Alteration of enzymes of hepatic glycerolipid synthesis in artificially reared rat pups fed high carbohydrate and high fat diets

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To study the effect of dietary modifications on hepatic microsomal enzyme activities of glycerolipid synthesis, rat pups were artificially reared on high fat (HF) or high carbohydrate (HC) formulas. The high fat diets contained both long and medium-chain triacylglycerols (HF-LCT/MCT) or medium chain triacylglycerols alone (HF-MCT) and were similar to rat milk with regard to calories derived from fat and carbohydrate during the suckling period. The formula high in carbohydrate provided 56% of calories from carbohydrate compared with 8% in rat milk. Activities of diacylglycerol acyltransferase, fatty-acid-CoA ligase, and monoacylglycerol acyltransferase in livers of 12-day old rats fed the HC formula decreased about 40%, 60%, and 75%, respectively. No significant change was observed in glycerol-P acyltransferase activity, the committed step of the glycerol-P pathway of triacylglycerol and phospholipid biosynthesis. When the HF-LCT/MCT or the HF-MCT formula was fed to artificially reared pups, diacylglycerol acyltransferase and fatty-acid CoA ligase activities did not change in livers of 12-day old rats, indicating that changes in enzyme activity were not caused by the artificial rearing procedure itself. In contrast, the developmentally expressed monoacylglycerol acyltransferase activity decreased about 50%, 70%, and 75% in rat pups fed the HF-LCT/MCT diet, HF-MCT diet, and HC diet, respectively. These results suggest that macronutrients in the diet influence the developmental patterns of hepatic enzymes of triacylglycerol synthesis pathway in the immediate postnatal period differentially.

Keywords: artificial rearing; glycerolipid biosynthesis; suckling rats; enzyme ontogeny; high-carbohydrate formula; medium-chain triacylglycerol

Introduction

The developing rat pup undergoes major changes in energy metabolism during the first 3 postnatal weeks. At birth the primary source of calories changes abruptly from maternally supplied glucose to milk triacylglycerol. During the first 2 weeks of the suckling period, milk triacylglycerol supplies approximately 70% of dietary calories, but after a gradual weaning transition during the third week, about 70% of the caloric intake is once again supplied from carbohydrate.[1,2] During the suckling period, 10-fold increases occur in enzymes of fatty acid oxidation and ketogenesis,[3-5] and gluconeogenesis.[6] In contrast, enzyme activities required for fatty acid and cholesterol synthesis decrease markedly from their high prenatal levels.[7,8]

In order to elucidate the processes that control neonatal triacylglycerol and phospholipid synthesis, we initiated a series of studies on the microsomal enzymes of glycerolipid synthesis in suckling rat liver. We have reported[9] that most of the microsomal enzymes of the glycerol-P pathway of triacylglycerol and phospholipid synthesis follow the developmental pat-

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tern typical of the late-fetal cluster of enzymes as outlined by Greengard.[10] Thus, each activity increases from the low levels present during the last 4 days before birth to near adult levels during the first postnatal week.[9]

Unlike the adult liver, liver from the late fetal and the suckling periods expresses an alternate pathway of diacylglycerol synthesis, the monoacylglycerol pathway.[9] In contrast to other hepatic enzymes in which ontogeny has been studied, the monoacylglycerol acyltransferase specific activity rises from low levels just before birth to high levels that peak during the second week after birth. The activity then declines during the third and fourth weeks, becoming virtually unmeasurable in the adult rat.[9] The specific activity of monoacylglycerol acyltransferase is more than 700-fold higher in the neonate than in the adult.[9] Because the developmental pattern of monoacylglycerol acyltransferase activity suggested that its expression might be regulated by the high triacylglycerol and low carbohydrate content of the rat-milk diet, we reared rat pups artificially on various formulas to determine whether nutrition might play a role in regulation. We also measured three other critical microsomal enzymes of glycerolipid synthesis: fatty acid-CoA ligase, an essential step in the activation of fatty acids that are destined for both glycerolipid synthesis and for β-oxidation, glycerol-P acyltransferase, the committed step of the glycerol-P pathway, and diacylglycerol acyltransferase, the activity unique to triacylglycerol synthesis.

Methods

Materials

Bovine serum albumin (essentially fatty acid free) and L-α-glycerol 3-phosphate were from Sigma Chemical Co. (St. Louis, MO, USA). Phosphatidylcholine, phosphatidylserine, *sn*-2-monooleoylglycerol, and *sn*-1,2-dioleoylglycerol were from Serdary Research Labs (London, Ontario, Canada). Palmitoyl-CoA was from P.L. Biochemicals (Milwaukee, WI, USA). (9,10-^{3}H)Palmitate, (1-^{14}C)palmitate, and (2-^{3}H)glycerol were from New England Nuclear (Boston, MA, USA). (^{3}H)Palmitoyl-CoA[11] and (^{3}H)glycerol 3-P[12] were synthesized by previously reported methods.

Animals and artificial rearing

Pups born to Sprague-Dawley rats (Zivic Miller Laboratories, Pittsburgh, PA, USA) were maintained with their dams until the time of cannulation. On the fourth postnatal day, rat pups were cannulated intragastrically under light ether anesthesia and were than raised in isolation from their dams using an artificial rearing system.[13,14] Control littermates in groups of eight to ten were reared by their dams. Cannulated pups were individually housed in styrofoam cups floating on a temperature-regulated water bath at 37° C. Twice daily pups were stroked to promote urination and defecation. They were cleaned and weighed daily. Using a syringe pump (Harvard apparatus, South Natick, MA, USA) rat milk-substitute formulas were delivered for 20–25-min periods every 2 hr at a rate that was adjusted to provide 0.45 kcal/g body weight per day.[14]

Diets and feeding protocol

Rat milk-substitute formulas were prepared as described previously[14] and stored frozen. After thawing, the formulas were kept refrigerated and used within 48 hr. For the developmental studies during the preweaning period, the high-carbohydrate (HC) formula (calorically) consisted of 20% fat, 24% protein, and 56% carbohydrate and contained skimmed evaporated cow milk (375 mL, Carnation, Los Angeles, CA, USA), hydrolysed casein (21.2 g), L-methionine (500 mg), corn oil (17 g, Best Foods, Englewood Cliffs, NJ, USA), sodium deoxycholate (85 mg), cholesterol (40 mg), vitamin supplement (185 mg consisting of 1.65 mg thiamin, 13.35 mg riboflavin, 80 mg vitamin C, 10.52 mg vitamin B6, 37 μg vitamin B12, 5 mg vitamin K, 60 IU vitamin E, 0.375 mg biotin, 10 mg p-aminobenzoic acid, 33 mg niacin, 500 μg folicin, and 16.8 mg pantothenic acid), 13.5 mg $FeSO_4.7H_2O$, 7.5 mg $CuSO_4.5H_2O$, 8.0 mg $ZnSO_4.7H_2O$, 750 μL linoleic acid, 67.3 g Polycose (Ross Laboratories, Columbus, OH, USA) and distilled water to a final volume of 500 mL.[15]

In a separate set of experiments designed to study the effect of dietary fat on hepatic enzyme activities, the preparation of milk formulas was slightly modified to maintain a low level of carbohydrate (8% of calories) in the formula. The composition of three milk formulas (high-fat both long and medium chain triacylglycerols, high-fat medium chain triacylglycerols, high carbohydrate, [HF-LCT/MCT, HF-MCT, and HC]) used in this set of experiments is given in *Table 1*. The caloric composition of the HC formula was similar to the first set of experiments. The milk formulas throughout these studies were isocaloric and isonitrogenous with rat milk.[16]

Enzyme assays

After the rat pups were killed by decapitation, the livers were rapidly removed and homogenized in five volumes of ice-cold buffer (250 mmol/L sucrose, 10 mmol/L Tris, pH 7.4, 1 mmol/L EDTA, 1mmol/L dithiothreitol). The homogenates were centrifuged at 12,000*g* for 20 min and the supernatant was then centrifuged at 100,000*g* for 60 min at 4° C. The pellets were rinsed with the homogenizing buffer to remove traces of cytosol, resuspended in 1 mL of the same buffer, and frozen in small aliquots at −70° C. Monoacylglycerol acyltransferase activity was measured with 25 μmol/L (^{3}H)palmitoyl-CoA and 50 μmol/L *sn*-2-monooleoylglycerol as previously described.[9] An aliquot of the heptane-soluble product was chromatographed on silica gel G thin layer plates in heptane/isopropyl ether/acetic acid (60:40:4, vol/vol) with standards to identify the percent of the label in the diacylglycerol and triacylglycerol products. Specific activity was calculated by adding the counts in diacylglycerol plus one-half of the counts in triacylglycerol. Typically, less than 18% of the total label was found as triacylglycerol. Diacylglycercol acyltransferase was measured with 30 μmol/L (^{3}H)palmitoyl-CoA and 200 μmol/L *sn*-1,2-dioleoylglycerol.[17] Fatty acid-CoA ligase was measured using 50 μmol/L (^{3}H)palmitate.[18] Glycerol-P acyltransferase was measured using 112.5 μmol/L palmitoyl-CoA, and 300 μmol/L (^{3}H)glycerol 3-P.[12] Enzyme activities are expressed as nmol of substrate used or product formed per min per mg of microsomal protein. Protein was determined by the method of Lowry et al.[19]

Thyroid hormone

After the rats were decapitated, trunk blood was collected in a solution (200 μL) containing trasylol (10 IU), 1 mmol/L EDTA, and 0.15 M NaCl and centrifuged in a microfuge for 5 min. The

Table 1 Composition of rat milk-substitute formulas (per 100 mL)

Ingredient	Rat milk*		HF(LCT + MCT)		HF(MCT)		HC	
		(% Cal)		(% Cal)		(% Cal)		(% Cal)
Carbohydrate (g)	3.16	(8)	3.16[a]	(8)	3.16[a]	(8)	22.1[a,b]	(56)
Protein (g)	9.36	(24)	9.36[c]	(24)	9.36[c]	(24)	9.36[c]	(24)
Lipid (g)	12.03	(68)	11.9[d]	(68)	11.9[e]	(68)	3.47[f]	(20)
Minerals[g] (g)			2.4		2.4		2.4	
Vitamins[h] (mg)			175		175		175	
Riboflavin[i] (mg)			0.6		0.6		0.6	
Choline chloride (mg)			50		50		50	
Calcium gluconate[i] (mg)			200		200		200	
Cholesterol (mg)			40		40		40	
L-Carnitine[i] (mg)			4		4		4	
Water			q.s.		q.s.		q.s.	
Kcal/mL			1.57		1.57		1.57	

[a] 1.31 g lactose + 0.6 maltodextrins from mineral mix + 0.17 g sucrose from vitamin mix, 1.06 g carbohydrate from Nutrisource protein (Sandoz Nutritional Corp., Minneapolis, MN, USA).
[b] 18.94 g Polycose, Ross Laboratories, Columbus, OH, USA.
[c] Nutrisource protein.
[d] 3.18 g MCT + 7.83 g corn oil + 0.874 g lipids from Nutrisource protein.
[e] 10.3 g MCT + 0.708 g linoleic acid + 0.874 g lipids from Nutrisource protein.
[f] 0.203 g medium chain triacylglycerol (MCT) (Nutrisource lipid) + 2.39 g corn oil + 0.874 g lipids from Nutrisource protein.
[g] Nutrisource minerals.
[h] Vitamin mixture (AIN-76, ICN Biochemicals, Cleveland, OH, USA).
[i] ICN; added as 2 mL of 10% solution.
* Taken from (16).

supernatants were kept frozen at −70° C until assayed. Two to three plasma samples were combined to obtain eight separate determinations. The radioimmune assay was performed with a kit from Becton-Dickinson, Paramus, NJ, USA.

Statistical Analysis

Results are presented as the means ± SD for groups of six to eight animals. The significance of differences between groups was determined by Student's *t* test or by analysis of variance (ANOVA).

Results and Discussion

Artificially reared rat pups were fed a HC formula containing 20% and 56% of total available calories from fat and carbohydrate, respectively, while the mother-reared pups consumed rat milk containing 8% and 68% calories from carbohydrate and fat, respectively.[1,2] The rate of body weight gain was identical in both groups during the 18-day study. The body weights of mother-fed and artificially reared HC pups were, respectively, 42.0 ± 1.2 g and 42.4 ± 1.1 g on day 18. We previously reported that liver protein and DNA content did not differ significantly.[15]

In the mother-reared control pups, the ontogeny and the specific activity of hepatic monoacylglycerol acyltransferase was similar to that previously reported (*Figure 1*).[9] Pups that were fed the HC formula showed a blunted rise in activity. Although the activity peaked on day 8, it declined rapidly to levels that were only 23% of control by day 12. Mixing studies showed that the activities from the mother-reared and the artificially reared pups were additive, eliminating the possibility that the changes were due to the presence of activators or inhibitors in the preparations. On day 12 the specific activity of this enzyme in liver from rats fed the HC formula was similar to the activity previously observed in mother-reared rats on the postnatal day 21.[9] The profound decrease observed in monoacylglycerol acyltransferase activity during artificial rearing on the HC formula suggests that the fat and carbohydrate composition of the diet or the secondary changes in plasma hormone concentrations may play a major role in regulating monoacylglycerol acyltransferase activity.

Three other critical hepatic enzyme activities of triacylglycerol synthesis were also measured in control and artificially reared rat pups. No difference was observed in glycerol-P acyltransferase activity, the committed step of the glycerolipid synthetic pathway (data not shown). Glycerol-P acyltransferase has a dual cellular location in microsomes and mitochondrial outer membrane.[20,21] Although both isoenzymes can catalyze the first reaction in the glycerolipid synthetic pathway, it is not known whether the isoenzymes differ in their specific functions. The lack of effect of the HC diet on the postnatal developmental profile of microsomal glycerol-P acyltransferase activity is consistent with an ongoing requirement for phospholipid biosynthesis and a continued flux of precursors into the pathway. In earlier studies, microsomal glycerol-P acyltransferase activity increased about 70-fold in liver during the fetal-to-suckling transition[17] and also during the differentiation of 3T3-L1 adipocytes[22] suggesting a role for the microsomal isoenzyme in the synthesis of triacylglycerol. In contrast, in the artifi-

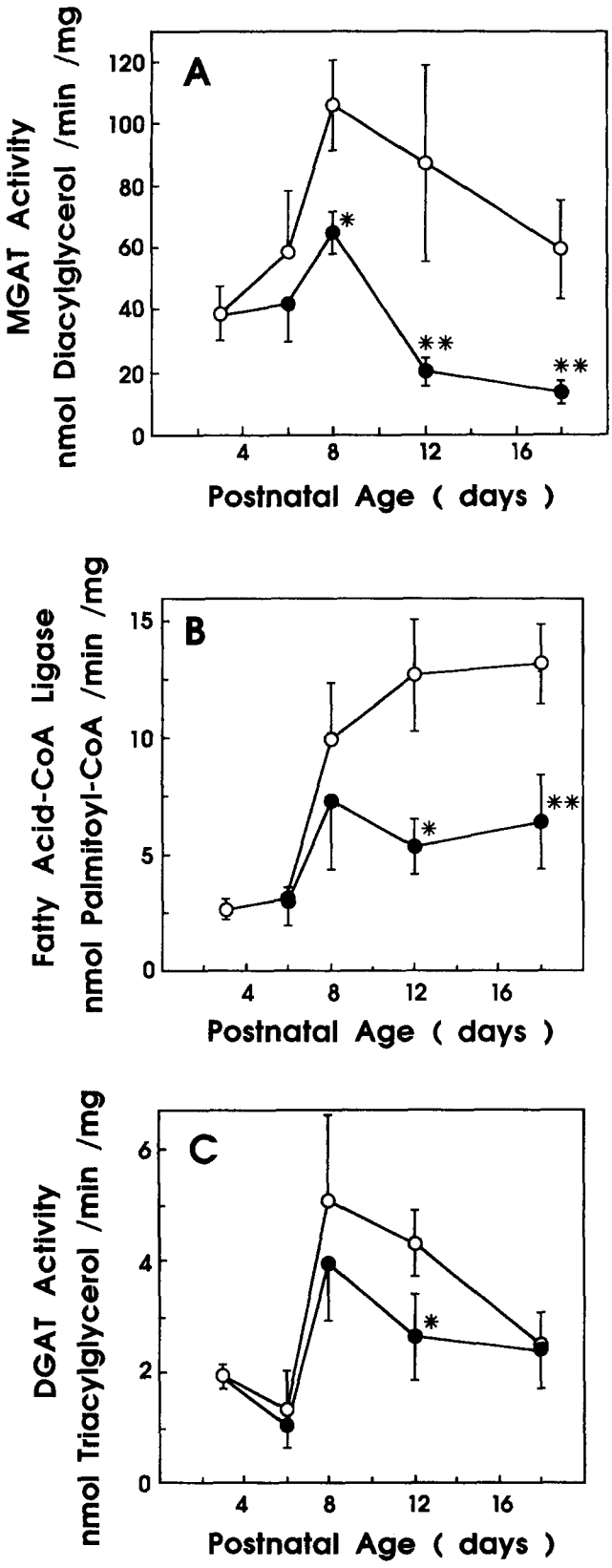

Figure 1 Hepatic monoacylglycerol acyltransferase (MGAT) activity (A), fatty acid-CoA ligase (B), and diacylglycerol acyltransferase (DGAT) activity (C) in pups artificially reared on a HC formula (●) and in mother-reared pups (○). Results are plotted as the means ± SD for six to eight animals per experimental point. *indicates $P < 0.05$; **indicates $P < 0.005$ by student's t test.

cially reared pups fed HC formula, monoacylglycerol acyltransferase activity decreased markedly while glycerol-P acyltransferase activity remained unaffected; this difference suggests that the high monoacylglycerol acyltransferase activity in neonatal liver may play a role that is unrelated to phospholipid biosynthesis.

As observed previously,[17] fatty acid-CoA ligase specific activity in the mother-reared control pups rose 4.8-fold to reach near-adult levels by postnatal day 12 (*Figure 1B*). In contrast, pups which had been fed the HC formula from day 4 showed a blunted increase: fatty acid-CoA ligase activity on day 12 was only 42% of that observed in the mother-reared animals. When day-12 samples were mixed, the activities were additive, thereby eliminating the possibility that these results were due to the presence of an inhibitor in the preparations. Long-chain fatty acid-CoA ligase activates fatty acids before they can be used for glycerolipid synthesis or for β-oxidation. Because the artificially reared pups gained weight normally[15] and because the liver protein content of similarly reared pups was normal, impairment of hepatic membrane synthesis seems unlikely. Thus, the decrease in fatty acid-CoA ligase activity suggests that the HC formula may have altered an activity that is specifically related to fatty acid oxidation.

In an earlier study,[17] the specific activity of the hepatic diacylglycerol acyltransferase on the postnatal day 7 was two-fold higher than observed in the adult. This observation was confirmed and extended in the mother-reared control pups: diacylglycerol acyltransferase specific activity peaked between postnatal days 8 and 12 and then declined, reaching the adult value by day 18 (*Figure 1C*). When artificially reared pups were fed the HC formula, diacylglycerol acyltransferase activity on day 12 was significantly reduced (39%) compared with the control animals. Mixing experiments showed that the activities were additive, and thus were not due to the presence of an inhibitor or activator in the preparations. By day 18, the activities in the mother-reared and high carbohydrate groups were similar, suggesting that the postnatal development of diacylglycerol acyltransferase activity is influenced by the HC diet. This result suggests that in mother-reared pups the normal postnatal increase in diacylglycerol acyltransferase activity depends on the high-fat diet. Whether this increase is regulated by diet-associated changes in plasma hormone levels remains to be determined.

To make certain that the changes observed in the critical enzymes of triacylglycerol synthesis did not result merely through some factor related to the artificial-rearing technique itself, three groups of rat pups were artificially nourished from day 4 until day 12 after birth, when maximal changes had been observed previously. Mother-reared rats were compared with rats that were artificially reared on a HC formula or on one of two different high fat (HF) formulas. In one high fat formula (HF-LCT/MCT), 48% of the calories were from corn oil (LCT) and 20% of the calories were

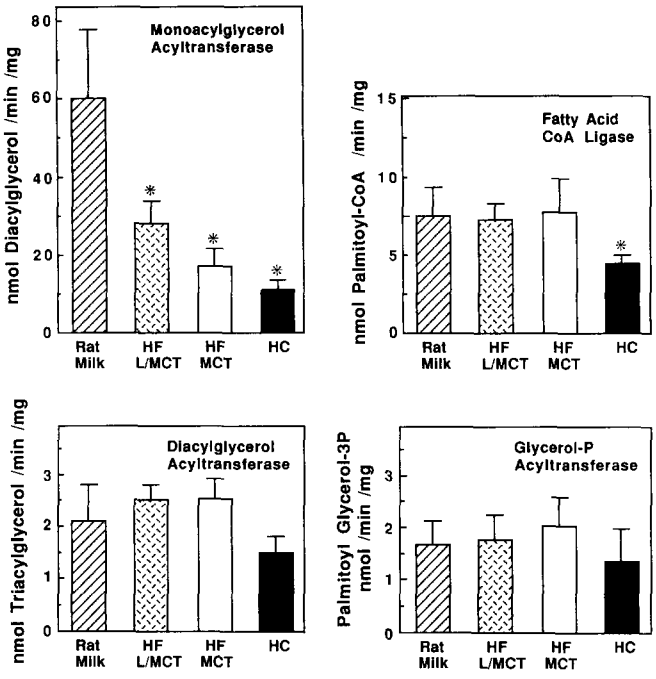

Figure 2 Hepatic fatty acid-CoA ligase, diacylglycerol acyltransferase, monoacylglycerol acyltransferase, and glycerol-P acyltransferase activities in pups that were artificially reared on HF-LCT/MCT, HF-MCT, and HC diets and in mother-reared pups. Results are the means ± SD for six to eight animals per experimental point. *indicates $P < 0.05$ by ANOVA. The results were virtually identical in a repeat study that compared mother-reared pups with pups reared artificially on HF-LCT/MCT or HF-MCT diet.

from medium chain triacylglycerol (MCT) (*Table 1*); in the second high fat formula (HF-MCT), 63.8% of the calories were from MCT and 4.2% of calories were from the linoleic acid. In this study, the growth of the pups was similar in all four groups.

Neither fatty acid-CoA ligase nor diacylglycerol acyltransferase activity was affected by either high fat formula, consistent with the conclusion that the artificial rearing procedure itself did not affect the levels of these two enzyme activities (*Figure 2*). As has been described above (*Figure 1*), the HC feeding caused these activities to decrease 41% and 29%, respectively, although the decrease in diacylglycerol acyltransferase did not reach statistical significance in this study (see *Figures 1B and C*). Again, none of the diets significantly altered total glycerol-P acyltransferase activity.

Previous studies of artificially reared rats have shown that the high carbohydrate formula results in two- to three-fold increases in plasma insulin concentrations.[15] It is likely that these altered insulin levels play a role in regulating the precocious induction of hepatic glucokinase and malic enzyme activities that were observed previously[15] and in postnatal changes in the activities of diacylglycerol acyltransferase, monoacylglycerol acyltransferase, and fatty acid-CoA ligase activities observed in this report. Since our preliminary data on the plasma free T4 levels in these four groups of pups show no alterations (average of 1.0 + 0.31 ηg/mL for all groups), thyroid hormone appears not to play a role in regulating the observed changes in the microsomal glycerolipid synthetic activities.

Activity of the monoacylglycerol acyltransferase is markedly decreased by both HC formula and HF formulas (*Figure 2*). These decreases are unlikely to have resulted from the artificial-rearing technique itself, because artificial rearing did not affect the other microsomal activities of glycerolipid synthesis. Unlike the early and transient increase that had been observed in hexokinase activity in response to both high fat and high carbohydrate diets,[15] the decrease in monoacylglycerol acyltransferase activity was measured eight days after the artificial rearing procedure was begun, a point at which hexokinase activity was identical in the experimental and control groups.[14]

Since the HF-MCT and HF-LCT/MCT formulas caused about 70% and 50% reductions, respectively, in monoacylglycerol acyltransferase activity, it appears that the normal high postnatal level of monoacylglycerol acyltransferase activity does not depend solely on the percent of dietary calories derived from fat or on the plasma insulin concentrations in the fat-fed pups. It is possible that the changes in metabolites in the liver and/or the alteration in plasma hormonal milieu under these dietary manipulations may play a role in regulating monoacylglycerol acyltransferase activity during development. For instance, in rat milk, medium-chain fatty acids are found only in the *sn*-3 position of triacylglycerol and comprise 35% of the total fatty acids.[23] After hydrolysis by gastric and pancreatic lipases, these medium chain fatty acids are absorbed directly into the portal vein, and enter the liver where they are further metabolized.[1] The commercial MCT that was used in this study, however, contains medium-chain fatty acids in all three positions; thus, hydrolysis of the *sn*-3 fatty acid releases a water-soluble, medium-chain *sn*-1,2-diacylglycerol. Medium chain diacylglycerol can activate protein kinase C[24] and thereby mediate a wide variety of cellular changes including cell proliferation and gene transcription.[25] If medium chain diacylglycerols are absorbed intact, activation of protein kinase C within hormone-secreting cells or within hepatocytes might lead to altered enzyme expression. Further studies will be required to determine the cause of the changes observed in glycerolipid enzyme activities during the postnatal period when the diet is modified.

Abbreviations

HF	high fat
HC	high carbohydrate
LCT	long chain triacylglycerol
MCT	medium chain triacylglycerol

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