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Monogalactosyldiacylglycerol: An abundant galactosyllipid of *Cirsium brevicaule* A. GRAY leaves inhibits the expression of gene encoding fatty acid synthase

Masashi Inafuku[a], Kensaku Takara[b], Naoyuki Taira[a,c], Ruwani N. Nugara[a,c], Yasuo Kamiyama[d], Hirosuke Oku[a,*]

[a] *Center of Molecular Biosciences, Tropical Biosphere Research Center, University of the Ryukyus, Okinawa 903-0213, Japan*
[b] *Faculty of Agriculture, University of the Ryukyus, Okinawa 903-0213, Japan*
[c] *United Graduate School of Agricultural Sciences, Kagoshima University, Kagoshima 890-0065, Japan*
[d] *Kobe Tokushukai Hospital, Hyogo 655-0017, Japan*

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ABSTRACT

Background: The leaves of *Cirsium brevicaule* A. GRAY (CL) significantly decreased hepatic lipid accumulation and the expression of fatty acid synthase gene (*FASN*) in mice.
Purpose: We aimed to purify and identify the active compound(s) from CL and determine the inhibitory mechanism of expression of *FASN*.
Methods: We purified monogalactosyldiacylglycerol (MGDG) from extracts of CL (CL-MGDG) and showed that it was the active CL component through analyses of its effects on the expression of genes of human breast cancer cell line, SKBR-3.
Results: The content and fatty acid composition of CL-MGDG are distinctly different from those of other vegetable-derived MGDGs. Treatment of SKBR-3 cells with MGDG decreased the level of *FASN* mRNA as well as the levels of mRNA encoding other protein involved in lipogenesis. Further, MGDG treatments significantly inhibited luciferase activities of constructs containing liver X receptor response element in *FASN* promoter region without altering the levels of mRNA encoding transcription factors. MGDG and the FASN inhibitor C75 decreased the viabilities of SKBR-3 cells in a concentration-dependent manner. CL-MGDG more potently inhibited cell viability than a commercial MGDG preparation.
Conclusions: CL represents a good source of glycoglycerolipids with potential as functional ingredients of food.

Introduction

Throughout southern Japan and Taiwan, *Cirsium brevicaule* A. GRAY is a wild perennial herb growing in rocky gravels or forest margins along maritime coastlines (Kadota 1990). Its leaves, stems, and roots have traditionally been used as food and medicine in the Okinawa and Amami Islands of Japan. We showed that dietary *C. brevicaule* A. GRAY leaves (CL) significantly decreased the expression of the gene encoding fatty acid synthase (FASN) in livers and white adipose tissues of mice fed a high-fat diet (Inafuku et al. 2013). Further, we detected a significant reduction in serum free fatty acid (FA) levels in mice fed a diet containing CL. An increase of free-FA influx into circulation, which leads to enhanced free-FA uptake by multiple tissues, is associated with the pathogenesis of metabolic syndromes such as obesity, non-alcoholic fatty liver disease, and type 2 diabetes. Moreover, dietary CL decreased hepatic lipid levels in mice fed a high-fat diet (Inafuku et al. 2013). Therefore, CL has recently been drawing attention as a new functional food material.

FASN is the enzyme that catalyzes the NADPH-dependent condensation of acetyl-CoA and malonyl-CoA to produce the saturated FA palmitate. The prognostic marker OA-519, which is expressed by breast cancer cells of patients with very poor prognosis, was identified as FASN (Kuhajda et al. 1994). Breast cancer is currently the

Abbreviations: ACACA, acetyl-CoA carboxylase α; ACTB, actin, beta; ALA, α-linolenic acid; CL, *Cirsium brevicaule* A GRAY leaves; EI-MS, electron ionization-mass spectrometry; FA, fatty acid; FASN, fatty acid synthase; LXR, liver X receptor; LXRE, liver X receptor response element; MGDG, monogalactosyldiacylglycerol; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; RXR, retinoid X receptor; SCD, stearoyl-CoA desaturase; SP1, stimulatory protein 1 site; SRE, sterol regulatory element; SREBF, sterol regulatory element binding transcription factor; SREBP, non-canonical sterol regulatory element binding protein recognition site.

* Corresponding author. Tel./fax: +81 98 895 8972.
E-mail address: okuhiros@comb.u-ryukyu.ac.jp, japanese.quail@gmail.com (H. Oku).

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leading cause of death in women, accounting for 23% of all cancer-related deaths (Donepudi et al. 2014). The expression of FASN increases dramatically at the transcriptional and posttranscriptional levels in patients with breast cancer (Alo et al. 1996; Wang et al. 2001) as well as other cancers, and the expression of FASN is undetectable in normal tissues other than liver, adipose and breast (Menendez and Lupu 2007). Further, overexpression of FASN is associated with advanced stage, metastasis and poor prognosis of cancer (Menendez et al. 2004; Wang et al. 2001). These differences in the expression of FASN between normal and cancer tissues imply that FASN represents a promising target for anti-tumor therapy. Further, inhibition of FASN catalytic activity by agents such as cerulenin and C75 preferentially induce apoptosis of cancer cells (Pizer et al. 1998; Puig et al. 2008) and inhibit tumor growth in xenograft models (Pizer et al. 2000). The potential of FASN as an anti-tumor target is indicated by analyses of RNA interference-mediated silencing of expression of *FASN* (Menendez et al. 2005).

These findings inspired us to purify and identify the bioactive compound from CL to determine how it inhibits expression of *FASN*. Further, we evaluated the antitumor effects of the bioactive compound on the human breast cancer cell line, SKBR-3.

Materials and methods

Reagents

Monogalactosyldiacylglycerol (MGDG) was purchased from Avanti Polar Lipids, Inc. (AL, USA). McCoy's 5A medium, C75, GW3965, and LG100268 were purchased from Sigma-Aldrich (MO, USA). FBS was obtained from AusGeneX PTY Ltd. (Oxenford, Australia) and inactivated at 56 °C for 30 min before use.

Preparation of extracts and a bioactive compound from CL

C. brevicaule A. GRAY (Kousyunsou®) was harvested on Tokunoshima Island in Kagoshima Prefecture, Japan. The freeze-dried and ground powder of CL was generously provided by Tokunoshima-cho (Kagoshima, Japan). The dried-CL powder was serially extracted by incubation with 10 volumes each of hexane, chloroform, ethanol and water for 2 h at 37 °C. The filtrates were evaporated or freeze-dried in vacuo, and stored at −80 °C. The CL chloroform extract was applied to a Hi-Flash silica-gel column (Yamazen Corp., Osaka, Japan) and eluted with chloroform/methanol (95/5, v/v). The eluate was evaporated to dryness, dissolved in chloroform, and purified using an HPLC with a silica gel column (Cosmosil 5SL-II, Nacalai Tesque, Inc., Kyoto, Japan) with chloroform/methanol/water/formic acid (92.0/7.1/0.4/0.5, v/v). The active fractions were further evaporated, dissolved in methanol, and further purified through an HPLC reverse-phase column (Cosmosil 5C22-AR-II, Nacalai Tesque) with methanol/water/trifluoroacetic acid (91.8/8.0/0.2, v/v). All extracts and isolated fractions were evaporated, dissolved in DMSO and stored at −80 °C.

Determination of the chemical structure and fatty acid composition of the bioactive compound isolated from CL

To identify the chemical structure of the purified active compound, NMR spectra were acquired using a Bruker UltraShield 400 MHz NMR spectrometer (Bruker Corporation, Billerica, MA, USA) with $CDCl_3$ as an internal standard. ESI-MS and MS/MS data were acquired using a Bruker Esquire 3000 Plus Ion Trap Mass Spectrometer.

To determine its FA composition, MGDG was methylated and FA methyl esters were analyzed using a gas chromatography system as described elsewhere (Wongtangtintharn et al. 2005).

Cell cultures

The human breast cancer cell line SKBR-3 was purchased from the JCRB Cell Bank (Tokyo, Japan) and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were grown in McCoy's 5A medium supplemented with 10% FBS and 1% penicillin–streptomycin.

Western blotting

Twenty-four hours after seeding the cells into a 24-well plate (1.5×10^5 cells per well), the cells were treated with purified fractions or chemicals for 24 h. Protein was extracted and equal amounts of protein were separated using SDS-PAGE. The proteins were electrophoretically transferred onto a polyvinylidene difluoride membrane. The blots were probed with antibodies against FASN and β-actin (Cell Signaling Technology, Tokyo, Japan) as well as liver X receptor (LXR)-α, LXR-β and retinoid X receptor (RXR)-α (Abcam, Cambridge, UK) according to the manufacturers' protocols. The membrane was washed and incubated with horseradish peroxidase-conjugated secondary antibody (Biosource International, Inc., CA, USA) for 2 h at room temperature. Band intensities were quantified using ImageJ (NIH, MA, USA), and the level of each protein was normalized to that of β-actin.

Analysis of mRNA levels

Twenty-four hours after seeding the cells in a 96-well plate (2×10^4 cells per well), cells were treated with CL extracts, MGDGs, α-linolenic acid (ALA) and 5 μM of the LXR agonist (GW3965) or 1 μM of the RXR agonist (LG100268) for 24 h, washed with PBS, and cDNA was synthesized using a Gene Expression Cell-to-CT kit (Life Technologies, CA, USA). The TaqMan primer/probe sets for *FASN* (Hs01005622_m1), acetyl-CoA carboxylase α (*ACACA*, Hs01046047_m1), sterol regulatory element binding transcription factor 1 (*SREBF1*, Hs01088691_m1), stearoyl-CoA desaturase (*SCD*, Hs01682761_m1), nuclear receptor subfamily 1, group H, member (NR1H) 3 (*LXR-α*, Hs00172885_m1), NR1H2 (*LXR-β*, Hs01027215_g1), RXR-α (Hs01067640_m1), and actin, beta (*ACTB*, Hs01060665_g1) were purchased from Applied Biosystems (CA, USA). To measure the relative abundance of target transcripts, amplifications were performed using TaqMan Fast Advanced Master Mix with the StepOne Real-Time PCR System (Applied biosystems), and the amounts of target transcripts were normalized to those of *ACTB*.

Construction of luciferase reporter plasmids

Fragments of the human FASN promoter were generated using nested-PCR. In brief, a PCR fragment (−1677/+128 bp) was amplified using human genomic DNA as a template (Promega Corp., WI, USA) and then used as a template for a second−round of PCR. To generate −1600, −835, −150, and −106 bp fragments, specific upstream primers were combined with the common downstream primer at +70 bp. These amplicons were sequenced and cloned into a firefly luciferase reporter vector [pGL4.23 (luc2/minP), Promega Corp.] that is digested with KpnI and HindIII using an In-Fusion HD Cloning Kit (Clontech Laboratories, Inc., CA, USA). The plasmids used for transfection experiments were purified from *E. coli* using a PureYield Plasmid Miniprep System (Promega Corp.).

Transient DNA transfection and luciferase assay

SKBR-3 cells were seeded at 4×10^4 cells per well in a 96-well plates 24 h before transfection. The cells were transiently transfected with the plasmids using the FuGENE HD transfection