Dietary Fructooligosaccharides Change the Concentration of Calbindin-D9k Differently in the Mucosa of the Small and Large Intestine of Rats

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ABSTRACT Previouly, we confirmed that dietary fructooligosaccharides (FOS) increase calcium absorption in rats. In this study, we examined the influence of FOS feeding on the concentration of calbindin-D9k of several intestinal segments in rats. Rats in the control group were fed a diet without FOS. Rats in the other two groups were fed the diet containing FOS at either 50 or 100 g/kg for 10 d and subjected to a calcium absorption study. On the final day of feeding, the rats were killed and the entire intestine was removed. The intestinal mucosa was collected from four segments, i.e., the proximal and distal segments of the small intestine, the cecum and the colorectum, respectively. The apparent absorption of calcium increased dose dependently (r = 0.9256, P < 0.0001). Significant positive correlations between apparent calcium absorption and the relative amounts of calbindin in both large intestinal segments were observed (cecum, r = 0.8956, P = 0.0011; colorectum, r = 0.8828, P = 0.0016). Also, significant negative correlations between apparent calcium absorption and the relative amounts of calbindin-D9k in both small intestinal segments were observed (proximal, r = −0.7149, P = 0.0304; distal, r = −0.8740, P = 0.0021). In conclusion, FOS feeding increases levels of calbindin-D9k in the large intestine, but decreases those in the small intestine. Moreover, these results suggest that part of the stimulatory effect of fructooligosaccharides relates to the transcellular route of calcium absorption in the large intestine of rats. J. Nutr. 128: 934–939, 1998.

KEY WORDS: dietary fructooligosaccharides · calcium binding protein · calcium · absorption · rats

Calbindin-D9k (CaBP), a cholecalciferol-induced calcium-binding protein has a high affinity for calcium, and the production of CaBP is stimulated by dietary vitamin D (Dufo et al. 1996) or calcium restriction (Freund and Bronner 1975, Thomasset et al. 1979). A high correlation between intestinal calcium-binding protein, including CaBP concentration, and calcium absorption has been observed (Staun and Jarnum 1988, Taylor and Wasserman 1969); therefore CaBP is thought to play an important role in intestinal calcium transport. Recently, the transcellular calcium transport system has been shown to consist of several steps, and the factors involved include not only CaBP but also the brush border membrane, calmodulin and a calcium pump as proposed previously by many investigators (Bronner 1987, Feher et al. 1989, Wasserman and Fullmer 1995). It seems likely that CaBP plays an important role in calcium transport via the transcellular route (Feher et al. 1989 and 1992, Wasserman and Fullmer 1995).

Recent studies suggest a novel way to improve calcium balance. Indigestible oligosaccharides (Ohta et al. 1993 and 1996), resistant starch (Schulz et al. 1993, Younes et al. 1996), guar gum hydrolysate (Hara et al. 1996) and inulin (Levrat et al. 1991, Rémysy et al. 1993) increase calcium absorption. Among these, the effect of fructooligosaccharides (FOS), a mixture of indigestible and fermentable sugars (Hosoya et al. 1988, Oku et al. 1984), has been well examined. We have confirmed that the main intestinal segment in which FOS exert a stimulatory effect on calcium absorption is the large intestine, i.e., the cecum, colon and rectum, in rats (Ohta et al. 1994, 1995 and 1997). However, further details of the mechanism of the stimulatory effect of indigestible carbohydrates on calcium absorption remain to be clarified. Much data support the following hypothesis. Indigestible carbohydrates reach the large intestine intact and are fermented by bacteria in the intestinal lumen, resulting in the production of organic acids such as acetate, propionate and butyrate. These acids may dissolve insoluble calcium salts in the luminal contents and increase diffusive calcium absorption via the paracellular route (Demigné et al. 1989, Schulz et al. 1993, Younes et al. 1996). According to this hypothesis, the indigestible carbohydrates do not elicit biochemical changes such as an increase in mucosal CaBP concentration or histological changes in the intestine such as the development of intestinal villi. To our knowledge, there is no reported evidence indicating that dietary components other than vitamin D (Dufo et al. 1996) or calcium influence the segmental distribution and/or concen-
was homogenized in 4 volumes of 13.7 mmol/L Tris-HCl buffer, pH 1F- tion, yielding three samples from nine rats of each group. Each sample

The mucosal scrapings of three rats were pooled for one determina-

tion of several intestinal segments in rats.

calcium and its influence on the intestinal CaBP concentra-

Also, there is no direct evidence confirming that indigestible experimental diets, the rats were subjected to a calcium (Ca) absorp-

mixture).

Chemical analysis

mmol/kg diet

1 Fructooligosaccharides (FOS, Meioligo-P, Meiji Seika Kaisha, Tokyo, Japan; concentration of oligosaccharides was >95% of total mixture).

2Prepared according to AIN-93 formulation (Reeves et al. 1993).

tration of mucosal calcium-binding proteins such as CaBP. Also, there is no direct evidence confirming that indigestible carbohydrates stimulate calcium absorption via the paracellular route. Therefore we thought that it might be possible to estimate the extent to which the transcellular route is involved in the mechanism of the stimulatory effect of indigestible carbohydrate on calcium absorption by measuring the levels of CaBP in each intestinal segment. In this study, we examined the influence of FOS feeding on the apparent absorption of calcium and its influence on the intestinal CaBP concentration of several intestinal segments in rats.

MATERIALS AND METHODS

Animals and diets. Five-week-old male Sprague-Dawley rats (Clea Japan, Tokyo, Japan) were housed in individual stainless steel metabolic cages with wire-mesh bottoms in a temperature- and humidity-controlled room (25°C and 55% relative humidity) with a 12-h light:dark cycle. Three experimental diets were used in this experiment. The composition of these diets is shown in Table 1.

Rats were divided into three experimental groups of nine. Rats in one group received a diet that contained sucrose at 100 g/kg diet (control diet). Rats in the second group received a diet that contained sucrose at 50 g/kg diet and FOS at 50 g/kg diet (5% FOS diet). The rats in the remaining group received a diet that contained FOS at 100 g/kg diet (10% FOS diet). All rats were allowed free access to water and the experimental diet for 10 d. On the final day of the experiment, the rats were anesthetized with diethyl ether. Whole blood was drawn by abdominal venous puncture and rats were killed. Ethical consideration. This study was approved by the Animal Committee of Meiji Seika Bioscience Laboratories, and the animals were maintained in accordance with their guidelines for the care and use of laboratory animals.

Tissue preparation. After washing out luminal contents with cold saline, mucosal cells were scraped from the following four segments of intestine with a glass slide: proximal small intestine (upper half), distal small intestine (the last half), cecum and colon-rectum. The mucosal scrapings of three rats were pooled for one determination, yielding three samples from nine rats of each group. Each sample was homogenized in 4 volumes of 13.7 mmol/L Tris-HCl buffer, pH 7.4, containing 0.12 mol/L NaCl and 4.74 mmol/L KCl. The superna-

tant fraction obtained by centrifugation at 39,000 × g for 30 min was measured for protein content by the method of Lowry et al. (1951), and the appropriate amount was used for Western blot analysis.

Western blot analysis. A CaBP protein expressed in bacteria was obtained by the method of Smith and Johnson (1988). For Western blot analysis, polyclonal antibody was prepared by injecting this protein into a rabbit. An aliquot of the supernatant fraction prepared as described above, containing 1 μg of protein for proximal intestine, cecum and colon-rectum or 10 μg of protein for distal intestine was analyzed by Tricine-SDS-PAGE as described by Schägger and Jagow (1987), with a 4% stacking gel prepared by the method of Laemmli (1970). All samples for each segment were electrophoresed on a separate gel; thus each gel consisted of nine sample lanes for three groups (three for each group) and a size marker lane. The proteins separated by Tricine-SDS-PAGE were transferred to a Hybond-C nitrocellulose membrane (Amersham, Arlington Heights, IL) using the Trans-Blot Cell apparatus (BioRad, Richmond, CA). For the immunoreaction, this membrane was incubated with polyclonal antibody by the method of Sesshu et al. (1995) and CaBP bands were detected by a light-emitting system, Renaissance (DuPont NEN, Boston, MA), according to the manufacturer’s instructions. Briefly, after the membrane was washed, immunogenic CaBP bands were identified using a second antibody conjugated to horseradish peroxidase followed by light emission from the oxidation of luminol. These chemiluminescence signals were detected on Fuji X-ray film (Fuji Shashin Film, Tokyo, Japan), and densities were quantified with a Shimadzu scanning densitometer CS-9000 (Kyoto, Japan). The concentrations of CaBP obtained by a densitometer were used for the calculation of total amount of CaBP. The mean values of the three lanes were calculated for each group and tested statistically.

Calcium absorption study. Six days after the start of feeding the experimental diets, the rats were subjected to a calcium (Ca) absorption study for 5 d. All feces and urine were collected for a 5-d period. The apparent absorption of Ca was calculated from the following formula: Apparent absorption (%) = (intake − fecal excretion)/ (intake) × 100.

The amount of Ca in the diets and feces was determined by

![Chemical structures of the fructooligosaccharides (FOS). FOS are a mixture of 34% 1-kestose, 53% nystose and 10% 1F-β-fructofuranosyl nystose (Meioligo-P, Meiji Seika Kaisha, Tokyo, Japan).](https://academic.oup.com/jn/article-abstract/128/6/934/4722383/FIGURE_1)
means of a sequential plasma spectrometer (ICPS-5000, Shimadzu) as described previously (Ohta et al. 1994). Before analysis, the diets and feces were dried and then micropulverized. Micropulverized samples (~100 mg) were ashed at 550°C for 24 h. The ashed samples, dissolved in 4 mL of 2 mol/L HCl, were diluted appropriately with distilled water for atomization.

**Chemicals.** Fructooligosaccharides (FOS) are a mixture of 34% 1-kestose, 53% nystose and 10% 1F-fructofuranosylnystose (Meio-P, Meiji Seika Kaisha, Tokyo, Japan). The chemical structure of FOS is shown in Figure 1. FOS were manufactured from sucrose using fructosyltransferase (Hidaka et al. 1988). Other dietary components were purchased from Oriental Yeast (Tokyo, Japan). All other reagents were of analytical grade and were purchased from Wako Pure Chemical Industries (Tokyo, Japan).

**Statistics.** Values were expressed as means ± SD for nine rats or three pools prepared from samples from three rats each. Data were analyzed by one-way ANOVA and Tukey’s test (Dawson-Saunders and Trapp 1994) (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**

**Body weight and food intake.** Initial and final body weights and total body weight gain did not differ among the experimental groups (data not shown). Total food intake in rats fed the control diet (200 ± 5 g/10 d) was significantly greater than that of rats fed either the 5% (186 ± 13 g/10 d) or 10% FOS (184 ± 10 g/10 d) diet.

**Intestinal mucosa analysis.** The weights of the mucosa of the proximal small intestine and cecum of rats fed the FOS diets, irrespective of dietary FOS level, were significantly higher than those in rats fed the control diet (Table 2). The weight of the mucosa of the colorectum in rats fed the 10% FOS diet was higher than that in rats fed the control diet. The total amount of mucosal protein in the cecum of rats fed the FOS diets, irrespective of dietary FOS level, was significantly higher than that in rats fed control diet. The total amount of mucosal protein in the colorectum of rats fed the 10% FOS diet was higher than that in rats fed the control diet.

The relative concentration of CaBP in the proximal segment of the small intestine of rats fed the FOS diets, irrespective of dietary FOS level, was significantly lower than that in rats fed the control diet (Fig. 2A, Table 2). The relative concentration of CaBP in the distal segment of the small intestine of rats fed the 10% FOS diet was significantly lower than that in rats of other groups (Fig. 2B, Table 2). The relative concentration of CaBP in the cecum of rats fed the 10% FOS diet was significantly higher than that in rats fed the 5% FOS or control diet (Fig. 2C, Table 2). The relative concentration of CaBP in the colorectal segment of the large intestine of rats fed the FOS diets, irrespective of dietary FOS level, was significantly higher than that in rats fed the control diet (Fig. 2D, Table 2).

Relative amounts of CaBP in the proximal segment of the small intestine of rats fed the 10% FOS diet were significantly higher than those in rats fed the control diet. Relative concentration of CaBP in the distal segment of the small intestine of rats fed the FOS diets, irrespective of dietary FOS level, were considered significant at *P* < 0.05.

<table>
<thead>
<tr>
<th>Mucosa, g/3 rats</th>
<th>Control diet</th>
<th>5% FOS diet</th>
<th>10% FOS diet</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small intestine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal intestine</td>
<td>3.414 ± 0.086b</td>
<td>4.063 ± 0.280a</td>
<td>4.463 ± 0.160a</td>
<td>0.002</td>
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<tr>
<td>Distal intestine</td>
<td>2.817 ± 0.085</td>
<td>2.653 ± 0.075</td>
<td>3.059 ± 0.385</td>
<td>0.179</td>
</tr>
<tr>
<td>Large intestine</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Cecum</td>
<td>0.291 ± 0.033b</td>
<td>0.534 ± 0.048a</td>
<td>0.647 ± 0.143a</td>
<td>0.007</td>
</tr>
<tr>
<td>Colorectum</td>
<td>0.427 ± 0.008b</td>
<td>0.486 ± 0.043ab</td>
<td>0.628 ± 0.084a</td>
<td>0.013</td>
</tr>
<tr>
<td>Total mucosal protein, mg/3 rats</td>
<td>232.4 ± 25.8</td>
<td>275.0 ± 52.2</td>
<td>315.7 ± 14.9</td>
<td>0.009</td>
</tr>
<tr>
<td>Small intestine</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Proximal intestine</td>
<td>100.0 ± 22.1a</td>
<td>64.7 ± 4.9b</td>
<td>52.5 ± 7.1b</td>
<td>0.013</td>
</tr>
<tr>
<td>Distal intestine</td>
<td>100.0 ± 18.9a</td>
<td>95.6 ± 17.1a</td>
<td>43.6 ± 8.4b</td>
<td>0.007</td>
</tr>
<tr>
<td>Large intestine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cecum</td>
<td>16.5 ± 5.7b</td>
<td>30.6 ± 4.6a</td>
<td>30.9 ± 2.3a</td>
<td>0.011</td>
</tr>
<tr>
<td>Colorectum</td>
<td>16.8 ± 4.3b</td>
<td>21.8 ± 1.0ab</td>
<td>28.3 ± 1.4a</td>
<td>0.006</td>
</tr>
</tbody>
</table>

1 Values are means ± SD, *n* = 3. The mucosa samples from three rats were pooled. *a,b* Values in a row with no common superscript letters are significantly different, *P* < 0.05.

2 Relative amount of CaBP (% control · mg protein) = concentration of CaBP (mol/100 mol) × total protein (mg).
lower than those in rats fed the control diet (Table 2). Relative amounts of CaBP in cecum of rats fed the 10% FOS diet were significantly higher than those in rats fed the control diet (Table 2). Relative amounts of CaBP in the colorectal region of rats fed the FOS diets, irrespective of dietary FOS level, were significantly higher than those in rats fed the control diet (Table 2).

**Ca absorption.** Dietary Ca intake did not differ among the three groups (Table 3). Fecal excretion of Ca in rats fed the control diet was significantly higher than that in the rats fed either of the FOS diets. Fecal excretion of Ca in rats fed the 10% FOS diet was significantly lower than that in rats fed the 5% FOS diet. Ca absorption in rats fed the control diet was significantly lower than that in rats fed the 5% FOS diet. FOS concentration in the experimental diet and apparent Ca absorption were dose dependent ($r = 0.9256, P = 0.0001$).

**Correlation between Ca absorption and the relative amount of CaBP.** Significant negative correlations between Ca absorption and the relative amounts of CaBP in either the proximal ($r = -0.7149, P = 0.0304$) or distal ($r = -0.8740, P = 0.0021$) segments of the small intestine were observed (Fig. 3A, B). There were significant positive correlations between Ca absorption and the relative amounts of CaBP in both the cecum ($r = 0.8956, P = 0.0011$) and the colorectum ($r = 0.8828, P = 0.0016$) (Fig. 3C, D).

**DISCUSSION**

To our knowledge, there is no reported evidence that dietary components other than vitamin D (Wasserman and Taylor 1966, Duflot et al. 1996) or calcium influence the segmental concentration and/or distribution of calbindin-D9k (CaBP) in the intestine. In this study, feeding fructooligosaccharides increased the levels of CaBP in the large intestine, i.e., the cecum and colorectum, whereas it decreased levels in the small intestine in both proximal and distal segments in a dose-dependent manner (Table 2 and Fig. 3). All of the rats were fed experimental diets containing sufficient levels of vitamin D and Ca (Reeves et al. 1993). Therefore, our findings demonstrate that FOS is a dietary factor that affects the concentration or amount of mucosal CaBP. Moreover, the results suggest that the concentration or amount of CaBP is regulated independently in the large and small intestine.

It has been reported that a high correlation is observed between the mucosal calcium-binding proteins, including CaBP concentration, and Ca absorption (Taylor and Wasserman 1969). Thus the increase in mucosal CaBP concentration is indicative of an improvement in Ca absorption from the intestinal segment. Previously, we confirmed that at least one-half of the stimulatory effect of FOS on calcium absorption takes place in the colon and rectum in rats (Ohta et al. 1995). Several authors have reported that other indigestible carbohydrates also enhance calcium absorption from the large intestine, i.e., the cecum, colon and rectum (Demenge 1989, Levrat et al. 1991, Rémesy et al. 1993, Schulz et al. 1993). In this study, the amounts of CaBP in both the cecum and colorectum were increased by FOS feeding. Moreover, positive correlations between Ca absorption and the amount of CaBP in either the cecum or the colorectum were observed. Results of this study, demonstrating the stimulatory effect of dietary FOS on levels of CaBP, are consistent with observations indicating that FOS exert a stimulatory effect on calcium absorption in the large intestine.

The mechanisms of the stimulatory effects of indigestible...
calcium absorption via the paracellular route (Deminge 1989, there is no information demonstrating that CaBP is involved in calcium salts in the luminal contents and increase diffusive absorption remains to be clarified, but it has been suggested that CaBP must influence intracellular Ca transport (Fullmer et al. 1979). Diffusive calcium absorption via the paracellular route likely is not regulated. The detailed role of CaBP in Ca absorption is not yet been clarified. At present, one hypothesis endorsed by several authors is as follows. Indigestible carbohydrates reach the large intestine intact and are fermented by bacteria in the intestinal lumen, resulting in the production of organic acids such as acetate, propionate and butyrate. These acids may dissolve insoluble calcium salts in the luminal contents and increase diffusive calcium absorption via the paracellular route (Demingle 1989, Schulz et al. 1993, Younes et al. 1996). However, some results show that a regulatory mechanism controlling Ca absorption from the large intestine exists (Ohta et al. 1997). It has been reported that calcium binding proteins, including CaBP, exist in the large intestine (Petith et al. 1979, Wilson et al. 1981) and that calcium absorption from the large intestine is regulated (Fevus et al. 1981, Nellans and Goldsmith 1981, Petith et al. 1979). Diffusive calcium absorption via the paracellular route likely is not regulated. The detailed role of CaBP in Ca absorption remains to be clarified, but it has been suggested that CaBP must influence intracellular Ca transport (Fullmer et al. 1996, Wasserman and Fullmer 1995). To our knowledge, there is no information demonstrating that CaBP is involved in diffusive calcium absorption via the paracellular route. Therefore from all of these findings, it seems likely that indigestible carbohydrates including FOS stimulate Ca absorption not only via the paracellular route, but also via the transcellular route. At the least, FOS feeding affects the state of intracellular Ca by increasing the levels of CaBP.

The amounts of CaBP in both the proximal and distal segments of the small intestine decreased in response to FOS feeding. A simple explanation is that Ca absorption from the small intestine decreased in rats fed the FOS diet. We speculate that the decrease in levels of CaBP in the small intestine may be a response compensating for the increase in Ca absorption from the large intestine. We previously reported that a similar amount of Ca was absorbed when Ca was supplied either to the stomach or to the cecum of rats (Ohta et al. 1997). After long-term FOS feeding, the stimulatory effect of FOS on Ca absorption disappeared (Shimura et al. 1991). It appears that the extent of Ca absorption is regulated throughout the entire intestine. We do not have an irrefutable explanation for the mechanism of the stimulatory effect of FOS on the levels of mucosal CaBP but propose one hypothesis as follows. In chick kidney primary cell cultures, it has been reported that sodium butyrate promotes an increase in levels of 1,25-cholecalciferol receptor activity and CaBP D28k (Anita and Anthony 1992). Butyrate is produced by luminal fermentation of FOS, and it is possible that this butyrate may stimulate CaBP D9k production by mucosal cells in the large intestine by a mechanism similar to the stimulation of CaBP D28k in the chick kidney primary cell.

Dietary indigestible carbohydrates enhance the proliferation of intestinal mucosal cells in both the large and small intestine (Goodlad 1987, Jacobs 1983). In this study, FOS feeding increased the wet weight and total protein of mucosa in both the large and small intestine. These results agree with previous findings. On the other hand, short-chain fatty acid supplementation also promotes the proliferation of mucosal cells in both the large and small intestine in rats (Sakata 1987). Therefore the stimulatory effect of indigestible carbohydrates on the proliferation of intestinal mucosal cells is based on the production of short-chain fatty acids from these carbohydrates by bacterial fermentation in the intestinal lumen. However, there is almost no information available concerning the functional changes occurring under these conditions, such

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**TABLE 3**

<table>
<thead>
<tr>
<th>Calcium absorption in rats fed control or fructooligosaccharide (FOS)-containing diets during the last 5 d of the 10-d experiment</th>
<th>Intake</th>
<th>5% FOS</th>
<th>10% FOS</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmol Ca/d</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intake</td>
<td>2.72 ± 0.04</td>
<td>2.60 ± 0.13</td>
<td>2.73 ± 0.17</td>
<td>0.073</td>
</tr>
<tr>
<td>Fecal excretion</td>
<td>1.02 ± 0.07a</td>
<td>0.58 ± 0.14b</td>
<td>0.29 ± 0.11c</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Apparent absorption</td>
<td>1.70 ± 0.05c</td>
<td>2.02 ± 0.16b</td>
<td>2.44 ± 0.15a</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

1 Values are means ± SD, n = 9. a,b,c Values in a row with unlike superscript letters are significantly different, P < 0.05.
as changes in nutrient absorption. FOS feeding leads to a decrease in Ca absorption from the small intestine as described above, and the increase in proliferation of intestinal mucosal cells does not directly indicate an improvement in intestinal functions such as nutrient absorption. Clinical trials to assess the improvement in intestinal function due to short-chain fatty acids such as acetate propionate and butyrate supplementation have been done (Frankel et al. 1994, Koruda et al. 1990, Kriple et al. 1989). Therefore, the above-mentioned point may be an important concern in the future.

In conclusion, FOS feeding leads to an increase in levels of CaBP in the large intestine, but a decrease in the levels in the small intestine. These results suggest that FOS stimulate Ca absorption not only via the paracellular route, but also via the transcellular route from the large intestine. Moreover, the amount of mucosal CaBP is regulated independently in the small and large intestine.

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LITERATURE CITED


