Administration of Cholic Acid Inhibits Equol Production from Daidzein in Mice

Hiroko Yoshioka¹, Masamichi Watanabe², Fumio Nanba², Toshio Suzuki², Satoru Fukiya³, Atsushi Yokota³ and Toshiya Toda¹*¹

¹ Department of Innovative Food Sciences, School of Food Sciences and Nutrition, Mukogawa Women’s University, Nishinomiya, Hyogo 663–8558, Japan
² Research and Development, Fujicco Co. Ltd., Kobe, Hyogo 650–8558, Japan
³ Laboratory of Microbial Physiology, Research Faculty of Agriculture, Hokkaido University, Sapporo, Hokkaido 060–8589, Japan

(Received May 20, 2020)

Summary Equol (Eq) is a metabolite of soy isoflavone daidzein (De) produced by the intestinal microbiota. The clinical effectiveness of soy isoflavone is considered to depend on the individual ability of Eq production. Previous studies have demonstrated that habitual dietary patterns may influence the production of Eq. For example, high Eq producers consumed less fat as a percentage of energy than low Eq producers. However, the inhibitory factors of Eq production are unknown. Recently, it was reported that bile acids induced by high-fat diet consumption may be a host-related factor controlling the composition of the intestinal microbiota. In this study, we investigated the effect of cholic acid (CA) administration, a mimic of the microbiota altered by a high-fat diet, on Eq production in mice. CA administration significantly decreased the levels of the De metabolites Eq, dihydrodaidzein, and O-desmethylandolensin in the serum of mice. However, CA administration did not affect the total molar concentration of De and its metabolites. Moreover, CA administration increased the levels of secondary bile acids, particularly deoxycholic acid (DCA), which has strong antibacterial activity in the cecum contents of mice. Thus, CA administration may increase the levels of DCA, a secondary bile acid, resulting in inhibition of Eq production. These findings may help to reveal the factors inhibiting Eq production and enhance the clinical effectiveness of isoflavone intake.

Key Words isoflavone, daidzein metabolite, bile acid, deoxycholic acid, gut microbiota

Equol is a metabolite of soy isoflavone daidzein produced by the intestinal microbiota and shows higher estrogenic activity than daidzein (1). Previous studies have demonstrated that the clinical efficacy of isoflavone depends on an individual’s ability to produce equol. Only 30–50% of the human population can produce equol (2). Several studies revealed a relationship between equol production and dietary factors. For example, equol producers consumed more dietary fiber than equol non-producers. Moreover, it was reported that high equol producers consumed less fat as a percentage of energy than low equol producers (3).

Cholic acid (CA) is synthesized from cholesterol in the liver and is among the primary bile acids accounting for more than 31% of the total bile acid in humans (4). Primary bile acids are present in the bile as glycine- and taurine-conjugated bile acids. Bile acids emulsify dietary fat and liposoluble dietary nutrients through interfacial activity in the small intestine and play a role in assisting in their digestion. A high-fat diet promotes bile secretion to facilitate lipid digestion (5). Most conjugated bile acids excreted into the bile are reabsorbed in the ileum and circulated back to the liver (6). However, some bile acids flow into the large intestine and are excreted with feces. During transit to the large intestine, bile acids are deconjugated and converted to secondary bile acids by the microbiota (7). Secondary bile acids show strong bactericidal activity because of their high hydrophobicity of molecule (8, 9). Previous studies reported that high-fat diets increase Firmicutes and decrease Bacteroidetes levels in the gut microbiota of mice (10–12). Recently, Islam et al. reported that diets supplemented with cholic acid show increased total bile acids and deoxycholic acid (DCA) in the feces of rats and an increased ratio of Firmicutes to Bacteroidetes in the cecal microbiota of rats (13). Thus, as a host factor, bile acid controls the composition of the gut microbiota in rats (14).

We predicted that equol producing-bacteria have higher sensitivity than the other gut microbiota to bile acids and secondary bile acids; additionally, we hypothesized that habitual high-fat diet intake decreases the number of equol producing-bacteria in the gut microbiota and decreases or abolishes equol production ability. In this study, we investigated the effect of administration of cholic acid, which mimics changes in the microbiota cause by a high-fat diet, on equol production in mice.

*To whom correspondence should be addressed.
E-mail: ttoda@mukogawa-u.ac.jp
MATERIALS AND METHODS

Reagents. Sodium cholate hydrate, β-glucuronidase from Helix pomatia, sulfatase from Helix pomatia, and O-desmethyloflavonolensin (O-DMA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Daidzein and equol were purchased from Wako (Osaka, Japan). Dihydrodaidzein (DHD) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). All other reagents used were of the highest grade available from commercial sources.

Animal treatment. All animal experiments were approved by and carried out according to the guidelines for the Care and Use of Laboratory Animals at Mukogawa Women’s University (Acceptance No. FSN-02-2018-01-A). Male C57BL/6J mice (8 wk old) were obtained from Japan SLC, Inc. (Shizuoka, Japan) and maintained in a temperature-controlled room (22˚C) with a 12-h : 12-h light/dark cycle (lights on at 08:00). The mice were acclimatized for 14 d with free access to drinking, the mice were sacrificed under anesthesia.

The mice were sacrificed under anesthesia.

Measurement of daidzein and its metabolites in the serum. A 200-µL aliquot of serum was incubated with 200 µL sulfatase (50 U/mL) in 0.1 M acetic acid buffer (pH 4.5) for 2 h at 37˚C. The mixture was incubated with 200 µL of 200 U/mL β-glucuronidase in 0.1 M acetic acid buffer for 2 h at 37˚C. The mixture was loaded into a Sep-Pak C18 Plus Light Cartridge® (Waters Co., Milford, MA, USA) conditioned with methanol followed by water. After washing with 15% methanol, the absorbed component was eluted with 1 mL methanol. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed using a Nexera2 LC system (Shimadzu Corporation, Kyoto, Japan) connected to a triple quadrupole mass spectrometer (LC-MS 8040; Shimadzu) equipped with an electrospray ionization source. The instrument was operated under positive electrospray ionization and multiple reaction monitoring modes. Chromatographic separation was performed with a Daisopak SP-120-5-ODS-RPS Column, with a 150 mm length×2.2 mm inner diameter and a 5-µm particle size (OSAKA SODA Co., Ltd., Osaka, Japan). Isocratic elution was performed with 0.1% formic acid and methanol (40 : 60, v/v) at a flow rate of 0.2 mL/min.

The injection volume was 5 µL. The total run time was 6 min per sample. The MS operating conditions were optimized as follows: interface voltage, 4.5 kV; interface temperature, 350˚C; desolvation line temperature, 250˚C; heating block temperature, 400˚C; drying gas (N2), 15 L/min; nebulizing gas (N2), 2 L/min and collision-induced dissociation gas (argon), 230 kPa. The m/z values for each analyte were as follows: daidzein, 123.05, 163.05 and 95.00; genistein and its derivatives (5.55%), and glycitin and its derivatives (27.99%). Food and water intake were measured once a day. After 2 wk of feeding and drinking, the mice were sacrificed under anesthesia. Blood and cecum samples were collected for analysis.

Measurement of bile acids in cecal contents. The concentration of each bile acid in the cecal contents was measured by ultra-performance liquid chromatography (UPLC)-MS as previously reported (15). Briefly, methanol extraction from freeze-dried cecal contents was repeated 3 times. The pooled extracts were evaporated, dissolved in methanol, and subjected to UPLC-MS analysis (Waters). The bile acids analyzed were cholic acid (5β-cholanic acid-3α,7α,12α-triol, CA), α-muricholic acid (5β-cholanic acid-3α,6β,7α-triol, αMCA), β-muricholic acid (5β-cholanic acid-3α,6β,7β-triol, BMA), α-muricholic acid (5β-cholanic acid-3α,6α,7β-triol, αMCA), chenodeoxycholic acid (5β-cholanic acid-3α,7α-diol, CDCA), deoxycholic acid (5β-cholanic acid-3α,12α-diol, DCA), hyocholic acid (5β-cholanic acid-3α,6α,7α-triol, HCA), hyodeoxycholic acid (5β-cholanic acid-3α,6α-diol, HDCA), ursodeoxycholic acid (5β-cholanic acid-3α,7β,12α-triol, UCA), ursodeoxycholic acid

Table 1. Composition of the control and isoflavone diets.

| Ingredient      | CD  | ID  | (g/kg diet) |
|-----------------|-----|-----|-------------|
| Casein          | 200 | 200 |
| l-Cystine       | 3   | 3   |
| Corn starch     | 506.2| 506.2|
| Maltodextrin    | 125 | 125 |
| Sucrose         | 68.8| 68.8|
| Cellulose       | 50  | 50  |
| Soybean oil     | 25  | 25  |
| Lard            | 20  | 20  |
| Mineral mix S10026 | 10  | 10  |
| Dicalcium phospathe | 13  | 13  |
| Calcium carbonate | 5.5 | 5.5 |
| Potassium citrate, 1 H2O | 16.5| 16.5|
| Vitamin mix V10001 | 10 | 10 |
| Choline bitartrate | 2   | 2   |
| FD&C Red Dye#40 | 0.04| 0.04|
| FD&C Blue Dye#1 | 0.01| 0.01|
| Total           | 1,055.05| 1,055.05|
| Fujiflavone P40 | —   | 5.3  |
| Total           | 1,055.05| 1,060.35|
(5β-cholanic acid-3α,7β-diol, UDCA), cholic acid (5β-cholanic acid-3α,ol, LCA), taurocholic acid [5β-cholanic acid-3α,7α,12α-triol-N-(2-sulphoethyl)]-amide, TCA), tauro-α-muricholic acid [5β-cholanic acid-3α,6β,7α-triol-N-(2-sulphoethyl)-amide, TaOMCA], tauro-β-muricholic acid [5β-cholanic acid-3α,6β,7β-triol-N-(2-sulphoethyl)-amide, TβMCA], tauro-α-muricholic acid [5β-cholanic acid-3α,6α,7β-triol-N-(2-sulphoethyl)-amide, TaOMCA], taurochenodeoxycholic acid [5β-cholanic acid-3α,7α-diol-N-(2-sulphoethyl)]-amide, TCDDA), taurosodeoxycholic acid [5β-cholanic acid-3α,7β-diol-N-(2-sulphoethyl)]-amide, TUDCA], taurodeoxycholic acid [5β-cholanic acid-3α,12α-diol-N-(2-sulphoethyl)-amide, TDCA], taurohyodeoxycholic acid [5β-cholanic acid-3α,7α,12α-triol-N-(carboxymethyl)-amide, GCA], 7-oxo-deoxycholic acid (5β-cholanic acid-3α,12α-diol-7-one, 7oDCA), 7-oxo-lithocholic acid (5β-cholanic acid-3α,ol-7-one, 7oLCA). 12-oxo-lithocholic acid (5β-cholanic acid-3α,ol-12-one, 12oLCA). 3-oxo-12α-5β-cholanic acid (5β-cholanic acid-12α,ol-3-one, 3o12α), and 23-nordeoxycholic acid (23-nor-5β-cholanic acid-3α, 12α-diol, NDCA). The standard reagents of these bile acids were purchased from Steraloids, Inc. (Newport, RI, USA). The concentration of each bile acid was calculated as a normalized value using NDCA as the internal standard.

Statistical analysis. Statistical analysis was performed using JMP version 14.0 (SAS Institute Inc., Cary, NC, USA) and Excel 2007 (Microsoft, Redmond, WA) with the add-in software Statcel 3 (OMS Inc., Tokyo, Japan). Data are shown as the means and standard error (SE). The statistical significance of body weight, consumption, cecum and bile acids were determined using two-way analysis of variance (ANOVA), followed by Tukey Kramer multiple comparison test for post hoc analysis. The statistical significance of daidzein and its metabolites were determined using Student’s t-test. The level of significance was set to p<0.05.

RESULTS

We first examined whether CA administration inhibited equol production and daidzein metabolism in mice. Daidzein is converted by specific gut bacteria via DHD to equol and O-DMA (16, 17). Daidzein and its metabolites were not detected in the serum of the CD+DW and CD+CA groups (data not shown). In the ID+DW group, daidzein and its metabolites were detected in the serum of mice (Fig. 1). The ID+CA group showed a significantly high serum level of daidzein compared to in the ID+DW group. The ID+CA group showed significantly decreased levels of DHD and O-DMA compared to the ID+DW group and no equol was detected in the serum of mice (Fig. 1). Additionally, in the ID+CA group, the total molar concentrations of daidzein and its metabolites were not significantly changed compared to in the ID+DW group (Fig. 1).

To identify the factor that altered equol production and daidzein metabolism following CA administration, bile acid components in the cecum of mice were investigated. CA administration significantly increased total bile acids and secondary bile acids (Fig. 2A). The levels of primary bile acids in the CD+DW and ID+CA groups were higher than those of ID+DW group (Fig. 2A). Furthermore, CA administration significantly increased levels of CA, DCA, 7oDCA, 12oLCA and 3o12α in cecum of mice (Fig. 2B and C). Particularly, DCA was shown remarkably high levels (Fig. 2C). The levels of αMCA and UCA in the ID+CA groups were higher compared to ID+DW group (Fig. 2B). Other bile acids showed much lower levels than bile acids shown in Fig. 2B and C and were not changed by CA administration (data not shown).

Isoflavone intake significantly decreased final body weight and water consumption and increased total cecum and cecum contents. CA administration significantly decreased feed and water consumption and increased cecum tissue (Table 2).

DISCUSSION

In this study, we demonstrated that administration of CA inhibited equol production in mice. CA significantly decreased the levels of the daidzein metabolites DHD, equol, and O-DMA in the serum of mice (Fig. 1). However, CA did not affect the total molar concentration of daidzein or its metabolites (Fig. 1). Moreover, CA increased the levels of secondary bile acids among the cecum contents (Fig. 2A). This result indicated that most CA administered was converted to secondary bile acids.

Most dietary isoflavones are as present in their glycoside forms. In the intestine, cleavage of the sugar chain from glycoside forms by β-glucosidase promotes their absorption in the body. Isoflavone glycosides are converted into aglycone by two enzymes, lactose phlorizin hydrolase and cytosolic β-glucosidase, in the small intestine. Some isoflavone glycosides are converted to
aglycone by microbiota-derived β-glucosidase. Additionally, some absorbed aglycones are metabolized by the microbiota through the enterohepatic circulation as conjugated aglycones (18, 19). Ingested daidzin is converted into daidzein by β-glucosidase and then metabolized to DHD, equol, and O-DMA by the microbiota (Fig. 3) (16, 17). Thus, the inhibition of β-glucosidase activity and/or disfunction of the microbiota in the gastrointestinal tract can inhibit the conversion of daidzein to equol. We investigated the effect of CA administration on equol production in mice fed daidzin. CA did not alter the total molar concentrations of daidzein, DHD, equol, and O-DMA in the serum (Fig. 1). This result suggests that CA did not affect the conversion of daidzin to
daidzein. Thus, we found that CA inhibited the conversion of daidzin to equol, and O-DMA without changing in β-glucosidase activity derived from the small intestine tissue or microbiota.

Bacteria such as Escherichia coli, Bacteroides ovatus, Ruminococcus productus, Streptococcus intermedius, Eggerthella sp. strain YY7918, and Adlercreutzia equolifaciens have been shown to be involved in daidzein metabolism (20–22). In previous studies, equol production was inhibited in cynomolgus monkeys by administration of the antibiotic kanamycin (23). Additionally, kanamycin administration was suggested to inhibit equol production by decreasing equol-producing bacteria (24). Generally, secondary bile acids are known to have strong bactericidal activity. Particularly, DCA, 12oLCA, and 3o12α have stronger bactericidal activities than CA, the major bile acid (25). CA significantly increased the levels of DCA, 7oDCA, 12oLCA, and 3o12α among the secondary bile acids in the cecum (Fig. 2C). Moreover, DCA accounted for the highest percentage among these molecular species in the cecum (Fig. 2C).

Previous study has been reported that ingestion of 0.5% Fujiflavone P40 detects equol in the serum of mice (26). Furthermore, it has been reported that CA administration for 2 wk produces an intestinal flora pattern similar to that of high fat diet intake. Hence, in this study, the concentration of Fujiflavone P40 and the administration period were determined.

Final body weight was decreased by intake of isoflavones for 2 wk. Furthermore, feed consumption was reduced by CA administration. Moreover, water consumption was reduced by isoflavone intake and/or CA administration. It was considered that these changes had no effect of CA on equol production because there was no change in the appearance and behavior of the mice during the experiment period. Additionally, there was almost no difference in the bile acid concentration in the cecum contents between CD+DW, CD+CA and ID+DW groups than in the CD+CA group (Fig. 2B). However, DCA that have strong bactericidal activities accounted for the highest percentage among bile acids increased in the cecum by CA administration (Fig. 2C). From this result, the difference of the amount of primary bile acids between the CD+DW, CD+CA and

| Table 2. Body weight, consumption and cecum. |
|-----------------------------------------------|
| ![Table](image) |

CD+DW, control diet and distilled water; CD+CA, control diet and 0.3% cholic acid; ID+DW, isoflavone diet and distilled water and ID+CA, isoflavone diet and 0.3% cholic acid. Each value represents mean±SE, n=6–7, and were analyzed by two-way ANOVA, followed by Tukey-Kramer’s test for post hoc analysis.
ID+DW groups might have little effect on equol production. Since the reason why the phenomenon was occurred is unclear yet, it is necessary to reveal in the future.

These results suggest that CA affects equol-producing bacteria and inhibits equol production by increasing DCA in the gut. These findings may help to reveal the factors inhibiting equol production and enhance the clinical effectiveness of isoflavone intake. To reveal the inhibitory mechanism of equol production, studies are needed to analyze the gut microbiota at administration of CA to mice fed daidzin in detail.

**Authorship**

Research conception and design: HY and TT; investigation: HY, MW and FN; statistical analysis on the data: HY, MW, TS, SF, AY and TT; writing of the manuscript: HY and TT.

**Disclosure of state of COI**

MW, FN and TS are employees of Fujicco Co., Ltd. All other authors declared no competing interests. This study was partly supported financially by Fujicco Co., Ltd.

**REFERENCES**

1) Atkinson C, Frankenfeld CL, Lampe JW. 2005. Gut bacterial metabolism of the soy isoflavone daidzein: exploring the relevance to human health. Exp Biol Med 230: 155–170.

2) Setchell KDR, Cole SJ. 2006. Method of defining equol-producer status and its frequency among vegetarians. J Nutr 136: 2188–2193.

3) Rowland BR, Wiseman H, Sanders TAB, Adlercreutz H, Bowey EA. 2000. Interindividual variation in metabolism of soy isoflavones and lignans: influence of habitual diet on equol production by the gut microflora. Nutr Cancer 36: 27–32.

4) Einarsson K, Ericsson S, Ewerth S, Reihnér E, Rudling M, Ståhlberg D, Angelin B. 1991. Bile acid sequestrants: mechanisms of action on bile acid and cholesterol metabolism. Eur J Clin Pharmacol 40(Suppl 1): S53–S58.

5) Hofmann AF, Borgrström B. 1964. The intraluminal phase of fat digestion in man: the lipid content of the micellar and oil phases of intestinal content obtained during fat digestion and absorption. J Clin Invest 43: 247–257.

6) Houten SM, Watanabe M, Auwerx J. 2006. Endocrine functions of bile acids. EMBO J 25: 1419–1425.

7) Ridlon JM, Kang DJ, Hylemon PB. 2006. Bile salt biotransformations by human intestinal bacteria. J Lipid Res 47: 241–259.

8) Kurdi P, Kawanishi K, Mizutani K, Yokota A. 2006. Mechanism of growth inhibition by free bile acids in lactobacilli and bifidobacteria. J Bacteriol 188: 1979–1986.

9) Akare D, Martinez JD. 2005. Bile acid induces hydrophobicity-dependent membrane alterations. Biochim Biophys Acta 1735: 59–67.

10) Hildebrandt MA, Hoffmann C, Sherrill-Mix SA, Keilbaugh SA, Hamady M, Chen Y, Knight R, Ahmed RS, Bushman F, Wu GD. 2009. High-fat diet determines the composition of the murine gut microbiome independently of obesity. Gastroenterology 137: 1716–1724. e2.

11) Turnbaugh PJ, Bäckhed F, Fulton L, Gordon JI. 2008. Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. Cell Host Microbe 3: 223–233.

12) Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. 2009. The effect of diet on the gut human microbiome: a metagenomic analysis in humanized gnotobiotic mice. Sci Transl Med 1: 6ra14.

13) Islam KBMS, Fukiya S, Hagi M, Fuji N, Ishizuka S, Ooka T, Ogura Y, Hayashi T, Yokota A. 2011. Bile acid is a host factor that regulates the composition of the cecal microbiota in rats. Gastroenterology 141: 1773–1781.

14) Yokota A, Fukiya S, Islam KBMS, Ooka T, Ogura Y, Hayashi T, Hagi M, Ishizuka S. 2012. Is bile acid a determinant of the gut microbiota on a high-fat diet? Gut Microbes Landes Biosci 3: 455–459.

15) Hagi M, Matsumoto M, Fukushima M, Hara H, Ishizuka S. 2009. Improved analysis of bile acids in tissues and intestinal contents of rats using LC/ESI-MS. J Lipid Res 50: 173–180.

16) Setchell KDR, Clerici C. 2010. Equol: history, chemistry, and formation. J Nutr 140: 1355S–136S.

17) Frankenfeld C. 2011. O-Desmethylangolensin: the importance of equol’s lesser known cousin to human health. Adv Nutr 2: 317–324.

18) Stevens JF, Maier CS. 2016. The chemistry of gut microbial metabolism of polyphenols. Phytochem Rev 15: 425–444.

19) Murota K, Nakamura Y, Uehara M. 2018. Flavanoid metabolism: the interaction of metabolites and gut microbiota. Biosci Biotechnol Biochem 82: 600–610.

20) Ueno T, Uchiyama S. 2001. Identification of the specific intestinal bacteria capable of metabolising soy isoflavone to equol. Am J Nutr Metab 45: 114.

21) Yokoyama S, Oshima K, Nomura I, Hattori M, Suzuki T. 2011. Complete genomic sequence of the equol-producing bacterium Eggerthella sp. Strain YY7918, isolated from adult human intestine. J Bacteriol 193: 5570–5571.

22) Maruo T, Sakamoto M, Ito C, Toda T, Benno Y. 2008. Adlercreutzia equolifaciens gen. nov., sp. nov., an equol-producing bacterium isolated from human faeces, and emended description of the genus Eggerthella. Int J Syst Evol Microbiol 58: 1221–1227.

23) Blair RM, Appt SE, Franke AA, Clarkson TB. 2003. Treatment with antibiotics reduces plasma equol concentration in cynomolgus monkeys (Macaca fascicularis). J Nutr 133: 2262–2267.

24) Katsumata SI, Fujioka M, Fujii S, Takeda K, Ishimi Y, Uehara M. 2016. Kanamycin inhibits daidzein metabolism and abilities of the metabolites to prevent bone loss in ovariectomized mice. BMC Res Notes 9: 334.

25) Watanabe M, Fukiya S, Yokota A. 2017. Comprehensive evaluation of the bactericidal activities of free bile acids in the large intestine of humans and rodents. J Lipid Res 58: 1143–1152.

26) Sugiyama F, Wu J, Fujioka M, Ezaki J, Takeda K, Miyaura C, Ishiida T, Yamada K, Ishimi Y. 2006. Soybean isolavones preserve bone mass in hindlimb-unloaded mice. J Bone Miner Metab 24: 439–446.

27) Chiang JYL, Ferrell JM. 2018. Bile acid metabolism in liver pathobiology. Gene Expr 18: 71–87.