Benzyl Isothiocyanate Produced by Garden Cress (Lepidium sativum) Prevents Accumulation of Hepatic Lipids

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Summary We determined the physiological effects of glucotropaeolin-rich lyophilized garden cress sprout powder (GC) administered to fasting and nonfasting mice. High-performance liquid chromatography analysis revealed glucotropaeolin (57.4±1.1 mg/g dry weight) as a major phytochemical constituent of GC. Decreasing tendency in body weight and feeding efficiency ratio were detected in the group of mice fed 0.05% (w/w) GC (GC0.05). Nonfasting mice exhibited significantly lower liver weights that were unchanged after fasting. Decreased total lipid (TL) and triglyceride (TG) levels in the liver were detected in the nonfasted GC0.01 and GC0.05 groups, but only in TLs of the fasted groups. The levels of plasma TGs and nonesterified fatty acids of the GC0.05 group, which remained unchanged during nonfasting, decreased after fasting. To determine its effects on the accumulation of lipids in the liver, the glucotropaeolin aglycone, benzyl isothiocyanate (BITC), was added to the liver-derived HepG2 human cell line cultured in a medium containing a high concentration of d-glucose (4,500 mg/L d-glucose) (HG group) or 1 mM oleic acid (SO group). Toxicity was not detected when cells were treated with as much as 5 mM BITC; however, lipid accumulation was inhibited by BITC in a concentration-dependent manner in the HG groups. The same effect was observed when 2 mM BITC was added to the diet of the SO groups. These results suggest that moderate levels of GC or BITC are useful for reducing liver and plasma TGs.

Key Words garden cress, glucotropaeolin, benzyl isothiocyanate, hepatic triglyceride accumulation, plasma triglyceride

Materials and Methods

Materials. Garden cress seed was purchased from Suba Seeds Co. S.p.A. (Longiano, Italy). Garden cress sprouts were germinated for 6 d under natural lighting at room temperature (20°C). GC was prepared as follows: Garden cress sprouts frozen at −60°C were lyophilized for 16 h using an FDU-1200 freeze dryer (Tokyo Rikakikai Co., Ltd., Tokyo, Japan). The dried...
standard product was crushed using a Vita-Mix Absolute Mill (Osaka Chemical Co., Ltd., Osaka, Japan) to magnitude level 6 for 3 min. and the final product was designated GC. The AIN-93G feed used here was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). Glucotropaeolin-potassium salt was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan).

Determination of glucotropaeolin. GC (50 mg) was suspended in 10 mL of 80% (v/v) methanol, stirred at room temperature for 60 min, and centrifuged at 12,000 rpm for 5 min at 10˚C. Thereafter, the supernatant was filtered using a Dismic syringe filter (13HP045CN; Toyo Roshi Kaisha, Ltd., Tokyo, Japan), and high-performance liquid chromatography (HPLC) was used to quantify GC. The eluent was analyzed using an Alliance HPLC System (Nihon Waters K.K., Tokyo, Japan) equipped with a Myhtysil RP-18 GP column (5 μm, i.d. 4.6 mm×150 mm, Kanto Chemical). The HPLC conditions were as follows: solvent A, 50 mm phosphoric acid in water; solvent B, acetonitrile; gradient, 0–15 min for 5–7.5% B linear (curve number 6, Waters Empower software), 15–16 min for 7.5% B isocratic; flow rate, 1.0 mL/min; column temperature, 40˚C; detection, 240 nm; and injection volume, 10 μL. The glucotropaeolin was identified according to its retention time compared with authentic compounds.

Experiments using mice. The Animal Welfare Committee of the Kobe BM Laboratory approved the protocol for using mice, which complies with the “Law on the Protection and Management of Animals,” “The Ordinance on Protection and Management of Animals,” and “Guidelines on How to Dispose of Animals” (Permission nos. 17K010-2 and 17K010-3). Mice were purchased from Charles River Laboratories Japan Inc. (Yokohama, Japan) and reared at 23˚C, 55% humidity, under light/dark periods 8:00–20:00 h and 20:00–4:00 h, respectively.

To exclude the effects of the estrous cycle, 8 wk-old male C57BL/6J mice were randomly assigned to three groups (8 or 6 mice per group), housed individually, and fed ad libitum for 28 d with AIN-93G (control group) or AIN-93G supplemented with 0.01% (w/w) or 0.05% (w/w) GC each (GC0.01 and GC0.05 groups, respectively). We measured body weights and food consumption.

Fasting and nonfasting mice (16 h: 8 and 6 mice per group, respectively) were killed using isoflurane inhalation, and liver weights and plasma markers were measured. Blood was collected into Capiject II tubes (with heparin lithium; Terumo Co., Ltd., Tokyo, Japan). After 30 min of inversion and mixing, the mixture was centrifuged at 5,000 rpm for 20 min at 4˚C to collect plasma. The concentrations of plasma glucose, TGs, nonesterified fatty acids (NEFAs), and total- and high-density lipoprotein (HDL) cholesterols were measured using the kits as follows: Glucose CII Test Wako, Triglyceride E-Test Wako, Cholesterol E-Test Wako, and HDL Cholesterol E-Test Wako (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), respectively. Skylight Biotech (Akita, Japan) measured the activities of alanine transaminase (ALT) and aspartate transaminase (AST).

Quantification of lipids in the liver. Lipids were extracted from the liver according to a modification of a published method (18). Briefly, liver pieces (0.1 g fresh weight) were weighed in a microtube, incubated in 200 μL of 1 M NaCl, and homogenized for 30 s on ice using a BC-G10 Microtube Homogenizer (12,000 rpm) (Biomedical Science, Tokyo, Japan). The homogenate was mixed with 700 μL of chloroform (Chr)/methanol (MeOH) (2 : 1 [v/v]) with dibutylhydroxytoluene 0.05% [w/v]). The mixture was subsequently stirred using a Cute Mixer CM-1000 (Wakenyaku Co., Ltd., Kyoto, Japan) set to magnitude level 18 at 100 rpm for 15 min at 20˚C. After centrifugation at 13,000 rpm for 10 min at 10˚C, the lower layer was transferred to a new tube. Wash solution (Chr/MeOH/distilled and deionized water [3 : 48 : 47 (v/v/v)]) was added, mixed for 30 s and centrifuged at 13,000 rpm for 10 min at 10˚C. The lower layer was transferred to a new tube and dried using nitrogen gas (0.5 atm. 20 L/min. 5 min). The dried residue was weighed and defined as the amount of total lipids (TLs), dissolved in 1 mL of Triton X-100/methanol (2 : 1 [v/v]) solution, and used for measuring TGs as well as those in plasma.

Cell culture. The HepG2 cell line derived from human hepatocytes was used to evaluate the effects of BITC on the accumulation of lipids in the liver according to a published method (19). Cells were added to the wells of a 96-well plate (1.0×10^4 cells/well) and cultured for 24 h in Dulbecco’s modified Eagle’s medium (DMEM) (FUJIFILM Wako) containing 10% fetal bovine serum, 1% penicillin-streptomycin, and 1,500 mg/L d-glucose (LG). LG was further modified to contain 4,500 mg/L d-glucose (HG group), or supplemented with 1 mM sodium oleate and 1% bovine serum albumin (SO group). These media were added to each well. BITC was diluted with dimethyl sulfoxide and added to the medium at final concentrations ranging from 1–5 μM. The media were changed every 48 h, and cytotoxicity testing and Oil Red O staining were performed.

A Cell Counting Kit-8 (FUJIFILM Wako) was used to determine cytotoxicity. Oil Red O staining was performed as follows: The medium was removed, the well was washed once with phosphate buffered saline, the cells were fixed using 10% neutral-buffered formalin solution (FUJIFILM Wako) for 4 h. The well was washed once with distilled-deionized water (DDW), replaced with 70% isopropanol for 1 min, the isopropanol solution was removed, and cells were incubated with 100 μL of 70% Oil Red O solution (3 mg/mL Oil Red O in isopropanol/DDW [70 : 30 (v/v/v)]) for 30 min at room temperature. After washing once with 70% isopropanol and DDW, the dye remaining in the cells was eluted with 100 μL of 70% isopropanol for 20 min, and absorbance at 492 nm was measured using a Multiskan Ascent microplate reader (Thermo Electron Co., Ltd., Rosemount, MN, USA).

Statistical analysis. Dunnett tests were performed
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using Microsoft Excel with the add-in software Statcel 4 (The Publisher OMS Ltd., Saitama, Japan). Differences were considered significant if \( p < 0.05 \).

**Results**

Raw garden cress sprouts (18.2±3.8 g) yielded 0.9±0.2 g of GC (calculated moisture content, 95.2±0.2%). HPLC analysis of GC detected BG as the major peak (Fig. 1) that represented 57.4±1.1 mg/g dry weight (DW) of the starting material. Other major peaks were not observed.

Mice were fed a control or an experimental diet supplemented with GC to determine the effects of GC on body weight and feeding efficiency during fasting. The feeding efficiencies of the control and GC0.01 groups were not significantly different. In contrast, the feeding efficiency of the GC0.05 group was significantly lower (Fig. 2A). Although no significant difference was observed in the GC0.01 group, a significant decrease in body weight was observed in the GC0.05 group 25 d after administration of GC compared with the control group (Fig. 2B). The nonfasting data, although not statistically significant, showed similar trends (Supplemental Online Material, Fig. S1). Liver and body weights were significantly reduced in the nonfasted GC0.05 group (Table 1). In contrast, there was no significant difference in liver weights of the GC-supplemented groups compared with those of the control groups (Table 2).

We next analyzed plasma markers of fasted and nonfasted mice. The TL and TG levels in the livers of both GC groups were significantly decreased (Table 1). Notably, the TG levels of both GC groups were reduced by approximately 50% (0.56- or 0.42-fold compared with the control). The TL levels contained in the liver after fasting were significantly reduced in both GC groups.
Fig. 3. Cytotoxicity (A) and effects on the accumulation of TGs (B) of BITC added to cultures of HepG2 cells. LG-C, low glucose (LG)-control; LG-1, LG-1 μM BITC; LG-2, LG-2 μM BITC; HG-C, high glucose (HG)-control; HG-1, HG-1 μM BITC; HG-2, HG-2 μM BITC; HG-5, HG-5 μM BITC; SO-C, sodium oleate (SO) control; SO-1, SO-1 μM BITC; SO-2, SO-2 μM BITC. Each value represents the mean±SE (n=6). **p<0.01 and *p<0.05 vs control group.
compared with those of the control groups, although there was no difference between the amounts of TGs (Table 2). Furthermore, there was not a significant change in plasma markers of nonfasted mice (Table 1). In contrast, there were significant reductions in the levels of plasma TGs, NEFAs, and ALT in the fasted GC0.05 group compared with those of the control group (Table 2). Notable, the TG level was reduced 0.65-fold compared with that of the control. These results suggest that the accumulation of TGs in the livers of nonfasted mice was suppressed by components of GC, resulting in reduced release of TGs into the bloodstream of fasted mice.

To evaluate the effect of BITC on the accumulation of lipids in hepatocytes, we used HepG2 cells cultured in DMEM supplemented with HG or SO. BITC-induced toxicity was not detected under these conditions (Fig. 3A). Although there was no significant change in lipid levels in the LG group, there was a clearly discernable decrease (Fig. 3B). In contrast, there was a significant and concentration-dependent antilipogenic effect of BITC in the LG group (Fig. 3B). Further, BITC (2 \( \mu \text{M} \)) had a significant \((p<0.05\) vs. control) inhibitory effect on lipid accumulation in the SO groups (Fig. 3B).

**Discussion**

In the present study, the maximum concentration of GC administered to mice was 0.05%, which is equivalent to 1.8 mg/kg/d of BITC. The cytotoxic dose of BITC for rats is 25 mg/kg/d (15–17). We did not detect cytotoxicity of GC or BITC at any concentration tested. Pharmacokinetic studies of humans show that the maximum blood concentration of BITC (including metabolites) is \(<2.4 \mu\text{M}\) (5). Thus, the concentrations of BITC tested here are considered safe and potentially suitable for use as a food supplement.

We show here that there was a significant decrease in liver weights in the nonfasted GC0.05 group. There was no difference in blood markers among the nonfasted groups, although hepatic TL and TG concentrations were reduced in a concentration-dependent manner by GC. Conversely there was no significant difference in liver weights and TG levels, although the levels of plasma ALT, TGs, and NEFAs were reduced in the fasted GC0.05 group. These results suggest that GC inhibited the conversion or accumulation of carbohydrates to lipids in the liver. Further, analysis of HepG2 cells showed that BITC suppressed the accumulation of lipids synthesized from sugars and fatty acids. These results suggest that the BITC present in GC reduces the endogenous accumulation of TGs in the liver, and consequently reduces fasting plasma TG levels.

Excess accumulation of TGs occurs in nonalcoholic fatty liver disease (20), and elevated plasma TG levels accelerate the aging of blood vessels to increase the risk of coronary heart and cerebrovascular disease caused by arteriosclerosis (21–23). Therefore, moderate levels of BITC, which reduce fatty liver and the levels of plasma TGs, may be useful for preventing such symptoms.

SFN (18, 24) and BITC (25) improve lipid metabolism by suppressing lipogenesis in adipose tissue via the AMP-activated protein kinase signaling pathway. Furthermore, SFN ameliorates abnormal lipid metabolism in a mitochondrial-dependent manner (26, 27). For example, SFN activates the sirtuin 1/peroxisome proliferator-activated receptor gamma coactivator 1\(\alpha\) and nuclear factor erythroid-2-related factor/nuclear respiratory factor 1 signaling cascades (26). Moreover, SFN activates the expression of adipose triglyceride lipase and hormone-sensitive lipase of adipocytes or hepatocytes, respectively, to increase the degradation of TGs (26, 27). In this way, SFN enhances mitochondrial biogenesis and promotes oxidation of NEFAs generated by the catabolism of TGs. In the present study, we did not analyze adipose tissues, although the proposed mechanism (26) may explain the mechanism through which GC or BITC decreases the levels of TGs.

Myrosinase is required for optimizing the yield of SFN, although the activity of this enzyme is primarily lost during the process of cooking or extraction of glucoraphanin (28). Furthermore, the bioavailability of SFN in raw broccoli is 10-fold higher than that in cooked broccoli (29). This may be true as well for the bioavailability of BITC in garden cress. For example, the myrosinase activity of maca is maintained by drying at 20˚C under vacuum (3), indicating that activity is maintained in GC. On the other hand, glucosinolates are also known to be metabolized to isothiocyanates by gut microbiota (30). To varying degrees, the glucosinolates may be absorbed by human body as isothiocyanates without endogenous myrosinase.

Our present findings strongly suggest that it is important to consider the daily intake of garden cress sprouts, which is expected to reduce the levels of TGs. The average daily dietary intake for mice (body weight, 30 g) is approximately 5 g/d. The dose of GC used here was approximately 6.8 mg/kg/d based on the human-equivalent dose (31). The amount of human equivalents (body weight, 60 kg) of GC added in the study cited above (31) is approximately 8.5 g day of raw garden cress sprouts and approximately 8.5 mg of BITC (23.4 mg of BG). Therefore, a sufficient reduction of the amount of TGs can be expected by ingesting a relatively small amount of BG or BITC as well as by ingesting a reasonable amount of fresh garden cress.

In conclusion, we suggest the supplementation of a diet with 0.05% (w/w) of GC suppresses the accumulation of lipids in the mouse liver, accompanied by reduced levels of plasma TGs. The mechanism may be extrapolated to that responsible for the effect of BITC in garden cress sprouts on the accumulation of TGs in hepatocytes. Thus, the incorporation of garden cress sprouts into a normal diet may reduce the levels of hepatic or plasma TGs, or both. Further preclinical research and clinical trials are required to validate these findings.

**Authorship**

Research conception and design: SY; experiments: MK, AH, and YU; statistical analysis of the data: MK.
and SY: interpretation of the data: SY, MN, and TO; writing of the manuscript: SY and MK.

Disclosure of state of COI
AH is an employee of Saladcosmo Co. Ltd. MN was an employee of Saladcosmo Co. Ltd and the present affiliation is Yasaidekenkou R&D Co. Ltd. All the other authors declared no competing interests.

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Supporting information
Supplemental online material is available on J-STAGE.

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