Alterations in hepatic lipogenic capacity in rat pups artificially reared on a milk-substitute formula high in carbohydrate or medium-chain triacylglycerides

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The effects of rat milk-substitute formulas that are high in carbohydrates or medium-chain triglycerides were evaluated in artificially reared rat pups by measuring hepatic lipid synthesis and two lipogenic enzymes. Four-day-old pups were fed one of three isocaloric formulas: high-carbohydrate formula (HC), high fat formula containing both long- and medium-chain triglycerides (HF/LCT+MCT), or high fat formula containing medium-chain triglycerides (HF/MCT). Compared with age-matched mother-fed control pups, pups reared artificially on HC formula had significantly enhanced in vitro lipogenic capacity as well as higher activities of hepatic fatty acid synthase and glucose-6-phosphate dehydrogenase from postnatal days 6–18. The artificial rearing procedure per se had no effect on hepatic lipogenic capacity because there was no difference between pups reared on the HF/LCT+MCT formula and mother-fed controls in any parameters measured. There was, however, a significant increase in hepatic lipogenic capacity in pups reared on the HC or HF/MCT formula. Plasma insulin levels were increased significantly in the HC group on day 12 compared with the other three groups, suggesting that hyperinsulinemia is not a necessary precondition for elevating hepatic lipogenesis during the suckling period. The mechanism responsible for increased lipogenic capacity in livers of the HF/MCT group appears to be a low level of polyunsaturated fatty acids in the diet. The lack of difference in body weights of 12-day-old pups reared artificially on any of these three formulas indicates that the total amount, rather than type of calories, is important in determining weight gain at this early postnatal age.

Keywords: rat neonates; artificial rearing; milk-substitute formulas; medium-chain triacylglycerides; hepatic lipogenesis; plasma insulin

Introduction

The capacity to synthesize lipids from glucose in the liver and other tissues of the mammalian fetus develops before birth because the transfer of free fatty acids from maternal circulation to the fetus via the placenta is insufficient to meet the lipid requirements of the rapidly growing fetus.[1,2] For instance, the capacity to incorporate glucose-carbon into fatty acids and sterols is well developed in the liver of the term rat fetus.[2] At birth, this hepatic lipogenic capacity declines rapidly following consumption of milk high in fat-derived calories during the suckling period.[3] This decrease in lipogenic capacity is reversed when the rat pup is weaned to a high-carbohydrate stock diet at the end of the third postnatal week, potentially indicating a diet-mediated metabolic adaptation.[2] To test the role of the diet in regulating the lipogenic potential of suckling pups, various groups have tried to wean pups

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prematurely to solid food. Due to numerous technical limitations this approach has met with limited success.[4,5] To circumvent these problems, a number of laboratories, including ours, have adopted the artificial rearing method.[6] We have previously reported that artificial rearing of the neonatal rat on a milk-substitute formula that is high in carbohydrate-derived calories (HC) resulted in hyperinsulinemia and precocious development of the lipogenic enzyme NADP-malate dehydrogenase (malic enzyme), and in the maintenance of high levels of glucose-6-phosphate dehydrogenase in the liver during the first few days of postnatal life.[7] Possible changes in hepatic lipogenic capacity, however, were not investigated in that study.[7] The present study was initiated to investigate the changes in lipogenic capacity in livers of artificially reared rats on the HC formula containing only 20% of its calories as fat, compared with about 68% in rat milk.[8] Our ability to rear the rat pup artificially on a substitute formula has allowed us to modify not only the caloric contribution of fat in the formula but also the type of fat (long- and medium-chain triacylglycerides, LCT and MCT) that can be added to the formula. We therefore examined the effect of different dietary fats (LCT and MCT) on the hepatic lipogenic capacity and on the activities of key lipogenic enzymes in the rat liver during the immediate postnatal period.

Materials and methods

Animals and artificial rearing

Pregnant Sprague-Dawley rats (Zivic Miller Laboratories, Pittsburgh, PA) were fed Purina rat chow (Ralston Purina Co., St. Louis, MO, USA) and water ad libitum. Pups were born naturally and remained with their dams until the time of cannulation on day 4. Intragastric cannulas were placed under light ether anesthesia and the pups were raised from that point on in isolation from their dams using an artificial rearing system.[9,10] Pups were individually housed in styrofoam cups floating on a temperature regulated water bath at 37° C. Age-matched pups in groups of 10 reared by dams served as controls. Pups of both sexes were randomly assigned to different dietary treatments. For artificially reared pups, milk-substitute formulas were delivered for 20- to 25-min periods every 2 hr at a rate of 0.45 kcal/g body weight per day.[7] Twice daily, pups were stroked to promote urination and defecation. The daily routine included cleaning, weighing, and adjusting the rate of formula delivery as reported previously.[7,10] Pups were reared artificially up to postnatal day 18. Survivability of these pups was about 60–70%; death usually occurred within 3–4 days of cannulation and was associated with trauma resulting from interorgan damage.[7,10] Pups from both the control and experimental groups were processed simultaneously at specified ages from days 6–18. Pups were killed by decapitation and trunk blood and liver specimens were processed as described below.

Rat milk-substitute formulas

Experiment 1 was designed to investigate the effect of a HC formula on hepatic lipogenic capacity in rat pups during the suckling period. A milk-substitute HC formula was prepared using skimmed evaporated cow milk as described previously[7] and stored frozen, then thawed as needed and refrigerated for up to 2 days before use. This HC formula (calorically) consisted of 20% fat, 24% protein, and 56% carbohydrate.

Experiment 2 was designed to include a group of pups reared artificially on a milk formula high in fat-derived calories from both LCT and MCT, and to investigate the effect of a high fat formula containing MCT as a predominant source of fat on hepatic lipogenic potential. The composition of three milk-substitute formulas, namely HC, high-fat formula containing both LCT and MCT (HF/LCT + MCT), and high-fat formula containing MCT (HF/MCT) used in Experiment 2 are given in *Table 1*. In all three preparations, skimmed evaporated cow milk was not used as it contributed a higher than required amount (about 8% calories) of carbohydrates in high fat formulas (HF/LCT + MCT and HF/MCT) to simulate rat milk composition.[10] The exact compositions of the three formulas were as reported previously.[11] Only relative amounts of carbohydrates and lipids were varied to achieve the desired caloric contributions; the protein content and the levels of micronutrients were constant in the

Table 1 Composition and caloric distribution of rat milk substitute formulas[1] used in experiment II

Source[2]	HC[1]	HF/MCT[1]	HF/LCT + MCT[1]	Rat milk[3]
		g/100 mL of formula (% calories)		
Carbohydrate	22.1 (56%)	3.16 (8%)	3.16 (8%)	3.16 (8%)
Protein	9.36 (24%)	9.36 (24%)	9.36 (24%)	9.36 (24%)
Lipids[4]	3.47 (20%)	11.9 (68%)	11.9 (68%)	12.03 (68%)

[1]Abbreviations used: HC, high-carbohydrate formula; HF/MCT, high-fat formula containing medium-chain triacylglycerides (MCT); HF/LCT + MCT, high-fat formula containing both long-chain triacylglycerides (LCT) and MCT.

[2]The sources of all nutrients and their amounts in the three formulas were as reported previously.[11] Briefly, carbohydrates: lactose, maltodextrins, and Polycose (Ross Laboratories, Columbus, OH, USA); lipids: corn oil, linoleic acid, and medium-chain triglycerides (Nutrisource lipid, Sandoz Nutrition Corp., Minneapolis, MN, USA); protein: Nutrisource protein (Sandoz Nutrition Corp.); vitamins: vitamin mixture AIN-76 (ICN, Cleveland, OH, USA); mineral mixture: Nutrisource minerals (Sandoz Nutrition Corp.).

[3]Rat milk composition is taken from Dymsza et al.[8]

[4]The calculated content of linoleic acid per 100 mL of formula was as follows: 1.27 g in the HC formula; 3.39 g in the HF(LCT + MCT) formula; and 1.05 g in the HF(MCT) formula. These values were within the range of linoleic acid content (0.73–1.37 g per 100 mL) in rat milk.[12] The content of linolenic acid in rat milk is extremely low (ranging from 0.07–0.16 g/100 g milk.[12] Based on 0.95% linolenic acid content in corn oil, linolenic acid content per 100 mL in the three formulas was: 0.031 g in the HC formula; 0.082 g in the HF(LCT + MCT) formula; and 0.01 g in the HF(MCT) formula. The calculated levels of polyunsaturated fatty acids in these diets were: 1.32 g in the HC formula, 3.53 g in the HF(LCT + MCT) formula, 1.06 g in the HF(MCT) formula, and approximately 2.25 g in rat milk.[12]

three formulas. The calculated content of linoleic acid, linolenic acid, and polyunsaturated fatty acids in the three formulas and in rat milk is shown in *Table 1*. The milk-substitute formulas were isocaloric and isonitrogenous with each other (*Table 1*). The caloric composition of HC formula in Experiment 2, however, was maintained similar to the HC formula used in Experiment 1.

Lipogenesis

Lipogenesis was measured as the rate of ^3H incorporation from ^3H_2O into both the saponifiable and nonsaponifiable lipid fractions from liver slices.[13] The liver slices were prepared as reported by Ballard and Oliver[14] and were rinsed in Krebs-Ringer bicarbonate buffer, pH 7.4. Liver slices (approximately 100 mg) were incubated in 3 mL of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 5 mmol/L glucose, approximately 0.25 mCi ^3H_2O, and 895 pmol insulin in stoppered 25 mL Erlenmeyer flasks in an atmosphere of 95% O_2/5% CO_2 for 2 hr at 37° C. Incubation was stopped by the addition of 0.5 mL of 6 N H_2SO_4. The slices were removed, rinsed in distilled water, and the lipids were extracted in chloroform/methanol (2:1 vol/vol) according to Folch et al.[15] After saponification of extracted lipids in methanolic KOH, nonsaponifiable lipids (sterol fraction) were extracted twice with petroleum ether (boiling point 40–60° C) and the two extracts were combined and washed several times with 0.2 N KOH until the aqueous wash was free of contaminating ^3H_2O radioactivity.[16] The saponified solutions were then acidified with HCl and extracted twice with petroleum ether for saponifiable lipids (fatty acid fraction). The extracts were combined and washed with 1 N HCl until the aqueous phase was free of contaminating ^3H_2O radioactivity.[16] Radioactivity in both the fractions was counted in a Beckman liquid scintillation counter. The results are expressed as cpm of ^3H incorporated in lipids/g of tissue/2 hr.

Enzyme assays

The liver tissues (about 0.3 g) were homogenized in 5 volumes of ice-cold buffer (250 mmol/L sucrose, 10 mmol/L Tris, pH 7.4, 1 mmol/L dithiothreitol, 1 mmol/L EDTA). The homogenates were centrifuged at 12,000*g* for 20 min and the supernatants were then centrifuged at 100,000*g* for 60 min at 4° C. The resulting 100,000*g* supernatants were assayed at 37° C for glucose-6-phosphate dehydrogenase[17] and fatty acid synthase[18] using spectrophotometric procedures.

Other assays

After decapitation, trunk blood was collected in a pre-weighed tube with 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.[11] The supernatants were kept frozen at −70° C until assayed. Plasma insulin[19] was measured by radioimmunoassay using rat insulin as standard. Cytosolic protein concentrations were measured by Lowry's method.[20]

Statistical analysis

Results are presented as means ± SEM for the number of animals indicated in figure legends. Significance of differences between groups was determined either by Student's *t* test (in Experiment 1) or by analysis of variance (ANOVA one-way) (for Experiment 2 with four dietary treatments). The levels of statistical significance were set as follows: a = $P < 0.05$; b = $P < 0.01$; and c = $P < 0.001$.

Results

Experiment I

During the preweaning period, the pups reared artificially and fed a HC formula (AR-HC group) grew at the same rate as the mother-fed pups (control group) (body weights in g on postnatal days 4, 8, 12, and 18: 10.5 ± 0.5, 15.5 ± 0.6, 27.5 ± 1.0, and 42.0 ± 1.2 for the control pups; and 11.7 ± 0.2, 15.7 ± 0.3, 26.2 ± 0.4, and 42.4 ± 1.1 for the AR-HC pups, respectively). These results show that the rate of formula delivery (0.45 kcal/g body weight per day) and the composition of the HC formula were able to support normal growth of rat pups reared artificially from postnatal day 4–18.

The hepatic lipogenic capacity was measured by quantifying the incorporation of ^3H from ^3H_2O into both nonsaponifiable and saponifiable lipids in liver slices from 6-, 8-, and 12-day-old AR-HC and control pups. Compared with the age-matched control (mother-fed) pups, feeding the HC formula from postnatal day 4 onward resulted in a significant increase (from 1.4- to 3.1-fold) in the rates of ^3H incorporation from ^3H_2O into the two lipid fractions (*Figure 1*). Activities of two

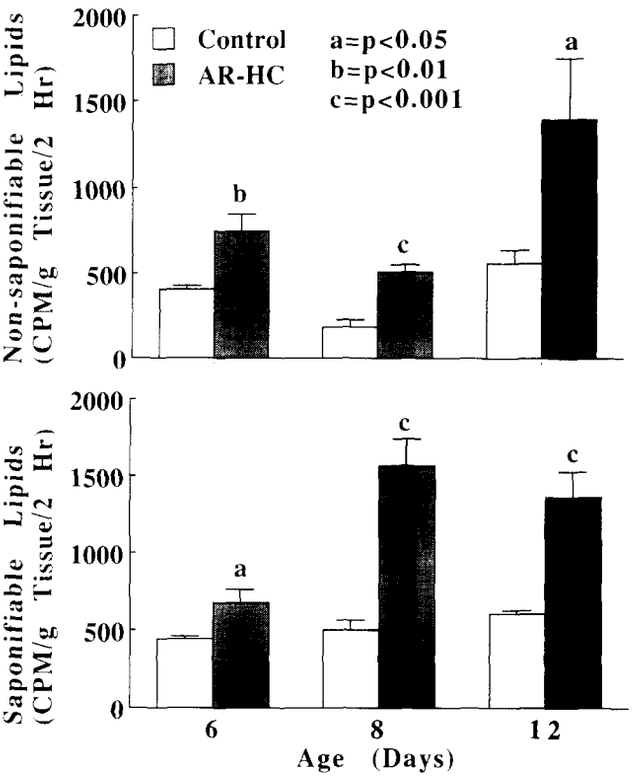

Figure 1 Postnatal changes in the incorporation of ^3H from ^3H_2O into non-saponifiable (sterol) and saponifiable lipid (fatty acids) fractions by liver slices from control (mother-fed) pups and pups artificially reared on a milk formula high in carbohydratre (AR-HC). Incubation conditions and lipid extraction procedures were as described in Materials and methods. The amounts of radioactivity incorporated in the lipid fractions were not corrected for slight variability in the specific radioactivity of ^3H_2O in the incubation medium used among different age groups. Values are means ± SEM (n = 5–8). Statistical analyses (P values as shown) between the two groups were determined by Student's *t* test.

key lipogenic enzymes, fatty acid synthase and glucose-6-phosphate dehydrogenase, were also increased significantly (from 2- to 3-fold) in the AR-HC group in comparison with the control group at all the age points studied (*Figure 2*). These results demonstrate an overall enhancement in hepatic lipogenic capacity of pups fed the HC formula compared with the control pups.

Experiment II

To ascertain that the enhancement of lipogenesis observed in the AR-HC group in Experiment I was not due to the artificial rearing procedure per se, a group of AR pups was fed a high fat formula (HF/LCT+MCT) containing the content of macronutrients similar to that of rat milk (*Table 1*). An additional group of AR pups received a high fat formula (HF/MCT) in which the caloric contribution from fat was derived largely from medium-chain triglycerides (*Table 1*). Four-day-old pups were started on one of three formulas (HC, HF/LCT+MCT, HF/MCT) and were killed on day 12. Mother-fed littermates (controls) served as an additional control group in this experiment. The body weights of 12-day-old AR pups on the three formulas were not significantly different from each other or from the mother-reared control pups (mean ± SEM, n = 6–12: 23.6 ± 1.2, 23.1 ± 1.3, 24.0 ± 1.6, and 25.6 ± 1.6 g for the AR-HF/LCT+MCT, AR-HF/MCT, AR-HC, and mother-fed control groups, respectively).

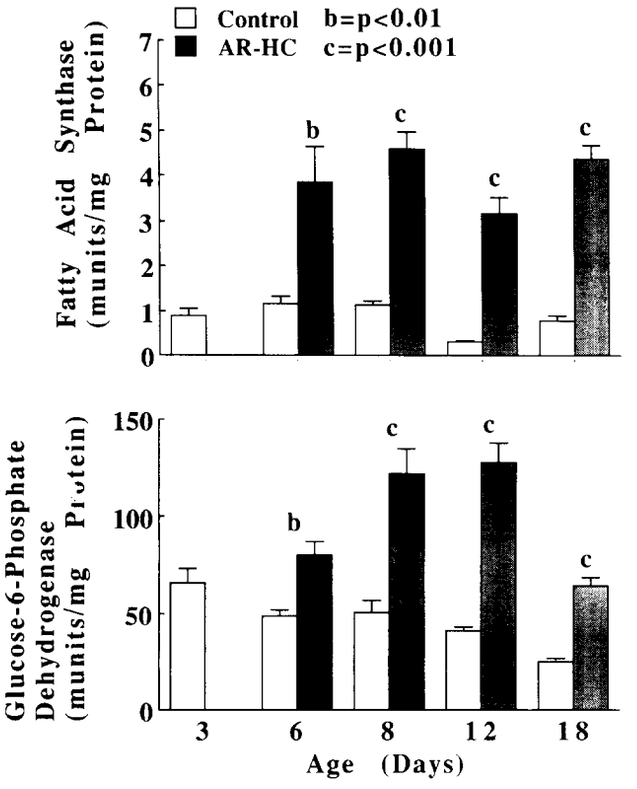

Figure 2 Postnatal changes in hepatic fatty acid synthase and glucose-6-phosphate dehydrogenase activities in mother-fed (control) pups and pups artificially reared on a milk formula high in carbohydrate (AR-HC). Activities of these two enzymes in the liver cytosolic fraction were assayed as described in Materials and methods. Values are means ± SEM (n = 5–8). Statistical analyses (P values as shown) between the two groups were determined by Student's t test.

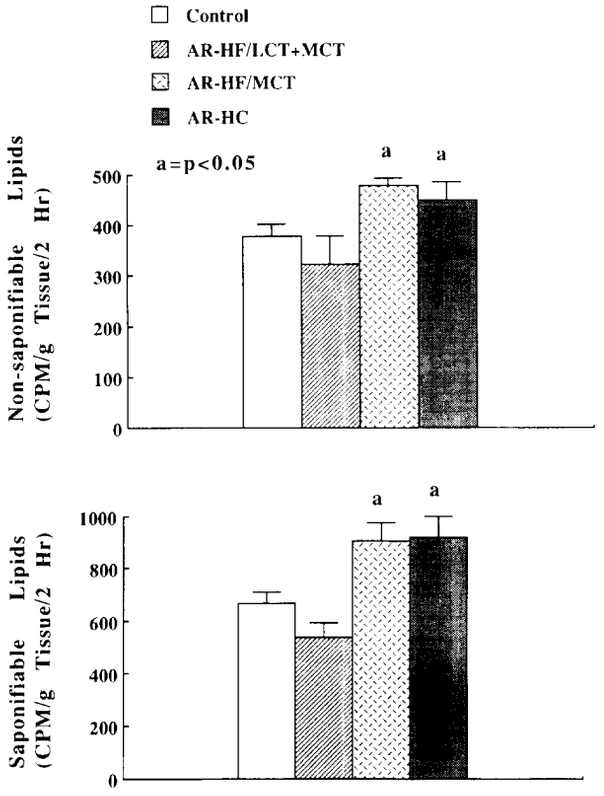

Figure 3 Incorporation of ^3H from ^3H_2O into non-saponifiable and saponifiable lipid fractions by liver slices from 12-day-old mother-fed (control) pups and pups artificially reared on three different milk formulas (HC, HF/LCT+MCT, and HF/MCT). Incubation conditions and lipid extraction procedures were as described in Materials and methods. Values are means ± SEM (n = 5–7). Statistical differences among the groups were determined by one-way analysis of variance.

In vitro hepatic lipogenic rates of the AR-HF/LCT+MCT group were not significantly different from those of the mother-reared control group (*Figure 3*), indicating that the artificial rearing procedure per se was not responsible for the observed increase in hepatic lipogenic capacity. The AR-HC group, as reported above, had significantly increased rates of lipid synthesis compared with either the mother-fed controls or the AR-HF/LCT+MCT group (*Figure 3*). The increase in lipid synthesis was associated with concomitant changes in the activities of fatty acid synthase and glucose-6-phosphate dehydrogenase (*Figure 4*). In contrast to the mother-fed control and AR-HC/LCT+MCT groups, which both derived fat calories from both LCT and MCT (in a 2:1 ratio), the AR-HF/MCT group, which derived fat calories largely from MCT (86%), had significantly increased rates of ^3H incorporation into both the nonsaponifiable and saponifiable lipid fractions (*Figure 3*) and a significant increase in fatty

acid synthase activity with a moderate, but not statistically significant, increase in glucose-6-phosphate dehydrogenase activity *(Figure 4)*.

Plasma insulin levels (0.107 ± 0.01 pmol/L) in the AR-HF/LCT+MCT group were not significantly different from those (0.156 ± 0.02 pmol/L) of the mother-fed control group. The AR-HF/MCT group appeared to have slightly higher (but not statistically significant) levels of plasma insulin (0.238 ± 0.04 pmol/L). The AR-HC group had significantly higher levels of plasma insulin (0.923 ± 0.34 pmol/L) compared with either the mother-fed controls or the two AR-HF groups.

Discussion

The results presented in this report support two significant observations on metabolic adaptations in artificially reared rat pups fed a milk-substitute formula high in either carbohydrate- or MCT-derived calories. These observations pertain to the effects of chronic hyperinsulinemia on weight gain and hepatic lipogenic capacity.

First, it should be noted that artificially reared pups on three different milk formulas (i.e., HF/LCT+MCT, HF/MCT, and HC) gained similar body weights from postnatal days 4–12. Of interest was the AR-HF/MCT group, which received its high level of fat-derived calories in the form of MCT (about 64% of metabolizable calories) but did not differ from other groups in body weight gain. It has been shown that the rates of digestion, absorption, and metabolism of MCT are different from those of LCT in both suckling and adult rats.[21,22] In adult rats, replacement of carbohydrate-derived calories by MCT (about 60% of metabolizable calories) in the diet caused a marked decrease in body weight gain.[23,24] However, lowering the caloric contribution by MCT to 30% and maintaining carbohydrate-derived calories at about 50% of metabolizable energy did not cause a reduction in body weight gain.[25] In the present study, changing either the contribution of carbohydrate- or fat-derived calories to the total metabolizable calories or the type of fat (LCT+MCT or MCT) in the formula, in the presence of isocaloric intake, did not affect the body weight gain in artificially reared rat pups from postnatal days 4–18. The reason for this differential response in weight gain between adult animals and pups is not entirely clear; however, it may be attributable to a lower level of carbohydrate-derived calories (8%) in the HF/LCT+MCT and HF/MCT formulas. In any case, the results show that a high caloric contribution by MCT did not have an adverse effect on weight gain in rat pups during the suckling period.

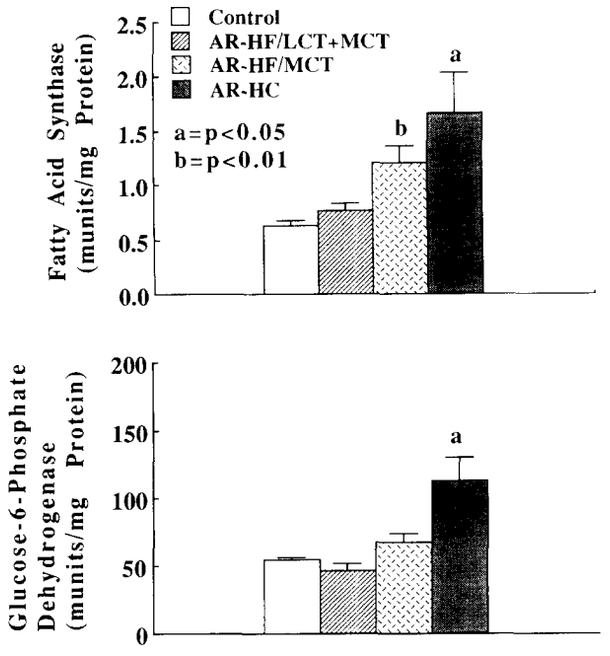

Figure 4 Activities of fatty acid synthase and glucose-6-phosphate dehydrogenase in livers from 12-day-old mother-fed (control) pups and pups artificially reared on three different milk formulas (HC, HF/LCT+MCT, and HF/MCT). Activities of these two enzymes in the liver cytosolic fraction were assayed as described in Materials and methods. Values are means ± SEM (n = 4–12). Statistical differences among the groups were determined by one-way analysis of variance.

In other studies, hyperinsulinemia resulted in increased weight gain in both the fetus and newborn animals.[26–28] For instance, when the litter size was reduced to four pups per nursing mother, suckling pups grew at a higher rate due to hyperinsulinemia as a result of increased daily caloric intake, compared with pups in a normal size litter.[28] Similarly, hyperinsulinemia resulting from chronic injection of insulin into fetuses or newborn animals led to increased weight gain compared with age-matched control animals.[26,27] In the present study, although the AR-HC group maintained chronic hyperinsulinemia, the pups did not grow at a higher rate compared with the AR-HF/LCT+MCT or the AR-HF/MCT pups. Because the three groups of pups received about the same number of total calories per day, the daily caloric intake appears to be more important than hyperinsulinemia as the primary determinant or the limiting factor in weight gain.

Second, although hepatic lipogenic capacity (quantified by the incorporation of labeled substrates into sterols and fatty acids and the activities of lipogenic enzymes) increases rapidly in near-term rat fetuses, this capacity decreases rapidly during the first postnatal day.[2] During the suckling period, lipogenic capacity in rat liver is maintained at low levels because rat milk consumed during this period is high in fat calories (68% of metabolizable calories) and extremely low (8%) in carbohydrate-derived calories.[8] Furthermore, the plasma insulin concentrations are maintained at low levels in suckling rat pups due to very low levels of carbohydrate in the milk.[8] During a gradual weaning transition from a milk diet to a solid diet (high in carbohydrate-derived calories) from 15–30 days postnatal, the hepatic lipogenic capacity is regained steadily.[2,29] Premature weaning of 16- or 18-day-old rat pups to a diet high in carbohydrate-derived calories causes an early increase in hepatic lipogenic capacity and also

a precocious induction of NADP-malate dehydrogenase activity.[4,5] In a previous study, compared with mother-reared pups (consuming only 8% of total calories from carbohydrates), artificially reared pups on a milk formula containing 22% of calories from carbohydrates had small, but significant, increases in the activities of glucose-6-phosphate dehydrogenase and malic enzyme.[7] Hepatic lipogenesis was, however, not measured in that study. The results of the present study show that the hepatic lipogenic capacity is maintained at higher levels in rat neonates within the first few days of postnatal life if they consume a milk-substitute formula high in carbohydrate and low in fat.

Although increased hepatic lipogenesis in rat neonates was predictable based on dietary studies performed on weaned and adult rats, it remained an experimentally difficult task to modify nutrient intake of rat neonates. Artificial rearing of rat pups on a modified milk formula, as reported here, allowed us to verify this prediction. More importantly, this approach allowed us to examine the effects of different dietary lipids on lipogenic capacity in the neonatal rat liver. The AR-HF/LCT + MCT group, like the mother-fed control group, maintained a markedly reduced hepatic lipogenic capacity, presumably due to a high level (68%) of caloric contribution from mixed dietary fats. However, when MCT provided the majority of fat-derived calories without altering the overall contribution of fat to the AR-HF/MCT group, a marked increase in hepatic lipogenic capacity was observed in this group compared with that of the AR-HF/LCT + MCT group. To our knowledge, this is the first documentation of the effect of dietary MCT on hepatic lipogenesis in rat neonates. Medium chain fatty acids are not readily deposited in tissue triglycerides. Instead they are oxidized to acetyl-CoA and converted to ketone bodies in the liver.[21,23] Under the dietary regimen in which very little carbohydrate is provided, it is possible that the acetyl-CoA generated from the oxidation of medium chain fatty acids is utilized for de novo fatty acid synthesis in the liver. It is also possible that, to a smaller extent, medium chain fatty acids are elongated to long chain fatty acids. Additional studies are warranted to elucidate possible contributions of these two pathways to the synthesis of hepatic long chain fatty acids in the AR-HF/MCT group.

The biochemical mechanisms responsible for the observed increases in hepatic lipogenic capacity in the AR-HC and the AR-HF/MCT groups appear to be different, however. In the AR-HC group, a higher level of plasma insulin resulting from the high carbohydrate content of the HC formula (containing 20% of total calories from LCT) was the principal contributing factor for the maintenance of an increased lipid synthesis and increased levels of lipogenic enzymes *(Figures 3 and 4)*. Dietary manipulations resulting in higher levels of plasma insulin are shown to cause increases in lipogenic capacity in neonatal rats.[28] In contrast, in the AR-HF/MCT group, in which there was not a significant change in plasma insulin levels, an increase in lipogenic capacity in the AR-HF/MCT group compared with the AR-HF/LCT + MCT group is most likely due to the absence of repressive or inhibitory effects of long chain fatty acids, especially the polyunsaturated fatty acids *(Table 1)*. Dietary polyunsaturated fatty acids are shown to be potent inhibitors of the synthesis of fatty acids and triglycerides[30] as well as the concentration of fatty acid synthase mRNA in both weanling and mature rats.[30–32] In summary, our results show that consumption of a formula high in either carbohydrate- or MCT-derived-calories enhances hepatic lipogenic capacity in artificially reared rat pups during the immediate postnatal period. Also, a high caloric contribution by MCT has no adverse effect on body-weight gain in rat pups in the period immediately postnatal.

Abbreviations

AR artificially reared
HC high carbohydrate
HF high fat
LCT long-chain triacylglycerides
MCT medium-chain triacylglycerides

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