The Effect of Fructooligosaccharides was Analyzed by cDNA Expression Arrays

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Summary We have already reported that indigestible fructooligosaccharides (FOS) increased calcium absorption in rat large intestines and that calbindin-D9k (CaBP), which is an intestine-specific calcium-binding protein, is involved in that increasing effect. In this study, not only the CaBP gene, every gene that changed expression profiles as the result of FOS feeding was identified by cDNA expression arrays. Sprague-Dawley male rats were fed an experimental diet containing 10% FOS for 10 d. To compare gene expression with rats fed a control diet, total mRNA was extracted from the colorectum and analyzed using a Rat cDNA Expression Arrays filter. This arrays filter contains probes of 588 genes, and 195 of them showed detectable changes in their expression by FOS feeding. There were six genes that increased their expression more than twice that of the control. Among them, genes related to the induction of cell growth such as Map kinase 1 and Max were included. Expressions that decreased to less than half were observed in 20 genes, such as somatostatin and prohibitin, which prohibit cell growth. These results are consistent with the other observation that FOS increases cell growth in the colorectum. This approach has revealed that cDNA array technology is an effective tool for nutritional sciences that involve the regulation of a large number of genes, especially for molecular mechanisms of regulation, by nutritional constituents.

Key Words cDNA arrays, calbindin-D9k, fructooligosaccharides, gene expression, rat

Recent technological advances have provided a powerful tool, DNA arrays, for parallel analysis of complex biological problems. It has enabled an important experimental approach for identifying changes at the level of the individual mRNA molecule during important cellular transitions. Before this technology, research on the physiological effects of dietary constituents was conducted gene by gene.

Concerning calcium absorption, there is very little evidence on the dietary constituents at the molecular level except vitamin D. Ohta et al. reported that a fructooligosaccharide (FOS) diet increased calcium absorption positively correlating to the amount of FOS that was fed to rats (1). Moreover, it was confirmed that at least one-half of the stimulatory effect of FOS on calcium absorption takes place in the colorectum in rats (2). Other indigestible carbohydrates have also been demonstrated to enhance calcium absorption from the cecum and colorectum (3–6).

The observed increase of calcium absorption is the result of the contribution of many factors, such as Ca channel protein (7), Ca transporter (8, 9), Ca-Na exchanger (10), Ca pump (11) and some other undetermined factors. Among them, we have evidence that calbindin-D9k (CaBP), which is the intestine-specific calcium-binding protein, is involved in increasing the effect of FOS diets (12).

There arises the crucial question of whether the increase of Ca absorption is the major effect of FOS ingestion or merely a side reaction of its metabolites. However, advancements in DNA array technology have allowed the introduction of a way to a massive parallel analysis of a DNA structure and its transcripts. In an earlier, outstanding study in 1996, the structure of mitochondrial DNA was analyzed using DNA chip technology that had 135,000 oligonucleotides, constructed on a chip (13). This is a revolutionary tool that can be used to see every effect of food or dietary constituent extensively at the gene expression level, and also provides the possibility of finding new unexpected physiological effects. In nutritional sciences, gene expression profiles were already analyzed using cDNA arrays, regarding the effect of dietary protein quantity and quality (14), fish oil feeding (15, 16) and other nutrients (17).

In the present study, we investigated the molecular basis of the effect of an FOS diet on the rat colorectum using the cDNA expression arrays filter.

MATERIALS AND METHODS

Animals and diets. Five week-old male Sprague-Daw-
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Table 1. Composition of the experimental diets.1,2

|                  | Control diet | FOS diet |
|------------------|--------------|----------|
| (g/kg Diet)      |              |          |
| Casein           | 200          | 200      |
| Dextrin          | 482          | 482      |
| Corn oil         | 70           | 70       |
| Vitamin mixture  | 10           | 10       |
| Mineral mixture  | 35           | 35       |
| Cellulose        | 50           | 50       |
| Sucrose          | 150          | 50       |
| FOS              | 0            | 100      |
| L-Cystine        | 3            | 3        |

1 Fructooligosaccharides (FOS, Meloligo-P; Meiji Seika Kaisha, Tokyo, Japan; concentration of oligosaccharides was higher than 95% of total mixture).
2 Prepared according to the AIN-93G formulation (18).

ley rats (Clea Japan, 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 AIN-93G formulation (18) and Table 1 shows the composition.

Rats were divided into two groups of seven, the experimental and control groups. The experimental diet contained FOS in a 100 g/kg diet (10% FOS diet) instead of sucrose in the control diet. All rats in both groups were allowed free access to water and given 20 g of diet a day for 10 d. The diet dosage (20 g) was determined to obtain a similar growth rate for the two groups, without any remaining diet. At the end of the experiment, on day 10, the rats were sacrificed under diethyl ether anesthesia and their colorectums removed immediately. All of the experiments reported here received prior approval from the Animal Care Advisory Committee of Kagawa Nutrition University.

RNA preparation. The mucosal cells were scraped from the colorectum with a glass slide. The scraping sample of each rat was weighed and Western and Northern blotting analyses were conducted individually. For cDNA array analysis, the scraped mucosal cells of seven rats were pooled. Total cellular RNA was isolated using a commercially available kit, Wako ISOGEN (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Western blotting analysis. Western blotting analysis was basically conducted as described previously by Ohta et al. (12) with some modifications. To transfer the proteins that were separated by Tricine-SDS-PAGE, Immobilon TM-P (Millipore Corporation, Billerica, MA, USA) was used. After immunoreaction, 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 by a LAS-1000plus luminescent imaging analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Northern blotting analysis. Northern blotting analysis. For ß-actin (21) detection, a full-length ß-actin cDNA probe was used as the probe for CaBP Northern blotting analysis. For ß-actin (21) detection, a full-length ß-actin cDNA was used. Quantitation was performed using a BAS-2000 Bio-imaging Analyzer (Fuji Photo Film Co., Ltd.).

cDNA preparation. RNA sample was treated with RNase-free DNase I (Promega Corporation, Madison, WI, USA), and 32P-labeled cDNA probes were prepared from 50 µg total RNA using the BD Atlas™ Pure Total RNA Labeling System (BD Biosciences Clontech, Palo Alto, CA, USA) and [α-32P]dATP. This system includes the functions of poly (A)+ RNA purification, reverse transcriptase reaction and 32P-labeling. The 32P-labeled cDNA probe was further purified using a NucleoSpin Extraction Spin Column (BD Biosciences Clontech), and fractions corresponding to cDNA were pooled and counted in a Beckman liquid beta-spectrometer (DU-600, Beckman Coulter, Inc., Fullerton, CA, USA).

cDNA array analysis. The BD Atlas™ Rat cDNA expression array (BD Biosciences Clontech) was prehybridized in BD ExpressHy™ (BD Biosciences Clontech) including 0.1 mg/mL sheared salmon testes DNA for 30 min at 68°C and hybridized with 32P-labeled cDNA probes (ca. 1.0×106 cpm) overnight at 68°C. The filters were washed three times with 2×SSC, 1% SDS at 68°C and once with 0.1×SSC, 0.5% SDS at 68°C. After washing, the filters were then exposed to an imaging plate (Fuji Photo Film Co., Ltd.) overnight and scanned with a BAS-2000 Bio-imaging Analyzer (Fuji Photo Film Co., Ltd.). Expression levels were quantified using a macro-array analysis program (Array Gauge™ Ver. 1.1.2, Fuji Photo Film Co., Ltd.). Each filter also contained nine housekeeping genes for normalizing the hybridization signals.

Statistical analysis. Data are expressed as means ± SD. The significance of the differences between the control and FOS-diet groups was determined using Student’s t-test. Statistical significance was defined as p<0.05.

RESULTS AND DISCUSSION

FOS-induced hypertrophy of mucosal cells in colorectum

Care was taken to obtain a similar growth rate for the two groups, as described above in Materials and Methods. There was no significant difference in body weight between the control and FOS-diet group rats on day 10, whereas the weight of mucosal cells of the colorectum showed an apparent increase in FOS-diet group rats (Table 2). We have previously shown that small intestinal mucosa also showed hypertrophy in FOS-diet group rats, but an increase in large intestinal mucosa was more prominent (22) in 1969. New evidence that the colon is also a region of calcium absorption (22) was shown in rats against the accepted notion that calcium absorption occurs predominantly in the small intestine.
Table 2. Body weight and weight of colorectum mucosal cells after 10 d of feeding control or FOS diets. 

|               | Control diet | FOS diet    |
|---------------|--------------|-------------|
| Body weight, g| 203±6        | 207±9       |
| Mucosa weight, mg | 526±37      | 631±84*     |

Values are means±SD, n=7.
* Different from the control group, p<0.05 (Student’s t-test).

It was suggested simply from this observation that FOS feeding stimulated cell proliferation in the colorectum; therefore the increase in cell mass or cell surface was followed by an increase in calcium absorption.

**Molecular mechanism of the increased calcium absorption caused by FOS diets**

Although the mechanism of how FOS diets affect the stimulatory effects on calcium absorption has not yet been clarified, several hypotheses have been suggested. First, FOS reaches the large intestine intact, free from enzymatic digestion, and is fermented by enteric bacteria to produce organic acids such as acetate, propionate, and butyrate (23, 24). Although these acids have important physiological effects in their own right, they also act to lower the pH of the lumen. This luminal environment may facilitate a change in calcium salts from the insoluble to soluble form and thus increase the diffusive absorption of calcium via the paracellular route (3, 6, 25).

The second hypothesis is that organic acids produced by fermentation directly affect calcium absorption in the large intestine, with the production of a highly absorbable calcium-acetate complex that could pass through cell membranes more easily than ionized calcium (26). The third hypothesis suggests that a hypertrophy of mucosa in the large intestine causes enhanced calcium absorption. Some organic acids produced by the fermentation of indigestible carbohydrates, especially butyrate, have the effect of promoting epithelial cell proliferation (27). For the third hypothesis unlike the first and second hypotheses, many biochemical factors might be involved in the intermediate process.

It has been reported that a high correlation is observed between the mucosal calcium-binding proteins, including CaBP concentration, and Ca absorption (28). We demonstrated that FOS is a dietary factor that affects the concentration or amount of mucosal CaBP, which is down-regulated in the small intestine and up-regulated in both the cecum and colorectum (12). Moreover, positive correlations between the level of Ca absorption and the amount of CaBP in either the cecum or colorectum were observed. The results of this study indicate that CaBP is involved in the process of increasing the effect of calcium absorption as the result of a FOS diet, and that the colorectum is the primary place for this favorable effect.

Figure 1 shows the level where the expression of CaBP is regulated by FOS: transcriptional, post-transcriptional, translational or post-translational. The determination of CaBP protein and mRNA was conducted by Western and Northern blot analysis, respectively. Values were calculated as a percent compared with the control group rats. The expression of both CaBP protein (Fig. 1A) and mRNA (Fig. 1B) increased more than twice in protein and a little less than 2-fold in mRNA. From this observation, we conclude that the FOS diet up-regulated CaBP expression in the colorectum at the transcriptional level.

**Application of cDNA array technology to the study of increasing effect as the result of a FOS diet**

It is believed that CaBP is involved in the process of increasing calcium absorption in the colorectum as the result of feeding a FOS diet. We made a preliminarily analysis of the change in gene expression profile in the colorectum of FOS-fed rats and confirmed increased expression in several genes related to cell growth such as cyclinD3, c-jun, A-raf, c-H-ras, and erbB2 (data not shown). This result was consistent with the hypertrophy of the colorectum as shown in Table 2.

In this study, using the Atlas Rat cDNA expression
Table 3. Genes that are up-regulated in the colorectum of FOS-diet group rats.\(^1\)

| Gene/protein name | Average ratio\(^2\) | Genebank accession no. | Classification(s) |
|-------------------|----------------------|------------------------|-------------------|
| Max               | 2.32                 | D14447                 | Basic transcription factors |
| Mitogen-activated protein kinase 1 | 2.54 | M61177 | Intracellular kinase network member |
| Calmodulin II     | 2.59                 | X13817                 | Calcium-binding proteins |
| Tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein, \(\xi\) polypeptide | 2.20 | D17615 | Amino acid metabolism, Kinase activator and inhibitors |
| Galanin           | 2.10                 | J03624                 | Neuropeptides      |
| Fatty acid amide hydrolase | 2.11 | U72497 | Metabolism of cofactors, vitamins, and related substances |

\(^1\)The relative level of change level of each transcript is represented as a ratio of FOS-diet group rats relative to the control-group rats. Genes that increased more than 2-fold are presented in the table.

\(^2\)Average ratio for each gene was calculated from two different comparisons of the gene expression patterns of the colorectum in FOS-diet group rats.

Table 4. Genes that are down-regulated in the colorectum of FOS-diet group rats.\(^1\)

| Gene/protein name | Average ratio\(^2\) | Genebank accession no. | Classification(s) |
|-------------------|----------------------|------------------------|-------------------|
| Prohibitin        | 0.27                 | M61219                 | Functionally unclassified |
| Retinoic acid receptor; \(\beta\) | 0.47 | M81766 | Nuclear receptors |
| LIM domain containing, protein kinase | 0.34 | D31873 | Other DNA binding and chromatin proteins |
| Mitogen-activated protein kinase kinase 1 | 0.33 | Z16415 | Intracellular kinase network member |
| Phospholipase C, \(\gamma\) 2 | 0.50 | J05155 | Intracellular kinase network member |
| Rab-related GTP-binding protein | 0.48 | M94043 | Phospholipase and phosphoinositol kinases, Endocytosis |
| Rapamycin and FKBP12 target-1 protein | 0.48 | U11681 | GTP/GDP exchangers and G-protein GTPase activity modulators |
| Neuruplin-2       | 0.34                 | AFO16297               | Other intracellular transducers/effectors/modulators |
| T-cell receptor CD3, subunit \(\zeta\) | 0.48 | L08447 | Other receptors |
| Cadherin 6 (K-cadherin) | 0.49 | D25290 | Immune system proteins |
| Protein tyrosine phosphatase, receptor-type, \(c\) polypeptide (lantigen Cd45, leukocyte-common antigen/T200 glycoprotein) also RT7 | 0.48 | M10072 | Cell surface antigens, Cell-cell adhesion receptors |
| Luteinizing hormone/choriogonadotropin receptor | 0.30 | M26199 | Cell surface antigens, Protein phosphatases |
| Insulin receptor  | 0.50                 | M29014                 | Immune system proteins |
| Gastric inhibitory peptide receptor | 0.44 | L19660 | Hormone receptors |
| Organic cation transporter | 0.36 | U76379 | Protein kinases |
| Solute carrier family 15 (oligopeptide transporter), member 1 | 0.34 | D50306 | Ligand-gated ion channels, Neurotransmitter receptors |
| \(\beta\)-nerve growth factor precursor | 0.43 | M36589 | Xenobiotic transporters |
| Somatostatin      | 0.29                 | M25890                 | Sympotors and antiporters |
| Tyrosine kinase receptor ligand 2 | 0.40 | U97143 | Growth factors, cytokines, and chemokines |
| Urinary-plasminogen activator, urokinase | 0.32 | X63434 | Hormones |

\(^1\)The relative level of change of each transcript is represented as a ratio of FOS-diet group rats relative to control-group rats. Genes increased less than 2-fold are presented in the table.

\(^2\)Average ratio for each gene was calculated from two different comparisons of the gene expression patterns of the colorectum in FOS-diet group rats.
array (BD Biosciences Clontech) that contained duplicate spots of each of the 588 known genes, gene expression was successfully determined for 195 genes and compared between two filters of the FOS-diet group rats and control rats. Regarding these 195 genes, Fig. 2 shows the level of change in gene expression in the FOS-diet group rats relative to the control diet rats. Six of 195 genes had up-regulated expression greater than 2-fold (Table 3), while 20 genes decreased to less than half of the control (Table 4). In Tables 3 and 4, classification of the function of genes is based on a review of literature. Results were shown as a ratio of the FOS-diet group to the control group. Among the six genes that showed more than 2-fold up-regulation as the result of the feeding of the FOS diet (Table 3), the increase was most prominent in calmodulin, a calcium-binding protein, which has a variety of functions in intracellular signal transduction. Figure 3A and B shows the blots of mitogen-activated protein (Map) kinase 1 and Max. Map kinase 1 is a signal-transducing enzyme and related to cell proliferation (Fig. 3A). Max also showed an increase (Fig. 3B). This transcription factor works for genes that work to induce cell proliferation by forming a heterodimer with Myc (10). The up-regulation of these genes is consistent with our other observation that a FOS diet increased the growth of mucosal cells in the colorectum (Table 2).

Table 4 shows the list of genes that down-regulated by the FOS diet. Among the 20 genes, Fig. 3C and D show the blots of prohibitin and somatostatin. The former is one of the tumor suppressor proteins and prohibits cell growth (29), and the latter is an intestine-specific peptide hormone and functions to restrain digestive tract movement and cell proliferation (30). The down-regulation of these genes is consistent with the results shown in Table 2. Though Map kinase 1 was up-regulated, Map kinase kinase 1 was down-regulated. Genes related to intracellular signal transduction, phospholipase C and LIM-kinase, were down-regulated. The decreased levels of insulin receptor (31) and glucose-dependent insulinotropic polypeptide (gastric inhibitory peptide) (32), which is a potent stimulator of insulin secretion, might be a reflection of the decrease in blood glucose levels caused by the FOS diet.

Among 195 genes including enzymes, hormones, transcription factors, hormone receptors, signal transduction factors, growth factors and other elements, we could not find a new correlation between several genes introducing any important physiological interaction. The evidence regarding the few genes related to cell proliferation coincided with the observation that a FOS diet increases the mucosal cell growth of the colorectum. However, the mechanism of how the short-chain organic acids that were produced by the fermentation of FOS affect cell growth still remains to be elucidated. Any change in gene expression that suggests the regulatory mechanism of calcium absorption or the increasing effect of FOS was not detected. For these purposes, an intestine-specific cDNA arrays filter must be developed or we have to prepare it ourselves. Most of the genes of the 588 cDNA on the filter do not seem intestine-specific in their expression. If a tissue-specific cDNA arrays filter for rats was available, the array technology would be a revolutionary tool in nutritional research to resolve the molecular mechanism of nutritional constituents.

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