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EFFECT OF EUMICROTREMUS ORBIS WATER EXTRACTS ON THE EXPRESSION OF PROTEINS RELATED TO LIPID METABOLISM IN OBESE MICE

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ABSTRACT

In this study, the lipolytic and anti-obesity effects of *Eumicrotremus orbis* water extracts (EOWE) were investigated in mice. Animals were randomized into three groups: CON (not supplemented with EOWE); SIL (low dose of supplemented EOWE); SIH (high dose of supplemented EOWE). The AST, ALT, and ALP serum levels were reduced in the EOWE groups compared to the CON group, with no significant difference ($p < 0.05$). There was a decrease in serum TG levels in the SIH group compared to the CON group ($p<0.05$). The HSL and p-HSL expression in the EOWE groups were increased compared to the CON group ($p<0.05$). The expression of MCAD in all organs of the EOWE groups were increased compared to the CON group ($p<0.05$). There was an increase in the UCP-2 expression in liver and muscle tissues of the EOWE groups, compared to the CON group ($p<0.05$). These results suggested that EOWE acts as a PPARα activator, and may regulate obesity by stimulating PPARα and UCP activity. Therefore, EOWE appeared to show potential as an anti-obesity material.

Keywords: lipolytic effect; anti-obesity; hormone-sensitive lipase; *Eumicrotremus orbis*; medium chain acyl-CoA dehydrogenase.

INTRODUCTION

Obesity is a result of various genetic, environmental, and social factors. Obesity is caused when the energy consumed by the body exceeds the energy spent; this excess energy is converted to triglycerides, which is accumulated in the body. The accumulated triglycerides, in turn, act as the primary cause for adult diseases, such as hypertension, cardiovascular diseases, hyperlipidemia, and diabetes (Spielgelman & Flier, 1996; Visscher & Seidell, 2001). There has been a sharp rise in the obese population in Korea, from 26.0% in 1998 to 32.4% in 2012. This trend is expected to continue because of the westernization of diet and changes in living conditions (Jin et al, 2013). Obesity prevention and treatment necessitates the regulation of food intake, inhibition of nutrient absorption, increased lipolysis and thermogenesis, and inhibition of adipogenesis and lipogenesis (Spiegelman and Flier, 2001; Ardevol et al, 2000; Balkau et al, 2007). Many previous studies have focused on the adipogenetic inhibition of adipocytes, fat absorption inhibition, and the increase in fat excretion; however, lipolysis, which requires considerable attention, has not been extensively studied. Therefore, it is believed that a greater diversity of studies focusing on the development of safe and effective obesity preventatives is required. Four forms of fat are known to exist in the body: triglyceride (TG), total cholesterol (TC), phospholipid (PL), and free fatty acid (FAA). Among these, ≥95% of the fat content in fat, liver, and muscle tissues are believed to be TG (Koppack, Jensen & Miles, 1994). Fat is broken down into fatty acids and glycerol via lipolysis to meet the energy demands of body organs. In other words, obesity can be prevented in part by the effective breakdown of fat accumulated in the body, and by raising its utility as a source of energy (Elbein, Leppert & Hasstedt, 1997).

Eumicrotremus orbis is a cold sea fish found in the waters of the North-Eastern Pacific Ocean surrounding Korea, including the Eastern coast of Korea, Sakhalin Island, and the Sea of Okhotsk. This fish is also called "Simtoongyi" in the Gangwon Province of Korea, and is a commonly used ingredient in soup because of its many positive effects, such as providing hangover relief and the recovery of liver functions. The primary active components include collagen and chondroitin sulfate; these components are known for their antimicrobial (Elbein, Leppert & Hasstedt, 1997), anti-cancer (Okutani & Morikawa, 1978; Okutani, 1982), immunomodulatory (Yamaguchi, 1992), and lipolysis (Chen et al, 2013; Wilsie et al, 2005; Panagosa et al, 2014) functions.

The possible obesity-preventive effects of EOWE were examined in this study by investigating its effect on

the expression of medium chain acyl-CoA dehydrogenase (MCAD; a beta oxidation enzyme acting on fatty acids existing in the mitochondria), uncoupling protein 2 (UCP-2; which is related to energy consumption), and PPARα target factors, in the adipose, liver, and skeletomuscular tissues of white mice. BaseGd on the results of these experiments, the potential of EOWE as an anti-obesity material was tested.

MATERIALS AND METHODS

PREPARATION OF EXTRACTS

Eumicrotremus orbis water extracts (EOWE) were extracted for six hours at 100°C by the addition of distilled water at 20 times the volume (w/v) of freeze-dried *Eumicrotremus orbis* powder. The EOWE extracts were centrifuged (Supra 22K, Hanil Science Industrial Co., Ltd., Daejeon, Korea) at 6000 rpm for 20 min, and the supernatant was filtered (No 2C Whatman, Buckinghamshire, UK). The filtered supernatant was freeze-dried (PVTFD30R, Ilshin Biobase Co., Ltd., Gyeonggi-Do, Korea). The freeze-dried powder was stored in a −70°C freezer until further analysis.

EXPERIMENTAL ANIMALS AND TREATMENT

Five-week old C57BL/6 mice (27.16 ± 1.56 g; Orient Bio Inc., Seoul, Korea) were used in this study. The mice were fed *ad libitum* for 1 week with dry feed, during the acclimatization period (to the breeding room). The white mice were divided into 3 groups based on a weight-based randomized block method: CON (not supplemented with EOWE), SIL (500 mg/kg body weight (BW)), and SIH (1000 mg/kg BW); each group consisted of 10 mice. Each breeding box housed 3 mice. The animals were bred under the following conditions: temperature, 22 ± 2°C, relative humidity, 65 ± 5%, and a 12 h light-dark cycle (from 7 am to 7 pm light period). Each specimen was provided with EOWE through a gavage, once a day at 3 pm. Two gavage doses were applied: 500 mg/kg BW and 1000 mg/kg BW; the EOWE were suspended in distilled water, with 200 μL gavaged each time using an oral syringe. The control group was gavaged with distilled water. The feed used in this study was an animal experiment-use dry feed (AIN-96, Clea Japan, Inc., Tokyo, Japan). The mice were provided with feed and water *ad libitum* for 6 weeks.

FORCED SWIMMING TEST

The mice (control and extract-administered groups) were forced to swim once a week for 6 weeks. Seventy percent of an acrylic plastic water tank (60 cm × 60 cm × 40 cm) was filled with 25 ± 2°C water; the mice were subjected to a forced swimming regimen in this tank once a week (one session per day, 10 min per session). The specimens were subjected to 6 weeks of feeding and supplementation; following this, the specimens were provided with only water for 16 h prior to killing.

BLOOD COLLECTION AND ORGAN HARVESTING

Blood was collected from the abdominal aorta of all mice (anesthetized with ether) upon completion of the experimental period (6 weeks). The blood samples were centrifuged for 10 min at 2000 × *g* in order to separate the serum, and storing in a −70°C freezer until further analysis. The specimens were dissected after forced swimming (for blood collection), and the visceral fat, liver, and skeletomuscular tissues were excised. These were also rapidly frozen with liquid nitrogen and stored at −70°C until further analysis.

ANALYSIS OF BLOOD BIOCHEMICAL PARAMETERS

After anesthetization with ether, whole blood samples were collected from mice in heparinized tubes via heart puncture. Plasma was prepared by centrifuging the blood samples at 2000 × *g*, 4°C, for 10 min; the samples were stored at −70°C in a deep freezer. The plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and triglyceride (TG) expression levels were analyzed using commercial kits (Sigma-Aldrich, St. Louis, MO, USA).

WESTERN BLOT ANALYSIS

Tissues excised from the different organs were washed twice with ice-cold phosphate buffered saline (PBS). Ice-cold lysis buffer (50 mM tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM ethylene diamine tetraacetic acid (EDTA), 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM sodium orthovanadate, 1 mM NaF, 1 g/mL aprotinin, 1 g/mL leupeptin, and 1 g/mL pepstatin) was added to these samples, and allowed to react at 4°C for 1 h. The samples were then centrifuged for 30 min at 14000 rpm, in order to separate the supernatant. The total protein content present in the cell lysate was quantified using the Bio-Rad protein quantification reagent (Bio-Rad, Hercules, CA, USA), as per the manufacturer protocols. Laemmli sample buffer (Bio-Rad) was mixed with the cell lysates in order to prepare the samples; the samples were separated using an 8% sodium dodecyl sulphate (SDS)-polyacrylamide gel via electrophoresis. The separated proteins were transferred to a nitrocellulose membrane (Bio-Rad) via electroblotting. The nitrocellulose membrane containing the separated proteins was blocked (for non-specific protein interactions) using 5% skim milk. The membrane was incubated with the primary antibody (1:1000 dilution, Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. The membrane was then conjugated with anti-rabbit IgG antibody (1:2500, Cell Signaling Technology) for 1 h at room temperature after washing with PBS-T. Following this, enhanced chemiluminescence solution (ECL, Amersham Life Science Corp; GE Healthcare) was applied to the membrane in a dark room; the membrane was sensitized using X-rays in order to quantitate the specific protein. The membranes were re-probed with antibodies against GAPDH (1:5000 dilution, Cell Signaling Technology) for normalization. Several exposure times were applied in order to obtain the signals in the linear range. Band intensities were quantified using equipment name (Manufacturer, City, Country)

STATISTICAL ANALYSIS

The mean and standard deviation of all data

obtained in this study was computed using a statistical analysis program (SPSS package program v18.0; SPSS Inc., Chicago, IL, USA). The average value analysis of the two groups verified the significant difference at $p < 0.05$ using the independent *t*-test. The average value analysis of three or more groups was performed according to the one-way analysis of variance (ANOVA) method. The significance of all mean values was tested using Duncan's multiple comparison test ($P < 0.05$).

RESULTS

SERUM BIOCHEMICAL PROFILE

The aminotransferase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) serum levels were decreased in mice supplemented with EOWE (SIL and SIH groups) compared to the CON group. However, no difference was observed between any of the groups ($p<0.05$; Table 1). The TG serum levels in the SIH group were decreased by approximately 11.5%, compared to the CON group. No difference was observed between the SIL and CON groups ($p < 0.05$; Table 1).

Table 1. Effect of Eumicrotremus orbis water extracts on the plasma biochemical parameters

Parameter [1)]	AST (U/L)	ALT (U/L)	ALP (U/L)	TG (mg/dL)
CON [2)]	90.00 ± 21.58[ns, 3)]	46.20 ± 5.60[ns]	50.25 ± 4.74[ns]	104.00 ± 7.55[ab, 4)]
SIL	83.40 ± 24.49	43.50 ± 5.39	48.75 ± 5.50	103.50 ± 6.36[ab]
SIH	82.50 ± 12.97	44.00 ± 1.73	47.50 ± 3.51	92.00 ± 6.24[a]

[1)]AST, aspartate aminotransferase; ALT, alanine amino transferase; ALP, alkaline phosphatase; TG, triglyceride
[2)]CON, group not supplemented with EOWE/control; SIL, low dose EOWE supplementation (500 mg/kg body weight); SIH, high dose EOWE supplementation (1000 mg/kg body weight)
[3)]ns: no significant difference
[4)]Data expressed as mean (bar) with/without standard deviation (SD; error bar) (n = 10). Different letters indicated a significant difference among the groups supplemented with EOWE, according to ANOVA using Duncan's multiple range test ($p < 0.05$).

p-HSL, HSL, UCP-2, AND MCAD EXPRESSION IN ADIPOSE TISSUES

In order to evaluate the lipolytic effect of EOWE in mice, we investigated the expression levels of hormone-sensitive lipase (HSL), phosphor-HSL (p-HSL), uncoupling protein-2 (UCP-2), and medium chain acyl-CoA dehydrogenase (MCAD) in murine adipose tissues, as shown in Figure 1. We observed an increase in the expression of p-HSL in the SIH group compared to the CON group; however, no difference was observed between the SIL and CON groups ($p<0.05$; Figure 1(a)). The HSL expression levels were increased by approximately 35.3% in the SIH group compared to the CON group. UCP-2 expression levels did not differ between any of the groups ($p<0.05$; Figure 1(a)). We also observed an increase in the MCAD expression in the SIL and SIH groups (19.3% and 49.2%, respectively) compared to the CON group ($p<0.05$; Figure 1(a)).

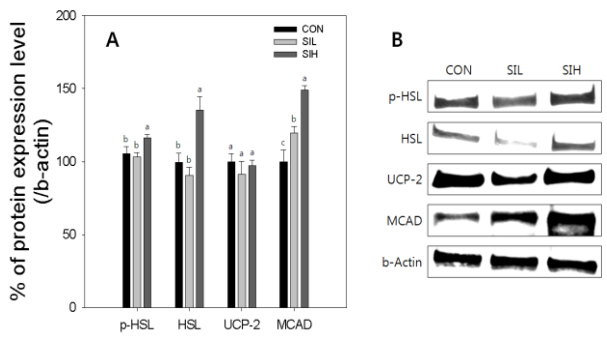

Fig. 1. Effect of *Eumicrotremus orbis* water extracts on the expression levels of lipid metabolism-related proteins in mouse adipose tissues. (A) Adipose tissue extracts were immunoblotted with p-HSL, HSL, UCP-2, and MCAD. The expression levels of lipid metabolism-related proteins were measured by western blot. The protein expression levels were normalized using beta-actin, and shown by the relative expression of proteins in the control group. (B) The representative bands of the molecules are shown. Data was expressed as mean (bar) with/without standard deviation (SD; error bar) of n = 10 mice; different letters indicated a significant difference among the groups supplemented with EOWE according to ANOVA using Duncan's multiple range test ($p < 0.05$). CON, group not supplemented with EOWE; SIL, low EOWE dose group (500 mg/kg body weight); SIH, high EOWE dose (1000 mg/kg body weight). HSL, hormone sensitive lipase; p-HSL, phosphorylated HSL; UCP-2, uncoupling protein-2; MCAD, medium chain acyl-CoA dehydrogenase.

p-HSL, HSL, UCP-2, AND MCAD EXPRESSION IN LIVER TISSUES

The expression levels of p-HSL, HSL, UCP-2, and MCAD were also investigated in murine liver tissues, as shown in Figure 2. p-HSL expression increased by 13.9% and 34.2% in the liver tissue of the SIL and SIH groups, respectively, compared to the CON group ($p<0.05$; Figure 2(a)). The HSL expression levels in the SIL and SIH group were increased by approximately 11.0% and 34.1% compared to the CON group, respectively ($p<0.05$; Figure 2(a)). In addition, the expression levels of UCP-2 in the SIL and SIH groups showed an approximately 17.6% and 47.8% increase compared to the CON group, respectively ($p<0.05$; Figure 2(a)). MCAD expression levels in the SIL and SIH groups increased by approximately 29.9% and 91.9%, respectively, compared to the CON group ($p<0.05$; Figure 2(a)).

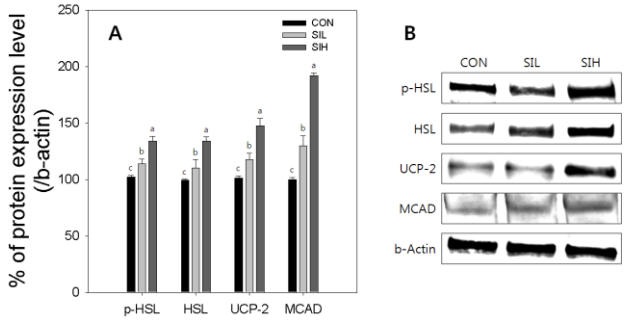

Fig. 2. Effect of *Eumicrotremus orbis* water extracts on the expression levels of lipid metabolism-related proteins in murine liver tissues. (A) Adipose tissue extracts were immunoblotted with p-HSL, HSL, UCP-2, and MCAD. The expression levels of lipid metabolism-related proteins were measured by western blot analysis. The protein expression levels were normalized using beta-actin, and shown by relative expression of proteins in the control group. (B) The representative bands of the molecules are shown. Data was expressed as mean (bar) with/without SD (error bar) of n = 10 mice; different letters indicated a significant difference among the groups supplemented with EOWE according to the ANOVA performed using Duncan's multiple range test ($p < 0.05$). CON, group not supplemented with EOWE; SIL, low EOWE dose group (500 mg/kg body weight); SIH, high EOWE dose group (1000 mg/kg body weight). HSL, hormone sensitive lipase; p-HSL, phosphorylated HSL; UCP-2, uncoupling protein-2; MCAD, medium chain acyl-CoA dehydrogenase.

p-HSL, HSL, UCP-2, AND MCAD EXPRESSION IN THE MUSCLE

The p-HSL, HSL, UCP-2, and MCAD expression in murine muscle tissue was also investigated, as shown in Figure 3. The p-HSL expression in the SIH group increased by approximately 27.9%, compared to the CON group; no difference was observed between the CON and SIL groups ($p<0.05$; Figure 3(a)). The HSL expression in the SIL and SIH groups increased by approximately 15.4% and 33.9%, compared to the CON group ($p<0.05$; Figure 3(a)). The UCP-2 expression in the SIH group increased by approximately 15.4%, compared to the CON group; no difference was observed between the CON and SIL groups ($p<0.05$; Figure 3(a)). The MCAD expression levels in the SIL and SIH groups increased by approximately 9.4% and 84.2%, respectively, compared to the CON group ($p<0.05$; Figure 3(a)).

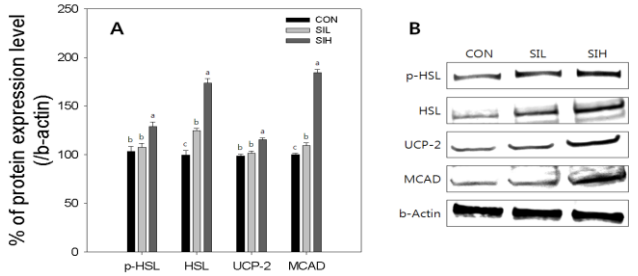

Fig. 3. Effect of the *Eumicrotremus orbis* water extracts on the expression levels of lipid metabolism-related proteins in the murine muscle tissues. (A) Adipose tissue extracts were immunoblotted with p-HSL, HSL, UCP-2, MCAD. The expression levels of lipid metabolism-related proteins were measured via western blot analysis. The protein expression levels were normalized using beta-actin, and shown via the relative expression of proteins in the control group. (B) The representative bands of all tested molecules are shown. Data was expressed as mean (bar) with/without SD (error bar) of n = 10 mice; different letters indicated a significant difference among the groups supplemented with EOWE as per the ANOVA performed using Duncan's multiple range test ($p < 0.05$). CON, control/group not supplemented with EOWE; SIL, group supplemented with low dose of EOWE (500 mg/kg body weight); SIH, high EOWE dose group (1000 mg/kg body weight). HSL, hormone sensitive lipase; p-HSL, phosphorylated HSL; UCP-2, uncoupling protein-2; MCAD, medium chain acyl-CoA dehydrogenase.

DISCUSSION

Appetite control, inhibition of fat digestion and absorption, promotion of energy consumption, and regulation of lipid metabolism are some of the known preventive measures for obesity (Ahn, Park & Do, 2007). HSL and lipoprotein lipase (LPL) are among the major enzymes involved in the regulation of lipid metabolism (Mead, Irvine & Rajani, 2002; Ryu, Daily & Cha, 2005). In particular, HSL, along with LPL, is an important regulatory enzyme that affects lipolysis (Sztalryd & Kraemer, 1995). HSL undergoes phosphorylation under the influence of protein kinase A (PKA) (Holm, 2003; Fredrikson et al, 1981), breaking down triglycerides into glycerol and free fatty acids (Langfort et al, 1999; Frayn et al, 1995; Morimoto et al, 2001). It also plays a leading role in intra-muscular triglyceride (IMTG) breakdown (Langfort et al, 2000; Donsmark et al, 2003), which is the breakdown of triglycerides stored in the muscles into glycerol and free fatty acids (Oscai, Essig & Palmer, 1990). PPARα is responsible for the decrease in energy storage through fatty acid oxidation in the liver and muscles (Evans, Barish & Wang, 2004). Many of the PPARα target genes encode important enzymes involved in the regulation of transport of fatty acids into cells and the oxidation of fat metabolites. CPT-1 regulates the mitochondrial transport process that is critical for the production of energy from MCAD breakdown (included in the mitochondrial oxidative pathway) and from the fatty acids transported into the cells; their expression is increased by the presence of the PPARα ligand (Brady et al, 1989). UCP, which is present in the mitochondrial inner membrane, increases the energy consumption, inhibits body fat production, and regulates obesity, by utilizing protons generated during oxidative phosphorylation in the mitochondria to generate heat, instead of ATP (Boss, Hagen & Lowell, 2000). In particular, UCP-2, along with UCP-1 and 3, is a regulatory factor for energy metabolism (Palou, Llado & Pons, 1999), and is expressed in most tissues (Fleury et al, 1997).

Skeletal muscles are composed of tissues that have a high basic energy demand; these also display a high-

energy demand during contraction, and the requisite energy is provided through UCP-2 and UCP-3 (Zurlo et al, 1990). Triglycerides accumulated in the tissues are converted to energy during exercise; UCP expression is known to play a positive role in this (Boss et al, 1998). Moreover, UCP-2 has been investigated as a target factor for obesity treatment, because of its association with energy consumption, metabolic efficiency, fat oxidation, and fat distribution (Elbein, Leppert & Hasstedt, 1997), and its function as a negative regulator for insulin secretion (Enerback et al, 1997).

This study investigated the potential of EOWE usage as a regulator of obesity, by affecting the expressions of MCAD (a mitochondrial beta-oxidation enzyme regulating fatty acids), UCP-2 (which is related to energy consumption), and PPARα target factors, in the adipose, liver, and skeletomuscular tissues. The control and experimental groups did not show any significant differences in the AST, ALT, and ALP levels. Therefore, EOWE did not appear to be toxic. The expression of HSL, which plays a leading role in lipolysis, was significantly increased in all tissues in the high concentration EOWE diet group, compared to the control group; the expression level of p-HSL was also significantly increased in the high concentration diet group compared to the control group. Significantly increased expression levels of MCAD (a fatty acid beta-oxidation enzyme) were also observed in all tissues of the high concentration diet group, compared to the control group. The expression level of UCP-2 (regulates obesity by promoting energy consumption) was significantly increased in the liver and muscle tissues of the high concentration EOWE diet group, compared to the control group. Based on the results of this study, it was determined that EOWE contributes to obesity prevention by promoting the expression of PPARα target genes and fatty acid oxidation in the adipose, liver, and skeletal muscles, while simultaneously increasing UCP-2 expression in the liver and skeletal muscles.

Based on the results of previous studies, the lipolytic activity seen in the body during exercise varies, depending on the type, intensity, and duration of exercise. In particular, the expression of HSL, an important proteolytic enzyme regulating TG metabolism, varies in the different tissues during exercise (Langfort et al, 2000; Donsmark et al, 2003; Enevoldsen et al, 2001; Petridou et al, 2007). So far, studies related to *Eumicrotremus orbis* have been limited to ecological purposes, with no studies focusing on its physiological activities. Glucuronic acid (GlcA), which is expressed in *Eumicrotremus orbis*, is known to positively affect adipogenesis or lipolysis (Chen et al, 2013; Wilsie et al, 2005; Panagosa et al, 2014). Therefore, further molecular analyses investigating the anti-obesity effects of *Eumicrotremus orbis* must be performed in the future.

CONCLUSION

In this study, proteins related to lipid metabolism and energy consumption-related UCP-2 were subjected to molecular analysis, in order to determine the potential of EOWE as an obesity-preventive agent. EOWE induced an increase in the p-HSL and HSL expression levels, and contributed to the promotion of MCAD and UCP-2 expressions. Based on these results, it was determined that EOWE plays a positive role in lipolysis and energy reaction, which confirmed its potential for use as an obesity-preventive agent. Therefore, exercise and consistent intake of EOWE (which contains high quantities of GlcA) is believed to facilitate obesity prevention and serum lipid metabolism via the breakdown of fat accumulated in the body and generation of energy.

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