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RESEARCH ARTICLE

Antioxidative, Hypolipidemic, and Anti-inflammatory Activities of Sulfated Polysaccharides from *Monostroma nitidum*

Minh Hien Hoang, Ji-Young Kim, Ji Hae Lee, SangGuan You, and Sung-Joon Lee

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Abstract Polysaccharides from seaweed have different biological activities. Two types of sulfated polysaccharides (SPs) were purified from *Monostroma nitidum* (MF1 and MF2) and investigated for biological activities *in vitro*. The MF1 and MF2 fractions exhibited strong antioxidant activities assessed using superoxide dismutase (SOD) assays. Stimulation of lipid-loaded hepatocytes by the MF1 and MF2 fractions significantly ($p<0.05$) reduced cellular lipid concentrations, compared with controls. Quantitative PCR analysis revealed that reductions in cellular lipid concentrations accompanied reduced expressions of cholesterol synthesis genes, and induced gene expressions for cholesterol degradation, LDL uptake, and peroxisomal β-oxidation. Gene expressions related to inflammation, including inducible NO synthase (iNOS), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8), and visfatin, were suppressed in lipid-loaded hepatocytes stimulated with MF1 and MF2. SPs from *M. nitidum* exhibited hypolipidemic and anti-inflammatory activities.

Keywords: *Monostroma nitidum,* sulfated polysaccharide, antioxidation, anti-inflammation, lipid metabolism

Minh Hien Hoang, Ji-Young Kim, Ji Hae Lee, Sung-Joon Lee (✉)
Department of Biotechnology, Graduate School of Biotechnology, Korea University, Seoul 136-713, Korea
Tel: +82-2-3290-3029; Fax: +82-2-3290-3653
E-mail: junelee@korea.ac.kr

Minh Hien Hoang, Ji-Young Kim, Ji Hae Lee, Sung-Joon Lee
Division of Food Bioscience and Technology, College of Life Sciences and Biotechnology, Korea University, Seoul 136-713, Korea

Minh Hien Hoang
Institute of Biotechnology, Vietnam Academy of Science and Technology, Hanoi 10000, Vietnam

SangGuan You
Department of Marine Food Science and Technology, Gangneung-Wonju National University, Gangneung, Gangwon 210-702, Korea

Introduction

Non-alcoholic fatty liver disease (NAFLD) ranges from simple steatosis to steatosis accompanied by inflammation and fibrosis (steatohepatitis, or NASH), which can further develop into cirrhosis or hepatocellular carcinoma. The progression of simple steatosis to NASH is thought to occur via what has been called a "2-hit hypothesis" (1) in which hepatic fat accumulation acts as a predisposing factor (1st hit) leading to further insults (2nd hit) of oxidative stress, cytokine accumulation, bacterial endotoxin, and/or endoplasmic reticulum stress as additional factors. NAFLD may be considered the hepatic event in the metabolic syndrome and is, therefore, linked to the risk factors of obesity, insulin resistance, hypertension, and dyslipidemia that are common to the metabolic syndrome (2-5). NAFLD has been estimated to affect up to 30% of the population of western countries. There is no exclusive treatment for NAFLD and, as a result, it is often treated using weight loss, an insulin sensitizer, lipid-lowering drugs, and/or antioxidant therapies that have side effects and are not designed to treat the specific NAFLD characteristics of hepatic steatosis and liver insulin resistance (6). Biologically active components from natural materials have been considered for reduction of the adverse effects of drugs without affecting physiological functions.

Recently, efforts have focused on isolation of novel bioactive compounds from marine resources because of beneficial health effects. Marine algae are valuable sources of structurally diverse bioactive compounds. Additionally, seaweed is an excellent source of dietary fiber, with a high proportion of soluble to total dietary fiber. The dietary fiber in seaweed is mainly composed of indigestible sulfated polysaccharides (SPs), which are resistant to human digestive enzymes. Several common structural SPs include fucoidans in brown algae, carrageenans in red algae, and ulvans in

green algae. These SPs exhibit the beneficial biological activities of anticoagulant, antiviral, antioxidative, anticancer, and immunomodulating activities, and hypolipidemic and hypocholesterolemic effects (7).

Polysaccharides are absorbed intestinally through Peyer's patch and are delivered to the circulation where they exert biological activities in metabolically active tissues, including the liver. Schipper *et al.* (8) reported that interactions of polysaccharides with the cell membrane result in a structural reorganization of tight junction-associated proteins, followed by enhanced transport through the paracellular pathway.

Different biological activities of SPs have been reported. Ulvan from *Ulva pertusa* is a potential antihyperlipidemic agent that significantly reduced serum triglyceride (TG) and total and low-density lipoprotein cholesterol (LDL-C) levels, and elevated high-density lipoprotein cholesterol (HDL-C) levels in mice (9). According to Saeid *et al.* (10), the antihyperlipidemic ulvan activity depends on the molecular weight (Mw) of ulvan fractions. High Mw fractions are more effective against serum total cholesterol and LDL-C levels, whereas low Mw fractions are more effective against TG and HDL-C levels. Ulvan contains uronic acid and sulphates and can sequester or bind bile acids (11). Additionally, SPs are reportedly soluble (7) and form viscous gels during passage through the gastrointestinal tract (8) Gels can restrict absorption of bile acids by the body as bile binds to the gel and is excreted. In turn, this forces the liver to scavenge cholesterol from the blood for synthesis of replacement bile, thus lowering cholesterol levels in the blood (12). Porphyran from the marine red algae *Porphyra yezoensis* can also be used as a potent antihyperlipidemic agent (13) due to reduction of apolipoprotein B100 (apoB100) secretion, mainly through suppression of lipid synthesis in human liver-derived cells (14). Furthermore, SPs from *Monostroma nitidum* were shown to be potent thrombin inhibitors mediated by heparin cofactor II, which exhibits a strong anticoagulant activity (14). Moreover, rats fed SPs from *M. nitidum* showed significantly lower levels of plasma total cholesterol (15), and Wong *et al*. (16) reported that seaweed-based diet using *M. nitidum* reduced serum cholesterol concentrations compared with those levels in control group. However, no study to date has investigated the mechanisms behind the hypolipidemic effects of SPs from *M. nitidum.* Therefore, the hypolipidemic mechanisms of MF1 and MF2 fractions were studied *in vitro*, focusing on effects in lipid-loaded hepatocytes, major tissues for regulation of the lipid metabolism.

Materials and Methods

Materials The green seaweed *M. nitidum* was harvested in the spring of 2009 from the coast of Wando, Chunnam Province, Korea. Immediately after collection, raw seaweed was washed with distilled water three times and air-dried at 60°C for 48 h. Dried raw material was milled using a blender, sieved (<0.5 mm), and stored at −20°C.

Extraction and fractioning Milled samples (20 g) were treated with 85% ethanol (EtOH, 200 mL) at room temperature overnight with constant mechanical stirring to remove lipophilic pigments (chlorophylls and carotenes) and low Mw proteins. Samples were then rinsed with acetone centrifuged at 18,500×*g* for 10 min at 10°C, then dried at room temperature. Dried biomass (20 g) was subjected to extraction twice using distilled water (400 mL) at 65°C with stirring for 2 h. Extracts were centrifuged at 18,500×*g* for 10 min, then the supernatant was collected and evaporated under reduced pressure at 60°C to obtain a volume of approximately 100 mL. Ethanol (EtOH, 99%) was added to the supernatant to obtain a final concentration of 30% EtOH, and the solution was maintained at 4°C for 4 h. After centrifugation at 18,500×*g* for 15 min, additional EtOH was added then supernatant was collected in a final concentration of 70% EtOH. The solution was maintained at 4°C overnight. Crude polysaccharide was obtained by filtration of the solution through a nylon membrane (0.45-μm pore size, Whatman International, Maidstone, UK) and washing with EtOH (99%) followed by acetone. After drying at room temperature overnight, the yield was calculated based on the weight of the dried biomass obtained after treatment of milled samples using 85% EtOH.

Crude polysaccharide (250 mg) dissolved in distilled water (10 mL) was fractionated using ion-exchange chromatography on a DEAE Sepharose fast-flow column (17-0709-01, GE Healthcare Bio-Science AB, Uppsala, Sweden) equilibrated using distilled water. The column was washed with distilled water, and the bound polysaccharide was eluted using a solution of distilled water and increasing concentrations of NaCl (from 0.5 to 1.0 M). Two fractions were obtained, referred to as MF1 and MF2. The fractions were dialyzed against distilled water for 3 days, then lyophilized. The carbohydrate elution profile was determined using the phenol-H_2SO_4 method (17) based on measurement of the absorbance at 490 nm.

Chemical composition The sulfate content of the polysaccharide was determined using the $BaCl_2$ gelatin method with K_2SO_4 as a standard (18) after hydrolyzing the polysaccharide in 0.5 M HCl at 105°C for 5 h. The total carbohydrate and protein contents were determined using the phenol-H_2SO_4 method with glucose as a standard (17) and the Lowry *et al.* method (19), respectively, using a commercial assay kit (DC Protein Assay kit, Bio-Rad, Hercules, CA, USA). The uronic acid content of the

polysaccharide was determined based on a sulfamate/m-hydroxydiphenyl assay using glucuronic acid as a standard (20).

Determination of monosaccharide composition Quantitative determination of the monosaccharide composition of the polysaccharide was performed using HPLC on a system consisting of a pump (Waters 510, Waters, Milford, MA, USA), an injection valve (Model 7010, Rheodyne, Rohnert Park, CA, USA) with a 20 μL sample loop, a column (carbohydrate analysis column, 4.6 × 250 mm, Waters), and a refractive index (RI) detector (Waters 2414). The polysaccharide (6 mg) was hydrolyzed for 90 min in 2 M trifluoroacetic acid (TFA, 0.3 mL) at 120°C. After removal of TFA from the sample solution using a stream of dried nitrogen, the hydrolyzed polysaccharide was injected into the HPLC system. A mixture of acetonitrile and water (80:20, v/v) was used as the mobile phase at a flow rate of 2 mL/min.

Cell culture and treatment HepG2 cells were obtained from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin before treatment. All cells were grown in 5% CO_2 at 37°C. Experimental cells were seeded into 100 mm culture dishes or 6 well culture plates. After confluence was reached, cells were cultured for 24 h in DMEM containing 10% FBS, 1% penicillin/streptomycin, and palmitate (PA, 600 μM) that was conjugated to 0.16% fatty acid-free BSA to mimic hyperlipidemic conditions. After intracellular lipid droplets formed in the HepG2 cells, the cells were washed with PBS, then incubated with either serum-free containing vehicle (1% distilled H_2O) or seaweed fractions (50, 100, and 200 μg/mL). Each treatment was performed at least in triplicate.

Determination of the superoxide dismutase (SOD) activity The superoxide anion is an important scavenger of intracellular free radicals. The disproportionation activity of the superoxide anion was examined using a Superoxide Dismutase Activity Colorimetric Assay kit (Abcam, Dawinbio, Inc., Seoul, Korea). Briefly, 0.2 mL of MF1 and MF2 (50, 100, and 200 μg/mL) was placed separately in a tube and 3 mL of Tris-HCl buffer (pH 8.5) and 0.2 mL of pyrogallol (7.2 mM) were added. The mixture was shaken and incubated at 25°C for 10 min, then 1 mL of a stop solution (1 N HCl) was added to the tube. The absorbance of the resulting solution was then measured spectrophotometrically at 420 nm.

Lipid extraction and measurement HepG2 cells were washed 3 times in 2 mL of ice-cold PBS, and soluble cell protein was dissolved in 200 μL of lysis buffer (10 mM Tris-HCl at pH 7.4, 0.1 M EDTA, 10 mM NaCl, 0.5% Triton X-100) and measured using Bradford reagent (Bio-Rad) with BSA as a standard. For determination of the intracellular TG and cholesterol levels, after washing 3 times with cold PBS, cells were treated with 1 mL of hexane/isopropanol (2:1) for 30 min at room temperature, then transferred to a test tube. Culture plate wells were washed with 1 mL of hexane/isopropanol, then the washing solutions were transferred to the corresponding test tubes. The organic solvent of hexane/isopropanol was removed under nitrogen, and lipids were resuspended in 95% EtOH. TG and cholesterol contents were quantified using an enzymatic method with a Cobas C111 automatic analyzer (Roche, Basel, Switzerland).

Table 1. Chemical composition of SPs from *M. nitidum*

(unit: %)

Component	SPs	
	MF1	MF2
Yield	38.0±0.3	36.7±0.4
Total carbohydrate	58.3±0.2	67.1±1.0
Sulfate content	10.5±2.7	17.7±1.0
Protein	9.4±0.4	1.6±0.1
Uronic acid	21.8±1.6	13.3±0.5
Rhamnose	61.8±3.6	95.7±1.6
Glucose	36.6±3.9	ND
Xylose	1.6±0.3	4.3±1.7

ND, not detected

Isolation of total RNA and real-time quantitative qPCR analysis Total RNA was extracted from HepG2 cells using an RNAiso Plus kit (Takara, Tokyo, Japan) according to the manufacturer's protocol after 1 day of treatment with vehicle as a positive control, or a seaweed fraction. For generation of cDNA, 2 μg of total RNA was reverse-transcribed using oligo $(dT)_{15}$ with M-MLV Reverse Transcriptase (Mbiotech, Seoul, Korea) according to the manufacturer's protocol, resulting in 20 μL of cDNA. Real-time qPCR was performed using Bio-Rad iQ SYBR Green Supermix reagent with a Bio-Rad iQ5 Cycler System. The reaction conditions were 95°C for 3 min, followed by 50 cycles of 95°C for 10 s, 57°C for 15 s, and 72°C for 20 s. Melting curve analysis for 71 cycles starting at 55°C and increasing by 0.5°C every 10 s was performed for determination of the primer specificity. Primers (Supplemental Table 1) were designed using OligoPerfect Designer software (Invitrogen, Carlsbad, CA, USA). Expression levels were normalized to the level of GAPDH expression and analyzed using iQ5 System Software (version 2) based on the normalized expression (CT) method according to manufacturer guidelines.

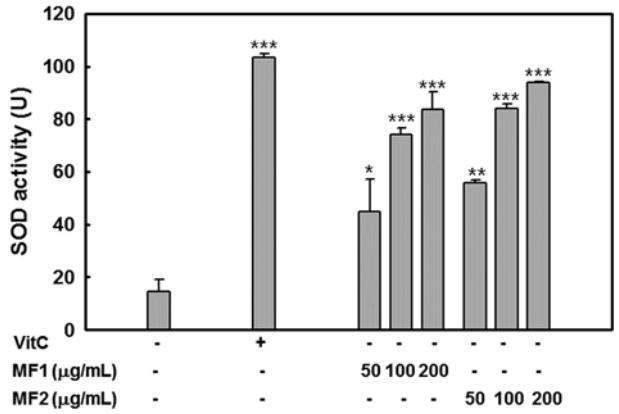

Fig. 1. Effects of the MF1 and MF2 fractions on SOD activities. A reaction mixture containing Tris-HCl buffer and SPs at different concentrations (50, 100, and 200 μg/mL) was incubated at 25°C for 10 min. The absorbance of the resulting solution was measured at 420 nm. The reference compound was ascorbic acid. Data are given as a mean±SEM (n=3). *, **, *** Significantly different from the control group, *p<0.05; **p<0.005; ***p<0.0005

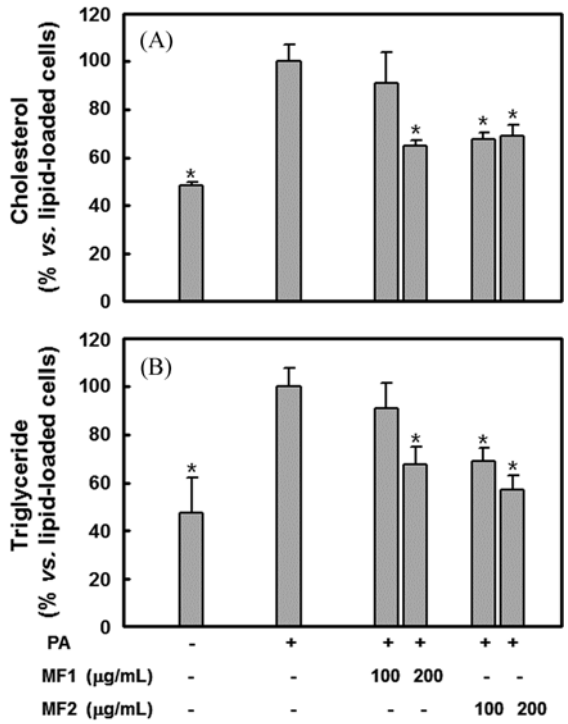

Fig. 2. Effects of the MF1 and MF2 fractions on lipid levels in lipid-loaded HepG2 cells. HepG2 cells were incubated with PA (600 μM) for 24 h, then stimulated using serum-free containing vehicle (1% water) or seaweed fractions (100 and 200 μg/mL) for another 24 h. Cellular cholesterol (A) and TG levels were measured using an enzymatic method (B). Data are given as a mean±SEM (n=5). * Significantly different from the lipid-loaded group, *p< 0.05

Statistical analysis Data were expressed as mean± standard error of the mean (SEM). Differences were considered statistically significant at p<0.05, assessed using the Student t-test function of the SAS software package (SAS Institute, Cary, NC, USA).

Result and Discussion

Chemical composition SPs extracted from *M. nitidum* yielded 38.0 and 36.7% MF1 and MF2 fractions, respectively. MF1 and MF2 contained sulfates (10.5 and 17.7%, uronic acid (21.8 and 13.3%), and carbohydrates (58.3 and 67.1%), respectively. Rhamnose was the major sugar in both fractions, along with small amounts of xylose and glucose.

Anti-oxidative activity of SPs SPs from seaweed exhibited antioxidant properties in a stress-induced rat model (7). Superoxide dismutase (SOD) is a major scavenger of reactive oxygen species (ROS). Therefore, the antioxidant activities of fractions MF1 and MF2 isolated from *M. nitidum* were evaluated using a SOD assay. Compared with an untreated control, the percent of SOD activity was significantly (p<0.05) increased after treatment with ascorbic acid, a reference compound, and by MF1 and MF2 fractions in a dose-dependent manner (Fig. 1). The MF1 and MF2 fractions (200 μg/mL) increased SOD activities by 650 and 579%, respectively.

SPs reduced lipid concentrations in lipid-loaded hepatocytes The hypolipidemic activities of the MF1 and MF2 fractions were analyzed. Cells were treated with 600 μM PA to mimic hepatic lipid accumulation for 24 h before treatment with and without SPs. The control group (cells alone) received neither PA nor SPs. When PA was added to HepG2 cells, the cholesterol and TG concentrations increased markedly, by 45 and 47%, respectively, versus the control (Fig. 2A, 2B). MF2 stimulation had a potent dose-dependent effect on cholesterol and TG accumulation inhibition, whereas MF1 reduced cholesterol and TG accumulation significantly (p<0.05) only at a high concentration (Fig. 2A, 2B) of 200 μg/mL, compared with controls. At 200 μg/mL, both MF1 and MF2 reduced cellular cholesterol concentrations by 33 and 36% and TG concentrations by 43 and 31%, respectively, consistent with previous reports (21-23). Thus, SPs isolated from *M. nitidum* suppressed cholesterol and TG accumulation in lipid-loaded cells.

Effect of SP fractions on cholesterol metabolism in lipid-loaded hepatocytes The hypocholesterolemic mechanisms induced by MF1 and MF2 in cultured hepatocytes were investigated due to significant (p<0.05) reductions in cholesterol levels in cells incubated with MF1 and MF2, compared with controls. Cholesterol levels in hepatocytes are regulated by a balance between

endogenous biosynthesis and catabolism that is primarily regulated by the activities of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase and cholesterol 7 α-hydroxylase (CYP7A1), respectively (24). Additionally, the LDL receptor (LDLR) plays a crucial role in uptake of plasma LDL from the circulation (25). In lipid-loaded HepG2 cells, HMG-CoA reductase expression was dramatically induced, whereas mRNA expressions of CYP7A1 and LDLR were significantly (p<0.05) reduced, compared with controls (Fig. 3A). However, lipid-loaded cells stimulated using MF2 (200 μg/mL) showed significantly (p<0.05) reduced mRNA levels of HMG-CoA reductase (29% decrease; Fig. 3A), compared with controls, but not lipid-loaded cells stimulated using MF1, similar to other reports (23). Thus, the mechanism by which polysaccharide extracts of *Ulva fasciata* reduce serum cholesterol is mediated through inhibition of HMG-CoA reductase.

The mechanism involved in lowering of cholesterol levels by polysaccharides is not fully understood; however, Huang *et al.* (26) proposed that the mechanism is related to the hepatic bile acid concentration in which a reduction in the bile acid concentration activates CYP7A1, which converts cholesterol into bile acids. This leads to a reduced hepatic cell cholesterol content that up-regulates LDLR synthesis, thereby accelerating transport of LDL-cholesterol from the blood into hepatocytes. This study demonstrated that MF1 and MF2 stimulation induced mRNA expressions of CYP7A1 and LDLR (Fig. 3A). In lipid-loaded cells stimulated using 200 μg/mL of MF1, mRNA expression levels of CYP7A1 and LDLR were increased by 2.4 and 21.5 times, respectively. In comparison, CYP7A1 and LDLR expression levels were induced by 5.4 and 13.5 times, respectively, using 200 μg/mL of MF2.

In this study, the mechanisms by which SPs from *M. nitidum* lower cholesterol levels were analyzed using lipid-loaded hepatocytes. Alterations in CYP7A1, LDL-R, and HMG-CoA expressions are herein reported for the first time to underlie this effect, at least in part. SPs apparently influence cholesterol handling at multiple points in the metabolic process. However, in this study, the differential affects of MF1 and MF2 on expressions of CYP7A1, LDL-R, and HMG-CoA were not explained. Further study is needed for determination of connections with and expressions of genes related to cholesterol metabolism. SPs rich in rhamnose may have an additional hypocholesterolemic effect due to reduction of cholesterol synthesis, as reported by Matloub *et al.* (23).

Hypotriglyceridemic mechanism in lipid-loaded HepG2 cells and induction of fatty acid oxidation and TG hydrolysis Yu *et al.* (27) suggested that polysaccharides from *Rosae Laevigatae Fructus* improved hyperlipidemia, possibly through up-regulation of lipoprotein lipase (LPL) expression. Additionally, Matloub *et al.* (23) reported that decreased serum TG concentrations in hyperlipidemic rats treated with isolated polysaccharides can be explained on the basis of increased clearance of TGs secondary to an increase in LPL activity. Thus, the TG-lowering effects of SPs were further studied in the context of regulation of fatty acid oxidation and VLDL-TG hydrolysis. Fatty acids removed from the circulation are subsequently synthesized into hepatic TGs for storage. TGs are lipolyzed to fatty acids in the fasting state to sustain gluconeogenesis, and LPL is responsible for hepatic TG hydrolysis (28). Resulting free fatty acids are subsequently converted into acyl-CoA, the initial substrate for β-oxidation by carnitine palmitoyl-transferase 1 (CPT1) or the uncoupling protein 2 (UCP2), in the mitochondrial membrane. Acyl-CoA oxidase (ACOX) transforms acyl-CoA to acetyl-CoA, which enters the TCA cycle (29). Lipid-loaded cells stimulated using MF1 and MF2 exhibited significant (p<0.05) induction by UCP2 at a concentration of 200 μg/mL, compared with controls, with no alteration of CPT1 expression (Fig. 3B). Apolipoprotein C3 (APOCIII) is an inhibitor of both LPL activity and remnant clearance. MF2 significantly (p<0.05) reduced APOCIII expression at a high concentration, compared with controls. (Fig. 3B). Accordingly, VLDL lipolysis was induced, and large LDL particles that could be more efficiently cleared via LDLR were generated (30). A reduction in the hepatic TG concentration apparently is caused by SPs from *M. nitidum,* resulting in induction of hepatic fatty acid oxidation and/or VLDL-TG hydrolysis. However, structural analysis is needed and cellular mechanisms remain to be determined.

Effect of SP fractions on inflammation in lipid-loaded HepG2 cells PA treatment can result in low-grade inflammation (31), which is characterized by activated transcription of the inammatory mediators NO synthase (iNOS), tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-8 (32,33). TNF-α is a key component in many metabolic diseases, including obesity, diabetes, dyslipidemia, and atherosclerosis (34). iNOS, IL-6, and IL-8 are also commonly viewed as destructive proinflammatory mediators (31). Lipid-lowering drugs, such as fenofibrate and simvastatin, used in hyperlipidemia can significantly lower the serum levels of TNF-α and IL-6 (35). Thus, prevention of low-grade inflammation by inhibition of proinflammatory cytokine activation may be an effective method for treatment of hyperlipemia. In this study, PA treatment increased the mRNA levels of the proinflammatory molecules iNOS, TNF-α, IL-6, and IL-8. However, the effects of PA on increased expressions of these genes were abolished after stimulation using SPs. In lipid-loaded HepG2 cells, MF1 and MF2 treatments reduced the mRNA expressions of iNOS, TNF-α, IL-6, and IL-8 in a

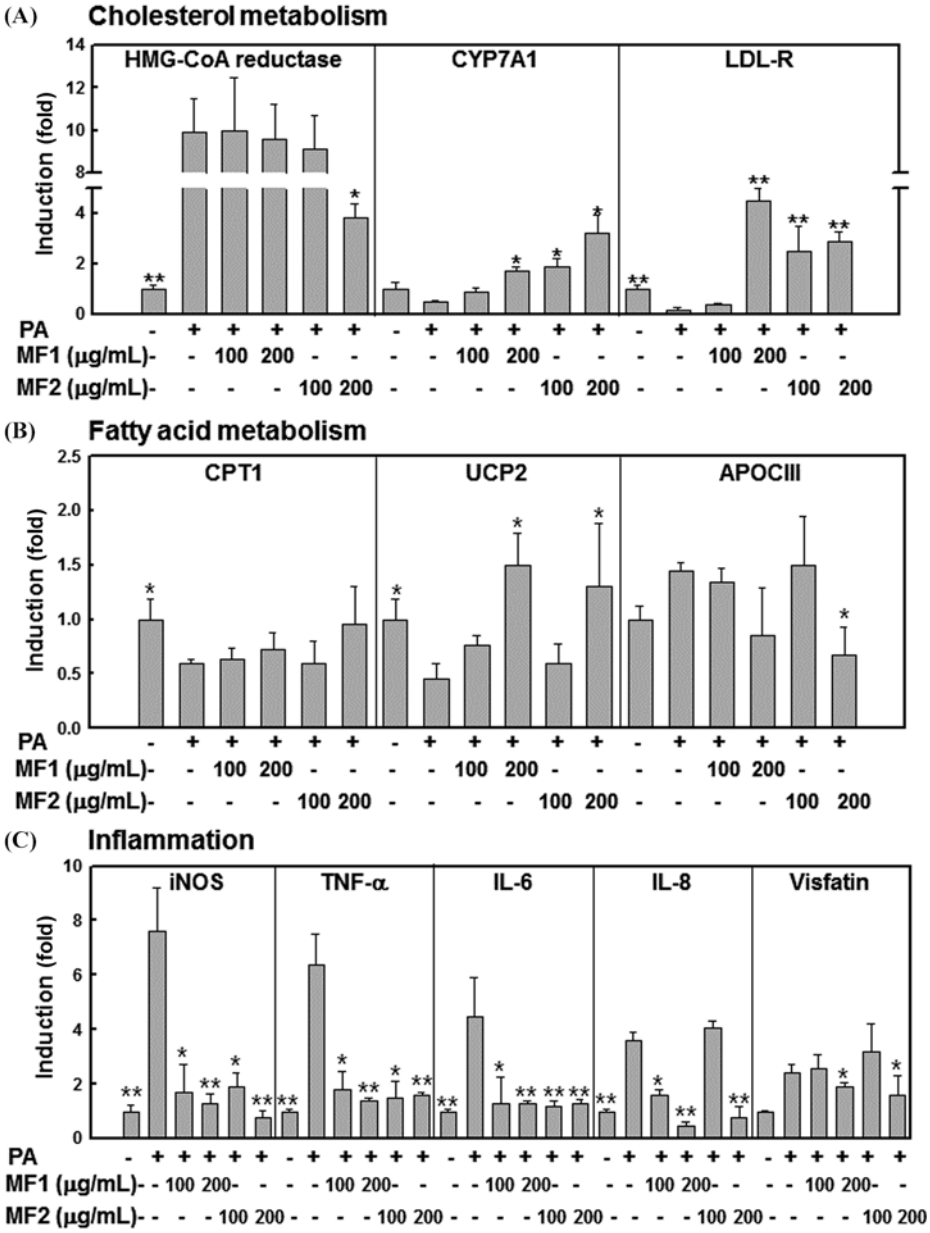

Fig. 3. Effects of the MF1 and MF2 fractions on expressions of genes involved in cholesterol (A), fatty acid metabolism (B), and inflammation (C) in lipid-loaded hepatocytes. HepG2 cells were incubated with PA (600 μM) for 24 h and stimulated with serum-free containing vehicle (1% water) or seaweed fractions (100 and 200 μg/mL) for another 24 h. Total RNA was extracted from cells, and gene expression levels were measured using real-time qPCR analysis. Data are given as a mean±SEM (n=5). *, ** Significantly different from the lipid-loaded group, *p<0.05; **p<0.01

dose-dependent manner (Fig. 3C).

Recently, several studies have reported proinflammatory effects of visfatin, which plays a role in obesity-related metabolic diseases and inflammatory conditions. Visfatin expression and protein synthesis were significantly increased in HepG2 cells treated with PA in both a time and a concentration-dependent manner. Additionally, visfatin-specific small interfering RNA significantly decreased PA-induced mRNA expression and protein synthesis of both IL-6 and TNF-α (26). Therefore, visfatin expression in lipid-loaded hepatocytes was assessed in this study. Incubation with MF1 and MF2 reduced visfatin gene expression significantly (p<0.05), compared with controls. MF1 and MF2 at 20 μM reduced visfatin gene expressions by 21% and 33%, respectively, compared with lipid-loaded cells (Fig. 3C). Thus, SPs probably exert a lipid lowering effect by reduction of increased levels of proinflammatory mediators and visfatin mRNA expression.

SPs from *M. nitidum* showed hypolipidemic effects in lipid-loaded hepatocytes, which may have nutritional implications for hyperlipidemia and atherosclerosis. The hypolipidemic effects of SPs are probably regulated by

multiple mechanisms in cholesterol, TG, and inflammation pathways.

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Disclosure The authors declare no conflict of interest.

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