Calcium is involved in many biochemical reactions as a main mediator of signal transduction, and calcium homeostasis is strictly regulated. Bone, considered a storehouse of calcium, has been intensely studied in both medical and nutritional contexts, and the role of bone is emphasized by the increasing number of patients with skeletal diseases, notably osteoporosis. To increase the calcium intake from diet, many nutritional guidelines and interventional trials have been implemented in Japanese society; however, calcium intake remains low (1). While increasing dietary calcium intake is important, increasing calcium absorption in the gastrointestinal tract also may be a critical approach. Indeed, over the years many foodstuffs have been developed with the aim of increasing calcium absorption; one example is that of indigestible fructooligosaccharides (FOS), which have been demonstrated to increase calcium absorption in the gastrointestinal tract (2). However, the mechanism whereby FOS promote calcium absorption remains unclear.

Calcium absorption by the gastrointestinal tract has two routes, transcellular and paracellular. Several molecules have been reported to be involved with the transcellular route, including: transient receptor potential vanilloid type 6 (TRPV6/CaT1/ECaC2), which is localized to the luminal side of small intestine mucosal epithelial cells, where the molecule promotes calcium uptake; calbindin-D9k, which binds and transports intracellular calcium; and plasma membrane calcium-ATPase 1b (PMCA1b), which is localized to the basolateral side of small intestine mucosal epithelial cells, where the molecule facilitates the transport of calcium from cells to blood (3).

The determination of calcium content within segments along the length of the intestine showed that the promotion of calcium absorption by the colon in rats (4). While increasing dietary calcium intake is important, increasing calcium absorption in the gastrointestinal tract also may be a critical approach. Indeed, over the years many foodstuffs have been developed with the aim of increasing calcium absorption; one example is that of indigestible fructooligosaccharides (FOS), which have been demonstrated to increase calcium absorption in the gastrointestinal tract (2). However, the mechanism whereby FOS promote calcium absorption remains unclear.

Calcium absorption by the gastrointestinal tract has two routes, transcellular and paracellular. Several molecules have been reported to be involved with the transcellular route, including: transient receptor potential

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**Note**

**Short-Chain Fatty Acids Increase the Level of Calbindin-D9k Messenger RNA in Caco-2 Cells**

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**Summary** Fructooligosaccharides (FOS) are indigestible oligosaccharides that increase the expression of calbindin-D9k and consequently increase calcium absorption by the colon in rats. The molecular mechanism of the increased expression of calbindin-D9k resulting from FOS ingestion has not been elucidated. Short-chain fatty acids (SCFAs), namely, fermentation products of FOS by intestinal bacteria have been hypothesized as direct effectors of calbindin-D9k gene expression. To test this hypothesis, SCFAs were added to Caco-2 human intestinal epithelial cells, and changes in the levels of transcription of genes for calbindin-D9k, and transcription factors (vitamin D receptor: VDR, caudal homeobox-2: Cdx-2, hepatocyte nuclear factor 1-α: HNF1-α) were determined by quantitative reverse transcription polymerase chain reaction. Addition of sodium propionate or sodium butyrate to cell cultures increased levels of calbindin-D9k mRNA to 731% ($p<0.05$) and 321% ($p<0.05$), respectively. However, addition of these SCFAs did not affect the levels of mRNA VDR, Cdx-2, or HNF1-α. In conclusion, addition of SCFAs to cultured Caco-2 cells results in elevation of calbindin-D9k mRNA, consistent with the expected role of SCFAs as mediators of the increase of calcium absorption in rats that were fed with FOS.

**Key Words** fructooligosaccharides, short-chain fatty acids, calbindin-D9k, calcium absorption, Caco-2 cells

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**Materials and Methods**

**Cell culture.** Caco-2 human intestinal epithelial cells...
Cell treatments and RNA isolation. Cells were plated at 1.0 × 10^5 cells/cm² in 6-well plates in quadruplicate and grown for 24 h before the addition of SCFAs to the culture medium, as indicated below. Sterile stock solutions of sodium salts of acetate, lactate, propionate, and butyrate were prepared in PBS. The medium was changed every other day.

For the time-course experiments (Fig. 2A, C), cells were cultured with or without 1.0 mmol/L sodium propionate or sodium butyrate. Levels of calbindin-D9k mRNA were measured 9, 11, 13, and 15 d after addition of the SCFA. For the dose-response experiments (Fig. 2B, D), cells were cultured in DMEM supplemented with 0, 0.5, 1.0, 2.0, or 5.0 mmol/L of sodium propionate or sodium butyrate for 11 d, and levels of calbindin-D9k mRNA then were measured.

Following experimental treatment, cells were washed three times with ice-cold PBS. Cells were harvested from each plate and total RNA was isolated. RNA preparation was conducted as described by Fukushima et al. (6). For each SCFA concentration or time point, four wells were assayed.

Northern blot analysis. Northern blot analysis was performed as described in our previous paper (7). Poly(A)^+ RNA was prepared using Oligotex-dT30 super (Takara Shuzo, Kyoto, Japan) in accordance with the manufacturer’s instructions. Calbindin-D9k cDNA (8) was kindly provided by Dr. H. F. DeLuca of the University of Wisconsin-Madison. A 270 bp EcoRI fragment from the plasmid plCaBP-270 carrying the coding sequences of calbindin-D9k was used as the probes for Northern blot analysis. For β-actin detection, full-length β-actin cDNA (9) was used.

Real-time reverse transcription polymerase chain reaction (RT-PCR). First-strand cDNA synthesis and real-time RT-PCR were performed using a High Capacity cDNA Reverse Transcription kit (Life Technologies Corp.) and TaqMan Universal Master Mix II (Life Technologies Corp.), respectively. Primers and probe for calbindin-D9k (Assay ID: Hs00187854_m1), vitamin D receptor (VDR; Assay ID: Hs00172113_m1), caudal homeobox-2 (Cdx-2; Assay ID: Hs01078080_m1), and hepatocyte nuclear factor 1α (HNF1-α; Assay ID: Hs00167041_m1) were obtained from TaqMan gene expression assays (Life Technologies Corp.). Analysis was conducted using an ABI PRISM 7700 Sequence Detector (Life Technologies Corp.). Gene expression was quantified using the comparative threshold cycle method (10). Target mRNA expression was normalized to that of 18s ribosomal RNA.

Statistical analysis. Results are expressed as mean ± SEM. All data were analyzed using SPSS 15.0J for Windows software (SPSS Japan Inc., Tokyo, Japan). Time-course experiments (Fig. 2A, C) were tested using Student’s t-test for comparing identical times. Dose-response experiments (Fig. 2B, D) were tested by 1-way ANOVA with a post hoc Dunnett’s test for comparing groups to the control without sodium propionate or butyrate.
Results

After 6 d of subculture, cell cultures became confluent and levels of calbindin-D9k mRNA were detectable (Fig. 1A). Experimental cells were cultured with sodium butyrate from the beginning and calbindin-D9k mRNA levels increased at 10, 12, and 14 d (Fig. 1A), compared with control cells (cultured without sodium butyrate). However, addition of sodium butyrate to confluent cells at 6 d did not increase the levels of calbindin-D9k mRNA (Fig. 1B).

Sodium acetate, lactate, propionate, and butyrate were added from the beginning of cell culture. Levels of calbindin-D9k mRNA were compared with control cells at 11 d after adding SCF A (Table 1). Sodium propionate and sodium butyrate increased calbindin-D9k mRNA 731% (p<0.05) and 321% (p<0.05), respectively (Table 1). However, sodium acetate and sodium lactate did not affect the levels of mRNA (Table 1).

Figure 2 shows the time-course experiments (Fig. 2A, C), and the dose-response experiments (Fig. 2B, D). Compared with control cells (incubated without sodium propionate or sodium butyrate), calbindin-D9k mRNA levels increased through 11 d after the addition of sodium butyrate (p<0.05) or sodium propionate (p<0.05) and decreased thereafter (Fig. 2A, C). Levels of calbindin-D9k mRNA were higher (p<0.05) than control in the presence of sodium propionate or sodium butyrate, peaking with supplementation at 1.0 mmol/L of SCFA.

Table 1. Effect of short-chain fatty acids (sodium acetate, lactate, propionate and butyrate) on mRNA levels of Calbindin-D9k in Caco-2 cells.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Sodium acetate</th>
<th>Sodium lactate</th>
<th>Sodium propionate</th>
<th>Sodium butyrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calbindin-D9k</td>
<td>100±4.40</td>
<td>115±9.50</td>
<td>100±13.0</td>
<td>731±86.1*</td>
<td>321±23.7*</td>
</tr>
</tbody>
</table>

Values are presented as mean±SEM (n=4).

* Different from control, p<0.05.
Table 2. Effect of short-chain fatty acids (sodium propionate and butyrate) on mRNA levels of vitamin D receptor, caudal homeobox-2 and hepatocyte nuclear factor 1-α in Caco-2 cells.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Sodium propionate</th>
<th>Sodium butyrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of control</td>
<td>100±1.96</td>
<td>103±3.53</td>
<td>102±4.48</td>
</tr>
<tr>
<td>VDR</td>
<td>100±6.28</td>
<td>104±3.50</td>
<td>125±7.66</td>
</tr>
<tr>
<td>Cdx-2</td>
<td>100±2.43</td>
<td>122±4.93</td>
<td>122±9.06</td>
</tr>
<tr>
<td>HNF1-α</td>
<td>9.06</td>
<td>3.50</td>
<td>7.66</td>
</tr>
</tbody>
</table>

Values are presented as mean±SEM (n=4).
VDR, vitamin D receptor; Cdx-2, caudal homeobox-2; HNF1-α, hepatocyte nuclear factor 1-α.

(Fig. 2B, D). However, addition of sodium propionate or sodium butyrate did not affect the levels of mRNA VDR, Cdx-2, and HNF1-α (Table 2).

Discussion

Indigestible oligosaccharides, FOS, reach the colon through the small intestine and produce SCFAs as products of fermentation (11). SCFAs lower intestinal lumen pH and the acidity increases calcium absorption by converting calcium to a soluble form (12). In the present study, it has been hypothesized that SCFAs promote the expression of the calbindin-D9k gene in the rat colon. The molecular mechanism of SCFA promotion of the expression of calbindin-D9k gene was tested using Caco-2 human intestinal epithelial cells.

Extensive experiments and analyses have shown that, in the rat, the transcellular absorption of calcium in the duodenum is a linear function of the cellular content of calbindin-D9k (13). The luminal contents remain in the colon for several hours, but reside for several minutes in the duodenum. During this long stay in the colon, SCFAs are produced by FOS fermentation; the resultant products are assumed to promote the calcium absorption mediated by increased levels of calbindin-D9k protein.

Levels of calbindin-D9k mRNA were not detected at the earliest stage of cell culture, when Caco-2 cell density was low (Fig. 1A). As cell proliferation achieved confluence, cell differentiation was initiated, along with the beginning of calbindin-D9k gene expression (Fig. 1A). The results showed that the expression of calbindin-D9k gene was significantly elevated in the presence of propionate or butyrate (Table 1). However, addition of these SCFAs to confluent cells did not increase the levels of calbindin-D9k mRNA (Fig. 1B). Therefore, these SCFAs were added to cells before they achieved confluence. To eliminate the possibility that the apparent increase of calbindin-D9k mRNA level was caused by a change in the cell proliferation, the DNA content per culture dish was not significantly different between the control and SCFA-supplemented cultures (data not shown). Thus, the increased levels of calbindin-D9k mRNA were not the effect of increased proliferation of cells. Furthermore, several studies showed that sodium butyrate at 1.0–2.0 mmol/L promoted differentiation of Caco-2 cells (14, 15). In our experiment, we suggest that propionate and butyrate enhanced cell differentiation, not cell proliferation.

Concerning the effect of propionate and butyrate on calbindin-D9k transcription, there are two possibilities. First, these SCFAs directly enter the cell and affect the gene expression, inhibiting histone deacetylase activity (16, 17). Second, these SCFAs bind SCFAs specific receptors and affect the gene expression by the intracellular signal transduction. Two G-protein-coupled receptors, GPR41 (FFA3) and GPR43 (FFA2), which are present on the cell membrane of the intestinal epithelial cells, are possible candidates for the regulator of calbindin-D9k transcription, since GPR41 and GPR43 bind propionate or butyrate (18, 19).

Transcription factors VDR, Cdx-2, and HNF1-α are direct activators of calbindin-D9k expression in the intestine (20–23). Previous studies demonstrated that the addition of 1.0 mmol/L Sodium butyrate to Caco-2 cells for 48 h induced the expression of Cdx-2 (24). However, the result in Table 2 in the present study showed that levels of mRNAs of VDR, Cdx-2, and HNF1-α were unaltered by the presence of SCFAs for 11 d. This difference may be caused by the length of treatment time. Thus, VDR, Cdx-2 and HNF1-α might not be members of the signal-transduction pathway from SCFAs to calbindin-D9k expression. We suggest the existence of some other regulatory factor. Further studies will be needed to clarify the mechanism of calbindin-D9k gene induction. Furthermore, it seems to be necessary to conduct an experiment with net apical-to-basolateral calcium transport across Caco-2 cell monolayers in the future.

Conclusions

In this study using Caco-2 human intestinal epithelial cells, we propose that SCFAs, namely, fermentation products of FOS, increase calbindin-D9k mRNA on a molecular basis through involvement in the promotion of calcium absorption by FOS in rats.

Acknowledgments

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