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Regulation of vitamin A metabolism-related gene expression

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Cellular retinol-binding protein, type II (CRBPII) is abundantly expressed in the small intestinal epithelial cells and plays a pivotal role in intestinal absorption and metabolism of retinol and β-carotene. In the 5′-flanking region of rat CRBPII gene, two DR-1 type elements which consist of a direct repeat of the AGGTCA-like motif spaced by a single nucleotide have been identified as putative binding sites for a heterodimer of peroxisome proliferator-activated receptor (PPAR) and retinoid X-receptor (RXR). We found that CRBPII levels were elevated in the residual jejunal segment of rats subjected to jejunal bypass operation, where a concomitant increase in the apoprotein B levels occurred. This result suggested that CRBPII expression was enhanced by a condition where fat absorption was stimulated. Indeed, dietary fat (especially unsaturated fatty acids) has been shown to induce CRBPII gene expression in the jejunum. Nuclear run-on assays revealed that this increase of CRBPII mRNA levels by a high-fat diet was the result of the induction of the gene transcription through the rise in PPARα expression level as well as the increase in its ligand levels. Electrophoretic mobility shift assay using the DR-1 type *cis*-elements of CRBP II gene showed that PPARα-RXRα heterodimer was capable of binding to these elements, and that nuclear extracts from the jejunum of rats fed the high-fat diet gave greater density of retarded bands than those of rats fed a fat-free diet. We also found that the expression of PPARδ was rather reduced by dietary fat. Thus, CRBPII gene expression is regulated predominantly by dietary fatty acids.

Cellular retinol-binding protein II: Gene expression: Peroxisome proliferator-activated receptor: Dietary fat

Vitamin A and its analogues are called retinoids. It is well known that vitamin A deficiency causes night blindness and many other disorders related to physiological actions of vitamin A, but their mechanisms have remained to be defined. Since the discovery of retinoid-binding proteins by the pioneering work of Goodman and Chytil in the 1970s (Ong *et al.* 1994) and the recent discovery of nuclear receptors by Chambon and Evans in 1987 (Mangelsdorf *et al.* 1994), there has been an explosion of new knowledge in the field of retinoid research. Nowadays, it is envisaged that the physiological action of retinoids is expressed via nuclear receptors that can bind retinoids and regulate the expression of various genes. As final forms of active metabolites of vitamin A, all-*trans* retinoic acid and 9-*cis* retinoic acid are used as ligands for nuclear receptors (RAR, RXR), which results in regulation of the expression of various genes at the transcription level.

CRBPII is essential for intestinal absorption and metabolism of retinol and β-carotene

The cytoplasmic retinoid-binding proteins were discovered in the search for retinoid binding receptors by using the sucrose gradient centrifugation approach. The approach demonstrated the existence of a 16-kDa protein that specifically bound labeled retinol or retinoic acid (Bashor *et al.* 1973). Thus two cytoplasmic retinol-binding proteins, CRBP and CRBPII, and two cytoplasmic retinoic acid-binding proteins, CRABPI and CRABPII, have been purified and extensively characterized (Ong *et al.* 1994). We have focused on and studied the CRBPII, which exhibited a distinct tissue distribution and physiological roles.

It is well accepted that vitamin A absorption is stimulated by intestinal fat absorption, but the theory may have to be revised by the evidence that the modulation of

Abbreviations: CRBP, cellular retinol-binding protein; L-FABP, liver-type fatty acid-binding protein; LRAT, lecithin:retinol acyltransferase; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor.

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intestinal vitamin A absorption is the result of a dietary fat-mediated change in the CRBPII gene expression. CRBPII is specifically and abundantly expressed in small intestinal epithelial cells, representing 0·4–1·0 % of soluble proteins in the small intestine. Therefore, we hypothesized that this protein might play an important role in the intestinal absorption and metabolism of retinol and β-carotene, and exert cytoprotection from the detergent action of retinol in the case of excessive uptake, in so far as the retinol entering enterocytes can bind to CRBPII. This protein–retinol complex serves as a substrate for microsomal lecithin: retinol acyltransferase (LRAT), which is one of the retinol-esterifying enzymes (Ong *et al.* 1987). CRBPII can also bind the retinal complexed with CRBPII, which then serves as a substrate for retinal reductase to produce retinol (Kakkad & Ong, 1988). Thus, CRBPII is essential for intestinal absorption and metabolism of retinol and β-carotene. We have shown that there are strong relationships between intestinal CRBPII expression levels and the activities of LRAT and β-carotene cleavage enzyme in the small intestine of developing chicks (Goda *et al.* 1993; Tajima *et al.* 1999*a*,*b*). These levels increased in parallel during the period from embryos to postnatal stages, being concomitant with the developmental increases in serum retinol and β-carotene concentrations of chicks (Takase & Goda, 1990; Takase *et al.* 1996).

Developmental CRBPII gene expression in chick duodenum

The CRBPII gene has DR-1 like elements which consist of a direct repeat of the AGGTCA-like motif spaced by a single nucleotide. To date, two elements (termed RXRE and RE3) have been identified as putative binding sites for a heterodimer of PPAR (peroxisome proliferator-activated receptor) and RXR (retinoid X receptor) in the 5′-flanking region of the rat CRBPII gene. Chick duodenum expressed constant levels of PPARα and RXRα mRNAs during development, whereas duodenal CRBPII mRNA level and arachidonic acid content increased abruptly around hatching (Suruga *et al.* 1997). The LRAT activity was elevated in parallel with the developmental changes of CRBPII gene expression in chick duodenum (Tajima *et al.* 1999*a*). Taken together, these results suggest that the developmental elevation of CRBPII levels is closely associated with the developmental induction of intestinal absorption and metabolism of retinol and β-carotene.

Effects of dietary fat on CRBPII and nuclear receptors gene expression

We unintentionally found that the jejunal-bypass operation led to a marked increase in the amounts of both CRBPII and apolipoprotein B in the residual jejunal segment of rats (Takase *et al.* 1993). This result suggested that stimulating fat absorption might enhance CRBPII expression. Indeed, we found in subsequent studies that jejunal CRBPII mRNA and its protein levels in rats fed a high-fat (corn oil) diet were more than two-fold greater than those in rats fed a low-fat diet (Goda *et al.* 1994). Unsaturated fatty acids, e.g. oleic, linoleic and α-linolenic acids enhanced CRBPII mRNA levels, whereas medium-chain fatty acids and saturated fatty acids had little effect on the CRBPII mRNA levels (Suruga *et al.* 1995). We then investigated the possibility that the increases in CRBPII gene expression induced by feeding a high-fat diet or unsaturated fatty acids are associated with activation of the nuclear receptors that are expressed in the small intestine and activated by some fatty acids or eicosanoids as their ligands. Such candidates are PPAR subtypes α and δ.

The mouse CRBPII gene has DNA binding sites for the nuclear receptors, which were termed as retinoid response elements (RE1 and RE3). These elements consist of two direct repeats of the AGGTCA-like motif with one intervening nucleotide, thus called ‘DR-1’ (Nakshatri & Chambon, 1994). Interestingly, a peroxisome proliferator response element (PPRE) which has been identified in the upstream regulatory sequence of the gene encoding acyl-CoA oxidase also consists of an almost perfect direct repeat of the sequence AGG(T/A)CA spaced by a single base pair. However, it remained unclear whether the CRBPII gene expression should be dominated by PPARs activation through a fatty acid signaling pathway or by RXR activation through a retinoid signaling pathway, or both.

In vivo study of CRBPII gene expression

We have clarified the effects of depletion of dietary fat on the CRBPII mRNA levels. Feeding a fat-free diet containing a sufficient amount of vitamin A repressed CRBPII mRNA accumulation by 50 % within a day, and this low level was sustained over the following 9 d (Takase *et al.* 1998). Furthermore, the amount of CRBPII protein levels in rats fed the fat-free diet for 10 d was 40 % less than that in rats fed the 10 % corn oil diet (Fig. 1). In parallel to a decrease of CRBPII mRNA level, PPARα mRNA level in rat jejunum was also reduced by a long-term (7 d) feeding of an isocaloric low-fat diet as compared to the 10 % corn oil diet (Takase *et al.* 1998). The hepatic total retinol content in rats fed the fat-free diet in this study was 14 % lower than that of the rats fed the 10 % corn oil diet (Tanaka K. *et al.* unpublished result). This result suggested the possibility that retinyl esters, incorporated into chylomicrons and exported to the lymph, were decreased due to the reduction of CRBPII protein level and the lack of fatty acid absorption. As mentioned above, the rats fed the fat-free diet exhibited lower expression of CRBPII and PPARα genes. There is little evidence from intact animals as to whether the rate of retinol absorption is correlated with CRBPII protein level in the small intestine. Results from an *in vitro* study using Caco-2 cells over-transfected with a CRBPII expression vector suggested that CRBPII level would be a key determinant of retinol absorption (Levin, 1993). Based on the results of our *in vivo* studies, we hypothesized that regulation of the expression of the CRBPII gene might be a determinant of vitamin A absorption as well as of its esterification status in animals.

We further investigated whether dietary retinol was necessary for the dietary fat-induced CRBPII gene expression. Oral administration of corn oil to vitamin A-deficient animals with serum retinol concentrations below 50 μg/l elicited approximately 3-fold accumulation of CRBPII

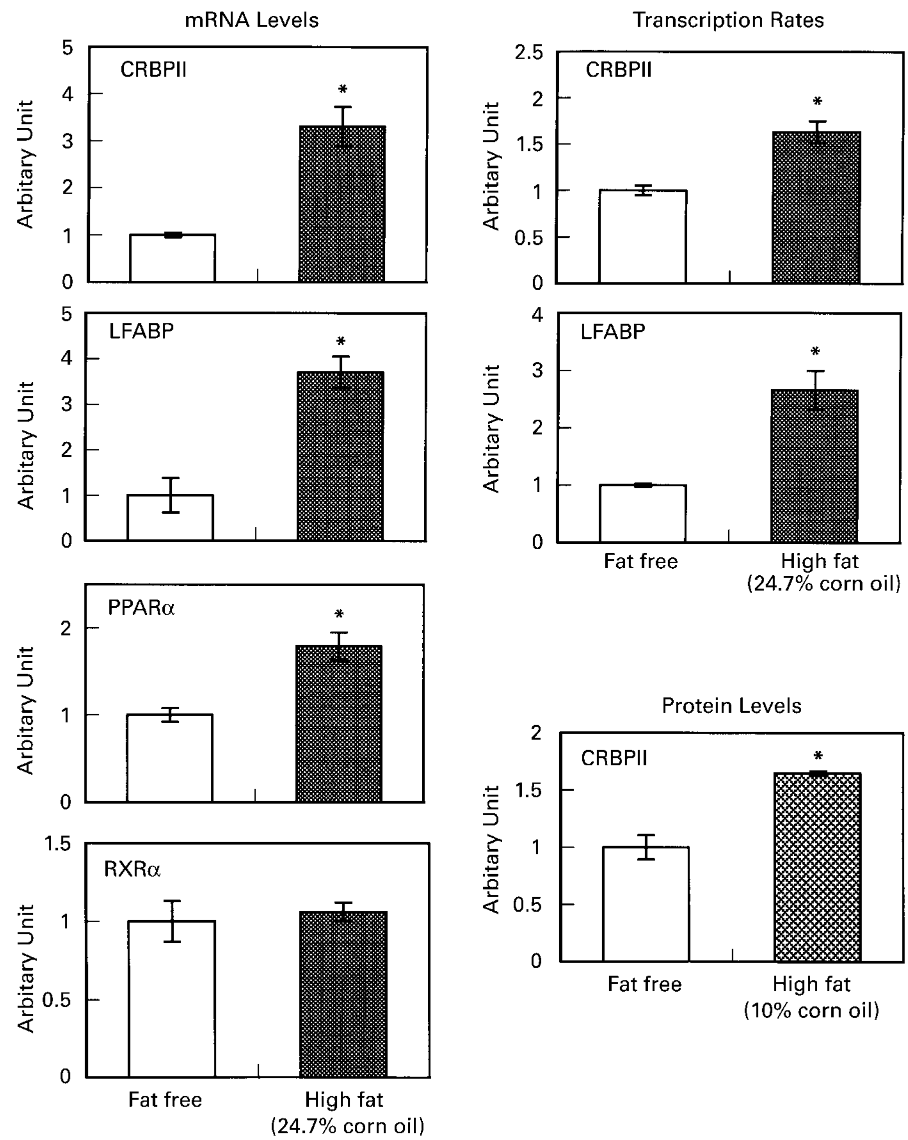

Fig. 1. Effects of high-fat diets on CRBPII, L-FABP, PPARα and RXRα expression in rat jejunum. Rats were fed a fat-free or a high-fat (10 % or 24·7 % corn oil) diet for a week. Nuclear run-on assays were performed using isolated rat jejunal nuclei. The mRNA levels were determined by Northern blot hybridizations. The CRBPII protein levels were estimated by western blotting. Results were expressed as relative values representing the mean values of the fat-free diet group as 1. Values are means ± SEM for three animals. *Denotes a significant difference compared with the fat-free diet group at $P < 0{\cdot}05$.

mRNA within 6 h (Takase *et al.* 1998). However, the administration of 9-*cis* retinoic acid to such animals brought about no accumulation of the CRBPII mRNA (Takase *et al.* 1998). Thus, in spite of vitamin A deficiency, oral administration of corn oil, but not 9-*cis* retinoic acid, caused an increase in jejunal CRBPII mRNA level. These results suggest that the CRBPII gene expression in rat jejunum may be regulated predominantly by dietary fatty acids, but little by dietary retinoids. These studies also provided evidence supporting the notion that RXRα is a silent partner of PPARα *in vivo*, and that signaling via fatty acids which results in the regulation of the CRBPII gene expression is mediated by a heterodimer of PPARα with a silent RXR partner.

Molecular mechanisms mediating the effects of fatty acids on the CRBPII gene expression

We have further studied the molecular mechanisms mediating the effects of fatty acids on CRBPII gene expression. As shown in Fig. 1, a nuclear run-on assay using isolated rat jejunal nuclei showed that the transcription rates of both CRBPII and L-FABP genes significantly increased in rats fed a high-fat diet (Suruga *et al.* 1999*a*). The increases in the transcription of CRBPII and L-FABP genes paralleled the rises in the respective mRNA levels (Fig. 1). The electrophoretic mobility shift assay (gel shift assay) revealed that jejunal nuclear proteins bound to the nuclear receptor response elements of the CRBPII gene

(RXRE and RE3). These elements resemble the peroxisome proliferator response elements (PPRE) on the liver-type fatty acid-binding protein (L-FABP) gene. The gel shift assay showed that the amount of jejunal nuclear proteins binding to the CRBPII-RXRE and RE3 was greater in rats fed the high-fat diet than in rats fed the fat-free diet, and was enhanced by addition of bacterially expressed PPARα protein (Suruga *et al.* 1999*a*). The heterodimer of PPARα-RXRα was capable of binding to the CRBPII-RXRE and RE3 elements, and these binding activities were enhanced by addition of some PPARα ligands in the gel shift assay (Suruga *et al.* 1999*a*,*b*). These findings indicate that dietary fatty acids elicit an induction of CRBPII gene transcription through an increase in the expression of PPARα as well as a rise in its ligand levels.

A transcriptional regulatory mechanism mediated by fatty acids has been investigated by luciferase reporter assays in transiently transfected CV-1 cells. In this study, the cells cotransfected with both PPARα and RXRα expression vectors, together with CRBPII-RXRE or -RE3 luciferase reporter vector, were treated with various fatty acids. The data demonstrated that PPARα ligands such as arachidonic acid and carbaprostacyclin particularly elevated the luciferase reporter activity (Suruga *et al.* unpublished data).

Relative amount of PPARα protein to PPARδ protein is important for the PPAR-target gene expression in the small intestine

Further investigation of the regulatory mechanism of CRBPII gene expression showed that dietary fat decreased PPARδ mRNA levels, whereas it increased PPARα mRNA levels (Kitagawa *et al.* unpublished data). Significant correlations were observed between the ratio of PPARα/PPARδ mRNA levels and the CRBPII mRNA level, as well as between the ratio of PPARα/PPARδ mRNA levels and the L-FABP mRNA level. This result suggested that the transcription of CRBPII and L-FABP genes might be controlled by the mutual competition of PPARα and PPARδ for their respective bindings to the PPREs of both genes. The gel shift assay showed that the amount of PPARα-RXRα heterodimer binding to the elements increased (or decreased) depending on the increase (or decrease) in the protein ratio of PPARα/PPARδ (Mochizuki *et al.* unpublished data). In the study using bacterially expressed proteins of RXRα, PPARα and PPARδ, we have demonstrated that PPARα competes with PPARδ not only for the ligand-binding, but also for the binding to their common heterodimer partner RXRα (Mochizuki *et al.* unpublished data). Thus the PPREs in promoter regions of CRBPII and L-FABP genes are possibly subjected to transcriptional regulation through the competition between PPARα and PPARδ for the common ligands such as fatty acids and their analogues. These findings suggest the importance of not only the absolute amount of PPARα protein, but also the relative amounts of PPARα and PPARδ proteins in the small intestinal absorptive cells.

Diurnal variation of CRBPII gene expression in the small intestine

It is now clear that dietary fat is capable of regulating intestinal CRBPII gene expression. Thus, we considered it pertinent to investigate the nutritional relevance of this diet-related CRBPII gene expression to the diurnal variation of CRBPII expression in rat jejunum. After starting feeding a laboratory chow diet at 18.00 hours, the levels of CRBPII mRNA and protein began to increase and reached maximal levels around 04.00 hours (Suruga *et al.* unpublished data). This diurnal variation in CRBPII expression may be explained by the involvement of dietary factor, e.g. fatty acids.

Conclusion

Our results suggest that CRBPII gene expression is regulated predominantly by dietary fatty acids, but little by dietary retinoids, and that feeding a high-fat diet also increases PPARα mRNA levels in the small intestine. Because the binding activities of the heterodimers of PPARα-RXRα to the CRBPII-RXRE and CRBPII-RE3 elements are increased by ligands for PPARα, it is suggested that the CRBPII gene expression in the small intestine is controlled by (1) the level of ligands for PPARα, and (2) the formation of a heterodimer of

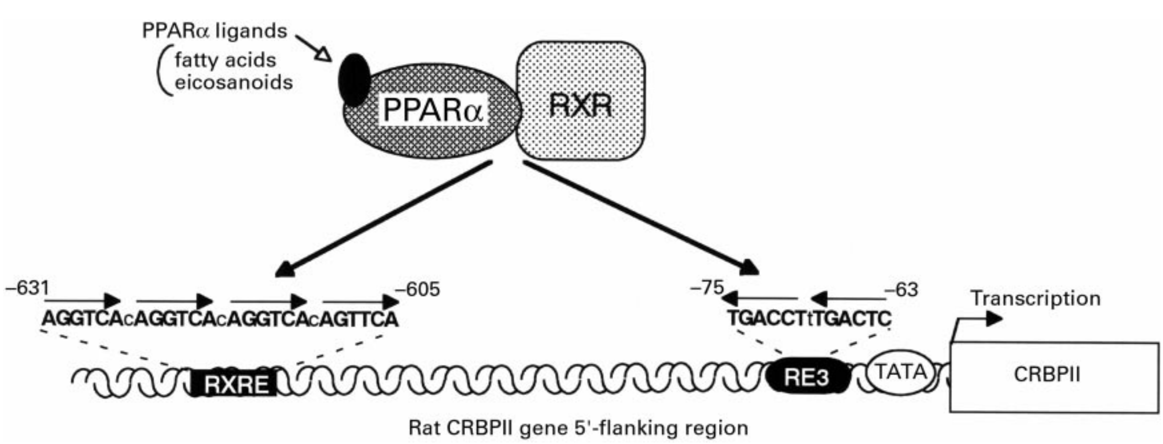

Fig. 2. Dietary fatty acids induce rat CRBPII gene transcription via PPARα-RXR heterodimer bound to the RXRE and/or RE3 element(s).

PPARα-RXRα which leads to the binding of the heterodimer to the *cis*-regulatory elements of CRBPII gene (Fig. 2), and it may be modulated by the relative amount of PPARα compared with its competitive subtype PPARδ.

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