Expression of Calbindin-D9k, VDR and Cdx-2 Messenger RNA in the Process by Which Fructooligosaccharides Increase Calcium Absorption in Rats

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Summary We have previously shown, through evidence that the expression of calbindin-D9k protein shows a dose-dependent change in the intestine, that calbindin-D9k plays a role in the ability of a fructooligosaccharide (FOS) diet to increase calcium absorption. This study shows that the regulation of calbindin-D9k expression occurs at the transcriptional level in a segment-specific manner, decreasing in the proximal intestine and increasing in the colorectal segment. To determine the transcriptional regulation of the FOS diet on calbindin-D9k expression, two transcription factors, vitamin D receptor (VDR) and cdx-2, were analyzed during 10 d feeding of the FOS diet. The mRNA expression of VDR and cdx-2 was influenced by the FOS diet and showed a segment-specific change. In the proximal small intestine, there was a significant correlation between the changes in both mRNAs (r=0.69, p<0.01), while the expression of calbindin-D9k correlated neither with VDR nor with cdx-2. This means that the transcriptional change induced by the FOS diet was not regulated by VDR and cdx-2. In the colorectal segment, there were significant correlations between gene expressions of calbindin-D9k vs. VDR, r=0.73, p<0.01 and calbindin-D9k vs. cdx-2, r=0.52, p<0.05. These results suggested that both transcription factors, VDR and cdx-2, were involved in the regulation of calbindin-D9k gene expression in the colorectal segment during the process through which the FOS diet enhanced calcium absorption.

Key Words fructooligosaccharides, calbindin-D9k, vitamin D receptor, cdx-2

Since osteoporosis is one of the most serious lifestyle-related diseases, calcium has long been a target of nutrition research. Absorptive enterocytes lining the intestinal villi are responsible for the terminal digestion and absorption of calcium and other nutrients. Active calcium absorption is by way of the transcellular path, with three primary components participating in series in the calcium absorption process. These components include epithelial calcium channels (CaT1), which enhance the luminal calcium in the absorbing enterocyte; intracellular calbindin-D9k, which binds calcium at high affinity and diffuses calcium across the cytoplasm of enterocytes from the brush border membrane to the basolateral side; and the ATP-activated basolateral membrane calcium pump, which transports cytosolic calcium into the extracellular fluid of the lamina propria (1). The expression of calbindin-D9k is largely regulated by the active form of vitamin D3, 1,25 dihydroxyvitamin D3 (2, 3), and stimulated by calcium restriction in the duodenal segment (3). A high correlation between its concentration and calcium absorption has been observed (4); therefore, calbindin-D9k is thought to play an important role in intestinal calcium absorption.

Though many diets have been recommended to improve calcium absorption, 1,25 dihydroxyvitamin D3 is the only dietary factor that increases calcium absorption; its mechanism of action has been analyzed at the molecular level (5). Vitamin D receptor (VDR) is also a transcription factor and its binding sequence (VDRE) on the promoter has been determined (5). Indigestible carbohydrates including fructooligosaccharides (FOS) (6, 7), resistant starch (8), guar gum hydrolysate (9) and inulin (10) have been intensively studied as dietary factors that can improve calcium absorption. Among them, FOS, a mixture of indigestible and fermentable sugars, has been well examined. Ohta et al. reported that FOS increased calcium absorption in balance studies of rats in vivo and that calbindin-D9k was involved in this effect (11). In the colorectal segment, the degree of enhancement of calcium absorption and the change of calbindin-D9k expression positively correlated with the amount of ingested FOS. Moreover, analysis of fecal pellets in the colorectal segment confirmed that this stimulatory effect of FOS on calcium absorption took place in this segment (7). The molecular mechanism by which these indigestible carbohydrates enhance calcium absorption remains to be clarified.

The calbindin-D9k gene was isolated and the 5′-regu-
Calbindin Expression by Fructooligosaccharides

MATERIALS AND METHODS

Animals and diets. Five-week-old male Sprague-Dawley rats (CLEA Japan, Inc., Tokyo, Japan) were individually housed in a temperature- and humidity-controlled room (25°C and 55% relative humidity) with a 12-h light-dark cycle. The control diet was prepared according to the AIN-93G formulation (19). Animals were housed individually in temperature and humidity-controlled conditions (25°C and 55% relative humidity) with a 12-h light:dark cycle. The control diet contained FOS in place of sucrose at a level of 100 g/kg diet (10% FOS diet). Table 1 shows the composition of both diets. FOS (Meioligo-P, Meiji Seika Kaisha, Tokyo, Japan) is a mixture of 42% 1-kestose, 46% nystose, and 9% 1F-β-fructofuranosylnystose. Other dietary components were purchased from Oriental Yeast Co., Ltd., Tokyo, Japan.

Rats were divided into three groups of 12 rats each, the two experimental groups and one control group. All rats were fed 15 g diet/d on day 1, 18 g diet/d on day 2, and 20 g diet/d on days 3–10 and were allowed free access to water throughout the experimental period. The diet dosage was designed to obtain a similar growth rate for the experimental and control groups without any diet remaining un consumed. The experimental diet group rats were sacrificed at days 5 and 10. The 12 control rats were sacrificed on day 10. Six rats from each group were used for protein and DNA determinations, and the remaining 6 rats were used for RNA analysis.

Table 1. Composition of experimental diets.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Control diet</th>
<th>FOS diet</th>
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<tbody>
<tr>
<td>Cassein</td>
<td>200</td>
<td>200</td>
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<tr>
<td>Cornstarch</td>
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<td>482</td>
</tr>
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<td>Corn oil</td>
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<td>35</td>
</tr>
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</tr>
<tr>
<td>Sucrose</td>
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</tr>
<tr>
<td>FOS</td>
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<td>100</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3</td>
<td>3</td>
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</tbody>
</table>

1Fructooligosaccharides (FOS, Meioligo-P, Meiji Seika Kaisha, Tokyo, Japan; concentration of oligosaccharides was >95% of total mixture).
2Prepared according to AIN-93 formation (19).

All of the experiments reported here received prior approval from the Animal Care Advisory Committee of Kagawa Nutrition University.

Analysis of colorectal contents. The pH of the colorectal contents was measured directly using a compact pH electrode (B-112, Horiba, Kyoto, Japan) before scraping the mucosal cells.

Preparation of intestinal samples. After the luminal contents were washed out with cold saline, mucosal cells were scraped from the proximal small intestine (upper half) and colorectal segment with a glass slide. Both samples were homogenized in four volumes of 13.7 mmol/L Tris-HCl buffer, pH 7.4, containing 0.12 mol/L NaCl and 4.74 mmol/L KCl. Appropriate amounts of the homogenate were subjected to DNA extraction by the method of Schmidt and Thannhauser (20). DNA content was determined by the diphenylamine reaction method (21). The protein content of the supernatant fraction obtained by centrifugation at 39,000×g for 30 min was measured by the method of Lowry et al. (22), and the appropriate amount was used for Western blot analysis.

Western blot analysis. Western blot analysis was basically conducted as described previously by Ohta et al. (11) with some modifications. To transfer the proteins that were separated by Tricine-SDS-PAGE, ImmobilonTM-P (Millipore Corporation, Billerica, MA) was used. After immunoreaction, immunogenic calbindin-D9k bands were identified using a second antibody conjugated to horseradish peroxidase followed by light emission from the oxidation of luminol. These chemiluminescence signals were quantified by a LAS1000plus luminescent imaging analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan). The means of the six lanes from each group on a membrane were calculated and compared statistically.

RNA isolation and Northern blot analysis. Total RNA and poly(A)+ RNA were prepared using Wako ISOGEN (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and Oligotex-dT30 super (Takara Shuzo, Kyoto, Japan), respectively. The RNA was then denatured and electrophoresed on 1% agarose-formaldehyde gels in 2.2 M formaldehyde. RNA was visualized by staining with ethidium bromide. The RNA was then transferred to a nylon membrane. After hybridization, the membranes were washed and exposed to X-ray film using an intensifying screen.
respectively, in accordance with the manufacturers’ instructions. Calbindin-D9k cDNA (23) and VDR cDNA (24) were kindly provided by Dr. H. E. DeLuca of the University of Wisconsin-Madison. A 270 bp EcoRI fragment from the plasmid pCaBP-270 and a 1,784 bp EcoRI fragment from the plasmid pUCVDR (1784) carrying the coding sequences of calbindin-D9k and VDR, respectively, were used as the probes for Northern blot analysis. A 279-bp probe for cdx-2 was amplified from rat intestinal cDNA using primers comprising bases 199–227 and 449–477 according to the published sequence (accession number AJ278466). For β-actin (25) detection, full-length β-actin cDNA was used. Quantitation was performed using a BAS-2000 Bioimaging Analyzer (Fuji Photo Film Co., Ltd.). Each value of the six lanes from each group was corrected for β-actin, and the means were compared statistically.

Statistical analysis. Values were expressed as means±SD. Data were analyzed by one-way ANOVA and Tukey’s test (26) (SPSS Version 6.0, SPSS, Chicago, IL). Differences were considered significant at p<0.05. A simple linear regression equation was calculated by the least-squares method using Microsoft Excel Version 7.0 (Microsoft, Tokyo, Japan).

RESULTS

The FOS diet increased weight of intestinal mucosa, and amount of mucosal DNA

There was no significant difference in body weight gain during 10 d feeding between rats fed the FOS diet and rats fed the control diet (data not shown). The contents of the colorectal segment of the large intestine increased in acidity after 5 d feeding of the FOS diet (p<0.001) (Table 2). The mucosal weights of the proximal small intestine and the colorectal segment of rats fed the FOS diet were 53.0% higher (p<0.001) and 20.0% higher than those of the control rats (p<0.001), respectively. The total mucosal DNA content was also higher than that of the control by 20.8% (p<0.005) in the proximal small intestine segment and 41.4% (p<0.001) in the colorectal segment.

The relative concentration of calbindin-D9k protein was expressed on both protein and DNA bases, each showing the same pattern of change resulting from the FOS diet; on a protein basis, 54.8% less in the proximal small intestine segment (p<0.001) and 239% more in the colorectal segment (p<0.001) (Table 2). Calbindin-D9k mRNA expression was differently regulated by the FOS diet in the proximal small intestine and colorectal segment

The pattern of calbindin-D9k mRNA expression in Northern blot analysis after 5 and 10 d feeding of the FOS diet is shown in Fig. 1, with the proximal small intestine segment in panel A and the colorectal segment in panel B. The pattern of β-actin is also shown as an internal control. The means of the six rats from each group are shown in Fig. 2 as a percentage of the control group; the values were corrected for β-actin. The effect of the FOS diet appeared after only 5 d of feeding in the colorectal segment (Fig. 2B), and there was a significant correlation (r=0.96, p<0.01 for the proximal small intestine, r=0.94, p<0.01 for the colorectal segment) with the effect on calbindin-D9k protein expression (Table 2). In the proximal small intestine segment, calbindin-D9k mRNA was 46.3% less (p<0.01) than in the control group, while the colorectal segment showed a 263% (p<0.01) increase at 5 d and 258% (p<0.01) at 10 d. Therefore, calbindin-D9k mRNA expression was regulated by the FOS diet and was segment specific.

VDR mRNA expression was differently regulated by the FOS diet in the proximal small intestine and colorectal segment

The patterns of Northern blot analysis of VDR and β-actin (an internal control) mRNA after 5 and 10 d feeding of the FOS diet are shown in Fig. 3, with the proximal small intestine segment in panel A and the colorectal segment in panel B. The means of the corrected values of six rats from each group are shown in Fig. 4 as a percentage of the control group. The expression of VDR mRNA in the proximal small intestine segment decreased by 48.1% (p<0.01) at 5 d and increased to the control level (p=0.74) at 10 d. In contrast, the colorectal segment showed a 102% higher level (p<0.001) at 5 d and 48.0% (p<0.001) at 10 d. Figure 4 indicates that VDR gene expression was regulated by the FOS diet and was segment specific. VDR mRNA expression was differently regulated by the FOS diet in the proximal small intestine and colorectal segment

Cdx-2 mRNA expression was differently regulated by the FOS diet in the proximal small intestine and colorectal segment

The patterns of Northern blot analysis of cdx-2 and
Fig. 1. Northern blot analysis of calbindin-D9k (CaBP) mRNA of intestinal segments: (A) proximal small intestine and (B) colorectal segment of rats fed control (without fructooligosaccharides, FOS) or 10% FOS diets for 5 or 10 d. Total RNA was extracted from mucosal cells of the proximal small intestine or colorectal segment, and mRNA was further purified. The mRNA fraction (1.0 µg) was analyzed on an agarose gel, transferred to a membrane, and hybridized with (α-32P)dCTP-labeled calbindin-D9k cDNA. Thereafter, all radioactivity was washed out from the membrane, and rehybridization was conducted for β-actin mRNA. The arrow indicates the position of the calbindin-D9k and β-actin mRNA.

Fig. 2. mRNA of calbindin-D9k from the proximal small intestine (A) and the colorectal segment (B) of rats fed control (without fructooligosaccharides, FOS) or 10% FOS diets for 5 or 10 d. Levels of mRNA were calculated as a percentage of the values of the control group and expressed as means±SD, n=6. Values in a row with no common superscript letters are significantly different, p<0.05.

Fig. 3. Northern blot analysis of the vitamin D receptor (VDR) mRNA of intestinal segments: (A) proximal small intestine and (B) colorectal segment of rats fed control (without fructooligosaccharides, FOS) or 10% FOS diets for 5 or 10 d. Total RNA was extracted from mucosal cells of the proximal small intestine or colorectal segment, and mRNA was further purified. The mRNA fraction (1.0 µg) was analyzed on an agarose gel, transferred to a membrane, and hybridized with (α-32P)dCTP-labeled VDR cDNA. Thereafter, all radioactivity was washed out from the membrane, and rehybridization was conducted for β-actin mRNA. The arrow indicates the position of the VDR and β-actin mRNA.

Fig. 4. mRNA of VDR from the proximal small intestine (A) and the colorectal segment (B) of rats fed control (without fructooligosaccharides, FOS) or 1.0% FOS diets for 5 or 10 d. Levels of mRNA were calculated as a percentage of the values of the control group and expressed as means±SD, n=6. Values in a row with no common superscript letters are significantly different, p<0.05.

internal control β-actin mRNA after 5 and 10 d feeding of the FOS diet are shown in Fig. 5. The means of the corrected values of six rats from each group are shown in Fig. 6 as a percentage of the control group. In the proximal small intestine segment (panel A), the expression was 31.2% less (p<0.01) after 5 d and significantly recovered to the control level (p=0.12) at 10 d. Conversely, the colorectal segment showed a gradual increase, with expression 108% higher at 10 d (p<0.001). It is clear that cdx-2 gene expression was regulated by the FOS diet differently in the proximal small intestine and colorectal segment.

Putting all results together, there were significant correlations in the colorectal segment between calbindin-D9k vs. VDR, r=0.73 (p<0.01) and calbindin-D9k vs. cdx-2, r=0.52 (p<0.05). Between the two transcription factors, there was a significant correlation in the proximal small intestine (VDR vs. cdx-2, r=0.69, p<0.01), while no correlation was evident in the colorectal segment.

DISCUSSION

As a novel tool to clarify the mechanism of calcium absorption, we used the fact that FOS increases calcium...
absorption and that this increasing effect takes place in the colorectal segment (7, 27). To analyze the mechanism by which FOS increases calcium absorption, the mRNA expression of calbindin-D9k and two transcription factors, VDR and cdx-2, was determined in the proximal small intestine and colorectal segment of rats fed an FOS diet. Observation of the tissues of rats fed the FOS diet indicated that all intestinal segments grew prominently compared to those of control rats. As shown in Table 2, the contents of the colorectal segment increased in acidity, and the weight of mucosal cells and mucosal DNA of both segments increased in rats fed the FOS diet more than in control rats. The correlation between the increase of weight of mucosal cells and DNA suggested the stimulated proliferation of intestinal mucosal cells. This result is consistent with our previous observation using cDNA expression array filters (28). We provided evidence that the FOS diet enhanced the expression of genes that are involved in cell proliferation. However, our observation was inconsistent with other evidence on the effects of butyrate. Among several short-chain fatty acids that were produced from FOS by colonic bacteria (29), butyrate was considered as the most likely potential factor to regulate expression of genes in the intestine (30). Moreover, butyrate was thought to be a direct factor in the action of dietary fibers that protect against colon carcinogenesis by stimulating cell differentiation and apoptosis and inhibiting cell proliferation (30). In contrast to these effects, butyrate is a stimulant of normal colonic cell proliferation in vivo (31, 32), utilizing butyrate as an energy substrate, and normal colonic mucosa in vitro. Although the molecular mechanisms responsible for the effects of butyrate in colonocyte biology are not known, butyrate works differently against normal colon cells and colon cancer cells.

The calbindin-D9k protein calculated on the basis of both protein and DNA showed the same change as a result of the FOS diet—a decrease in the proximal small intestine and an increase in the colorectal segment. Moreover, there was a significant correlation between changes of protein and mRNA and transcriptional regulation was suggested. Furthermore, these results suggest that the increase of calcium absorption resulting from FOS was caused by the transcellular active transport of calcium rather than paracellular passive transport.

Several intestinal transcription factors: HNF-1α, VDR, and cdx-2, and their corresponding cis-elements have been analyzed (33–36). Their binding sites in the calbindin-D9k gene are adjacent to the TATA box and to each other (33–36). Thus, it is tempting to speculate that these transcription factors are important regulators of calbindin-D9k gene expression. During the process by which the FOS diet increases calcium absorption, the interrelationship between calbindin-D9k and VDR and cdx-2 would be reflected as changes in their mRNA levels. Although the absorption of ingested calcium mostly occurs in the proximal small intestine (37), the increase of calcium absorption due to the FOS diet occurred in the colorectal segment (7); for this reason, mRNA was extracted from the proximal small intestine and colorectal segments.

The conclusion of our study is that calbindin-D9k is involved in the process by which FOS increases calcium absorption in the colorectal segment. The expression of Calbindin-D9k in the proximal small intestine segment seemed to be regulated in a different manner from that in the colorectal segment without the effect of VDR and cdx-2. Although neither factor correlated with calbindin-D9k in the proximal small intestine, they showed similar changes as a result of the FOS diet. The expres-
sion profile of calbindin-D9k is specific both developmentally from fetus to adult (3, 38) and regionally along the longitudinal axis of the intestine (39), with the most abundant expression in the proximal small intestine (37). A very precise and complicated network of transcription factors and butyrate has been suggested by many lines of evidence. Cdx-2 was stimulated by butyrate (40), and the cis-element that interacts with cdx-2 was identified in the VDR promoter region (41). Co-transfection of Caco-2 cells and several transgenic mice with various combinations of transcription factors has revealed a complex pattern of effectiveness that differs from the sum of the activation of any of these factors alone (39). For the same promoter, some combination among the network decides the developmental and region-dependent regulation that is specific for the genes of the intestine including calbindin-D9k (18), sucrase-isomaltase (17) and lactase-phlorizin hydrolase (42).

Even though all of the transcription factors and cis-elements were determined, complete resolution of their network would be necessary for the appropriate diet guideline to avoid calcium deficiency from the nutritional viewpoint.

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