Repression of Fat-Dependent Intestinal Apo A-IV mRNA Abundance by Medium Chain Triacylglycerols and Proteins, and Elevation by Carbohydrates of Fat-Dependent Apo A-IV Transport in Suckling Rat Pups

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Summary Nutrients regulating intestinal apo A-IV synthesis and secretion in developing rats have not been clearly defined. We examined the effect of ingestion of fats, carbohydrates and proteins on the abundance of intestinal apo A-IV mRNA and the serum concentration of apo A-IV in 14-day-old suckling rat pups fasted overnight. In pups ingesting long-chain fatty acid-fat (soybean oil, triolein: LCT), there was a prompt elevation of the mRNA at 1.5 h after ingestion, although a graded dose of soybean oil did not result in a comparable elevation of the apo A-IV mRNA. In pups on MCT, but not trilaurin, there was a repression of the LCT-dependent elevation of the message. In pups on Intralipid (composed of soybean oil, lecithin and glycerol), mRNA was elevated at 6 h after ingestion. Administration of Intralipid with lactose, glucose, fructose and sucrose induced a rapid elevation of mRNA together with elevation of serum apo A-IV, although administration of casein, whey proteins and soybean proteins resulted in repression of the Intralipid-dependent mRNA elevation. The message correlated weakly to the serum apo A-IV and triacylglycerols and with no correlation to intestinal fat accumulation. These results suggest that metabolic events following the ingestion of milk components modulate intestinal apo A-IV expression in developing rats, possibly through mucosal fatty acid utilization.

Key Words expression of apo A-IV, apo A-IV mRNA, rat pups, intestine, medium chain fatty acids, carbohydrates, proteins, triacylglycerols

Apo A-IV was first discovered in rat plasma HDL, and was later found in the chylomicrons and HDL in the mesenteric lymph in rats (1,2) and humans (3).

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Although apo A-IV seems to have various roles such as the activation of lecithin cholesterol acyltransferase (4), binding of HDL to cell membranes (5), and regulation of satiety (6), the precise role of this protein is unclear.

Apo A-IV synthesis in the intestine and its transport to the lymphatics are regulated developmentally; the abundance of the apo A-IV mRNA in the intestine and the plasma concentration of apo A-IV are higher in suckling than in adult rats (7). During the first 2 weeks of life, the suckling rat must obtain necessary nutrients from milk which is rich in triacylglycerols, contributing 70% of total ingested calories (8). Because fatty acid metabolism plays a critical role in providing much of the energy requirement during the suckling period, apo A-IV in the intestine may be related to the transport of a large amount of ingested fatty acids.

We showed in previous experiments that the administration of Intralipid, composed of soybean oil, lecithin and glycerol, resulted in an elevation to the prefasting level of the intestinal apo A-IV mRNA abundance in 14-day-old rat pups fasted overnight (9). In the same experiment, we also observed that the administration of milk components modified the intestinal apo A-IV mRNA abundance in the pups: administration of casein and lactose interfered with the Intralipid-dependent elevation of the message; returning the fasted pups to their dams only resulted in a transient elevation of the intestinal apo A-IV mRNA; administration of casein alone, as compared to that of lactose alone, lowered the apo A-IV mRNA. From these observations, we speculated that a milk component might modify the fat-dependent elevation of intestinal apo A-IV mRNA.

In the present study, we examined the effect of milk components—long chain fatty acids, medium-chain fatty acids, lecithin, carbohydrates, proteins—on the abundance of the intestinal apo A-IV mRNA, concentration of serum apo A-IV, and absorption and transport of ingested fats in rat pups fasted overnight. As a source of fat, long-chain fatty acid fats (LCT) as well as medium-chain fatty acid fats (MCT) were used, since medium-chain fatty acids are composed of approximately 35% of milk-fat fatty acids (8). The administration of these milk components exerted different effects on fat-dependent elevation of the intestinal apo A-IV mRNA and the serum apo A-IV.

**MATERIALS AND METHODS**

**Animals and diets.** Seven-week-old female Wistar rats with 8–10 pups per dam were obtained from Seiwa Experimental Animals Co (Fukuoka, Japan). The pups, 14 days old, were separated from their dams for 15 h, during which time they were given 0.75 ml of physiological saline every 3 h through a silicon stomach tube to minimize fat-independent or nonspecific elevation of apo A-IV mRNA (9). The pups were then given the liquid diet every 3 h by stomach tube: 0.5 and 1 ml soybean oil (Nacalai Tesque Inc., Kyoto, Japan); 0.5 ml triolein (Nacalai Tesque Inc.); 0.5 ml MCT composed of either trioctanoate (Fuji Oil Co., Osaka, Japan) alone or

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mixed with tridecanoate (Fuji Oil Co.) and trioctanoate (43.5:56.5, wt/wt); 0.5 ml Intralipid (Kabivitrum AB, Sweden) composed of 10% (wt/vol) soybean oil, 1.2% (wt/vol) egg yolk lecithin and 2.5% (wt/vol) glycerol; 1 ml of equal volume of MCT (mixture of trioctanoate and tridecanoate) and soybean oil; 1 ml of an equal volume of trilaurin (Nacalai Tesque Inc.) and soybean oil; 0.5 ml Intralipid containing either 3.0% (wt/vol) carbohydrates (lactose, glucose, fructose and sucrose), or 9.2% (wt/vol) proteins (casein, whey protein and soybean protein) equivalent to concentrations in dam’s milk, respectively (10). The whey protein (whey protein concentrate) and soybean protein (soy protein isolate) were a gift from the Technical Research Institute of Snow Brand Milk Products Co. (Saitama, Japan) and Fuji Oil Co., respectively. The pups were killed by withdrawal of aortic blood, while under diethyl ether anesthesia, at 0, 3, 6 and 9 h after refeeding. The whole intestine was carefully flushed with physiological saline and immediately placed in liquid nitrogen. All aspects of the experiment were approved by the Kyushu University Animal Policy and Welfare Committee.

**Determination of intestinal apo A-IV abundance.** Preparation of intestinal total RNA and determination of specific mRNA were carried out as described elsewhere (9). Briefly, the intestine was homogenized with 4 M guanidinium thiocyanate (Nacalai Tesque Inc., Kyoto, Japan) and total RNA was prepared by ultracentrifugation with CsCl₂. The concentration of RNA was estimated spectrophotometrically, assuming that an OD₂₆₀ of 1 corresponds to 40 μg of RNA/ml. The ratio of OD₂₆₀/OD₂₈₀ was used to estimate the purity of the RNA. In the ratio of OD₂₆₀/OD₂₈₀ was below 1.7, samples were extracted again with a chloroform:phenol mixture (1:1, vol/vol). RNA preparations (0.2–1.2 μg) supplemented with yeast RNA (BMY, Tokyo, Japan) to provide 1.2 μg of total RNA (10 μl) were blotted onto nitrocellulose filters prewetted with 3 M NaCl-1 M sodium citrate solution (pH 7.0) with a vacuum-manifold apparatus (Bio Rad Japan), according to the manufacturer’s instructions. After pre-hybridization of the filters, hybridization was done in hybridization solution containing cDNA labeled with [α-³²p]-deoxycytidine triphosphate (dCTP) (1.48 MBq/mmol, Amersham Japan, Tokyo) by a multiprime DNA-labeling system, according to the manufacturer’s instructions (Amersham Japan) for 18 h at 42°C. The cDNA for rat apo A-IV was a gift from Dr. J. I. Gordon (Washington Univ. School of Med., St. Louis, MO, USA). After exposing the blots to X-ray film (Fuji Film Industry Co., Tokyo, Japan) for 3 days, the autoradiograms of the blots were analyzed by quantitative scanning densitometry in the linear range of film sensitivity (Desital Densitorol DMU-33C, Toyo Science Industrial Co., Tokyo, Japan).

**Determination of serum triacylglycerols and apo A-IV, and intestinal triacylglycerols.** Serum apo A-IV was measured by rocket immunoelectrophoresis, as described previously (11). The concentration of apo A-IV in the serum was determined by comparing rocked heights with those in a series of dilutions of pooled serum obtained from 5-week-old male Sprague-Dawley rats, and expressed using arbitrary units. Serum triacylglycerols were measured using enzyme assay
kits (Triglyceride G-Test, Wako Pure Chemical Industries, Osaka, Japan). The lipids in the whole intestine were extracted in chloroform:methanol mixture (2:1, vol/vol) \((12)\), and the glyceride glycerols were determined chemically \((13)\). In some experiments, a portion of the lipid extracts was spotted on thin-layer plates (Silica gel G, Merck), developed with a solvent system composed of hexane:diethyl ether:formic acid \((80:20:2, \text{vol/vol})\) \((14)\). The plates were sprayed with 3\% (wt/vol) Cu acetate-7\% (wt/wt) phosphoric acid solution and colorized by heating at 150\°C for 30 min \((15)\).

Statistical analysis. Data were analyzed using Duncan’s new multiple-range test to determine the exact nature of the differences among the killing-time points \((16)\). The effects of the diet and time and the interaction were analyzed by two-way ANOVA \((17)\). Linear regression was used to identify significant correlations between variables.

RESULTS AND DISCUSSION

In earlier work, we found that suckling rats fasted for 15 h had a markedly lower abundance of mRNAs for apo A-IV and B, without an accompanying reduction in \(\beta\)-actin and apo A-I mRNAs, and that Intralipid administration to these pups restored the levels of intestinal apo A-IV and B mRNAs \((9)\). The primary objective of the present study was to examine the effects of major components of rat milk on LCT-dependent apo A-IV mRNA abundance in the intestine and the apo A-IV concentration in the serum, and to search for a possible link between the message and the accumulation of intestinal triacylglycerols or transport of the triacylglycerols, and between the message and the transport of the translational product. Serum apo A-IV and triacylglycerols in the suckling rats were assumed to be primarily derived from the intestine, since expression of apo A-IV mRNA \((7)\) and secretion of the translational product and triacylglycerols \((18)\) are extremely low in the liver.

Effect of long chain triacylglycerols. In experiments to determine the fat-dependent abundance of apo A-IV mRNA, soybean oil and triolein were used, since oleic acid and linoleic acid are major fatty acids in the milk of rats fed commercial rat chow. As shown in Fig. 1, the major glycerides in the intestine of pups fasted overnight or pups ingesting different nutrients were triacylglycerols with a small amount of monoacylglycerols. Diacylglycerols, which would be just above free cholesterol, were not detected in any appreciable amount. In accordance with previous findings \((19)\), developing rats had a fairly large amount of free fatty acids in the intestinal mucosa, under conditions of overnight fasting or refeeding. As shown in Fig. 2, the accumulation of intestinal triacylglycerols was more rapid and occurred to a greater extent in pups on triolein than in pups on soybean oil. In the pups on triolein, there was a transient elevation of serum triacylglycerols at 1.5 h, after which it returned to prefeeding levels. Refeeding the soybean oil and triolein resulted in a prompt elevation of intestinal apo A-IV mRNA at 1.5 h after
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Fig. 1. Thin-layer chromatography of the lipids extracted from the intestinal mucosa. Column A–F are for fasted pups, and pups on triolein, Intralipid, mixture of trioctanoate and tridecanoate, lactose and casein, respectively, at 6 h after administration. CE: cholesteryl esters, TG: triacylglycerols, FFA: free fatty acids, FC: unesterified cholesterol, MG: monoacylglycerols, PL: phospholipids.

administration and the elevation was maintained throughout the 9-h feeding period. In contrast, apo A-IV in the serum was transiently higher in pups on triolein, thereby suggesting that the level of apo A-IV mRNA and the translation or transport of the translation product are not coupled. It is also possible that the serum apo A-IV secreted from the intestine was cleared faster in pups on soybean oil than in those on triolein. Judging from the pattern of accumulation of the intestinal triacylglycerols, LCT-dependent elevation of the mRNA did not appear to be due to the amount of absorbed LCT.

As shown in Fig. 3, the concentrations of serum and mucosal triacylglycerols tended to be higher in pups given an increased dose of soybean oil (1 ml vs. 0.5 ml) within 3 h after administration. However, a graded dose of soybean oil resulted in a comparable elevation of the apo A-IV mRNA with no significant elevation of serum apo A-IV. These results suggest that LCT-dependent elevation of the intestinal apo A-IV mRNA have a threshold level over which apo A-IV mRNA abundance would not increase.

Effect of medium-chain triacylglycerols. As shown in Figs. 1 and 2, the administration of MCT—trioctanoate and an MCT mixture composed of tri-
Fig. 2. Intestinal apo A-IV mRNA and triacylglycerols, and serum apo A-IV and triacylglycerols in pups given long-chain fatty acid-fats, medium-chain fatty acid-fats and Intralipid. Values are M±SEM for 3–4 rats. *Significantly different from data on pups fasted at p<0.05. abc Values at the same time with different superscripts are significantly different (p<0.05). Results of ANOVA in pups on triolein, soybean oil, trioctanoate, mixture of trioctanoate and tridecanoate: significant influence of diet×time (p<0.05), diet (p<0.05) and time (p<0.05) on serum triacylglycerols; diet (p<0.05) and time (p<0.05) on intestinal triacylglycerols; diet×time (p<0.05) and diet (p<0.01) on serum apo A-IV; diet (p<0.01) and time (p<0.01) on apo A-IV mRNA.

octanoate and tridecanoate—resulted in accumulation of intestinal triacylglycerols with increase in time, although it has been reported that MCT, as compared to LCT, is absorbed efficiently and is transported mainly through the portal vein system (20). In contrast, serum triacylglycerols tended to be lower in pups on MCT than in pups on LCT. Administration of MCT did not elevate apo A-IV mRNA in the intestine, rather there was a lower level of apo A-IV mRNA and its translation products with time.

The effect of MCT on LCT-dependent abundance of apo A-IV mRNA was subsequently measured by giving pups an equal volume of soybean oil. As a control, pups were also administered trilaurin mixed with soybean oil. Longer chain fatty acid-containing fats such as trimyristate or tripalmitate were not tested as a control fat, since these fats are difficult to maintain in a liquid state, even when mixed with soybean oil. As shown in Fig. 4, trilaurin with soybean oil, as compared
Fig. 3. Intestinal apo A-IV mRNA and triacylglycerols, and serum apo A-IV and triacylglycerols in pups given different doses of soybean oil. Values are M±SEM for 3-4 rats. *Significantly different from the pups fasted at p<0.05. **Values at the same time with different superscripts are significantly different (p<0.05). Results of ANOVA: Significant influence of diet×time (p<0.05), diet (p<0.01) on serum triacylglycerols; diet (p<0.05) on intestinal triacylglycerols; diet (p<0.05) and time (p<0.05) on serum apo A-IV.

to soybean oil alone, resulted in a greater accumulation of intestinal triacylglycerols and a prompt elevation of the serum triacylglycerols; but, the serum concentration of triacylglycerols was decreased with time. In contrast, administration of MCT with soybean oil did not result in an elevation of serum triacylglycerols, as compared to administration of soybean oil alone, although MCT administration increased the accumulation of intestinal triacylglycerols. Thus, MCT inhibited LCT transport, possibly by enhancing LCT oxidation in the intestine (21). A challenge with trilaurin did not affect soybean oil-dependent elevation of apo A-IV mRNA and serum apo A-IV concentration. In contrast, a challenge with MCT resulted in repression of the soybean oil-dependent elevation of apo A-IV mRNA, with no influence on the serum apo A-IV concentration. Thus, MCT probably does not suppress LCT-dependent apo A-IV translation and/or transport.

It has been reported that a condition stimulating fatty acid oxidation, where ketone body formation concomitantly increases (22), is involved in an altered level of hepatic apo A-IV mRNA in rats: fenofibrate, commonly described as a lipid-
lowering agent by increasing fatty acid oxidation through induction of peroxisomal proliferation, reduces the level of apo A-IV mRNA (23); a fish oil diet, which also induces elevation of fatty acid oxidation in the liver (24), reduces net production of apo A-IV in lean Zucker rats and the abundance of apo A-IV mRNA in the hepatocytes (25). In suckling rats, MCT provides 70–80% of the substrate for ketogenesis (8), and formation of ketone bodies from LCT is also greater due to larger quantities of carnitine and the activity of carnitine palmitoyltransferase in suckling rats (26), as compared to that in adult rats (27). In addition, it has been reported that administration of MCT results in a concomitant increase of LCT-derived ketone body formation in rats (21). Therefore it is considered that ingestion of MCT decreased the amount of apo A-IV mRNA, possibly through a metabolic event which drives intestinal fatty acids into oxidative utilization rather than into an esterification process.

Effect of Intralipid. As shown in Fig. 2, in pups on Intralipid, there was a
prompt elevation on the serum concentration of triacylglycerols at 1.5 h, and there was a trend toward continuation of a high level of triacylglycerols compared to pups on soybean oil, although the accumulation of intestinal triacylglycerols was similar between the pups on Intralipid and soybean oil, except for the level at 1.5 h. In pups given Intralipid alone, apo A-IV mRNA was elevated, but this elevation occurred at a later time, as compared to pups given triolein or soybean oil alone. Intralipid administration did not result in a significant elevation of serum apo A-IV concentrations. Why the soybean oil-emulsion, as compared to soybean oil alone, caused a delay in the elevation of the message is not clear. The ingredients of Intralipid—glycerol and lecithin—may be relevant to this phenomenon, because glycerol stimulates triacylglycerol synthesis in the intestinal mucosa (28), and lecithin improves lymphatic triacylglycerol transport with less cholesterol (29) and affects intestinal apoprotein synthesis in adult rats with bile fistulas (30).

**Effect of carbohydrates.** To determine the effect of water-soluble nutrients in rat milk on fat-dependent apo A-IV expression in the intestine, Intralipid was used
as a source of fat. As shown in Figs. 1 and 5, the pups on carbohydrates, in comparison with Intralipid alone, tended to have higher accumulation of intestinal triacylglycerols, except the pups on glucose, although the carbohydrate tended to induce a lower concentration of serum triacylglycerols. In contrast to Intralipid administration alone, challenge with various carbohydrates with Intralipid resulted in prompt elevation of the message shortly after ingestion, and maintained the message at a high level throughout the refeeding period. In addition, the carbohydrate ingestion enhanced the Intralipid-dependent elevation of the serum apo A-IV concentration after 3 h, suggesting that carbohydrate administration potentiated LCT-dependent intestinal apo A-IV mRNA expression and its translation.

Sucrose-rich diet has been reported to stimulate hepatic apo A-IV mRNA in adult rats (31). A high plasma insulin/glucagon ratio was seen to increase apo A-IV mRNA abundance (7) and its translation (32) in rat hepatocytes. The concentration of plasma insulin, which is known to decrease ketogenesis in suckling rats and starved rats (22), has been reported to be lower in overnight fasted rat pups than

Fig. 6. Intestinal apo A-IV mRNA and triacylglycerols, and serum apo A-IV and triacylglycerols in pups given Intralipid-proteins. Values are M±SEM for 3-4 rats. *Significantly different from the pups fasted at p<0.05. abc Values at the same time with different superscripts are significantly different (p<0.05). Results of ANOVA in pups on proteins: significant influence of diet (p<0.05) and time (p<0.05) on intestinal triacylglycerols; diet (p<0.01) and time (p<0.05) on serum apo A-IV.
in fed pups (33), and a high dose of glucose enhances insulin secretion (34). Consequently, the high insulin/glucagon ratio may also favor the flow of fatty acid into the esterification pathway rather than into the oxidation pathway in the intestine of the developing rat. Therefore, it is considered that carbohydrate administration exerted an effect on intestinal apo A-IV expression (apo A-IV mRNA abundance and its translation) by inhibiting mucosal fatty acids from enhanced oxidation.

**Effect of proteins.** As shown in Figs. 1 and 6, the type of administered protein influenced differently the Intralipid-dependent elevation of intestinal accumulation of triacylglycerols and the concentration of serum apo A-IV. Casein resulted in a greater accumulation of intestinal triacylglycerols than did Intralipid alone. Casein administration also led to an elevation of serum triacylglycerols as compared with soybean or whey proteins after 3 h. In contrast to carbohydrate administration, the protein challenge resulted in a repression of the Intralipid-dependent elevation of apo A-IV mRNA after 6 h and tended to elevate the serum apo A-IV concentration, except in pups on casein after 1.5 h. Because the type of protein has been reported to influence plasma insulin (35) and thyroxine (36) in experimental animals, it is also possible that protein administration to fasted pups resulted in an altered abundance of the apo A-IV mRNA and the concentration of serum apo A-IV through hormonal events.

**Correlation between apo A-IV expression and triacylglycerol level.** Table 1 shows the relationship between the intestinal apo A-IV mRNA or triacylglycerols and the serum apo A-IV or triacylglycerols. A statistically significant correlation was observed between the message and serum triacylglycerols or serum apo A-IV, and between serum apo A-IV and serum triacylglycerols. Therefore, it is considered that secretion of triacylglycerol-rich lipoproteins from the intestine rather than absorption of fats by the intestine appeared to be related to the expression of intestinal apo A-IV. However, since the observed correlation coefficient was relatively low, metabolic events such as oxidation and/or esterification of the absorbed fatty acids may also influence apo A-IV expression in the intestine.

Reue et al. (37) showed that both transcription and turnover contribute to steady-state hepatic apo A-IV mRNA concentration in different strains of mice. In our previous experiment, we showed that elevation of apo A-IV mRNA in suckling rat pups given Intralipid was not due to increased transcription but rather to the

| Table 1. Correlation coefficient between serum triacylglycerols or apo A-IV and intestinal apo A-IV mRNA or triacylglycerols.1 |
|---------------------------------------------------------------|
| Apo A-IV mRNA | Serum triacylglycerols | Intestinal triacylglycerols | Serum apo A-IV |
|----------------|------------------------|-------------------------------|----------------|
| 0.180 (141)a   | 0.155 (142)            | 0.313 (137)b                 |
| Serum apo A-IV | 0.391 (146)b           | 0.042 (147)                  |

1 Figures in parentheses shows the number of animals. a,b Values are statistically significant at p < 0.05 and p < 0.01, respectively.
stability of the message (9). Thus, the nutrients examined in the present experiment possibly affected the stability of the apo A-IV message in the intestine.

In summary, our observations showed that the major components in rat milk-MCT, lecithin, casein and lactose-influenced apo A-IV expression in the developing rat intestine, possibly through utilization of mucosal fatty acids. These observations may be relevant to nutritional intervention for premature babies who require adequate energy from dietary fats.

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