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Inhibitory Effects of Marine Algae Extract on Adipocyte Differentiation and Pancreatic Lipase Activity

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Abstract – Obesity, which is characterized by excessive fat accumulation in adipose tissues, occurs by fat absorption by lipase and sequential fat accumulation in adipocyte through adipocyte differentiation. Thus, inhibition of pancreatic lipase activity and adipocyte differentiation would be crucial for the prevention and progression of obesity. In the present study, we attempted to evaluate anti-adipogenic activity of several algae extracts employing preadipocytes cell line, 3T3-L1 as an in vitro assay system. The effects on pancreatic lipase activity in vitro were also evaluated. Total methanolic extracts of *Cladophora wrightiana* and *Costaria costata* showed significant inhibitory activity on adipocyte differentiation as assessed by measuring fat accumulation using Oil Red O staining. Related to pancreatic lipase, *C. wrightiana* and *Padina arborescens* showed significant inhibition. Further fractionation of *C. wrightiana*, which showed the most potent activity, suggested that $CHCl_3$ and n-BuOH fraction are responsible for adipocyte differentiation inhibition, whereas *n*-BuOH and H_2O fraction for pancreatic lipase inhibition. Our study also demonstrated that *n*-BuOH fraction was effective both in early and middle stage of differentiation whereas $CHCl_3$ fraction was effective only in early stage of differentiation. Taken together, algae might be new candidates in the development of obesity treatment.

Key words – Marine algae, *C. wrightiana*, Adipocyte differentiation, Pancreatic lipase, 3T3-L1 cells, Obesity

Introduction

Obesity is a major health problem in both developed and developing countries (Brug and Crawford, 2009). It is no longer considered only a cosmetic problem but associated with several pathological disorders, including diabetes, hypertension, atherosclerosis and cancer (Kopelman, 2000). Obesity is characterized by abnormal increase in the number and/or size of adipocytes in adipose tissue (Rosen and Spiegelman, 2006; Bastard *et al.*, 2006). Therapeutics for obesity can be developed by various ways such as lipase inhibition, suppression on food intake, stimulation of energy expenditure, inhibition on adipocyte differentiation and regulation on lipid metabolism (Yun, 2010). Synergic action of these mechanisms is preferred for the most effective way to treatment of obesity.

Energy intake starts from fat absorption through digestion of fat into monoglycerides and fatty acids. Lipase is a key enzyme for lipid absorption. Among lipase, pancreatic lipase is responsible for the hydrolysis of 50 - 70% of total dietary fats (Birari and Bhutani, 2007). Reduction of fat absorption by the inhibition of pancreatic lipase is known to be beneficial for the regulation of obesity (Yun, 2010). Orlistat, a specific pancreatic lipase inhibitor, has been clinically used for the prevention of obesity (Hill *et al.*, 1999; Ballinger and Peikin, 2002). Absorbed fat is further accumulated into adipose tissue. Adipose tissue is as a metabolic and endocrine organ which plays an essential role in regulating energy balance. In obesity, adipocytes undergo abnormal growth characterized by increased numbers of fat cells storing their lipids through excessive adipocyte differentiation. Therefore, inhibition of fat absorption and/or fat accumulation by the disturbance of lipase and adipocyte differentiation is suggested to be important therapeutics in obesity.

Marine algae are rich sources of bioactive compounds with diverse biological activities (Kim and Karagozlu, 2011). Because of harsh underwater environment, algae produces characteristic constituents by unique metabolic pathways compared to aerial plants. Marine algae exhibit

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diverse biological activities, such as anticancer, anticoagulant, antiviral, antioxidant, and anti-inflammatory activity (Athukorala *et al*., 2007; Artan *et al.*, 2008; Park *et al*., 2005; Kim *et al*., 2009; Kim and Thomas, 2011; Mayer and Gustafson, 2008). Recently, beneficial effects of marine algae against obesity have been reported (Maeda *et al*., 2007; Kong *et al*., 2010; Park *et al*., 2011). In the present study, we attempted to investigate the anti-adipogenic activity of algae extract by measuring adipocyte differentiation and pancreatic lipase activity.

Experimental

Plant material – The marine algae samples were collected by hand from a depth of 10 m using scuba equipment off the shore in the South Korea. *Hypnea flexicalis* and *S. micracanthum* were collected from Wando, Cheonnam Province, Korea, at April, 2010. *C. costata*, *A. clathratum* and *D. viridis* were collected from Goseong, Gangwon Province, Korea, at March, 2010. *C. wrightiana*, *M. papulosa*, *Pachymeniopsis lanceolata*, *P. atborescens* and *H. clathratus* were collected from Seogwipo, Jeju Province, Korea, at May, 2010. They were identified by the herbarium of Wildlife Genetic Resources Center, National Institute of Biological Resource, where voucher specimens, *C. costata* (BAE20100330E3), *A. clathratum* (BAE20100330E2), *D. viridis* (BAE 20100330E1), *C. wrightiana* (BAE20100508E7), *M. papulosa* (BAE20100508E6), *Pachymeniopsis lanceolata* (BAE20100508E5), *P. atborescens* (BAE20100508E4), and *H. clathratus* (BAE20100508E3) were deposited.

Extraction and isolation – Fresh algae were dried for three days at the room temperature. Then, they were extracted 3 times with MeOH at room temperature for 2 days, which yielded the methanolic extract. The methanolic extract was then suspended in H_2O and partitioned successively with *n*-hexane, $CHCl_3$, EtOAc and *n*-BuOH to yield *n*-hexane, $CHCl_3$, EtOAc, *n*-BuOH and H_2O fractions.

Sample preparation – Fresh algae were washed two or three times with tap water to remove salts and epiphytes from the surface and air dried for two or three days ant the room temperature. Its extract and fractions were dissolved in DMSO and diluted with water. Sample solutions were filtered in membrane filter (0.2 μm) before treatment.

Culture of 3T3-L1 cells – 3T3-L1 mouse embryo fibroblasts were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) until confluence. Two days after confluence (day 0), cells were stimulated to differentiate with differentiation medium containing DMEM with 10% FBS, 0.5 mM 3-isobutyl-1-methyl-xanthine, 1 μM insulin and 1 μM dexamethasone for 2 days (day 2). Cells were then maintained in DMEM supplemented with 10% FBS and 1 μM insulin for another 2 days (day 4), followed by culturing with DMEM with 10% FBS for an additional 4 days (day 8). All media contained 100 IU/mL penicillin and 100 μg/mL streptomycin. Cells were maintained at 37 ºC in a humidified atmosphere of 95% air-5% CO_2.The cultures were treated with test samples for whole culture period (day 0 - 8) for general experiment. In some experiment, test samples were treated after differentiation was induced (fully differentiated cells) for three day. For the evaluation of time dependent activity, test samples were treated for indicated time-periods.

Oil Red O staining – Lipid droplets in cells were stained with Oil Red O (Choi *et al*., 2011). Eight days after differentiation induction, cells were washed three times with PBS and fixed with 10% formalin at room temperature for 1 hr. After fixation, cells were washed once with PBS and stained with freshly diluted Oil Red O solution (3 parts of 0.6% Oil Red O in isopropyl alcohol and 2 parts of water) for 15 min. Cells were then washed twice with water and visualized. For quantitative analysis, Oil Red O staining was dissolved with isopropyl alcohol and optical density was measured at 520 nm by ELISA plate reader.

Assessment of pancreatic lipase activity – Pancreatic lipase inhibitory activity was evaluated using previously reported methods with a minor modification (Nakai *et al*, 2005; Lee *et al*., 2010). Briefly, enzyme solution was prepared by the reconstitution of porcine pancreatic lipase (Sigma, St. Louis, MO) in 0.1 M Tris-HCl buffer (pH 8). Then, test sample was mixed with enzyme buffer, and incubated for 15 min at 37 ºC. After incubation, 10 mM*p*-nitrophenylbutyrate (*p*-NPB) was added and the enzyme reaction was allowed to proceed for 15 min at 37 ºC. Pancreatic lipase activity was determined by measuring the hydrolysis of *p*-NPB to *p*-nitrophenol at 405 nm using a microplate reader. Relative pancreatic lipase activity (%) was calculated as (activity of compound w/ substrate – negative control of compound w/o substrate) / (activity of w/o compound and w/ substrate – negative control of w/o compound and substrate) × 100.

Statistical analysis – The evaluation of statistical significance was determined by the Student's *t*-test with a value of $p < 0.05$ or less considered to be statistically significant.

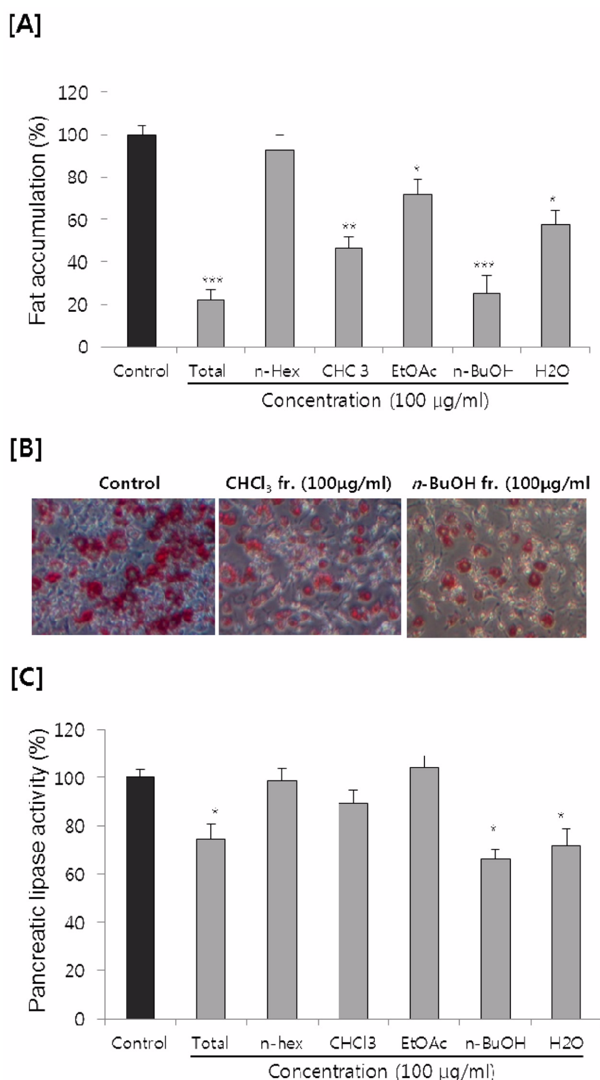

Fig. 1. Effects of *C. wrightiana* fractions on adipocyte differentiation and pancreatic lipase activity. 3T3-L1 cells were treated with samples for whole differentiation process (days 0-8). Cultures were then subjected to Oil Red O staining, photographed (B) and quantitated (A). Pancreatic lipase activity was measured using porcine pancreatic lipase *in vitro* (C). Results are expressed as mean ± SD of three independent experiments, each performed using triplicate wells. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared with control.

Results and Discussion

Obesity arises from chronic imbalance between energy intake and energy expenditure, which results in excessive total fat mass. Energy intake starts from fat absorption through digestion of fat into monoglycerides and fatty acids, catalyzed by lipases. Absorbed fat is further accumulated into adipose tissue, which is called adipocyte differentiation. Therefore, inhibition of fat absorption and/or fat accumulation by the disturbance of lipase and

Table 1. Effect of algae extracts on adipocyte differentiation and pancreatic lipase activity

Algae (100 μg/ml)	Fat accumulation (% of control)	Pancreatic lipase activity (% of control)
Control	100.0 ± 4.9	100.0 ± 3.2
Hypnea flexicalis	105.0 ± 9.9	97.3 ± 4.9
Sargassum micracanthum	102.9 ± 9.2	104.2 ± 6.0
Costaria costata	61.8 ± 7.8*	93.6 ± 8.4
Agarum clathratum	Toxic	93.1 ± 3.8
Desmarestia viridis	88.5 ± 4.9	94.0 ± 9.7
Cladophora wrightiana	22.0 ± 4.9***	73.3 ± 6.5*
Meristotheca papulosa	95.2 ± 6.6	93.6 ± 3.8
Pachymeniopsis lanceolata	86.1 ± 4.9	87.7 ± 6.4
Padina arborescens	77.4 ± 5.9	70.6 ± 9.3*
Hydroclathrus clathratus	96.8 ± 9.1	76.6 ± 7.7

$^{*}p<0.05$, $^{***}p<0.001$ compared with differentiated control.

adipocyte differentiation is suggested to be important therapeutics in obesity.

Adipocyte differentiation was induced by the differentiation mixture including IBMX, insulin and dexamethasone. Differentiated adipocytes showed accumulation of lipid droplets in their cytoplasm and easily distinguished by microscopic observation after Oil Red O staining (Fig. 1). For the screening of anti-obesity activity, algae extracts were treated to 3T3-L1 cells during whole differentiation period and fat accumulation was measured. As shown in Table 1, *C. wrightiana* significantly reduced in differentiated 3T3-L1 cells. At a concentration of 100 mg/ml, *C. wrightiana* extract inhibited fat accumulation up to 22.0% of fully differentiated cells. *C. costata*also inhibited fat accumulation.

We next measured the effect of algae on pancreatic lipase activity. Pancreatic lipase activity *in vitro* was determined using porcine pancreatic lipase by measuring the hydrolysis of *p*-NPB to *p*-nitrophenol. Among algae tested, *C. wrightiana* and *P. arborescens* showed significant inhibition on pancreatic lipase (Table 1).

We further fractionated total methanolic extract of *C. wrightiana* into *n*-hexane, $CHCl_3$, EtOAc, *n*-BuOH and H_2O fraction using sequential solvent fractionation and compared their activity. Among the fractions of *C. wrightiana, n*-BuOH fraction showed strong inhibitory activity against fat accumulation, followed by $CHCl_3$ fraction (Fig. 1A). Microscopic observation also showed the decreased fat droplet in 3T3-L1 cells by the treatment of $CHCl_3$ and *n*-BuOH fraction samples (Fig. 1B). However, *C. wrightiana* extract showed little activity when treated to fully differentiated adipocytes (data not

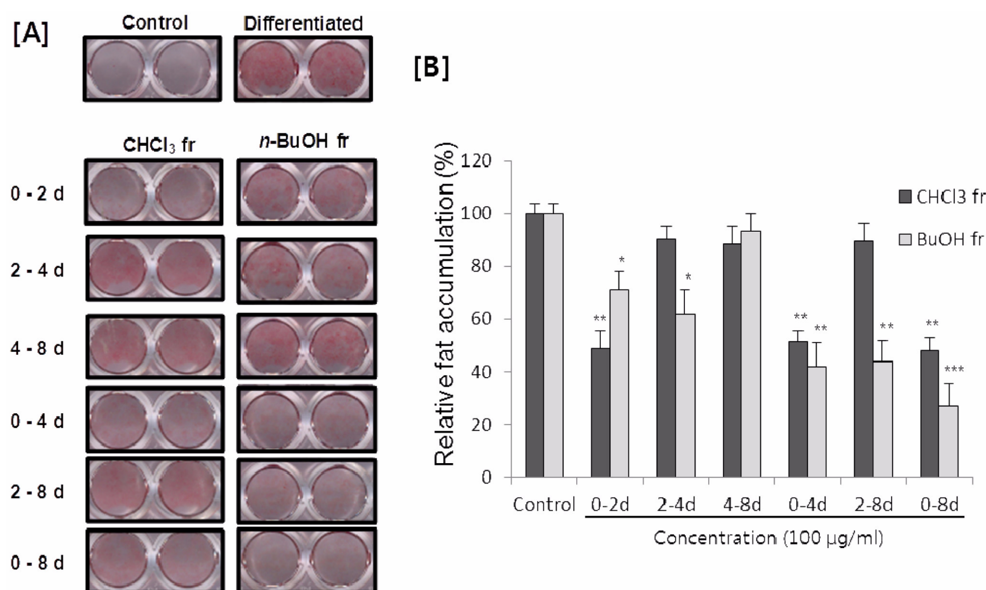

Fig. 2. Effects of *C. wrightiana*on adipocyte differentiation during differentiation process. Cultures were induced to differentiate and samples (100 μg/ml) were added at different periods of differentiation, namely, during days 0 - 2, 2 - 4, 4 - 8, 0 - 4, 2 - 8 and 0 - 8. On day 8, cells were stained with Oil Red O. Values are expressed as means ± SD of triplicate experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared with control.

shown). These results suggest that *C. wrightiana* might be effective in the prevention of adipogenesis but not in lipolysis. Related to pancreatic lipase activity, *n*-BuOH and H_2O fraction showed inhibitory activity (Fig. 1C).

Adipogenesis, the development of mature fat cells from preadipocytes, is an intensely studied model of cellular differentiation. The process of adipogenesis includes alteration of cell shape, growth arrest, clonal expansion, and a complex sequence of changes in gene expression and storage of lipid, which occurs in several stages (Gregoire *et al*., 1998; Lefterova and Lazar, 2009). For differentiation, preadipocytes enter growth arrest and continue to subsequent differentiation process by appropriate mitogenic and adipogenic signals. During the initial stage of differentiation, a dramatic decrease of preadipocyte factor-1 (Pref-1) expression accompanies a rapid increase in the expression of PPARγ and CCAAT/enhancer-binding proteins (C/EBPs). During the terminal stage of differentiation, enzymes involved in triacylglycerol metabolism such as fatty acid synthase and glycerol-3-phosphate dehydrogenase increase to a great extent (Farmer, 2006; White and Stephens, 2010). Therefore, we further examined which stage of adipogenic differentiation was effectively inhibited by *C. wrightiana*. During differentiation, 3T3-L1 cells were incubated with 100 μg/ml of $CHCl_3$ and *n*-BuOH fractions of *C. wrightiana* during days 0 - 2, 2 - 4, 4 - 8, 0 - 4, 2 - 8 and 0 - 8. On day 8, cells were stained with Oil Red O and quantitated. $CHCl_3$fraction inhibited adipocyte differentiation when treated during days 0 - 2, 0 - 4 and 0 - 8 whereas no significant inhibitory effect observed when treated during days 2 - 4, 4 - 8 and 2 - 8 days. These results suggest that $CHCl_3$ fraction could effectively inhibit the early stage of adipocyte differentiation. Interestingly, *n*-BuOH fraction reduced fat accumulation not only by the treatment during days 0 - 2, 0 - 4 and 0 - 8 but also by the treatment during days 2 - 4 and 2 - 8. However, treatment during days 4 - 8 failed to inhibit fat accumulation. Therefore, *n*-BuOH fraction might act on early and middle stage of adipocyte differentiation. Taken together, *n*-BuOH fraction showed more potent inhibition and wide stage selectivity as compared to $CHCl_3$fraction.

Therapeutics for obesity can be developed by various ways such as lipase inhibition, suppression on food intake, stimulation of energy expenditure, inhibition on adipocyte differentiation and regulation on lipid metabolism (Yun, 2010). Synergic action of these mechanisms is preferred for the most effective way to treatment of obesity. Natural products contain diverse constituents, which allow multiple activities. In our present study, *C. wrightiana* showed inhibition both fat accumulation and pancreatic lipase activity. Our study also showed that $CHCl_3$ and *n*-BuOH fractions of *C. wrightiana* were more effective in the prevention of adipogenesis whereas *n*-BuOH and H_2O fractions were effective in the inhibition of pancreatic lipase activity. Therefore, we suggest that

diverse constituents of *C. wrightiana* might exert synergic action in the treatment of obesity by combinatorial action of the inhibition of fat absorption and accumulation.

Marine algae generally produce diverse compounds from different metabolic pathways to defense against highly competitive environment. Therefore, importance as a source of novel bioactive substances is growing rapidly. Although antioxidant and anticancer activity of *C.* species have been investigated (Murugan and Harish, 2007; Feng*et al*., 2007), little is known about *C. wrightiana.* Our present study suggested the beneficial effects of *C. wrightiana*. Further study needs to be carried to characterize bioactive constituents.

In conclusion, several algae extract showed significant inhibition on fat accumulation in 3T3-L1 cells and/or pancreatic lipase activity. Among algae tested, *C. wrightiana* inhibited both fat accumulation and pancreatic lipase activity. Therefore, further study for the potential of algae in the prevention of obesity is strongly needed.

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