Ontogenic development-associated changes in the expression of genes involved in rat bile acid homeostasis

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Abstract Ontogenic changes in the rat bile acid (BA) pool, measured enzymatically and by GC-MS, and expression of enzymes (5α-reductase, 5β-reductase, and cytochrome P450 enzymes Cyp7a1, Cyp8b1, Cyp27 and Cyp3a11), transporters [bile salt export pump, sodium taurocholate-cotransporting polypeptide, apical sodium-dependent bile acid transporter, and organic solute transporter α/β (Ostα/Ostβ)], and nuclear receptors [fetoprotein transcription factor (Ftf), farnesoid X receptor (Fxr), small heterodimer partner (Shp), and hepatic nuclear factor 4α (HNF-4α)], determined by quantitative PCR, were investigated. The absolute size of the BA pool increased progressively up to adulthood, whereas the complexity of its composition was high in fetuses, decreased after birth, increased again progressively up to adulthood, and decreased in aged animals. *Allo*-cholic acid only appeared early in development, in spite of low 5α-reductase expression. The relative size of the BA pool, corrected by liver weight, was maintained from 1 week after birth, except at weaning, when a transient peak accompanied by Shp downregulation and Cyp7a1 upregulation was observed. An imposed weaning delay of 1 week had no effect on the time course of the BA pool size but decreased the proportion of chenodeoxycholic and α-muricholic acids, whereas the proportion of cholic acid was increased, probably as a result of Cyp8b1 upregulation. In conclusion, changes in the expression of genes involved in BA homeostasis may play a role in physiological adaptations to digestive functions during the rat life span.—Cuesta de Juan, S., M. J. Monte, R. I. R. Macias, V. Wauthier, P. B. Calderon, and J. J. G. Marin. **Ontogenic development-associated changes in the expression of genes involved in rat bile acid homeostasis. *J. Lipid Res.* 2007.** 48: **1362–1370.**

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Bile acids (BAs) are amphipathic steroids synthesized in the liver from cholesterol and are involved in important physiological functions. Among these, the best known is their role in fat digestion and absorption. This is mainly performed by their detergent activity, which is closely related to their chemical structure, which varies among many different molecular species present in the BA pool. The composition of the BA pool is not the same in all species, and in each species it probably changes during its life span. All BA variants share the presence of the steroid ring and the side chain, but they differ mainly in the degree of hydroxylation and the position of their hydroxyl groups. The total amount of BAs in the body and the composition of this pool are determined by the expression and activity of enzymes involved in their synthesis and biotransformation in concerted action with plasma membrane transporters, which account for their retention in the enterohepatic circulation. BA metabolism by intestinal bacteria and elimination by the kidney may also contribute to the final BA pool size and composition.

Owing to the key role of the liver in BA homeostasis, it is easy to understand why phenotypic changes in this organ affect the BA pool. Thus, proliferative processes occurring in the liver after partial hepatectomy or during hepatocarcinogenesis are accompanied by important changes in the composition of the BA pool (1–3). Such changes are closely related to changes in the expression profiles of enzymes involved in BA metabolism (4, 5). Although ontogenic development also involves proliferation and differentiation, in addition to adaptive changes to modifications in the diet, little is known about the expression of enzymes

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Abbreviations: Asbt, apical sodium-dependent bile acid transporter; BA, bile acid; CYP, cytochrome P450 enzymes; Ftf, fetoprotein transcription factor; Fxr, farnesoid X receptor; Hnf4α, hepatic nuclear factor 4α; Ntcp, sodium taurocholate-cotransporting polypeptide; Ost, organic solute transporter; Shp, small heterodimer partner.

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involved in BA synthesis associated with this development (6); more is known about the expression of transporters under these circumstances (7, 8). In light of the foregoing, the aim of this study was to investigate the relationship between changes in the BA pool size and composition during fetal life, postnatal development, youth, maturity, and senescence and modifications in the expression profiles of the enzymes, transporters, and nuclear receptors involved in the control of BA pool size and composition. Particular attention is paid to the weaning period, when important nutritional, nervous, and endocrine factors that may affect BA homeostasis occur.

MATERIALS AND METHODS

Chemicals

BAs and 5β-cholestane used as standards in GC-MS (>95% pure by thin-layer chromatography) as well as cholylglycine hydrolase, 3α-hydroxysteroid dehydrogenase, diaphorase, and resazurin were purchased from Sigma-Aldrich (Madrid, Spain). [^{3}H]taurocholic acid (specific radioactivity, 3.0 Ci/mmol) was obtained from New England Nuclear (Pacisa, Madrid, Spain). All other chemicals were from Sigma-Aldrich or Merck Eurolab (Barcelona, Spain). They were of high purity and were used as purchased without any further purification.

Animals and experimental design

Pregnant Wistar CF rats and their offspring were obtained from the University of Salamanca Animal House, except for aged animals, which were from Harlan Iberica (Barcelona, Spain). Dams and weaned animals were fed on commercial pelleted rat food (Harlan Iberica) and water ad libitum. Lighting was controlled by a timer that permitted light between 8:00 AM and 8:00 PM. All animals received humane care as described in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication). Experimental protocols were approved by the Ethical Committee for the Use of Laboratory Animals of the University of Salamanca. All litters were weaned on day 21 unless stated otherwise. Samples were obtained from fetuses (gestation days 14 and 20), neonates (on the first day after delivery), young animals of 1, 2, 3, or 4 weeks of age, adult rats of 8 weeks of age, and aged/senescent rats 9 and 24 months old. Fetuses and neonates were used without separation between males and females. Only males were used in groups of older animals. Eight weeks of age was defined as adult stage based on previous studies by us and others (9, 10).

Sample collection was always performed at 3:00 PM on nonfasted animals anesthetized with sodium pentobarbital (5 mg/100 g body weight, ip). After laparotomy, a small sample (<30 mg) of liver tissue was obtained to measure gene expression. To extract the total BA pool, the liver, small intestine, and mesenterium were removed and processed together as reported previously (11). Briefly, these organs were washed in saline, weighed, and homogenized in saline (1:2, w/v). A trace amount of radiolabeled [^{3}H]taurocholic acid was added to the homogenate and used as an internal standard to evaluate the yield of the extraction procedure. BAs were extracted from the homogenate by the addition of 2 volumes of ethanol and incubation at 65°C for 2 h, followed by centrifugation (3,500 rpm for 10 min). This process was repeated once, and both supernatants were pooled and passed through filter paper before being used to measure radioactivity and to carry out BA analysis.

To obtain fetal samples, on day 14 or 20 of pregnancy a cesarean incision was made under pentobarbital anesthesia. Fetuses were then removed and weighed, and their livers and small intestines were processed as described above. The BA pool was not measured in 14 day old fetuses, owing to the large amount of fetuses needed to obtain the required amount of tissue. At this time, only hepatic gene expression was measured by pooling the livers of three to five fetuses together.

In separate experiments, designed to investigate the influence of the time of weaning, the litters were weaned on either day 21 or day 28. In both groups, samples were obtained at 21, 24, 28, and 35 days of age. In these animals, a sample from the terminal ileum (1 cm distant from the ileal-cecal junction) was also obtained to measure ileal expression of BA carriers.

Quantitation of gene expression by real-time RT-PCR

Freshly obtained rat liver or small intestine samples were immersed in the RNA-stabilization reagent RNAlater (Ambion, biNova Cientifica, Madrid, Spain) and stored at −80°C until RNA extraction within 7 days. Total RNA was isolated from these samples using RNAeasy spin columns from Qiagen (Izasa, Barcelona, Spain). After treatment with RNase-free DNase I (Roche Diagnostics, Barcelona, Spain), RNA was quantified fluorimetrically with the RiboGreen RNA-Quantitation kit (Molecular Probes, Leiden, The Netherlands). DNA was synthesized using random nonamers and avian myeloblastosis virus reverse transcriptase (Enhanced Avian RT-PCR kit; Sigma-Genosys, Cambridge, UK). Primer oligonucleotides obtained from Sigma-Genosys were designed with the assistance of Primer Express software (Perkin-Elmer Applied Biosystems, Madrid, Spain) for DNA fragments in published sequences, and their specificity was checked using BLAST. The primer sequences have been reported elsewhere (4, 12), except for the following genes: apical sodium-dependent bile acid transporter (Asbt) (forward primer, TCA GTT TGG AAT CAT GCC TCT CA; reverse primer, ACA GGA ATA ACA AGC GCA ACCA), Cyp3a11 (forward primer, TGA CTG CTC TTG ATG CAT GGTT; reverse primer, ATC ACA GAC CTT GCC AAC TCCT), hepatic nuclear factor 4α (Hnf4α) (forward primer, CCA GCC TAC ACC ACC CTG GAG TT; reverse primer, TTC CTC ACG CTC CTC CTG AA), organic solute transporter α (Ostα) (forward primer, GCT GCC CAC CCC TCA TAC TT; reverse primer, GAT GCC ATC AGG AAT GAG AAA CA), and Ostβ (forward primer, AGA AAC CAG GGC CGC TCT A; reverse primer, TCC TTC TGC ACT GTG GTC CAT). Real-time quantitative PCR was performed using AmpliTaq Gold polymerase (Perkin-Elmer Applied Biosystems) in an ABI Prism 5700 Sequence Detection System (Perkin-Elmer Applied Biosystems). Thermal cycling conditions were as follows: 1 cycle at 95°C for 10 min followed by 45 cycles at 95°C for 15 s and 60°C for 60 s. Detection of amplification products was carried out using SYBR Green I (Perkin-Elmer Applied Biosystems). The absence of artifacts or nonspecific products of PCR, checked with 2.5% agarose gel electrophoresis and melting temperature curves, permitted the use of SYBR Green I detection in all cases. The results of mRNA abundance for each target gene in each sample were normalized using both polyubiquitin-C and 18S rRNA as housekeeping genes (4, 5). The latter was measured with the TaqMan® rRNA Control Reagents kit (Perkin-Elmer Applied Biosystems).

Determination of BA pool size and composition

The BA pool size was determined by analysis of total BAs in ethanolic extracts from liver and small intestine. BA concentrations were measured enzymatically (13) as described previously (9), and the BA pool size was corrected by taking into account

the recovery in the extraction procedure of the radioactivity measured both in the initial homogenate and in the final extract. The average yield was 90%. For quantitative analysis, BA species in the ethanolic extracts were separated and measured by GS-MS, as described previously (1). Briefly, after adding nordeoxycholic acid as a first internal standard, conjugated BAs were deamidated enzymatically (14, 15). Unconjugated BAs were extracted from the reaction mixture by liquid-solid extraction in C18 cartridges (Sep-Pak; Waters-Millipore, Madrid, Spain). Methyl ester derivatives were prepared by reaction with ethereal diazomethane. Trimethylsilyl ether derivatives were prepared in pyridine-hexamethyldisilazane-trimethylchlorosilane (3:2:1, v/v) for 45 min at 55°C (16). Before injection into the gas chromatograph, 5β-cholestane was added to the samples as a second internal standard. GC-MS analyses were carried out on a gas chromatograph (HP 5890 series II; Hewlett-Packard, Madrid, Spain) connected to a mass spectrometer (HP 5972; Hewlett-Packard) using a method described previously (14).

Statistical analysis

Results are expressed as means ± SD. To calculate the statistical significance of the differences among groups, Student's *t*-test or the Bonferroni method of multiple-range testing was used, as appropriate.

RESULTS AND DISCUSSION

Ontogenic changes in the BA pool size

To express the values of changes in the BA pool by body weight and liver weight, these were measured in all experimental groups (data not shown). The values of both parameters increased approximately in parallel up to adulthood. After this, liver weight increased less than body weight. Thus, the liver-to-body weight ratio decreased from 4.2% in adult animals (8 weeks old) to 2.1% in aged animals (9 months old). The absolute size of the BA pool increased progressively from 0.32 ± 0.07 μmol/animal in fetuses (20 days of pregnancy) to 89.2 ± 12.9 μmol/animal in adult rats (8 weeks of age) (**Fig. 1A**). In older rats, the rate of increase in the BA pool size was markedly slower. When the BA pool size was corrected by liver weight (Fig. 1B) a steady-state value was reached at 1 week of age that persisted through the life span of the animals, except after weaning, when a transient and marked peak (250% of the value in adults) was found. The values of the BA pool size in adult rats found in this study by collecting the liver and intestine and subsequently extracting BAs were similar to those obtained previously (12 μmol/g liver) in our laboratory by bile drainage for 12 h (17).

Owing to the importance of the stability of the abundance of the housekeeping genes used to correct the measurements of PCR before carrying out comparisons of expression levels between groups of rats with different ages, we first investigated the abundance of polyubiquitin C in all groups (18) with respect to 18S rRNA. Because no significant variations among groups for both genes were observed (data not shown), polyubiquitin C was used as a normalizer throughout this study. The stability in the expression of polyubiquitin C found here is consistent with previous studies showing similar results during liver

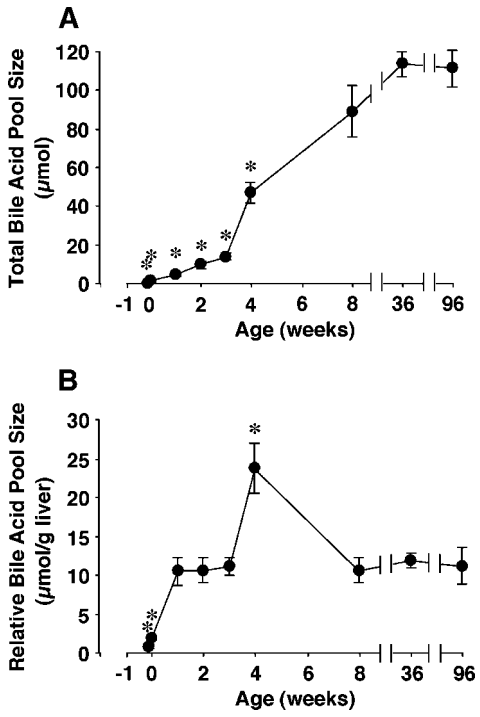

Fig. 1. Time course of the absolute (A) and relative to liver weight (B) size of the bile acid (BA) pool during rat development, adulthood, and senescence. Values are means ± SEM from six animals per group taken from three different litters. * $P < 0.05$, compared with the group of adult rats (8 weeks old) by the Bonferroni method of multiple range testing.

cell proliferation, such as that occurring during liver regeneration and carcinogenesis (4, 5).

When the expression of the key enzymes involved in BA synthesis was determined, we observed that the maturation of Cyp7a1 (**Fig. 2A**) was preceded by that of Cyp27 (Fig. 2B), suggesting that the acidic pathway may play a more important role in young animals than in adults. However, Cyp27 does not seem to be responsible for the increase in the BA pool after weaning, because no change in the expression of Cyp27 was seen, whereas that of Cyp7a1 was enhanced. The early evolution of the degree of Cyp7a1 expression was similar to that reported previously using Northern blot (6), although with this less sensitive technique the authors were not able to detect Cyp7a1 mRNA in 18 day old fetuses.

Ontogenic changes in BA pool composition

Study of the proportions of molecular species in the BA pool revealed that complexity was high during intrauterine life (**Fig. 3**). This was probably favored by the transfer across the placenta of maternal BAs, some of which cannot be produced by the fetus. This is evident for secondary BAs, which cannot be produced in the aseptic fetal intestine but that have been found in the fetal BA pool in rats and also in humans (19). It has been shown that the placenta expresses transport proteins able to transport BAs (20). Some of these mechanisms are potentially bidirectional (21) and do not have similar affinities for

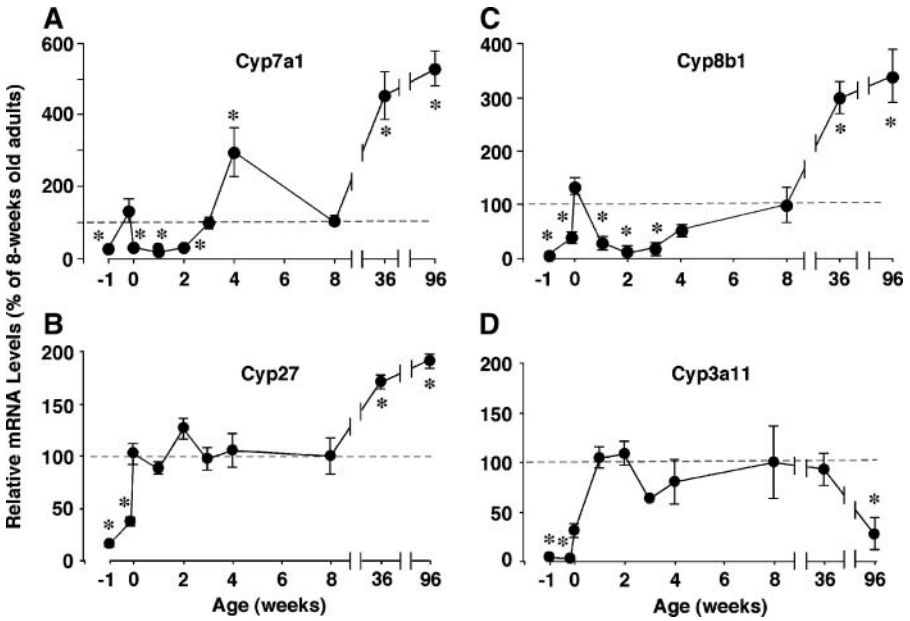

Fig. 2. Time course of the relative abundance of the mRNA of Cyp7a1 (A), Cyp27 (B), Cyp8b1 (C), and Cyp3a11 (D) during rat development, adulthood, and senescence. Values are means ± SEM from six animals per group taken from three different litters. * $P < 0.05$, compared with the group of adult rats (8 weeks old) by the Bonferroni method of multiple range testing. Threshold cycle (ct) values for measurements carried out on calibrator tissue (8 week old rat liver) were as follows: Cyp7a1 = 24.8, Cyp27 = 25.6, Cyp8b1 = 28.8, and Cyp3a11 = 22.0.

different species of BAs (22), and this could be involved in determining the selective transfer of BA molecular species from the mother to the fetus. The complexity of the composition of the BA pool declined immediately after birth but was recovered progressively afterward, its maximum being attained at between 3 and 8 weeks of age (Fig. 3). The complexity of the BA pool composition declined again in old animals. The reduction in the diversity of BA species observed in neonates and old rats was characterized by an enhanced predominance of cholic acid, which accounted for 77% and >85% of the BA pool, respectively (Fig. 3). In both cases this was related to *i*) upregulation of Cyp8b1 (Fig. 2C), suggesting the existence of stimulated hydroxylation at C12 (23), which is needed for the synthesis of cholic acid, and *ii*) downregulation of Cyp3a11 (Fig. 2D), whose human ortholog CYP3A4 is involved in the synthesis of 6α/6β-hydroxylated BAs such as hyodeoxycholic acid or muricholic acids (24–26).

One issue of particular interest in this study was the presence of "flat" BA *allo*-cholic acid in the BA pool of the rat during ontogenic development. This BA was found more abundantly in the perinatal period, disappearing during youth. The transformation of 3-oxo-7α-hydroxy-4-cholestene into 3-oxo-7α-hydroxy-5β-cholestane during BA synthesis is mediated by 3-oxo-Δ^4-steroid-5β-reductase (EC 1.3.1.23; henceforth designated 5β-reductase). A lower rate of this metabolic process may result in an increase in the amount of Δ^4-BAs (27). In contrast, steroid-5α-reductase type I (EC 1.3.99.5; henceforth designated 5α-reductase), which catalyzes the reduction of $\Delta^{4,5}$ double bonds in a variety of steroid substrates, including BAs, may account for the conversion of 3-oxo-Δ^4-steroid intermediates to their respective 3-oxo-5α-structures (28). This enzyme has been found expressed at high levels in male rat liver (4, 5, 29). In humans, 5α-reductase activity has been suggested to be responsible for the appearance of *allo*-BAs in children with 5β-reductase deficiency (27). Because during experimental rat hepatocarcinogenesis a significant relationship between the appearance of flat BAs and the 5α-reductase-to-5β-reductase expression ratio has been reported (5), we studied this relationship here during rat ontogenic development (**Fig. 4**). The results indicate that *allo*-cholic acid was absent in adults but surprisingly was present in the perinatal period, when the 5α-reductase-to-5β-reductase expression ratio was below values found in adult rat liver (Fig. 4). The reason for these findings is unclear, but it can be suggested that the mechanism accounting for the appearance of this BA in normal liver during ontogenic development would not be dependent only on the 5α-reductase-to-5β-reductase expression ratio.

It has been shown previously that the nuclear receptors involved in bile formation are developmentally regulated and are poorly developed during the fetal stage, whereas their expression reaches gradual maturity during the postnatal period (10). Our results confirmed this concept. Thus, the expression of farnesoid X receptor (Fxr) (**Fig. 5A**), small heterodimer partner (Shp) (Fig. 5B), Ftf (Fig. 5C), and Hnf4α (Fig. 5D) was very low at 1 week before birth and increased progressively over the first postnatal week. The subsequent decrease in Fxr was accompanied by a continuous increase in Shp mRNA

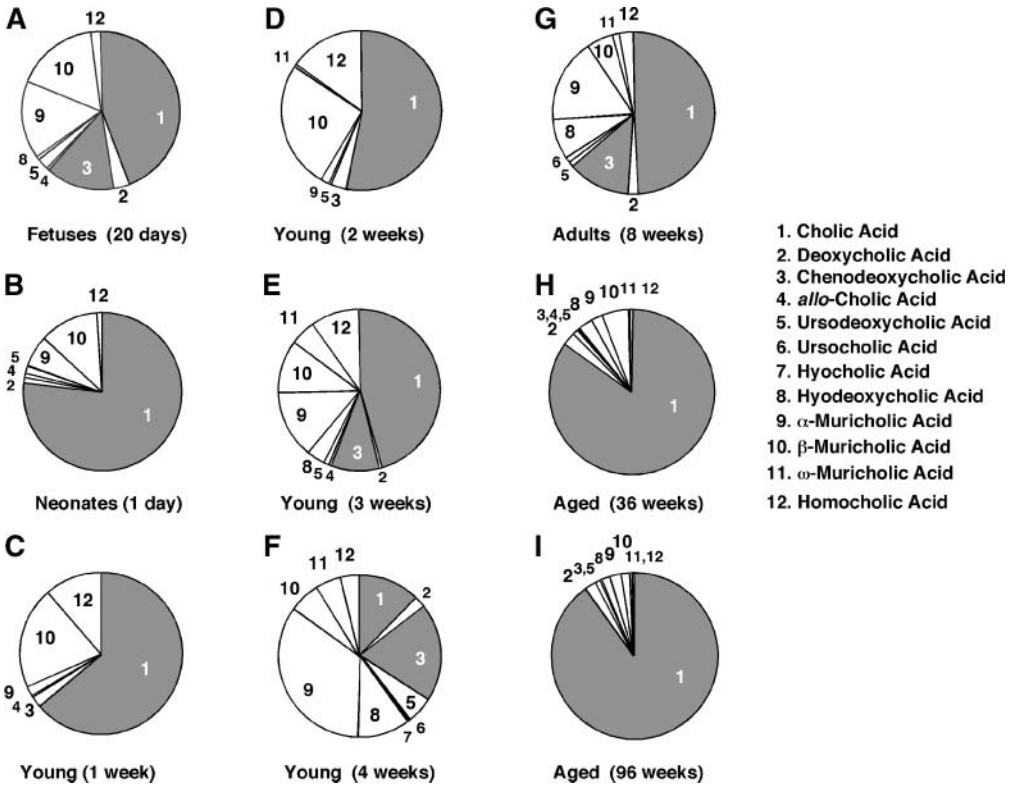

Fig. 3. Proportion of BA species in the BA pool of the rat during development, adulthood, and senescence (A–I). Values are means from six animals per group taken from three different litters.

up to the third week, when downregulation of this nuclear receptor preceded the upregulation of Cyp7a1 (Fig. 2A). The expression levels of these four nuclear receptors remained at similar values from youth to adulthood but increased during senescence, except for Hnf4α, which decreased.

The well-known relatively hydrophilic BA pool of the rodent is mainly attributable to the high proportion of

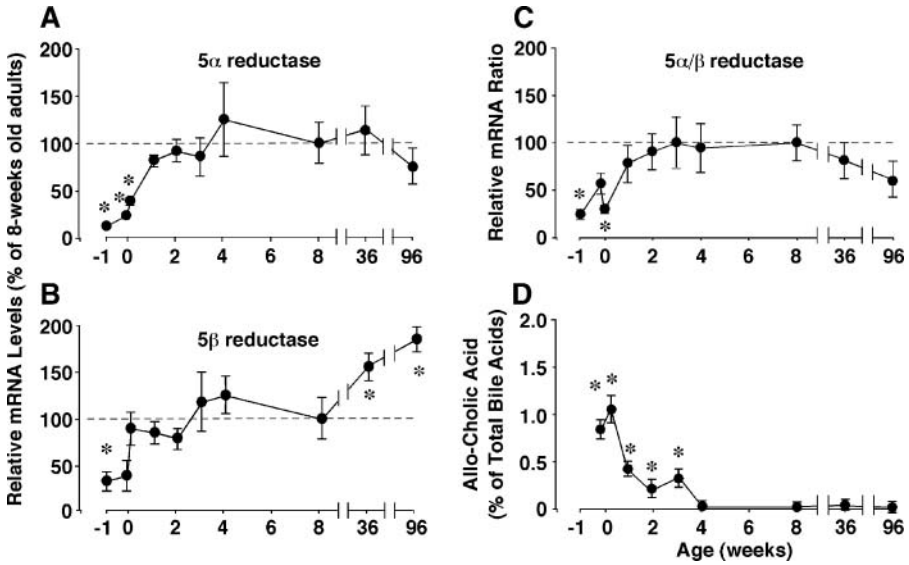

Fig. 4. Time course of the relative abundance of the mRNA of 5α-reductase (A), 5β-reductase (B), the 5α-reductase-to-5β-reductase ratio (C), and the proportion of *allo*-cholic acid in the BA pool (D) during rat development, adulthood, and senescence. Values are means ± SEM from six animals per group taken from three different litters. * $P < 0.05$, compared with the group of adult rats (8 weeks old) by the Bonferroni method of multiple range testing. Threshold cycle (ct) values for measurements carried out on calibrator tissue (8 week old rat liver) were as follows: 5α-reductase = 26.4 and 5β-reductase = 28.9.

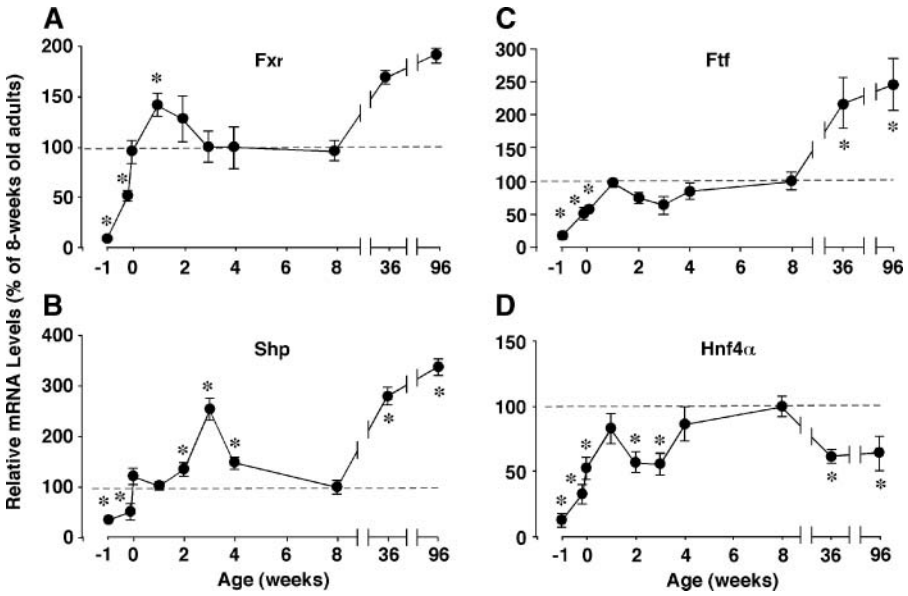

Fig. 5. Time course of the relative abundance of the mRNA of the nuclear receptors farnesoid X receptor (Fxr) (A), small heterodimer partner (Shp) (B), Ftf (C), and hepatic nuclear factor 4α (Hnf4α) (D) during rat development, adulthood, and senescence. Values are means ± SEM from six animals per group taken from three different litters. * $P < 0.05$, compared with the group of adult rats (8 weeks old) by the Bonferroni method of multiple range testing. Threshold cycle (ct) values for measurements carried out on calibrator tissue (8 week old rat liver) were as follows: Fxr = 25.3, Shp = 27.0, Ftf = 25.5, and Hnf4α = 22.7.

muricholic acids, which are believed to be poor ligands for Fxr. Our results revealed a progressive decrease in the amount of muricholic acids in the BA pool during senescence (Fig. 3). Thus, the marked increase in Fxr expression in aged rats may be related to the increasing hydrophobicity of their BA pool.

When the expression profiles of the nuclear receptors (Fig. 5) were compared with those of enzymes (Fig. 2), it appeared that during senescence the stimulating effect of Ftf on the transcription of Cyp7a1 and Cyp8b1 could overcome the inhibition caused by Shp, whose upregulation could be attributed to the high level of Fxr and the fact that the amount of its ligands remained constant (Fig. 1), resulting in overexpression of these enzymes in the liver of old animals (Fig. 2). However, the influence of other regulatory mechanisms cannot be ruled out. Finally, whether the coincidence in time of the decrease in the expression of Hnf4α and Cyp3a11 in senescence indicates the existence of a cause-and-effect relationship or is merely an epiphenomenon requires further investigation. In this respect, it has been shown that during fetal liver development in mice the expression of Cyp3a11 requires Hnf4α (30).

Weaning-induced changes in BA pool size and composition

Important hormonal changes have been observed during the third week of age, characterized by increasing circulating concentrations of glucocorticoids and thyroxine (31). Regarding the control of BA pool size and composition, the changes occurring during this period of ontogeny include a peak in Shp expression before weaning, followed by a decrease to values close to those of adults over the next week (Fig. 5B). This has also been observed by others (10), together with the existence of a hormone-dependent peak in conjugating activity at weaning in the rat, which could be induced precociously on day 14 by cortisone acetate injection on days 10–13 (32). Moreover, although the maturation of the expres-

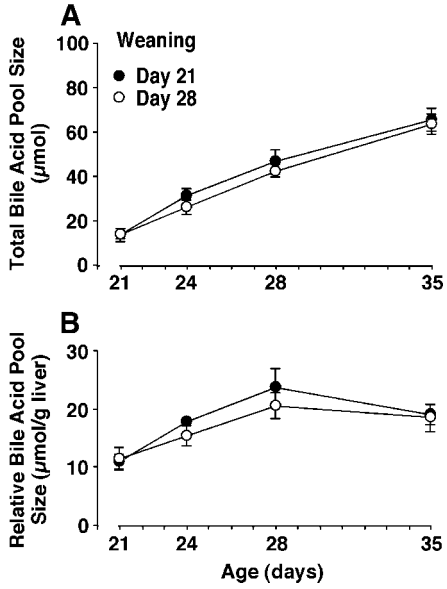

Fig. 6. Effect of time of weaning on the absolute (A) and relative to liver weight (B) rat BA pool size. Values are means ± SEM from six animals per group taken from three different litters. * $P < 0.05$, comparing groups of rats weaned at 21 and 28 days with the Student's *t*-test.

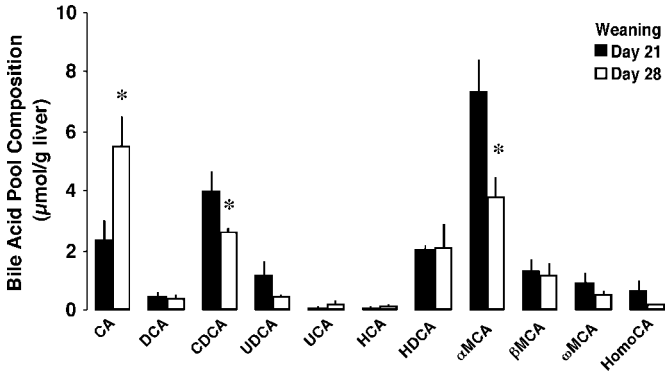

Fig. 7. Effect of time of weaning on the abundance of BA species in the rat BA pool in 4 week old animals. Values are means ± SEM from six animals per group taken from three different litters. * $P < 0.05$, comparing groups of rats weaned at 21 (closed bars) and 28 (open bars) days with the Student's *t*-test. CA, cholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; UDCA, ursodeoxycholic acid; UCA, ursocholic acid; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; MCA, muricholic acid; HomoCA, homocholic acid.

sion of sodium-dependent BA uptake by the rat liver via sodium taurocholate-cotransporting polypeptide (Ntcp) occurs early after birth (at ~1 week) (7), BA transport in rat ileum via Asbt appears abruptly at weaning (33). Ileal BA binding protein is a cytosolic protein involved in the absorption of conjugated BAs. Northern and Western blot analyses have suggested that the maturation of ileal BA binding protein expression occurs at weaning and is influenced by glucocorticoids but not by thyroxine (34). Therefore, we decided to further explore BA homeostasis during this period of life in the rat.

To elucidate whether the changes were attributable to chronologically programmed events or to changes caused by nutritional, nervous, and endocrine factors affecting the pups after separation from their dams, two different experimental groups were investigated. In one group, weaning was imposed on day 21 after birth. In the second group, weaning was delayed until day 28. This had no effect on body weight (on day 28 of age, 21 day weaning group = 49.8 ± 4.3 g and 28 day weaning group = 52.9 ± 3.7 g; on day 35 of age, 21 day weaning group = 79.5 ± 3.4 g and 28 day weaning group = 82.3 ± 6.2 g). Moreover, the time course of the absolute or relative BA pool size was not affected (**Fig. 6**). However, it induced a profound change in the BA composition. The delay in weaning was accompanied by increased amounts and proportions of cholic acid in the BA pool and a decreased relevance of chenodeoxycholic acid and α-muricholic acid (**Fig. 7**). It is

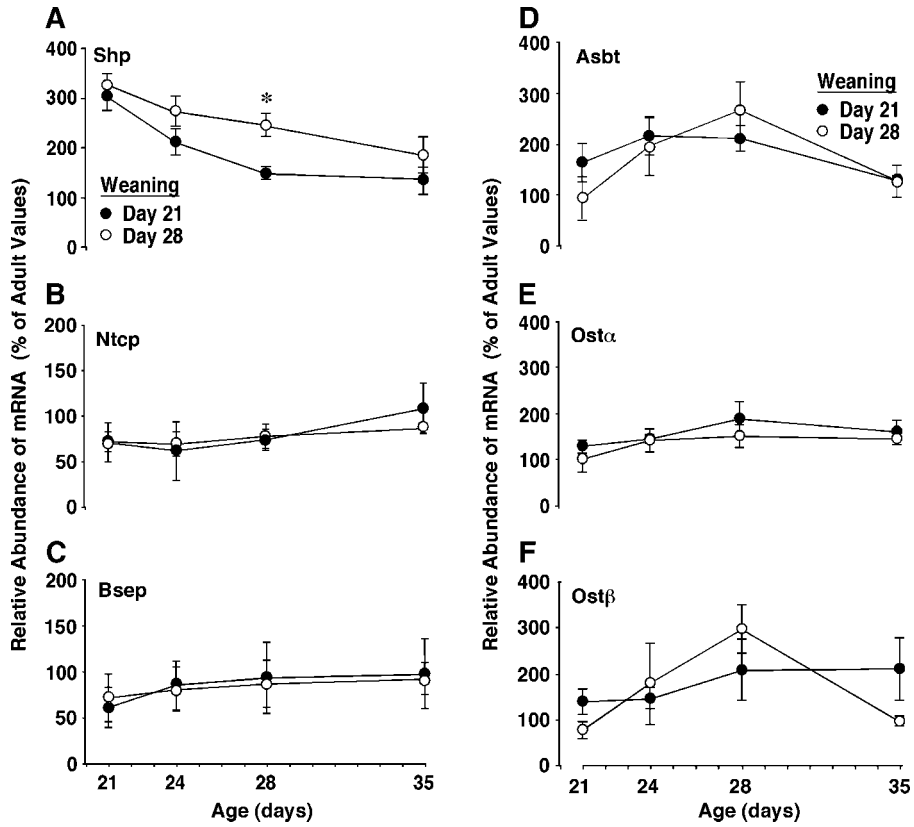

Fig. 8. Effect of time of weaning on the relative abundance of the mRNA of Shp (A), sodium taurocholate-cotransporting polypeptide (Ntcp) (B), and bile salt export pump (Bsep) (C) in rat liver and apical sodium-dependent bile acid transporter (Asbt) (D), organic solute transporter α (Ostα) (E), and Ostβ (F) in rat ileum. Values are means ± SEM from six animals per group taken from three different litters. * $P < 0.05$, comparing groups of rats weaned at 21 (closed circles) and 28 (open circles) days with the Student's *t*-test. Threshold cycle (ct) values for measurements carried out on calibrator tissue (8 week old rat liver or ileum as appropriate) were as follows: Shp = 27.0, Ntcp = 23.2, Bsep = 23.4, Asbt = 24.2, Ostα = 23.2, and Ostβ = 22.6.

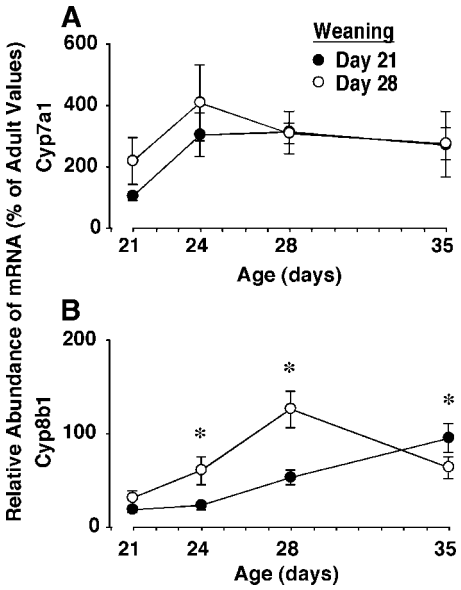

Fig. 9. Effect of time of weaning on the relative abundance of mRNA by real-time quantitative RT-PCR of Cyp7a1 (A) and Cyp8b1 (B) in rat liver. Values are means ± SEM from six animals per group taken from three different litters. * $P < 0.05$, comparing groups of rats weaned at 21 (closed circles) and 28 (open circles) days with the Student's *t*-test. Threshold cycle (ct) values for measurements of Cyp7a1 and Cyp8b1 mRNA carried out on calibrator tissue (8 week old rat liver) were as follows: 24.8 and 28.8, respectively.

unlikely that this would have been attributable to changes in the transporters involved in enterohepatic circulation that are already expressed at adult levels at this stage (35). In this study, no changes were observed in the abundance of mRNA of liver Ntcp and bile salt export pump or intestinal Asbt and Ostα/Ostβ, although the decrease seen at that time of life in Shp mRNA was slower in the group weaned at 28 days (**Fig. 8**). Moreover, the expression of Cyp7a1 was not affected by delaying weaning either (**Fig. 9A**). This, together with the absence of changes in the expression of transporters determining the enterohepatic circulation, is consistent with the lack of changes in the BA pool size. However, the delay in weaning caused a significant upregulation of Cyp8b1 (Fig. 9B), which may account for the increased proportion of cholic acid in the BA pool of these animals (Fig. 7).

In sum, during postnatal rat liver development, and more markedly around weaning, important quantitative and qualitative changes are observed in the BA pool. Although the quantitative changes seem to be age-programmed, and hence not dependent on the time of weaning, modifications in BA pool composition are probably related to this dietary change and can be modulated experimentally by delaying weaning.

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