Expression of key enzymes in bile acid biosynthesis during development: CYP7B1-mediated activities show tissue-specific differences

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Abstract The developmental variation of cytochrome P450 (CYP)7A1, CYP7B1, CYP27A1, and 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase, key enzymes in bile acid biosynthesis, were investigated in pigs of different ages. As part of these studies, peptide sequences from a purified pig liver oxysterol 7α-hydroxylase were analyzed. The sequences showed a high degree of identity with those of murine and human CYP7B1. Enzymatic activities and mRNA levels of CYP27A1 and 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase were similar in livers of newborn and 6-month-old pigs. Enzymatic activity mediated by CYP7A1 increased several-fold between infancy and adolescence. Hepatic CYP7A1 and CYP7B1 mRNA levels increased several-fold with age. Hepatic microsomal 7α-hydroxylation of 27-hydroxycholesterol and dehydroepiandrosterone, substrates typical for CYP7B1, increased about 5-fold between infancy and adolescence whereas the activities in kidney microsomes decreased at least 10-fold. In conclusion, the results indicate that the expression of CYP27A1 and 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase are similar in livers of newborn and 6-month-old pigs whereas the levels of CYP7A1 increase. The finding that the levels of CYP7B1 increase with age in the liver but decrease in the kidney suggest a tissue-specific developmental regulation of CYP7B1. The age-dependent variation in the liver and kidney suggests that hormonal factors are involved in the regulation of CYP7B1.—Norlin, M. **Expression of key enzymes in bile acid biosynthesis during development: CYP7B1-mediated activities show tissue-specific differences.** ***J. Lipid Res.*** **2002.** 43: **721–731.**

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Primary bile acids are formed from cholesterol in the liver either through the "neutral pathway", considered to be quantitatively most important in humans, or the "acidic pathway" (1). Bile acids are essential for normal absorption of lipids and lipid-soluble vitamins. The formation of bile acids is the quantitatively most important way to metabolize cholesterol and is therefore important in the regulation of cholesterol homeostasis. Several inborn errors of bile acid forming enzymes leading to disease have been reported (2–4). Recently, Setchell et al. (4) described a newborn child with severe neonatal cholestasis with a mutation in the cytochrome P450 (CYP)7B1 gene. The finding that lack of CYP7B1, an enzyme active as an oxysterol 7α-hydroxylase in the acidic pathway (1), proved to be lethal, strongly indicates a quantitative importance of the acidic pathway for bile acid formation in early human life. This is in contrast to the findings in mice by Ishibashi et al. (5) and Schwarz et al. (6) showing that murine hepatic CYP7B1 is not present in the newborn but may be upregulated later in life. Instead, CYP7A1 (cholesterol 7α-hydroxylase) appears to be critical for normal lipid absorption in the newborn mouse (5).

Although a limited amount of data are available on developmental changes of CYP7A1 (7–9), the variation during different periods of life of the bile acid forming enzymes is largely unknown. A controlled study of the development of these enzymes in humans is hampered by the difficulty in obtaining tissue material from individuals of different ages. Several of the key enzymes in bile acid biosynthesis have been purified and characterized in the pig (10–14). The pig, which is probably biochemically more related to humans than rodents are, may be more useful for studies of bile acid forming enzymes than rats and mice.

Whereas CYP7A1 is found only in the liver, CYP7B1 is expressed also in many extrahepatic tissues (15). The role of CYP7B1 in extrahepatic tissues remains uncertain. In addition to oxysterols, CYP7B1 catalyzes 7α-hydroxylation of dehydroepiandrosterone and pregnenolone (15, 16). Dehydroepiandrosterone and pregnenolone as well as their 7α-hydroxylated derivatives are considered to have partly unclear functions in various organs such as the brain (16).

cDNA encoding CYP7B1 has been isolated from mouse and human (15–17) but the enzyme has not been isolated or characterized from tissues of these species. In previous reports, we described the properties of a purified CYP en-

Abbreviations: CYP, cytochrome P450.

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zyme fraction, isolated from pig liver, with the same enzymatic activities as those of recombinantly expressed murine CYP7B1 (12–14). It is at present not clear whether the pig liver oxysterol 7α-hydroxylase used in these studies is the porcine ortholog of the murine and human CYP7B1 or another CYP species.

CYP27A1 (sterol 27-hydroxylase) is active in both the neutral and acidic bile acid forming pathways (1). This enzyme, which is expressed in liver and many extrahepatic tissues, is reported to play important roles also in other processes such as the bioactivation of vitamin D and the elimination of intracellular cholesterol from certain cells (18–21).

In this article the developmental variation of 7α-hydroxylating and 27-hydroxylating cytochromes P450 (CYP7A1, CYP7B1 and CYP27A1) and 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase, key enzymes in bile acid biosynthesis, are investigated in pigs of different ages. The current investigation also addresses the question whether the purified pig liver oxysterol 7α-hydroxylase fraction contains an enzyme identical with murine and human CYP7B1.

MATERIALS AND METHODS

Chemicals

DEAE-Sepharose CL6B, aminohexyl-Sepharose, and oligo(dT)-cellulose were purchased from Pharmacia. Hydroxylapatite (Macroprep Ceramic HTP) and horseradish peroxidase conjugated goat anti-mouse immunoglobulin were purchased from BioRad. 27-Hydroxycholesterol was a kind gift from Dr L. Tökes, Syntex, Palo Alto, CA. [4-^{14}C]cholesterol (52 mCi/mmol), [α-^{32}P]dCTP (3000 Ci/mmol), reagents for ECL (enhanced chemiluminescence) detection, the Megaprime labeling system, and Hybond-N nylon filters used in Northern blotting were purchased from Amersham International. [4-^{14}C]dehydroepiandrosterone (56.2 mCi/mmol) was purchased from PerkinElmer Life Sciences. Unlabeled dehydroepiandrosterone and Amberlite XAD-2 were obtained from Sigma Chemical Co. 7α-Hydroxycholesterol was purchased from Steraloids Inc. (Wilton, NH). 1α-Hydroxyvitamin D_3 was a generous gift from Dr. Lise Binderup, Leo (Copenhagen, Denmark). 5β-[7β-^{3}H]cholestane-3α,7α,12α-triol (500 mCi/mmol) was synthesized as described (22). The RNeasy total RNA isolation system was obtained from Qiagen. Oligonucleotides were obtained from Interactiva. Taq polymerase and oligo dNTP mix were from PE Applied Biosystems. Other chemicals, reagents, and materials were those used previously in this laboratory (12, 23).

Animals

The livers and kidneys from 6-month-old male castrated, otherwise untreated, domestic pigs were obtained from the local slaughterhouse. Livers and kidneys from male pigs aged from a few days to 3 months were obtained from the Funbo-Lövsta Research Centre, Department of Animal Breeding and Genetics, Swedish University of Animal Sciences, Ultuna. The piglets were castrated within the first week of life and weaned at about 5 weeks of age. The newborn piglets (≤5 days) used in the present study were not castrated. The domestic pig is considered to reach sexual maturity at about 6 months of age.

Preparation of microsomes and mitochondria from liver and kidney

Livers and kidneys from pigs of varying ages (newborn to 6 months) were minced in 10 mM Tris-HCl buffer, pH 7.4, containing 250 mM sucrose and 1 mM EDTA. Microsomes and mitochondria were prepared from the tissues according to standard methods (23). Microsomes used for assay of enzymatic activities were suspended in 50 mM Tris-acetate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA and stored at −20°C until analysis. Mitochondria were suspended in the same buffer and used immediately in incubation experiments.

Mitochondrial CYP active in 27-hydroxylation was partially purified from liver mitochondria from newborn and 6-month-old pigs, respectively. The mitochondrial preparations used were mixtures of liver tissue from three individuals. The procedure was similar to the methodology described previously (24, 25). Preparation of cytochrome P450 from livers of newborn and 6-month-old pigs were performed in parallel and the samples were treated similarly throughout the preparation.

Purification of oxysterol 7α-hydroxylase from pig liver

CYP catalyzing the 7α-hydroxylation of 27-hydroxycholesterol and dehydroepiandrosterone but not cholesterol was purified from liver microsomes from 6-month-old pigs as previously described by Norlin and Wikvall (12). SDS-PAGE of this purified CYP fraction showed one major and two to three minor protein bands (12).

Incubation procedures

Incubations were carried out at 37°C for 5, 10, 20, or 30 min. 27-Hydroxycholesterol (6 μg, unlabeled), [4-^{14}C]dehydroepiandrosterone (8.5 μg), or [4-^{14}C]cholesterol (5 μg) dissolved in 25 μl of acetone were incubated with varying amounts of microsomes (0.2–1 mg) and 1 μmol NADPH in a total volume of 1 ml of 50 mM Tris-acetate buffer, pH 7.4, containing 20% glycerol, and 0.1 mM EDTA. Incubations with cholesterol contained 0.05% (w/v) Triton X-100. 7α-Hydroxycholesterol and 7α,27-dihydroxycholesterol (20 μg of substrate) dissolved in 25 μl of acetone were incubated with 0.2 mg of microsomes and 1 μmol NAD^+ in a volume of 1 ml of 100 mM phosphate buffer pH 7.4, containing 20% glycerol and 0.1 mM EDTA. Incubations with liver mitochondria (3 mg) and unlabeled cholesterol (6 μg) or 7α-hydroxy-4-cholesten-3-one (6 μg) were carried out in presence of isocitrate (8 μmol) and $MgCl_2$ (10 μmol), and 50 mM Tris-acetate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA. 5β-[7β-^{3}H]Cholestane-3α,7α,12α-triol (20 μg) or 1α-hydroxyvitamin D_3 (10 μg), dissolved in 25 μl of acetone, were incubated with mitochondrial CYP fractions (0.06–0.4 mg), 4 nmol of ferredoxin, 0.4 nmol of ferredoxin reductase, and 1 μmol NADPH in a volume of 1 ml of 50 mM Tris-acetate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA.

Incubations with 27-hydroxycholesterol, cholesterol, 7α-hydroxycholesterol, 7α,27-dihydroxycholesterol, 7α-hydroxy-4-cholesten-3-one, and 1α-hydroxyvitamin D_3 were terminated with 5 ml of trichloroethane-methanol (2:1) and incubations with dehydroepiandrosterone with 5 ml of ethyl acetate. The incubations with 5β-cholestane-3α,7α,12α-triol were terminated with 5 ml of ethanol.

Analysis of incubation mixtures

Formation of 7α-hydroxycholesterol, 7α,27-dihydroxycholesterol, and 7α-hydroxydehydroepiandrosterone was analyzed by HPLC as previously described (12–14). The formed 7α-hydroxycholesterol and 7α,27-dihydroxycholesterol were converted to the respective 3-oxo-Δ^4 derivative by incubation with cholesterol oxidase prior to analysis. Analysis of formation of 7α-hydroxy-4-cholesten-3-one from 7α-hydroxycholesterol and 7α,27-dihydroxy-4-cholesten-3-one from 7α,27-dihydroxycholesterol was carried out as described by Furster et al. (11, 26). Incubations with 1α-hydroxyvitamin D_3 were analyzed by HPLC as described by Axén et al. (27). The incubations with 5β-cholestane-3α,7α,12α-triol

were extracted with acidified ether and the extracted steroids were applied on silica gel TLC plates. The chromatoplates were developed once in a solvent system consisting of ethyl acetate-isooctane-acetic acid, 50:50:17 (v/v/v) and scanned for radioactivity using a Berthold Tracemaster 20 TLC scanner.

SDS-PAGE

SDS-PAGE experiments with CYP27A1 were performed according to Laemmli (28) with 15% acrylamide and 0.09% bisacrylamide slab gels (15 cm × 10 cm × 0.1 cm) containing 0.1% (w/v) SDS. The gels were polymerized by addition of 0.1% (v/v) tetramethylenediamine and 0.1% (w/v) ammonium hydrogen sulfate. The experiments with 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase were performed with modifications as described (11, 26).

Immunoblotting

Immunoblotting was performed as described (29) except that enhanced chemiluminescence (ECL) was used to detect the immunoreactive bands. The monoclonal antibodies against porcine CYP27A1 and 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase, used in immunoblotting, were those produced and described in previous reports from this laboratory (10, 26). The bands on the film were scanned and the relative intensity was quantified using the NIH Image (1.62) program, which is a public domain image processing and analysis program for the Macintosh (http://rsb.info.nih.gov/nih-image/index.html).

Peptide sequence analysis of pig liver oxysterol 7α-hydroxylase

Purified oxysterol 7α-hydroxylase protein (12) was treated with Amberlite XAD-2 to remove detergent. Proteolytic peptides were generated by cleavage with trypsin (Boehringer) and sequence analysis was performed by mass spectrometry quadrupole-time-of-flight (30).

Isolation of RNA and Northern blot analysis

Total RNA was isolated from liver tissue with the RNeasy total RNA Midi isolation kit (Qiagen) according to the manufacturers instructions. Poly(A)$^+$ RNA was prepared by oligo(dT)-cellulose chromatography according to standard procedures (31).

Northern blot analysis was carried out following electrophoresis of either total RNA (20 μg) or poly(A)$^+$ RNA (12 μg) on denaturing 1.2% agarose gels containing formaldehyde. The RNA was transferred to a nylon filter (Hybond N) and hybridization was carried out at 42°C for 16 h with a ^{32}P-labeled cDNA probe. The probes were labeled with [α-^{32}P]dCTP with the Megaprime labeling system, according to the manufacturers protocol. The cDNA probes used were a 1.7 kb CYP27A1 pig kidney cDNA fragment (32), a 1.5 kb *Nco*I-*Nco*I fragment of human CYP7A1 cDNA excised from a pJL vector containing human CYP7A1 (33), and a 1.6 kb *Eco*RI-*Not*I fragment of human CYP7B1 cDNA excised from a pCMV6 vector containing human CYP7B1. The pJLH7α1.5 and the pCMV-hCYP7B1 vectors were kind gifts from Dr. J. Y. L. Chiang, Northeastern Ohio Universities, and Dr. D. W. Russell, University of Texas, Dallas, respectively. The hybridization buffer contained 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate), 1× PE (50 mM Tris-HCl, pH 7.5, 0.1% sodium pyrophosphate, 1% SDS, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 5 mM EDTA, and 0.2% BSA), 100 μg/ml salmon sperm DNA and 100 μg/ml tRNA. After hybridization the filters were washed in 2× SSC containing 0.1% SDS at room temperature for 10 min and then in 0.1× SSC containing 0.1% SDS at 42°C for 20 min. Filters hybridized with the CYP27A1 cDNA probe were subjected to further washing at 55°C for 40 min. After washing, the filters were exposed to Fuji RX film with an intensifying screen for 1 to 5 days at −80°C. The filters were then stripped and probed with a 1.7 kb *Bam*HI-*Sal*I fragment of human β-actin cDNA.

The bands on the film were scanned and the relative intensity was quantified using the NIH Image program as described above.

PCR experiments

RT-PCR experiments on pig liver 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase were performed using the following oligonucleotide primers: forward primer, 5′-GGCAATGAAAATACCCCATATGA-3′; reverse primer, 5′-ATCCTCACCGGCCTGCACCCA-3′. These primers, designed from sequences of an internal and a C-terminal peptide obtained from the purified pig liver 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase (11, Furster and Wikvall, unpublished results) were a kind gift from Drs. Catrin Furster and Kjell Wikvall, Uppsala. First-strand cDNA synthesis was conducted using 1 μg of pig liver RNA and Reverse Transcription System (Promega) with 0.5 μg Oligo(dT)$_{15}$ primer according to the manufacturer's instructions. PCR was performed with 30 pmol of each primer and 2.5 units of Taq polymerase, in a total volume of 50 μl (reaction buffer: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 0.2 mM dNTP). The PCR reaction was run as follows: 95°C for 10 min, followed by 30 cycles (94°C for 2 min, 50°C for 1 min, 72°C for 1 min), and 72°C for 10 min. The 700 bp PCR product was analyzed by electrophoresis on 1% agarose gels containing 0.5 μg/ml ethidium bromide and by DNA sequence analysis using Dye-labeled Terminator for the sequence reaction and a ABI377 DNA sequencer for analysis.

The levels of 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase mRNA were assessed by RT-PCR involving reverse-transcribing 1 μg of total RNA from livers of pigs of varying ages to generate a pool of cDNA representing the RNA in the original sample. The resulting cDNA pool for each RNA sample was divided into aliquots (5 μl) with one aliquot used in each PCR. The oligonucleotide primers and conditions for PCR were as described above. The expression of CYP27A1 for each sample was also determined, and this was used as an internal control for the efficiency of each RT-PCR. The primers used for porcine CYP27A1 were those described previously (32). The number of cycles used for PCR were determined to be in the exponential phase of the amplification process. The number of cycles was 25 for 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase and 30 for CYP27A1. The agarose gel with PCR products were photographed and the negatives were scanned to determine the relative intensity of the bands in the same way as for the immunoblotting and Northern blot experiments.

Other methods

Ferredoxin and ferredoxin reductase were prepared from bovine adrenal mitochondria as described by Wikvall (34). Protein concentrations in microsomal and mitochondrial fractions were determined by the method of Lowry (35). The concentration in purified

TABLE 1. Summary of the changes in levels of CYP27A1, CYP7A1, CYP7B1, and 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase with age

	CYP27A1	3β-Hydroxy-Δ^5-C_{27}-Steroid Dehydrogenase	CYP7A1	CYP7B1	
				Liver	Kidney
mRNA levels	↔	↔	↑	↑	ND[a]
Catalytic activity	↔	↔	↑	↑	↓
Immunodetectable protein	↓	↔	ND	ND	ND

The table shows a comparison of the levels of protein and mRNA in pigs aged from newborn to 6 months old. For detailed data see Tables 2, 4, 5, and 6.

[a] Not determined.

TABLE 2. 7α-Hydroxylase activities in liver microsomes from pigs of different ages

Age	7α-Hydroxylation of Cholesterol	7α-Hydroxylation of 27-Hydroxycholesterol	7α-Hydroxylation of Dehydroepiandrosterone
	pmol/mg/min	*pmol/mg/min*	*pmol/mg/min*
Newborn (0–5 days)	0.3 + 0.1[a]	80 ± 17[b]	439 ± 57[c]
2 weeks	ND[d]	183 ± 24	1313 ± 4
12 weeks	1.5 ± 0.1[b]	242 ± 46	1474 ± 285
24 weeks	1.9 ± 0.2	331 ± 63	2687 ± 267

Incubations were performed as described in Materials and Methods. Duplicate incubations (triplicates for 7α-hydroxylation of cholesterol) were performed with microsomes from two to five individuals of each age. The data represent the means and standard deviation. A difference in enzyme activity from that of 24-week-old pigs was interpreted as significant if $P < 0.05$. The values for different ages were compared with the activity of 24-week-old pigs.

[a] $P < 0.01$.
[b] $P < 0.05$.
[c] $P < 0.02$.
[d] ND, not determined.

protein fractions was estimated by measuring the absorbance at 280 nm (concentration in mg/ml = absorbance of protein at 280 nm).

RESULTS

Studies on the expression of bile acid biosynthetic enzymes during development

The levels of CYP7A1, CYP7B1, 3β-hydroxy-Δ^5-C_{27} steroid dehydrogenase, and CYP27A1 were examined in pigs of different ages by assay of enzymatic activities and mRNA levels. CYP27A1 and 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase levels were analyzed also with immunoblotting. The general findings of this study are summarized in **Table 1**.

Hepatic 7α-hydroxylation

Microsomal 7α-hydroxylase activities in livers from pigs of different ages. The 7α-hydroxylation of cholesterol, 27-hydroxycholesterol, and dehydroepiandrosterone in liver microsomes from pigs, varying in age from newborn to 6 months, was examined. Since the domestic pig reaches sexual maturity at about 6 months, the results would reflect the variation between infancy and adolescence. The activities toward these substrates were lowest in the newborn and increased

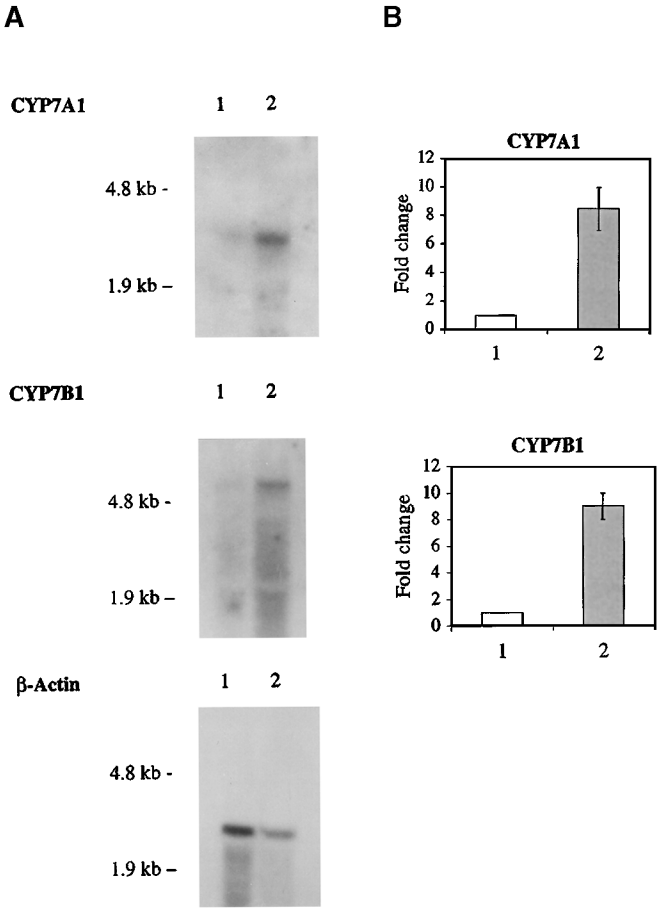

Fig. 1. Northern blot analysis of cytochrome P450 (CYP)7A1 and CYP7B1 mRNA in pig liver. A: Poly(A)$^+$ RNA (12 μg) isolated from livers of newborn (≤5 days old) (lane 1) and livers of 6-month-old (lane 2) pigs. Northern blotting was performed as described in Materials and Methods initially with a CYP7A1 cDNA probe. The filters were then stripped and rehybridized with a CYP7B1 cDNA probe and subsequently with a β-actin cDNA probe. Note that the three photos in Fig. 1 show the same RNA samples hybridized with different probes. The hybridization signal for the CYP7B1 cDNA probe was weak and required long exposure times. The experiment with β-actin shows that the RNA is not degraded. The samples were mixtures of RNA from four individuals, respectively. B: The bands on the film were scanned using the NIH Image program and the relative intensity was expressed as fold change after normalization to β-actin. Error bars represent mean and range of two experiments. 1, newborn; 2, 6-month-old pigs.

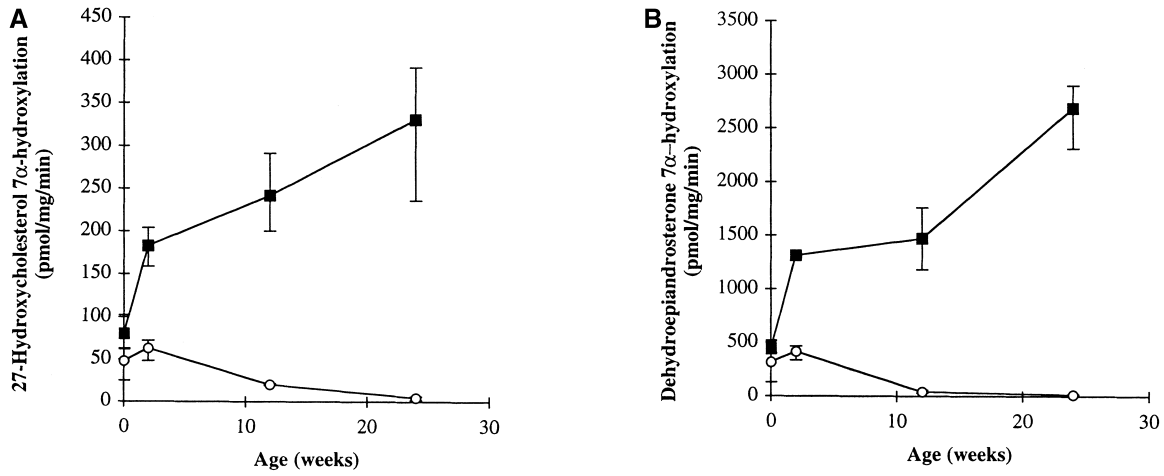

Fig. 2. Age-dependent variations of the 7α-hydroxylase activity toward 27-hydroxycholesterol (A) and dehydroepiandrosterone (B) in microsomes from liver (closed square) and kidney (open circle). Duplicate incubations were performed with microsomes from two (for 2–12-week-old pigs) or five (for newborn and 6-month-old pigs) individuals. The data represent the means and range. In most cases the liver and kidneys used were from the same individuals. The renal 27-hydroxycholesterol 7α-hydroxylase activity was 48 pmol/mg/min for newborn and 5 pmol/mg/min for 6-month-old pigs. The renal dehydroepiandrosterone 7α-hydroxylase activity was 319 pmol/mg/min for newborn and 15 pmol/mg/min for 6-month-old pigs.

with age (Table 1 and **Table 2**). The 7α-hydroxylase activities toward cholesterol, 27-hydroxycholesterol, and dehydroepiandrosterone increased over time to become about 4- to 6-fold higher in livers from 6-month-old pigs than in livers from newborns.

Levels of CYP7A1 mRNA. Northern blot hybridization was carried out with poly(A)$^+$ RNA from livers of newborn and 6-month-old pigs. The samples were mixtures of RNA from four individuals of each age. The probe was a ^{32}P-labeled human 1.5 kb CYP7A1 cDNA fragment. The levels of CYP7A1 mRNA were about 8-fold higher in livers of 6-month-old pigs as compared with livers of newborns (**Fig. 1**).

Levels of CYP7B1 mRNA. Northern blot hybridization was carried out with poly(A)$^+$ RNA from livers of newborn and 6-month-old pigs. The samples were mixtures of RNA from four individuals of each age. The probe was a ^{32}P-labeled human 1.6 kb CYP7B1 cDNA fragment. The levels of CYP7B1 mRNA were about 9-fold higher in livers of 6-month-old pigs as compared with livers of newborns (Fig. 1).

TABLE 3. Comparison of porcine oxysterol 7α-hydroxylase peptide sequences with human and murine CYP7B1

Amino Acid Sequence		Position of Similar Sequence	Identity with Porcine Sequence
Peptide 1			
porcine	YITFILDPFQYQSVIK		
human	YITFILDPFQYQLVIK	89–104	94%
murine	YITFVLNPFQYQYVTK	89–104	75%
Peptide 2			
porcine	FTQLASGFPIELLGNIK		
human	FAYLVSNIPIELLGNVK	223–239	65%
murine	FPYLVSDIPIQLLRNEE	216–232	41%
Peptide 3			
porcine	EQLDSLVYLESTILESLR		
human	EQLDSLICLESSIFEALR	344–361	72%
murine	EQLDSLVCLESTILEVLR	342–359	89%

Amino acid sequences of three internal peptide fragments obtained from the protein in the purified porcine oxysterol 7α-hydroxylase fraction. Alignments with human and murine CYP7B1 are shown.

Renal 7α-hydroxylation

The catalytic activity toward 27-hydroxycholesterol and dehydroepiandrosterone was examined in kidney microsomes from pigs varying in age from newborn to 6 months. In contrast to the results with liver microsomes, the 7α-hydroxylation of these substrates by kidney microsomes decreased with age (**Fig. 2**). The 7α-hydroxylase activities in kidneys from 6-month-old pigs were about 10–20 times lower than in kidneys from newborns. Figure 2 shows a comparison of the age-dependent variations in the 7α-hydroxylation of 27-hydroxycholesterol and dehydroepiandrosterone in liver and kidney.

Structural analysis of peptides from purified porcine oxysterol 7α-hydroxylase

In previous reports we described the properties of a purified CYP enzyme fraction, isolated from pig liver, show-

TABLE 4. 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase activities towards 7α-hydroxycholesterol and 7α,27-dihydroxycholesterol in liver microsomes from pigs of different ages

Age	7α-Hydroxycholesterol	7α,27-Dihydroxycholesterol
	pmol/mg/min	*pmol/mg/min*
Newborn (0–5 days)	1032 ± 585 (n = 5)	632 ± 114 (n = 4)
2 weeks	636 ± 375 (n = 7)[a]	307 ± 11 (n = 4)[a]
12 weeks	1015 ± 430 (n = 12)	510 ± 191 (n = 4)
24 weeks	1164 ± 179 (n = 9)	525 ± 131 (n = 4)

Incubations were performed as described in Materials and Methods. Incubations were performed with microsomes from two to five individuals of each age. The data represent the means and standard deviation. n, number of incubation experiments.

[a] The difference in enzyme activity was not statistically different from that of the 24-week-old pigs ($P > 0.1$).

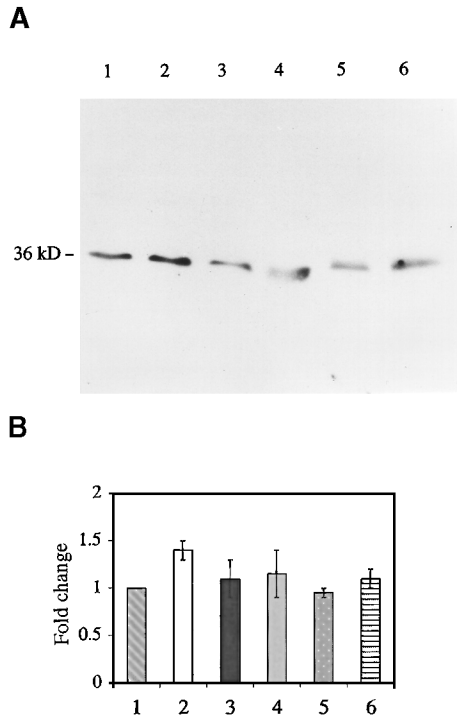

Fig. 3. Immunoblotting of 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase in liver microsomes from pigs of different ages. A: Immunoblotting was performed with microsomes (50 μg) from livers of 2-week-old (lanes 1 and 2), 12-week-old (lanes 3 and 4), and 24-week-old pigs (lanes 5 and 6) with a monoclonal antibody mixture raised against 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase as described (11, 26) except that enhanced chemiluminescence (ECL) was used to detect the immunoreactive bands. The concentration of the primary antibodies was 0.1 μg/ml. The secondary antibody (horseradish peroxidase conjugated goat anti-mouse IgG) was diluted 1:3,000. Fetal bovine serum (7.5%, w/v) was added in all solutions to minimize nonspecific antibody binding. B: The bands on the film were scanned using the NIH Image program and the relative intensity was expressed as fold change with the value for one of the newborns set to 1. Error bars represent mean and range of two experiments. 1 and 2, 2-week-old; 3 and 4, 12-week-old; 5 and 6, 24-week-old pigs.

ing the same catalytic properties as those of recombinantly expressed murine CYP7B1 (12–14). In order to compare the protein with CYP7B1, the highly purified porcine "oxysterol 7α-hydroxylase" preparation (12) was used for sequence analysis. Three peptide fragments generated by proteolytic cleavage of the protein were subjected to amino acid sequence analysis. These peptides showed high sequence homology with human (65–94% identity) and murine (41–89% identity) CYP7B1 (**Table 3**).

Hepatic microsomal 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase

Hepatic microsomal 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase activities in livers from pigs of different ages. The dehydrogenase/isomerase activity toward 7α-hydroxycholesterol, a substrate of the neutral pathway, and 7α,27-dihydroxycholesterol, a substrate in the acidic pathway, was measured in liver microsomes from pigs varying in age from newborn to 6 months. The rates of formation of 7α-hydroxy-4-cholesten-3-one and 7α,27-dihydroxy-4-cholesten-3-one were not significantly changed during this age period (Table 1 and **Table 4**).

Immunoblotting with antibodies directed against microsomal 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase. A mixture of monoclonal antibodies raised against hepatic microsomal 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase (26) were used in immunoblotting experiments with liver microsomes from pigs of different ages. The antibodies recognized a protein of the same apparent M_r as 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase (36 kDa) in all preparations. The amount of immunoreactive protein was similar in all ages (**Fig. 3**).

Levels of 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase mRNA. The purification and properties of a pig liver microsomal 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase were described in previous reports from this laboratory (11, 26). Sequences for an internal peptide and the C-terminal part have been obtained from the purified enzyme (Furster and Wikvall, unpublished results). In the present study, oligonucleotide primers designed from this sequence information were used in PCR experiments with pig liver RNA. A DNA product of about 700 bp was amplified and sequenced. Alignment of this porcine microsomal 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase sequence with the corresponding sequence (475–1119) of a recently cloned human 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase (36) revealed 86% identity (**Fig. 4**). In order to examine the levels of 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase mRNA in livers from pigs of different ages, the oligonucleotide primers for the porcine enzyme were used in RT-PCR experiments. The samples were mixtures of RNA from three individuals of each age. **Figure 5** shows that the mRNA levels were similar in newborn and adolescent pigs. As an internal control, the levels of CYP27A1 mRNA were examined in the same samples. There was no age-dependent difference in CYP27A1 mRNA levels (see Fig. 7).

Hepatic mitochondrial CYP27A1

CYP27A1-mediated activities in livers from pigs of different ages. 27-Hydroxylation of cholesterol (substrate in the acidic pathway) and 7α-hydroxy-4-cholesten-3-one (substrate in the neutral pathway) were measured in liver mitochondria from newborn, 12-week-old, and 24-week-old pigs (**Table 5**). There was no significant age-dependent difference in the activity. 27-Hydroxylation of cholesterol, 7α-hydroxy-4-cholesten-3-one, 5β-cholestane-3α,7α,12α-triol, and 25-hydroxylation of 1α-hydroxyvitamin D_3 were measured in partially purified CYP extracts, isolated from liver mitochondria of newborn (5 days old) and 6-month-old pigs. These reactions are all considered to be mediated by CYP27A1 in mitochondria (1, 18, 32). The catalytic activity toward these substrates were about the same in livers from newborns as in livers from 6-month-old pigs (Table 1 and **Table 6**).

Immunoblotting with an antibody directed against CYP27A1. A monoclonal antibody raised against CYP27A1 (10) was used in immunoblotting experiments with partially purified liver mitochondrial protein from newborn and 6-month-old pigs. The antibody recognized a protein of the same

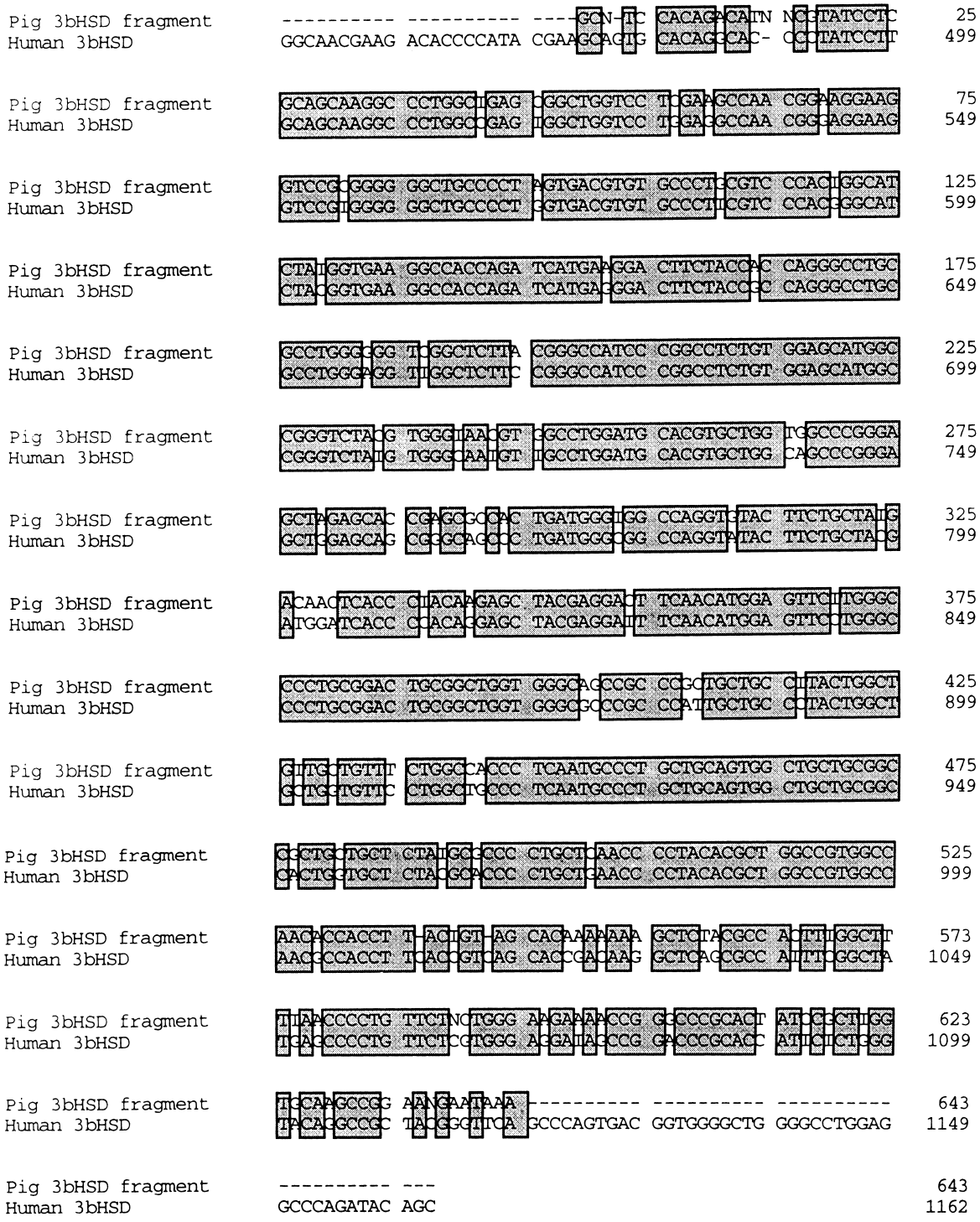

Fig. 4. Sequence of a DNA fragment of porcine 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase and alignment with human 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase.

apparent M_r as CYP27A1 (52 kDa) in both preparations. Scanning of the intensity of the bands indicated that the amount of immunoreactive protein was about 1.5 times larger in livers from newborns than in livers from 6-month-old pigs (**Fig. 6**).

Levels of CYP27A1 mRNA. Northern blot hybridization was carried out with samples of RNA prepared from livers of newborn and 6 months old pigs (**Fig. 7**). The samples were mixtures of RNA representing five individuals of each age. The probe was a ^{32}P-labeled 1.7 kb CYP27A1 pig kidney cDNA fragment (32). The levels of CYP27A1 mRNA in newborn and 6-month-old pigs were found to be similar. Two (or three) closely spaced hybridizing mRNAs appeared to be present in both samples. The amount of each RNA transcript varied between different individuals (data not shown). This pattern, however, did not show a consis-

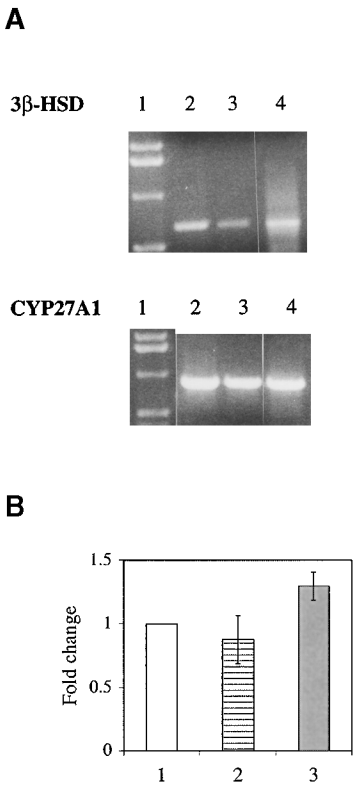

Fig. 5. RT-PCR experiments on mRNA for 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase and CYP27A1 in liver from pigs of different ages. The experiments were carried out with the primers for porcine 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase and CYP27A1, respectively, as described under Materials and Methods. A: The amplified PCR products (700 bp for 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase and 833 bp for CYP27A1) were analyzed by electrophoresis on 2% agarose gels containing 0.5 μg/ml ethidium bromide. The samples of total RNA used for first strand cDNA synthesis were mixtures from three individuals of each age. Each lane contain 5 μl of the respective first strand cDNA synthesis reaction mixture as described under Materials and Methods. Lane 1, DNA size ladder (from top to bottom; 2036, 1635, 1018, 516 bp), lane 2, newborn; lane 3, 2-week-old; lane 4, 24-week-old pigs. Negative controls containing water instead of cDNA were included in each experiment and did not show any product formation. B: The gel was photographed and the bands on the negative film were scanned using the NIH Image program and the relative intensity was expressed as fold change after normalization to CYP27A1. Error bars represent mean and standard deviation of three experiments. 1, newborn; 2, 2-week-old; 3, 24-week-old pigs.

tent variation with age but appeared more to reflect differences between individuals. A pattern of more than one CYP27A1 mRNA transcript has previously been reported in tissues from pig, rat, and rabbit (32, 37, 38).

DISCUSSION

The bile acid pool of human newborn infants is reported to be reduced as compared with normal adults. It has been proposed that the mechanisms that control bile acid metabolism and turnover may be one of the factors responsible for the inefficient fat absorption observed in human newborn infants (39). Studies in pigs also indicate that the bile acid pool size is smaller in suckling piglets as compared with older animals (40, 41). It might be assumed that the expression of bile acid forming enzymes would increase with age in parallel with the maturation of the lipid absorption system. However, the present study shows that this is only true in part. Of the enzymes studied, only hepatic CYP7A1 and CYP7B1 showed an increase with age. In contrast, the levels of CYP27A1 and 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase remained similar throughout the studied age period. Surprisingly, the developmental expression pattern for CYP7B1 in kidney was found to be opposite to the pattern in liver.

TABLE 5. 27-Hydroxylase activities of liver mitochondria from pigs of different ages

Age	27-Hydroxylation of Cholesterol	27-Hydroxylation of 7α-Hydroxy-4-cholesten-3-one
	pmol/mg/min	*pmol/mg/min*
Newborn (0–5 days)	18 ± 1	156 ± 3
12 weeks	12 ± 3[a]	125 ± 34[a]
24 weeks	18 ± 3	142 ± 35

Incubations were performed as described in Materials and Methods. The data are given as the means of three experiments with SD.

[a] The difference in enzyme activity was not statistically different from that of the 24-week-old pigs ($P < 0.1$).

CYP7A1

CYP7A1, an enzyme generally considered to be rate-limiting in bile acid biosynthesis (1), increased several-fold with age both at the enzyme activity level and the mRNA level. The developmental pattern found for CYP7A1 is partly in agreement with previously reported findings (7, 8). Kwekkeboom et al. (7) also reported an increase of cholesterol 7α-hydroxylase activity with age but found the highest activity in 4 to 8-week-old female pigs. In rats, cholesterol 7α-hydroxylase is reported to be low in newborns but increased at weaning (7, 42).

CYP27A1

In contrast to CYP7A1, liver mitochondrial CYP27A1 was not significantly increased with age. Levels of CYP27A1 mRNA and enzymatic activities were about the same in newborns and 6-month-old pigs. In the Western blot experiments, the levels of CYP27A1 protein were even slightly higher in the newborns. Taken together the results of the present study indicate that there is no age-dependent difference in the expression of CYP27A1 in pig liver. This is in contrast to the results of Lewis et al. (8), who reported that sterol 27-hydroxylase activity was not detectable in pig liver mitochondria within 2 days of term. CYP27A1 is reported to be involved not only in bile acid biosynthesis but also in other important processes such as the bioactivation of vitamin D (18, 19) and the elimination of intracellular cholesterol (20). A mutation in the CYP27A1 gene results in the disease cerebrotendinous xanthomatosis (3). The presence of similar levels of CYP27A1 in the newborn and in the mature state is in agreement with multiple important roles for this enzyme.

TABLE 6. Catalytic activities of partially purified liver mitochondrial CYP

Age	27-Hydroxylation of Cholesterol	27-Hydroxylation of 7α-Hydroxy-4-cholesten-3-one	27-Hydroxylation of 5β-Cholestane-3α,7α,12α-triol	25-Hydroxylation of 1α-Hydroxyvitamin D_3
	pmol/mg/min	*pmol/mg/min*	*pmol/mg/min*	*pmol/mg/min*
Newborn (5 days)	186 (150–122)	1769 (1710–1793)	2516 (2342–2691)	158 (138–179)
24 weeks	164 (145–190)	2998 (2810–2995)	1912 (1869–1956)	156 (146–167)

Incubations were performed as described in Materials and Methods. The data are given as the means of two or three experiments with range.

3β-Hydroxy-Δ^5-C_{27}-steroid dehydrogenase

The present results show high levels of microsomal 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase in the newborn. The levels of mRNA, protein, and enzymatic activities associated with this enzyme did not increase any further but remained similar in newborns and 6-month-old pigs. These results indicate an important role for the enzyme in early life. In consistence with this concept, a mutation in the human 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase gene (36) results in neonatal cholestasis and progressive liver disease (3).

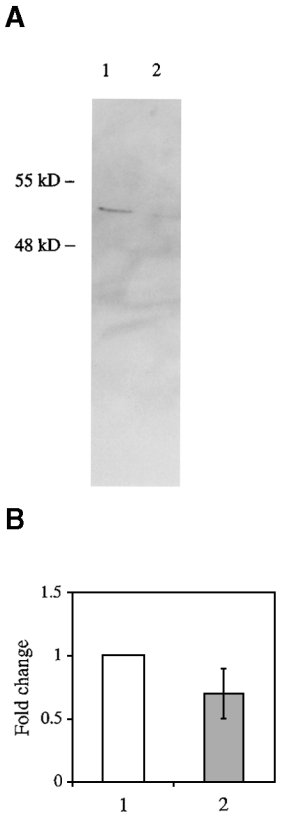

Fig. 6. Immunoblotting of partially purified CYP from liver mitochondria. A: Immunoblotting was performed with purified mitochondrial protein (25 μg) from livers of 5-day-old (lane 1) and 6-month-old pigs (lane 2) with a monoclonal antibody raised against liver mitochondrial CYP27A1 (10). The samples were mixtures of material from three individuals, respectively. The concentration of the CYP27A1 antibody was 0.1 μg/ml. The secondary antibody (horseradish peroxidase conjugated goat anti-mouse IgG) was diluted 1:3,000. Fat-free milk (10%, w/v) was added in all solutions to minimize nonspecific antibody binding. The experiment was performed twice with similar results. B: The bands on the film were scanned using the NIH Image program and the relative intensity was expressed as fold change. Error bars represent mean and range. 1, 5-day-old; 2, 6-month-old pigs.

CYP7B1

The developmental pattern of hepatic CYP7B1 expression appears to differ strongly between humans and mice. In contrast to the findings by Setchell et al. (4) on human CYP7B1, Schwarz et al. (6) showed that murine hepatic CYP7B1 is not present in the newborn liver but is upregulated later in life. From the current investigation it may be concluded that the porcine form of CYP7B1 appears to be more similar to the human CYP7B1 than the murine ortholog is. The sequences of three peptides obtained from a highly purified pig liver oxysterol 7α-hydroxylase fraction, with the same catalytic properties as CYP7B1 (12), showed a high degree of identity with both murine and human CYP7B1 sequences. The sequence identity was

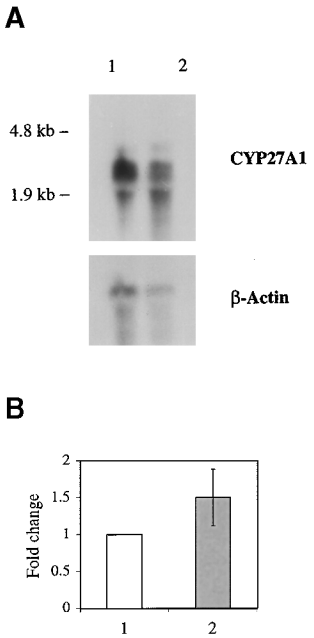

Fig. 7. Northern blot analysis of CYP27A1 mRNA in pig liver. A: Total RNA (20 μg) isolated from livers of newborn (lane 1) and livers of 6-month-old (lane 2) pigs. Northern blotting was performed as described in Materials and Methods with a CYP27A1 cDNA probe. Blots were stripped and rehybridized with human β-actin cDNA. The samples were mixtures of RNA representing five individuals of each age. B: The bands on the film were scanned using the NIH Image program and the relative intensity was expressed as fold change after normalization to β-actin. Error bars represent mean and standard deviation of three experiments. 1, newborn (≤5 days old); 2, 6-month-old pigs.

the highest with human CYP7B1. The ability of a human CYP7B1 cDNA probe to hybridize with a pig liver RNA transcript also strongly indicates that a protein belonging to the CYP7B1 subfamily is expressed in pig liver. The size of the porcine CYP7B1 transcript was considerably larger than the CYP7A1 and CYP27A1 transcripts, which is also the case with human CYP7B1 RNA (15).

Age-dependent changes in the enzymatic activity toward substrates typical for CYP7B1 showed different patterns depending on the tissue studied. Whereas incubations with 27-hydroxycholesterol and dehydroepiandrosterone and liver microsomes showed an increase of 7α-hydroxylase activity with age, the same experiments with kidney microsomes showed a marked age-dependent decrease. The contrasting patterns in liver and kidney may have different reasons. One possibility is that 7α-hydroxylation of 27-hydroxycholesterol and dehydroepiandrosterone in the liver is performed also by enzymes other than CYP7B1, some of which are not present in the kidney. Three hepatic 7α-hydroxylases have been described so far: CYP7B1, CYP7A1, and CYP39A1. CYP7A1 was recently shown to be able to catalyze 27-hydroxycholesterol 7α-hydroxylation, but it should be noted that CYP7A1 does not 7α-hydroxylate dehydroepiandrosterone (14). CYP39A1, a 7α-hydroxylase active toward 24-hydroxycholesterol was recently described by Li-Hawkins et al. (43). CYP39A1 avidly metabolized 24-hydroxycholesterol but had little or no activity toward other oxysterols, including 27-hydroxycholesterol (43). From these findings it appears very unlikely that either CYP7A1 or CYP39A1 should contribute to the contrasting developmental patterns observed in kidney and liver in this study. Another possible, and more likely, explanation is that the results are due to tissue-specific developmental regulation of CYP7B1. The finding that CYP7B1 mRNA levels were several-fold higher in livers of 6-month-old pigs than in newborns supports this explanation.

The function of CYP7B1 in the kidney remains to be investigated. Some information was obtained in a study on the disruption of the Cyp7b1 gene in mice (44). From the results of that study it was suggested that the major physiological role of CYP7B1 is to inactivate 25- and 27-hydroxycholesterol, compounds that play several roles in lipid metabolism. In vivo cholesterol biosynthesis was reduced in kidneys of male but not female mice. The authors suggested that a failure to catabolize oxysterols in the male kidney may lead to a decrease in sterol biosynthesis (44). The present data, showing a strong decrease with age, suggest that the renal enzyme is important for the newborn. An important role for CYP7B1 in the newborn is consistent with the results obtained by Setchell et al. (4). These authors described an infant with a mutation in the CYP7B1 gene who suffered from severe cholestasis. As shown by the autopsy, this patient also had enlarged and histologically abnormal kidneys.

The regulation of CYP7A1 has been extensively studied (1, 45) and some information is available on the regulation of CYP27A1 (46–48). However, little data have yet been presented on the regulatory mechanisms for CYP7B1 (1). The results of the present study indicate a tissue-specific developmental regulation of this enzyme. The age-dependent variation in liver and kidney suggest that hormonal factors are involved in the regulation of CYP7B1.

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