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Abstract

Nostoc commune var. *sphaeroides* Kützing (*N. commune*), a blue-green alga, has been used as both a food ingredient and in medicine for centuries. To determine the effect of *N. commune* on cholesterol metabolism, *N. commune* lipid extract was incubated at increasing concentrations (25–100 mg/L) with HepG2 cells, a human hepatoma cell line. The addition of *N. commune* lipid extract markedly reduced mRNA abundance of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) and LDL receptor (LDLR) ($P < 0.05$), with a concomitant decrease in their protein expression ($P < 0.001$). Reduced HMGR activity by 90% with *N. commune* lipid extract confirmed the inhibitory role of *N. commune* in cholesterol synthesis ($P < 0.006$). To elucidate a molecular mechanism underlying the repression of HMGR and LDLR by *N. commune* lipid extract, expression of sterol regulatory element binding protein 2 (SREBP-2) was assessed. Whereas mRNA for SREBP-2 remained unchanged, SREBP-2 mature protein was reduced by *N. commune* ($P < 0.009$). In addition, *N. commune* lipid extract also decreased SREBP-1 mature protein by ~30% ($P < 0.002$) and reduced the expression of SREBP-1-responsive genes such as fatty acid synthase and stearoyl CoA desaturase 1 (SCD-1) ($P < 0.05$). Therefore, our

results demonstrate that *N. commune* lipid extract inhibits the maturation process of both SREBP-1 and -2, resulting in a decrease in expression of genes involved in cholesterol and fatty acid metabolism.

Abbreviations: BGA, blue-green algae; CHD, coronary heart disease; Ct, threshold cycle; FAS, fatty acid synthase; HMGR, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase; INSIG, insulin-induced gene; LDLR, LDL receptor; SCAP, SREBP cleavage-activating protein; SCD-1, stearoyl CoA desaturase 1; S1P, site-1-protease; SREBP, sterol regulatory element binding protein.

Introduction

Coronary heart diseases (CHD) are the leading cause of death in the United States and other Westernized countries. A direct correlation exists between the concentration of plasma cholesterol and the severity of atherosclerosis (1). According to the 2001 report of the National Cholesterol Education Program, an expert panel estimated that 65 million US adults should be recommended to have therapeutic lifestyle changes, including diet, and 36 million of that population also need drug therapy for treatment of elevated cholesterol levels (2). Currently, statin drugs are widely prescribed to lower plasma cholesterol concentration. Statins primarily reduce plasma cholesterol levels by inhibiting 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), the rate-limiting enzyme in the cholesterol biosynthetic pathway. Although statins have proven to be effective at lowering plasma cholesterol level, potential adverse effects may occur (1,3).

Increased public interest in natural and herbal medicine has prompted the identification of natural products with heart protection. Several herbal and functional food supplements have been used for primary prevention of heart disease. Consumption of garlic (4), soy protein (5), and artichoke (6) has been reported to moderately decrease plasma cholesterol levels. In addition, soluble fibers from barley, beans, oat bran, and psyllium reduced plasma cholesterol concentration when consumed by healthy hyperlipidemic patients on a low-fat diet (7). To meet the public's demand for natural foods for the prevention of CHD, continuous efforts need to be made to discover and develop new bioactive natural products.

Blue-green algae (BGA) are among the most primitive life forms on earth (8). Edible BGA, such as *Spirulina* and *Aphanizomenon flos-aquae*, are currently marketed as dietary supplements with various health claims for immune function, inflammation, and heart disease. *Spirulina* is known to be rich in vitamins, minerals, essential and nonessential amino acids, trace elements, and essential fatty acids (9). Currently, *Spirulina* is harvested from controlled ponds in dozens of countries and is being sold as an organic dietary supplement. In contrast to *Spirulina*, most of *A. flos-aquae* products are naturally harvested from the Upper Klamath Lake in southern Oregon and therefore it is called "a natural dietary supplement." Although the term "natural" could attract consumers, concerns over naturally grown BGA have been raised because they are easily contaminated with *Microcystis aeruginosa*, which generates potent hepatotoxin microcystins, as well as with heavy metals (10). In response to this concern, Health Canada performed broad sampling of BGA

supplements available on the Canadian market in May 1999 to test their microcystin contents. The survey revealed that only *Spirulina* has no detection of microcystins and many non-Spirulina BGA products harvested from natural lakes contain microcystins above levels considered acceptable by Health Canada and WHO. In addition, the quality of naturally grown BGA can vary depending on their growing environments, e.g., altitude, temperature, and sun exposure (11).

Nostoc commune var. *sphaeroides* Kützing (*N. commune*) is an edible BGA that has been used as a food delicacy as well as an herbal medicine in countries to which it is native for thousands of years. It has been historically suggested that *N. commune* can treat a variety of medical conditions, including inflammation, night blindness, burns, anxiety, and chronic fatigue. Studies have also demonstrated various health benefits from *N. commune*, including antiviral and anticancer activities (12–14). Importantly, a cholesterol-lowering effect of *N. commune* was reported in rats fed a high-cholesterol diet, and the effect was attributed to its high-fiber content (15).

Although previous research has shown *N. commune* confers health benefits, the investigation into the mechanisms behind the biological effects have been limited. In this study, we investigated the ability of *N. commune* lipid extract to influence key regulators of both cholesterol and fatty acid metabolism in HepG2 cells, a human hepatoma cell line. Because sterol regulatory element binding proteins (SREBP) are transcriptional factors that play a central role in regulating both cholesterol and fatty acid metabolism (11,16), the effect of *N. commune* on the expression as well as maturation of SREBP was investigated.

Experimental Procedures

Preparation of Nostoc *lipid extract*

Fresh *N. commune* was obtained from Algaen Corporation. *N. commune* was lyophilized and ground for lipid extraction by the Bligh-Dyer method (17). Briefly, 42 mL of chloroform:methanol (1:2, v:v) was added to ~3 g of dry *N. commune* and vortexed. After the addition of 14 mL of chloroform, the sample was vortexed and filtered to remove solids. Subsequently, 6.3 mL of 0.05% H_2SO_4 was added and vortexed. To separate phases, the sample was centrifuged at 500 × *g*; 5 min and the lower liquid phase was recovered. Tubes containing lipid extract in chloroform were purged with N_2 and tightly sealed. The lipid extract was stored at –80°C with the oxygen absorber AGELESS (Mitsubishi Gas Chemical America) to prevent oxidation until use. To incorporate the lipid extract into cell culture medium, chloroform was evaporated under nitrogen and cell culture medium was added for subsequent sonication at room temperature for 5 min.

Cell culture

HepG2 cells (ATCC) were maintained in minimal essential medium containing 10% fetal bovine serum, 100 kU/L penicillin, 100 mg/L streptomycin, 1× vitamins, and 2 mmol/L L-glutamine in a humidified chamber at 37°C with 5% CO_2. All cell culture supplies were purchased from MediaTech. Cells were plated at a density of 3×10^5 cells per well in 12-well plates. When cells reached ~90% confluence, *N. commune* lipid extract (0–100 mg/L) was added to culture medium for 24 h.

Cytotoxicity measurement

Cytotoxicity of *N. commune* lipid extract was determined using Cell Counting Kit-8 (Dojindo Molecular Technologies) according to the manufacturer's instructions. SDS (0.5 mmol/L) was also added as a positive control for cytotoxicity. Data are expressed as cell viability (%) relative to a control that was not incubated with *N. commune* lipid extract.

Total RNA isolation and quantitative real-time PCR

HepG2 cells were washed twice with cold PBS and 1 mL of TRIZOL reagent (Invitrogen) was added to each well of 12-well plates for total RNA isolation following the manufacturer's protocol. One microgram of total RNA was treated with DNase I (Promega) to remove any genomic DNA. The RNA samples were reverse transcribed by Moloney murine leukemia virus reverse transcriptase (Promega) for subsequent real-time PCR analysis using the Sybr Green procedure and an ABI 7300 instrument (Applied Biosystems). Primers for HMGR, LDL receptor (LDLR), fatty acid synthase (FAS), stearoyl CoA desaturase 1 (SCD-1), SREBP-1c, SREBP-2, and glyceraldehyde 3-phosphate dehydrogenase were designed according to GenBank database using the Primer Express software provided by ABI. Primer sequences are given in Table 1. Expression of mRNA values was calculated using the threshold cycle (C_t) value, i.e. the number of PCR cycles at which the fluorescent signal during the PCR reaches a fixed threshold. For each sample, $\Delta C_{t,\,sample}$ was calculated by subtracting the C_t value of glyceraldehyde 3-phosphate dehydrogenase, a house-keeping gene, from that of the gene of interest to normalize the data. The expression levels relative to control were estimated by calculating $\Delta\Delta C_t$ ($\Delta C_{t,\,sample} - \Delta C_{t,\,control}$) and subsequently using the $2^{-\Delta\Delta Ct}$ method (18).

Table 1. Quantitative Realtime PCR primers

Gene	Forward Primer	Reverse Primer
HMGR	5′-CCCAGTTGTGCGTCTTCCA-3′	5′-TTCGAGCCAGGCTTTCACTT-3′
LDLR	5′-ACTGGGTTGACTCCAAACTTCAC-3′	5′-GGTTGCCCCCGTTGACA-3′
SREBP-2	5′-TCCGCCTGTTCCGATGTAC-3′	5′-TGCACATTCAGCCAGGTTCA-3′
SREBP-1c	5′-TCAGCGAGGCGGCTTTGGAGCAG-3′	5′-CATGTCTTCGATGTCGGTCAG-3′
FAS	5′-CGCTCGGCATGGCTATCT-3′	5′-CTCGTTGAAGAACGCATCCA-3′
SCD-1	5′-CCGACGTGGCTTTTTCTTCT-3′	5′–TGGGTGTTTGCGCACAAG-3′
GAPDH	5′-GGTGGTCTCCTCTGACTTCAACA-3′	5′-GTTGCTGTAGCCAAATTCGTTGT-3′

Western blot analysis

HepG2 cells were incubated with 100 mg/L *N. commune* lipid extract for 24 h. After removing cell culture medium, cells were washed twice with cold PBS and scraped in 1 mL of PBS. Cells were transferred into a 1.5-mL microfuge tube and centrifuged at 12,000 × *g*; 5 min at 4°C. The supernatant was removed and 25 mL of cell lysis buffer (150 mmol/L NaCl, 25 mmol/L Tris-HCl, pH 7.4, 1% Triton X-100) containing Protease Inhibitor Cocktail set III (Calbiochem) was added to the cell pellet. The cells were resuspended and incubated on ice for 20 min, after which the cell lysates were centrifuged at 12,000 × *g*; 5 min at 4°C. We collected the supernatant and measured protein concentration by BCA assay (Pierce) using bovine serum albumin as a standard.

Fifty micrograms of cell protein was subjected to 4–16% SDS-PAGE for Western blot analysis using antibodies for HMGR (Upstate), LDLR (Abcam), SREBP-1 (Santa Cruz), SREBP-2 (Santa Cruz), and β-actin (Sigma). The blots were developed using a horseradish peroxidase system (Pierce) and densitometry analysis was performed using a Chemidoc XRS (Biorad) and Quantity One software (Biorad). β-Actin was used as a loading control to normalize the data.

HMGR activity measurement

HMGR activity was measured according to the methods by Brown et al. (19,20) with modification. Cell lysate was prepared as described above after 24 h incubation in the absence (control) or presence of *N. commune* lipid extract (100 mg/L). Two hundred microliters of reaction mixture was prepared as follows: 100 mg cellular protein, 0.1 mol/L potassium phosphate buffer (pH 7.5), 20 mmol/L glucose-6-phosphate, 2.5 mmol/L $NADP^+$, 1 U glucose-6-phosphate dehydrogenase, 5 mmol/L dithiothreitol, and 6 nmol [^{14}C]HMG-CoA. The reaction was initiated with the addition of [^{14}C]HMG-CoA, and the mixture was incubated for 2 h at 37°C. The reaction was stopped by the addition of 20 μL of 5 mol/L HCl on ice. Subsequently, 0.2 μCi of [^{3}H]mevalonolactone was added as an internal standard for correct recovery and incubated at 37°C for 30 min to ensure lactonization of mevalonate. The mevalonolactone was extracted into 5 mL of diethyl ether twice and isolated by TLC with acetone:benzene (1:1). Mevalonolactone was identified by comigration with a mevalonolactone standard and visualized by iodine vapor. Liquid scintillation counting was performed to measure ^{14}C and ^{3}H counts for mevalonolactone formation and for correction of recovery, respectively. HMGR activity was expressed as pmol mevalonate synthesized · mg protein^{-1} · min^{-1}.

Statistical analysis

ANOVA and Tukey's pairwise comparison or unpaired *t*-test with Welch's correction for unequal variance when appropriate were used to identify significant differences of treatments, with $P < 0.05$ considered significant by GraphPad InStat 3 (GraphPad Software). Data are expressed as means ± SEM.

Results

Cytotoxicity of* N. commune *lipid extract in HepG2 cells

Cytotoxicity of *N. commune* lipid extract was assessed by incubating HepG2 cells with increasing concentrations of lipid extract for 24 h and subsequently measuring cell viability. Cells incubated with 25–100 mg/L of *N. commune* lipid extract showed no reduction in cell viability compared with control (data not shown). Although the percentage of viable cells was lowered at 200 mg/L of *N. commune* lipid extract ($P < 0.05$) compared with control, > 80% of cells were still viable. Because there were no decreases in cell viability in incubations with *N. commune* lipid extract concentrations of up to 100 mg/L, these concentrations were used for the cell culture experiments.

Effect of* N. commune *lipid extract on the expression of HMGR and LDLR

Quantitative real-time PCR analysis demonstrated that *N. commune* lipid extract significantly reduced mRNA abundance of HMGR and LDLR in HepG2 cells (Fig. 1A). Protein levels of HMGR and LDLR were measured after incubation with 100 mg/L of *N. commune* lipid extract for 24 h. Western blot analysis revealed that both HMGR and LDLR protein levels were significantly diminished by the addition of *N. commune* lipid extract by ~70% and ~60%, respectively (Fig. 1B). The inhibitory effect of *N. commune* lipid extract on HMGR was further confirmed by reduced HMGR activity by ~90% compared with control (Fig. 2). This result indicates a potential reduction in cholesterol biosynthesis by *N. commune* lipid extract.

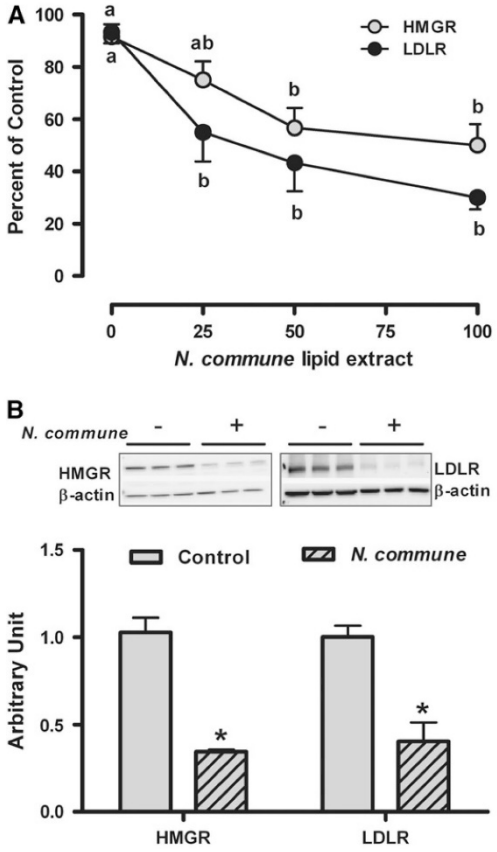

Figure 1. *N. commune* lipid extract reduces mRNA and protein expression of genes involved in cholesterol metabolism in HepG2 cells. (A) Real-time PCR analysis for mRNA expression of HMGR and LDLR in HepG2 cells incubated with *N. commune* lipid extract (0–100 mg/L) for 24 h. Data are expressed as percentage of control that was not treated with *N. commune* lipid extract (0 mg/L). Values are means ± SEM, $n = 6$. Means without a common letter differ, $P < 0.05$. (B) Western blot analysis for HMGR and LDLR protein levels in HepG2 cells incubated with 100 mg/L *N. commune* lipid extract for 24 h. Data are expressed as arbitrary units. Values are means ± SEM, $n = 6$. *Different from control, $P < 0.005$ for HMGR, $P = 0.0014$ for LDLR.

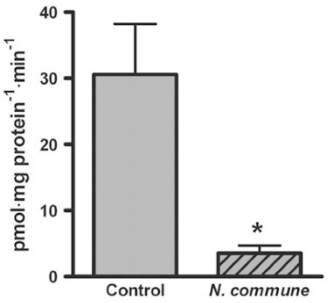

Figure 2. *N. commune* lipid extract reduces HMGR activity in HepG2 cells. HepG2 cells were incubated in the absence (control) or presence of *N. commune* lipid extract (100 mg/L) for 24 h and cell lysates were used for HMGR activity. Data are expressed as pmol mevalonate synthesized · mg protein^{-1} · min^{-1}. Values are means ± SEM, n = 6. *Different from control, P = 0.02.

Inhibition of SREBP maturation by N. commune *lipid extract*

To determine the molecular mechanisms underlying the inhibition of HMGR and LDLR expression by *N. commune* lipid extract, we evaluated the modulation of SREBP-2 by *N. commune*. At 25–100 mg/L, *N. commune* lipid extract did not affect SREBP-2 mRNA abundance (data not shown). Because transcriptional activity of SREBP is largely regulated by the maturation process of precursor proteins (21),Western blot analysis was performed to determine the level of mature SREBP-2 protein. At 100 mg/L, *N. commune* lipid extract reduced SREBP-2 mature protein levels by 35% (P = 0.02) (Fig. 3). Our next question was whether *N. commune* lipid extract specifically targets the SREBP-2 pathway or if SREBP-1 could also be inhibited by *N. commune* lipid extract. To address this question, expression and maturation of SREBP-1 as well as mRNA levels of SREBP-1-responsive genes were measured. Similar to SREBP-2, mRNA level of SREBP-1c was not significantly changed by incubation with *N. commune* lipid extract (data not shown). However, the SREBP-1 mature protein level was significantly reduced by ~30% (Fig. 4A). From 25–100 mg/L of *N. commune* lipid extract, both FAS and SCD-1 mRNA levels were significantly decreased (Fig. 4B), indicating that *N. commune* lipid extract represses the expression of genes involved in fatty acid biosynthetic pathways through the inhibition of SREBP-1 maturation. These data combined indicate that *N. commune* lipid extract can widely affect the expression of genes involved in cholesterol and fatty acid metabolism by inhibiting the maturation process of both SREBP-1 and SREBP-2.

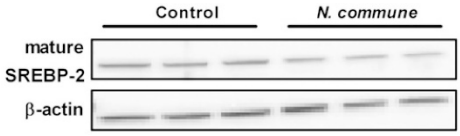

Figure 3. *N. commune* lipid extract reduces mature protein levels of SREBP-2 in HepG2 cells. HepG2 cells were incubated with 100 mg/L *N. commune* lipid extract for 24 h, after which Western blot analysis for mature SREBP-2 was performed. A representative blot of 2 separate experiments is shown.

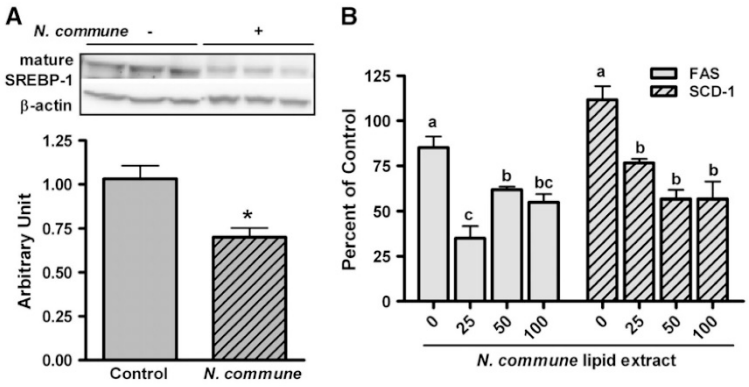

Figure 4. *N. commune* lipid extract reduces mature protein levels of SREBP-1 and the expression of SREBP-1-responsive genes in HepG2 cells. (A) Western blot analysis for SREBP-1 in HepG2 cells incubated with 100 mg/L *N. commune* lipid extract for 24 h. The upper panel shows a representative blot of 3 separate experiments and the lower panel is densitometry analysis of the blots. Data are express as arbitrary units. Values are means ± SEM, $n = 9$. *Different from control, $P = 0.003$. (B) Real-time PCR analysis of SREBP-1-responsive genes in HepG2 cells incubated with 0–100 mg/L *N. commune* lipid extract for 24 h. Data are expressed as percentage of control that was not treated with *N. commune* lipid extract. Bars without a common letter differ, $P < 0.05$. Values are means ± SEM, $n = 6$. Means without a common letter differ, $P < 0.05$.

Discussion

Consumption of foods with bioactive compounds that may confer a beneficial effect on health is increasingly popular. Although *N. commune* has been consumed for centuries for its purported health benefits, not all claims have been substantiated. In this study, we investigated the effect of *N. commune* lipid extract on the expression and function of key regulatory proteins involved in cholesterol and fatty acid metabolism in HepG2 cells. Our results demonstrate for the first time, to our knowledge, that *N. commune* lipid extract represses the expression of both cholesterol and fatty acid biosynthetic genes by inhibiting the maturation process of SREBP-1 and -2.

SREBP are key transcriptional regulators in cholesterol and fatty acid metabolism. They were first discovered as specific transcription factors that bind to the 10-base pair sterol regulatory element within the promoter of the gene encoding LDLR (22,23). When cellular cholesterol is depleted, SREBP are activated to increase the transcription of genes involved in the cholesterol biosynthetic pathway, including HMGR, HMG-CoA synthase, farnesyl diphosphate synthase, and squalene synthase (24–26). Enzymes involved in fatty acid synthesis and uptake, such as acetyl-CoA carboxylase, FAS, SCD-1, and lipoprotein lipase, are also under the transcriptional regulation of SREBP (27–31). Later studies using cell lines and transgenic animals revealed that 2 isoforms of SREBP, i.e., SREBP-1 and SREBP-2, have different functions. SREBP-1 plays an important role in fatty acid metabolism, whereas SREBP-2 plays a greater regulatory role in cholesterol metabolism than fatty acid metabolism (32–35). SREBP are primarily regulated at the posttranscriptional level and 2 major

regulatory proteins are involved in the activation of SREBP, i.e., insulin-induced genes (INSIG) and SREBP cleavage-activating protein (SCAP). SCAP/SREBP precursor complex is retained in the endoplasmic reticulum membrane via sterol-induced interaction of SCAP with INSIG (36,37). Upon the signal for low cellular cholesterol level, SCAP is released from INSIG and guides SREBP to the Golgi where a mature form of SREBP is released through 2-step proteolytic cleavages by site-1-protease (S1P) and site-2-protease (S2P) (38). The mature SREBP enters the nucleus and enhances the expression of genes involved in cholesterol and fatty acid metabolism. Therefore, alterations in the SREBP maturation process could have a wide range of effects on body lipid metabolism. It needs to be further determined which regulatory proteins participating in the maturation process of SREBP are targeted by *N. commune* lipid extract. Nonetheless, our observation that *N. commune* lipid extract did inhibit SREBP maturation implicates its potential as a functional food to reduce lipogenesis as well as cholesterol biosynthesis.

HMGR is the key rate-limiting enzyme in cholesterol synthesis. Repression of HMGR by competitive inhibitors such as statins in humans and by inhibition of the regulatory transcription factor SREBP-2 in rats has been shown to decrease the plasma cholesterol level (39,40). This study shows that *N. commune* lipid extract reduces HMGR mRNA, protein, and activity by inhibiting the maturation process of SREBP-2. *N. commune* lipid extract also significantly reduced LDLR mRNA and protein levels. LDLR is responsible for the uptake of LDL from circulation and decreased LDLR expression is associated with increased plasma LDL cholesterol concentration (41). Therefore, *N. commune* lipid extract may not induce a favorable change in plasma total cholesterol concentration due to reduced LDLR expression despite its effect on decreased cholesterol biosynthesis. Alternatively, depending on the body's cholesterol status, inhibition of cholesterol biosynthesis by *N. commune* lipid extract could be enough to lower the plasma total cholesterol level. However, evidence exists showing that inhibition of SREBP-2 pathway with diminished expression of both HMGR and LDLR decreases plasma total cholesterol level. When the abundance of nuclear SREBP-2 was reduced in rats, which resulted in diminished mRNA abundance of HMGR and LDLR, plasma total cholesterol concentration as well as liver cholesterol content decreased (40). In addition, inhibition of SREBP maturation by knocking down S1P in mice induced reductions in SREBP expression, cholesterol, and fatty acid biosynthesis as well as plasma cholesterol concentration (42). These studies suggest that the SREBP-2 maturation pathway can be a therapeutic target to lower plasma cholesterol level. In addition to its role in regulating cholesterol metabolism through SREBP-2, *N. commune* lipid extract inhibited key enzymes in fatty acid biosynthetic pathways, such as FAS and SCD-1, through repressed maturation of SREBP-1 in HepG2 cells. Therefore, our results strongly suggest that *N. commune* lipid extract contains bioactive compounds that can inhibit the SREBP maturation process. A study is underway in our laboratory to determine bioactive compounds responsible for the repressed SREBP pathway in *N. commune* lipid extract.

Atherosclerosis is a progressive and continuous process throughout life and its progression can be accelerated by dyslipidemia, including high plasma total cholesterol and triglyceride concentrations. Despite extensive efforts to reduce the risk of CHD for the last several decades, CHD remain as the leading cause of death in the United States. Given that

the public's interest in the prevention and/or therapy for CHD has shifted from the use of drugs to that of natural products, discovery and development of natural sources bearing atheroprotective properties are needed. Our current study indicates that *N. commune* lipid extract contains bioactive compounds that can decrease the expression and function of key regulatory genes involved in cholesterol and fatty acid biosynthetic pathways through the repression of the SREBP maturation process. This property of *N. commune* lipid extract could contribute to lower plasma total cholesterol as well as triglyceride concentrations. Although further studies are required to ensure the biological functions of *N. commune*, particularly in vivo, this study suggests a strong potential of *N. commune* to be developed as a new natural food to reduce the risk of CHD.

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