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Report

Myoga (*Zingiber Mioga*) Modulates Hepatokine Production by Improving Steatosis in PXB-Cells LA, a Humanized MASLD Cell Model

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Oral administration of aqueous extracts of myoga (*Zingiber mioga*) alleviates fatty liver disease by decreasing liver weight and hepatic lipogenic gene expression in high-fat diet-fed mice. In the present study, we investigated whether aqueous myoga extract affects biomarkers of metabolic dysfunction-associated steatotic liver disease (MASLD) and insulin resistance-inducing hepatokines in human hepatocytes with MASLD features derived from humanized murine livers *in vitro*. The results showed that myoga extract decreased intracellular triglyceride content, leading to a reduction in leucine-rich α2 glycoprotein, an MASLD progression marker. Furthermore, myoga extract favorably modulated the secretion of hepatokines, suppressing insulin resistance-promoting hepatokines such as selenoprotein P, while enhancing the secretion of the beneficial hepatokine fibroblast growth factor 21. These findings demonstrated that the myoga extract restores hepatokine balance in MASLD-like hepatocytes in the PXB-cells LA model and facilitated the practical application of this regional specialty in advanced functional foods for the management of MASLD and related metabolic disorders.

Key words *Zingiber mioga*, NAFLD/MASLD, insulin resistance, hepatokine, fibroblast growth factor 21

INTRODUCTION

Obesity, particularly visceral fat accumulation resulting from energy imbalance due to poor dietary habits and physical inactivity, serves as the primary trigger by promoting a continuous influx of fatty acids into the liver, directly driving fatty liver disease (hepatic dysfunction). This condition functions as a central pathological link that induces insulin resistance, which impairs the body's ability to regulate blood glucose levels, ultimately resulting in the onset or exacerbation of diabetes. Furthermore, the combination of hyperglycemia and the release of pro-inflammatory substances from the fatty liver accelerates vascular damage, leading to arteriosclerosis and an increased risk of life-threatening systemic complications such as myocardial infarction and stroke.[1, 2] We previously developed a novel assay system for screening antilipidemic agents by profiling lipoprotein in freshly isolated human hepatocytes derived from humanized murine livers (PXB-cells).[3] Recently, we also established metabolic dysfunction-associated steatotic liver disease (MASLD)-like hepatocytes derived from PXB-cells (PXB-cells LA) through continuous incubation in a proprietary lipid maintenance medium.[4] More recently, we developed assessment platforms using PXB-cells LA to screen candidate agents with anti-arteriosclerotic and insulin resistance-ameliorating potential.[5, 6]

Myoga (*Zingiber mioga*) is a perennial ginger family plant whose flower buds are widely consumed in Japan. Kochi Prefecture accounts for over 90% of domestic production, followed by regions such as Akita and Gunma (FY2022).[7] Several previous studies have investigated the preventive effects of myoga against lifestyle-related diseases.[8, 9] Lee *et al*. reported that an aqueous extract of the plant inhibits preadipocyte differentiation and mitigates weight gain and elevated blood lipid levels in mice fed a high-fat diet.[10] Notably, the extract was shown to prevent the progression of fatty liver by suppressing lipogenic genes in the mouse liver, while also demonstrating multifaceted effects such as improving insulin resistance and alleviating hepatic inflammation. Therefore, we evaluated the direct effects of an aqueous extract of Akita-grown myoga (hereafter referred to as "myoga extract") on hepatocyte function and lipid metabolism using PXB-cells LA.

MATERIALS AND METHODS

Freeze-dried powder (1 g) of commercially available myoga flower buds was extracted with 50 mL distilled water. After centrifugation, the resulting aqueous extract was lyophilized. PXB-cells LA-derived MASLD-like human hepatocytes were prepared as described previously.[4] Briefly, PXB-cells were seeded at a density of 4×10^5 cells/well in collagen-coated 24-well microplates (Day 0). The cells were cultured in lipid-maintained medium (Phoenix Bio, Higashi-Hiroshima,

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Japan) from seeding until Day 6, followed by maintenance in hepatocyte growth medium (HCGM, Phoenix Bio) for 2 days. On day 8 of culture, PXB-cells LA were either treated with 100 μg/mL myoga extract or left untreated for two days. Intracellular protein and triglyceride amounts were measured using a Bio-Rad Protein Assay Kit II (Bio-Rad Laboratories, Hercules, CA, USA) and Cholestest TG (Sekisui Medical Co., Ltd., Tokyo, Japan) assay kits, respectively. The culture medium was collected for enzyme-linked immunosorbent assay (ELISA).

ELISA Albumin levels in the culture medium were quantified using the LZ-Test "Eiken" U-ALB (Eiken Chemical, Taito-ku, Tokyo, Japan). Human fibroblast growth factor 21 (FGF21) and leucine-rich α2 glycoprotein (LRG) levels were measured using specific ELISA kits (Immuno-Biological Laboratories, Fujioka, Japan) according to the manufacturer's instructions. The levels of both hepatic protein in the culture medium were normalized to that of total intracellular protein content.

Real-Time RT-PCR Total RNA was isolated using QuickGene RNA Cultured Cell Kit S (Fujifilm Wako, Osaka, Japan). Template cDNA was synthesized from 5 μg of total RNA using the PrimeScript RT reagent Kit (Takara Bio, Kusatsu, Japan). In a fluorescent temperature cycler (CFX Connect™ Real-Time PCR Detection System; Bio-Rad Laboratories), 2.5% of each RT reaction was amplified in 25 μL of 1× SYBR® Premix Ex Taq (Takara Bio) containing 0.2 μM of each primer. The samples were incubated in the thermal cycler for initial denaturation at 95°C for 10 s, followed by 40 cycles of PCR. Each cycle consisted of 95°C for 5 s and 60°C for 30 s. The relative expression levels of both mRNAs were normalized to that of glyceraldehyde-3-phosphate dehydrogenase. The primer sequences used in this study are listed in Table 1.

Statistical Analyses Statistical analyses were conducted using EZR (Jichi Medical University, Japan), a graphical user interface for R. EZR builds upon the R Commander by adding specialized functions tailored for biostatistical research. Data are expressed as mean ± standard deviation (SD). The significance of the differences was analyzed using the Mann-Whitney U-test. Statistical significance was set at $p < 0.05$.

RESULTS

Myoga Improves MASLD Markers First, we evaluated the effects of myoga extract on hepatic or MASLD markers in PXB-cells LA (Fig. 1). The extract significantly suppressed intracellular triglyceride levels, a hallmark of fatty liver disease, by 31%, whereas the protein content remained unaffected.

Next, we investigated the effects of the extract on the expression of albumin, a marker of hepatic function, and found that it did not negatively affect albumin production. LRG is a secreted protein involved in the progression of hepatosteatosis and fibrosis, positioning it as a key biomarker and therapeutic target in metabolic dysfunction-associated steatohepatitis (MASH).[11, 12] We previously demonstrated that LRG production was higher in PXB-cells LA than in control PXB-cells. Myoga extract suppressed LRG production in PXB-cells LA by 35% compared with untreated cells.

Myoga Modulates Gene Expression of Lipid Droplet (LD)-Associated Proteins LDs serve as dynamic metabolic platforms for triglyceride storage and are regulated by specific surface proteins.[13] Fat-specific protein 27 (FSP27) facilitates energy storage by promoting LD contact and lipid transfer. It also functions together with perilipin (PLIN) to sequester adipose triglyceride lipase (ATGL), the rate-limiting lipolytic enzyme, from LD substrates. However, under high energy demand, phosphorylation triggers structural changes in PLIN that recruits ATGL to the LD surface, thereby initiating rapid lipolysis. Dysregulation of the interplay between FSP27, PLIN, and ATGL is a key driver of metabolic diseases, including ectopic fat accumulation and insulin resistance.

To elucidate the mechanism by which myoga extract influences intracellular triglyceride degradation, we investigated its effect on the gene expression of LD surface proteins in PXB-cells LA (Fig. 2). The extract upregulated ATGL expression by 1.9-fold compared to that in untreated PXB-cells LA, while downregulating the expression of FSP27 (73% in untreated cells) and PLIN2 (82%), both of which are essential for LD maintenance.

Myoga Regulates Hepatokine Balance in PXB-Cells LA Hepatokines are liver-derived secreted proteins that regulate systemic metabolism. They are categorized as "beneficial" hepatokines, which improve glucose metabolism, and "detrimental" hepatokines, which induce insulin or exercise resistance.[14] During fatty liver disease progression, the secretory balance shifts; an excess of detrimental hepatokines trigger systemic conditions, such as diabetes and arteriosclerosis. Specifically, selenoprotein P induces exercise resistance by attenuating the metabolic benefits of physical activity, in addition to causing insulin resistance,[15] whereas leukocyte cell-derived chemotaxin 2 (LECT2) reduces insulin sensitivity in the skeletal muscle.

In the present study, we investigated the effects of myoga extract on the mRNA expression of these detrimental hepatokines in PXB-cells LA (Fig. 3-a). As expected, treatment with the extract suppressed the expression of selenoprotein P and LECT2 by 18% and 57%, respectively. Furthermore,

Table 1. Primer Sequences Used in the Study

Gene	Forward primer	Reverse primer
ATGL	5′- ATGGTGGCATTTCAGACAACC-3′	5′- CGGACAGATGTCACTCTCGC-3′
FGF21	5′-GGGAGTCAAGACATCCAGGT-3′	5′-GGCTTCGGACTGGTAAACAT-3′
FSP27	5′-ATTGATGTGGCCCGTGTAACG-3′	5′-CAGCAGTGCAGATCATAGGAAA-3′
GAPDH	5′-GCACCGTCAAGGCTGAGAAC-3′	5′-TGGTGAAGACGCCAGTGGA-3′
LECT2	5′-GTGTTCGAATATCTGGAAGAGGT-3′	5′-AAGGGCAATAGAGTTCCAAGT-3′
PLIN2	5′-TTGCAGTTGCCAATACCTATGC-3′	5’-CCAGTCACAGTAGTCGTCACA-3′
SPP	5′-TCATCAAGGAATCTCTTCTCG-3′	5′-CAAGACGGCCACATCTATCA-3′

Abbreviations: ATGL, adipose triglyceride lipase; FGF 21, fibroblast growth factor 21; FSP 27, fat-specific protein 27; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LECT2, leukocyte cell-derived chemotaxin 2; PLIN, perilipin; SPP, selenoprotein P

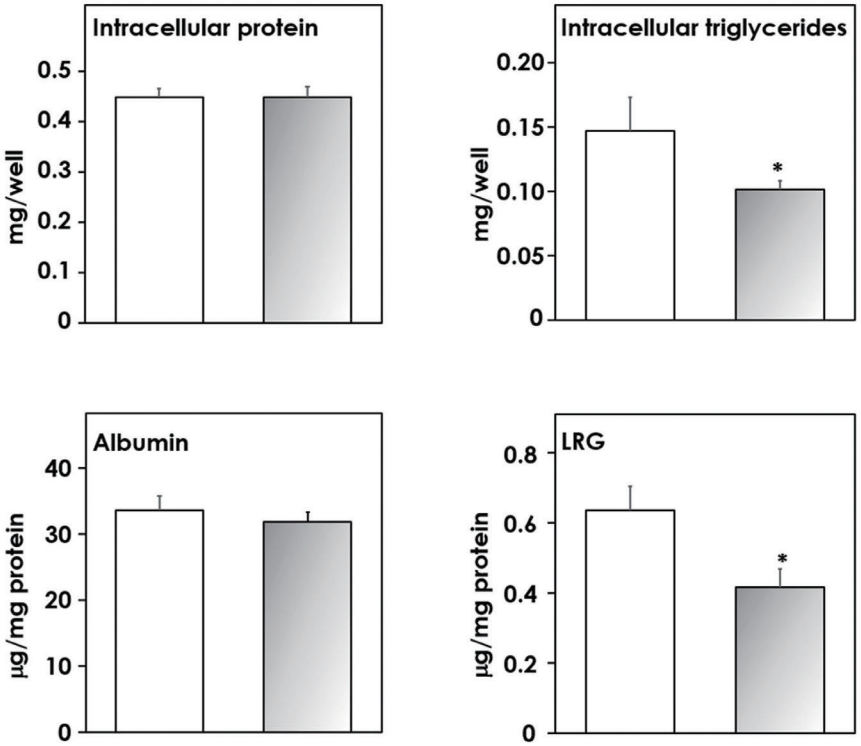

Fig. 1. Effects of Myoga Extract on Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD) Markers in PXB-Cells LA

PXB-cells LA were incubated without (white bar) or with 100 μg/mL myoga extract (gray bar) for two days. Intracellular protein and triglyceride levels per well were determined using a protein assay kit and Cholestest TG, respectively. Extracellular albumin and LRG levels, normalized to intracellular protein amounts, were measured using LZ-Test "Eiken" U-ALB and an LRG-specific ELISA kit, respectively. * $p < 0.05$ (n = 4)

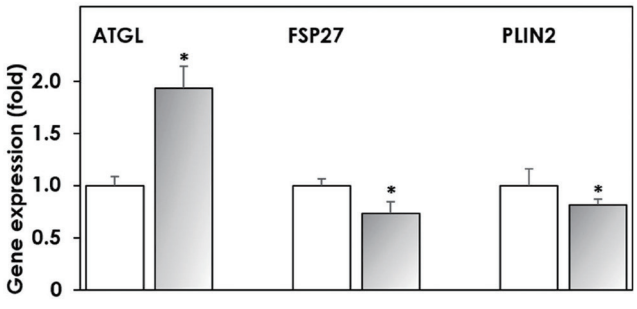

Fig. 2. Effects of Myoga Extract on Gene Expression of Lipid Droplet-Associated Proteins in PXB-Cells LA

PXB-cells LA were incubated without (white bar) or with 100 μg/mL myoga extract (gray bar) for two days, and gene expression was determined using realtime-PCR. * $p < 0.05$ (n = 4)

the extract increased the expression of FGF21, a beneficial hepatokine that enhances systemic insulin sensitivity, by 1.3- and 1.5-fold at the mRNA and protein levels, respectively (Fig. 3-b). These results suggest that myoga extract not only directly inhibits triglyceride accumulation in PXB-cells LA but may also improve fatty liver-associated insulin resistance by modulating the secretory balance between detrimental and beneficial hepatokines.

DISCUSSION

Because PXB-cells inherently exhibit steatosis upon isolation from humanized liver chimeric mice,[16] the PXB-cells LA

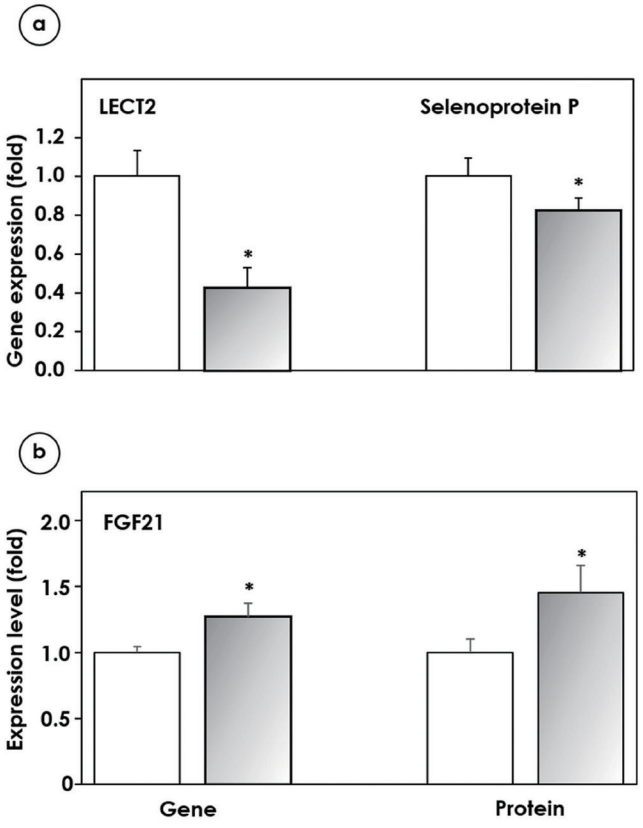

Fig. 3. Effects of Myoga Extract on Hepatokines in PXB-Cells LA

PXB-cells LA were incubated without (white bar) or with 100 μg/mL myoga extract (gray bar) for two days, and gene expression of detrimental hepatokines (a) and gene expression and protein levels of beneficial hepatokines (b) were measured. * $p < 0.05$ (n = 4)

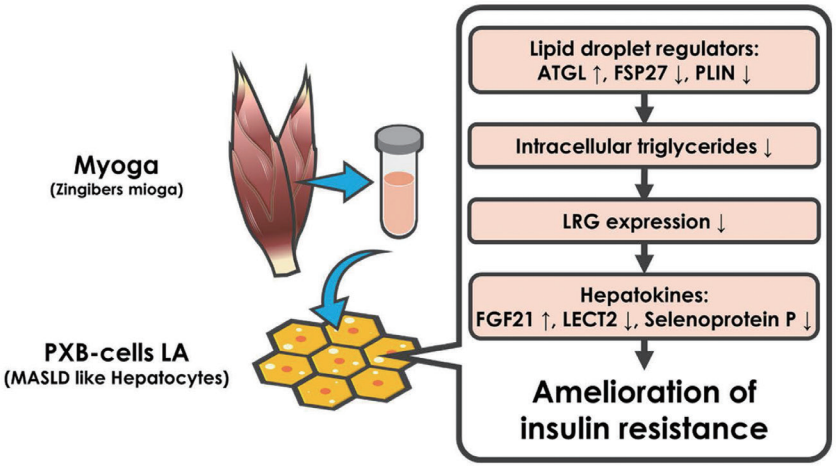

Fig. 4. Myoga Extract Regulates Lipid Metabolism and Hepatokine Profiles to Improve Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD)

model was uniquely developed to preserve this post-isolation fatty liver state through a proprietary culture method. In the present study, we used PXB-cells LA model to demonstrate that myoga extract effectively ameliorates steatotic conditions characteristic of MASLD.

Our present data indicate the possibility that myoga extract is involved in triggering triglyceride degradation within LDs, thereby potentially contributing to the reversal of the cellular state associated with steatosis (Fig. 4). Treatment of this *in vitro* MASLD model with myoga extract significantly attenuated the expression of LRG, a marker of liver injury progression. Furthermore, resolution of the steatotic state by myoga extract led to normalization of the hepatokine balance. Lee *et al*. reported that myoga extract improves insulin resistance by regulating gluconeogenesis, hepatic lipid accumulation, and inflammation in high-fat diet-fed mice [10]. Our current findings extend these observations by demonstrating that the extract restores hepatokine balance in MASLD-like human hepatocytes. Although identifying the active compounds and conducting clinical trials are essential next steps, our goal in the present study was to apply local food ingredients in advanced functional foods for the management of MASLD and related metabolic disorders.

Conflicts of interest Masaki Takahashi and Masakazu Kakuni are employees of PhoenixBio Co., Ltd.

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