Note

Cholestyramine, a bile acid sequestrant, increases cecal short chain fatty acids and intestinal immunoglobulin A in mice

Saki Nishida, Ayumu Horinouchi, Yasuki Higashimura, Reina Akahori, Kenji Matsumoto*

Department of Food Science, Ishikawa Prefectural University
1-308 Suematsu, Nonoichi, Ishikawa 921-8836, Japan

*Corresponding author: Kenji Matsumoto, Professor (Ph.D.)
Department of Food Science, Ishikawa Prefectural University
1-308 Suematsu, Nonoichi, Ishikawa 921-8836, Japan
Tel: +81-76-227-7458
Email: kmatsu@ishikawa-pu.ac.jp
Summary

Bile acid sequestrants are used as medicinal drugs to treat dyslipidemia and type 2 diabetes. We found that cholestyramine, a bile acid sequestrant, increases cecal short-chain fatty acid (SCFA) production and intestinal immunoglobulin A (IgA) in C57BL/6J mice. In a 12-week high-fat diet study, feeding cholestyramine (2% w/w) significantly promoted C2-C4 SCFAs in the cecum by ~1.6-fold and fecal IgA by 1.8-fold. In an 8-week normal-fat diet study, feeding cholestyramine (1% and 2%) increased the cecal propionic acid content by ~2.0-fold. Fecal IgA was also significantly increased at 4 weeks (1%: 1.7-fold; 2%: 2.1-fold) and 8 weeks (1%: 1.8-fold; 2%: 2.0-fold) in the normal-fat diet study. These results indicate that bile acid sequestrants may exert their physiological functions, such as intestinal IgA production, through SCFA-dependent signaling pathways.

Keywords: bile acid sequestrants, cholestyramine, short chain fatty acids, intestinal immunoglobulin A
Introduction

Bile acid sequestrants promote fecal bile acid excretion by interfering with the enterohepatic bile acid circulation, thus upregulating bile acid synthesis in the liver.\textsuperscript{1)} This increased bile acid synthesis demands hepatic cholesterol, thus taking in low-density lipoprotein (LDL) from the blood and decreasing LDL-cholesterol. Bile acid sequestrants also exert hypoglycemic effects by working on the bile acid receptors, the farnesoid X receptor and the G protein-coupled receptor TGR5.\textsuperscript{2)} Their signaling induces glucagon-like peptide 1 (GLP-1) secretion, increased energy expenditure, alterations in the gut microbiome, and improved hepatic glucose homeostasis.\textsuperscript{3, 4)}

Intestinal short-chain fatty acids (SCFAs), particularly, acetate, propionate, and n-butyrate, produced by the intestinal microbiota from dietary fibers, have received much attention because of their physiological properties through their receptors, G protein-coupled receptors GPR41 and GPR43.\textsuperscript{5)} GPR41 and GPR43 exist in intestinal epithelial cells, adipocytes, and immune cells. Thus, SCFA signaling through these receptors exerts anti-inflammatory, anti-diabetic, and anti-obesity effects.\textsuperscript{5, 6)}

Immunoglobulin A (IgA) is the most abundant antibody in mucosal secretions. IgA excludes pathogens in the intestinal lumen and neutralizes microbes and toxins within intestinal epithelial cells without damaging the tissue.\textsuperscript{7)} Intestinal IgA also regulates commensal bacterial diversity.\textsuperscript{8)} Thus, IgA plays important roles in the intestinal barrier system, and recent studies have reported that intestinal SCFAs promote intestinal IgA production.\textsuperscript{9, 10)}

In this study, we examined the effect of bile acid sequestrants on energy metabolism and found that cholestyramine ingestion increased the amounts of cecal SCFAs and fecal intestinal IgA in high-fat diet (HFD)-fed mice. These effects were confirmed in normal-fat diet (NFD)-fed mice.
Materials and Methods

Ethical approval of the study protocol

Experiments were conducted in accordance with the guidelines for the appropriate conduct of animal experiments set by the Scientific Council of Japan (2006). The Animal Experimentation Ethics Committee of Ishikawa Prefectural University (Ishikawa, Japan) approved the study protocol (No.29-14-9 and 1-14-15).

HFD study

Six-week-old male C57BL/6J mice were purchased from Charles River Laboratories Japan (Yokohama, Kanagawa, Japan). After a one-week acclimation, the mice were divided into two groups of eight by body weight. Mice in the control group (HF-C group) or the cholestyramine group (HF-Chol 2% group) were fed a high-fat D12451 diet (45 kcal% fat content, 4.73 kcal/g; Research Diets, New Brunswick, NJ, USA) supplemented with 2% cellulose or 2% cholestyramine (Sigma-Aldrich, St Louis, MO, USA). The mice were individually housed and fed their assigned diets for 12 weeks. At 10 weeks, feces were collected for 48 h, then the mice were fasted for 14 h and blood was collected from the tail vein into heparinized capillary tubes. Body weight and food intake were recorded, and the daily average food intake was calculated. At the end of the 12 weeks, the animals were euthanized with CO₂ gas without fasting, and then cecal contents were collected.

NFD study

Six-week-old male C57BL/6J mice were purchased from Charles River Laboratories Japan. The mice were fed a normal-fat D12450H diet (10 kcal% fat content, 3.85 kcal/g; Research Diets) for 2 weeks, then divided into three groups of six by body weight and fecal IgA content. Mice in each group were fed the D12450 diet (NF-C group) or a
cholestyramine-supplemented diet (1% or 2%, w/w; NF-Chol 1% group or NF-Chol 2% group) for 8 weeks. Because accidental disorder was observed at 4 weeks in one mouse in the NF-Chol 1% group, that mouse was removed, leaving 5 mice in that group. Body weight and food intake were recorded, and the daily averages food intake was calculated. Feces were collected for 48 h before cholestyramine feeding, at 4 weeks, and at 8 weeks. At the end of 8 weeks, the mice were euthanized by CO2 gas without fasting, then the blood samples, cecal contents, and large intestine were collected.

**Blood chemistry**

Plasma samples were prepared from blood samples and stored at -80°C until use. Total cholesterol, high-density lipoprotein cholesterol (HDL-C), and triacylglycerol levels in the plasma were analyzed using Cholesterol E, HDL-cholesterol E, and TG E kits (Fuji Film Wako Pure Chemical, Osaka, Japan), respectively. Plasma non-HDL cholesterol levels were calculated by subtracting the HDL cholesterol concentration from the total cholesterol concentration.

**Analyses of feces**

Feces from the 48-hour collection were lyophilized and powdered using a Multi-beads Shocker machine (Yasui Kikai, Osaka, Japan). Bile acids were extracted from the dried feces by heating at 65°C for 2 h in 90% ethanol. Bile acid content was analyzed using a TBA kit (Wako Pure Chemical). Total lipid was extracted from 100 mg of dried feces using the method described by Bligh and Dyer. Fecal IgA was extracted using phosphate-buffered saline containing a protein inhibitor cocktail (cOmplete tablets; Roche, Manheim, Germany) as previously described. Fecal IgA content was measured using a Mouse IgA kit (Bethyl Laboratories, Montgomery, TX, USA).
Measurement of SCFAs in cecal contents

SCFAs in the cecal contents were analyzed as per our previous study. The SCFA content in each sample was calculated using an internal standard method with 2-ethylbutyrate as the standard.

Morphological analysis of the large intestine

Dissected large intestine samples were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Samples were dehydrated with ethanol and embedded in paraffin wax. Paraffin sections were cut using a microtome, and stained with hematoxylin and eosin (H&E). The prepared specimens were observed using a BX50 microscope (Olympus, Tokyo, Japan).

Statistical analyses

The results are presented as the mean ± standard deviation (SD). Differences between two groups were analyzed using t-test. Differences between three groups were analyzed directly using the Tukey-Kramer multiple comparison test or following a two-way repeated-measures analysis of variance. Statistical analyses were performed using BellCurve Excel-Toukei software (SSRI, Tokyo, Japan). Differences were considered significant at P<0.05.

Results and Discussion

HFD study

Cholestyramine feeding in the HFD study increased the fecal bile acid excretion by 3.8-fold (Figure 1-A) and exerted anti-obesity, hypocholesterolemic, and hypotriglyceridemic
effects (Table 1). These effects by bile acid sequestrants have been reported in other animal
experiments.\(^{11}\)

To examine the effects of cholestyramine on intestinal conditions, we analyzed the cecal
SCFAs. Cholestyramine feeding increased the cecal and fecal contents by \(~1.5\)-fold. The
C2-C4 SCFAs and total SCFAs were significantly increased by \(~1.6\)-fold in the HF-Chol 2%
group compared with those in the HF-C group. C2-C4 SCFAs are reported to induce
intestinal IgA production through B-cell activation or GPR43 signaling,\(^9,^{10}\) thus, we
analyzed the fecal IgA content. In accordance with the increased SCFAs, fecal IgA production
was significantly increased in the HF-Chol 2% group. These results suggest that the increase
in SCFAs following cholestyramine feeding promotes intestinal IgA production.

**NFD study**

To confirm that cholestyramine feeding promoted SCFAs in the cecal contents and
induced intestinal IgA, we performed an additional NFD study. Because cholestyramine
treatment in the NFD study may cause side effects via lipid and fat-soluble vitamin deficiency,
the experimental period of the NFD study (8 weeks) was designed shorter than that of the
HFD study (12 weeks). Cholestyramine feeding in the NFD study increased the fecal volume,
which was consistent with the results of the HFD study (Figure 2-A). The increases in fecal
bile acid levels were almost the same between the NF-Chol 1% and NF-Chol 2% groups
(Figure 2-B). Moreover, the NF-Chol 1% and NF-Chol 2% groups did not significantly differ
in their anti-obesity, hypocholesterolemic, and hypotriglyceridemic effects (Table 2). These
results suggest that the cholestyramine dosage used in the NFD study was saturated even at
1% supplementation.

Figure 3 shows the amounts of the cecal SCFAs in the NFD study. Only propionic acid
was significantly increased in both the NF-Chol 1% and NF-Chol 2% groups, but a similar
tendency was observed for acetic acid and n-butyric acid. The total SCFAs in the NF-Chol 2% group was significantly higher than that in the NF-C group. In accordance with the increased SCFA production, fecal IgA was significantly increased in the NF-Chol 1% and NF-Chol 2% groups (Figure 2-C). These results indicate that cholestyramine feeding also promoted intestinal SCFA and IgA production in the NFD study.

Mucosal inflammation in the large intestine also induces intestinal IgA production.14) Thus, the large intestinal histopathology was observed (supplemental Figure 1). H&E staining showed no disorders, such as inflammation, in the large intestine in any group. Although we did not examine the expression of inflammatory cytokines in the large intestine, H&E staining demonstrated that intestinal induction of IgA by cholestyramine may not depend on mucosal inflammation.

In the present study, cholestyramine feeding induced cecal SCFAs in both the HFD and NFD studies. Moreover, intestinal IgA production, which is promoted by SCFAs, was significantly increased via cholestyramine feeding. SCFAs are the major metabolites produced by the enterobacteria; thus, cecal SCFA production depends on intestinal microbiota.5, 9, 10) HFD and NFD contain only cellulose, which is poorly fermented by the microbiota, as a dietary fiber. On the other hand, cholestyramine inhibited lipid absorption because increased fecal total lipid by cholestyramine treatment was observed (Table 1 and Table 2). Therefore, increased lipids, such as long-chain fatty acids, in cecum may be metabolized to SCFAs by the enterobacteria. In any case, cholestyramine feeding may have altered the intestinal microbiota compositions in both the HFD and NFD studies, leading to the increase in cecal SCFAs. To validate this hypothesis, we are currently investigating the influence of cholestyramine feeding on the intestinal microbiota profile.

Bile acid sequestrants are reported to exert hypoglycemic effects through TGR5 signaling, which promotes GLP-1 secretion.3,4) Intestinal SCFAs also promotes GLP-1
secretion via GPR41 and GPR43 signaling. Thus, using bile acid sequestrants to increase the SCFAs may contribute to glycemic control through GPR41 and GPR43 signaling.

Acknowledgements

We thank Traci Raley, MS, ELS, for editing a draft of this manuscript.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Materials

The online version of this article contains supplementary materials.
References


15) Koh A, De Vadder F, Kovatcheva-Datchary P, Bäckhed F. From Dietary Fiber to Host
**Table 1.** Physiological data of the high-fat diet study

<table>
<thead>
<tr>
<th></th>
<th>HF-C</th>
<th>HF-Chol 2%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Daily intake (kcal/d)</td>
<td>15.5 ± 0.5</td>
<td>16.6 ± 0.9*</td>
</tr>
<tr>
<td>Total intake (kcal)</td>
<td>1400 ± 40</td>
<td>1500 ± 80*</td>
</tr>
<tr>
<td>Initial body weight (g)</td>
<td>23.4 ± 1.0</td>
<td>23.3 ± 0.9</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>45.8 ± 2.2</td>
<td>38.6 ± 1.7**</td>
</tr>
<tr>
<td>Body weight gain (g)</td>
<td>22.4 ± 1.9</td>
<td>15.3 ± 1.5**</td>
</tr>
<tr>
<td>Blood chemistry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.20 ± 0.31</td>
<td>3.55 ± 0.19**</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/L)</td>
<td>2.77 ± 0.11</td>
<td>2.58 ± 0.15*</td>
</tr>
<tr>
<td>nonHDL-cholesterol (mmol/L)</td>
<td>1.43 ± 0.22</td>
<td>0.97 ± 0.15**</td>
</tr>
<tr>
<td>Triacylglycerol (mmol/L)</td>
<td>0.940± 0.194</td>
<td>0.747± 0.090*</td>
</tr>
<tr>
<td>Amount of cecal contents (mg/cecum)</td>
<td>183 ± 34</td>
<td>268 ± 27**</td>
</tr>
<tr>
<td>Amount of dried feces (mg/d)</td>
<td>386 ± 33</td>
<td>637 ± 56**</td>
</tr>
<tr>
<td>Total lipid in feces (mg/d)</td>
<td>22 ± 3.1</td>
<td>89.7 ± 14.1**</td>
</tr>
</tbody>
</table>

Abbreviations: HDL, high-density lipoprotein.

*P<0.05, **P<0.01
### Table 2. Physiological data of the normal-fat study

<table>
<thead>
<tr>
<th></th>
<th>NF-C</th>
<th>NF-Chol 1%</th>
<th>NF-Chol 2%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number</strong></td>
<td>6</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Daily intake (kcal/d)</td>
<td>15.5 ± 0.7</td>
<td>15.2 ± 1.1</td>
<td>14.8 ± 0.5</td>
</tr>
<tr>
<td>Total intake (kcal)</td>
<td>930 ± 43</td>
<td>909 ± 67</td>
<td>891 ± 33</td>
</tr>
<tr>
<td>Initial body weight (g)</td>
<td>25.2 ± 0.6</td>
<td>24.6 ± 1.5</td>
<td>24.5 ± 0.6</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>38.0 ± 2.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.2 ± 2.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32.9 ± 1.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Body weight gain (g)</td>
<td>12.8 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.62 ± 1.27&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.35 ± 1.48&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Blood chemistry</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.18 ± 0.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.80 ± 0.63&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.66 ± 0.30&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/L)</td>
<td>2.84 ± 0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.87 ± 0.38&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.49 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>nonHDL-cholesterol (mmol/L)</td>
<td>2.34 ± 0.71&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.932 ± 0.488&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.17 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Triacylglycerol (mmol/L)</td>
<td>1.38 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.424 ± 0.125&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.401 ± 0.106&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Amount of cecal contents (mg/cecum)</td>
<td>240 ± 18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>371 ± 57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>401 ± 62&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total lipid in feces (mg/d)</td>
<td>18.9 ± 2.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>50.9 ± 11.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>50.9 ± 13.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Abbreviations: HDL, high-density lipoprotein.
Different letters in the same row denote statistical significance (P < 0.05).
Figure 1. Analysis of the feces and cecal contents in the HFD study. A: Amount of fecal bile acid. B: Amount of SCFAs in the cecal contents. C: Amount of fecal IgA. The results represent the mean ± SD. **P < 0.01 vs. HF-C group.
Figure 2. Fecal analysis in the NFD study. A: Amount of feces. B: Amount of fecal bile acid. C: Amount of fecal IgA. The results represent the mean ± SD. Different letters denote statistical significance through the experimental period (P<0.05).
Figure 3. Cecal SCFAs in the NFD study. Different letters denote statistical significance (P<0.05).