Adult sterol metabolism is not affected by a positive sterol balance in

the neonatal Golden Syrian hamster

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Summary

Dietary components impact metabolism early in life. Some of the diet-induced effects are long lasting, and can lead to various adult-based diseases. In the current studies, we examined the short term effects of dietary cholesterol on neonatal hepatic sterol metabolism, and the long term effects that those early life diets had on sterol metabolism in adulthood. Neonatal hamsters began consuming solid food as a supplement to milk by 5 days of age; diets contained 0 or 2% added cholesterol (wt/wt). By 10 days of age, plasma and liver cholesterol concentrations were 3.2- and 2.5-fold greater, respectively, in the neonates fed cholesterol. Hepatic sterol synthesis rates were suppressed 65% in cholesterol-fed neonates as compared to control neonates. By 20 days of age, plasma and liver cholesterol concentrations were still greater and sterol synthesis rates were now suppressed maximally in neonates fed cholesterol as compared to control neonates. Expression level of an apoB-containing lipoprotein receptor (LDL receptor-related protein; LRP) was greater and the mature form of the sterol regulatory element binding protein-2 (SREBP-2) was similar in livers of 20 day old control neonates as compared to control neonates at 10 days of age. To test if the change in sterol balance in the neonatal period had a lasting effect upon hepatic sterol metabolism, all animals were weaned onto a low cholesterol diet. At 70 days of age, hepatic sterol synthesis rates, plasma lipoprotein and liver cholesterol concentrations, and bile acid pool sizes and compositions were measured. Sterol balance in the adults was similar between animals fed either diet early in life as demonstrated by a lack of difference in any parameter measured. Thus, even though dietary cholesterol suppressed hepatic sterol synthesis rates dramatically in the neonatal hamster, the change has little impact upon sterol balance later in life.
Introduction

There are times during early life when a positive or negative metabolic stimulus can have a long lasting impact upon metabolism. (2). Studies over the past several years indicate that susceptibility to various diseases, including atherosclerosis, stroke, hypertension, and non-insulin dependent diabetes, can indeed be “programmed” by early life environments (reviewed in (1, 3, 4)). It has been hypothesized that “programming” occurs during periods of rapid cell division (2), such as during fetal or neonatal development.

Numerous regulators and stimuli are involved in metabolic control. Some of the most common regulators of metabolism are dietary components. In the neonate, dietary components originate in either breast milk or formula, commonly a cow’s milk-based formula. The compositions of the breast milk and cow’s milk-based formulas are quite similar with respect to some components, but markedly different with respect to others (41). The most recent and widely used cow’s milk-based formulas have similar composition and content of the macronutrients, carbohydrate, protein, and triglyceride, as compared to breast milk. In contrast to formulas, however, breast milk contains numerous human-based whey proteins, including gamma globulins, and living cells. Additionally, cholesterol is present in much greater amounts in breast milk as compared to cow’s milk-based formulas. Previous studies have found that whole body sterol synthesis rates were suppressed and plasma cholesterol concentrations were increased in human infants fed breast milk versus formula, (5, 46), most likely due to a difference in cholesterol content within the diets.

To test if manipulation of sterol metabolism early in life has long lasting effects on metabolism, an elegant series of studies was completed in which infant primates were fed either breast milk or formulas containing different levels of cholesterol or different fatty acid
compositions (29, 30). As expected, infant baboons fed cholesterol as either breast milk or as cholesterol-supplemented formula had altered sterol metabolism as compared to those fed cholesterol-free formula (31). Differences in metabolism persisted into adulthood between animals fed either the breast milk or formula, but not between those fed high or low levels of cholesterol-supplemented formulas, regardless of diet fed to the adult (29, 30). Though these studies were invaluable in determining baseline variables in the animals fed the different diets early in life and other processes not described here (26), they did not examine metabolic factors that regulate plasma cholesterol concentrations in response to exogenous sterol, specifically hepatic sterol synthesis rates (9, 42, 44, 45).

Thus, the purpose of the current studies was to evaluate the effect of dietary cholesterol on hepatic sterol metabolism in the neonatal hamster, and to follow any metabolic changes through to adulthood. The physiology of hamsters makes it a useful animal model for studying sterol metabolism for numerous reasons. These include: 1) in both humans and hamsters, hepatic sterol synthesis occurs at rates similarly proportional to whole body synthesis rates and thus both respond the same way to dietary sterol (9), 2) unlike rats and mice, neither hamsters nor humans upregulate bile acid synthesis in response to a positive sterol balance across the liver (17, 18, 34, 39) and 3) neonatal hamsters will begin to consume solid food early in life and thus consumption of dietary components can be readily manipulated early in age. To accomplish our goals, newborn hamsters were exposed to different levels of dietary cholesterol. By 10 days of age, sterol metabolism was markedly different between hamsters fed high and low levels of cholesterol, thus making these animals an excellent model to study the impact of neonatal diets on neonatal and adult metabolism; cholesterol-fed neonates had suppressed hepatic sterol synthesis rates and greater lipoprotein and liver cholesterol concentrations as compared to
control neonates. The differences were maintained at 20 days of age. When adult animals were in steady state, sterol balance was similar between all animals regardless of diets fed early in life as depicted by similar hepatic sterol synthesis rates, plasma and liver cholesterol concentrations, bile acid pool sizes and compositions, and responsiveness to moderate levels of dietary cholesterol.
Materials and Methods

Animals and diets

Non-pregnant female and male Golden Syrian hamsters weighing 90-100g (Charles River, Inc., Kingston, NJ) were housed in cages and kept in a temperature and humidity controlled room with alternating light and darkness. All animals were fed a pelleted chow diet (7102, Harlan Teklad, Madison, WI). Female hamsters were fed ground chow (LC; 7102CM) for 2 weeks prior to mating. Hamsters were mated as described previously (28). At 14 days of gestation, 50% of the dams were maintained on the LC diet whereas the other 50% were fed a diet containing 2% added cholesterol (HC; wt/wt). The HC diet was not fed throughout the whole pregnancy since dietary cholesterol can affect sterol balance in the fetus (28), and the purpose of this study was to examine the effect solely of neonatal diet on adult sterol balance. Pups were born at 15.5 days of gestation. At birth, litters were assigned to four different groups. Neonates were exposed to the same diets fed to the dams until pups were weaned at 20 days of age. The first group of litters was studied at 10 days of age when the pups had been exposed to the two different diets for several days. The second group of litters was studied at 20 days of age just prior to weaning. The third group of animals consisted of litters that were fed either LC or HC from birth to 20 days of age and switched to pelleted chow (7102) when weaned. The animals consumed the pelleted chow until 70 days of age when metabolism was examined. The fourth group of animals consisted of litters that were fed either LC or HC from birth to 20 days of age, were fed pelleted chow until 70 days of age, and then were fed 0.12% cholesterol (wt/wt) from 70 to 91 days of age to test their responsiveness to exogenous cholesterol. All protocols were approved by Institutional Animal Care and Use Committee at the University of Cincinnati.
Cholesterol concentrations

Hamsters were anesthetized, exsanguinated from the abdominal aorta, and livers were excised rapidly. Plasma cholesterol concentrations were measured enzymatically (Roche Diagnostics Corporation, Indianapolis, IN). Appropriate plasma samples were pooled, and plasma separated into various lipoprotein fractions by FPLC (7). Cholesterol content within each fraction was measured enzymatically. Hepatic cholesterol concentrations were determined by GLC using stigmastanol as an internal standard (43). The foodstuffs within the stomach were collected and cholesterol contents were determined by GLC.

In vivo hepatic sterol synthesis rates

Animals were injected with 5-50 mCi of $[^3]$H$_2$O, depending on body mass. After 1 h, the liver was collected, saponified and digitonin precipitable sterols (DPS) were isolated as previously described (8). The rates of sterol synthesis are expressed as the nmol of $[^3]$H$_2$O incorporated into DPS per h per g liver.

Bile acid pool size and composition

Hamsters were anesthetized and exsanguinated. The livers, including the gallbladder, were removed and placed in 100 % ethanol. The small intestines were isolated with care taken so as not to lose contents and placed in 100% ethanol with the liver. This sample constituted the bile acid pool size (BAPS). Tissue samples were cut into 3 x 3 mm pieces and processed as described previously (47). Briefly, bile acids were extracted, samples centrifuged, and supernates filtered. The remaining pellets were homogenized, the extraction process was repeated, and the extracts were added to the initial sample. A portion of the combined sample was dried down under the nitrogen using 5β-cholanic acid-7α, 12α-diol as an internal standard. The bile acids were separated and quantitated using an Evaporative Light Scattering Detector.
Bile acids were identified based on the retention time of known standards.

**Protein expression levels**

Livers were collected from neonates. Microsomes were isolated as described (21), except pellets were stored in 10% glycerol. Nuclei were isolated from separate liver samples (22). For each gel, equal amounts of protein from every litter were pooled. Proteins were separated on Bio-Rad 4-15% Tris-HCl Ready gels (Biorad Laboratories, Hercules, CA), transferred to a PVDF membrane and detected with antibodies to the LDL receptor-related protein (LRP; SC-25469, Santa Cruz Biotechnologies, Santa Cruz, CA) and the mature form of the sterol regulatory element binding protein-2 (SREBP-2; SC-5603, Santa Cruz Biotechnologies) using ECL Plus (Amersham Bioscience, Little Chalfont Buckinghamshire, UK). Relative differences were quantitated by NIH Scion Image software.

**Statistical analyses**

Data are presented as means ± SEM. Male and female data from each litter were averaged for the 10 and 20 day old neonates since there was no sex difference at these early ages. Data were analyzed between two different diets at each age by a student’s t-test or by the Mann-Whitney Rank Sum Test; normality failed in all the statistical tests for the neonates. Significance occurred at P<0.05 when data were used for one comparison and P<0.025 when data were used for two comparisons.
Results

Neonates

Since the purpose of these studies was partly to determine the appropriateness of using the hamster as a model to study the effect of early life diet on adult metabolism, it was necessary to first establish whether or not the animals would consume the two different diets early in life. Animals were exposed to the diets at birth; diets were not fed to the dam during gestation since we have previously shown that fetal cholesterol concentrations and sterol synthesis rates are affected by maternal hypercholesterolemia in the hamster (28). As expected, solid food was detected in the stomachs of the neonates by 5 days of age. By 10 days of age, the cholesterol content of the stomach foodstuffs at sacrifice was markedly greater in the neonates exposed to the HC diet (0.17±0.02 vs 5.81±0.62 mg). Thus, since the neonates consumed solid food by 5 days of age, we could evaluate the effects of cholesterol on neonatal metabolism.

At 10 days of age, body mass was similar in the LC-fed as compared to the HC-fed neonates (12.9±0.2 vs 14.0±0.5 g for LC- and HC-fed neonates, respectively), whereas sterol metabolism was markedly different. Neonates fed LC had a plasma cholesterol concentration of 98±3 mg/dl (Fig. 1), and plasma cholesterol concentrations of neonates fed HC were 3-fold greater (P<0.001). This difference in plasma cholesterol concentrations was due to greater VLDL- and LDL-cholesterol concentrations in the neonates fed the HC diet (Fig. 2). Hepatic cholesterol concentrations were also greater (P<0.001) in HC-fed neonates by 10 days of age (Fig. 3A) (3.0±0.1 vs 7.6±0.8 mg/g). Associated with the increase in cholesterol concentration was a marked suppression (P<0.001) of hepatic sterol synthesis rate in the HC-fed neonates (173±27 vs 61±8 nmol [3H]H2O converted to sterol/h per g) (Fig. 3B).
As neonates matured to 20 days of age, weight gain was similar in neonates fed either diet (33.3±1.3 vs 34.9±0.6 g for LC- and HC-fed neonates, respectively). Plasma cholesterol concentrations in 20 day old LC-fed neonates changed minimally from the concentrations in 10 day old neonates (115.6±6.0 mg/dl) (Fig. 1). Concentrations were 2.1-fold greater in the HC-fed as compared to LC-fed 20 day old neonates (P<0.001). As at 10 days of age, the differences in circulating levels of cholesterol were due to variations in the VLDL- and LDL-cholesterol concentrations (Fig. 2). Liver cholesterol concentrations in 20 day old LC-fed neonates (2.3±0.2 mg/g) (Fig. 3) were much lower than those in HC-fed neonates (43.4±2.9 mg/g; P<0.001). Hepatic sterol synthesis rates increased ≈20-fold between 10 and 20 days of age (3045±299 nmol $[^3]$H$_2$O converted to sterol/h per g) (Fig. 3) in animals fed control. Synthesis rates in 20 day old HC-fed neonates were markedly suppressed and were only 4% of rates in LC-fed control neonates (P<0.001).

Expression profiles of various proteins involved in sterol metabolism were measured in the control neonates (Fig. 4); the mature form of SREBP-2 was measured as a key regulator of sterol metabolism and LRP was measured as a receptor known to take up apoB-containing lipoproteins. Expression level of LRP increased significantly between 10 and 20 days of age, whereas expression level of SREBP-2 remained relatively constant.

*Adults*

Once it was determined that the hamster neonatal sterol metabolism could be markedly affected by diet, it was necessary to determine if the early life diets had any lasting effects on sterol metabolism. To test this, neonates were weaned onto a typical low cholesterol-containing pelleted diet until a new steady state was reached by 70 days of age. Body mass was slightly greater in males fed the LC diet early in life as compared to HC-fed animals (128±4 vs 118±2 g
in LC- vs HC-fed animals early in life; P<0.05,) whereas body mass in adult females was similar, regardless of early life diet (124±4 vs 117±2 g in LC- vs HC-fed animals early in life). To test sterol balance, plasma, lipoprotein and liver cholesterol concentrations, liver sterol synthesis rates and bile acid pool sizes were measured. Additionally, animals were fed a moderate level of dietary cholesterol to determine if the animals would respond similarly to dietary cholesterol if exposed to low vs high cholesterol diets early in life.

Sterol metabolism was similar between adult males fed LC or HC diets early in life, and between adult females fed the same two diets early in life. Plasma cholesterol concentrations were similar for all the adult males, regardless of early life diet, and for all the adult females; plasma cholesterol concentrations averaged 112.0±6.5 mg/dl for adult males fed control and 105.6±9.9 mg/dl for adult males fed cholesterol early in life (n=9-10), and 120.4±6.7 mg/dl for adult females fed control and 112.0±5.1 mg/dl for adult females fed cholesterol early in life (n=9). Cholesterol was carried in similar lipoprotein classes regardless of diet fed early in life (Fig. 5). Liver cholesterol concentrations averaged 2.3±0.1 and 2.3±0.1 mg/g for all the males and all the females, respectively (Fig. 6), and did not vary with animals fed different early life diets. Hepatic sterol synthesis rates averaged 411±42 and 781±81 nmol [3H]H2O converted to sterol per h per g in the adult males and females, respectively (Fig. 6), regardless of early life diet. Finally, BAPS was similar in males and females fed either diet early in life (41.6±5.5, 56.0±11.9, 45.7±3.5 and 50.4±1.4 µmol in males and females fed LC early in life and males and females fed HC early in life, respectively); bile acid composition also was similar in the pools and consisted of 89.4±1.1, 88.3±2.2, 89.5±0.5 and 90.3±0.4% cholic acid in males and females fed LC early in life and males and females fed HC early in life, respectively. As expected, plasma cholesterol concentrations were similar in animals fed 0.12% (wt/wt) cholesterol for 21
days, and were increased as compared to concentrations in control-fed hamsters, regardless of
amount of cholesterol fed during the neonatal period; plasma cholesterol concentrations averaged
197.2±7.5 mg/dl for adult males fed control and 187.2±7.3 mg/dl for adult males fed cholesterol
early in life (n=4-7), and 262.2±18.4 mg/dl for adult females fed control and 232.6±17.3 mg/dl
for adult females fed cholesterol early in life (n=4-5).
**Discussion**

*Neonates*

Since neonatal hamsters begin to consume solid food at an early age, the effect of feeding cholesterol early in life had a dramatic effect on sterol metabolism in the neonatal hamster. Plasma and liver cholesterol concentrations were markedly greater in the neonates exposed to exogenous cholesterol by 10 days of age while hepatic sterol synthesis rates were suppressed, as previously shown in humans fed bioavailable cholesterol (5, 6, 46). These results are consistent with those obtained in adults; plasma cholesterol concentrations increased simultaneously with liver cholesterol concentrations (9, 42).

Interestingly, plasma cholesterol concentrations tended to decrease \( (P=0.045) \) between 10 and 20 days of age in the neonatal hamster fed high levels of cholesterol at a time when consumption of the cholesterol-containing solid food increased. Most of the decrease in circulating cholesterol appeared to be in the form of cholesterol carried as apoB-containing lipoproteins. The age-related decrease in plasma sterol concentrations could be the result of an increase in LRP expression levels. Though not measured here, expression levels of the LDL receptor have also been shown to increase with maturation (38). As these lipoprotein receptor levels increase, lipoproteins are cleared more rapidly and LDL-cholesterol concentrations decrease.

Age-related increases in expression levels/activities of proteins involved in sterol metabolism, such as lipoprotein receptors, could be affected by at least two different mechanisms. First, expression levels/activities of sterol-related proteins could be affected by various transcription factors. One of the key regulators for proteins involved in sterol metabolism is the sterol regulatory element binding protein-2 (SREBP-2) (19). When in a
negative sterol balance, SREBP-2 levels are elevated resulting in an upregulation of transcription of various genes, including the LDL receptor and HMG-CoA synthase (37). Interestingly, there were similar amounts of the active (mature) form of SREBP-2 in the 10 and 20 day old hamster liver. These results were unexpected since there was such a marked increase in hepatic sterol synthesis rates between the 10 and 20 day old hamster. The age-related changes in expression levels/activities of proteins involved in sterol synthesis must have been the result of variations in other transcription factors for sterol synthesis enzymes (10, 11, 13) or in post-translational modifications of HMG-CoA reductase (reviewed in (16, 25)). A second mechanism which could affect age-induced changes in expression levels/activities in sterol-related proteins could be an intrinsic timing mechanism, similar to that which occurs during induction of sucrase activity in the late neonatal period (24, 32).

Adults

Although we have shown that exposure to cholesterol early in life can affect sterol metabolism, the question remains: Will this effect persist into adulthood? A number of studies have attempted to answer this question in humans and have shown variable results (14, 15, 27, 35). In these past studies, the early life diets consisted of either breast milk or formulas, and thus, their composition differed with respect to more than one dietary component. Consequently, it is impossible to determine the role of one specific dietary component, such as cholesterol, on the risk of adult-based diseases from these studies.

In the current research, there was no effect of an early life cholesterol-containing diet upon sterol balance in the adult animals and consequently their responsiveness to exogenous sterol. It has been demonstrated previously in adult rodents and primates that the ability of animals to respond to dietary cholesterol is, at least partially, dependent upon hepatic sterol synthesis rates
If synthesis rates are high, exogenous sterol will initially suppress synthesis rates without affecting liver or plasma cholesterol concentrations. Only when enough cholesterol is fed to fully suppress sterol synthesis rates will hepatic and plasma cholesterol concentrations increase, and if cholesterol is fed at levels significantly greater than that synthesized in the liver, then all animals could respond similarly.

The effect of feeding high or low levels of cholesterol early in life had no effect on hepatic sterol synthesis rates in either adult male or female hamsters. As expected, there was no effect of early life diet on plasma or liver cholesterol concentrations, and responsiveness to a moderate level of dietary cholesterol. The similar increase in plasma cholesterol with the 0.12% cholesterol diet indicates that the dietary cholesterol in animals fed the different diets early in life was bioavailable and absorbed similarly. Thus, these studies suggest that sterol metabolism is not programmed by cholesterol during the neonatal period.

Summary

Three major conclusions can be made from the current studies. First, plasma cholesterol and liver concentrations increase and hepatic sterol synthesis rates decrease in hamster neonates fed cholesterol. As neonates age to 20 days, plasma cholesterol concentrations of cholesterol-fed neonates actually decrease, even when more cholesterol is consumed, possibly due to changes in expression/activity levels of proteins involved in sterol metabolism.

Second, these studies demonstrate that adults that had consumed high amounts of cholesterol early in life should not be at greater risk to develop heart disease as compared to adults that consumed low amounts of cholesterol as infants, possibly due to the lack of expression or ability to regulate proteins involved in sterol metabolism early in life. These data are promising for certain infants fed cholesterol-supplemented diets, such as neonates with the Smith-Lemli-Opitz

(9, 42, 44, 45).
Syndrome (SLOS), or other infants that require an exogenous source of cholesterol, possibly as a substrate for bile acids. Infants with SLOS do not synthesize cholesterol at normal rates (23, 40), and thus are fed supplemental cholesterol from the time of diagnosis to improve cognitive functions (12, 20, 33).

Third, these studies demonstrate the promise of using the hamster as a model to study adult-based diseases. Since the hamster neonate will begin to consume solid food by 5 days of age, a time when rapid growth occurs, they can be used to study a number of different dietary components that might affect adult metabolism. Hamsters also can be used for fetal studies since it has already been shown that the hamster fetus is affected by components circulating in the maternal plasma (28). Although these studies suggest that cholesterol supplementation during neonatal development does not affect adult sterol metabolism, cholesterol supplementation during in utero development could have an impact on adult metabolism and therefore should also be examined. Various factors that could affect fetal and subsequently adult metabolism include altered maternal support in the form of nutrient supply and/or anomalous placentas (1).
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**Figure 1.** Plasma cholesterol concentrations in 10 and 20 day old neonates fed either 0 or 2% added cholesterol (wt/wt). Diets containing no added cholesterol (LC) or higher levels of cholesterol (HC) were made available to neonates at birth. Plasma cholesterol concentrations (mg/dl) were measured at either 10 or 20 days of age in the neonates. Each value represents the mean ± 1 SEM (n=9-13); results from males and females in one litter were pooled and each litter represents an n value of one. Asterisks represent significant differences (P<0.05) between neonates fed different diets.
Figure 2. Plasma lipoprotein cholesterol concentrations in 10 and 20 day old neonates fed either 0 or 2% added cholesterol (wt/wt). Diets and dietary regimes are the same as those described in Fig. 1. Plasma was collected, like samples were pooled and lipoprotein fractions from 10 and 20 days of age separated by size using gel exclusion chromatography.
Figure 3. Hepatic cholesterol concentrations and sterol synthesis rates in 10 and 20 day old neonates fed either 0 or 2% added cholesterol (wt/wt). Diets and dietary regimes are the same as those described in Fig. 1. Liver cholesterol concentrations are presented as mg/g. Sterol synthesis rates are presented as nmol of [3H]H2O converted to sterol per h per g. Each value represents the mean ± 1 SEM (n=10-23); results from males and females in one litter were pooled and each litter represents an n value of one. Asterisks represent significant differences (P<0.05) between neonates fed different diets.
**Figure 4.** Expression profiles of proteins involved in sterol metabolism in the 10 and 20 day old neonatal hamster fed the control diet. Samples from each of the litters were pooled (n=4-5) and expression levels determined for SREBP-2 and LRP. Proteins were detected with ECL Plus.
Figure 5. Plasma lipoprotein cholesterol concentrations in adult male and female hamsters fed either 0 or 2% cholesterol for the first 20 days of life. Plasma collected from animals described in Fig. 4 was pooled between like days and diets, and lipoproteins separated by size using size exclusion chromatography.
**Figure 6.** Hepatic cholesterol concentrations and sterol synthesis rates in adult male and female hamsters fed either 0 or 2% cholesterol for the first 20 days of life. Diets and dietary regimes are the same as those described in Fig. 4. Cholesterol concentrations are presented as mg/g. Sterol synthesis rates are presented as nmol of $[^3H]H_2O$ converted to sterol per g per h. Each value represents the mean ± 1 SEM (n=7-10); each n represents a different litter.
References


