Possible Role of Fatty Acids in Milk as the Regulator of the Expression of Cytosolic Binding Proteins for Fatty Acids and Vitamin A through PPARα in Developing Rats

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(Received June 4, 2007)

Summary Fatty acids in milk are thought to play an important role in intestinal maturation and gene expression in the postnatal small intestine. In this study, we determined the jejunal mRNA levels, in rats, of peroxisome proliferator-activated receptor α (PPARα) and PPARδ which are nuclear receptors for fatty acids. We also measured expression of their target genes during the postnatal period, namely liver type fatty acid-binding protein (L-FABP) and cellular retinol-binding protein, type II (CRBPII). The mRNA levels of PPARα, L-FABP and CRBPII, but not PPARδ, gradually increased during the suckling period and then sharply declined to a low level at the end of the weaning period. Rat pups at 17 d of age, weaned to a high-fat diet, showed significantly greater mRNA levels of PPARα, L-FABP and CRBPII than those weaned to a low-fat diet. Oral administration of PPARα ligand, WY14,643 during four consecutive days of the weaning period caused a parallel increase in the mRNA levels of PPARα, L-FABP and CRBPII genes. Furthermore, caprylic acid and oleic acid, which are major components of fatty acids in milk, induced jejunal PPARα, L-FABP and CRBPII gene expression. Our results suggest that fatty acids in milk may play a pivotal role in maintaining an enhanced level of expression of L-FABP and CRBPII genes in the small intestine, presumably by acting as inducers of PPARα gene expression.

Key Words PPAR, fatty acid, development, small intestine

Rat pups ingest fatty acids from milk as major nutrients during the 4 wk following birth while they are suckled. Fatty acids in milk are important for the development of many organs in rat pups during the suckling-weaning period. During this period, the small intestine matures rapidly to take on the adult form in preparation for the nutritional change from a diet rich in fat (milk) to one rich in carbohydrate (solid food) (1–3). Because fatty acids in milk are absorbed from the small intestine, the signaling pathway for fatty acid absorption and metabolism in the small intestine during the suckling-weaning period is thought to be important in development (1–4). Several studies have shown that genes related to fatty acid metabolism and absorption are highly expressed in the small intestine during the suckling-weaning periods (5–7). It is probable that fatty acids in milk regulate absorption and metabolism of fatty acids in the postnatal small intestine by inducing gene expression. However, it is not certain whether expression of genes related to fatty acid metabolism and absorption is directly regulated by fatty acids in milk.

Recent studies have shown that the signaling of fatty acid is mediated by one of the nuclear receptors, the peroxisome proliferator-activated receptor (PPAR) (8, 9). To date, three subtypes of PPAR have been cloned in amphibians, rodents and humans: PPARα (10, 11), PPARδ (also called PPARβ, NUC-1, or FAAR) (12), and PPARγ (13). Various types of fatty acids, some eicosanoids, and some hypolipidemic and antidiabetic drugs have been shown to activate PPARs by acting as their ligands (14–16). PPARs are thought to play key roles in different aspects of lipid metabolism and homeostasis. These PPARs share a common binding specificity for the “DR1 type” element (17), which has been designated as the peroxisome proliferator-activated receptor response element (PPRE) since it enhances the transcription of the PPAR-target genes. Gene expression of the target genes for PPAR is thought to be differently activated through PPAR subtypes, which are activated by fatty acids or eicosanoids, differentially recruited on the PPRE (14–17). In the small intestine, both PPARα and PPARδ are explicitly

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Abbreviations: CRBPII, cellular retinol-binding protein, type II; L-FABP, liver-type fatty acid-binding protein; PPAR, peroxisome proliferator-activated receptor.
expressed (18, 19). We have reported that the transcription of liver-type fatty acid-binding protein (L-FABP) and cellular retinol-binding protein type II (CRBP-II) genes, which are cytosolic binding proteins for fatty acids and vitamin A facilitating their absorption from lumen to enterocyte, were up-regulated by a high-fat diet through PPARα (20, 21). Our own and other groups have shown that expression of L-FABP and CRBP-II genes were highly detectable during the suckling-weaning period (5, 6, 10, 22–24). Moreover, our recent studies have shown that clofibrate, which is known to be an activator for PPARα, induced expression of L-FABP and acyl-CoA oxidase genes in weanling rats (22).

In this study, we examined jejunal gene expression of PPARα as well as L-FABP and CRBP-II in developing rats. Moreover, we demonstrated that fatty acids in milk induced expression of not only L-FABP and CRBP-II, but also of PPARα in weanling rats. Our study indicates that the signaling of the presence of fatty acids which then leads to intestine gene expression in the small intestine during the suckling-weaning period is mediated by a change in expression of PPARα.

MATERIALS AND METHODS

Animals. Sprague-Dawley suckling rats (Japan SLC, Hamamatsu, Japan) were kept with their mothers, and both mothers and pups were given free access to a standard laboratory chow diet (ME Oriental Yeast Co., Ltd., Tokyo, Japan) throughout the experimental period. Rat pups were killed by decapitation between 14:30 and 15:00 at the age of 5, 13, 20, 27 and 42 d. To assess the effect of dietary fat during the postnatal stage on intestinal gene expression, rat pups were removed from their mothers at the age of 17 d, and they then received free access to either a low-fat diet containing 2.4% (w/w) corn oil or a high-fat diet containing 24.7% (w/w) corn oil (Table 1) (25). They were killed at the ages of 21 and 28 d. In a further experiment, rats weaned from mother at 27 d of age, were orally administered with 1 mL/kg body weight of 20% glycerol in water (control), 180 μmol/kg BW of WY14,643 and various fatty acids (caprylic acid, oleic acid, linoleic acid and arachidonic acid) suspended in 20% glycerol once a day for four consecutive days starting at 27 d of age (between 9:00–9:30). These rats were killed between 14:30 and 15:00 at 30 d of age. The experimental procedure used in the present study met the guidelines of the Animal Usage Committee of the University of Shizuoka.

RNA analysis. The entire small intestine was flushed with ice-cold 0.9% NaCl solution. The jejunoileum extending from the ligament of Treitz to the ileocecal valve was divided into two equal parts along its length. A portion (100 mg) was excised from the middle of the proximal half of the jejunoileum (jejunum) and immediately used for RNA extraction. Total RNA was extracted by the acidified guanidinium thiocyanate method as described by Chomczynski and Sacchi (26). Northern blot analysis of PPARα, PPARδ, L-FABP, CRBP-II mRNAs and 18S rRNA was performed using 32P-labeled cDNA probes as described previously (25). The cDNA probes used in the Northern blot analysis were described elsewhere for rat PPARα (20), PPARδ (20), CRBP-II (25), L-FABP (27) and 18S rRNA (28). The specific mRNA signals from Northern blots were quantified using an image analyzer (BAS 2000, Fuji Film, Tokyo, Japan) for Northern blot analysis, and the signals were standardized for 18S rRNA signal.

Statistical analysis. Results are expressed as means ± SE. The significance of differences between groups was determined by ANOVA followed by Tukey’s multiple range test or Student’s t-test where appropriate.

RESULTS

Postnatal changes in gene expression of PPARα, PPARδ, L-FABP and CRBP-II in rat jejunum

In the first experiment, the rat pups were kept with the mother until weaning was completed. At the age of 5, 13, 20, 27 and 42 d, the jejunum was excised and was subjected to Northern blot hybridization for PPARα, PPARδ, L-FABP and CRBP-II mRNAs. PPARα mRNA levels gradually increased during the suckling period (5–13 d after birth), and reached their maximal level at 20 d after birth. At their peak, PPARα mRNA levels were twice those in 5-d-old rats. The PPARα mRNA level then declined to a low basal level which was observed at the end of weaning period (at 27 d after birth), as well as at 42 d of age (Fig. 1). On the other hand, the PPARδ mRNA level did not change markedly during the postnatal period, showing only a small and transient rise at 20 d after birth (Fig. 1). Similarly, both L-FABP and CRBP-II mRNA levels were temporally elevated during the period of 13–20 d after birth and they subsequently declined to low levels by the end of the weaning period (at 27 d after birth) (Fig. 1).

Dietary fat-induced PPARα, PPARδ, L-FABP and CRBP-II gene expression in the postnatal small intestine

Suckling rats receive milk rich in fatty acids from their mother. To investigate whether the expression of PPARα, L-FABP and CRBP-II genes observed in the suck-
fat diet throughout the postnatal period (Fig. 2).

Effects of WY14,643 on expression of PPARα, L-FABP and CRBPII genes in the rat postnatal small intestine

To examine whether PPARα-specific ligand, WY14,643 up-regulates gene expression of L-FABP and CRBPII in the postnatal small intestine, we orally administered WY14,643 to weanling rats for 4 d from 27 d of age. L-FABP mRNA levels in the jejunum were 1.7-fold higher (p<0.05) in WY14,643-treated animals than in controls. Similarly, the jejunal CRBPII mRNA level was 2.2-fold higher (p<0.05) in WY14,643-treated rats than in controls (Fig. 3). Interestingly, the PPARα mRNA level in the jejunum was 2.8-fold higher (p<0.05) in WY14,643-treated animals than in controls, but there was no difference in the PPARδ mRNA level (Fig. 3).

Expression of jejunal PPARα, L-FABP and CRBPII genes in weanling rats supplemented with fatty acids

To examine which type of fatty acids in milk up-regulate gene expression of PPARα, L-FABP and CRBPII in the rat postnatal small intestine, we orally administered caprylic acid, oleic acid, linoleic acid and arachidonic acid to weaning rats for 4 d from 27 d of age. The PPARα mRNA level was up-regulated by caprylic acid (2.5-fold, p<0.01) and oleic acid tended to enhance the PPARα mRNA level. On the other hand, the PPARδ mRNA level was not enhanced by any of the fatty acids tested in this experiment (Fig. 4). Jejunal L-FABP mRNA levels were induced 1.9-fold by caprylic acid (p<0.01) and 1.6-fold by oleic acid (p<0.05). The level of jejunal CRBPII mRNA in the jejunum was 2.8-fold higher (p<0.01) in caprylic acid- or oleic acid-treated animals than in control animals. Arachidonic acid induced the jejunal CRBPII mRNA level 2.2-fold (p<0.05) (Fig. 4).

DISCUSSION

Rat pups ingest fatty acids as major nutrients from milk during the suckling-weaning period, which normally lasts until 27 d after birth in the rats (1, 3). During this period, genes related to fatty acid absorption and metabolism are highly expressed in the small intestine (5–7, 22, 24, 29). It is thought that fatty acids in milk regulate these genes during this period to ensure effective absorption of fatty acids from milk in the small intestine. However, the molecular mechanism of small intestinal regulation of fatty acid absorption and metabolism-related genes in this period has not been elucidated. Our own and other laboratories have demonstrated that liver-type (L-FABP) and intestine-type (L-FABP) fatty acid-binding proteins, which are proteins mediating fatty acid absorption from lumen to enterocyte, are induced by dietary fat in adult rats (19, 20, 22, 30). Additionally, previous studies have demonstrated that the gene expression of cellular retinol binding protein (CRBPII), which is a protein allowing absorption of retinol from lumen to enterocyte, is up-regulated by dietary fat (21, 22, 25, 31–33). Recent studies have shown that these genes, including L-FABP and CRBPII, possess a direct repeat with one space (DR-1) of AGGTCA-like motifs in their promoter regions (11, 34,
Fig. 2. Effects of dietary fat on the expression of jejunal PPARα, PPARδ, L-FABP and CRBPII genes in the weaning period. 17-d-old rat pups were removed from their mothers and they received a high-fat diet or a low-fat diet for 5 and 11 d. Each mRNA level was normalized to 18S rRNA abundance. Values represent means±SE for 4 animals. Asterisks indicate significant differences as compared with the levels in control rats (*p<0.05, **p<0.01).

Fig. 3. Effects of WY14,643 on mRNA levels of PPARα, PPARδ, CRBPII and L-FABP genes in the jejunum of weanling rats. Ten micrograms of total RNA were analyzed for PPARα, PPARδ, L-FABP and CRBPII by Northern blot hybridization. Northern blots of RNA derived from the jejunum of 30-d-old rats which were treated orally with WY14,643 in 20% glycerol or vehicle (control) at 27–30 d of age. mRNA levels were normalized for 18S rRNA abundance. Values represent means±SE for 4 animals. Asterisks indicate significant differences as compared with the levels in control rats (*p<0.05, **p<0.01).

Fig. 4. Effects of fatty acids in milk on mRNA levels of PPARα, PPARδ, L-FABP and CRBPII in the jejunum of weanling rats. Ten micrograms of total RNA were analyzed for PPARα, PPARδ, CRBPII and L-FABP mRNAs by Northern blot hybridization. Northern blots of RNA were derived from the jejunum of 30-d-old rats which had been orally administered with caprylic acid, oleic acid, linoleic acid or arachidonic acid in 20% glycerol or vehicle (control) at 27, 28, 29, 30 d of age. Values represent means±SE for 4 animals. Asterisks indicate significant differences as compared with levels in control rats (*p<0.05, **p<0.01).
35). The DR-1-type cis-element is known to be bound with peroxisome proliferator-activated receptor (PPAR)- retinoid X receptor (RXR) heterodimer (17). PPARs are subdivided into three subtypes, termed α, δ, and γ (10–13). In the small intestine, PPARα and PPARδ are coexpressed (18, 19). Studies in our own and other laboratories have demonstrated that expression of the genes involved in β-oxidation and absorption of fatty acids and retinol in the small intestine are regulated by PPAR (19, 21, 30, 36, 37). Moreover, our previous studies suggest that dietary fat-induced alterations of small intestinal L-FABP and CRBPII gene expression may be regulated by the expression change of PPARα (20, 25, 31, 33). Recently, we have shown that the expression of small intestinal PPAR-dependent genes are up-regulated by clofibrate which is a known PPARα activator (22). It is most likely that fatty acid absorption-related genes as well as PPAR-dependent genes in the postnatal small intestine are regulated by PPARs. Therefore, we considered it pertinent to closely examine the time course of the developmental changes in the expression of PPARα and PPARδ genes as well as L-FABP and CRBPII as the PPAR-dependent genes. By measuring mRNA levels at the ages of 5, 13, 20, 27 and 42 d, we have shown in this study that expression of the PPARα gene in the small intestine is temporally elevated for a particular period extending from the day just prior to the onset of weaning (13 d) through to the middle of the weaning period (20 d). We also found that mRNA levels of L-FABP and CRBPII were also temporally elevated during this particular period (Fig. 1). We also confirmed that acyl-coA oxidase, which is an enzyme related to β-oxidation and a typical target gene of PPARs, is temporally elevated in this particular period (data not shown). On the other hand, the PPARδ gene was expressed at a relatively constant level throughout the experimental period (Fig. 1). These results indicate that expression of fatty acid and retinol absorption-related genes as well as PPAR-dependent genes are associated with expression of PPARα in the postnatal period.

During the suckling and weaning periods, rat pups receive dietary fat predominantly from the milk. To investigate whether dietary fatty acids are able to modulate PPARα, L-FABP and CRBPII gene expression during this period, we removed the rat pups from their mothers at 17 d of age to wean them to a low- or high-fat diet. In the rats fed a high-fat diet, PPARα, L-FABP and CRBPII mRNA levels were all enhanced, but not PPARδ (Fig. 2). These results suggest that fatty acids in milk may be one of the primary nutritional factors that maintain an enhanced level of expression of PPARα, L-FABP and CRBPII genes in the suckling period.

To confirm that expressions of L-FABP and CRBPII genes are regulated by PPARα during the suckling-weaning period, we measured jejunal PPARα, L-FABP and CRBPII mRNA levels when weaning rats at the age of 27 d were orally administered with the PPARα-specific ligand, WY14,643, for 4 d. As shown in Fig. 3, L-FABP and CRBPII gene expression were up-regulated by WY14,643. Interestingly, PPARα mRNA level was strongly induced by WY14,643. On the other hand, PPARδ mRNA levels were not affected by WY14,643. Previous studies by others have shown that L-FABP is up-regulated by WY14,643 in the small intestine (37, 38). Moreover, our own previous studies have shown that PPAR-associated gene expression, including that of L-FABP, is enhanced by the PPARα activator, clofibrate, in weanling rats (22). Our data suggest that postnatal induction of L-FABP and CRBPII genes by WY14,643 must be regulated by PPARα.

The milk produced by rats is rich in fatty acids, especially medium-chain fatty acids, n-6 fatty acid and n-9 fatty acids (39–41). Several studies have demonstrated that medium-chain fatty acids and unsaturated fatty acids may be important for development of the infant (42, 43). Additionally, it has already been demonstrated that the percentage of medium-chain fatty acids in total milk fatty acids during the suckling-weaning transient period is considerably higher than that during the suckling period (44). Thus, we focused in this study on these fatty acids, although other fatty acids such as long-chain saturated fatty acids are present in milk (40, 41). We hypothesized that certain types of fatty acids in milk would induce these genes during the suckling-weaning period. We therefore examined expression of these genes following oral administration of a range of fatty acids, namely the medium-chain fatty acid, caprylic acid, the n-9 fatty acid, oleic acid and the n-6 polyunsaturated fatty acids, linoleic acid and arachidonic acid, all of which are components of the milk of weaning rats. PPARα, L-FABP and CRBPII were induced by caprylic acid and oleic acid to similar extents (Fig. 4). Additionally, arachidonic acid tended to induce CRBPII expression, but not that of PPARα. The difference in fatty acid selectivity for expression of these genes has not previously been reported. Recent studies have shown that arachidonic acid strongly induced PPARα transactivation as a ligand in many cells (15, 16, 45). The elevation of CRBPII gene expression by arachidonic acid could be caused by the transactivation of PPARα as a ligand because PPARα expression is not markedly changed by arachidonic acid. On the other hand, our own and other groups have shown that caprylic acid does not act as a PPARα ligand (15). Additionally, transactivity of PPARα by oleic acid is lower than that by arachidonic acid in cells of the intestinal cell line, Caco-2 (46). Interestingly, we found in this study that caprylic acid and oleic acid induced PPARα expression in the postnatal small intestine. Therefore, the induction of L-FABP and CRBPII by caprylic acid and oleic acid may have been caused by a change in expression of PPARα. The mechanism of PPARα up-regulation by caprylic acid and oleic acid in the postnatal small intestine is, as yet, unclear. The medium-chain fatty acids tend to be converted to energy by β-oxidation in mitochondria to a greater extent than long chain fatty acids (47). In a previous study we showed that RXRα, which is a heterodimer partner of PPARα, was up-regulated by a diet containing medium-chain triacylglycerols (25). Additionally, we confirmed that p300, which is a major
coactivator for PPARα in small intestine (48), was also up-regulated by caprylic acid in weaning rats (49). Considering that PPARα activates β-oxidation in many organs (50), caprylic acid may have some function in activating β-oxidation in the postnatal small intestine through enhancing gene expression of PPARα as well as p300 and RXRα which are components of the PPARα-dependent transcriptional machinery. The results of the current study suggest that fatty acids in milk may alter the function of the small intestine to allow effective absorption of dietary fat and vitamin A by inducing L-FABP and CRBPII through PPARα. Further work should investigate the effect of fatty acids on jejunal gene expression and should include a large range of fatty acids from short-chain fatty acids to long-chain fatty acids during the suckling-weaning period.

In conclusion, the results of this study suggest that expression of small intestinal L-FABP and CRBPII genes in postnatal development is regulated by a change in expression of PPARα which transmits the signaling of fatty acids derived from milk.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (09670074, 11670076, 18790171), a grant from Uehara Memorial Foundation and Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists, the COE program in the 21st century, the Center of Excellence for Evolutionary Human Health Sciences, from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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