB

Gene	Accession no.		Fold change	*t* test
Nuclear receptor subfamily 1, group 1, member 2 (Nr1i2)	NM_052980	Dam's liver	0.901	0.265
		Fetal liver	1.000	0.998
		Placenta	0.982	0.957
Nuclear receptor (CAR) (Nr1i3)	NM_022941	Dam's liver	1.167	0.390
		Fetal liver	1.263	0.127
		Placenta	1.752	0.189

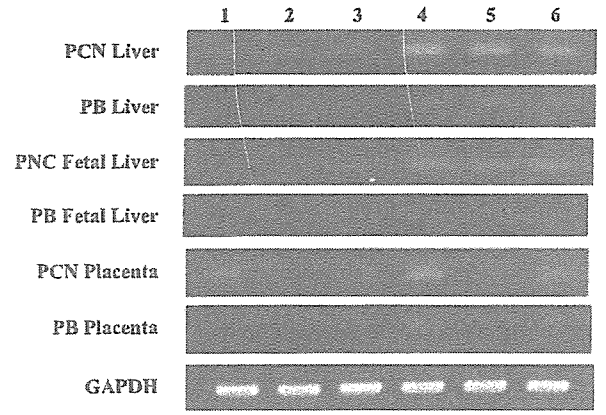

Fig. 2. The expression of CYP3A1 mRNA by RT–PCR in dam's liver, fetal liver, and placenta. Agarose gel electrophoresis. 1–3, rat number of control group; 4–6, rat number of treated group.

tendency of significant increase (Table 3). On the other hand, the gene expression of cytochrome P-450 ISF/BNF-G (CYP 1A subfamily) and Cyp4a1 (CYP4A subfamily) decreased in PCN group (Table 3). In PB group, the gene expression of Cyp3A1 and cytochrome P-450e prominently increased (Table 4), and that of Cyp2f1, Cyp2a1, Cyp2d2 (CYP2D subfamily), and cytochrome P450 PB1 (CYP2C subfamily) also showed a tendency of significant increase (Table 4). On the other hand, the gene expression of cytochrome P-450 ISF/BNF-G and Cyp2e1 (CYP2E subfamily) decreased in PB group (Table 4).

In fetal liver, the gene expression of Cyp3A1, pregnenolone 16-alpha-carbonitrile-inducible cytochrome P450 (CYP3A subfamily), and p450Md mRNA for cytochrome P-450 prominently increased in PCN group (Table 3), and that of Cyp2a1 also significantly increased (Table 3). On the other hand, significantly down-regulated genes coding CYPs were not detected in PCN group (Table 3). In PB group, the gene expression of Cyp3A1 and cytochrome P-450e prominently increased (Table 4), and that of Cyp2a1 showed a tendency of increase. The expression of CYP4A3 gene (CYP 4A subfamily) showed a tendency of decrease (Table 4).

In placenta, the expression of Cyp3A1 gene showed a non-significant increase in PCN group (Table 3), while it showed a significant increase in PB group (Table 4). There were no significantly down-regulated genes detected in either PCN or PB groups (Tables 3 and 4).

The expression of Nr1i2 gene was significantly elevated only in dam's liver of PCN group (Table 5), and the expression of Nr1i3 gene did not show significant changes in any groups (Table 6).

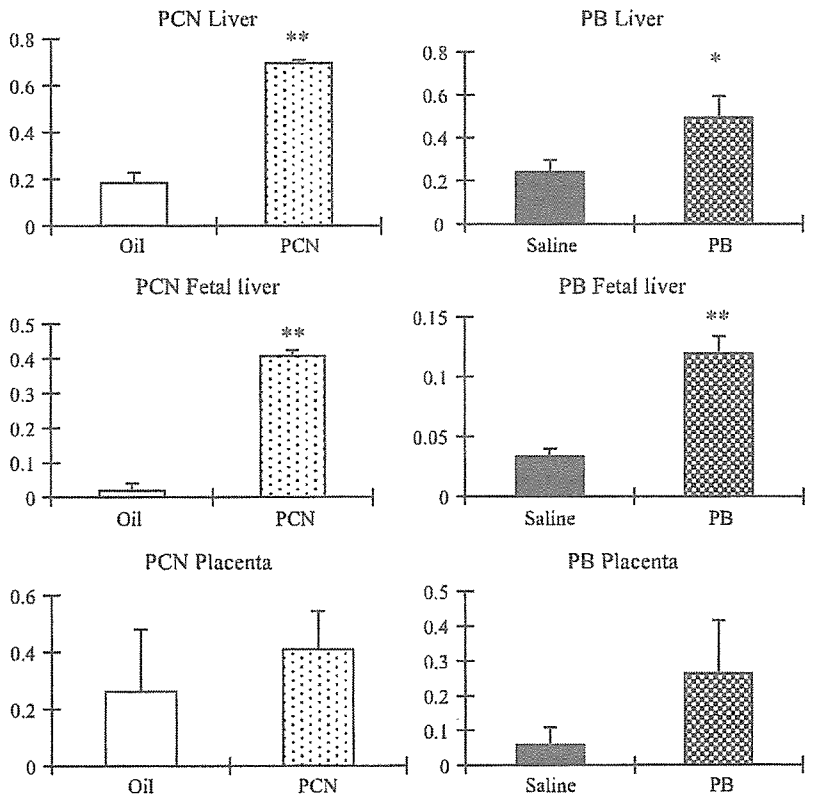

Fig. 3. The relative CYP3A1 band density to GAPDH by RT–PCR in dam's liver, fetal liver, and placenta. (□): CO group; (▨): PCN group; (■): Sa group; (▩): PB group. *$P < 0.05$ and **$P < 0.01$: significantly different from the control group.

Findings of RT–PCR for CYP3A1 mRNA

We performed RT–PCR on CYP3A1 mRNA, because it was the only gene which showed an up-regulation commonly in dam's and fetal livers and placenta. As shown in Figs. 2 and 3, a significant increase in CYP3A1 mRNA expression was observed in dam's and fetal livers. Although not significant, CYP3A1 mRNA expression also increased in placenta.

Discussion

Previously, we have reported the expression of nine CYP isozymes protein, against which antibodies were commercially available, in pregnant rat's liver, placenta, and fetal liver after treatment with dexamethasone, PCN or PB by Western blot analysis and immunohistochemistry (Ejiri et al., 2003, in press). In this study, the expression of CYPs genes was examined after PCN or PB treatment by microarray analysis, which can analyze 40 drug metabolizing CYP isozymes gene expression at one time.

In PCN group, the expression of Cyp3A1 gene was mainly elevated in both dam's and fetal livers, and its degree was more prominent in the latter. These findings of Cyp3A1 gene expression corresponded to the results of CYP3A1 protein expression (Ejiri et al., 2003). In placenta, although CYP3A1 protein was hardly induced by PCN (Ejiri et al., 2003), Cyp3A1 gene expression showed a mild but not significant increase. On the other hand, the expression of cytochrome P-450e and Cyp2b3 genes increased in dam's liver but not in fetal liver. This may be due to the difference of drug metabolizing enzyme profiles between dams and fetuses.

In PB group, the expressions of Cyp3a1 and cytochrome P-450e genes were elevated mainly in dam's and fetal livers. In our previous study on the inductions of CYPs proteins by PB, CYP3A1 protein was clearly induced in fetal liver by Western blot analysis, while CYP2B1 protein was not detected by either Western blot analysis or immunohistochemical staining (Ejiri et al., in press). Cyp3A1 gene expression in placenta significantly increased in PB group in the present study, but significant changes were not observed in Western blot analysis and immunohistochemical staining (Ejiri et al., in press).

In our previous report, the expression of CYP2B1 protein in dam's liver was observed in PB group but not in control group, and it was not detected in fetal liver even in PB group (Ejiri et al., in press). Among four probes for CYP2B subfamily, only cytochrome P-450e (phenobarbital-inducible) gene showed an apparently significant increase in this study. The relation between the increased gene expression of cytochrome P-450e and the protein expression of CYP2B1 in our previous report is still obscure.

As mentioned above, the existence of the orphan nuclear receptors, which induce CYPs by some inducers such as PCN or PB, is attracting researchers' attention (Masuyama et al., 2000; Mikamo et al., 2003; Xiong et al., 2002; Zhang et al., 1999). For example, it is said that Nr1i2 is concerned with an induction of CYP3A subfamily by PCN, and Nr1i3 is concerned with an induction of CYP2B subfamily by PB. In this study, a significant elevation in Nr1i2 gene expression was observed only in dam's liver of PCN group, while Nr1i3 gene expression showed no significant changes in all groups. In dam's liver, as mentioned above, Nr1i2 was induced in PCN group, followed by significant increase of Cyp3A1 gene expression. However, in fetal liver, Nr1i2 was not induced in PCN group, but a prominent induction of Cyp3A1 was observed. In addition, in dam's liver, although the expressions of cytochrome P-450e and Cyp3A1 genes were induced in PB group, the expression of Nr1i3 gene did not show significant change. Further study should be done to clarify the relationship between orphan receptors and CYPs gene expression.

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Microarray analysis on Phase II drug metabolizing enzymes expression in pregnant rats after treatment with pregnenolone-16α-carbonitrile or phenobarbital

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Abstract

We previously reported the expression profiles of 9 cytochrome P450 isozymes (CYPs) proteins and those of 40 CYPs genes in pregnant rat's liver, placenta and fetal liver after treatment with pregnenolone-16α-carbonitrile (PCN) or phenobarbital (PB). This study was carried out focusing on the gene expression profiles of Phase II drug metabolizing enzymes, Glutathione *S*-transferase isozymes (GSTs) and UDP-glycosyltransferase isozymes (UDPGTs). Fischer 344 (F344) pregnant rats were daily treated intraperitoneally with 50 mg/kg of PCN or 80 mg/kg of PB from 13 to 16 days of gestation (DG). They were sacrificed on 17 DG, and microarray analysis using Affymetrix Rat Expression Array 230 A was performed. Among 16 GSTs genes examined in this study, 7 genes were significantly induced in dam's liver and 3 genes in fetal liver, respectively, in the PCN-group, while 8 genes were significantly induced in dam's liver and 1 gene in fetal liver, respectively, in the PB-group. On the other hand, among 11 UDPGTs genes examined, 5 genes were significantly induced in dam's liver and 3 genes in fetal liver, respectively, in the PCN-group, while 5 genes were significantly induced in dam's liver and 1 gene in fetal liver, respectively, in the PB-group. There were no significant changes in the placenta of all groups. This is the first report of the gene expression profiles of Phase II drug metabolizing enzymes in pregnant rat and fetal livers and placenta after treatment with typical inducers of drug metabolizing enzymes.

Keywords: GSTs; Microarray analysis; Pregnenolone-16α-carbonitrile (PCN); Phenobarbital; Pregnant rat; UDPGTs

Introduction

There are two steps for drug metabolism; Phase I and Phase II reactions. Cytochrome P450 isozymes (CYPs) are one of the representative enzymes in Phase I reaction. Previously, we reported that CYP3A1 was detected in rat placenta through pregnancy (Ejiri et al., 2001). After that, we examined the induction of CYPs proteins in pregnant rat liver, placenta and fetal liver by Western blot analysis and immunohistochemistry using commercially available antibodies against nine CYPs after treatment with pregnenolone-16α-carbonitrile (PCN), dexamethasone (DEX) and phenobarbital (PB) (Ejiri et al., 2003, 2005b). As a result, CYP3A1 protein was induced by PCN and DEX in dam's and fetal livers, with no prominent induction in the placenta (Ejiri et al., 2003). In addition, after PB injection, CYP3A1 protein was significantly induced, CYP2B1 protein was detected and CYP2D1 protein was reduced in dam's liver, and CYP3A1 and CYP2C6 proteins were induced in fetal liver (Ejiri et al., 2005b). In the placenta, no significant induction of CYPs was observed after PB treatment. After those studies, using DNA microarray method, we examined the gene expressions of CYPs in dam's liver, placenta and fetal liver after treatment with PCN and PB (Ejiri et al., 2005a). Ten genes expression significantly increased in dam's liver in the PCN-group, and seven genes expression in the PB-group, respectively. On the other hand, four genes expression increased in fetal liver in the PCN-group, and three genes in the PB-group, respectively. Being common to dam's and fetal livers, the gene expression of CYP3A subfamily and CYP2E subfamily increased in both PCN and PB-groups. In the placenta, only Cyp3A1 gene expression was significantly induced in the PB-

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group, and it also showed a tendency to increase in the PCN-group (Ejiri et al., 2005a).

In this study, we carried out DNA microarray analysis focusing on the expression of Phase II drug metabolizing enzymes, especially Glutathione *S*-transferase isozymes (GSTs) and UDP-glycosyltransferase isozymes (UDPGTs), in dam's liver, placenta and fetal liver after treatment with PCN and PB. Both PB and PCN are known to induce not only CYPs but also GSTs and UDPGTs (Bulera et al., 2001: Longueville et al., 2002; Parkinson, 1996). The protocol of this study was approved by the Animal Care and Use Committee of Graduate School of Agricultural and Life Science, The University of Tokyo.

Materials and methods

Animals

Twelve pregnant rats of the Fischer 344 (F344) strain were purchased from Saitama Experimental Animal Co. (Saitama, Japan). The animals were individually housed in plastic cages in an animal room under controlled conditions (temperature: 23 ± 2°C; relative humidity: 55 ± 5%; light/dark cycle: 14/10 h), and fed commercial pellets (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water ad libitum. The day of a vaginal plug being recognized was designated as 0 day of gestation (0 DG).

Chemicals

Pregnenolone-16α-carbonitrile (PCN) was purchased from Sigma Chemical Co. (St. Louis, MO), and phenobarbital sodium (PB) and corn oil from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). PCN was dissolved in corn oil and PB was dissolved in physiological saline immediately before use, and the concentration was adjusted to 50 mg/ml for PCN and 80 mg/ml for PB, respectively.

Treatments

From 13 to 16 DG, 3 dams were daily treated intraperitoneally with 50 mg/kg of PCN (PCN-group), 3 dams with 80 mg/kg of PB (PB-group), 3 dams with 0.1 ml/kg of corn oil alone (CO-group) as control for PCN-group and 3 dams with 0.1 ml/kg of saline alone (Sa-group) as control for PB-group, respectively. All animals were sacrificed by exsanguination under ether anesthesia on 17 DG.

RNA extraction and microarray analysis

Dam's liver, fetal liver and placenta were cut into slices less than 5 mm thick as soon as possible. Then, the slices were submerged in the RNA*later* RNA Stabilization Reagent. After incubated at 4°C for overnight, samples were stored at −80°C until used. Total RNA was extracted using the RNeasy Mini Kit (QIAGEN Inc., CA, USA) from frozen tissues according to the manufacturer's instructions. Microarray analysis was performed according to the Affymetrix protocol. Briefly, of total RNA, 10 μg was used for cDNA synthesis using the T7-$(dT)_{24}$ primer (Primer sequence: 5′-GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGG CGG -$(dT)_{24}$-3′). Following this, biotin-labeled cRNA was synthesized from the cDNA using the Enzo High Yield RNA Transcription Labeling Kit (Enzo Diagnostics, NY, USA). Then 25 μg of biotin-labeled cRNA was fragmented and stored at −20°C until ready to perform hybridization. The hybridization solution was prepared using GeneChip Eukaryotic Hybridization Control Kit (Affymetrix), and was hybridized to the Affymetrix Rat Expression Array 230 A at 45°C for 16 h in GeneChip Hybridization Oven 640 (Affymetrix). The chips were washed and stained using the Fluidics Station (Affymetrix) and scanned with GeneArray Scanner.

Data analysis

The microarray imaging data were analyzed using the Microarray Suite 4.0 (Affymetrix) and Spotfire Pro Version 4.2 program (Spotfire Inc., MA, USA).

Table 1
GST and UDPGT isozymes examined

		Genes	Probe ID	Accession no.
GST	Alpha	Glutathione *S*-transferase Yc1 subunit	1367774_at	NM_031509
		Glutathione *S*-transferase Yc2 subunit	1371089_at	AA945082
		Glutathione *S*-transferase mRNA	1368180_s_at	NM_017013
		Similar to GLUTATHIONE *S*-TRANSFERASE 8 (GST 8–8)	1372297_at	AI234527
	Mu	Glutathione *S*-transferase, mu 1 (Gstm1)	1386985_at	M28241
		Glutathione *S*-transferase Yb4 gene (GstYb4)	1369921_at	NM_020540
		Glutathione *S*-transferase, mu type 2 (Yb2) (Gstm2)	1370952_at	AI169331
		Glutathione *S*-transferase, mu type 3 (Yb3) (Gstm3)	1387023_at	NM_031154
		Glutathione *S*-transferase, mu 5 (Gstm5)	1370813_at	U86635
	Pi	Glutathione *S*-transferase, pi 1 (Gstp1)	1388122_at	X02904
	Theta	Glutathione *S*-transferase 1 (theta) (Gstt1)	1368354_at	NM_053293
		Glutathione *S*-transferase, theta 2 (Gstt2)	1368409_at	NM_012796
	Kappa	Similar to GTK1 RAT GLUTATHIONE *S*-TRANSFERASE	1398378_at	AI231779
	Unknown	Microsomal glutathione *S*-transferase 3	1388300_at	AA892234
		Microsomal glutathione *S*-transferase 2	1372599_at	BI290559
		Microsomal glutathione *S*-transferase 1 (Mgst1)	1367612_at	NM_134349
UDPGT	UDPGT-1	UDP glycosyltransferase 1 family, polypeptide A1 (Ugt1a1)	1370613_s_at	AF461738
		UDP glycosyltransferase 1 family, polypeptide A1 (Ugt1a1)	1387759_s_at	J02612
	UDPGT-2	UDP glycosyltransferase 2 family, polypeptide A1 (Ugt2a1)	1369850_at	NM_022228
		UDP-glucuronosyltransferase, phenobarbital-inducible form (UDPGTR-2)	1370698_at	M13506
		UDP-glucuronosyltransferase	1370615_at	U27518
		Androsterone UDP-glucuronosyltransferase (Ugt2b2)	1387825_at	NM_031533
		EST	1385247_at	AA858993
		UDP-glucuronosyltransferase mRNA, complete cds	1387955_at	M31109
		UDP-glucuronosyltransferase (UGT2B12)	1368397_at	NM_031980
	UDP-galactose	Ceramide UDP-galactosyltransferase	1368858_at	L21698
		UDP-glucuronosyltransferase 8 (Ugt8)	1368857_at	NM_019276

Table 2
Primer sequences, cycle numbers and annealing temperature

Gene	Sequence		Cycle number			Annealing temperature
			Liver	Fetal liver	Placenta	
GST alpha	Sense	GCATCAAACTCTTCAACATA	21	25	29	55°C
	Antisense	CTCAACTACATCGCCACCAA				
UDPGT-2	Sense	CTGCGGAAAGGTGTTGGTAT	22	25	30	61°C
	Antisense	GGAGAGAAGCGAAGACTGTA				
GAPDH	Sense	GAGTATGTCGTGGAGTCTACTG	22	22	22	58°C
	Antisense	GCTTCACCACCTTCTTGATGTC				

After global normalization was performed in each experimental datum, fold changes (average of signals of treated groups/average of signals of control groups) were calculated. Student's *t* test or Welch's *t* test was done. In this study, we picked up the probes focusing on Phase II drug metabolizing enzymes, especially GSTs and UDPGTs (Table 1). Among those isozymes which we examined, probes of which significance level was $P < 0.05$ and Absolute Call was present were picked up.

Reverse transcriptase polymerase chain reaction (RT-PCR) for GST and UDPGT mRNAs

Total RNA was prepared as described above. For RT-PCR analysis, we selected GST alpha and UDPGT-2 genes because they were up-regulated in both dam's liver and fetal liver. PCR was performed with pairs of oligonucleotide primers corresponding to the cDNA sequences of the rat mRNA. PCR was carried out with 1 μl of cDNA sample in a 100 μl reaction mixture containing 50 pM of sense and antisense primers, 1.25 U of rTaq, 10× PCR buffer and dNTP mixture (Takara, Ohtsu, Japan). This was immediately followed by preheating at 95°C for 7 min, denaturation at 95°C for 1 min, annealing for 1 min and extension at 72°C for 1 min using Takara PCR Thermal Cycler SP (Takara). Annealing temperatures and cycle numbers are shown in Table 2. Optimal cycle numbers were determined in a preliminary experiment to ensure that the amplification was in the linear range and not during the plateau phase. PCR products were identified by electrophoresis on 2% agarose gel (Nippon Gene Co. Ltd.) followed by ethidium bromide (Invitrogen) staining. Fluorescent-gel imaging was carried out using an ultraviolet-CCD video system Fas-III (Toyobo, Tokyo, Japan). The relative band density against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was represented as the mean ± standard deviation (SD) for three dams, and statistical analysis was carried out using Student's *t* test or Welch's *t* test.

Results and discussion

As mentioned before, we have reported the expression profiles of 9 CYPs proteins and those of 40 CYPs genes in pregnant rat's liver, placenta and fetal liver after treatment with PCN or PB by Western blot analysis, immunohistochemistry and DNA microarray analysis (Ejiri et al., 2003, 2005a,b). This study was carried out focusing on the gene expression profiles of Phase II drug metabolizing enzymes, GSTs and UDPGTs.

The selected results of microarray analysis on GSTs and UDPGTs in dam's liver, placenta and fetal liver are shown in Tables 3 and 4. Among 16 probes for drug metabolizing GSTs and 11 probes for UDPGTs, probes showing significant changes in their expression were picked up.

In dam's liver of the PCN-group, the expression of 5 GSTs genes was strongly increased; Glutathione *S*-transferase mu 1 (Gstm1) and Glutathione *S*-transferase mu type 2 (Yb2) (Gstm2) (GST mu class), Glutathione *S*-transferase Yc2 subunit (GST Yc2), Glutathione *S*-transferase (GST) and

Table 3
Gene expression changes of Phase II drug metabolizing enzymes in the dam liver, placenta and fetal liver treated with PCN

		Genes	Fold changes	*t* test	Accession no.
Dam's liver					
Up-regulated	GST mu	Glutathione *S*-transferase, mu 1 (Gstm1)	8.416	0.000	M28241
		Glutathione *S*-transferase, mu type 2 (Yb2) (Gstm2)	4.482	0.000	AI169331
	GST alpha	Glutathione *S*-transferase Yc1 subunit	1.286	0.020	NM_031509
		Glutathione *S*-transferase Yc2 subunit	6.472	0.001	AA945082
		Glutathione *S*-transferase	3.152	0.000	NM_017013
		Glutathione *S*-transferase 8 (GST 8–8) (CHAIN 8) (GST CLASS-ALPHA)	1.965	0.005	AI234527
	Unknown	Microsomal glutathione *S*-transferase 2	1.943	0.002	BI290559
	UDPGT-1	UDP glycosyltransferase 1 family, polypeptide A1 (Ugt1a1)	3.958	0.000	AF461738
		UDP glycosyltransferase 1 family, polypeptide A1 (Ugt1a1)	4.637	0.000	J02612
	UDPGT-2	UDP glycosyltransferase 2 family, polypeptide A1 (Ugt2a1)	2.472	0.000	NM_022228
		UDP-glucuronosyltransferase, phenobarbital-inducible form (UDPGTR-2)	3.231	0.000	M13506
		UDP-glucuronosyltransferase (UGT2B12)	1.806	0.001	NM_031980
Down-regulated	Unknown	Microsomal glutathione *S*-transferase 3	0.277	0.020	AA892234
Fetal liver					
Up-regulated	GST pi	Glutathione *S*-transferase, pi 1 (Gstp1)	1.469	0.024	X02904
	GST alpha	Glutathione *S*-transferase	4.791	0.018	NM_017013
		Glutathione *S*-transferase 8 (GST 8–8) (CHAIN 8) (GST CLASS-ALPHA)	1.293	0.006	AI234527
	UDPGT-1	UDP glycosyltransferase 1 family, polypeptide A1 (Ugt1a1)	1.437	0.009	AF461738
	UDPGT-2	UDP-glucuronosyltransferase, phenobarbital-inducible form (UDPGTR-2)	2.353	0.002	M13506
		UDP-glucuronosyltransferase (UGT2B12)	1.842	0.000	NM_031980

No significant change was observed in the placenta.

Table 4
Gene expression changes of Phase II drug metabolizing enzymes in the dam liver, placenta and fetal liver treated with PB

		Genes	Fold changes	*t* test	Accession no.
Dam's liver					
Up-regulated	GST mu	Glutathione *S*-transferase, mu 1 (Gstm1)	5.769	0.016	M28241
		Glutathione *S*-transferase, mu type 2 (Yb2) (Gstm2)	2.124	0.001	AI169331
	GST alpha	Glutathione *S*-transferase Yc1 subunit	1.214	0.032	NM_031509
		Glutathione *S*-transferase Yc2 subunit	4.493	0.005	AA945082
		Glutathione *S*-transferase	2.712	0.000	NM_017013
		Glutathione *S*-transferase 8 (GST 8–8) (CHAIN 8) (GST CLASS-ALPHA)	1.516	0.043	AI234527
	GST theta	Glutathione *S*-transferase 1 (theta) (Gstt1)	1.708	0.023	NM_053293
	Unknown	Microsomal glutathione *S*-transferase 2	1.686	0.006	BI290559
	UDPGT-1	UDP glycosyltransferase 1 family, polypeptide A1 (Ugt1a1)	1.654	0.003	AF461738
		UDP glycosyltransferase 1 family, polypeptide A1 (Ugt1a1)	1.704	0.009	J02612
	UDPGT-2	UDP-glucuronosyltransferase, phenobarbital-inducible form (UDPGTR-2)	3.047	0.000	M13506
		UDP-glucuronosyltransferase	1.575	0.043	U27518
		UDP-glucuronosyltransferase (UGT2B12)	1.806	0.001	NM_031980
Fetal liver					
Up-regulated	GST mu	Glutathione *S*-transferase, mu 5 (Gstm5)	1.239	0.025	U86635
	UDPGT-2	UDP-glucuronosyltransferase (UGT2B12)	1.358	0.042	NM_031980

No significant change was observed in the placenta.

Glutathione *S*-transferase 8 (GST 8–8) (GST alpha class) (Table 3). The expression of microsomal Glutathione *S*-transferase 2 was also strongly increased (Table 3). In addition, the expression of Glutathione *S*-transferase Yc1 subunit (GST Yc1) (GST alpha class) showed a slight but significant increase (Table 3). The expression of 5 UDPGTs genes was strongly increased; two probes of UDP glycosyltransferase 1 family polypeptide A1 (Ugt1a1) (UDPGT-1 family), and UDP glycosyltransferase 2 family polypeptide A1 (Ugt2a1), UDP-glucuronosyltransferase phenobarbital-inducible form (UDPGTR-2) and UDP-glucuronosyltransferase (UGT2B12) (UDPGT-2 family) (Table 3). On the other hand, the expression of microsomal Glutathione *S*-transferase 3 was significantly decreased, and there were no UDPGTs genes which were significantly down-regulated (Table 3). In dam's liver of the PB-group, the expression of 7 GSTs genes was strongly increased; Gstm1 and Gstm2 (GST mu class), GST Yc2, GST and GST 8–8 (GST alpha class), Glutathione *S*-transferase 1 (theta) (Gstt1) (GST theta class) and the genes which were similar to microsomal Glutathione *S*-transferase 2 (Table 4). GST Yc1 (GST alpha class) showed a slight but significant increase (Table 4). The expression of 5 UDPGTs genes was strongly increased; two probes of Ugt1a1 (UDPGT-1 family), and UDPGTR-2, UDP-glucuronosyltransferase and UGT2B12 (UDPGT-2 family) (Table 4). On the other hand, there were no GSTs and UDPGTs genes which showed a significant decrease (Table 4).

In the placenta, there were no significant changes in the expression of GSTs and UDPGTs genes in both PCN- and PB-groups (Tables 3 and 4).

In fetal liver of the PCN-group, the expression of GST alpha class, and UDPGTR-2 and UGT2B12 (UDPGT-2 family) was strongly increased (Table 3). The expression of Glutathione *S*-transferase pi 1 (Gstp1) (GST pi family), GST8-8 (GST alpha

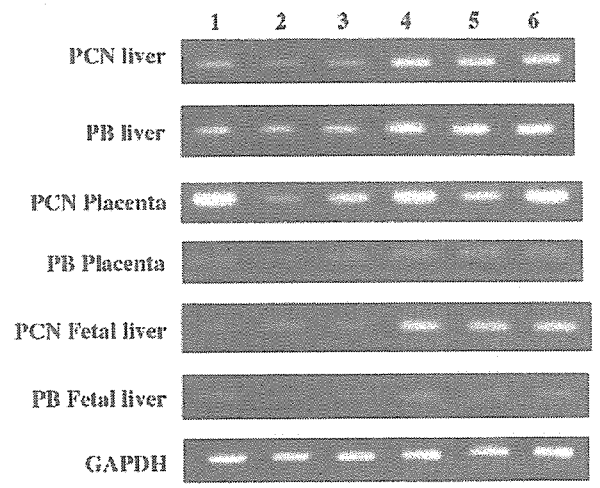

Fig. 1. The expression of GST alpha mRNA by RT-PCR in the dam's liver, fetal liver and placenta. Agarose gel electrophoresis. 1–3: rat number of control group. 4–6: rat number of treated group.

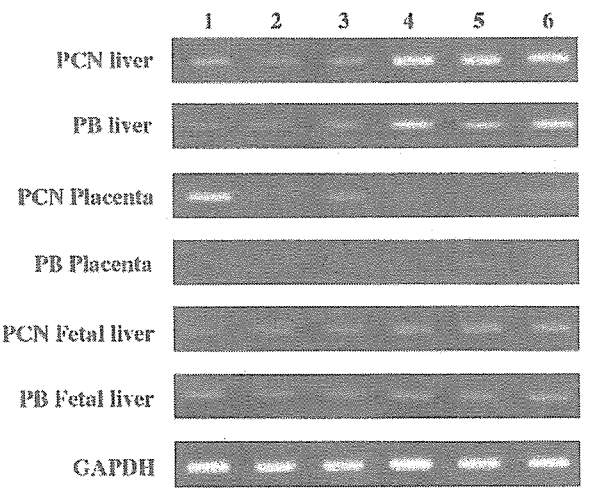

Fig. 2. The expression of UDPGT-2 mRNA by RT-PCR in the dam's liver, placenta and fetal liver. Agarose gel electrophoresis. 1–3: rat number of control group; 4–6: rat number of treated group.

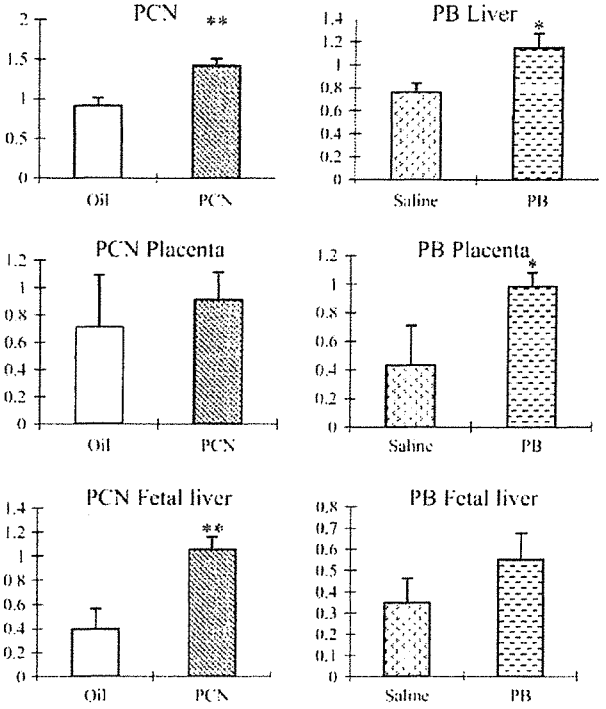

Fig. 3. The relative GST alpha band density to GAPDH by RT-PCR in the dam's liver, placenta and fetal liver. ☐: CO-group; ▨: PCN-group; ▩: Sa-group; ▤: PB-group. *$P < 0.05$ and **$P < 0.01$: significantly different from the control group.

class) and Ugt1a1 (UDPGT-1 family) showed a significant increase (Table 3). On the other hand, there were no GSTs and UDPGTs genes which showed a significant decrease (Table 3). In fetal liver of the PB-group, although no genes showing a remarkable induction, the expression of Glutathione *S*-transferase mu 5 (Gstm5) (GST mu class) and UGT2B12 (UDPGT-2 family) showed a slight but significant increase (Table 4). There were no genes showing decreased expression (Table 4).

As mentioned above, among 16 GSTs genes examined in this study, 7 genes were significantly induced in dam's liver and 3 genes in fetal liver, respectively, in the PCN-group, while 8 genes were significantly induced in dam's liver and 1 gene in fetal liver, respectively, in the PB-group. There were no significant changes in the placenta of both PCN- and PB-groups. On the other hand, among 11 UDPGTs genes examined, 5 genes were significantly induced in dam's liver and 3 genes in fetal liver, respectively, in the PCN-group, while 5 genes were significantly induced in dam's liver and 1 gene in fetal liver, respectively, in the PB-group. There were no significant changes in the placenta of both PCN- and PB-groups. The number of genes showing significant changes in their expression was much larger in dam's liver than in fetal liver, suggesting the difference in profiles of drug metabolizing enzymes between them.

In dam's liver, the expression of GST alpha class was widely induced and that of GST mu class was strongly induced in the PCN-group. In PB-group, the expression of GST alpha and mu classes was mainly induced, and that of GST theta class was also increased. GSTs are arranged into four classes, alpha, mu, pi and theta, based on amino acid identity (Parkinson, 1996). It is said that the members of the alpha and mu classes of GSTs are inducible by PB and corticosteroids (Parkinson, 1996). It was reported in the liver of female rats that GST Ya (GST alpha class) was induced by PB and PCN and GST theta 5 (GST theta class) tended to show an increased expression after PB treatment (Bulera et al., 2001; Longueville et al., 2002). Those reports corresponded well to our present results in the pregnant rat liver. In fetal liver, the expression of GST alpha class was mainly induced in the PCN-group, while only GST mu class was slightly induced in the PB-group.

UDPGTs are classified into two families based on amino acid sequence homology (Kumar and Surapaneni, 2001; Parkinson, 1996). It is said that the PB-inducible UDPGT enzymes and the PCN-inducible UDPGT enzymes are included in UDPGT-1 family, and the enzymes of UDPGT-2 family, especially UDPGT 2B subfamily (forms 2 and 12), are induced by PB (Parkinson, 1996). In the present study, the expression of UDPGT-1 and UDPGT-2 genes was strongly increased in dam's liver in both PCN- and PB-groups. In fetal liver, the enzymes of UDPGT-2 family were induced in both PCN- and PB-groups. UDPGT1a (UDPGT-1 family) is said to be induced by PCN in female rats (Longueville et al., 2002), and, in the present study, UDPGT1a was also strongly induced in dam's liver. In fetal liver, UDPGT1a was significantly but slightly induced in the PCN-group. Although the pi class of GSTs is said to be expressed in the rat placenta (Parkinson, 1996), the significant expression was not observed in the placenta in this study.

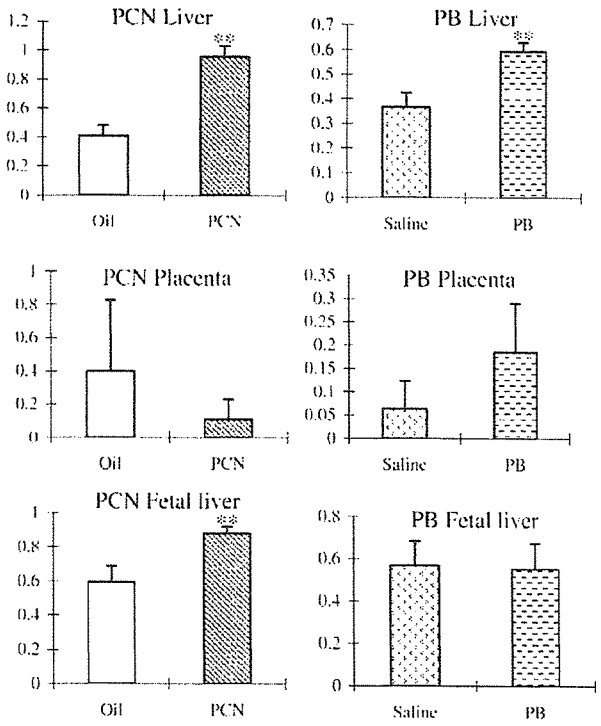

Fig. 4. The relative UDPGT-2 band density to GAPDH by RT-PCR in the dam's liver, placenta and fetal liver. ☐: CO-group; ▨: PCN-group; ▩: Sa-group; ▤: PB-group. **$P < 0.01$: significantly different from the control group.

RT-PCR was performed on GST alpha and UDPGT-2 mRNAs. Those genes were detected to be commonly up-regulated in both dam's liver and fetal liver by DNA microarray analysis.

In dam's liver, the expression of GST alpha and UDPGT-2 mRNAs was significantly elevated in both PCN- and PB-groups (Figs. 1–4).

In the placenta, a significant increase of GST alpha mRNA was observed in the PB-group (Figs. 1 and 3). The expression of UDPGT-2 mRNA did not show a significant change in both PCN- and PB-groups (Figs. 2 and 4).

In fetal liver, the expression of GST alpha and UDPGT-2 mRNAs was significantly elevated in the PCN-group (Figs. 1–4). In the PB-group, the expression of GST alpha and UDPGT-2 mRNAs showed no significant changes (Figs. 1–4).

The results of RT-PCR analysis on the expression of GST alpha and UDPGT-2 in dam's liver were in agreement with those of DNA microarray analysis. In fetal liver, differing from the results of DNA microarray analysis, UDPGT-2 did not show a significant change in the PB-group by RT-PCR. In the placenta, the expression of GST alpha mRNA in the PB-group was a significant but slight change.

In conclusion, this is the first report of the gene expression profiles of Phase II drug metabolizing enzymes in pregnant rats and fetal livers and placenta after treatment with typical inducers of drug metabolizing enzymes.

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Effects of pregnancy on CYPs protein expression in rat liver

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Abstract

A body of evidence suggests that pregnancy may be responsible for the depression in the microsomal enzyme activity and the reduction in the total content of cytochrome P450 (CYP) in the rat liver. However, changes in expression of individual CYP isozyme remain poorly known. The current study was designed to examine the changes in CYPs protein expression in the liver of F344 rats in midpregnancy and late pregnancy by Western blot analysis and immunohistochemistry. Total nine antirat CYPs antibodies (CYP1A1, CYP2B1/CYP2B2, CYP2C6, CYP2C12, CYP2D1, CYP2D4, CYP2E1, CYP3A1, and CYP4A1) were used. In comparison with age-matched nonpregnant control rats, there were significant decreases in hepatic levels of CYP2B2, CYP2C6, and CYP4A1 in midpregnancy (day 13) and CYP2B2, CYP2C6, CYP4A1, CYP1A1, CYP2B1, and CYP2E1 in late pregnancy (day 19). The expression of CYP2C12, CYP2D1, and CYP 3A1 did not differ between nonpregnant and pregnant rats, and CYP2D4 was not detectable in microsomal proteins obtained from nonpregnant and pregnant rats at a protein loading of 20 μg total protein per lane. Immunohistochemistry showed that there were no differences in the distribution and degree of immunostainability for the abovementioned antibodies to nine CYPs between pregnant and nonpregnant rats.

Keywords: CYPs; F344 rat; Immunohistochemistry; Pregnancy; Western blot analysis

Introduction

Cytochrome P450 isozymes (CYPs) are the collective term for a large superfamily of heme-containing proteins that play an important role in the oxidative metabolism of numerous endogenous and foreign compounds (Nelson et al., 1996). Four of the CYP450 families, families CYP1 to CYP4, defined on the basis of their amino acid sequence similarities, are involved in drug metabolism and are preferentially expressed in the liver.

Expression of CYPs is known to be influenced by a variety of endogenous and foreign factors such as inflammation, age, gender, nutritional status, pregnancy, and chemical exposure. Pregnancy is a physiological state accompanied by a high metabolic demand. This appears to be involved in changes in activity of hepatic cytochrome CYPs monooxygenase. Previous studies have demonstrated that normal pregnancy is associated with a decrease in total CYP450 content and/or reduced activity of microsomal drug-metabolizing enzyme in the liver (Dean and Stock, 1975, 1989; Feuer and Liscio, 1969; Guarino et al., 1969; Neale and Parke, 1973). Change in total CYP450 content or drug metabolism, however, does not reflect changes in expression of individual CYPs, because CYPs metabolize xenobiotics and endogenous substances with differing affinities; that is, each individual CYP is characterized by diverse substrate specificity and significant differences in regulation. Genetic variability among individual family members further contributes to significant intersubject differences in metabolic capacity and pharmacological response (Rogers et al., 2002). To date, only few individual CYPs have been investigated during pregnancy. The findings obtained in hamsters have shown that in comparison to nonpregnant controls, pregnant hamsters showed

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markedly decreased hepatic levels of both CYP2E and CYP2B mRNAs, which correlated with the results seen in immunoblot studies for these isozymes (Miller et al., 1992). Borlakoglu et al. (1993) investigated the alterations in rat hepatic drug metabolism during pregnancy and lactation. In their data, Western blot analysis of microsomal proteins obtained from pregnant rats shows that the expression of CYP1A1, CYP1A2, CYP2A1, CYP2B1, and CYP4A1 was not detectable in pregnant rats at a protein loading at 3 μg total protein per well. Casazza et al. (1994) demonstrated that CYP2E1 expression is suppressed in females particularly in late pregnancy in both acetone-treated and untreated rats by using immunoblot and Northern blot analyses. In human, there is an increase in activity of CYP2C6 enzyme during pregnancy, as assessed with dextromethorphan phenotyping test (Wadelius et al., 1997). This has been thought to be due to an induction of CYP2C6 enzyme. In the study by Czekaj et al. (2000), pregnancy was associated with decreased levels of CYP2B1/CYP2B2 protein in the liver in control and tobacco smoke-treated rats. Recently, Ejiri et al. (2001, 2003) investigated the changes in the expression and localization of CYPs protein in rat placenta at 9, 11, 13, 16, and 19 days of gestation by Western blot analysis and immunohistochemical staining. Only CYP3A1 was clearly detected during a long period of pregnancy.

According to the above-cited studies, changes in expression of most hepatic CYPs during rat pregnancy remain poorly known. In the present study, a total of nine CYPs throughout CYP1 to CYP4 subfamilies were designed to investigate the changes in expression in midpregnancy (day 13) and late pregnancy (day 19) by using Western blot analysis. The nine CYPs are thought to be concerned with drug-metabolizing system. CYP1A1 is involved in the oxidation of a wide spectrum of endogenous compounds and xenobiotics; CYP2B1 and CYP2B2 are involved in the activation of arenes, arylamines, and nitrosamine; CYP2C metabolizes the endogenous compound, arachidonic acid, and as such may play an important physiological role via the generation of bioactive eicosanoids; CYP2D has been shown to be one of determinants of polymorphic drug oxidations in human and rats, and it mainly catalyzes reaction of debrisoquine 4-hydroxylation; CYP2E1 is the major component of the microsomal ethal-oxidazing system (MEOS) and is responsible for the majority of acetone monooxygenase activity in rats; CYP3A catalyzes the 6β-hydroxylation of testosterone and metabolizes several drugs; CYP4A catalyzes the hydroxylation of the ω or ω-1 carbon of saturated and unsaturated fatty acids and prostaglandins.

In the present study, we also investigated the localization and distribution of these CYPs in the liver of pregnant and nonpregnant rats. The protocol of this study has been approved by the Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, The University of Tokyo.

Materials and methods

Animals

Twelve pregnant and six age-matched nonpregnant female rats (11 weeks of age) were purchased from Saitama Experimental Animal Co. (Saitama, Japan) and used in this study. The day of a vaginal plug being recognized was designated as 0 day of gestation. The rats were individually housed in plastic cages in an animal room controlled at 23°C ± 2°C and at 55% ± 5% humidity condition with 14 h/10 h light/dark cycle, and fed pellets (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and water ad libitum. On days 13 and 19 of gestation, six dams were sacrificed. Livers were removed and used for Western blot analysis and immunohistochemical staining. Nonpregnant were used as controls.

Immunohistochemistry

Immunohistochemical staining for CYPs was carried out on the paraffin sections using a LSAB method with streptavidine (DAKO, Carpinteria, CA) (1:300). Monoclonal goat antirat CYP1A1, CYP2C6, CYP2E1, and CYP4A1 antibodies (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) (1:200) and monoclonal rabbit antirat CYP2B1/2B2, CYP2C12, CYP2D1, CYP2D4, and CYP3A1 antibodies (Chemicon International Inc., Temecula, CA) (1:200) were used as the first antibody. Biotinylated antirabbit and antigoat antibodies (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD) (1:400) were used as the second antibody.

After having been deparaffinized, the sections were pretreated with trypsin solution (0.1% trypsin and 0.1% calcium chloride/Tris buffer) at 37°C for 30 min, then treated with 0.3% H_2O_2/methanol for 30 min to inactivate endogeneous peroxidase, and incubated in 8% skim milk/TBS at 37°C for 40 min to block nonspecific binding of the antibodies. The tissue sections were then incubated overnight at 4°C with the first antibodies diluted in 8% skim milk/TBS. Following washing in 3× TBS, the sections were incubated with the second antibodies for 30 min at room temperature and then in the streptavidine for 30 min. Finally, the sections were visualized by 0.05% 3,3′-diaminobenzidine (DAB) with 0.03% hydrogen peroxide in Tris HCl buffer, followed by counterstaining with methyl green.

Western blot analysis

Livers were homogenized in ice-cold 0.1 M phosphate buffer, pH 7.4, containing 150 mM KCl, 1 mM EDTA Na, and 1 mM DTT, and microsomes were prepared by differential centrifugation. Briefly, the liver homogenates were centrifuged at 9000 × *g* for 20 min at 4°C, and the resulting supernatant spun at 105,000 × *g* for 1 h at 4°C. After discarding the supernatant, the pellets were suspended in the same buffer and recentrifuged. The pellets were resuspended with 0.1 M PB, pH 7.4, containing 150 mM KCl, 20%

glycerol, 1 mM EDTA Na, and 1 mM DTT and stored at −80°C until used. Protein concentration of the samples was measured using bovine serum albumin (BSA) as the standard. Microsomal proteins (20 or 40 μl) were separated using SDS–PAGE in 10% polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane (BIO-RAD, Richmond, CA), and the plate was blocked with 8% skim milk/TBS for 1 h at room temperature. The membrane was then incubated with the abovementioned antirat CYP1A1, CYP2B1/2B2, CYP2C6, CYP2C12, CYP2D1, CYP2D4, CYP2E1, CYP3A1, and CYP4A1 antibodies diluted in 8% skim milk/TBS (1:200) overnight at 4°C, followed by another 1-h incubation with horseradish peroxidase-conjugated secondary antibodies [donkey anti-rabbit IgG (Amersham Pharmacia Biotech Ltd., Arlington Heights, IL) and rabbit antigoat IgG (Cappel, Aurora, OH)]. The protein bands were visualized by ECL plus Western blotting detection system (Amersham Pharmacia Biotech Ltd.) followed by a brief exposure to Hyperfilm (Amersham Biosciences UK Ltd.). Quantity One v3.0 software (PDI, Inc., NY, USA) was used to quantitate the band intensities.

Statistical analysis

Results were presented as the mean ± standard deviation (SD). Student *t* test was employed to calculate the statistical significance between control and midpregnancy (day 13) or late pregnancy (day 19) groups.

Results

Change in body and liver weights

There were significant increases in the body and liver weights on day 19 of gestation (Table 1). On day 13 of gestation, there was no significant increase in the liver weight compared with nonpregnant controls, although there was a significant increase in maternal body weight (Table 1).

Findings of Western blot analysis

The results of Western blot analysis are shown in Figs. 1 and 2. The expression of CYP2C12, CYP2D1, and CYP 3A1 proteins did not differ between nonpregnant and pregnant (both days 13 and 19 of gestation) rats. CYP2D4 was not detectable in microsomal proteins obtained from nonpregnant and pregnant rats at a protein loading of 20 μg total protein per lane. Fig. 2 shows significant decreases in the CYP1A1, CYP2B1, and CYP2E1 contents in pregnant rats on day 19 of gestation (70.6%, 21.9%, and 61.0% of nonpregnant control values, respectively) and no significant change in pregnant rats on day 13 of gestation when compared with control. CYP2B2, CYP2C6, and CYP4A1 contents showed significant decreases on days 13 and 19 of gestation when compared with controls. Namely, on day 13 of gestation, CYP2B2, CYP2C6, and CYP4A1 proteins decreased to 29.2%, 67.4%, and 60.0% of control values, and on day 19 of gestation, they decreased to 25.8%, 69.8%, and 70.4% of control values, respectively.

Table 1
Body and liver weights

Gestation day	Body weight (g)	Liver weight (g)
Nonpregnant	144.35 ± 3.72	5.69 ± 0.39
Day 13 of gestation	161.83 ± 5.94*	6.06 ± 0.35
Day 19 of gestation	201.13 ± 8.66**	8.91 ± 0.19**

Data are represented as mean ± SD of 6 rats.
* $P < 0.01$; significantly different from controls.
** $P < 0.001$ significantly different from controls.

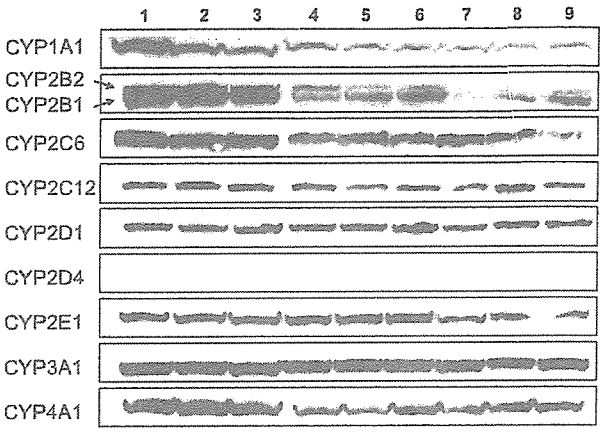

Fig. 1. Western blot analysis of liver microsomes from nonpregnant, midpregnant, and late pregnant rats. The amount of protein per lane was 20 μg (CYP2B1/2B2, CYP2C6 CYP2C12, CYP2D1, CYP2D4, CYP2E1, and CYP3A1) and 40 μg (CYP1A1 and CYP4A1). Lanes 1 to 3: Age-matched nonpregnant rats; lanes 4 to 6: midpregnant rats (day 13 of gestation); lanes 7 to 9: late pregnant rats (day 19 of gestation).

Immunohistochemical findings

Immunohistochemical analysis with antibodies to CYPs revealed that CYP1A1 was expressed in endothelial cells of both sinusoids and veins in the liver (Figs. 3a–c) There was a very slight expression of CYP4A1 in hepatocytes and endothelial cells in the liver. CYP2C6 (Figs. 3d–f), CYP2E1 (Figs. 3g–i), CYP 3A1 (Figs. 3j–l), and other CYPs were mainly expressed in centrilobular hepatocytes. CYP2D4 was not detectable in either pregnant or nonpregnant rat liver by immunohistochemical analysis. As shown in Fig. 3, there were no differences in the distribution and degree of immunostainability for antibodies against nine CYPs between pregnant and nonpregnant rat livers. Table 2 shows the distribution and degree of immunostainability for nine CYPs.

Discussion

In the present study, in comparison to age-matched nonpregnant control rats, pregnant rats showed significantly

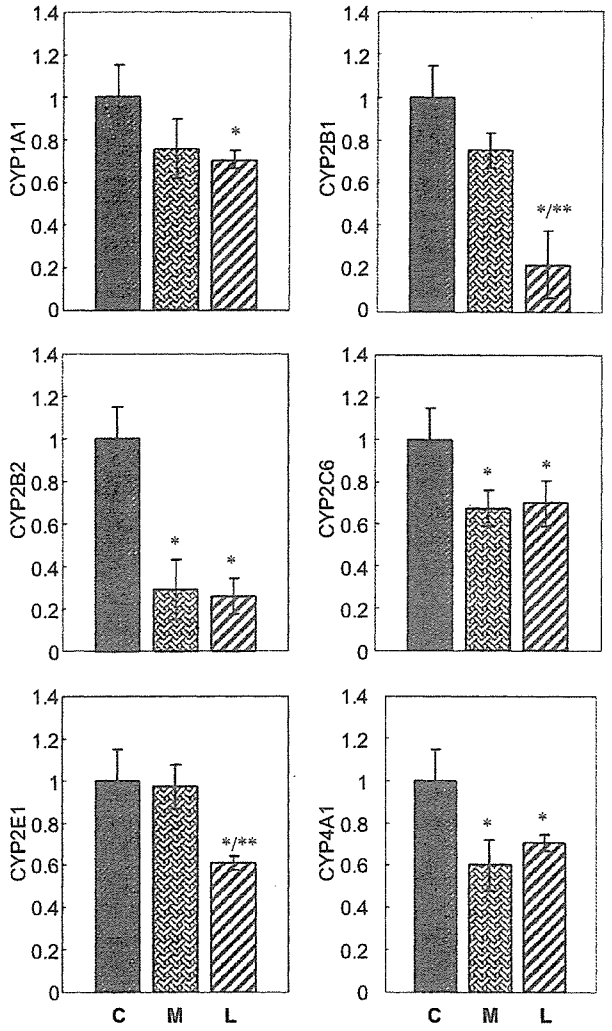

Fig. 2. Densitometry of Western blotting using monoclonal antibodies against rat hepatic CYP1A1, CYP2B1/2B2, CYP2C6, CYP2E1, and CYP4A1. Values are expressed as the ratio of pregnancy/nonpregnancy in arbitrary densitometric units of proteins amount and reported as the means ± SD of six rats. C indicates control nonpregnancy; M, midpregnancy; L, late pregnancy. *Significantly different from nonpregnant control at $P < 0.05$. **Significantly different from midpregnant rats at $P < 0.05$.

decreased hepatic levels of six out of nine CYPs proteins (CYP1A1, CYP2B1, CYP2B2, CYP2C6, CYP2E1, and CYP4A1) in midpregnancy (day 13) and/or late pregnancy (day 19).

Although, earlier reports have demonstrated that there was a decrease in rat hepatic total CYP450 content during pregnancy (Dean and Stock, 1975, 1989; Feuer and Kardish, 1975; Feuer and Liscio, 1969; Guarino et al., 1969), the mechanism of the effect of pregnancy on regulation of CYPs is still far from being fully clarified.

In the present study, pregnancy was linked to an increase in liver weight by up to 36.1% on day 19 of pregnancy. It has been hypothesized that a differential synthesis of hepatic proteins occurs during pregnancy, with little or no synthesis of the CYPs (Dean and Stock, 1975). It has also been thought that decrease in mixed-function oxidase activity during pregnancy is due to reduction in the hepatocellular capacity to metabolize drugs with an increase in liver size (Symons et al., 1982). However, in the present study, decreases in protein levels of CYPs (CYP2B2, CYP2C6, and CYP4A1) were not accompanied with an increase in liver weight, when detected in midpregnancy (day 13). Furthermore, Starkel et al. (2000) have demonstrated that early down-regulation of CYP3A and CYP2E1 in the regenerating rat liver is not related to the process of cellular proliferation. Therefore, liver enlargement might not be involved in the decrease in CYPs during rat pregnancy.

During pregnancy in rats, high plasma levels of both progesterone and its metabolites are produced. The depressed hepatic drug metabolism has been attributed to the pronounced hormonal changes that occur during pregnancy, in particular, to the higher plasma levels of both progesterone and its metabolites. Dean and Stock (1975) suggested that lower levels of hepatic microsomal enzyme activity might reflect a biological control mechanism to ensure the elevated levels of progesterone required to maintain the pregnant state. It has been known that the reduction in the activities of the microsomal drug-metabolizing enzymes in the rat is paralleled by a similar reduction in the total content of CYP450 (Dean and Stock, 1975; Feuer, 1979; Guarino et al., 1969; Neale and Parke, 1973). Thus, the increased production of progesterone and its metabolites can be implicated as a causative agent in pregnancy-induced regulation of CYPs; they seem to play a vital role in direct down-regulation of CYP450 expression and indirect reduction of the enzymatic activities.

Oxidative stress may be one of the factors which are responsible for the regulation of CYPs. In rats, lipid peroxidation remains low until midpregnancy and begins to rise after day 15 of pregnancy (Sugino et al., 1993). This role seems to be involved in our data; three out of nine CYPs decreased in midpregnancy (day 13), and up to six CYPs decreased in late pregnancy (day 19). Moreover, a measurable decrease in glutathione peroxidase in the liver and placenta, which play an important role in reducing the effects of oxidative stress in pregnancy, has been reported in pregnant rats (Mover-Lev and Ar, 1997). These observations indicate that there is an increase in oxidative stress during pregnancy. Oxidative stress has been suggested to result in the reduction of total CYP450 levels and drug metabolism activities in vivo (Gatti et al., 1993; Liu et al., 1993; Mannering and Deloria, 1986; Peristeris et al., 1992). Furthermore, Barker et al. (1994) have investigated the possibility that oxidative stress may influence inducer-dependent expression of CYP1A1 and CYP1A2, and demonstrated that hydrogen peroxide suppresses the accumulation of CYP1A1 and CYP1A2 mRNAs in isolated hepatocytes through a transcriptional mechanism. Pahan et al. (1997) demonstrated that there is a down-regulation of

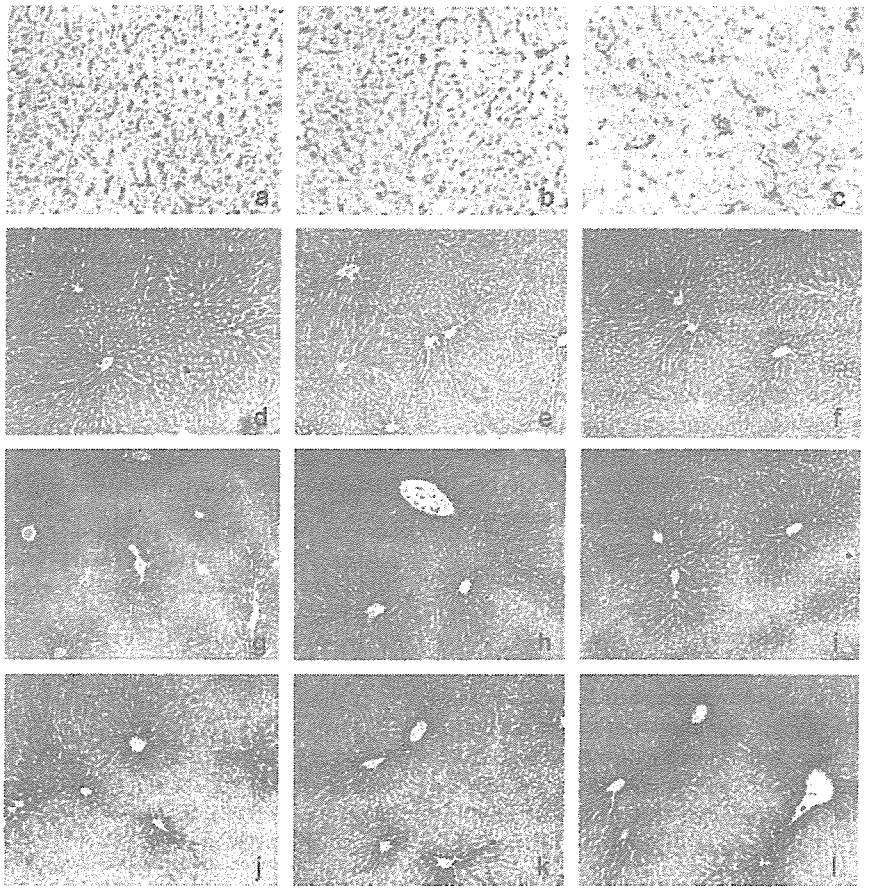

Fig. 3. Immunohistochemical distribution of CY1A1 (a–c), CYP2C6 (d–f), CYP2E1 (g–i), and CYP3A1 (j–l) proteins in the liver of nonpregnant rats (a, d, g, and j), midpregnant rats (b, e, h, and k), and late pregnant rats (c, f, i, and l). Immunostaining, magnification ×100.

CYP2E1 in rat liver peroxisomes by a mechanism of ischemia/reperfusion-induced oxidative stress. Decreases in CYP 1A1 and CYP2E1 protein levels in rat late pregnancy were also observed in the present study. Reduction of hepatic CYPs mRNAs expression during oxidative stress may be part of and adaptive response by the liver to minimize cell damage (Barke et al., 1994). To our knowledge, the relationship between CYPs expression and oxidative stress during pregnancy has not been investigated. It remains to be determined what extent changes in hepatic CYPs expression are regulated by increased oxidative stress during pregnancy.

Endogenous nitric oxide (NO), a potent vasodilator and a platelet antiaggregating factor, might also be involved in down-regulation of hepatic CYPs expression during rat pregnancy. Evidences provided by Conrad et al. (1993), McLaughlin and Conrad (1995), and Xu et al. (1996) show that the biosynthesis of NO increases during rat gestation as determined with plasma level and urinary excretion. Increased NO production has been suggested to play a role in the hemodynamic alterations of pregnancy (Xu et al., 1996). As one of the oxidative stresses, an increase in NO could also be thought to be an adaptive response during pregnancy by inhibiting CYP450 expression. Using human hepatocytes as an experimental model, Gonzalez (1990) demonstrated that exogenously added NO inhibited the CYP1A2 activity. In liver extracts, NO decreased the mRNA and protein expression of the phenobarbital-inducible CYP2B1 and CYP2B2 (Khatsenko et al., 1997), and it also decreased the constitutive expression of CYP3A2 (Minamiyama et al., 1997). In rat hepatocytes, high concentrations of bacterial lipopolysaccharide (LPS) evoked

Table 2
Distribution and degree of immunostainability for CYPs in the liver

CYPs	Cell type	
	Hepatocytes	Endothelial cells of sinusoids and veins
CYP1A1	–	++
CYP2B1/2	+	–
CYP2C6	++	–
CYP2C12	+++	–
CYP2D1	+++	–
CYP2D4	–	–
CYP2E1	++	–
CYP3A1	+++	–
CYP4A1	±	±

Note: –, negative; ±, very slight; +, slight; ++, moderate; +++, marked.

a rapid down-regulation of phenobarbital-induced CYP2B1 protein that was NO-dependent (Ferrari et al., 2001). Moreover, in a very recent study (Wang et al., 2003), it was demonstrated that a decrease in renal microsomal CYP4A1/CYP4A3 expression is dependent on NO in rat late pregnancy. These results, down-regulations of CYP2B1, CYP2B2, and CYP4A1 induced by NO, are partially consistent with our data. Therefore, these observations raise the possibility that NO may participate in down-regulation of hepatic CYPs during rat pregnancy. To date, it remains to be assessed that NO is involved in regulation of hepatic CYPs during rat pregnancy.

Pregnancy is a dynamic condition with major endocrine and physiological changes. At the present time, it is not clear how pregnancy regulates CYP450 expression. The abovementioned and other unknown mechanisms may be operating together to some extent, resulting in the decreased CYP450 expression. However, it should be pointed out that some CYPs were not affected during rat pregnancy, at least in the present study.

The localization and distribution of the CYP1A1, CYP2B1/2B2, CYP2C6, CYP2C12, CYP2D1, CYP2D4, CYP2E1, CYP3A1, and CYP4A1 proteins in the rat liver were also investigated by using immunohistochemistry with anti-CYPs antibodies. We observed that CYP1A1 is expressed in endothelial cells of both sinusoids and veins in the liver. The localization of CYP1A1 observed here confirms the findings of Annas and Brittebo (1998) who reported a colocalization of CYP1A1 immunoreactivity and covalent binding of 3H-Trp-P-1 in endothelial linings of capillaries and veins of heart, skeletal, muscle, and uterus in β-naphthoflavone (BNF)-treated rodents. Thus, endothelial cells appear to be the main cell type that expresses CYP1A1 in the rat liver. It has been suggested that CYP1A1 may be a factor in the regulation of vascular tone at these sites (Annas and Brittebo, 1998). CYP2B1/2B2, CYP2C6, CYP2C12, CYP2D1, CYP2E1, CYP3A1, and CYP4A1 were mainly expressed in centrilobular hepatocytes of hepatic acinus. This supports the general knowledge that most CYPs are expressed mainly in this region and some of them throughout the acinus in the rat liver. CYP4A1 was also detected in endothelial cells of both sinusoids and veins. We failed to show the CYP2D4 proteins in either pregnant or nonpregnant rat liver by using immunohistochemical staining.

In conclusion, the present study showed that pregnancy was associated with down-regulation of protein levels of CYP1A1, CYP2B1/2B2, CYP2C6, CYP2E1, and CYP4A1 in the rat liver but did not affect those of CYP2C12, CYP2D1, and CYP3A1. CYP1A1 and CYP4A1, especially the former, were expressed in endothelial cells of both sinusoids and veins. We are now conducting the study on the potential effects of oxidative stress and nitric oxide on the regulation of CYPs during pregnancy.

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Changes in cytochrome P450 isozymes (CYPs) protein levels during lactation in rat liver

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Abstract

The effects of pregnancy on CYPs protein level in the liver have been investigated in our previous study. Since pregnancy was associated with a decrease in CYPs protein level, the objective of this study was to investigate whether CYPs protein can revert to the virgin control level after delivery. Western blot analysis was performed to investigate the changes of total nine CYPs protein (CYP1A1, CYP2B1/CYP2B2, CYP2C6, CYP2C12, CYP2D1, CYP2D4, CYP2E1, CYP3A1 and CYP4A1) at three distinct phases: delivery (postpartum day 0, PPD 0), peak lactation (PPD 14) and on weaning (PPD 28). By PPD 0, CYP1A1, 2B1, 2B2, 2C6, 2E1 and CYP4A1 were markedly down-regulated when compared with virgin controls. By PPD 14, however, CYP1A1, 2B1, 2B2 and CYP2C6 returned to the virgin control level. All the decreased CYPs during lactation were at the virgin control level at PPD 28. The expression of CYP2C12, CYP2D1 and CYP 3A1 did not differ between lactating, post-lactation and virgin control rats. CYP2D4 was not detectable in microsomal proteins obtained from virgin control rats at a protein loading of 20 μg total protein per lane.

Keywords: CYPs; F344 rat; Western blot analysis; Lactation

Introduction

The cytochrome P450 (CYP) enzyme system is extremely important for the metabolism of xenobiotics as well as endogenous substances, such as fatty acids, prostaglandins and steroids (Lind et al., 2003). Expression of CYP isozymes (CYPs) is also known to be influenced by a variety of endogenous and foreign factors such as inflammation, age, gender, nutritional status, pregnancy and so on. Pregnancy is a physiological state accompanied by a high metabolic demand and an increased requirement for tissue oxygen (Spatling et al., 1992). A body of evidence suggests that pregnancy may be responsible for the depression in the microsomal enzyme activity and the reduction in the total content of CYP in the rat liver (Feuer and Liscio, 1969; Guarino et al., 1969; Neale and Parke, 1973; Dean and Stock, 1975, 1989). We have previously demonstrated that pregnancy is associated with decreased hepatic levels of some CYPs proteins in midpregnancy and/or late pregnancy (He et al., 2005). It has been suggested that changes in the physiological state of an animal may alter CYPs ability to metabolize foreign chemicals (Borlakoglu et al., 1993). The hormonal changes that occur during pregnancy and at the start of lactation are complex, and the balance between the various hormones during lactation is different from that during pregnancy and different again from that in the virgin animal (Smith, 1975). In the lactating animals, milk production introduces an additional metabolic system which competes for the available nutrients with other processes such as the formation of body reserves.

A few studies have been performed on the effects of lactation on tissue metabolism and activities of some enzymes. Smith (1975) has demonstrated that the activities of some enzymes in the rat liver could be influenced by pregnancy and lactation. Based on their observations, Abel et al. (1979) have suggested that drug disposition may be altered during lactation. Review by Williamson (1986) suggests that the main aim of changes in tissue metabolism during rat lactation is to preserve the increased intake of nutrients from diet for milk production. Smith et al. (1998) have demonstrated that major changes in hepatic lipid metabolism must occur to maintain cholesterol homeostasis during rat lactation.

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Table 1
Body and liver weights

Postpartum day (PPD)	Body weight	Liver weight
Virgin control	143.70 ± 1.66	5.78 ± 0.34
PPD 0	156.70 ± 6.61*	7.38 ± 1.17
PPD 14	184.50 ± 5.91***	8.23 ± 0.40**
PPD 28	188.03 ± 17.39*	7.60 ± 1.13

Data are represented as mean ± SD of 6 rats. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; significantly different from virgin controls.

It is assumed that, therefore, lactation may also be involved in the regulation of hepatic CYPs expression like pregnancy. Borlakoglu et al. (1993) investigated the alterations of some CYPs in the rat liver during lactation. Western immunoblot analysis of microsomal proteins obtained from pregnant and lactating rats showed that only CYP2C6 and CYP3A1 proteins are expressed at detectable levels, while the expression of others was not detectable in pregnant and lactating rats at a protein loading of 3 μg total protein per well. In their study, comparison was not performed between lactating and virgin control rats. During the past few decades, much less attention has been given to the effect of lactation on the regulation of hepatic CYPs expression. It was therefore of interest to investigate the effect of lactation on the expression of hepatic CYPs proteins. Attention is also paid to some CYPs, which were down-regulated during pregnancy in our previous study, whether and when they could return to the control virgin level. For this purpose, changes in protein levels of nine CYPs in the lactating and post-lactation rat liver were investigated by Western blot analysis at three distinct phases: delivery (postpartum day 0, PPD 0), peak lactation (PPD 14) and on weaning (PPD 28).

Materials and methods

Animals

Twelve pregnant and six age-matched virgin control rats (11 weeks of age) were purchased from Saitama Experimental Animal Co. (Saitama, Japan) and used in this study. The day of delivery recognized was designated as postpartum day 0 (PPD 0). The rats were individually housed in plastic cages in an animal room controlled at 23 ± 2°C and at 55 ± 5% humidity condition with 14 h/10 h light/dark cycle and fed pellets (MF, Oriental Yeast Co., Ltd.,

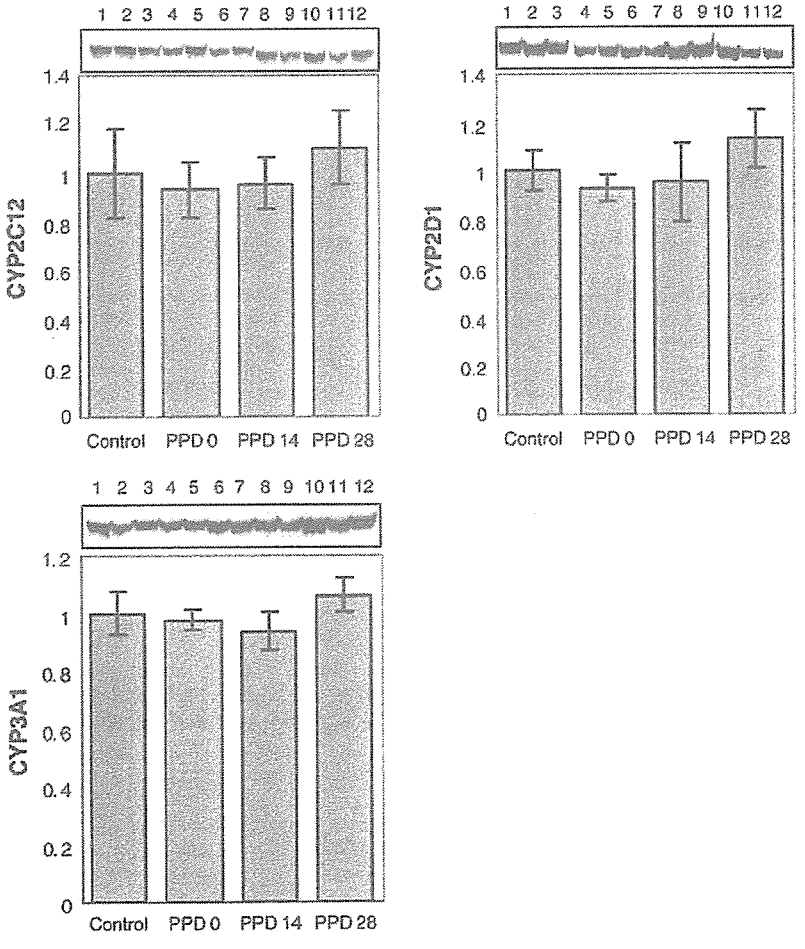

Fig. 1. Western blot analysis of liver microsomes from virgin control, lactating (PPD 0, PPD 14) and post-lactation (PPD 28) rats. The amount of protein per lane was 20 μg (CYP2C12, CYP2D1 and CYP3A1). Lanes 1 to 3: age-matched virgin control rats; lanes 4 to 6: PPD 0 rats; lanes 7 to 9: PPD 14 rats; lanes 10 to 12: PPD 28 rats. Densitometry of Western blotting using monoclonal antibodies against rat hepatic CYP2C12, CYP2D1 and CYP3A1 was performed. Values are expressed as the ratio of lactation (PPD 0 and PPD 14) and post-lactation (PPD 28)/virgin control in arbitrary densitometric units of protein amounts and reported as the means ± SD of 6 rats.

Tokyo, Japan) and water ad libitum. On PPD 0, PPD 14 (peak lactation) and PPD 28 (7 days post-lactation), 6 dams were sacrificed under ether anesthesia, respectively. Livers were removed and used for Western blot analysis. Age-matched virgin rats were used as controls.

Western blot analysis

Livers were homogenized in ice-cold 0.1 M phosphate buffer (PB), pH 7.4, containing 150 mM KCl, 1 mM EDTA Na, 1 mM DTT and microsomes were prepared by differential centrifugation. Briefly, the liver homogenates were centrifuged at 9000 × *g* for 20 min at 4°C, and the resulting supernatant was centrifuged at 105,000 × *g* for 1 h at 4°C. After discarding the supernatant, the pellets were suspended in the same buffer and re-centrifuged. The pellets were re-suspended with 0.1 M PB, pH 7.4, containing 150 mM KCl, 20% glycerol, 1 mM EDTA Na, 1 mM DTT, and stored at −80°C until used. Protein concentration of the samples was measured using bovine serum albumin (BSA) as the standard. Microsomal proteins (20 or 40 μl) were separated using SDS-PAGE in 10% polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Richmond, CA), and the plate was blocked with 8% skim milk/TBS for 1 h at room temperature. The membrane was then incubated with the abovementioned anti-rat CYP1A1, CYP2B1/2B2, CYP2C6, CYP2C12, CYP2D1, CYP2D4, CYP2E1, CYP3A1 and CYP4A1 antibodies diluted in 8% skim milk/TBS (1:200) overnight at 4°C followed by another 1 h incubation with horseradish-peroxidase-conjugated secondary antibodies (donkey anti-rabbit IgG; Amersham Pharmacia Biotech Ltd., Arlington Heights IL) and rabbit anti-goat IgG (Cappel, Aurora, OH)). The protein bands were visualized by ECL plus Western blotting detection system (Amersham Pharmacia Biotech Ltd., Arlington Heights, IL) followed by a brief exposure to Hyperfilm (Amersham Biosciences UK Ltd.). Quantity One v3.0 software (PDI, Inc, NY, USA) was used to quantitate the band intensities.

Statistical analysis

Results were presented as the mean ± standard deviation (SD) of six rats. Student's *t* test was employed to calculate the statistical significance between virgin control and postpartum (PPD 0, PPD 14 and PPD 28) groups.

Results

Change in body and liver weights

There were significant increases in the body weights on PPD 0, PPD 14 and PPD 28 compared with virgin controls.

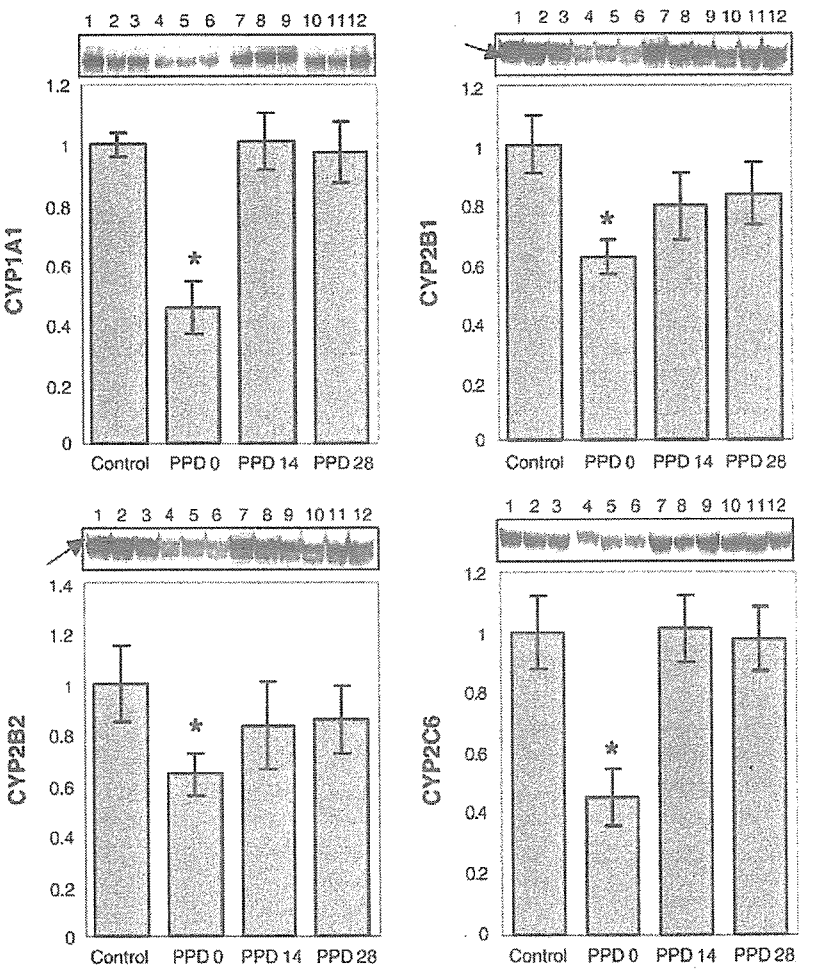

Fig. 2. Western blot analysis of liver microsomes from virgin control, lactating (PPD 0, PPD 14) and post-lactation (PPD 28) rats. The amount of protein per lane was 20 μg (CYP2B1/2 and CYP2C6) and 40 μg (CYP1A1). Lanes 1 to 3: age-matched virgin control rats; lanes 4 to 6: PPD 0 rats; lanes 7 to 9: PPD 14 rats; lanes 10 to 12: PPD 28 rats. Densitometry of Western blotting using monoclonal antibodies against rat hepatic CYP1A1, CYP2B1/2 and CYP2C6 was performed. Values are expressed as the ratio of lactation (PPD 0 and PPD 14) and post-lactation (PPD 28)/virgin control in arbitrary densitometric units of protein amounts and reported as the means ± SD of 6 rats. * Significantly different from virgin controls at $P < 0.05$.

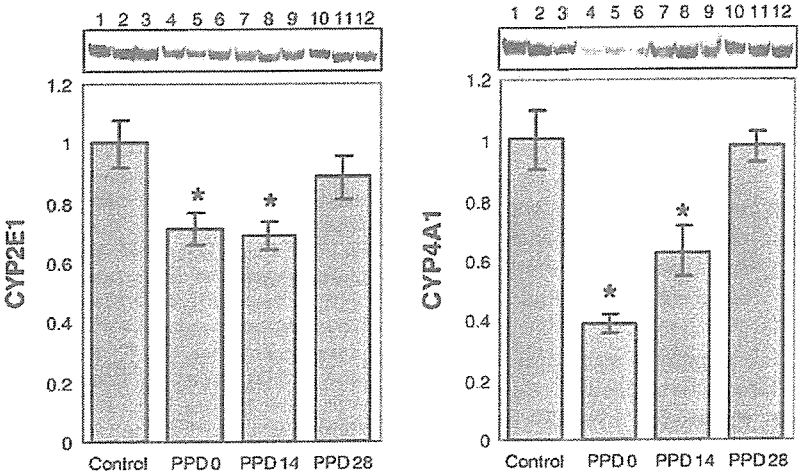

Fig. 3. Western blot analysis of liver microsomes from virgin control, lactating (PPD 0, PPD 14) and post-lactation (PPD 28) rats. The amount of protein per lane was 20 μg (CYP2E1) and 40 μg (CYP4A1). Lanes 1 to 3: age-matched virgin control rats; lanes 4 to 6: PPD 0 rats; lanes 7 to 9: PPD 14 rats; lanes 10 to 12: PPD 28 rats. Densitometry of Western blotting using monoclonal antibodies against rat hepatic CYP2E1 and CYP4A1 was performed. Values are expressed as the ratio of lactation (PPD 0 and PPD 14) and post-lactation (PPD 28)/virgin control in arbitrary densitometric units of protein amounts and reported as the means ± SD of 6 rats. * Significantly different from virgin controls at $P < 0.05$.

However, the increase in body weight was not accompanied with the increase in liver weight when detected on PPD 0 and PPD 28 (Table 1). On PPD 14, there was a significant increase in the liver weight compared with virgin controls (Table 1).

Findings of Western blot analysis

The results of Western blot analysis are shown in Figs. 1–3. The expression of CYP2C12, CYP2D1 and CYP 3A1 proteins did not differ between age-matched virgin control and experimental rats (at PPD 0, PPD 14 and PPD 28) (Fig. 1). CYP2D4 was not detectable in microsomal proteins obtained from virgin controls and experimental animals at a protein loading of 20 μg total protein per lane. Fig. 2 shows significant decreases in the CYP1A1, CYP2B1, CYP2B2 and CYP2C6 contents when detected on postpartum day 0 (45.3%, 61.9%, 63.8% and 45.3% of age-matched virgin control values, respectively). They returned to the virgin control levels by PPD 14 and kept constant levels on PPD 28 (Fig. 2). Decreases in CYP2E1 and CYP4A1 protein levels (71.3% and 39.0% of age-matched virgin control levels) were also found on PPD 0 (Fig. 3), and they were still decreased on PPD 14 (69.3% and 62.3% of virgin control levels). By PPD 28, CYP2E1 and CYP4A1 protein levels returned to virgin control levels.

Discussion

In the present study, in comparison with age-matched virgin control rats, lactating rats showed significantly decreased hepatic levels of six out of nine CYPs proteins (CYP1A1, CYP2B1, CYP2B2, CYP2C6, CYP2E1 and CYP4A1) at day of delivery (PPD 0). By PPD 21 (peak lactation), CYP1A1, CYP2B1, CYP2B2 and CYP2C6 proteins returned to the virgin control levels. All of the hepatic CYPs proteins were at virgin control level at 7 days post-lactation (PPD 28).

We previously demonstrated that pregnancy is associated with decreased hepatic levels of three (CYP2B2, CYP2C6 and CYP4A1) and six CYPs proteins (CYP1A1, CYP2B1, CYP2B2, CYP2C6, CYP2E1 and CYP4A1) in midpregnancy and late pregnancy, respectively. Based on the marked decreases in the same six CYPs proteins at PPD 0, it is obvious that they kept decreased protein levels even though the physiological state has been changes.

In the present study, peak lactation (PPD 14) was linked to an increase in liver weight by up to 42.4%. Although it has been suggested that a decrease in mixed-function oxidase activity during pregnancy is due to a reduction in the hepatocellular capacity to metabolize drugs with an increase in liver size (Symons et al., 1982), it could not be used, however, for interpreting during lactation. Since six CYPs proteins were decreased on PPD 0, not accompanying with an increase in liver weight. Of six decreased CYPs proteins, four returned to the virgin control levels on PPD 14. Based on these observations, it is clear that liver enlargement is not involved in the decrease in CYPs protein during rat lactation.

Progesterone and its metabolites have been suggested to be involved in regulation of activities of the hepatic drug metabolizing enzymes during pregnancy (Feuer, 1979). However, once lactation begins, prolactin is increased, and progesterone is decreased and disappears immediately. Prolactin is essential not only for the initiation of lactation after parturition but also for the maintenance of lactation. In addition to prolactin, successful lactation also requires the hormone oxytocin (Heil and Subramanian, 1998). Dean and Stock (1975) have suggested that lower levels of hepatic microsomal enzyme activity might reflect a biological control mechanism to ensure the elevated levels of progesterone required to maintain the pregnant state. Similarly, down-regulation of CYPs protein probably occurs in response to the hormonal demand for milk production during lactation. In the present study, six out of nine CYPs were down-regulated at