Early Diet Influences Hepatic Hydroxymethyl Glutaryl Coenzyme A Reductase and 7α-Hydroxylase mRNA But Not Low-Density Lipoprotein Receptor mRNA During Development

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Plasma cholesterol levels increase after birth, and to a greater extent in breast-fed versus formula-fed infants. This increase is believed to be due to the high fat and cholesterol content of the infant diet, but little is known about the effects of early diet on the expression of proteins involved in regulating cholesterol metabolism. This study examined changes in the expression of hepatic proteins regulating cholesterol metabolism during development. Newborn piglets were fed sow milk or one of four formulas for 18 days. The formulas had similar levels of palmitic acid (16:0) as in milk, supplied as palm olein oil with 16:0 esterified predominantly to the *sn*-1,3 position or as synthesized triglyceride (TG) with 16:0 esterified mainly to the *sn*-2 position of glycerol, each with no cholesterol (<0.10 mmol/L) or 0.65 mmol/L cholesterol added. Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of mRNA levels was used to assess the effects of diet on hepatic hydroxymethyl-glutaryl coenzyme A (HMG-CoA) reductase, low-density lipoprotein (LDL) receptor, and 7α-hydroxylase (C7H). LDL receptor mRNA levels showed no appreciable difference between milk- and formula-fed piglets. However, the levels of HMG-CoA reductase and C7H mRNA were higher ($P < .05$) in all formula-fed versus milk-fed piglets, irrespective of the formula TG source or cholesterol content. The lower levels of HMG-CoA reductase and C7H mRNA in milk-fed piglets were accompanied by higher ($P < .05$) plasma total, high-density lipoprotein (HDL), and apolipoprotein (apo) B–containing cholesterol. These studies show that the levels of hepatic HMG-CoA reductase and C7H mRNA, but probably not LDL receptor mRNA, are altered by early diet.

PLASMA TOTAL, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) cholesterol concentrations increase rapidly in human infants and most other animal species after birth.[1] This increase is higher in breast-fed than in formula-fed infants and animals.[2-4] Differences in the lipid composition between milk and formula may reasonably be considered responsible for these differences in plasma cholesterol concentrations. Milk and most formulas supply about 50% of energy as fat,[5] with about 20% to 30% of the fatty acids supplied as palmitic acid (16:0). However, about 60% of 16:0 in milk is esterified at the *sn*-2 position of the milk triglyceride (TG), whereas about 94% of 16:0 in formula is esterified at the *sn*-1,3 position of the TG.[5-7] Further, infant formulas are low in cholesterol, usually containing less than 0.10 mmol/L, versus about 0.52 to 0.78 mmol/L in milk.[6] Whether these differences in 16:0 distribution and cholesterol content contribute to the differences in plasma cholesterol between breast-fed and formula-fed infants is not known.

The higher plasma cholesterol in breast-fed compared with formula-fed infants may be secondary to alterations in cholesterol metabolism. For example, the higher plasma cholesterol of breast-fed infants could be the result of increased hepatic cholesterol synthesis. However, results of studies in other species suggest that hepatic cholesterol synthesis decreases after birth and is low during milk feeding.[4,8-10] In addition, decreased activity of hepatic hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol synthesis, has been reported for milk-fed compared with formula-fed piglets.[4] Consistent with this, studies using deuterated water to estimate rates of cholesterol synthesis have reported that cholesterol fractional synthetic rates are lower in infants who are breast-fed than in infants fed formula.[8] High saturated fat and cholesterol intakes have been shown to result in downregulation of the activity and mRNA levels of HMG-CoA reductase in adult animals.[11] This suggests that the cholesterol content and possibly the positional distribution of 16:0 in milk could be responsible for the apparent lower rate of cholesterol synthesis in breast-fed versus formula-fed infants.

Similar to HMG-CoA reductase, the expression of the LDL receptor, which is responsible for clearance of plasma LDL, is downregulated at the level of mRNA, protein, and activity by high fat and cholesterol intakes.[12-14] This suggests that hepatic LDL receptor expression may be downregulated in infants fed milk. In contrast to this, studies in baboons have found higher LDL receptor mRNA levels in animals fed milk than in those fed formula,[15] whereas studies in piglets found no difference in LDL receptor protein levels between milk-fed and formula-fed animals.[4] Although inconsistent, this information suggests that the higher plasma cholesterol associated with milk feeding is not due to reduced LDL receptor–mediated clearance of plasma cholesterol. Alternatively, then, the higher plasma cholesterol concentrations in milk-fed infants could also involve decreased conversion of hepatic cholesterol to bile acids, the rate of which is controlled by cholesterol 7α-hydroxylase (C7H). Dietary cholesterol has been found to increase C7H mRNA and activity levels in rodents,[11] but it decreases C7H mRNA and activity levels in monkeys.[16]

This study examined the effect of milk compared with formula feeding on LDL receptor, HMG-CoA reductase, and C7H mRNA levels. To elucidate the potential effects of the cholesterol content and the positional distribution of 16:0 in milk TG on cholesterol metabolism, piglets were fed formula with and without added cholesterol and with and without 16:0

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positioned to resemble milk fat. The studies were performed in piglets because plasma cholesterol levels are higher with milk than with formula feeding[4] and the lipid metabolism of pigs and the lipid composition of sow milk are similar to those in the human.[17] Hepatic and bile lipid concentrations were also determined because of the known regulatory effect these have on the expression of LDL receptor, HMG-CoA reductase, and C7H.[11,18-21] As well, breast milk contains various hormones and growth factors, such as thyroid hormone, that are not found in formula.[22] Thyroid hormone concentrations have been found to differ between milk- and formula-fed infants[23] and animals,[24] and have also been shown to have an effect on bile acid metabolism.[19,25,26] Therefore, plasma concentrations of thyroid hormones, insulin, glucagon, and free fatty acids were also determined in the piglets.

MATERIALS AND METHODS

Animals and Diets

Male Yorkshire piglets with birth weight greater than 1 kg were obtained from Peter Hill Holdings (Langley, British Columbia, Canada). Groups of piglets were taken at birth (day 0) and randomly assigned to be fed milk by their natural mother or fed one of four formulas, six piglets each, until 18 days of age. Piglets assigned to receive formula were bottle-fed, with passive immunity provided during the first 72 hours after birth by addition of bovine colostrum–derived immunoglobulins (IgG; LaBelle Associates, Bellingham, WA) to the formula.[27]

The four formulas were similar in composition, with the exception of the positional distribution of 16:0 in the TG and the cholesterol content (Table 1). Two formulas (palm olein) contained palm olein oil as the source of saturated fatty acid and had about 23% 16:0, with 93.7% of total 16:0 esterified to the *sn*-1,3 position of the TG. The other two formulas contained synthesized TG (Betapol; Loders Croklaan, Wormerveer, The Netherlands)[28] and had 22% to 23% 16:0, with 47% of the total 16:0 esterified to the *sn*-2 position of the TG. Levels of the other saturated fatty acids, oleic acid (18:1n-9), linoleic acid (18:2n-6), and linolenic acid (18:3n-3), were similar in the palm olein and synthesized TG formula (Table 1), although the enrichment of 18:1n-9, 18:2n-6, and 18:3n-3 at the *sn*-2 position of the TG varied inversely with the enrichment of 16:0.[28,29] The formulas contained no carbon chain (C) 20 or 22 n-6 or n-3 fatty acids. The sow milk had similar levels of 18:1n-9 (38%), but had lower 18:2n-6 (11%) and 18:3n-3 (1.1%) than the formulas (Table 1). Each formula was made without (−) and with (+) added cholesterol (0.52 mmol/L cholesteryl oleate + 0.13 mmol/L unesterified cholesterol). Other details of the sow milk and formula compositions have been previously reported.[29]

Table 1. Sow Milk and Formula Fatty Acid Composition

Parameter	Sow Milk	Palm Olein Formula		Synthesized-TG Formula	
		(−)	(+)	(−)	(+)
Fatty acid (% of total)					
12:0 + 14:0	3.8	9.1	9.1	10.1	9.9
16:0	30.5	23.3	23.3	22.4	22.6
18:0	9.4	4.7	4.7	4.1	4.1
18:1n-9	37.5	36.8	36.8	37.3	37.3
18:2n-6	11.1	20.3	20.3	20.0	20.0
18:3n-3	1.1	2.2	2.2	2.1	2.1
Cholesterol (mmol/L)	0.52	<0.10	0.65	<0.10	0.65
Position of 16:0*					
sn-2	60.4	6.3	6.3	47.0	47.0
sn-1,3	39.6	93.7	93.7	53.0	53.0

NOTE. (−) and (+) indicate formula without and with added cholesterol, respectively. Other details of the sow milk and formula composition have been described previously.[29] The formulas contained 58 g total fat, 56 g protein, and 62 g carbohydrate per liter.

*Percent of total 16:0 in *sn*-2 or *sn*-1,3 position of the milk or formula TG.

At 18 days of age, between 9:00 and 10:30 AM, the piglets were anesthetized with ketamine:rompun 37.5:3.75 mg/kg (MTC Pharmaceuticals, Cambridge, Ontario, and Bayvet Division, Chenango, Etobicoke, Ontario, Canada, respectively) by intramuscular injection. Blood samples were drawn by cardiac puncture using syringes rinsed with 0.4 mmol/L EDTA. The animals were killed by intracardiac injection of 1 mmol/L KCl. Plasma samples were prepared by centrifugation, and with the exception of aliquot samples for HDL cholesterol, the samples were frozen at −80°C. After laparotomy, the liver was removed, bile was drawn from the gallbladder, and the gallbladder was removed. The liver was then weighed and a portion was taken, immediately frozen in liquid nitrogen, and stored at −80°C until preparation of RNA. The abundance of LDL receptor, HMG-CoA reductase, and C7H mRNA was determined by reverse transcriptase–polymerase chain reaction (RT-PCR) analysis.[30] The remaining organ was homogenized in saline, and aliquots were stored at −80°C for later tissue lipid analysis.

All procedures involving the piglets were approved by the Animal Care Committee of the University of British Columbia and conformed to the guidelines of the Canadian Council on Animal Care.

Lipid Analysis

Plasma and liver total cholesterol and TG concentrations were determined using enzymatic reagents (Diagnostic Chemical, Charlottetown, Prince Edward Island, and Boehringer-Mannheim Diagnostics, Montreal, Quebec, Canada). Total lipids were extracted from the liver, and the aliquots were reconstituted in isopropanol and used for analysis of cholesterol and TG.[27] HDL cholesterol was determined in plasma following precipitation of apolipoprotein B (apo B)–containing lipoproteins with heparin–manganese chloride.[31] The amount of cholesterol associated with apo B–containing lipoproteins (chylomicron + LDL) was calculated as the difference between total and HDL cholesterol. Bile lipid was extracted using chloroform:methanol 1:2 (vol/vol) and exposed to fluorescent light overnight to permit photodegradation of biliary pigments before quantifying total cholesterol,[27] total bile acids,[32] and phospholipid.[33] Total protein was determined according to the method of Lowry et al[34] using bovine serum albumin as a standard. Plasma free fatty acid concentrations were determined using enzymatic reagents (Wako Chemicals, Richmond, VA). Plasma thyroid hormone, insulin, and glucagon concentrations were determined using radioimmunoassays (Incstar, Stillwater, MN, Immunocorp, Montreal, Quebec, Canada, and ICN Biochemicals, Costa Mesa, CA, respectively).

RT-PCR Quantitation of mRNA

The RT-PCR method[30] used to assess the levels of LDL receptor, HMG-CoA reductase, and C7H mRNA involved reverse-transcribing a known quantity of total RNA to generate a pool of cDNA representing the RNA in the original sample. The PCR was used to amplify cDNA corresponding to the mRNA of interest. The expression of β-actin for each sample was also determined, and this was used as an internal control for the efficiency of each RT-PCR. Intraassay and interassay variability between RT-PCR was less than 10% (see Table 5).

Total RNA was isolated from liver tissue using guanidine thiocyanate followed by cesium chloride density-gradient centrifugation.[35] RNA preparations were treated with 10 U RQ1 RNase-free DNase (Promega, Madison, WI) to ensure no genomic DNA contamination. Total RNA (2.5 μg) was used for first-strand cDNA synthesis using M-MLV reverse transcriptase (GIBCO BRL, Burlington, Canada) in 50 mmol/L Tris hydrochloride, 75 mmol/L KCl, 10 mmol/L DTT, 3 mmol/L $MgCl_2$, 2 mmol/L dNTPs (GIBCO BRL), and 10 U recombinant RNase inhibitor

Table 2. Plasma Lipid Concentrations (mmol/L) in Piglets Fed Sow Milk or Formula for 18 Days

Lipid	Sow Milk	Palm Olein Formula		Synthesized-TG Formula	
		(−)	(+)	(−)	(+)
Cholesterol					
Total*†	3.42 ± 0.42	2.16 ± 0.11	2.30 ± 0.09	1.93 ± 0.08	1.97 ± 0.08
HDL*	1.52 ± 0.13	1.00 ± 0.06	1.02 ± 0.04	0.89 ± 0.05	0.94 ± 0.06
Apo B	1.90 ± 0.29	1.16 ± 0.07	1.29 ± 0.08	1.04 ± 0.07	1.04 ± 0.04
TG*†	0.80 ± 0.12	0.37 ± 0.04	0.44 ± 0.6	0.28 ± 0.04	0.29 ± 0.04

NOTE. Data are the mean ± SEM; n = 6 per group. (−) and (+) indicate formula without and with added cholesterol, respectively.
*Sow-fed *v* formula-fed ($P < .05$).
†Significant effect of positional distribution of 16:0 in the formula TG ($P < .002$).

(Clontech, Palo Alto, CA) with 250 pmol random hexamers (GIBCO BRL) in a total volume of 25 μL. The reaction time was 10 minutes at 20°C followed by 60 minutes at 37°C. The resulting cDNA pool for each RNA sample was divided into aliquots (3 μL), with one aliquot used in each PCR. Separate PCRs were performed for LDL receptor, HMG-CoA reductase, C7H, and β-actin.

The PCR contained 20 mmol/L Tris hydrochloride, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.8 mmol/L dNTP (Gibco BRL), 12 pmol of each gene-specific primer, and 2.5 U Taq DNA Polymerase (Gibco BRL). The primer sequences used for each gene were as follows: LDL receptor,[36] *LDL-2* (forward) 5′-GACAACCCCGTCTATCAGAAGAC-3′ and *LDL-P* (reverse) 5′-GACCATCTGTCTCGAGGGGTAGG-3′, generating a 98-bp band; HMG-CoA reductase,[37] *HMG-L* (forward) 5′-ATTATGTGCTGCTTTGGCTGCATG-3′ and *HMG-R* (reverse) 5′-TTGAGGAGAAGGATCAGCTATCCA-3′, generating a 267-bp band; C7H,[38] *7AH-L* (forward) 5′-AATCCTCTTGAGTCCTCAGAGC-3′ and *7AH-R* (reverse) 5′-CCATCCATCGGGTCAATGCTTCT-3′, generating a 206-bp band; and β-actin,[39] *AC-2* (forward) 5′-TGATCCACATCTGCTGGAAGGTGG-3′ and *AC-3* (reverse) 5′-GGACCTGACTGACTACCTCATGAA-3′, generating a 524-bp band. The PCR amplification cycle was 94°C for 30 seconds followed by 65°C for 1.5 minutes. The number of cycles used for PCRs for LDL receptor, HMG-CoA reductase, C7H, and β-actin were gene-specific and were determined to be in the exponential phase of the amplification process.[30] The number of cycles for each gene was as follows: LDL receptor, 35; HMG-CoA reductase, 35; C7H, 35; and β-actin, 25. The PCRs (10 μL each) were resolved on a 1.5% agarose–1 × TBE gel and stained with ethidium bromide. A photograph was taken with Kodak TRI-X pan film (Eastman Kodak, Rochester, NY). The negatives were scanned with a video densitometer (model 620; Bio-Rad, Mississauga, Ontario, Canada) to determine the relative intensity of the bands, and this was expressed per microgram RNA used in the original RT reaction.

Statistical Analysis

An analysis to determine the effect of formula feeding in comparison to milk feeding from birth to 18 days of age was made with one-way ANOVA. Two-way ANOVA was used to examine the effect of the formula cholesterol content and the positional distribution of 16:0 in the formula TG. Formal tests of differences among groups used Fisher's least-square difference and were based on the least-square mean ± SE calculated from the ANOVA. All calculations were performed using the Number Cruncher Statistical System, version 5.1 (Kaysville, UT).

RESULTS

Plasma Lipids and Hormones in Milk- and Formula-Fed Piglets

The 18-day-old piglets fed milk had significantly higher ($P < .05$) plasma concentrations of total and HDL cholesterol and TG than piglets fed formula (Table 2). Inclusion of cholesterol in the palm olein and synthesized-TG formulas was associated with higher plasma total and HDL cholesterol and TG; however, the differences were not of statistical significance. The positional distribution of 16:0 in the formula TG, on the other hand, did significantly affect plasma total cholesterol and TG concentrations: piglets fed the palm olein formulas, with most of the 16:0 esterified to the TG *sn*-1,3 position, had significantly higher plasma cholesterol and TG concentrations than piglets fed the synthesized-TG formulas.

Plasma levels of total triiodothyronine (T_3), free T_3, glucose, insulin, and lactate were consistently higher and levels of total thyroxine (T_4) were consistently lower, although not significantly lower, in milk-fed than in formula-fed piglets. However, plasma free T_4 levels were significantly lower and glucagon and free fatty acid concentrations were higher ($P < .005$) than in milk-fed piglets (Table 3). Neither the TG fatty acid distribution nor the cholesterol content of the formula had any significant effect on plasma hormone or free fatty acid concentrations in formula-fed piglets.

Table 3. Plasma Thyroid Hormone, Insulin, Glucagon, Glucose, Free Fatty Acid, and Lactate Levels in Piglets Fed Milk and Formula for 18 Days

Parameter	Sow Milk	Palm Olein Formula		Synthesized-TG Formula	
		(−)	(+)	(−)	(+)
Thyroid hormones					
Total T_3 (nmol/L)	1.87 ± 0.4	1.28 ± 0.2	1.84 ± 0.1	1.66 ± 0.2	1.53 ± 0.1
Free T_3 (pmol/L)	1.83 ± 0.5	0.89 ± 0.2	1.34 ± 0.3	1.40 ± 0.2	1.09 ± 0.2
Total T_4 (nmol/L)	56.7 ± 3.5	58.0 ± 7.7	69.7 ± 7.8	71.4 ± 3.8	66.8 ± 8.8
Free T_4 (pmol/L)*	19.8 ± 1.9	26.1 ± 2.5	36.6 ± 3.3	41.4 ± 2.0	35.0 ± 4.0
Insulin (pmol/L)	65.1 ± 4.8	45.0 ± 7.1	55.7 ± 11	41.9 ± 4.2	53.3 ± 13
Glucagon (pmol/L)*	419 ± 52.2	162 ± 64.7†	249 ± 53.2[b]	344 ± 40.1[b]	324 ± 105.8[a]
Glucose (mmol/L)	7.0 ± 0.4	5.0 ± 0.3	5.4 ± 0.6	5.7 ± 0.5	5.6 ± 0.3
Free fatty acid (mEq/L)*	0.41 ± 0.04	0.11 ± 0.04	0.15 ± 0.04	0.08 ± 0.0	0.13 ± 0.0
Lactate (mmol/L)	3.0 ± 0.3	2.5 ± 0.4	3.1 ± 0.3	2.2 ± 0.4	2.6 ± 0.4

NOTE. Data presented as mean ± SEM, n = 6, except for glucagon where, [a]n = 3 and [b]n = 5. (−), (+) indicates formula without and with added cholesterol, respectively. *Values for piglets fed milk significantly different than values for piglets fed formula ($P < .05$).

Table 4. Liver and Bile Lipid Concentration in Piglets Fed Sow Milk or Formula for 18 Days

Parameter	Sow Milk	Palm Olein Formula		Synthesized-TG Formula	
		(−)	(+)	(−)	(+)
Liver (mmol/g protein)					
Total cholesterol	45.7 ± 3.2	41.3 ± 1.7	42.9 ± 1.1	36.5 ± 0.8	43.1 ± 2.2
TG*δ	43.8 ± 6.6	16.3 ± 1.9	18.6 ± 1.3	14.4 ± 1.2	21.3 ± 3.4
Bile (mmol/L)					
Bile acids*†	99.3 ± 11.2	56.3 ± 6.1	51.4 ± 6.4	27.2 ± 2.5	47.4 ± 5.6
Cholesterol*	3.6 ± 0.6	2.2 ± 0.5	2.5 ± 0.2	1.8 ± 0.3	1.7 ± 0.3
Phospholipid*	33.9 ± 5.8	18.9 ± 4.4	14.0 ± 0.8	9.7 ± 1.1	16.4 ± 1.3

NOTE. Data presented as the mean ± SEM, n = 6. (−), (+) indicates formula without and with added cholesterol, respectively.
*Values for sow-fed piglets significantly different from values for piglets fed formula ($P < .05$).
δSignificant effect of formula (+) cholesterol compared to formula (−) cholesterol ($P < .03$).
†Significant effect of positional distribution of 16:0 in the formula triglyceride ($P < .01$).

Effects of Formula and Milk Feeding on Hepatic and Bile Lipid Composition

The piglets fed milk had significantly ($P < .05$) higher hepatic TG, but not total cholesterol, concentrations and higher ($P < .05$) concentrations of bile acids, total cholesterol, and phospholipid in bile than piglets fed formula (Table 4). Addition of cholesterol to the formulas resulted in a significant increase in liver total cholesterol and TG concentrations but had no significant effect on bile cholesterol, phospholipid, or bile acid concentrations in formula-fed piglets. However, piglets fed the synthesized-TG formulas had significantly lower concentrations of bile acids in the bile than piglets fed palm olein formulas.

Effects of Formula and Milk Feeding on Hepatic LDL Receptor, HMG-CoA Reductase, and C7H RNA

The integrity of the total RNA isolated from the piglet liver is shown in Fig 1. A RT-PCR assay was developed and used to determine mRNA levels for HMG-CoA reductase, LDL receptor, and C7H, because of the greater degree of sensitivity and accuracy afforded with this method compared with Northern blot hybridization.[30,40] The interassay and intraassay variability and examples of each RT-PCR for each of the genes studied are shown in Table 5 and Fig 2, respectively. LDL receptor mRNA levels were similar in milk-fed and formula-fed piglets (Fig 3). The levels of HMG-CoA reductase mRNA and C7H mRNA in milk-fed piglets, in contrast, were significantly lower than in formula-fed piglets. Comparisons within groups of piglets fed the different formulas showed no statistically significant effect of the formula cholesterol content or TG fatty acid distribution on HMG-CoA reductase or C7H mRNA levels.

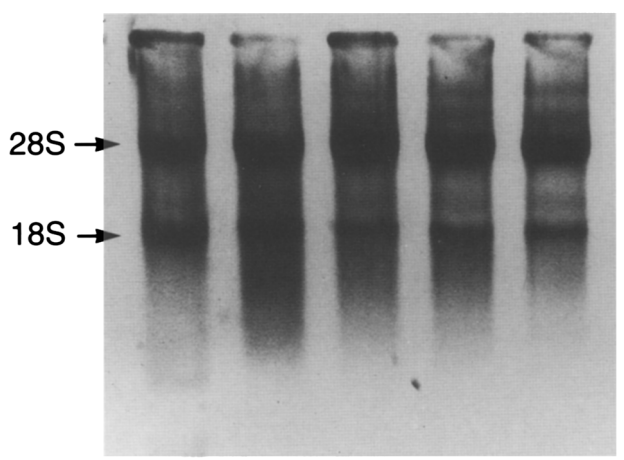

Fig 1. Representative photograph illustrating the integrity of total RNA isolated from the liver of piglets fed milk or formula for 18 days. Five micrograms of total RNA was resolved on a 1.5% agarose 1× TBE gel and stained with ethidium bromide. Ribosomal bands are marked. Lane 1, milk; lane 2, palm olein (−); lane 3, palm olein (+); lane 4, synthesized TG (−); lane 5, synthesized TG (+).

DISCUSSION

The results of this study show that despite large differences in plasma cholesterol concentrations, the level of hepatic LDL receptor mRNA measured using a RT-PCR assay was not different among piglets fed milk and piglets fed formula. Consideration of potential differences in expression of the hepatic LDL receptor appeared reasonable because it is estimated that about 70% of plasma LDL clearance occurs via LDL receptors, with 80% to 90% of this clearance via the liver.[41,42] Some in vitro studies have found changes in LDL receptor activity despite no change in protein mass, mRNA relative abundance, or rates of transcription of the LDL receptor gene.[43] This suggests the possibility that developmental or diet-induced differences in LDL receptor activity could occur due to regulation at a posttranslational level. However, previous studies with piglets fed formula and milk also found no difference in LDL receptor protein mass,[4] but whether LDL receptor activity changes with the type of diet fed in early life was not measured.

The finding of no difference in LDL receptor mRNA between piglets fed milk and those fed formula is in contrast to results of studies showing that high dietary cholesterol intake in adult and young (weaned) animals is accompanied by high plasma cholesterol together with a decreased receptor-mediated LDL fractional catabolic rate and decreased LDL receptor mRNA relative abundance.[12,15,41] Downregulation of LDL receptor activity is believed to result from a negative-feedback effect of increased hepatic cholesterol concentration on LDL receptor

Table 5. RT-PCR Intraassay and Interassay Variability

Gene	Intraassay Variability		Interassay Variability	
	Mean ± SEM*	%	Mean ± SEM*	%
β-Actin				
HMG-CoA reductase	241.8 ± 4.2	1.7	244.2 ± 1.7	1.0
LDL receptor	242.7 ± 7.4	3.1	229.4 ± 10.8	4.7
C7H	237.7 ± 5.3	2.2	238.8 ± 8.6	3.6

*Absorbance units per μg RNA.

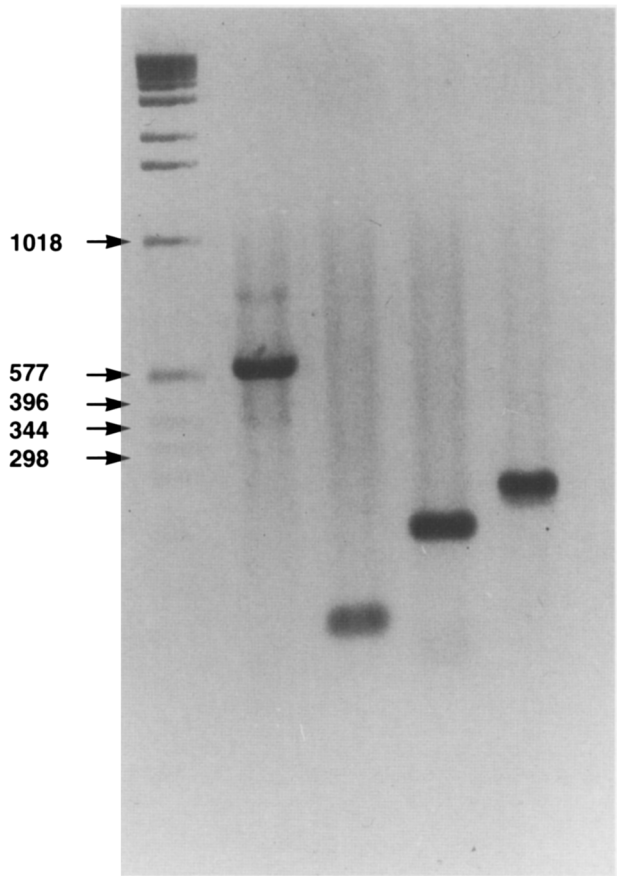

Fig 2. A representative photograph of examples of RT-PCR products obtained for each gene analyzed on a 1.5% agarose 1× TBE gel stained with ethidium bromide. Each lane contains 10 μL of each PCR. Lane 1, ladder; lane 2, β-actin; lane 3, LDL receptor; lane 4, C7H: lane 5, HMG-CoA reductase.

gene expression.[18] Large differences in hepatic cholesterol concentrations were not found between milk- and formula-fed piglets, although plasma cholesterol was about 28% to 70% higher in piglets fed milk than in those fed formula. Possibly, the absence of a sustained and different effect of the milk and formula diets on hepatic cholesterol concentrations explains the lack of difference in LDL receptor mRNA levels. It is also possible that low hepatic LDL receptor clearance, involving low hepatic expression of the LDL receptor gene at birth, could limit detection of further diet-induced negative feedback on LDL receptor gene expression. This may explain the postnatal increase in plasma cholesterol in response to the high-fat milk and formula diets.

The studies reported here clearly show that hepatic HMG-CoA reductase mRNA levels were higher in formula-fed piglets, irrespective of the formula cholesterol content, than in piglets fed milk. In agreement with this, previous studies have shown lower hepatic HMG-CoA reductase activity in piglets fed milk versus formula.[4] Also consistent with this, studies in human infants have found lower cholesterol fractional synthetic rates in breast-fed than in formula-fed infants.[8] These studies with piglets and infants are again in contrast to the results of studies showing that a high-fat and cholesterol diet downregulates cholesterol synthesis and LDL receptor clearance of plasma LDL.[11-14] However, discoordinate regulation of LDL receptor and HMG-CoA reductase activity with decreased cholesterol synthesis but no change in LDL receptor activity has been reported for rats fed a diet supplemented with cholesterol and saturated fat.[44] It is not clear why addition of cholesterol to the formula did not increase plasma cholesterol in the formula-fed piglets in these studies. However, some recent studies in infants

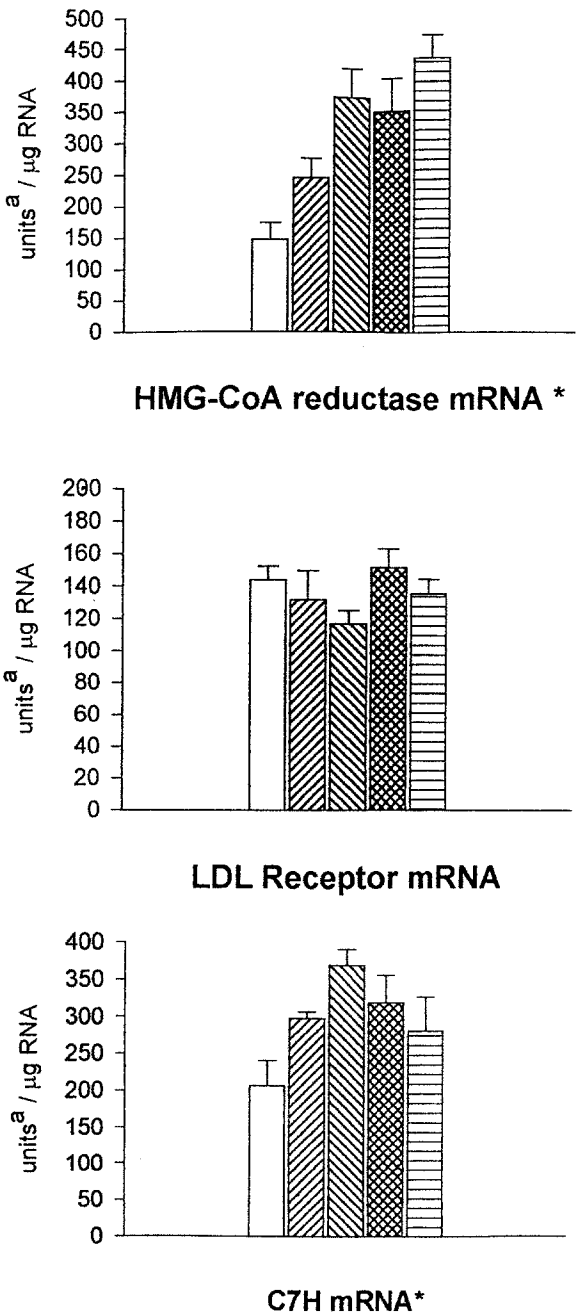

Fig 3. Hepatic HMG-CoA reductase, LDL receptor, and C7H mRNA levels. Data are the mean ± SEM (n = 6, except for synthesized TG (−) and (+), n = 4). *Milk-fed *v* formula-fed ($P < .01$). [a]densitometric units per μg RNA. (□) Milk; (▨) palm olein (−); (▧) palm olein (+); (▩) synthesized TG (−); (▤) synthesized TG (+).

have also reported that addition of cholesterol to the formula did not increase serum cholesterol or reduce cholesterol fractional synthetic rates.[45] Future studies should consider if the absorption of cholesterol added to the formula is equivalent to the absorption of cholesterol from milk fat, or if the ability to maintain plasma cholesterol homeostasis in response to increased dietary cholesterol intake during development is influenced by other factors in milk that are absent from formula.

Studies by others have shown that mRNA levels reflect the activity of C7H.[11] Results of the studies show lower levels of C7H mRNA in the liver of milk-fed versus formula-fed piglets, suggesting that C7H activity is reduced by milk feeding. This interpretation is supported by information showing that CH7 activity is low in the suckling rat.[10] The lower hepatic HMG-CoA reductase and C7H mRNA levels in milk-fed versus formula-fed piglets was accompanied by higher cholesterol, phospholipid, and bile acid concentrations in bile. A 25% lower rate of bile acid synthesis and lower bile acid excretion have been reported for baboons who were breast-fed rather than fed formula with or without cholesterol as infants.[46,47] Bile acids are known to regulate C7H expression at the level of transcription via a negative-feedback mechanism,[19] and have also been shown to decrease both HMG-CoA reductase and C7H activities, rates of transcription, and mRNA levels.[11,20,21] Possibly, lower rates of bile secretion or increased intestinal bile acid and cholesterol absorption could explain the lower HMG-CoA reductase and C7H mRNA and higher bile cholesterol and bile acid concentrations in mild-fed versus formula-fed animals. However, thyroid hormone has also been shown to stimulate expression of C7H.[25] It seems possible, then, that the higher levels of serum free T_4 in formula-fed versus milk-fed piglets could also be involved in the differences in C7H mRNA levels. However, differences in other hormones or growth factors could also be involved.

The absence of significant differences in LDL receptor, HMG-CoA reductase, and C7H mRNA levels between piglets fed the palm olein and synthesized-TG formulas suggests that the positional distribution of 16:0 in milk does not explain the large differences in HMG-CoA reductase and C7H mRNA between milk-fed and formula-fed piglets. Plasma cholesterol and the concentration of bile acids were lower in piglets fed formula with synthesized TG rather than palm olein. The mechanism by which the positional distribution of 16:0 in dietary TG influences plasma cholesterol and bile acid metabolism in piglets is not known. However, previous studies have reported that the positional distribution of fatty acids in plasma TG influences the rate of clearance via lipoprotein lipase, and possibly the rate and composition of remnant particles cleared by the liver.[48,49] A relationship between the amount of 16:0 in the 2-position of dietary TG and plasma saturated (16:0) cholesterol esters has also been reported.[29]

In summary, this study appears to be the first to show that formula feeding alters the levels of HMG-CoA reductase and C7H mRNA in piglets by a mechanism apparently unrelated to the proportion of dietary energy from fat or dietary cholesterol intake. The reason(s) for the lower levels of HMG-CoA reductase and C7H mRNA in piglets fed milk rather than formula with a similar content of fat, 16:0, and cholesterol is unknown. Direct or indirect effects of other milk components absent from the formula could be involved. Whatever the reason, these findings indicate that the increase in plasma cholesterol after birth with milk feeding is not due to increased hepatic cholesterol synthesis, and may not involve decreased hepatic LDL receptor–mediated clearance. Studies in other species have reported lasting effects of early diet–related changes in cholesterol metabolism.[3,46] It would seem worthwhile to consider if early diet–related differences in gene expression persist beyond infancy into adulthood in piglets as well.

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