Uptake and Excretion of Sodium Taurocholate by the Isolated Perfused Neonatal Sheep Liver

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Abstract □ We present a model for perfusion of the isolated perfused neonatal sheep liver which allows examination of drug disposition by the intact organ. We studied the disposition of sodium taurocholate (TC) in seven neonatal lambs (ages 2–11 days) and compared the results with earlier data from the perfused fetal sheep liver (Ring, J. A. et al. *Biochem. Pharmacol*. **1994**, *48*, 667–674). Measurements of perfusion pressure, oxygen consumption, lactate:pyruvate ratio, bile flow, and liver histology indicated that the preparation was both viable and stable over a 2 h period. [^{14}C]-labeled TC was added to the reservoir by constant infusion (30 μmol/h) and the ductus venosus shunt quantitated by injection of [^{153}Gd]-labeled microspheres. Shunt-corrected hepatic extraction ratio of TC was 0.56 ± 0.14 (fetal 0.23 ± 0.16, $p < 0.005$) and clearance of TC was 0.92 ± 0.35 mL/min/g liver (fetal 0.44 ± 0.23 mL/min/g, $p < 0.01$). We conclude that the isolated perfused neonatal sheep liver is a useful experimental model which will facilitate the study of the developmental physiology and pharmacology of the liver. There is considerable maturation of the biliary excretion of TC between the late fetal and early neonatal periods in the lamb.

Introduction

Sodium taurocholate (TC) is a principal bile salt in many mammalian species, including humans.[1] In the adult, TC undergoes an efficient enterohepatic circulation from bile to small intestine, active reabsorption from the terminal ileum, and then hepatic uptake and re-excretion into bile. TC is highly cleared from portal plasma by the liver cell and concentratively excreted into bile.[2] In contrast to many highly cleared drugs, TC is taken up by the liver by an active or facilitated transport process[3] and is then actively secreted into bile.[2]

Several studies have examined TC transport in the developing liver, both in late fetal and neonatal life.[4−7] In general they have shown, in several species, that hepatic transport processes of TC appear to be immature in the fetus and neonate. The same can also be said of many drug metabolic processes.[8,9] One of the difficulties encountered in studying fetal hepatic function in vivo is the inability to obtain samples consistently from, for example, hepatic outflow vessels or the bile duct. This leads to difficulties in defining fetal hepatic clearance and differentiating this from placental transfer and subsequent clearance by maternal organs. To overcome these problems we have recently developed the in-situ isolated, perfused fetal sheep liver model.[4] This has allowed detailed study of TC transport and the metabolism of a number of drugs by the fetal liver near term.[10,11]

The present study details the development of an analogous model in the neonatal sheep. This allows direct comparison of a variety of hepatic functions at various times before and after birth, a period of dramatic physiological change. We present here the findings on neonatal TC transport and compare them with our earlier data from late fetal life.

Experimental Section

Materials and Reagents—[^{14}C]TC (99% pure by TLC, 1.7 GBq/mmol) and [^{153}Gd] microspheres (37-185 MBq/g) were purchased from NEN (Boston, MA). D-Glucose and unlabeled TC (sodium salt) were obtained from Sigma Chemical Co. (St. Louis, MO), and bovine serum albumin (fraction V) from the Commonwealth Serum Laboratories (Melbourne, Victoria, Australia). Hartman's solution and sodium heparin were purchased from David Bull laboratories (Melbourne, Victoria, Australia).

Animals and Surgical Procedure—The experiments were approved by the Austin and Repatriation Medical Centre (ARMC) Animal Welfare Ethics Committee. Experiments were conducted in seven neonatal sheep between 2 and 11 days of age (2, 2, 3, 6, 8, 10, and 11 days). Pregnant ewes were supplied by a contracted local farmer and delivered to the animal house at least 2 weeks before the scheduled parturition date. All lambs were born by natural delivery at the ARMC and fed naturally by their mothers.

Anaesthesia was induced with intravenous sodium thiopental, a tracheostomy performed, and the lamb intubated and ventilated. Anaesthesia was maintained with halothane. A midline abdominal incision was performed and the remnant of the umbilical vein ligated. The portal vein, hepatic artery, bile duct, and superior mesenteric vein were located, and the gall bladder was isolated by ligating the cystic duct. The bile duct was cannulated (internal diameter (i.d.) 1.0 mm, external diameter (o.d.) 1.5 mm) and left to drain externally into a collection tube. Loose ties were applied around the portal vein, hepatic artery, and superior mesenteric vein. The inferior vena cava above the renal veins was located and a loose tie applied. A midline thoracotomy was performed to expose the suprahepatic inferior vena cava, and a loose tie was applied. A silastic inflow cannula (o.d. 6 mm, i.d. 4.5 mm, with an in-line small preliver bubble trap) primed with Hartman's solution at 37 °C, containing sodium heparin (5 units/mL), was then prepared for portal vein cannulation.

The superior mesenteric vein was ligated distally and cannulated with the inflow cannula via a proximal incision. The cannula was passed down the superior mesenteric vein to the portal vein, visually confirmed to be in the correct position and then secured. The supra-hepatic inferior vena cava was cannulated (o.d. 8 mm, i.d. 6 mm) and left to drain externally. The loose ties around the hepatic artery and inferior vena cava above the renal veins were secured, isolating the liver, which was flushed with heparinized Hartman's solution until it had uniformly blanched. Perfusion was commenced by connecting the primed perfusion circuit to the

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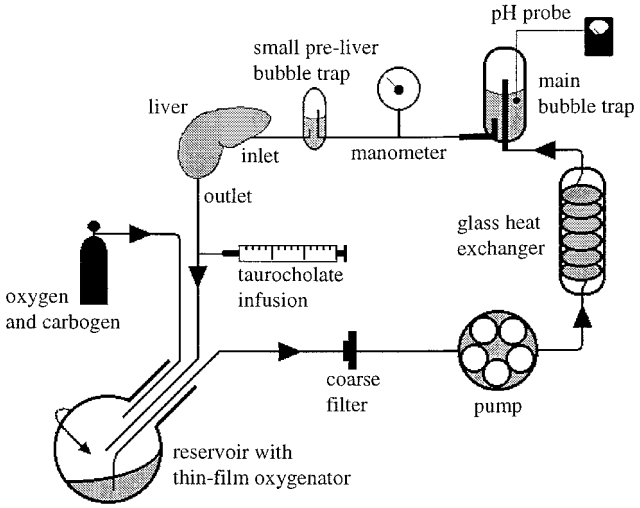

Figure 1—Diagram of the isolated neonatal liver perfusion circuit.

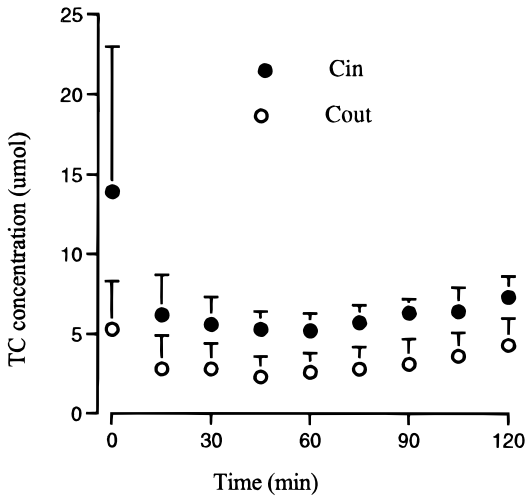

Figure 2—Mean concentration (and standard deviation) of TC in inflow and outflow cannulae.

silastic inflow cannula, and the distal end of the outflow catheter was connected to the reservoir to complete the recirculating system. The liver was perfused in situ and covered with saline-soaked gauze. The lamb was sacrificed by an overdose of sodium thiopental injected into the left ventricle.

Perfusion Circuit—The perfusate volume was 1000 mL and comprised Krebs-Henseleit buffer pH 7.4, bovine serum albumin 1% w/v, D-glucose 0.1% w/v, washed human red blood cells 20% v/v, and Hepes 5 mM. The perfusion circuit (Figure 1) was based on our perfused fetal liver preparation.[4] Modifications were made as follows to improve and simplify the method of perfusate oxygenation and perfusate sampling. The reservoir was a 3 L spherical glass flask with a ground glass neck. To this was connected a hollow glass stalk 30 cm long (i.d. 25.4 mm, o.d. 31.75 mm) with a ground glass connector to the reservoir. The reservoir with stalk was mounted at a 60° angle using a Corning model 349/2 rotary evaporator apparatus which constantly rotated at 40 rpm to mix the perfusate and form a thin perfusate film on the inside of the reservoir for oxygenation. Entering the stalk were three stiff, nonrotating Teflon tubes; one tube was immersed into the perfusate as the liver portal supply, another tube as the venous return from the liver, and the third tube positioned so as to constantly oxygenate the reservoir air space.

A peristaltic pump (Masterflex No. 7521-25 Cole-Palmer Instrument Co., Chicago, IL) provided a flow rate of approximately 200 mL/min, and flow was accurately quantitated at the conclusion of each experiment. The perfusate was kept at a temperature of 37 °C by recirculating water through a glass heat exchanger from a 15 L water bath. Perfusate pH was maintained at 7.40 by mixing humidified carbogen (95% oxygen, 5% carbon dioxide) in addition to the delivered oxygen via a T-piece, as required. TC was delivered into the reservoir at 30 μmol/h (4 mL/h of 7.5 mM TC solution) by a syringe pump (Sage Instruments No. 355, Cambridge, MA).

Viability and Stability—An initial equilibration time of 15 min was followed by a perfusion time of 120 min. The perfusion pressure of the liver was determined by subtracting the circuit pressure obtained before connection of the circuit to the liver from the pressure reading with the liver in place. Oxygen delivery and consumption were calculated using an AVL 995 blood gas analyzer (AVL Medical Instruments, Switzerland) every 30 min by sampling perfusate inflow to and outflow from the liver. At the conclusion of each experiment, samples of the left and right lobes of the liver were taken and fixed in 10% formalin solution for later histological examination. The perfusate lactate:pyruvate ratio was measured spectrophotometrically in samples collected at 0, 30, 60, 90 and 120 min using a commercially available kit (kit nos. 826-A and 726, Sigma Chemical Co, St Louis, MO).

Experimental Design—After the initial 15 min equilibration, a bolus loading dose of 30 μmol (4 mL of 7.5 mM solution, specific activity 111Bq/mmol) [^{14}C]TC was injected into the reservoir, followed by a constant infusion of 30 μmol/h of [^{14}C]TC (4 mL/h of 7.5 mM solution) for 120 min. Samples (5 mL) from the inflow and outflow cannulae were then taken every 15 min for the duration of the experiment. Reservoir volume was maintained by replacing sampled perfusate with an equal volume of blank perfusate. All bile was collected via the common bile duct cannula into preweighed tubes in 30 min aliquots. Volume of bile was calculated by weight, assuming a specific gravity of 1.0 g/mL. Perfusate (whole blood perfusate including red blood cells) and bile radioactivity was determined by liquid scintillation counting on a Packard 1900CA liquid scintillation analyzer as described previously.[12]

Hepatic Distribution of Perfusate Flow—Latex microspheres 15 μm in diameter labeled with [^{153}Gd] were used to determine the ductus venosus flow shunting through the liver, the proportion of flow to left and right lobes, and the evenness of liver perfusion as previously described.[13] Briefly, 200 μL [^{153}Gd] of the microsphere preparation (approximately 2 million microspheres) were injected into the inflow cannula, and the outflow perfusate was collected in 10 s aliquots for 60 s, by which time all microspheres not distributed to the liver had eluted. The liver was then removed from the carcass and divided into left and right lobes (via Cantlie's line, a line joining the middle of the gallbladder fossa to the left of the inferior vena cava posteriorly), and the lobes were individually weighed. The lobes were then divided further into 2−4 g sections which were subjected to γ counting using a Packard Cobra 5005C γ counter at an energy window of 85−115 keV. The proportion of perfusate shunted through the ductus venosus was taken as the ratio of total counts in outflow perfusate to the sum of total counts in outflow perfusate and liver tissue.

Calculations and Statistics—The hepatic extraction ratio (E) at steady state (using the 60−90 min interval, Figure 2) was calculated as:

$$E = C_{in} - C_{out}/C_{in}$$

where C_{in} is TC concentration in the inflow cannula, and C_{out} is TC concentration in the outflow cannula from the liver. Where a ductus venosus shunt was present, a corrected hepatic extraction ratio (E^*) was calculated to take into account the fraction of perfusate shunting through the ductus venosus:

$$E^* = E/(1 - S)$$

where S is the fraction of the perfusate shunted, calculated from the portal injection of microspheres. Total clearance of TC from perfusate (CL) was calculated as:

$$CL = EQ$$

where Q is perfusate flow rate. Biliary clearance of TC at steady state (using the 60−90 min interval, CL_{bile}) was calculated as:

$$CL_{bile} = v/C_{ss}$$

where v is the mean excretion rate of TC into bile at steady state,

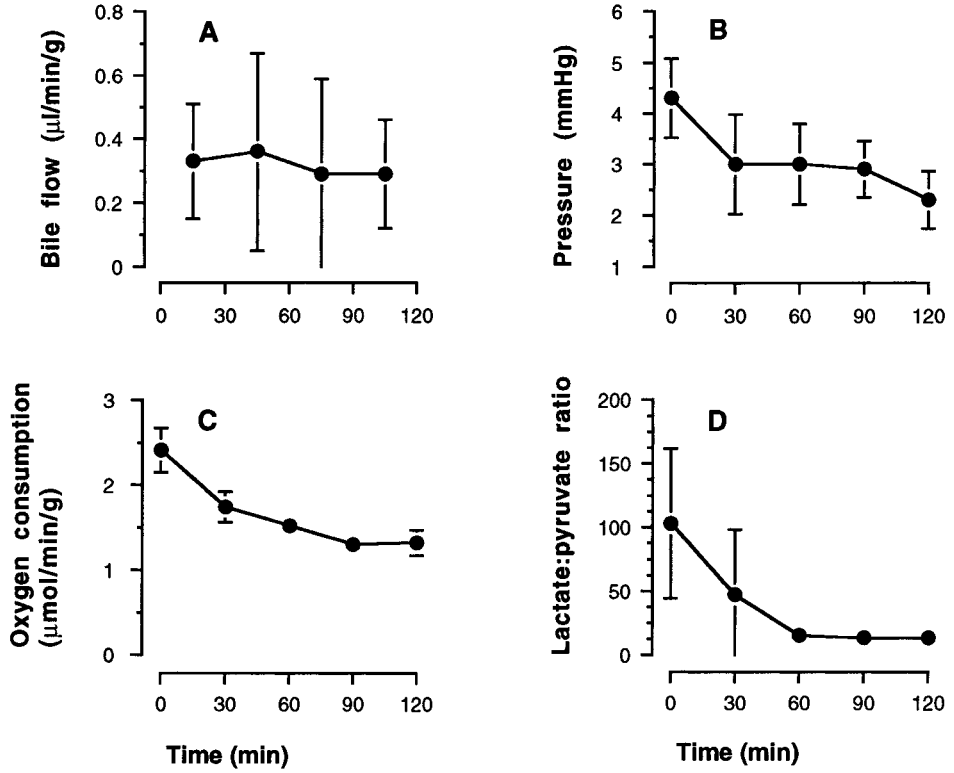

Figure 3—Physiological parameters (mean and standard deviation) versus time: (A) bile flow, (B) perfusion pressure, (C) oxygen consumption, (D) perfusate lactate:pyruvate ratio.

Table 1—Distribution of Perfusate Flow in Neonatal Liver

		liver weight (g)			perfusate flow (mL/g/min)	
neonatalage (days)	shunt (%)	left lobe	right lobe	total	left lobe	right lobe
2	15.2	45.8	52.8	96.6	1.67	2.96
2	18.2	41.3	52.2	91.5	1.83	2.92
3	1.6	72.8	90.3	163.1	1.17	1.37
6	1.4	73.8	62.9	136.7	1.31	1.53
8	2.0	41.3	50.2	91.5	1.83	2.92
10	0	71.2	67.1	138.4	0.84	2.21
11	3.0	56.3	52.6	108.9	1.58	2.72
Mean ± SD	5.9 ± 7.5	62.6 ± 14.7	66.4 ± 17.7	129.0 ± 30.8	1.39 ± 0.33	2.11 ± 0.79

and C_{ss} is the mean steady-state perfusate concentration in the inflow cannula.

Data are expressed as mean ± standard deviation. Correlations between variables were examined by linear regression analysis, and differences between groups of data were examined by unpaired *t*-test. A p value less than 0.05 was considered statistically significant.

Results

Liver Viability and Stability—Liver appearance was satisfactory in that the liver remained a uniform red color throughout the perfusion and there were no areas of hepatic capsular distension or exudation. Light microscopy of liver tissue showed no evidence of hypoxic damage or sinusoidal congestion. The whole liver was shown to have been grossly perfused by the presence of microspheres in all liver samples. Bile flow throughout the experiment was stable at 0.32 ± 0.24 µL/min/g of liver (as shown by linear regression) (Figure 3 A). Mean perfusion pressure was 3 mmHg and did not change significantly (as shown by linear regression) throughout the experimental period (Figure 3B). Overall mean oxygen consumption was 1.67 ± 0.44 µmol/min/g, falling from an initial rate of 2.41 ± 0.68 µmol/min/g liver to be stable over the latter 90 min of the perfusion at a rate of 1.48 ± 0.40 µmol/min/g liver (Figure 3C). The lactate:pyruvate ratio did not rise during the experiment, indicating that hepatic oxygenation did not deteriorate (Figure 3D).

Hepatic Distribution of Perfusate Flow—Ductus venosus flow as a proportion of total perfusate flow is shown in Table 1. The two neonates aged 2 days had ductus venosus shunts of 15.2 and 18.2%, and the five older lambs had shunts of less than 3% of perfusate flow (Table 1). Perfusate flow was 39.5 ± 8.5% to the left lobe of the liver and 60.5 ± 8.5% to the right lobe. The right lobe received a somewhat greater flow per gram of tissue (2.11 ± 0.79 mL/g/min) than the left lobe (1.39 ± 0.33 mL/g/min, $p < 0.05$).

Taurocholate Elimination—Mean C_{in} and C_{out} concentrations are depicted in Figure 2. Data for steady-state calculations were taken from the time period 60–90 min. Mean steady-state hepatic extraction of TC, calculated after taking into account shunted perfusate flow, was 0.56 ± 0.14; TC was concentratively secreted into bile (bile:plasma concentration ratio up to 5000). The total clearance of TC at steady state was 0.92 ± 0.35 mL/min/g liver (Table 2),

Table 2—Kinetics of Taurocholate Elimination

neonatal age (Days)	TC extraction	shunt-corrected TC extraction	bile/perfusate TC ratio	total TC clearance (mL/min/g)	biliary clearance of TC (mL/min/g)
2	0.43	0.51	785	1.01	0.65
2	0.46	0.56	1787	1.12	0.80
3	0.44	0.45	5241	0.56	0.60
6	0.32	0.33	2308	0.45	0.34
8	0.70	0.71	5828	0.85	0.53
10	0.61	0.61	3906	0.92	0.55
11	0.71	0.73	5529	1.51	1.49
Mean ± SD	0.52 ± 0.15	0.56 ± 0.14	3626 ± 2014	0.92 ± 0.35	0.71 ± 0.37

which was significantly higher than biliary clearance of TC (0.71 ± 0.37 mL/min/g liver, $p < 0.05$, Table 2). There was no significant correlation between hepatic extraction ratio, total clearance or biliary clearance of TC, and neonatal age over the age range studied.

Discussion

The isolated perfused liver (IPL) has proved a valuable experimental model in enhancing our understanding of the pharmacology and physiology of the liver.[14] Studies with this model complement those using isolated organelles or hepatocytes, and studies in whole animals. The IPL preserves the structural and functional integrity of the liver, while eliminating the confounding elements of metabolism and elimination by other organs.[14] Moreover, it allows ready access to hepatic inflow and outflow vessels and to the biliary tree.

The IPL model which we recently established to examine hepatic function in the fetal sheep[4] has proved particularly valuable in facilitating access to fetal hepatic blood vessels and bile ducts and in eliminating placental transport and maternal metabolism as confounding factors. The development of the neonatal IPL model described in the present study represents a modification of the approach used to establish the fetal model. The neonatal IPL is equally robust and stable over a 2 h experimental period, and the two preparations allow direct comparison of fetal and neonatal hepatic function.

The neonatal IPL has a stable bile flow comparable to other perfused liver models,[4,15] and maintains a low perfusion pressure,[16] a low lactate:pyruvate ratio, and a steady oxygen consumption after an early fall from a high initial level (Figure 3). The high initial reading was probably occasioned by increased tissue oxygen extraction consequent upon a transient acidosis after flushing the system with Hartman's solution. The absence of any later increase in lactate:pyruvate ratio, and normal histology, indicate that there has been no significant tissue hypoxia.[17] Moreover the mean level of oxygen consumption was significantly greater than that in the fetus (1.67 ± 0.44 versus 0.86 ± 0.07 μmol/min/g liver $p < 0.005$)[4] and approximates that previously reported in the neonatal and adult sheep liver in vivo.[18]

In the early neonatal period there was still appreciable shunting of blood through the ductus venosus (Table 1), although the degree of shunting was considerably less than in the near-term fetus (35 ± 18%[4]). By 6 days the ductus venosus had all but closed. This accords with previous observations in the sheep in vivo.[16,19] Nonetheless, it will be important in using this neonatal model, as in the fetal IPL, to measure and correct for the degree of ductus venosus shunting.

It is of interest that, on a weight basis, the right lobe of the liver appeared to be more generously perfused than the left (Table 1) whereas in the fetus, perfusion rates are similar.[4] This may reflect increasing resistance to flow in the portal sinus, and one consequence may be the disproportionate growth of the right lobe that is seen in the neonatal period.[16]

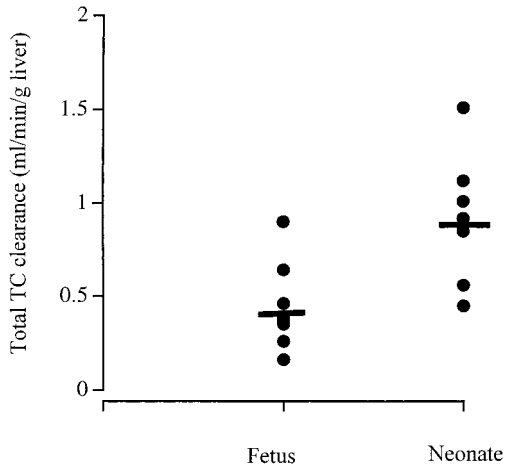

Figure 4—Total clearance of TC in neonatal liver (this study) and fetal liver (data from: Ring, J. A. et al. *Biochem. Pharmacol*. **1994**, *48*, 667–674).

In the adult human and rat, TC extraction approaches unity,[20,21] but no data are available in the adult sheep. In late fetal life, the hepatic extraction ratio (E^*) of 0.23 ± 0.16[4] indicates that at this stage of the liver's development TC is a low clearance compound. Within days of birth, TC extraction ratio had risen 2-fold to 0.56 ± 0.14 ($p < 0.005$), which represents an intermediate level of clearance. This increase in hepatic extraction ratio around the time of birth was accompanied by an increase in mean total TC clearance from 0.44 to 0.92 mL/min/g liver ($p < 0.01$, Figure 4). These results indicate a substantial change in the efficiency of hepatic TC transport, although there is further development in TC uptake and biliary excretion before the functions are fully mature.[21]

Values for total TC clearance were significantly greater than biliary clearance of TC in this study. The likely explanation is that although TC levels had reached steady state in perfusate (Figure 4), transport of TC into bile may not have reached steady state.

We conclude that the sheep neonatal IPL is a useful experimental model which will facilitate the study of the developmental physiology and pharmacology of the liver. Our study shows that there is considerable maturation of the biliary excretion of TC between the late fetal and early neonatal period in the lamb.

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