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Communication

## Absorptive Activity of Calcium in the Isolated Cecal Epithelium Adaptively Increased by 2 Week's Feeding of Difructose Anhydride III in Rats

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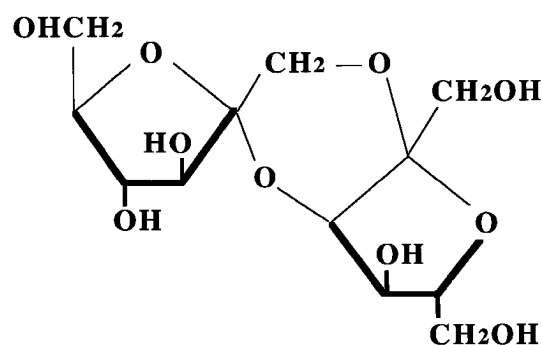
**We compared net Ca absorption and Lucifer Yellow (LY), a paracellular passage dye, permeability in the epithelium isolated from the rat small intestine, cecum, and colon after feeding with control and difructose anhydride (DFA) III diets for 14 days using the Ussing chamber system. Feeding of DFA III increased net Ca transport and LY passage in the cecal but not in small intestinal or colonic epithelium. Ability of paracellular Ca passage via Tight-junction (TJ) in the cecum was changed adaptively by feeding of DFA III. Changes in microbial fermentation may affect the functional changes of Ca transport in cecal epithelium itself.**

**Key words:** Ca absorption; indigestible saccharide; Ussing chamber; intestinal epithelium; Tight junction

Difructose anhydride (DFA) III (Fig. 1), an indigestible saccharide, consists of two fructose residues and is prepared from inulin with *Arthrobacter* sp. H65-7 fructotransferase.<sup>1)</sup> Ingestion of DFAIII increases Ca absorption in male rats as demonstrated by *in vivo* balance studies.<sup>1)</sup> Feeding DFAIII has restored Ca absorption in female rats with ovariectomy, which induces ovarian hormone deficiency followed by a reduction in Ca absorption.<sup>2)</sup> Thus, the ingestion of DFAIII might play a beneficial role in the absorption and retention of Ca in the body. In our recent report, Ca transport in the isolated intestinal epithelium was increased by luminal DFAIII application and this increase in Ca absorption occurred *via* the paracellular route, which is regulated by the Tight-junction (TJ).<sup>3)</sup> In *in vivo* balance studies using rats,<sup>1,2)</sup> DFAIII was repeatedly

ingested for at least 2–4 weeks, and the small and large intestinal mucosa was continuously exposed to this polyol through the feeding period. When DFAIII is fed for extended periods in the body, the question of whether the repeated ingestion of DFAIII adaptively changes intestinal epithelium function, especially in terms of Ca absorption activity, arises. The problem of which portion of the intestine is affected and which mechanism is involved in the functional changes in the mucosal tissue is also yet to be clarified.

Thus, the aim of this study was to examine the effects of DFAIII ingestion for 14 days on Ca transport function in the epithelial tissue of the small and large intestine in rats *in vitro*. Ca transport activity and the permeability of Lucifer Yellow (LY), a paracellular passage marker through TJs, in isolated epithelial tissue from the small intestine, cecum, and



**Fig. 1.** Structural Formula of Di-D-fructosefuranose 1,2'; 2,3' Dianhydride (difructose anhydride III) Produced from Inulin with *Arthrobacter* sp. H65-7 Inulase II (EC2.4.1.93).

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Abbreviations: DFA, difructose anhydride; TJ, Tight junction; LY, Lucifer Yellow; HBS, HEPES buffer solution; SCFA, Short-chain fatty acid

**Table 1.** Composition of Control and Test Diets

	Control diet	Test diet
Casein <sup>1</sup>	200	200
Dextrin <sup>2</sup>	400	400
Soybean oil <sup>3</sup>	70	70
Mineral mixture <sup>3</sup>	35	35
Vitamin mixture	10	10
Choline bitartrate	2.5	2.5
L-cystine	3.0	3.0
Tert-butylhydroquinone	0.014	0.014
Crystallized cellulose <sup>4</sup>	80	50
DFAI <sup>5</sup>		30
Sucrose	to make 1 kg	

<sup>1</sup> Casein (ALACID; New Zealand Daily Bond, Wellington, New Zealand).

<sup>2</sup> Dextrin (Pine-dex 4; Matsutani Chemical Industry, Tokyo, Japan).

<sup>3</sup> Mineral and vitamin mixtures were prepared according to the AIN-93G formulation.

<sup>4</sup> Crystallized cellulose (AVICEL; Asahi Kasei Ltd. Osaka, Japan).

<sup>5</sup> DFAI<sup>5</sup> (Nippon Beet Sugar MGF., Ltd. Obihiro, Japan).

colon of rats fed a control or DFAI<sup>5</sup>-containing diet was evaluated. As DFAI<sup>5</sup> itself is known to stimulate Ca absorption directly and promptly in the intestinal epithelium *in vitro*,<sup>3)</sup> we compared the Ca absorption in isolated epithelial tissue with or without the addition of DFAI<sup>5</sup> into the mucosal medium using an Ussing chamber system.<sup>4)</sup>

This study was approved by the Hokkaido University Animal Committee and the animals were maintained in accordance with the Hokkaido University guidelines for the care and use of laboratory animals. Twenty male Sprague-Dawley rats (5 wk old; Japan SLC, Shizuoka, Japan) were housed in individual stainless-steel metabolic cages placed in a room with controlled temperature (22–24°C), relative humidity (40–60%) and lighting (light 0800–2000 h). The animals were fed the stock (control) diet shown in Table 1 for an acclimation period of 7 days and then assigned to two groups with almost the same body weight. Composition of the test diet is shown in Table 1. In our previous balance studies, the ingestion of a 3% DFAI<sup>5</sup> diet increased net Ca absorption in the normal male<sup>1)</sup> and the ovariectomized female rats.<sup>2)</sup> DFAI<sup>5</sup> was kindly provided by Nippon Beet Sugar MGF, Ltd. (Obihiro, Japan). One group (control group, body weight, 203.9 ± 4.2 g, n = 10) was fed the control diet while the other group (DFAI<sup>5</sup> group, body weight, 207.7 ± 4.6 g, n = 10) was fed the test diet containing DFAI<sup>5</sup>. Rats were fed the assigned test diets for 2 weeks. All animals were allowed free access to deionized water throughout the test period. Body weight and food intake were measured every day.

On the last day of the experiment, the rats were anesthetized with pentobarbital sodium (30 mg/kg). The entire length of the small intestine (from the ligament of Treitz to the ileocecal junction), the entire cecal sac, and the entire length of the colon were

quickly removed. The outside surface and contents of the isolated intestine were washed with ice-cold (4°C) saline (154 mmol/l NaCl), and the wet weight of each specimen was measured. Segments of the small intestine (a 6-cm section from the center), cecum (whole sac) and colon (a 6-cm section from the middle and distal portion) were quickly isolated and rinsed with ice-cold HEPES buffer solution (HBS). The HBS used in this study consisted of 125 mmol/l NaCl, 4 mmol/l KCl, 6 mmol/l L-glutamine, 30 mmol/l 2-[4-(2-hydroxymethyl)-1-piperazyl] ethanesulfonic acid (HEPES), and 1.25 or 10 mmol/l CaCl<sub>2</sub>·2H<sub>2</sub>O, and it was gassed with 100% O<sub>2</sub> to maintain a constant pH of 7.4. HBS containing 1.25 mmol/l Ca was used as the bathing solution for the serosal component during the experiment and as a stabilizing solution for both the mucosal and the serosal sides between experiments. The serosa and muscle layers were removed, and stripped preparations, consisting of the mucosa and the submucosa, were mounted onto Ussing chambers (Diffusion chamber system, Corning Costar Co., Cambridge, UK) that exposed a circular area of the epithelium of 0.64 cm<sup>2</sup>. The serosal and mucosal sides of the segments were bathed in 1 ml of HBS containing 1.25 mmol/l Ca and continuously exposed to 100% O<sub>2</sub> gas. After a 30-min stabilization period, the medium on both sides of the tissue was removed by aspiration and 1 ml portions of the appropriate solutions were added to the mucosal and serosal sides.

In our preliminary evaluation using the same chamber system, a maximal Ca transport response was obtained with 100 mmol/l DFAI<sup>5</sup> on the luminal side.<sup>5)</sup> Lucifer Yellow CH dilithium salt (LY: FW 457.2, Sigma Chemical Co., St. Louis, MO) was used as the paracellular permeable marker at a concentration of 21.8 μmol/l in the 10 mmol/l Ca HBS mucosal medium. The HBS used in this study was found to remain at the constant pH of 7.4.

The prepared segments of the intestine, including segments of the small intestine, cecum, and colon, were used for experiments. Fresh HBS containing 1.25 mmol/l Ca was put into the serosal bath and 10 mmol/l Ca plus LY-HBS with or without 100 mmol/l DFAI<sup>5</sup> was put into the mucosal bath in the experiments using the small intestine, cecum, and colon preparations. After a 30-min incubation period, the serosal solution was transferred to a polyethylene test tube. The Ca concentrations in the serosal medium were measured by a colorimetric method using a commercial kit (Calcium C-Test, Wako Chemical Co., Osaka, Japan). The LY in the serosal solution was measured fluorometrically at 430 nm for excitation and 540 nm for emission (FP-550, JAS. Co., Tokyo, Japan) after the appropriate dilution of the solution with purified water.

**Table 2.** Effects of Luminal Application of DFAIII on Net Ca Absorption of Isolated Intestinal Epithelium in Rats Fed the Control or DFAIII Diet for 14 Days

Group of Diet	Application of DFAIII	Net Ca absorption (nmol min <sup>-1</sup> cm <sup>-2</sup> )		
		small intestine	cecum	colon
Control diet	DFAIII (-)	2.79 ± 0.38 <sup>b</sup>	3.59 ± 0.40 <sup>c</sup>	3.90 ± 0.59 <sup>a</sup>
	DFAIII (+)	6.48 ± 0.41 <sup>a</sup>	5.25 ± 0.38 <sup>b</sup>	4.56 ± 0.50 <sup>a</sup>
DFAIII diet	DFAIII (-)	3.21 ± 0.46 <sup>b</sup>	4.75 ± 0.49 <sup>bc</sup>	4.49 ± 0.48 <sup>a</sup>
	DFAIII (+)	5.87 ± 0.68 <sup>a</sup>	6.97 ± 0.43 <sup>a</sup>	5.41 ± 0.40 <sup>a</sup>
Two-way ANOVA				
Group of diet (G)		0.8586	0.0017	0.1590
Application of DFAIII (A)		<0.001	<0.001	0.1241
G × A		0.3065	0.5162	0.7955

Values are mean ± SEM (n = 10). Values not sharing a superscript letter are significantly different according to Duncan's test ( $P < 0.05$ ).

**Table 3.** Effects of Luminal Application of DFAIII on LY Permeability of Isolated Intestinal Epithelium in Rats Fed the Control or DFAIII Diet for 14 Days

Group of Diet	Application of DFAIII	LY permeability (pmol min <sup>-1</sup> cm <sup>-2</sup> )		
		small intestine	cecum	colon
Control diet	DFAIII (-)	2.63 ± 0.31 <sup>b</sup>	3.38 ± 0.40 <sup>c</sup>	2.85 ± 0.80 <sup>b</sup>
	DFAIII (+)	8.36 ± 1.54 <sup>a</sup>	7.17 ± 0.78 <sup>b</sup>	5.46 ± 0.84 <sup>a</sup>
DFAIII diet	DFAIII (-)	3.14 ± 0.47 <sup>b</sup>	4.75 ± 0.63 <sup>bc</sup>	3.21 ± 0.89 <sup>b</sup>
	DFAIII (+)	7.92 ± 0.92 <sup>a</sup>	10.32 ± 1.38 <sup>a</sup>	6.71 ± 1.01 <sup>a</sup>
Two-way ANOVA				
Group of diet (G)		0.9695	0.0141	0.3678
Application of DFAIII (A)		<0.001	<0.001	0.0014
G × A		0.6175	0.3152	0.6192

Values are mean ± SEM (n = 10). Values not sharing a superscript letter are significantly different according to Duncan's test ( $P < 0.05$ ).

All results are expressed as means ± SEM (n = 10). Statistical analyses were done by an unpaired *t*-test for comparison of body weight, food intake, and weights of the three intestinal portions between the two diet groups. For comparison of the two diet groups (control and DFAIII diets, G), luminal application of DFAIII (control and sugar application, A), and interaction of G × A analyses were done by two-way ANOVA following Duncan's multiple range test. A difference with  $P < 0.05$  was taken to be statistically significant.

There were no significant differences in body weight gain (8.2 ± 0.4 vs. 8.2 ± 0.7 g) or food intake (22.1 ± 0.5 vs. 21.4 ± 0.4 g) over the experimental period between groups fed the control and DFAIII diets. Similarly, there were no differences in wet weights of the small intestine (9.38 ± 0.51 vs. 9.16 ± 0.33 g) or the colon (1.27 ± 0.06 vs. 1.34 ± 0.05 g) between the two diet groups. The weights of the cecum in rats fed the DFAIII diet, however, were significantly greater (0.82 ± 0.03 vs. 1.19 ± 0.06 g,  $P < 0.001$ ) than those in rats fed the control diet. These phenomena are similar to our previous observations on the action of DFAIII.<sup>1,2</sup>

Regression analysis for the relation between the net Ca absorption and LY permeation was evaluated for three portions of the intestine, respectively. Statistically significant positive linear relations ( $P < 0.001$ )

were observed between net Ca absorption and LY passage (n = 40) in the small intestine ( $r = 0.775$ ), cecum ( $r = 0.815$ ) and colon ( $r = 0.763$ ). Thus, the increase in Ca transport and LY passage induced by the mucosal application of DFAIII occurred *via* the paracellular route in the intestinal tissue both in the control and DFA III diet groups. These results are consistent with those of our recent studies, and support the hypothesis that DFAIII directly affects the mucosal tissue of the intestine and increases net Ca transport *via* the paracellular route in which it is regulated by the function of TJ.<sup>3</sup>

The ingestion of DFAIII for 14 days affects net absorption of Ca ( $P < 0.002$ ) and permeability of LY ( $P < 0.001$ ) in isolated cecal epithelium, but not in the small intestinal and colonic epithelium (Tables 2 and 3). There were no differences in basal Ca transport or LY passage in the cecum between the control and DFAIII diet groups. However, the increase in net Ca transport and LY permeability induced by luminal DFAIII application was significantly greater ( $P < 0.05$ ) in the cecal epithelium isolated from DFAIII-fed rats than in that isolated from control diet-fed rats. The feeding of DFAIII did not affect basal Ca absorption or LY passage in the small intestinal and colonic preparations of rats fed the control or DFAIII diet. Net Ca transport and LY permeability were increased by the application of DFAIII in the

mucosal side of the small intestinal and colonic epithelial preparations in rats fed the control or DFAIII diet *in vitro*. Luminal application of DFAIII increased LY permeability, but not Ca transport in the colon isolated from rats fed the control or DFAIII diet. There were no differences in degree in the increase in Ca transport and LY passage between the diet groups. These results indicate that the ingestion of DFAIII changes Ca transport ability in the intestinal tissue itself, only in the cecum but not in the small intestine or colon.

The decrease in pH and the changes in the solubility of Ca are reported to be important factors affecting the rate of passive Ca absorption in rats *in vivo*.<sup>6,7)</sup> In the present *in vitro* experiment, pH in the mucosal medium was kept at 7.4. Since CaCl<sub>2</sub> is used as a Ca source in this study, most of the Ca exists in a soluble or ionized form in the incubation medium. The addition of DFAIII into the mucosal medium of the chamber did not affect the pH or Ca solubility. Thus, the increase in Ca transport induced by continuous DFAIII feeding might be due to functional change in the intestinal epithelium itself. Since increases in Ca absorption with the continuous feeding of DFAIII only occurred only in isolated cecal tissue, the microbial fermentation of this saccharide might be involved in this particular phenomenon in the cecum before it was isolated from the rats. Some products produced by microbial fermentation of DFAIII and/or changes in microbial flora itself may affect the permeability of the epithelial tissue and open the paracellular route. There was no significant effect of DFAIII feeding on Ca absorption and LY passage in the colon. The main function of the colon, especially the middle and distal portions used in this study, are formation of feces and its excretion. The different responses to the DFAIII ingestion between the cecal and colonic epithelium might reflect their different functions.

The infusion of short-chain fatty acids (SCFAs) into the colon or rectum stimulates Ca absorption in humans *in vivo*.<sup>8)</sup> Luminal perfusion of SCFAs also increases Ca absorption in the colon in rats.<sup>9)</sup> In our previous study using the same chamber technique, luminal application of SCFA increased net Ca transport from the mucosal side to the serosal side in isolated epithelial tissue of the cecum and colon in rats *in vitro*.<sup>10)</sup> Continuous ingestion of DFAIII obviously increased the production of SCFAs in the cecum in rats *in vivo*.<sup>1,2)</sup> When mucosal tissue is exposed to high concentrations of SCFAs produced by a DFAIII-containing diet, ordinary and basal Ca transport might be changed in the cecum in rats *in vivo*. In balance studies *in vivo*, a positive correlation exists between apparent Ca absorption and total SCFA, acetic, propionic or butyric acid concentration in the cecum of rats.<sup>11,12)</sup> Other factors caused by the fermentation process might be involved in this

functional change in the large intestine. Enlargement of the cecum and the colon induced by fermentation products extends the absorptive area and contributes to an increase in Ca absorption.<sup>13)</sup>

In conclusion, the increase in Ca absorption induced by the feeding of DFAIII occurs *via* two different mechanisms. DFAIII flowing into the lumen of the intestine directly affects the epithelial tissue, and promotes Ca absorption. Prompt and direct action of DFAIII mainly occurs in the small intestine and to a minor extent in the large intestine *in vivo* because DFAIII is thought to be fermented by microbes. Chronic and, probably, indirect action of DFAIII occurs in the large intestine, mainly in the cecum. Some factors (SCFAs, some organic acids, or unknown factors) derived from the microbial fermentation of DFAIII affect the ability of Ca transport in the mucosal tissue of the cecum. The paracellular route *via* TJ function is involved in the promotion of Ca transport in both cases.

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