博士論文の要約

学位論文題名

Prevention of fatty liver by dietary intervention via regulation of 12α-hydroxylated bile acid metabolism: Studies on oligosaccharide and dairy products in rats

（食餌による12α水酸化胆汁酸代謝の調節を介する脂肪肝の予防：

オリゴ糖および乳製品を用いたラットにおける研究）

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There is a growing concern about fatty liver worldwide. Excessive energy consumption increases biosynthesis of fatty acid or sterols in the liver. In this condition, biosynthesis of 12α-hydroxylated bile acids (12αOH-BAs) is also enhanced in the liver. Such alteration of BA metabolism is successfully mimicked in rats by a supplementation of cholic acid (CA) to diet, without any change in the proportion of lipids or carbohydrate contents in the diet. Moreover, the CA diet induces lipid accumulation or leaky gut. Some studies have already shown that oligosaccharides or dairy products mitigate lipid metabolism, but such preventive effects seem diverse depending on those types and almost no information is available not only in BA metabolism but also the mechanisms underlying. The purpose of the study is to investigate relationship between lipid metabolism include in those dietary interventions.

1. Ingestion of difructose anhydride III partially suppresses the deconjugation and 7α-dehydroxylation of BAs in rats fed with a CA-supplemented diet

Difructose anhydride III (di-D-fructofuranose-1,2′:2,3′-dianhydride, DFAIII), a non-digestible disaccharide, is mainly derived from inulin (Saito and Tomita. 2000). It can be used as an artificial sweetener since DFAIII has 40 to 50% of sweetness as sucrose (Kikuchi *et al*. 2004) with low energy availability (Tamura *et al*. 2006).

DFAIII enhances short-chain fatty acid (SCFA) production, including acetate, butyrate and lactate, via microbial fermentation in the large intestine (Zhu *et al*. 2018). DFAIII supplementation to diet (Minamida *et al*. 2005; Tamura *et al*. 2006) increases DFAIII-assimilating bacteria such as *Ruminococcus productus* accompanied by intestinal acidification and decreases fecal secondary BA concentration in humans (Minamida *et al*. 2006). Furthermore, as a physiological influence on host, DFAIII increases intestinal calcium absorption by fermentation in the large intestine in humans (Shigematsu *et al*. 2004) and rodents (Saito and Tomita. 2000).

Despite those physiological effects, little information is available in BA metabolism. It was difficult to discriminate between unconjugated and conjugated form of BA since conjugated

BAs disappear in the derivatization process in gas chromatography/mass spectrometry (MS) (Minamida *et al*. 2006). Liquid chromatography (LC)/MS enables us to analyze those conjugated BAs as well due to no derivatization step with high accuracy. By using LC/MS, it was investigated to specify how DFAIII influences on BA metabolism and symptoms that induced by the subsequent CA-supplementation model.

1-1. Materials & methods

1 – 2 – 1. Animals and diets in DFAIII experiment

The animal experiment was approved (permission No.14-0026) by the Institutional Animal Care and Use Committee of National University Corporation of Hokkaido University. All animals were maintained according to the Hokkaido University Manual for Implementing Animal Experimentation. Male Wistar rats (n = 32; 3-week old; Japan SLC Inc, Hamamatsu, Japan) were individually housed in stainless steel cages in a controlled environment of temperature (22 ± 2°C), humidity (55 ± 5%), and light (from 8:00-20:00). Rats had free access to water and a control diet (C) based on AIN-93G (Reeves *et al*. 1993). during the acclimation period for one week. The rats were then divided into two dietary groups, namely, C (n = 16) and D (DFAIII-supplemented diet) (n = 16). After feeding for 2 weeks, each group of rats was further divided into two groups and were fed diet with or without CA supplementation at 0.5 g/kg diet. Body weight and food intake were measured every other day. On day 33 of the test period, the rats were orally administered with a chromium (III) chloride hexahydrate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) (Downes and McDonald, 1964) solution with ethylenediamine tetra acetate 2Na (Dojindo Laboratories, Kumamoto, Japan) for the determination of urinary chromium (Cr) excretion. Urine samples for 24 h were obtained after 24 h from oral administration for gut permeability test. Fecal samples were collected from day 34 to 35 and stored at −30°C for the evaluation of BAs. On day 35, the rats were sacrificed under anesthesia by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight, Somnopentyl, Kyoritsuseiyaku Corporation, Tokyo, Japan). Blood plasma samples were collected using syringe from both abdominal aorta and portal vein with aprotinin (final concentration of 500 KIU/mL) and heparin (final concentration of 50 IU/mL). The blood samples were centrifuged, and the supernatant collected. Cecum was collected and the weights of whole cecum and cecal contents were measured. The cecal contents were diluted four times with deionized water and homogenized with Teflon homogenizer. The pH of homogenates was measured using a semiconducting electrode (ISFET pH sensor Argus; Sentron, Roden, Netherlands). Cecal contents were kept in liquid nitrogen and stored at −80°C for BA analysis. Epididymal adipose tissue was dissected and weighed. Liver tissue weight was measured, and the tissues were kept in liquid nitrogen and stored at −80°C for BA analysis.

1 – 2 – 2. Gut permeability

The concentration of Cr in urine samples was measured using an atomic absorption spectrophotometer (Z-5310, Hitachi High-Technologies Corporation, Tokyo, Japan). The proportion of urinary Cr excretion following oral administration of Cr-ethylenediaminetetraacetic acid solution was calculated as an indicator of gut permeability (Ten Bruggencate *et al*. 2005).

1 – 2 – 3. BA analysis

BA extraction and LC separation were performed with Dionex UltiMate 3000 UPLC system (Thermo Fisher Scientific corporation, San Jose, CA, USA) according to our previous study (Hagio, *et al*. 2009) with some modifications. MS was performed using Orbitrap mass spectrometer Q Exactive^{TM} (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an electrospray ionization probe under negative-ion mode. Full-scan MS spectra (from *m/z* 200–700) were acquired with Orbitrap analyzer after accumulation to a target value of 1e6 in the linear ion trap. Resolution in Orbitrap system was set to r = 17,500. Standard mass spectrometric conditions for all experiments were as follows: spray voltage, 3.8 kV; sheath gas flow rate, 35 L/h; aux gas flow rate, 10 L/h; heated capillary temperature, 250°C; aux gas heater temperature, 450°C; s-lens RF level, 100%. BA concentration was measured using nordeoxycholic acid (23-nor-5β-cholanic acid-3α,12α-diol) as an internal standard.

1 – 2 – 4. Fecal DFA III excretion

Fecal DFAIII excretion in the DFAIII-fed rats was measured based on the method as previously described (Minamida *et al*. 2005). Briefly, homogenized fecal samples were added to deionized water and sonicated. After centrifugation, the supernatant was collected and degreased with chloroform. The amount of DFAIII was analyzed using high-performance LC (Hitachi High-Tech Science Co., Tokyo) with a TSKgel Amide-80 column (4.6 × 250 mm).

1 – 2 – 5. Liver lipid parameters analysis

Lipids from liver were extracted by using Folch's method(Folch *et al.* 1957) and TAG and Chol concentrations were analyzed using Wako TAG E-test and Chol E-test (Wako pure chemical industries, Ltd., Osaka, Japan).

1 – 2 – 6. Statistical analysis

Data are presented as the mean with standard error of means (SEM). Statistical significance was evaluated using two-way analysis of variance (ANOVA). Student's *t*-test was used for comparison of DFAIII excretion between two groups of the rats fed diet with or without the CA supplementation. A P-value less than 0.05 is considered to be significant. All statistical analysis was performed using JMP software version 14.0 (SAS Institute Inc., Cary, NC, USA).

1 – 3. Results

1 – 3 – 1. Reduction of fecal DFAIII concentration

To investigate whether the ingested DFAIII was assimilated by the intestinal microbes, the fecal DFAIII excretion was determined. Fecal excretion of DFAIII was the highest on day 8 and gradually decreased thereafter. On day 21, the rats were divided into two groups and fed with or without the CA-supplemented diet to evaluate effect of the CA supplementation on fecal DFAIII excretion. As a result, the fecal DFAIII was detected at trace levels under the CA supplementation condition. A significant difference was observed between the groups on day 27, but negligible level was detected.

1 – 3 – 2. Alteration of BA metabolism in rats fed with the DFAIII diet

Thirty molecular species of BAs were analyzed using LC-MS at the end of the experiment.

TCA was the most abundant BA in the liver, intestinal contents, and portal plasma. The CA supplementation increased the concentration of TCA at these sites. Two-way ANOVA analysis revealed a synergetic effect of the CA and DFAIII supplementation on TCA concentration in the portal plasma. A similar tendency was observed in the ileal contents. The CA supplementation increased the concentration of TDCA in the liver; a significant interaction was found between CA and DFAIII. Similar changes were observed in the cecal and fecal DCA concentrations, as shown in the liver TDCA. A synergetic effect of the CA and DFAIII supplementation was observed on both CA and TCA concentrations in the cecal contents and feces. The trend in fecal 3o12α excretion resembled that observed for DCA. On the other hand, the concentration of ωMCA decreased after CA and DFAIII supplementation. Similar tendency was also observed in the feces. There was increase in 12αOH-BAs in aortic plasma.

1 – 3 – 3. Biochemical parameters in the rats fed the DFAIII diet

DFAIII-supplemented diets decreased the total food intake, final body weight, and epididymal adipose tissue weight but no significant difference was observed in liver TAG and Chol among groups. The weights of whole cecum and its contents were higher in the rats fed with DFAIII-supplemented diets than in those from the other groups and a reduction in the pH of the cecal content was observed by the DFAIII-supplementation. The pH reduction was significantly affected by the CA supplementation. The CA supplementation increased gut permeability, as observed with urinary Cr excretion, but no difference was observed in the rats fed with the DFAIII-supplementation.

1 – 4. Discussion

Extracted BAs are required to be derivatized for gas chromatography/MS analysis. Such derivatization process breaks taurine or glycine conjugation of BAs, which unable us to discriminate between conjugated and unconjugated BAs (Minamida *et al*. 2006). In the present study, we analyzed not only unconjugated BAs but also the conjugated forms of BAs using LC/MS (Hagio *et al*. 2009) that enabled us to elucidate the effect of DFAIII ingestion on BA metabolism, especially in the organs related with enterohepatic circulation.

In the rats fed control diet, the proportion of 12αOH-BAs was almost comparable that of non-12αOH-BAs in liver, ileal contents, and portal plasma. On the other hand, an increase in the proportion of non-12αOH-BAs was observed in large intestine and in feces such as βMCA and ωMCA. Those results suggest that 12αOH-BAs are selectively reabsorbed in ileal epithelial cells. This notion is supported by observations in mice (Fu and Klaassen, 2013) that show enormous excretion of ωMCA in feces rather than 12αOH-BAs. As expected, the CA-supplementation raised the concentration of 12αOH-BAs, for example TCA in enterohepatic circulation and DCA in cecal contents. The DFAIII-supplementation reduced cecal and fecal ωMCA concentrations. βMCA is converted into ωMCA in the large intestine by anaerobic bacteria, such as *Eubacterium lentum* strain (Eyssen *et al*. 1983), suggesting that the ingestion of DFAIII reduces conversion of βMCA into ωMCA by the gut microbes. Interestingly, in combination of CA with DFAIII, massive increase of TCA concentration was observed in the organs related with enterohepatic circulation accompanied by decreased fecal DCA excretion. These results suggest that 12αOH-BAs were selectively reused in enterohepatic circulation.

Simple feeding with CA-in the diet resulted in an increase in BA concentration mainly in the

organs associated with enterohepatic circulation. The CA supplementation increased DCA concentration in the large intestine and feces, indicating that the ingested CA was completely dehydroxylated into DCA by the gut microbes. On the other hand, the elevated DCA concentration by the CA-supplementation was normalized in the rats fed with DFAIII that resulted in the reduction in the pH of the cecal contents. Minamida and colleagues (Minamida *et al*. 2006) measured the conversion of CA to DCA using thin-layer chromatography and found that DCA production was significantly suppressed at pH 5.8 as compared to that observed at pH 7.5, suggesting an association between low pH and reduction in 7α-dehydroxylation under DFAIII-fed condition. The decrease in pH of the large intestine is thought to inhibit 7α-dehydroxylase activities in the luminal contents, responsible for the reduction in DCA concentration. In addition, DFAIII ingestion increased acetate and other organic acid levels in the cecal contents that may serve to reduce pH in the cecal contents (Minamida *et al*. 2005). A significant increase in the population of *Ruminococcus productus* was observed in the cecal contents of the rats fed with DFAIII. It was negatively correlated with the reduction in DFAIII excretion in the feces (Minamida *et al*. 2005). On the other hand, *R. productus* was undetected in control rats. These results suggest that DFAIII is consumed by *R. productus*, leading to their increase in the cecal contents, as was shown to assimilate DFAIII under in vitro conditions. Almost all BAs of the feces in the control rats were secondary BAs but those in the DFAIII-fed rats were primary BAs (Minamida *et al*. 2005), suggesting a less ability of 7α-dehydroxylation by altered gut microbiota in the DFAIII supplementation.

Deconjugation of BA is catalyzed by BSHs in the intestinal bacteria that hydrolyze the amino bond and liberate taurine or glycine moiety from the steroid ring of BA (Begley *et al*. 2006). In general, an enormous decrease in conjugated BA levels was observed in the large intestine. Such deconjugated BAs were subjected to secondary BA production in the subsequent dehydroxylation step (Hagio *et al*. 2009). A high concentration of TCA was observed in the large intestine of the DB-fed rats, wherein BAs underwent 7α-dehydroxylation by the gut microbiota. The suppression of BA deconjugation by the DFAIII ingestion was responsible for the subsequent reduction in secondary BA production.

A decrease in body weight was observed in the rats fed with DFAIII supplementation as compared to those fed with DFAIII-free diets. An increase in acetic acid concentration was observed in the cecum that serves as a potent stimulator to secrete anorexic gut hormones such as glucagon-like peptide-1 and peptide YY (Hira *et al.* 2017). It was recently revealed that acetic acid reduces appetite via direct influence on hypothalamus in the brain without induction of these gut hormones (Frost *et al*. 2014). In both cases, acetic acid is one of the candidates that suppresses appetite in the rats fed with DFAIII, resulting in the reduction in body weight. Furthermore, we observed a different influence of CA supplementation on BA concentrations in portal and aorta blood. The CA supplementation increased the concentration of portal BAs but not that of aorta BAs. The absorbed BAs in the ileal epithelia entered the enterohepatic circulation, as reflected in the BA profiles of the portal blood. The supplemented CA possibly enters this route rather than systemic blood as shown in the difference of BA concentration between portal and aorta plasma. This observation suggests that effect of the CA supplementation on the host may be restricted in the organs related with enterohepatic circulation. We observed that the CA supplementation might increase gut permeability. DCA is reported to disrupt gut barrier in human large intestine (Münch *et al*. 2007) and cultured

epithelial cells (Raimondi *et al*. 2008). Regardless of the significant reduction in DCA concentration observed in the cecal contents and feces following DFAIII supplementation, no influence was observed on the gut permeability. In the rats fed with the CA-supplemented diet, an increase in DCA or TCA was found in cecal contents or ileal contents, respectively. High concentration of TCA also might be involved in leaky gut in the small intestine, not in the large intestine.

BA metabolism especially in the gut induced by the CA supplementation (Islam *et al*. 2011) resembles that observed in the rats fed with a high-fat diet (Yoshitsugu *et al*. 2019), suggesting that the consumption of energy-dense diet selectively increase DCA levels in the large intestine. Such increases in DCA levels in the stool were reported in the patient with colorectal cancer, suggesting a relationship between DCA production and cancer development in the large intestine (Reddy *et al*. 1977). Only limited data are available on conjugated BAs in feces. Under normal conditions, almost no conjugated BAs were observed in the stool of humans and feces in experimental animals (Korpela *et al*. 1988; Nagengast *et al*. 1988) because of technical limitation of GC/MS. In our previous study, we successfully measured the levels of conjugated BAs in the feces of rats fed with soy pulp and *Bacillus coagulans* and found that conjugated BAs were absent in the feces (Lee *et al*. 2016). It may be rare to observe fecal-conjugated BAs following dietary intervention, as observed in the present study.

In conclusion, DFAIII altered BA metabolism, especially in the gut, through the suppression of deconjugation and 7α-dehydroxylation, which results in the reduction in DCA level in the large intestine probably through the modulation of gut microbiota. Hence, DFAIII may serve as a prebiotic source that reduces secondary BA production via suppression of 7α-dehydroxylation and deconjugation.

2. Ingestion of dairy products partially recovers the CA-induced disorders accompanied by modulation of BA metabolism

There are some reports showing that consumption of ripened cheese or fermented milk reduces hepatic lipid contents. In the following experiments, the involvement of 12αOH-BA was investigated in the reduction of liver lipids by consumption of dairy products in the CA-induced lipid accumulation model.

2-1. Cheese

A skimmed milk cheese was prepared and the ground sample was added to diet as protein source. The cheese diet partially ameliorated the CA-induced symptoms such as hepatic lipid accumulation and leaky gut. Also, the cheese diet improved plasma level of adiponectin. A negative correlation was observed between the gut permeability and the plasma adiponectin concentration. In BA metabolism, a significant decrease of CA was observed in feces. There was a massive reduction in unconjugated 12αOH-BA excretion in aortic plasma and feces of the rats fed the cheese diet. 12αOH-BA in aorta and feces might be a predictive biomarker for lipid accumulation.

2-2. Fermented milk powder (FMP)

The ingestion of FMP mitigated lipid accumulation and leaky gut, accompanied by a partial

reduction of serum aminotransferase activities. A decrease in food intake by FMP resulted in a positive correlation between portal DCA concentration and serum aminotransferase activities. A reduction of fecal DCA concentration by FMP might contribute to alleviate leaky gut. Although 12αOH-BAs remain at high level in the enterohepatic circulation there is an improvement in lipid accumulation and leaky gut by the FMP ingestion.

Taken together, significant relationships were observed between the symptoms in lipid accumulation and 12αOH-BA metabolism. Dietary intervention that regulates 12αOH-BA metabolism especially in the organs related with enterohepatic circulation is possible to ameliorate lipid accumulation and related symptoms.