Preferential utilization of newly synthesized cholesterol for brain growth in neonatal lambs

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Turley, Stephen D., Dennis K. Burns, and John M. Dietschy. Preferential utilization of newly synthesized cholesterol for brain growth in neonatal lambs. Am. J. Physiol. 274 (Endocrinol. Metab. 37): E1099–E1105, 1998.—These studies used the suckling lamb as a model to determine the sources of cholesterol that are utilized for development of the central nervous system in the neonate. Lambs were studied at 1.3 and 16.4 days after birth. Over this 15-day interval, 14 g of new brain tissue were formed. About 9–10 mg of cholesterol were utilized daily for this new tissue growth. To determine the source of this cholesterol, the absolute rates of low-density lipoprotein clearance and cholesterol synthesis were measured in vivo in nine separate regions of the central nervous system. Low-density lipoprotein clearance throughout the brain was very low and at most could have contributed only 0.3–0.4 mg cholesterol daily for new brain growth. In contrast, the brain synthesized 7–8 mg of cholesterol/day. There were pronounced regional differences in the concentration of cholesterol throughout the brain, and these correlated closely with the rate of sterol synthesis (r = 0.95) in these same regions. We conclude that the principal source of sterol for brain growth in suckling lambs is de novo synthesis.

Currently, there are two main avenues of investigation into the control of brain cholesterol metabolism. One of these is focusing on the question of how cholesterol turnover in the adult brain is regulated. Early experiments in baboons and more recent studies in humans have shown that the turnover of cholesterol in the adult brain is very slow and usually amounts to much less than 1% of the total tissue pool of sterol per day (22, 38). There is now evidence that this turnover is facilitated by the conversion of cholesterol to a hydroxylated derivative that passes the blood–brain barrier more readily than cholesterol itself (22).

The other major area of study concerns the origin of cholesterol in CNS tissue. The brain is the most cholesterol-rich organ in the body (7), and it acquires most of its sterol during myelination, which takes place in the early stages of development (29). Ultimately, this cholesterol must have been either synthesized locally and/or delivered there through the uptake of specific lipoproteins. The presence of mRNA for the LDL receptor in the brains of neonatal rats and rabbits implies that LDL from the plasma might be a source of cholesterol for brain growth in the developing animal (24, 31). However, such measurements are technically feasible in lambs that weigh ~4 kg at birth and have, by 2–3 wk of age, plasma LDL cholesterol concentrations that are sufficiently great to permit detailed metabolic measurements in multiple regions of the brain. The present studies thus describe the application of techniques for the measurement in vivo of the fraction of cholesterol contained in newly formed CNS tissue in suckling lambs that is derived from de novo synthesis and from the uptake of LDL from the plasma. The data support other lines of evidence that newly synthesized cholesterol is preferentially utilized for the growth and differentiation of the CNS in the neonate.

MATERIALS AND METHODS

Animals. Timed-pregnant crossbred Rambouillet ewes (Ovis aries) carrying either single or twin fetuses were obtained from the Department of Veterinary Science, University of Texas M. D. Anderson Cancer Center (Bastrop, TX), and maintained as described (5). After natural birth, the lambs had unrestricted access to their dams until taken for study. A total of 18 early neonatal lambs (14 males, 4 females) at ages ranging from 0.2 to 3 days and 11 late neonatal animals (3 males, 8 females) aged 16–18 days were used for different types of experiments. All procedures were approved by the...
Institutional Animal Care and Research Advisory Committee.

Measurement of the rate of LDL clearance by the liver and brain in vivo. LDLs were isolated by density gradient ultracentrifugation in the density range of 1.020–1.055 g/ml from the plasma of donor adult sheep that had been fed a cholesterol-free commercial diet and grass. Gradient gel electrophoresis revealed apolipoprotein B-100 to be the only protein in this fraction (11). The LDL was labeled either with 125I-labeled tyramine cellulose or directly with 131I as described (34). The labeled lipoproteins were used within 48 h and were filtered through 0.45-µm Millipore filters (Millipore, Bedford, MA) immediately before use. Five early neonatal lambs (3 males, 2 females) ranging in age from about 1 to 3 days and five late neonatal animals (2 males, 3 females) aged 16–18 days were sedated with xylazine (1 mg/kg body wt, given im; Gemini SA, Rugby Laboratories, Rockville Center, NY), fitted with two jugular vein catheters, and given a bolus injection of 125I-tyramine cellulose-LDL. This was followed by a continuous infusion of the same labeled LDL preparation over the next 5 h, during which time the lambs were kept sedated under warm covers. The plasma level of labeled LDL was measured at several points throughout this period. Ten minutes before the end of the infusion, a bolus injection of 131I-labeled LDL was administered as a marker for tissue blood contamination. The lambs were then given an overdose of pentobarbital sodium (50 mg/kg body wt; Nembutal, Abbott Laboratories, Chicago, IL) and were exsanguinated from the abdominal aorta. The liver and brain were removed, rinsed, and weighed. The 125I and 131I contents of aliquots of plasma and of tissue from each organ, including multiple regions of the brain (dissection is detailed in Dissection of brain into various regions), were then measured (34). Plasma was fractionated to obtain the LDL cholesterol level in each animal. The rate of LDL clearance by each organ was expressed as microliters of plasma cleared of LDL content per hour per gram of tissue (µL·h⁻¹·g⁻¹). Multiplication of this value by the whole organ weight and by the plasma LDL cholesterol concentration (µg/µl) yielded the amount of LDL cholesterol cleared by the liver or brain (µg/h for each organ).

Measurement of the rate of sterol synthesis by the liver and extrahepatic organs in vivo. A total of 13 early neonatal lambs (11 males, 2 females) aged from 0.2 to 2.5 days were sedated, fitted with a jugular vein catheter, and placed under a well-ventilated fume hood. After the intravenous administration of a bolus of [3H]water (750 mCi/kg body wt; ICN Biomedicals, Irvine, CA), the lambs were kept sedated under warm covers for the next 60 min, after which time they were given an intravenous overdose of pentobarbital sodium and exsanguinated. The liver, whole small intestine, adrenal glands, kidneys, spleen, and whole brain, as well as a section of spinal cord, were removed, rinsed, and weighed. Aliquots of tissue were taken for the measurement of cholesterol concentration and [3H]sterol content (32). The entire remaining carcass was also digested in alcoholic KOH, and its total [3H]sterol content was similarly determined. In the case of the brain, the tissue aliquots were taken from multiple regions as described in Dissection of brain into various regions. Before the brain was removed, cerebrospinal fluid (CSF) was collected directly from the cisterna magna. Alliquots of CSF and plasma were taken for the measurement of water and [3H]content so that the specific activity (SA) of water in both fluids could be determined. The SA of water in the plasma and CSF averaged 15.0 ± 1.0 and 14.6 ± 1.0 counts·min⁻¹·nmol⁻¹, respectively. The rate of sterol synthesis in each organ was expressed as the nanomoles of [3H]water incorporated into digitonin-precipitable sterols per hour per gram of tissue (nmol·h⁻¹·g⁻¹). These data and the organ weights were used to calculate whole animal sterol synthesis (µmol·h⁻¹·kg body wt⁻¹). The rate of incorporation of [3H]water into sterol by certain organs such as the brain and also by the whole body was converted to an equivalent milligram quantity of cholesterol, assuming 0.69 µg atoms were incorporated into the sterol molecule per carbon atom entering the biosynthetic pathway as acetyl-CoA (13).

Dissection of brain into various regions. After death, the cranial vault of the lamb was opened superiorly, and the rostral spinal cord was exposed via a posterior vertebral laminectomy. Cranial nerves and vascular connections were severed, the brain and spinal cord were removed, and the brains were immediately weighed. The cerebral hemispheres, brain stem, and cerebellum were sectioned in the fresh state in the coronal, horizontal, and sagittal planes, respectively, at ~5-mm intervals. The following regions were obtained for biochemical and radioisotopic analyses: superior frontal neocortex, superior parietal neocortex, white matter from corpus callosum and centrum ovale, corpus striatum, thalamus posterior to corpus striatum, midbrain at the level of the superior colliculus, midportion of the basis pontis, midportion of the medulla oblongata, cerebellar vermis, and cervical spinal cord. The proportion of total brain mass distributed among the nine regions that were studied was determined in a separate lot of organs taken from four late fetal animals. The weight of tissue in each region was recorded and expressed as a percentage of the total brain weight, which averaged 59.2 ± 2.7 g. The relative proportions of each region were as follows: frontal cortex, 20.5 ± 1.5%; parietal cortex, 18.3 ± 1.8%; cerebellar folia, 9.2 ± 0.2%; corpus striatum, 2.5 ± 0.2%; thalamus, 4.0 ± 0.4%; midbrain, 4.1 ± 0.3%; cerebral white matter, 8.7 ± 0.3%; brain stem, 3.7 ± 0.2%; and remaining brain parenchyma including temporal and occipital neocortex (these were combined and designated "other regions"), 29.0 ± 1.5%.

Analytical procedures. Plasma and tissue total cholesterol concentrations and the level of esterified and unesterified cholesterol in liver and brain were measured as described elsewhere (16, 35). Although noncholesterol sterols such as desmosterol are found in brain tissue in fetal sheep (S. D. Turley and J. M. Dietschy, unpublished findings) and in the neonates of other species such as the rat (17), in the lambs studied here, cholesterol was the only sterol detected throughout all regions of the brain.

Analysis of data. The data are presented either as values for individual animals or as means ± SE of values obtained from the number of lambs specified. The data for male and female lambs were combined because no gender-related differences in any of the metabolic parameters studied were apparent. Differences between mean values were tested for statistical significance using the two-tailed unpaired Student's t-test.

RESULTS

The average age of all the early and late neonatal lambs studied here was 1.3 and 16.4 days, respectively. Over this 15-day interval, body weights more than doubled as did plasma LDL cholesterol levels, and brain mass increased by 14 g (Table 1). Because the mean concentration of cholesterol in brain tissue was similar at both stages of development, the increment in brain cholesterol content, which averaged ~9–10 mg/ day, was due entirely to the growth of new CNS tissue. The first experiment was designed to determine the extent to which the cholesterol utilized for this CNS
tissue growth was derived from plasma LDLs. This was done by measuring in vivo the clearance of radiolabeled homologous LDL by various regions of the brain in groups of early and late neonatal lambs. For comparative purposes, the clearance of LDL by the liver was measured in these same animals. The data in Fig. 1 show that in the older lambs, hepatic LDL clearance (27 ± 4 µl·h⁻¹·g⁻¹) was far lower than in the early neonatal group (85 ± 11 µl·h⁻¹·g⁻¹). A distinctly different result was seen in the brain where no region manifested LDL clearance rates of more than 0.5 µl·h⁻¹·g⁻¹ in either group of animals. The values given in Fig. 1 for brain represent the average clearance rates for all nine regions. With the assumption of an average plasma LDL cholesterol concentration of 46 mg/dl and an average whole brain mass of 66 g over this 15-day interval, it can be calculated from these clearance values that, at most, only 0.3–0.4 mg of the 9–10 mg of cholesterol used daily in the formation of new brain tissue could have been derived from LDL.

The finding that essentially none of the cholesterol utilized for brain growth in neonatal lambs was derived from LDL prompted us to make a detailed study of sterol biosynthetic activity and its relationship to tissue cholesterol content in the brain of these animals. These studies, which were carried out only in young neonatal lambs in the age range of 0.2–2.5 days, involved the measurement in vivo of the rate of incorporation of [³H]water into sterols by all the organs, including nine different regions of the brain and a section of the spinal cord. The rate of sterol synthesis in each organ, expressed per gram wet tissue, is shown in Fig. 2A. Although the adrenal gland manifested an exceptionally high rate of synthesis, all the other

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Early Neonatal</th>
<th>Late Neonatal</th>
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<tr>
<td>Age, days</td>
<td>1.3 ± 0.2</td>
<td>16.4 ± 0.2</td>
</tr>
<tr>
<td>Gender, male/female</td>
<td>14/4</td>
<td>3/6*</td>
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<tr>
<td>Body wt, kg</td>
<td>4.2 ± 0.2</td>
<td>8.9 ± 0.3*</td>
</tr>
<tr>
<td>Brain wt, g</td>
<td>59.0 ± 1.4</td>
<td>73.3 ± 1.8*</td>
</tr>
<tr>
<td>Plasma LDL cholesterol concn, mg/dl</td>
<td>29.4 ± 3.2</td>
<td>62.7 ± 4.1*</td>
</tr>
<tr>
<td>Brain mean cholesterol concn, mg/g</td>
<td>11.8 ± 0.2</td>
<td>11.5 ± 0.4</td>
</tr>
<tr>
<td>Brain cholesterol concn, mg/brain</td>
<td>698 ± 22</td>
<td>841 ± 30*</td>
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Values represent means ± SE of data from combined number of males and females specified. After natural birth, lambs had unrestricted access to their dams until they were used in various metabolic studies. Age range of early and late neonatal groups was 0.2–3 days and 16–18 days, respectively. *P < 0.05 vs. corresponding value for early neonatal group.

Fig. 1. Rate of low-density lipoprotein (LDL) clearance by liver and brain in neonatal lambs at 2 stages of development. Rates of LDL clearance by liver and brain in 5 animals from each age group were measured in vivo as described in MATERIALS AND METHODS. Age of younger lambs (3 males, 2 females) ranged from about 1 to 3 days, and older group (2 males, 3 females) was aged 16–18 days. These values represent microliters of plasma cleared of its LDL content per hour per gram of tissue. Values for brain are averages of clearance rates found in 9 separate regions. Each point represents data from a single animal.

Fig. 2. Rate of sterol synthesis in liver and extrahepatic organs in early neonatal lambs. Lambs were given an intravenous bolus injection of [³H]water and killed 1 h later. [³H]Sterol content of liver and several extrahepatic organs including brain and whole remaining carcass was then determined as described in MATERIALS AND METHODS. These contents were taken as a direct measure of rate of sterol synthesis in each tissue (A), which was expressed as nmol of [³H]water incorporated into sterols per hour per gram of tissue. This rate was used in turn to calculate whole organ sterol synthesis (B). Whole animal sterol synthesis was determined as sum of synthesis in all organs normalized per kilogram body wt (whole animal sterol synthesis = 52.4 ± 5.9 µmol·h⁻¹·kg body wt⁻¹). Values represent means ± SE of data from 13 lambs (11 males, 2 females) ranging in age from 0.2 to 2.5 days.
organs also exhibited significant levels of biosynthetic activity. The rate for the brain, which represents the average of the values for all the regions studied, was higher than that for all other organs except the adrenal and small intestine. These synthesis data were also expressed on a whole organ basis (Fig. 2B). Although the bulk of the synthetic activity was manifest in the tissues of the residual carcass, the brain accounted for ~4.5% of whole animal sterol synthesis. With the use of the value for the rate of \[^{3}H\]water incorporation into sterol (Fig. 2B) together with a previously determined value for the number of \[^{3}H\] atoms that are incorporated into the sterol molecule per carbon atom entering the biosynthetic pathway as acetyl-CoA (13), it can be calculated that these lambs (average body wt 4.2 kg) synthesized a total of ~165 mg of cholesterol/day. Within the whole brain itself, ~7–8 mg of cholesterol were synthesized daily. This largely accounts for the daily increment of 9–10 mg in whole brain cholesterol content (Table 1).

The importance of de novo synthesis as a source of cholesterol for brain development was evident not only from the similarity in the values for the amount of cholesterol that the brain synthesized and laid down in new tissue each day but also from the remarkably close correlation (r = 0.95) that existed between tissue cholesterol concentration and the rate of cholesterol synthesis throughout all the major regions of the brain and spinal cord (Fig. 3). The values for both synthesis and concentration varied about threefold throughout the CNS, with those regions richest in white matter manifesting the highest levels of sterol synthetic activity and the greatest cholesterol content.

The relationship between tissue cholesterol content and synthesis in the brain was different than in the other organs, particularly the liver. Unlike brain tissue, which contained only unesterified cholesterol, in the liver, both unesterified cholesterol and esterified cholesterol were present. Among the 13 early neonatal animals used in these sterol synthesis experiments, some were studied as soon as 0.2 days after birth, whereas others were ~2.5 days old at the time of study. Generally, the lambs that had suckled longer had higher total cholesterol concentrations in their livers, with the additional cholesterol being present only in the esterified fraction. The relationship between the rate of synthesis and the respective concentrations of esterified and unesterified cholesterol in the livers of all 13 lambs is shown in Fig. 4. In those animals that had suckled the most, the concentration of esterified cholesterol increased severalfold, and this was accompanied by a dramatic compensatory reduction in the rate of hepatic cholesterol synthesis (Fig. 4A). There was no correlation between synthesis and unesterified cholesterol levels, which generally remained at 2.5–3.0 mg/g irrespective of how long the lambs had suckled (Fig. 4B). Thus, unlike the brain, in which cellular cholesterol content was determined by the rate at which cholesterol was synthesized locally, in the liver, the rate of synthesis changed in accordance with cellular cholesterol ester content, which in turn varied as a function of the amount of chylomicron cholesterol that was delivered to the liver from the small intestine.

**DISCUSSION**

In the adult human, about one-third of all the cholesterol in the body is present in the CNS, where it is contained primarily within myelin, the formation of which is completed in the early stages of development (7, 21). Although developing CNS tissue manifests a very high rate of cholesterol synthesis (29), the question of how much brain cholesterol ordinarily originates from plasma lipoproteins remains the subject of investigation. The expression of mRNA for the LDL receptor in the brains of neonatal rabbits and rats and the finding that transcytosis of LDL across the blood-brain barrier occurs under in vitro conditions suggest that cholesterol from LDL might be utilized for CNS tissue growth in the developing animal (10, 24, 31). The present studies were designed to determine the magnitude of this contribution by applying a primed constant infusion technique to directly measure, in vivo, the rate of LDL clearance by all the major regions of the brain in...
the suckling lamb. By studying lambs just after birth and again at ~2 wk postpartum, we were able to directly compare the daily increment in CNS tissue cholesterol content with the amount of LDL cholesterol cleared from the plasma by the whole brain. In neither the 1- nor the 16-day-old lambs did any region of the CNS manifest LDL clearance rates above 0.5 µl·h⁻¹·g⁻¹. Values <1 µl·h⁻¹·g⁻¹ are within the error of measurement of this method. Even if these low clearances represented true net LDL cholesterol uptake, it can be calculated that, at most, only 3–4% of the 9–10 mg of cholesterol utilized daily for brain growth during this phase of the lamb’s development could have come from LDL. Such a minimal contribution is also apparent from the finding that there were no regional differences in LDL clearance, yet there was a threefold difference in cholesterol concentration throughout different parts of the brain. Two points should be emphasized here. First, the trivial rates of LDL clearance by the brain were found in the same animals in which the liver actively transported LDL at rates that were regulated in accordance with the amount of cholesterol absorbed from the gastrointestinal tract (14). Second, the lack of significant LDL transport by the brain in these lambs cannot be attributed to suckling because similar results were found previously for fetal sheep, even at gestational ages preceding closure of the blood-brain barrier (33).

These clearance data provide direct evidence in vivo that the supply of cholesterol for new CNS tissue growth in the neonate is not dependent on the receptor-mediated transport of LDL out of the plasma. This conclusion is fully consistent with the previous finding that brain growth and development are normal in mice and rabbits genetically lacking the LDL receptor (12, 25). It also fits well with the observation that in young mice with Niemann-Pick (type C) disease, in which there is a defect in the mechanism for redistributing LDL cholesterol from the lysosomes, brain cholesterol levels do not increase, whereas in other organs, particularly the liver and spleen, the levels are markedly elevated (23). Although the function of the LDL receptor in brain remains to be elucidated, another type of lipoprotein receptor, gp330/megalin, which is a member of the LDL-receptor gene family, is essential for normal CNS development. In mice lacking this receptor, forebrain development is defective and the animals die perinatally from respiratory problems (37). Thus there is at least one kind of lipoprotein receptor that plays a crucial role in transporting essential nutrients needed during early development.

The data from the sterol synthesis studies in lambs aged 0.2–2.5 days established that the bulk, if not all, of the 9–10 mg of cholesterol used daily for new brain growth was synthesized locally. In all organs, including the brain, the absolute rates of cholesterol synthesis were calculated from several experimentally determined values, including one for the average SA of water in the plasma over the 60-min period after administration of the [³H]water bolus. In the calculations for the brain, we assumed that the average SA of CSF water over the 60 min after injection of the [³H]water was the same as that for plasma water. Although the SA of water in the plasma and CSF was the same at the end of the 60-min circulation time, it is possible that the equilibration of the [³H]water with CSF water was slower than that with plasma water. Therefore, to the extent that this might have been the case, the average SA of intracellular water in brain tissue would have been overestimated. In turn, the absolute rates of cholesterol synthesis would have been slightly underestimated. Although any such error would likely have been small, it should nevertheless be recognized that the value of 7–8 mg of cholesterol synthesized by the whole brain per day is possibly an underestimate of the true amount of sterol that was generated. Be that as it may, the near-perfect positive correlation between the rate of cholesterol synthesis and the tissue concentra-

![Fig. 4. Rate of sterol synthesis in liver as a function of hepatic esterified and unesterified cholesterol concentrations in early neonatal lambs. These data were derived from study described in Fig. 2. Each point represents data from a single animal.](http://ajpendo.physiology.org/)
tion of cholesterol across all regions of the brain provides further compelling evidence that the requirement for additional sterol by the rapidly growing CNS of the neonate is met fully through local synthesis. This conclusion is in agreement with the results of studies in the rat and rabbit (6, 15, 18) and with data from children with the Smith-Lemli-Opitz syndrome, in which an inherent block in the terminal enzymatic step in the sterol biosynthetic pathway causes profound changes in brain cholesterol metabolism that result in major neurological dysfunction (28).

In addition to providing a measure of the quantity of cholesterol generated daily by the brain, the data from the sterol synthesis experiments also showed how different the regulation of cholesterol levels in CNS tissue is from that in the other major organs, particularly the liver. From other published data, it can be calculated that the dietary cholesterol intake of the lambs in the present studies was ~180 mg·day−1·kg body wt−1 (4, 5), which exceeded by more than fourfold their rate of whole body cholesterol synthesis (39.2 mg·day−1·kg body wt−1). This resulted in an expansion of the cholesteryl ester pool in the liver and a striking compensatory downregulation of local cholesterol synthesis. This response has been well documented in several species (36). The lack of any correlation between unesterified cholesterol content and the rate of cholesterol synthesis in the liver was in marked contrast to the relationship between these two parameters in the brain. Although developing CNS tissue has been widely reported to contain minute amounts of esterified cholesterol (2, 6, 20, 27), none was detected in any region of the brain in these young neonatal lambs. Irrespective of the reason for this difference, it is clear from the present data that tissue cholesterol content in the CNS is dictated by the rate of local cholesterol synthesis. In contrast, in the liver and all the major extrahepatic organs, the rate of sterol synthesis is adjusted in accordance with the intracellular cholesteryl ester content, which fluctuates depending mainly on the amount of cholesterol that is transported into the cells through the clearance of lipoproteins from the plasma (14, 36).

One additional feature of cholesterol metabolism in the CNS is that, although its rate of cholesterol synthesis falls precipitously after myelination is complete, the brain continues to synthesize sterol at a very low basal rate in adulthood (30). Recent studies involving the measurement of cholesterol turnover in the brain of adult humans demonstrated not only that the minute daily fractional loss of sterol was facilitated through its conversion to 24S-hydroxycholesterol but that the quantity of cholesterol lost from the mature brain is within the range of the theoretical amount of sterol that it synthesizes, based on data from a primate model (22). The determination of what regulates the conversion of cholesterol to this particular hydroxylated derivative and the elucidation of the roles that each of the lipoprotein receptors play in regulating cholesterol homeostasis in the developing and mature brain will be important steps in better understanding the causes of neurological disease in humans.

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REFERENCES


