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Dietary fat regulates cellular retinol-binding protein II gene expression in rat jejunum

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Abstract

Cellular retinol-binding protein II (CRBP II) is an abundant cytosolic protein of intestinal absorptive cells. In this study, we examined whether dietary fat modulates the expression of CRBP II in the small intestine. In the rats fed a diet rich in long-chain triacylglycerols (LCT), both CRBP II mRNA and CRBP II protein levels in the jejunum were more than two-fold greater than in the rats fed a low fat diet and a diet rich in medium-chain triacylglycerols (MCT). The mRNA abundance of a retinoid X receptor (RXRα), which is thought to interact with the *cis*-element located in the CRBP II promoter, was elevated in the jejunum of rats fed high-LCT and high-MCT diets as compared with that of animals fed a low-fat diet, but the levels of RXRα mRNA of the LCT diet group was similar to that of MCT diet group. These results suggest that the expression level of the CRBP II gene is not directly related to the RXRα expression, and that it might be modulated by long-chain fatty acids or their metabolites.

Key words: Cellular retinol-binding protein, Type II; Dietary fat; Jejunum; (Rat)

1. Introduction

Cellular retinol-binding protein II (CRBP II) is an abundant cytosolic protein found in the small intestine of rats [1,2], human [3] and chickens [4]. The CRBP II has been suggested to play an important role in directing the absorbed retinol to the retinol acyltransferase [5,6] located in the microsomes where re-esterification of retinol and chylomicron formation occur. Thus, one may consider that the alteration of CRBP II levels in the small intestine may lead to changes in its capacity to absorb and transport retinol. However, information on the regulation of CRBP II gene expression is scarce. Recently, we found that the jejunum-bypass operation led to a marked increase in the amounts of both CRBP II and apolipoprotein B in the residual jejunal segment [7], suggesting that CRBP II expression might be enhanced in the small intestinal segment where fat absorption is stimulated. Although 9-*cis*-retinoic acid responsive element (RXRE) has been demonstrated in the promoter of mouse CRBP II gene [8], it is still unclear whether the change in CRBP II levels are accompanied by an alteration in RXRα mRNA levels in the small intestine. The present study presents evidence that CRBP II mRNA levels are not directly related to the RXRα expression, and they are dependent on the intake of long-chain triacylglycerols.

2. Materials and methods

Animals and diets. 7-week-old male rats of Sprague-Dawley strain (Japan SLC, Hamamatsu) received for 7 days one of three isoenergetic synthetic diets: a low fat (7 energy% corn oil)/high starch (70 energy% cornstarch) diet; a high medium-chain triacylglycerols (MCT) (66 energy% MCT and 7 energy% corn oil)/low starch (5 energy% cornstarch) diet; or a high long-chain triacylglycerols (LCT) (73 energy% corn oil)/low starch (5 energy% cornstarch) diet. These diets were solidified with 2% agar solution and served as cake diets [9].

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Abbreviations: CRBP II, cellular retinol-binding protein (type two); LCT, long-chain triacylglycerols; MCT, medium-chain triacylglycerols; RXR, retinoid X receptor; RXRE, retinoid X responsive element.

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Table 1
Composition of diets (g/kg)

Ingredient	Diet		
	low fat	MCT	LCT
Vitamin-free casein	157	159	159
Cornstarch	526	36	36
Corn oil	24	24	247
MCT [a]	–	223	–
Mineral mix [a]	28	28	28
Vitamin mix [b]	8.0	8.0	8.0
DL-methionine	2.4	2.4	2.4
Choline bitartrate	1.6	1.6	1.6
2% Agar	253	518	518

[a] Medium-chain triacylglycerols were generously supplied by Nisshin Oil Manufacturer (Yokohama, Japan).
[b] AIN-76 mineral mix and AIN-76 vitamin mix (J. Nutr. 107, 1340–1348) purchased from Oriental Yeast, Co., Tokyo, Japan.
All diets are isocaloric (3.0 kcal/g).

The details of the diet compositions are shown in Table 1. Fatty acid constituents of the MCT used were 75% octanoic acid and 25% decanoic acid as determined by gas-liquid chromatography. All diets contained at least 2.4% corn oil by weight to supply essential fatty acids. The animals were fed ad libitum and had unrestricted access to water. The rats were killed by decapitation between 10:00 a.m. and 11:00 a.m. The experimental procedures used in the present study met the guidelines of the animal usage committee of the University of Shizuoka.

Preparation of intestinal samples. The entire small intestine was removed and the duodenum extending from the pylorus to the ligament of Treitz was discarded. The jejunoileum was divided into three segments of equal length. The proximal third (jejunum) of the jejunoileum were flushed with 10 ml of ice-cold 0.9% NaCl solution. A 1.0-cm segment (100 mg) was excised from the middle region of the jejunal segment and immediately used for RNA extraction.

The mucosa was scraped from the remaining part of the jejunal segment with a glass microscope slide. Intestinal mucosa was homogenized in 6 ml of ice-cold 2 mM Tris/HCl (pH 7.1), containing 50 mM mannitol, 50 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride and 3 μg/ml aprotinin. After centrifugation of homogenates at 105 000 $\times g$ for 1 h (4°C), the clear supernatant fraction (cytosol) was frozen at −70°C and used for assay of CRBP II.

Enzyme immunoassay of CRBP II. CRBP II was purified to a homogeneity from jejunum of 3-month-old Sprague-Dawley rats as described previously [7]. Monospecific rabbit anti-rat CRBP II antiserum was prepared as described previously [7]. CRBP II was quantified by sandwich-type enzyme-linked immunosorbent assay (ELISA) according to the procedure reported previously [7]. Briefly, the sample was applied to wells of a microtitre plate (A/2; Costar, Cambridge, MA) that had been precoated with IgG of anti-rat CRBP II serum. Immobilized CRBP II was then detected by sequential incubations of biotinylated anti-rat CRBP II IgG, avidin-conjugated peroxidase (Vector Laboratories, Burlingame, CA), and 2,2′-azino-bis(3-ethylbenzthiazoline sulfonic acid) diammonium salt (Sigma, St. Louis, MO). Purified rat CRBP II was used as a standard.

Polymerase chain reaction amplification, cloning and sequencing of cDNA. CRBP II cDNA was synthesized with Molony murine leukemia virus reverse transcriptase (Superscript RNase H^-, Bethesda Research Laboratories) and random hexamer (Takara Shuzo Co., Kyoto, Japan) using 20 μg of rat jejunal total RNA as a template according to the procedure described previously [10]. The cDNA was amplified by polymerase chain reaction (PCR) using *Taq* DNA polymerase (AmpliTaq, Perkin-Elmer), and the gene-specific primers as described by Scharf [11]. The gene-specific primers for the PCR were designed from the rat CRBP II cDNA sequence [12]: PCR 5′ oligo (base 1–22), 5′-ATGAATTCGCAGCTTGTTCCTTCACGGTCA-3′ (*Eco*RI site underlined); PCR 3′ oligo (base 440–461), 5′-ATGGATCCATCACTTCTTTTTGAACACTTG-3′ (*Bam*HI site underlined). The reaction mixture was denatured at 94°C for 1 min, annealed at 50°C for 2 min, and polymerized at 72°C for 3 min. 25 cycles were performed, followed by a 7-min extension at 72°C. The PCR product was digested with *Eco*RI and *Bam*HI, subcloned into pBluescript II SK^+ (Stratagene) and sequenced by the dideoxynucleotide termination method [13] using modified T_7 DNA polymerase (Sequenase 2.0, U.S. Biochemical), and [^{35}S]dCTP as described by the manufacturer. The rat RXRα cDNA clone, corresponding to + 76 – + 837 of human RXRα mRNA [14], was a generous gift of Dr. Shigeaki Kato (Tokyo University of Agriculture). The cDNA probes were labeled with [α-^{32}P]dCTP (3000 Ci/mmol, Du Pont) using the random primer DNA labeling system (Takara Shuzo Co., Kyoto, Japan).

RNA extraction and Northern blot hybridization. Total RNA was extracted as described by Chomczynski and Sacchi [15]. Poly-adenylated RNA was extracted from total RNA using oligo-(dT)-latex (Oligotex-dT30, Japan Roche). For Northern blot analysis, aliquots (20 μg) of total RNA were denatured with 2.2 M formaldehyde, fractionated by electrophoresis on 1% agarose gels, and transferred to a nylon membrane (Hybond-N +, Amersham, Arlington Heights, IL) using 0.05 M NaOH as described by the manufacturer. RNA transfer was complete in 4h. The filters were rinsed in 2 × SSPE (1 × SSPE = 0.18 M NaCl, 0.01 M sodium phosphate, 1 mM EDTA (pH 7.7)). Prehybridization was performed at least 2h in a solution containing 50% deionized formamide, 5 × SSPE, 5 × Denhardt's solution (0.1% Ficoll, 0.1% bovine serum albumin, 0.1%

polyvinylpyrrolidone), 0.5% SDS at 42°C. The hybridization buffer consisted of the above buffer plus 20 μg ml heat-denatured salmon sperm DNA and ^{32}P-labeled probes. After hybridization (16 h at 42°C), membranes were washed twice with 2 × SSPE, 0.1% SDS at 60°C for 15 min, twice with 1 × SSPE, 0.1% SDS at 60°C for 30 min, and twice with 0.1 × SSPE, 0.1% SDS at room temperature for 15 min. The washed membranes were exposed to an image plate (Fuji Film) for 4 h at room temperature and analyzed with an image analyzer (BAS 2000, Fuji Film). Control hybridization was carried out using a human β-actin cDNA (400 bp) purchased from Nippon Gene Co. (Tokyo, Japan). Dot blotting was performed by spotting denatured RNA on a nylon membrane (Hybond-N +, Amersham Corp., Arlington Heights, IL) in three concentrations using a microfiltration apparatus (Immunodot, Atto, Tokyo, Japan): 2, 8 and 16 μg of total RNA or 0.3, 0.6 and 1 μg of poly (A)$^+$ RNA. The filters were treated with 0.05 M NaOH for 5 min, prehybridized in the presence of 50% formamide at 42°C, hybridized with ^{32}P-labeled cDNA probes for 16 h at 42°C exactly as described above for Northern blot hybridization. The washing and the detection of radioactivity on the nylon membranes were performed as described for Northern blot hybridization.

Statistical analysis. All results were subjected to one way ANOVA. Differences in mean values among groups were tested using Tukey's multiple range test [16], and were considered statistically different at $P < 0.05$.

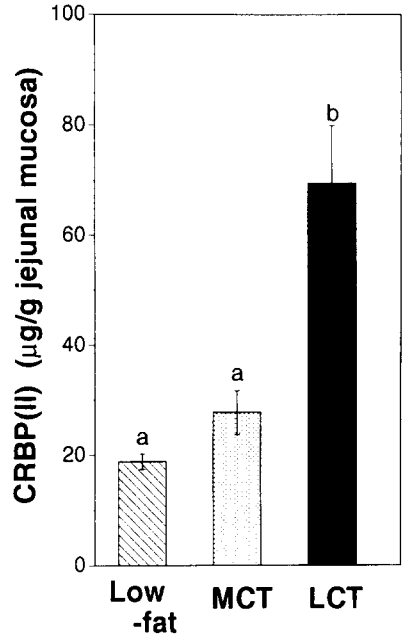

Fig. 1. Effect of feeding low-fat, high-MCT and high-LCT diets on CRBP II content in the jejunum. The rats were fed the various diets for 7 days. Cytosol was isolated from mucosal homogenates of the proximal one-third of jejunoileum, and CRBP II was measured by enzyme-linked immunosorbent assay. Values represent mean ± S.E. for four animals. (a,b) Values not sharing a common superscript letter are significantly different at $P < 0.05$.

3. Results

CRBP II content in jejunum

Food intake did not differ significantly among the dietary groups, and the mucosal total proteins in both upper and lower jejunum were unaffected by the dietary regimens (data not shown). The animals fed the high-LCT diet showed a 3.7-fold greater CRBP II content in the jejunum than the animals fed the low fat diet, whereas the animals fed the high-MCT diet exhibited no significant alteration in CRBP II content in this segment as compared with those fed the low fat diet (Fig. 1).

Northern blot hybridization analysis of rat CRBP II mRNA

The amplified and cloned CRBP II cDNA was 470 base long, consistent with the expected size of rat CRBP II cDNA according to the reported rat CRBP II cDNA sequence [12]. The identity of the cloned CRBP II cDNA was confirmed by sequencing; a complete identical nucleotide sequence was found. As indicated in Fig. 2, the rat CRBP II cDNA recognized a mRNA of approx. 800 base in RNA sample prepared from rat jejunum. This mRNA was identical in size to the mRNA for rat CRBP II reported previously [12]. The

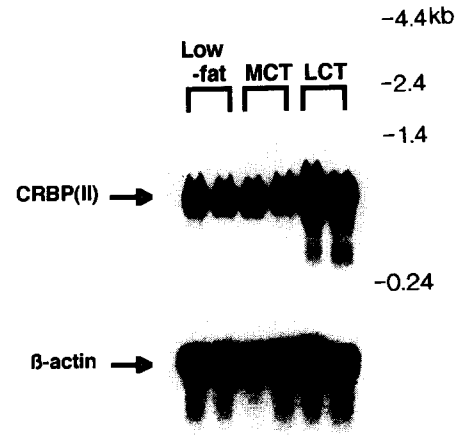

Fig. 2. Analysis of CRBP II mRNA by Northern blot hybridization. Total RNA was extracted from the jejunal segment of two individual rats fed low-fat, high-MCT and high-LCT diets, and 20 μg of total RNA were fractionated in formaldehyde/1% agarose gels. After Northern blotting, a ^{32}P-labeled cDNA for rat CRBP II was used to probe for CRBP II mRNA. After stripping the CRBP II cDNA probe, the blot was hybridized with a ^{32}P-labeled cDNA for human β-actin, which was used to control for variations in the amount of applied RNA. The positions of the RNA ladder markers are indicated in kilobases.

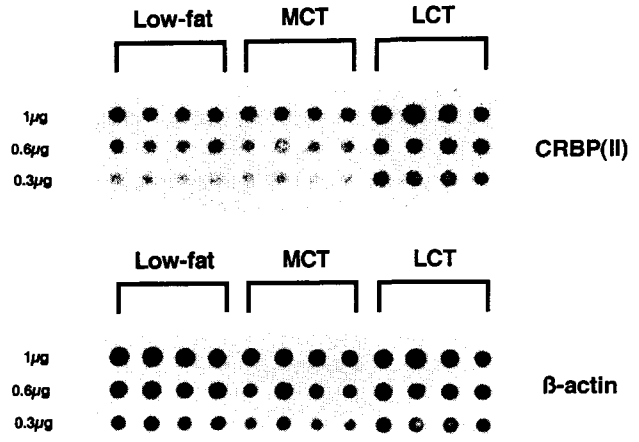

Fig. 3. Quantitative dot blot analysis of jejunal RNA samples using a cDNA probe for rat CRBP II. Animals were the same as described in Fig. 1. Polyadenylated RNA was extracted from the proximal one-third of jejunoileum. RNA was spotted on a nylon membrane filter in the amounts indicated, and hybridized with ^{32}P-labeled cDNA for rat CRBP II (top). After appropriate exposure to an image plate, the same RNA blots were rehybridized with a β-actin cDNA probe to show the integrity of RNA samples (bottom).

levels of CRBP II mRNA in the LCT-fed rats were greater than in low fat-fed rats and MCT-fed rats (Fig. 2).

Quantitative comparison of CRBP II mRNA

To quantitatively compare the levels of CRBP II mRNA among the dietary groups, dot blot analysis was performed. Linearity of the three concentrations of RNA was confirmed for each sample. As shown in Fig. 3, the abundance of CRBP II mRNA in the jejunum was greater in the rats fed the high-LCT diet than in the rats fed low fat diet or the high-MCT diet, whereas dot blot hybridization for β-actin mRNA showed no diet-related variations in β-actin mRNA levels. After normalizing on the basis of β-actin mRNA, relative abundance of CRBP II mRNA was compared among the dietary groups (Fig. 4). CRBP II mRNA levels in the upper jejunum of rats fed the high-MCT diet were similar to those in the rats fed low fat diet, but the animals fed the high-LCT diet exhibited a 3 times greater CRBP II mRNA levels than the animals fed the low fat diet (Fig. 4).

Northern blot hybridization analysis of RXRα mRNA

Because retinoid X (9-*cis*-retinoic acid) responsive element (RXRE) is known to be present in the promoter region of mouse CRBP II gene [8], we have determined the relative abundance of RXRα transcripts using rat RXRα cDNA probe. Northern blot analysis of RNA prepared from the jejunum of rats fed the various diets showed a single species of mRNA hybridized with the ^{32}P-labeled RXRα cDNA probe (Fig. 5). The amount of radioactivity hybridized to the

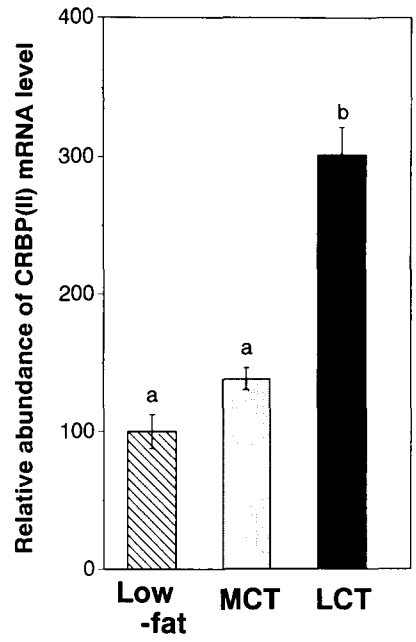

Fig. 4. Graphic representation of quantitative dot blots of jejunal CRBP II mRNA. Autoradiographic images were quantified using laser densitometry, and the results for each sample normalized for the β-actin mRNA abundance were expressed as arbitrary units, representing the mean value in the jejunum of the animals fed the low-fat diet as 100%. Difference in hybridization efficiency between filters were accounted for by including a RNA sample on each blot as internal standard. Values not sharing a common superscript letter are significantly different at $P < 0.05$ (Tukey's test).

RXRα mRNA was quantified by the image analyzer and the relative abundance in the RXRα mRNA in the jejunum of animals fed the various dietary regimens was compared, following the correction for relative abundance of β-actin mRNA. As shown in Fig. 6, both high-LCT and high-MCT diets produced signifi-

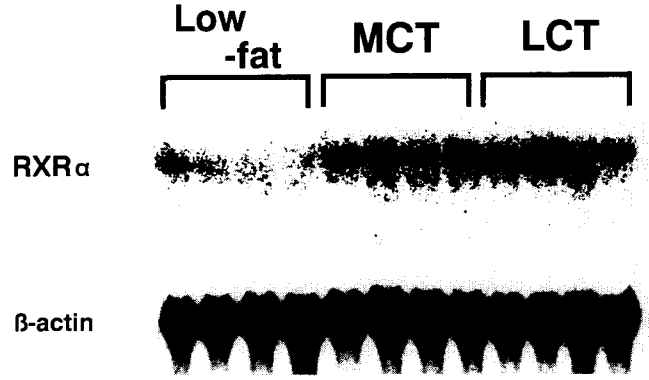

Fig. 5. Analysis of RXRα mRNA by Northern blot hybridization. Animals were the same as described in Fig. 1. Total RNA was extracted from the jejunal segment, and 20 μg of total RNA were fractionated in formaldehyde/1% agarose gels. After Northern blotting, a ^{32}P-labeled cDNA for rat RXRα was used to probe for RXRα mRNA. After stripping the RXRα cDNA probe, the blots were hybridized with a ^{32}P-labeled β-actin cDNA, which was used to control for variations in the amount of applied RNA.

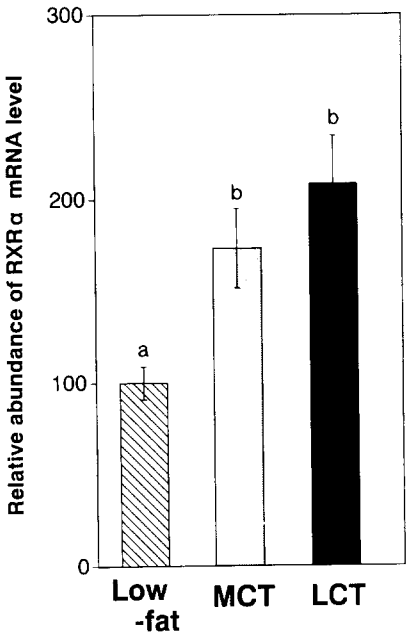

Fig. 6. Graphic representation of quantitative dot blots of jejunal RXRα mRNA. After dot blot hybridizations, autoradiographic images were quantified using laser densitometry, and expressed as in Fig. 4. The same arrangements as in Fig. 4.

cantly elevated RXRα mRNA levels in the jejunum (2.1 × and 1.7 ×, respectively) as compared with low fat diet. However, the levels of RXRα mRNA in the LCT diet group was similar to those in the MCT diet group. (Fig. 6).

4. Discussion

The studies presented in this paper demonstrate that dietary fat plays an important role in the induction of CRBP II in the small intestine. Previous studies showed that CRBP II is expressed abundantly in the small intestine of rat [1,2], human [3] and chicken [4]. During the embryonic development, CRBP II is abruptly induced a few days before birth [4,17]. The developmental changes of CRBP II mRNA accumulation in the proximal to distal axis of fetal rat small intestine during the late stage of gestation was shown to be very similar to those of intestinal fatty acid-binding protein and apolipoprotein A-I mRNAs [18]. In the 5′ nontranscribed region of CRBP II [19], intestinal fatty acid-binding protein [20] and apolipoprotein A-I [20], a closely related *cis*-element that consists of nearly perfect tandem repeat(s) of the sequence AGGTCA spaced by a single nucleotide, termed retinoid X response element (RXRE) [8] has been found. Therefore, it was conceivable that these three genes might be coincidentally expressed by the action of a common nuclear factor, presumably retinoid X receptors (RXRs). However, no evidence has been provided whether RXRs expression and/or production of its putative ligand, 9-*cis*-retinoic acid [21,22] are changeable in the small intestine during the development or in adult animals.

In the present study, we found that RXRα mRNA was expressed in the jejunum of adult rats, and that the levels of RXRα mRNA in the jejunum was significantly elevated in the rats fed the high-MCT and high-LCT diets as compared with that in the rats fed the low-fat diet. We have no explanation at present about the mechanism whereby the RXRα mRNA level was affected by the dietary manipulation. However, it should be noted that the alteration of RXRα mRNA accumulation did not necessarily correspond with the change in the levels of CRBP II mRNA in the jejunum; the animals fed the high-MCT diet showed a higher level of RXRα mRNA than in the rats fed the low-fat diet, but the CRBP II mRNA level in the rats fed the high-MCT diet was similar to the level seen in the rats fed the low-fat diet (Figs. 4 and 6). These results suggest that the change in the amount of RXRα mRNA does not necessarily produce the alteration of CRBP II mRNA level in the jejunum. However, we cannot exclude at present the possibility that other types of RXRs, i.e., RXRβ and RXRγ, might be involved in the regulation of CRBP II gene expression, although both RXRβ and RXRγ were reported to express at lower levels in small intestine in adult mice [23].

The mechanism whereby CRBP II gene expression is regulated in vivo has been unclear. Only a few studies demonstrated the alterations of CRBP II levels in the small intestine of adult animals; an increase of CRBP II level was found in the small intestine of the rats during pregnancy and lactation, with concomitant increase of food intake [24]. Recently, we observed that jejunum-bypass operation in the rat caused an elevated CRBP II level in the residual proximal jejunum, where the amount of apolipoprotein B was also elevated, suggesting that CRBP II expression might be stimulated in the intestinal segment where fat absorption is enhanced [7]. Unlike a ubiquitous cellular retinol-binding protein, CRBP I, which is reduced in vitamin A-deficient animals, CRBP II mRNA abundance in the small intestine is rather slightly elevated by vitamin A deficiency [25]. In the early ages of hatched chicks, feeding vitamin A-depleted diet decreased the intestinal CRBP II levels to approx. 70% of the levels of the chicks pair-fed the vitamin A-supplemented diet [26], but as the chicks grew, the effect of vitamin A depletion on intestinal CRBP II was less clear [26]. Therefore, we considered that some factor(s) other than retinoids should be involved in the modulation of CRBP II mRNA levels in the small intestine. In the present study, we have demonstrated that the intake of a diet rich in long-chain triacylglycerols (LCT) leads to an

increase in CRBP II protein level as well as CRBP II mRNA abundance in the small intestine, supporting the notion that CRBP II gene expression is under the control of a putative factor related with intestinal fat absorption.

It is unclear at present whether fat-induced increase in CRBP II mRNA is of physiological significance. However, it is possible that fatty acid-binding proteins and CRBP II are included in a family of genes that are regulated through a common nuclear factor. Indeed, it was shown that feeding a high-fat diet in the rat caused increased levels of fatty acid-binding proteins in the small intestine [27] and in the liver [28]. Both intestinal fatty acid-binding protein and CRBP II belong to a family of abundant intracellular proteins that are thought to facilitate the transport of amphiphilic lipids including fatty acids and retinoids [29]. Overall amino acid sequence is similar (31% identity) between intestinal fatty acid-binding protein and CRBP II, and replacement of a single amino acid at a key position can convert the ligand binding specificity of intestinal fatty acid-binding protein to that of CRBP II, and visa versa [30].

Alternatively, another nuclear factor might be responsible for the difference in the levels of CRBP II mRNA between the animals fed LCT diet and those fed MCT diet. Recently, Kliewer et al. [31] reported that peroxisome proliferator-activated receptor (PPAR; 32, 33) can form heterodimers with RXRα. The peroxisome proliferator responsive element located in the rat acyl-CoA oxidase promoter was shown to be composed of two direct AGG(A/T)CA repeats separated by a single nucleotide [34,35], thus conforming with RXRE. Cotransfection of expression plasmids for both PPAR and RXRα in the presence of a peroxisome proliferator, clofibric acid, and 9-*cis*-retinoic acid resulted in a synergistic increase in the activity of acyl-CoA oxidase promoter [31]. It is conceivable that 9-*cis*-retinoic acid and peroxisome proliferator might regulate an overlapping set of target genes through the RXRα-PPAR complex, because RXRα-PPAR complex was capable of interacting strongly with the CRBP II promoter as well as the acyl-CoA oxidase promoter [31]. More recently, Keller et al. [36] demonstrated that polyunsaturated fatty acids, including linolenic acid and linoleic acid, both present in corn oil used as a source of LCT in the present study, activated PPARs as potently as the peroxisome proliferator Wy 14643, the most effective activator known so far, and raised a possibility that the endogenous ligand for PPAR might be polyunsaturated fatty acids and/or their derivatives. Unfortunately, information regarding the tissue distribution of PPARs is scarce, and it is unknown whether PPARs are expressed in the small intestine. It will be interesting to determine in the future study whether PPAR is expressed in the small intestinal epithelial cells, and whether it interacts with RXRs in the small intestine.

In conclusion, the intake of a diet rich in long-chain triacylglycerols, which is known to increase intestinal fatty acid-binding protein in the small intestine as well as liver fatty acid-binding protein and peroxisome β-oxidation enzyme activity in the liver, led to an increase in CRBP II mRNA in the small intestine, strongly suggesting that fat metabolites might play an important role in the regulation of gene expression of CRBP II in the small intestine.

Acknowledgments

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