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Uptake of Taurocholate by Hepatocytes Isolated from Developing Rats

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Summary

To further define developmental changes in bile acid metabolism, we determined the kinetics of taurocholate uptake by hepatocytes isolated from Sprague-Dawley rats at 7, 14, 21, 28, and 56 days of age. There was a progressive increase in taurocholate uptake with age. The uptake process exhibited saturable kinetics in every age group with a maximum uptake velocity attained above a taurocholate concentration of 200 μM. There were no differences in Km values but Vmax increased progressively between 7 and 56 days of age. These data suggest that the deficit in hepatic excretory function observed in immature mammals of several species may, in part, be related to decreased transport of bile acids.

Speculation

Impaired transport of bile acids by the liver may limit bile flow during development and lead to inefficient fat digestion, altered hepatic excretion of drugs, and an increased susceptibility to cholestasis. Comparable Km values for the uptake process indicate that hepatocyte affinity for taurocholate remains constant during development; whereas the rise in Vmax with postnatal age may reflect an increase in the number of binding sites. Specific changes in the liver cell plasma membrane are likely to be important determinants of the ontogeny of bile acid transport and bile flow.

Bile acids are major synthetic and excretory products of the liver and are of primary importance in the generation of bile flow (11, 15, 20). There is increasing evidence in humans and in experimental animals that the enterohepatic circulation of bile acids is not fully developed at birth and that the perinatal liver is normally subject to a period of "physiological cholestasis" (13, 14, 41). In the normal human infant and in the suckling rat, bile acid pool size is decreased (42, 44) and the concentration of bile acids in serum is elevated (5, 6, 41). As a consequence of decreased bile secretion, biliary elimination of many drugs and organic anions such as bilirubin may be impaired (30, 43, 46) and the concentrations of bile acids reaching the intestine may be inadequate for optimal fat digestion (45). This immaturity of liver function may also place the infant at increased risk for clinical cholestasis as is commonly observed during gram negative infection or during parenteral nutrition (2, 8, 18). It is our hypothesis that during development, efficient enterohepatic cycling of bile acids may, in part, be limited by immaturity of bile acid transport by the liver. We, therefore, sought maturational changes in the uptake of taurocholate by hepatocytes isolated from suckling and weanling rats.

METHODS AND MATERIALS

Animals. Suckling and weanling Sprague-Dawley rats were obtained from Charles River Breeding Laboratories and were housed in a temperature-controlled room at 22°C with alternating 12 h light-dark cycles. Mothers and weanlings were maintained on Purina rat chow and water *ad libitum.* Male rats were utilized for hepatocyte isolation and all studies were conducted at midday.

The chemicals used in this study were: tauro-[carbonyl-^{14}C] cholic acid, (52.0 mCi/mmoles; greater than 98% pure by thin layer chromatography) and [^{3}H]-inulin, (1 mCi/6.9 mg) both from New England Nuclear Corp. (Boston, MA); collagenase (type II), Sigma Chemical Corp. (St. Louis, MO); sodium taurocholate (greater than 98% pure by thin layer chromatography) from Calbiochem (San Diego, CA); silicone oil, density, 1.05 g/ml, Aldrich Chemical Co, (Milwaukee, WI); trypan blue, 0.4%, Gibco Laboratories, (Grand Island, NY); bovine serum albumin (fraction V) from Sigma Chemical Co. (St. Louis, MO); and Insta-Gel from Packard (Downers Grove, IL). All reagents used in the preparation of the buffers were commercially available and of analytical grade.

Hepatocyte Isolation. Hepatocytes were isolated by a recirculating perfusion technique in rats at 14, 21, 28 and 56 days of age according to the method of Bissel *et al.* (9). Each rat liver was perfused *in situ* through the portal vein at a rate of approximately 2 ml/g of liver/min with oxygenated calcium-free Hank's buffer at pH 7.4 and at 37°C for a total volume of 1 ml/g body weight and then for 25 min with a 0.05% solution of collagenase in the same buffer. In pups of 7 days of age, the liver was perfused retrograde through the thoracic portion of the inferior vena cava as described by Ziegler (47). After perfusion, the livers were removed, the tissue gently dispersed with scissors, and incubated for an additional 10 min in 25–50 ml of 0.05% collagenase-buffer solution in a rotatory water bath at 37°C. The resulting cell preparation was then filtered through two layers of cheese cloth, washed and separated three times by low speed centrifugation (50 × *g* for 2 min) to yield a preparation of greater than 95% hepatocytes. The final cell pellet was resuspended in 10 ml of buffer (137 mM NaCl, 5.2 mM KCl, 0.9 mM $MgSO_4 \cdot 7\ H_2O$, 0.12 mM $CaCl_2$, 10 mM Na_2HPO_4, 25 mM $NaHCO_3$, and 1% glucose) containing 2% bovine serum albumin. This concentration of albumin was the minimum necessary to maintain cell viability in suspension for a period of greater than 3 hr. The number and homogeneity of the isolated cells were estimated in a hemocytometer. Hepatocytes were diluted with buffer to a final concentration of 1.5×10^6 cells/ml and maintained in a rotatory water bath at 37°C under 95% O_2/5% CO_2.

Freshly isolated hepatocytes from each age group were 90–95% viable as assayed by trypan blue exclusion before and at the completion of each experiment (32). Plasma membrane integrity was also suggested by minimal loss of intracellular enzyme activity into the incubation medium (rate of lactic acid dehydrogenase release less than 3% of intracellular activity per hour) (9). The rate of incorporation of [^{14}C]-leucine into cellular protein was linear in isolated hepatocytes (28).

Taurocholate uptake. After preincubation for 20 min at 37°C under 95% O_2/5% CO_2, 2 ml of the diluted cell suspension were added to a prewarmed vial containing [^{14}C]-taurocholate tracer (90 nCi) and varying concentrations of unlabeled taurocholate (5, 10, 25, 50, 100, 200 μM). Tritiated inulin was included in the

medium as a marker for adherent extracellular fluid. Two hundred μl aliquots were taken at 15, 30, 45 and 60 sec and placed into 0.5 ml microfuge tubes. In initial experiments, samples were obtained at 90 and 300 sec to establish accurate data regarding the time course of uptake. Hepatocytes were immediately separated from the incubation medium by microfuge centrifugation (10,000 X *g* for 5 sec) through a layer of silicone oil (100 μl) into 3 M KOH (50 μl) (3, 35, 36, 37). After dissolution of the cell pellet in the KOH (18–24 h), the bottom of each microfuge tube was cut off at the silicone oil-KOH interface and placed in a scintillation vial. Seventy-five μl of 2 N HCl and 10 ml of Insta-Gel scintillation cocktail were added to each vial and mixed thoroughly. The dissolved cell pellets and a sample of the incubation medium were then subjected to radioassay for ^{14}C and ^{3}H content in a Beckman liquid scintillation spectrophotometer. No significant radioactivity appeared in the oil phase and 1% or less of the total ^{3}H-inulin counts were associated with the sedimented cell fraction. Protein was determined according to the method of Lowry *et al.* (27). Results are expressed in nmoles of taurocholate per mg of cellular protein.

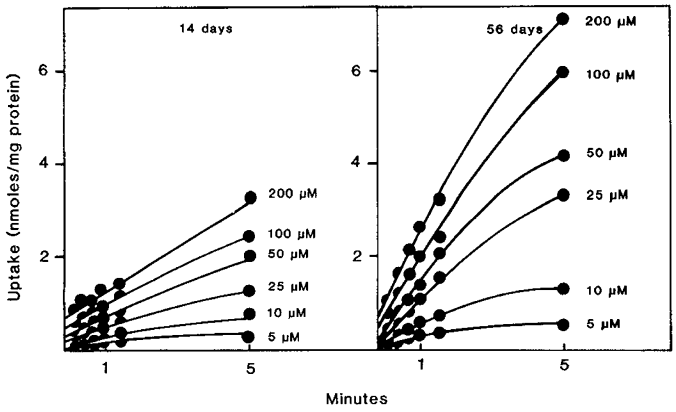

Fig. 1. Time course of taurocholate uptake by hepatocytes isolated from 14- and 56-day-old rats.

The Km and Vmax for taurocholate uptake in each experiment were derived from a double reciprocal plot of the initial uptake velocity *versus* taurocholate concentration according to Lineweaver-Burk (29). The kinetic values at each age were expressed as the mean ± S.E. Student's *t* test was used for statistical comparison.

RESULTS

Representative time courses for taurocholate uptake by hepatocytes isolated from 14- and from 56-day-old rats are shown in Figure 1. Uptake of taurocholate at concentrations from 5–200 μM was linear in excess of 90 sec, similar to data reported by other investigators using hepatocytes from adult rats (4, 35). Extrapolation of the uptake *versus* time plot yielded a positive intercept suggesting rapid initial adsorption of taurocholate to the plasma membrane. This adsorption apparently occurs independently from influx and has been observed with other amphiphilic compounds such as bromosulfophthalein (36) and estrone sulfate (38).

Initial rates of taurocholate uptake were subsequently calculated from the uptake during the first min of each experiment. On Figure 2 the initial uptake velocity (Vo) (nmoles/min/mg protein) is plotted as a function of taurocholate concentration. Each curve represents the mean and S.E. of six separate hepatocyte preparations from each age group. There was a progressive increase in taurocholate uptake by hepatocytes isolated from rats between 7 and 56 days of age. The uptake process was found to be saturable in each group with a maximum uptake velocity attained above a taurocholate concentration of 200 μM.

Rate constants were determined from a double reciprocal plot of each experiment and are shown graphically in Figure 3. Vmax increased with postnatal age. At 7 days the Vmax was only 23% of that attained in animals of 56 days of age ($P < 0.001$); the Vmax at this time was also significantly reduced in comparison to rats studied on day 21 and 28 ($P < 0.01$) but did not differ from that of the 14-day-old pups. The maximum velocities on days 14, 21 and 28 remained significantly less than the 56-day-old rats ($P < 0.01$). A 2-fold increment in Vmax occurred between weaning (21 days) and 56 days. In contrast there were no significant

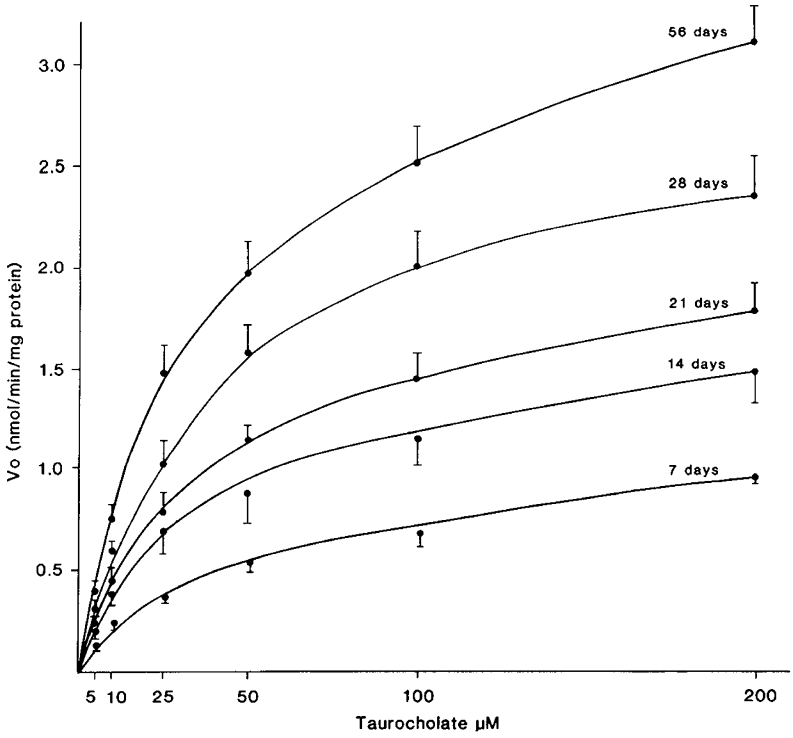

Fig. 2. Initial velocity of taurocholate uptake (Vo) plotted against substrate concentration for hepatocytes isolated from developing rats. Each point represents the mean ± S.E. of data obtained from six animals.

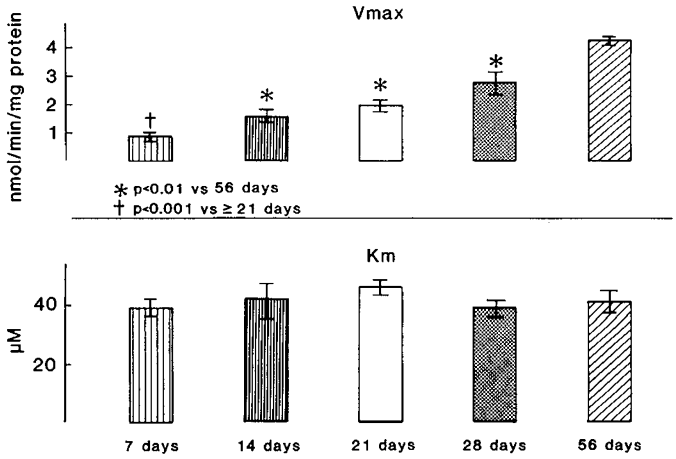

Fig. 3. Rate constants for uptake of taurocholate by hepatocytes isolated from developing rats. Each bar represents the mean ± S.E. of data obtained from six animals.

differences in Km values for taurocholate uptake for any of the age groups studied.

DISCUSSION

Studies performed in the isolated perfused rat liver and in isolated hepatocytes indicate that bile acid uptake is an active, sodium-dependent process (4, 33). The initial interaction between bile acids and the liver cell occurs at the sinusoidal face of the surface membrane where a putative bile acid receptor has been identified (1). A Na^+K^+ATPase has been localized to the basolateral portion of the hepatocyte and maintains a low intracellular sodium concentration by pumping sodium out of the cell in exchange for potassium (10). It is hypothesized that the sodium gradient that is created drives a sodium-anion coupled carrier that enables coupled anions such as bile acids to accumulate within the hepatocyte against an electrochemical gradient (11). Subsequent intracellular and canalicular transport is poorly understood but bile acids may be extruded into bile by active transport or in response to an electrochemical gradient (11).

The maturation of bile acid transport by the immature liver has not been directly examined. The use of an isolated hepatocyte system allows the study of functions specific to this cell type without the variability of blood flow and the interference of supporting tissue. Our data has demonstrated age-related changes in the uptake of taurocholate, the primary conjugated bile acid of the rat and of the human infant, in hepatocytes isolated from suckling and weanling rats. The uptake process was saturable and increased progressively between 7 and 56 days of age. Km values for taurocholate transport did not change appreciably with postnatal age, suggesting that the affinity of the liver cell plasma membrane for taurocholate remains constant during this period. However, the 4-fold increase in Vmax may reflect an increase in the number of transport or binding sites. The rate constants derived in 56-day-old rats are similar to those reported by other authors for the mature rat, *e.g.*, a Km of 20–40 μM and a Vmax of 1.8–3.8 nmoles/min/mg protein (4, 31, 35). Stacey and Klaassen (40) have observed a similar pattern of development for ouabain uptake in isolated rat hepatocytes; Vmax increased gradually with postnatal age whereas Km values remained constant.

Impaired hepatic transport of bile acids is supported by the finding of elevated concentrations of cholylglycine and conjugates of chenodeoxycholate in the serum of normal infants during the first months of life (5, 41). Similarly, serum concentrations of total cholate conjugates were markedly elevated in the serum of suckling and weanling rats (6). An exaggerated postprandial rise in the serum bile acid concentration in infants may reflect defective uptake by the liver, a finding that is especially striking in light of the probable inefficiency of intestinal bile acid reabsorption in the first months of life, which would tend to blunt the serum response (41). Studies in fetal dogs (19), monkeys (25), sheep (39), and in rat pups (24) have also shown delayed clearance of labeled taurocholate from serum. Decreased excretion of other organic anions by the developing liver may not only indicate immaturity of carrier mechanisms specific to these compounds but may also be related to impaired bile secretion. It has been suggested that concentrative transport of a variety of drugs and organic anions into bile may result from their incorporation into mixed micelles would be directly influenced by the availability of bile acids within the canalicular lumen (34).

The ontogeny of taurocholate uptake by the liver may be examined in the context of other developmental changes in the enterohepatic circulation of bile acids. Although uptake was a saturable process in hepatocytes isolated from rat pups even at 7 days of age, active ileal transport of bile acids was not demonstrated in two recent studies until after the second wk of life (26, 42). The rapid increase in bile acid pool size and the enhanced intestinal conservation of bile acids around the time of weaning may overwhelm the capacity of the developing liver to clear bile acids from the portal blood (26, 42). Bile acids would then spill over into peripheral blood as occurs during liver disease. Maximally elevated serum cholic acid levels are in fact detected in developing rats at 21–28 days and can be related to this increased bile acid load in the face of impaired transport by the liver (6). Progressive maturation of liver function possibly including uptake, intracellular binding, conjugation and secretion of bile acids leads to eventual "normalization" of serum bile acids in the rat during the second month of life (6).

The factors modulating the development of taurocholate uptake by the liver are unknown. The surges in plasma thyroxine and corticosterone known to occur during the mid-suckling period are likely to effect clusters of enzymes including those involved in hepatic lipid metabolism and in the biosynthesis of membranes (16, 17). The final steps of biochemical and structural differentiation in the liver may, in part, be related to the major change in diet associated with weaning (23). Since a Na^+K^+ ATPase and a proposed bile acid receptor have been localized to the liver plasma membrane, the organization of this membrane in particular should play a key role in the maturation of bile acid transport (1, 3; 10). The influence of the lipid composition and fluidity of a membrane system on membrane transport, enzyme activity, and on ligand-receptor interactions have been demonstrated in many studies involving liver and other tissues (7, 12, 21, 22).

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48. This paper was presented in part at the Annual Meeting of the American Association for the Study of Liver Diseases in Chicago, IL on November 7, 1980 and has been published in abstract form (Gastroenterology, *79:* 1056 1980).
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