

Anti-obesity effects of *Lysimachia foenum-graecum* characterized by decreased adipogenesis and regulated lipid metabolism

Jong Bae Seo^{1*}, Sung Sik Choe^{1*},
Hyun Woo Jeong², Sang Wook Park¹,
Hyun Jung Shin², Sun Mi Choi¹, Jae Young Park¹,
Eun Wook Choi¹, Jae Bum Kim^{2,3},
Dong Seung Seen¹, Jae-Yeon Jeong¹
and Tae Gyu Lee^{1,4}

¹R&D Center, BRN Science Co., Ltd.

Biotechnology Incubating Center, Golden Helix

²Institute of Molecular Biology and Genetic

School of Biological Sciences

³Department of Biophysics and Chemical Biology

Seoul National University

Seoul 151-742, Korea

⁴Corresponding author: Tel, 82-2-875-8998;

Fax, 82-2-887-5998; E-mail, tglee@brnsci.com

*These authors contributed equally to this work.

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Abbreviations: AMPK, AMP-activated protein kinase; HFD, high-fat diet; ND, normal diet; PPAR γ , peroxisome proliferator-activated receptor γ ; FAS, fatty acid synthase; ACC1, acetyl-CoA carboxylase 1; SCD1, stearyl-CoA desaturase 1; RXR α , retinoid X receptor α ; BADGE, bisphenol A diglycidyl ether; ADD1, adipocyte differentiation and determination factor 1; SREBP1c, sterol regulatory element binding protein 1c; ACO, acyl-CoA oxidase; CPT1, carnitine palmitoyltransferase 1; C/EBP, CCAAT/enhancer-binding protein; AICAR, aminoimidazole carboxamide ribonucleotide

Abstract

Lysimachia foenum-graecum has been used as an oriental medicine with anti-inflammatory effect. The anti-obesity effect of *L. foenum-graecum* extract (LFE) was first discovered in our screening of natural product extract library against adipogenesis. To characterize its anti-obesity effects and to evaluate its potential as an anti-obesity drug, we performed various obesity-related experiments *in vitro* and *in vivo*. In adipogenesis assay, LFE blocked the differentiation of 3T3-L1 preadipocyte in a dose-dependent manner with an IC₅₀ of 2.5 μ g/ml. In addition, LFE suppressed the expression of lipogenic genes, while increasing the ex-

pression of lipolytic genes *in vitro* at 10 μ g/ml and *in vivo* at 100 mg/kg/day. The anti-adipogenic and anti-lipogenic effect of LFE seems to be mediated by the inhibition of PPAR γ and C/EBP α expression as shown in *in vitro* and *in vivo*, and the suppression of PPAR γ activity *in vitro*. Moreover, LFE stimulated fatty acid oxidation in an AMPK-dependent manner. In high-fat diet (HFD)-induced obese mice (n = 8/group), oral administration of LFE at 30, 100, and 300 mg/kg/day decreased total body weight gain significantly in all doses tested. No difference in food intake was observed between vehicle- and LFE-treated HFD mice. The weight of white adipose tissues including abdominal subcutaneous, epididymal, and perirenal adipose tissue was reduced markedly in LFE-treated HFD mice in a dose-dependent manner. Treatment of LFE also greatly improved serum levels of obesity-related biomarkers such as glucose, triglycerides, and adipocytokines leptin, adiponectin, and resistin. All together, these results showed anti-obesity effects of LFE on adipogenesis and lipid metabolism *in vitro* and *in vivo* and raised a possibility of developing LFE as anti-obesity therapeutics.

Keywords: adipocyte differentiation; fatty acid oxidation; fatty acid synthesis; lipid metabolism; *Lysimachia foenum-graecum*; obesity

Introduction

Obesity has become a global health problem due to its association with various metabolic disorders such as type 2 diabetes, cardiovascular diseases, and certain types of cancer (Kopelman, 2000; Spiegelman and Flier, 2001; Yanovski and Yanovski, 2002; Hong and Park, 2010). Current medications used to treat obesity can be grouped into two categories (Bray and Tartaglia, 2000; Korner and Aronne, 2004). The first includes appetite suppressants or anorexics that control food intake by modulating the central nervous system. Sibutramine, a serotonin and noradrenaline re-uptake inhibitor, and rimonabant, a cannabinoid receptor antagonist, belong to this category. However, sibutramine is known to increase blood pressure, and may cause cardiac arrhythmias, constipation, and

headache with minimum body weight loss (Luque and Rey, 1999). Furthermore, the use of rimona-bant has been suspended due to its serious psychiatric side-effects (Leite *et al.*, 2009). Drugs in the second category inhibit the absorption of specific nutrients in food. For example, orlistat reduces fat intake by inhibiting pancreatic lipase, but often causes steatorrhea and is associated with a risk of developing deficiencies in lipid-soluble vitamins and essential fatty acids (Heck *et al.*, 2000).

Due to the undesirable side-effects associated with the currently available anti-obesity medications and limited efficacy, much attention has been focused on developing drugs that directly modulate energy metabolism without affecting the central nervous system. Some natural products such as berberine, resveratrol, and curcumin are known to have anti-obesity effects (Lee *et al.*, 2006; Zang *et al.*, 2006; Lee *et al.*, 2009). These natural compounds ameliorate obesity either by increasing energy expenditure or by inhibiting adipocyte differentiation.

Lysimachia foenum-graecum, an herbal plant, has been primarily used as a spice, insectifuge, and pest repellent. It has also been used as a traditional oriental medicine for the treatment of cold, rheum, headache, toothache, and diseases of the digestive system (Shen *et al.*, 2005; Li *et al.*, 2009). Although the chemical profile of the *L. foenum-graecum* has not been published yet to the best of our knowledge, triterpene saponins are found in abundance from the aerial part of *L. foenum-graecum*. Ten out of eleven triterpene saponins isolated from *L. foenum-graecum* were found to have novel structures and have been named as foenumoside A-E (Shen *et al.*, 2005), lysimachiagenoside A, C-F (Li *et al.*, 2009a, 2009b; 2010). Anti-inflammatory effect of foenumoside E (Shen *et al.*, 2005), and anti-oxidant effects of the methanol extract of *L. foenum-graecum* were reported recently (Li *et al.*, 2009), but anti-obesity effect of *L. foenum-graecum* has not been investigated yet.

In our high-throughput screening of natural product extract library against 3T3-L1 preadipocyte differentiation, we found a potent anti-adipogenic effect of *L. foenum-graecum* extract (LFE). To further characterize the anti-adipogenesis activity in detail, we conducted a series of *in vitro* and *in vivo* obesity-related assays. In this report, we demonstrate that LFE suppresses adipogenesis and shifts lipid metabolism towards lipid breakdown and suggest the potential use of LFE in treating obesity.

Results

LFE inhibits adipogenesis *in vitro*

To develop an anti-obesity drug that primarily regulates lipid metabolism without affecting the central nervous system, we performed a high-throughput adipocyte differentiation assay using 3T3L1 cells. 3T3L1 cells were seeded in 96-well plates and differentiation of preadipocyte was induced as described previously (Seo *et al.*, 2004) in the absence or presence of natural plant extract at 10 $\mu\text{g/ml}$. Six days later, cells were stained with Nile Red, a lipophilic fluorescent dye, and intracellular lipid content was measured using EnVision Multilabel Plate Reader. LFE was selected from 2,000 natural plant extracts for its potent anti-adipogenic activity (data not shown). To confirm the anti-adipogenic effect of LFE, preadipocyte 3T3-L1 cells were differentiated into mature adipocytes in the presence of various concentrations of LFE, and lipid accumulation was measured at day 6 by staining with Nile Red. As shown in Figure 1, LFE suppressed lipid accumulation with an IC₅₀ of 2.5 $\mu\text{g/ml}$ (Figure 1A), and reduced the number and size of cytosolic lipid droplets (Figure 1B). Cell viability was determined using MTT assay, and we observed no difference in LFE-treated and untreated cells, even at concentrations as high as 10 $\mu\text{g/ml}$ LFE (data not shown).

To investigate whether the reduced lipid accumulation in LFE-treated cells was due to the inhibition of adipocyte differentiation, we examined the expression of adipogenic markers in these cells. The expression of peroxisome proliferator-activated receptor γ (PPAR γ) and CCAAT/enhancer binding protein α (C/EBP α), master regulators of adipogenesis, was markedly suppressed by the addition of LFE (Figure 1C). In consistent with this result, expression of aP2 and adiponectin, downstream targets of PPAR γ and C/EBP α , was also reduced significantly in LFE-treated cells as revealed by qPCR (Figure 1D). In addition, LFE efficiently suppressed the induction of other mature adipocyte markers fatty acid synthase (FAS), acetyl-CoA carboxylase 1 (ACC1), and stearoyl-CoA desaturase 1 (SCD1). These data confirmed the inhibitory effect of LFE on adipocyte differentiation.

LFE regulates the expression of PPAR γ and C/EBP α and the activity of PPAR γ in mature adipocytes

Since PPAR γ and C/EBP α are also key regulators of lipogenesis, we asked whether LFE could also influence the expression of these proteins in differentiated adipocytes. Mature 3T3-L1 adipocytes were treated with LFE for 24 h and the expression level

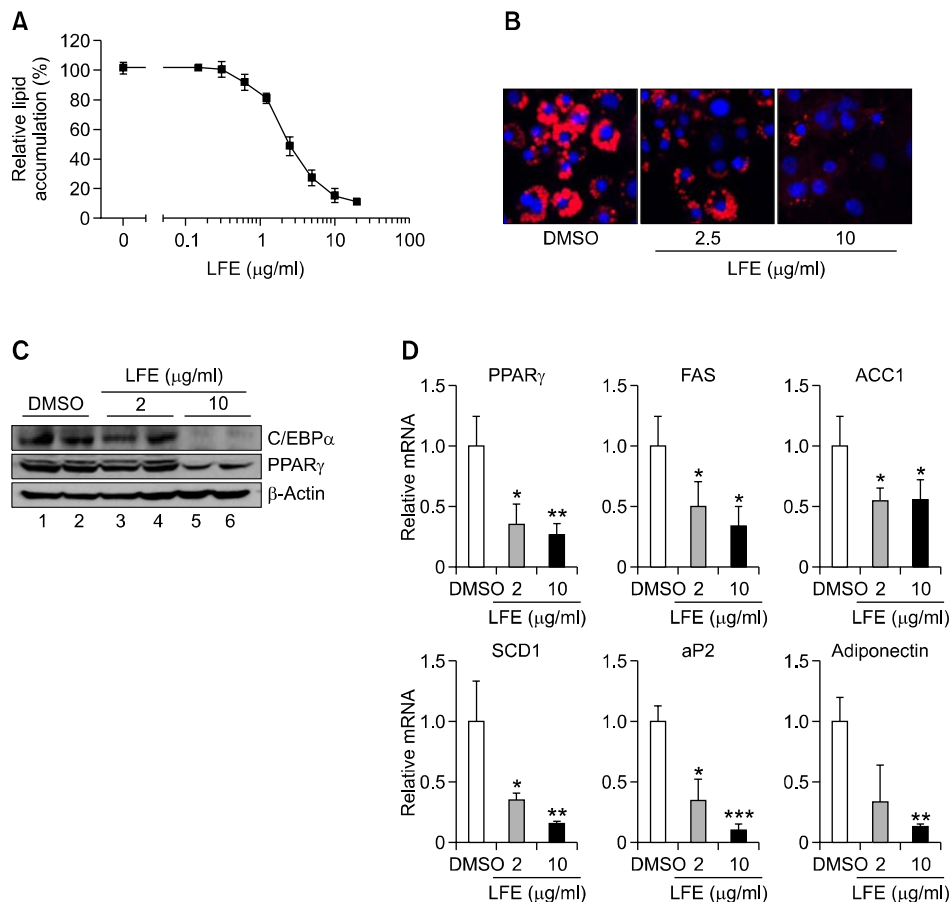


Figure 1. LFE inhibits adipocyte differentiation. (A) Mouse 3T3-L1 preadipocytes were differentiated into adipocytes in the presence of various concentrations of LFE for 6 days. Intracellular lipids were stained with Nile Red and fluorescence intensity was measured. Relative lipid accumulation was calculated as follows: (fluorescence intensity of treated cells / fluorescence intensity of DMSO-treated control cells) \times 100. (B) After staining with Nile Red, cells were counter-stained with Hoechst 33342 and photographed with IN Cell Analyzer 1000 (GE Healthcare). (C) Cells were harvested at day 6 and the expression of PPAR γ and C/EBP α was analyzed by Western blotting. (D) Total RNA was extracted at day 6 and the expression level of PPAR γ , FAS, ACC1, SCD1, aP2, and adiponectin mRNA was analyzed by qPCR. Each bar represents mean \pm SD of triplicate PCR reactions. Similar results were obtained from two independent experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

of PPAR γ and C/EBP α was determined by Western blotting. LFE at 10 μ g/ml was sufficient to repress the expression of PPAR γ and C/EBP α in these cells (Figure 2A). To address if LFE could also modulate the activity of PPAR γ directly, we transfected HEK 293 cells with PPAR γ and its dimerization partner retinoid X receptor α (RXR α). The activity of PPAR γ /RXR α was increased by the treatment of PPAR γ agonist rosiglitazone and abrogated by the co-treatment of PPAR γ antagonist bisphenol A diglycidyl ether (BADGE) (Figure 2B) (Bishop-Bailey *et al.*, 2000). Interestingly, LFE abolished the activation of PPAR γ /RXR α by rosiglitazone as efficiently as BADGE did, indicating the suppressive effect of LFE on PPAR γ activation. Taken together, these results demonstrate the inhibitory effect of LFE on the expression of PPAR γ

and C/EBP α and activation of PPAR γ , and suggest LFE prohibits lipogenesis by regulating PPAR γ and C/EBP α .

LFE decreases the expression of lipogenic genes while increasing that of lipolytic genes

We next asked if LFE could modulate the expression of genes involved in lipid metabolism in mature adipocytes and muscles. When adipocytes were treated with 10 μ g/ml of LFE, the expression of lipogenic genes such as adipocyte differentiation and determination factor 1 (ADD1)/sterol regulatory element binding protein 1c (SREBP1c), FAS, ACC1, and SCD1 were significantly reduced (Figure 3A). Since these are essential enzymes for fatty acid synthesis, it is likely that treatment of LFE

directly impairs the ability of adipocytes to synthesize fat.

To analyze the effect of LFE on the expression of lipolytic genes, the amount of PPAR α and PPAR δ , major transcription regulators of fatty acid beta-oxidation, was measured in C2C12 myotubes

in the absence or presence of 10 μ g/ml of LFE. The expressions of PPAR α and PPAR δ , as well as their downstream targets, acyl-CoA oxidase (ACO) and carnitine palmitoyltransferase 1 (CPT1), were significantly increased in these cells (Figure 3B). These data imply a role of LFE on stimulating lipid

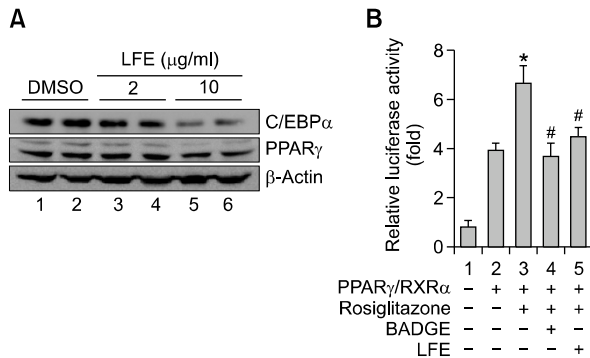


Figure 2. LFE suppresses the expression and activation of PPAR γ . (A) Differentiated 3T3-L1 adipocytes were treated with DMSO or LFE (2 or 10 μ g/ml) for 24 h. Total protein was extracted and the expression of PPAR γ and C/EBP α was analyzed by Western blotting. Experiment was done in duplicate. (B) HEK 293 cells were transfected with DR-1-luciferase reporter along with PPAR γ and RXR α expression vectors. After 6 h, cells were treated with DMSO, rosiglitazone (1 μ M), BADGE (100 μ M), or LFE (10 μ g/ml) and incubated for 24 h. Experiments were done in quadruple and luciferase activity was measured and expressed as mean \pm SD. Similar results were obtained from two independent experiments. * P < 0.05 vs. lane 2, # P < 0.05 vs. lane 3.

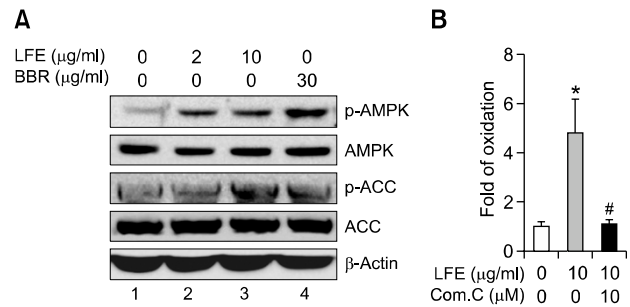


Figure 4. LFE stimulates fatty acid oxidation by activating AMPK. (A) Differentiated 3T3-L1 adipocytes were treated with DMSO, LFE (2 and 10 μ g/ml) or berberine (30 μ g/ml) for 30 min after starving cells in DMEM containing 0.1% BSA for 16 h. Berberine was used as a positive control for activating AMPK. Total cell lysates were subjected to Western blot analysis using antibodies specific for phospho-AMPK, phospho-ACC, total AMPK, total ACC, and β -actin. (B) Differentiated C2C12 cells were treated with or without LFE (10 μ g/ml) or compound C (10 μ M) and fatty acid oxidation assays were performed. Each bar represents mean \pm SD from three independent experiments. * P < 0.05 vs. DMSO, # P < 0.05 vs. LFE.

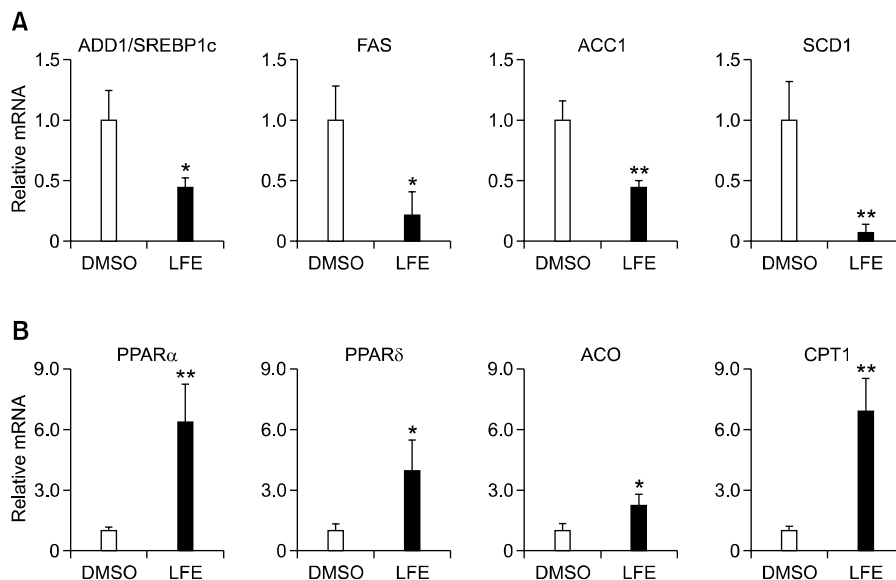


Figure 3. LFE modulates the expression of genes involved in lipid metabolism. Differentiated 3T3-L1 adipocytes (A) or C2C12 cells (B) were treated with 10 μ g/ml LFE for 24 h. Total RNA was isolated and analyzed by qPCR for the expression of lipogenic genes (ADD1/SREBP1c, FAS, ACC1 and SCD1) or lipolytic genes (ACO1, CPT1, PPAR α and PPAR δ). Each bar represents mean \pm SD of triplicate PCR reactions. Similar results were obtained in two independent experiments. * P < 0.05, ** P < 0.01.

consumption in muscles.

LFE promotes fatty acid oxidation by activating AMPK

Activation of AMPK and subsequent inhibition of ACC is known to play a crucial role in fatty acid oxidation by regulating mitochondrial availability of fatty acids (Winder *et al.*, 1997; Viollet *et al.*, 2007). When differentiated 3T3-L1 cells were treated with LFE for 30 min, phosphorylation of both AMPK and its substrate ACC was increased in a dose-dependent manner, without affecting the protein levels of total AMPK and ACC (Figure 4A). The rapid phosphorylation of AMPK by LFE suggests a direct regulatory role of LFE on AMPK activation in adipocytes.

To examine if the LFE-induced activation of AMPK and concomitant inhibition of ACC correlates

with fatty acid oxidation, C2C12 myotubes were treated with LFE in the presence or absence of specific AMPK inhibitor compound C. Addition of LFE alone increased fatty acid oxidation significantly, but the increase was abrogated completely by compound C (Figure 4B). These results show that LFE promotes lipid breakdown in an AMPK-dependent manner.

LFE reduces body weight gain and fat mass in HFD-induced obese mice

To investigate anti-obesity effects of LFE *in vivo*, mice were fed a HFD for 6 weeks in the presence of various amounts of LFE and body weight gain was monitored twice a week. Oral administration of LFE significantly reduced body weight gain by HFD in all doses of LFE tested (Figure 5A). Interestingly, LFE at 100 mg/kg or higher suppressed the body

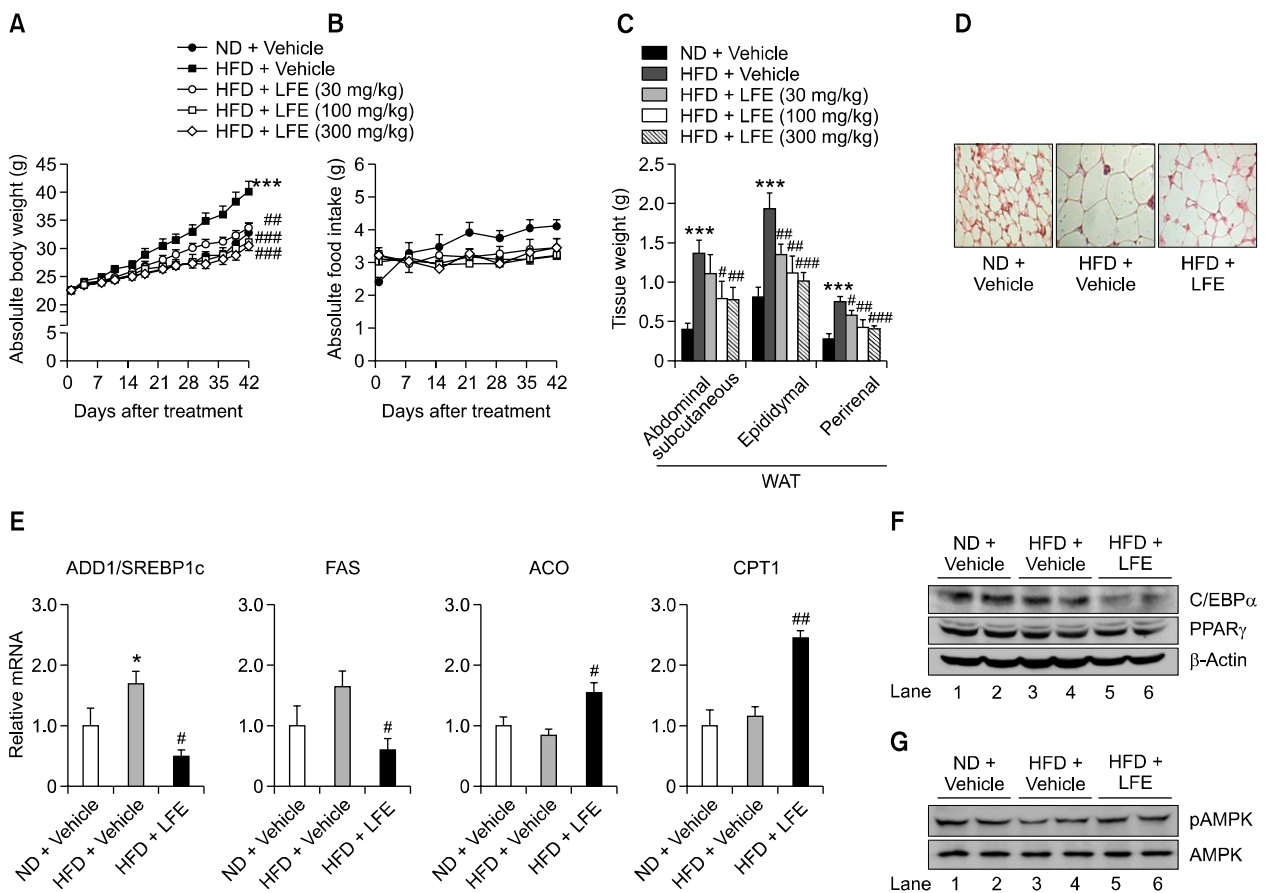


Figure 5. LFE ameliorates obesity induced by HFD. Mice were fed either a ND or HFD for 6 weeks in the presence (30, 100, and 300 mg/kg/day) or absence of LFE ($n = 8$). (A) Changes in body weight. (B) Absolute amount of food intake per mouse. (C) Comparison of fat pad weight from abdominal subcutaneous, epididymal, and perirenal WATs. (D) Histological analysis of the epididymal WATs after staining with hematoxylin and eosin. (E) Effect of LFE on the expression of genes involved in lipid metabolism in the epididymal WAT. (F) Effect of LFE on the expression of C/EBP α and PPAR γ proteins in the epididymal WAT. Epididymal WATs were obtained from two animals of each group and each lane represents the protein level of individual WAT. (G) Effect of LFE on the phosphorylation of AMPK in the epididymal WAT. Each bar represents mean \pm SD from eight mice. * $P < 0.05$ vs. ND+Vehicle, *** $P < 0.001$ vs. ND + Vehicle, # $P < 0.05$ vs. HFD + Vehicle, ## $P < 0.01$ vs. HFD + Vehicle, ### $P < 0.001$ vs. HFD + Vehicle.

weight gain in HFD mice similar to the level in vehicle-treated ND mice. Importantly, no significant difference in food intake was observed between the vehicle- and LFE-treated HFD groups (Figure 5B), confirming that the reduction in body weight gain in LFE-treated mice was not due to reduced caloric intake in these animals. To examine if the reduced body weight gain in LFE-treated group is related to decreased fat accumulation, weight of the abdominal subcutaneous, epididymal, and perirenal white adipose tissues and the sizes of epididymal adipocytes were examined. HFD-induced fat accumulation was significantly ameliorated in all of the white adipose tissues examined (Figure 5C) and adipocyte size was greatly reduced in LFE-treated group (Figure 5D), showing that LFE-induced body weight loss is mainly due to decreased fat accumulation in white adipose tissue.

LFE modulates metabolic gene expression and stimulates AMPK activation *in vivo*

We showed that LFE suppresses adipogenesis and lipogenesis by inhibiting the expression of PPAR γ and C/EBP α in undifferentiated and differentiated 3T3L1 adipocytes (Figures 1C and 2A). In consistent with these findings, the expression of C/EBP α and PPAR γ was reduced markedly in the epididymal WATs obtained from LFE treated HFD mice compared with those from vehicle-treated HFD mice (Figure 5F).

To check if LFE could also regulate metabolic gene expression *in vivo*, the expression of ADD1/SREBP1c, FAS, ACO, and CPT1 in epididymal tissue was measured by qPCR. Consistent with our observation *in vitro* (Figure 3), LFE inhibited the expression of lipogenic genes ADD1/SREBP1c and FAS, and promoted the expression of lipolytic genes ACO and CPT1 *in vivo* (Figure 5E). It has been known that HFD suppresses AMPK activation (Kim *et al.*, 2008; Pang *et al.*, 2008). Interestingly, LFE treatment restored AMPK phosphorylation in HFD mice (Figure 5G), further suggesting that anti-obesity effect of LFE partly relies on the activation of AMPK *in vivo*.

LFE improves the levels of triglyceride, glucose, and adipocytokines in plasma

Next, we measured plasma levels of obesity-related biomarkers in these mice. Forty five % reduction in circulating triglycerides and 35% reduction in glucose was observed in mice treated with LFE compared with mice fed a HFD alone (Figure 6A). Plasma levels of the adipocytokines leptin and resistin were also reduced significantly

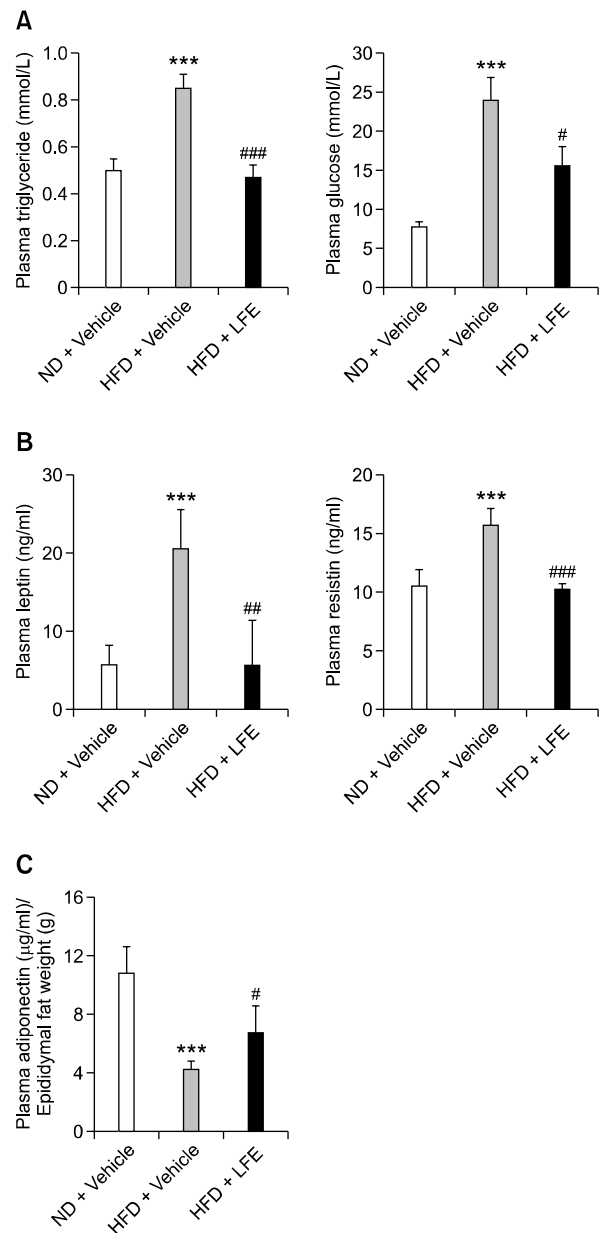


Figure 6. LFE improves plasma lipid parameters. Plasma was obtained after fasting for 16 h from ND or HFD mice treated with either vehicle or 100 mg/kg LFE as described in Figure 5. (A) Concentration of triglyceride and glucose in plasma. (B) Plasma concentration of leptin and resistin. (C) Plasma adiponectin levels adjusted to epididymal fat mass. Each bar represents mean \pm SD from eight individual animals. *** P < 0.001 vs. ND + Vehicle, # P < 0.05 vs. HFD+Vehicle, ## P < 0.01 vs. HFD + Vehicle, ### P < 0.001 vs. HFD+Vehicle.

in LFE-treated group, comparable to those in ND group (Figure 6B). In addition, treatment of LFE partially restored the plasma level of adiponectin in HFD group (Figure 6C). It has been known that plasma levels of leptin and resistin are proportional to body fat mass (Friedman and Halaas, 1998;

Steppan *et al.*, 2001), whereas the level of adiponectin is inversely correlated with body fat percentage (Yamauchi *et al.*, 2001). The decrease in leptin and resistin and the increase in adiponectin in LFE-treated mice, together with reduced triglycerides and glucose level in plasma, strongly support the anti-obesity effects of LFE in HFD-fed mice.

Discussion

Although *L. foenum-graecum* has been used as an oriental medicine to treat a variety of diseases, its effect on obesity or metabolic diseases has not been known so far. In this report, we investigated anti-obesity effect of LFE and showed that LFE efficiently blocks adipogenesis and lipogenesis in 3T3L1 adipocytes, promotes lipid catabolism in cultured cells of different tissue types, and inhibits body and adipose tissue weight gain in HFD-induced obese mouse model.

LFE was first identified in a high-throughput screening for natural product extracts with anti-adipogenic effect. The effect was further confirmed by the reduced expression of various adipogenesis-related genes (Figure 1D). Interestingly, LFE inhibited the expression of PPAR γ and C/EBP α in differentiating adipocytes (Figure 1C), mature adipocytes (Figure 2A), and the epididymal WATs obtained from LFE-treated HFD mice (Figure 5F). In addition, LFE efficiently blocked rosiglitazone-induced activation of PPAR γ (Figure 2B). In agreement with these results, the expression of PPAR γ target genes aP2 and adiponectin was also significantly decreased in LFE-treated adipocytes (Figure 1D). PPAR γ and C/EBP α are known to be the most crucial transcription factors in adipogenesis and lipogenesis (Tontonoz *et al.*, 1994a, 1994b). Ectopic expression of PPAR γ is sufficient to induce C/EBP α and to fulfill the differentiation of the preadipocytes to mature adipocytes. Although the initial induction of C/EBP α is dependent on PPAR γ , but C/EBP α , in turn, is known to reinforce the expression of PPAR γ and many specific genes required for adipocyte differentiation and lipogenesis. This positive feedback loop between PPAR γ and C/EBP α is believed to be essential for the maintenance of adipocytes in the terminally differentiated state (Brun *et al.*, 1996; Rosen *et al.*, 2000). Considering the importance of these factors, it seems likely that LFE exerts its anti-adipogenic and anti-lipogenic effects by primarily suppressing PPAR γ and C/EBP α *in vitro* and *in vivo*.

Our data showed an activation of AMPK and AMPK-dependent fatty acid oxidation by the

treatment of LFE *in vitro* and *in vivo* (Figures 4 and 5G). As a master regulator of glucose and lipid metabolism, AMPK orchestrates a cohort of cellular responses upon nutritional stresses (Hardie *et al.*, 2003, 2006; Kahn *et al.*, 2005). Activation of AMPK is known to switch anabolic pathways to catabolic pathways by inducing the expression of lipolytic transcription factor PPAR γ and suppressing the expression of lipogenic transcription factor ADD1/SREBP1c (Zhou *et al.*, 2001; Lee *et al.*, 2006; Yoon *et al.*, 2006). Here we showed that LFE treatment causes an induction of PPAR γ and its downstream targets CPT1 and ACO, and a reduction of ADD1/SREBP1c and its target FAS, ACC1, and SCD1 (Figure 3). Considering the stimulating effect of LFE on AMPK activation, it is plausible that LFE regulates the expression of the key metabolic regulators PPAR γ and ADD1/SREBP1c through activating AMPK.

It is intriguing to think the underline mechanism of PPAR γ , C/EBP α , and ADD1/SREBP1c suppression by LFE. It was reported that AMPK agonist aminoimidazole carboxamide ribonucleotide (AICAR) inhibits adipocyte differentiation by down-regulating PPAR γ , C/EBP α , and ADD1/SREBP1c (Giri *et al.*, 2006), suggesting that LFE inhibits the expression of these transcription factors indirectly through AMPK activation as observed in mature adipocytes. It has been also known that AICAR induces KLF2 expression, a repressor of PPAR γ activity, in mouse embryonic stem cells and fibroblasts (Adamo *et al.*, 2009), along with the fact that AMPK acts as an upstream positive regulator of p38 MAPK (Xi *et al.*, 2001) and 38 MAPK represses PPAR γ transcriptional activity by phosphorylating PPAR γ (Hu *et al.*, 1996; Diradourian *et al.*, 2005). These reports suggest a possible link between LFE-induced activation of AMPK and AMPK-mediated suppression of PPAR γ transcriptional activity.

High level of blood leptin in obese individual is known to elicit glucose tolerance in type II diabetes and hepatic steatosis by acting at pancreas and liver, respectively (Maffei *et al.*, 1995; Friedman and Halaas, 1998; Petersen *et al.*, 2002; Javor *et al.*, 2005). On the other hand, adiponectin is known to play a role in the suppression of type II diabetes and obesity (Yamauchi *et al.*, 2001; Ukkola and Santaniemi, 2002; Diez and Iglesias, 2003). Treatment of LFE not only blocked obesity, but also restored normal levels of blood adipokines leptin, resistin, and adiponectin as well as triglyceride and glucose in HFD-induced obesity model (Figure 6), suggesting a beneficial effect of LFE in other metabolic diseases. In accordance with this, we also found LFE significantly relieved fatty liver symptoms in HFD mice (data not shown). Interestingly,

LFE has been shown to have anti-oxidant and anti-inflammatory activities (Shen *et al.*, 2005; Li *et al.*, 2009). Since chronic diseases such as metabolic diseases and cardiovascular diseases are known to be associated with oxidative stress and inflammation (Lusis, 2000; Hansel *et al.*, 2004), the anti-oxidant and anti-inflammatory activities of LFE together with its anti-obesity effect may further contribute to modify metabolic diseases such as obesity, diabetes, and hepatic steatosis.

In conclusion, this study shows anti-obesity effects of *L. foenum-graecum* for the first time. LFE efficiently blocked adipogenesis and lipogenesis *in vitro*, and total body and adipose tissue weight gain in HFD-induced obesity model. The mechanism of LFE action involves, at least in part, the activation of AMPK and suppression of PPAR γ and C/EBP α . Although the precise mechanisms and active components in LFE need to be elucidated further, LFE provides novel therapeutic potential against obesity and possibly other metabolic diseases.

Methods

Plant material and preparation of LFE

Dried whole plant of *L. foenum-graecum* was purchased from Jinheung pharmacy (Kyoungdong Oriental Herbal Market, Seoul, Korea) and identified from their external appearance according to Hu and Kelso (Hu and Kelso, 1996). Sequencing analysis was conducted as previously described (Hao *et al.*, 2004) and the sequences of the internal transcribed spacer region of nuclear ribosomal DNA and the chloroplast *trnL-F* region from the purchased plant specimen were found to be identical with the known sequences of *L. foenum-graecum* (AF547771 and AF547716, data not shown), further confirmed the identity of *L. foenum-graecum*. A voucher specimen is deposited in our laboratory (R&D center, BRN Science Co., Ltd., Seoul, Korea). Plant material (200 g) was extracted two times with 95% ethanol at room temperature for 2 d and was subsequently filtered. The combined filtrate was concentrated under vacuum at 60°C, and completely dried by freeze drying. The yield was 8% and LFE powder was dissolved in DMSO at 20 mg/ml for *in vitro* studies and in 0.5% methylcellulose for *in vivo* study. LFE was identified by foenumoside B, α -linolenic acid, and linoleic acid from HPLC elution profile (Supplemental Figure 1). In brief, an HPLC system (Agilent 1200) equipped with Agilent ZORBAX Eclipse XDB-C18 column (4.6 \times 150 mm) was used for analysis. Acetonitrile gradient (0% to 100%) was used as a mobile phase with a flow rate of 1 ml/min. The absorption peaks were detected at 205 nm and compared with authentic standards.

Cell culture

3T3-L1 and C2C12 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA). 3T3-

L1 preadipocytes were maintained in Dulbecco's Modified Essential Medium (DMEM, Hyclone) supplemented with 10% bovine calf serum (Hyclone) at 37°C with 10% CO₂. Differentiation of 3T3-L1 cells was induced as described previously (Seo *et al.*, 2004). C2C12 cells (ATCC) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS, Hyclone) at 37°C with 5% CO₂. For differentiation, C2C12 cells were grown to confluence and were induced with DMEM containing 2% FBS for 7 d.

Nile red staining

Cells were washed with phosphate-buffered saline (PBS) three times and fixed with 3.7% formaldehyde at room temperature for 1 h. Cells were washed two times with PBS and were stained with 0.5 μ g/ml Nile Red (Sigma, St. Louis, MO) for 10 min. After washing with PBS, lipid content was measured using an EnVision Multilabel Plate Reader (Perkin Elmer, Wellesley, MA). Cells were subsequently stained with Hoechst 33342 (Invitrogen, Carlsbad, CA) and photographed using an IN Cell Analyzer 1000 (GE Healthcare, Buckinghamshire, UK).

Quantitative real-time RT-PCR (qPCR)

Total RNA was isolated from differentiated 3T3-L1, C2C12 cells, and epididymal adipose tissue using Trizol reagent (Invitrogen). Total RNA concentration and purity was measured using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Equal amount of total RNA (1 μ g) was used to synthesize cDNA using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative real-time PCR was performed on iCycler iQ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) with iQTM SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. The reaction was conducted as follows; (95°C for 5 min followed by 35 repetitive thermal cycles (94°C for 30 s, 60°C for 30 s, 72°C for 30 s). Primers were purchased from Bioneer (Daejeon, Korea). The sequences of the primers used in this study are as follows: PPAR γ (sense 5'-GAACGTGAAGCCCATCGAGG-3' antisense 5'-GTCCTTGTAGATCTCCTGGA-3'); FAS (sense 5'-CGGTAGCTCTGGGTGTA-3' antisense 5'-TGCTCCA GCTGCAGGC-3'); ACC1 (sense 5'-GAGTGACTGCCGAA ACATCTCTG-3' antisense 5'-GCCTTCTCTGACAAACGAGT-3'); SCD1 (sense 5'-TGGGTTGGCTGCTTGTG-3' antisense 5'-GCGTGGCAGGATGAAG-3'); aP2 (sense 5'-CAAAAT GTGTGATGCCTTTGTG-3' antisense 5'-CTCTTCTTTGG CTCATGCC-3'); adiponectin (sense 5'-GGCAGGAAAGGA GAACCTGG-3' antisense 5'-GCCTTGCTCTTCTTGAAGAG-3'); ACO (sense 5'-TGTTAAGAAGAGTGCCACCAT-3' antisense 5'-ATCCATCTCTCATAACCAAATTT-3'); CPT1 (sense 5'-ACTCCTGGAAGAAGAAGTTCAT-3' antisense 5'-AGTA TCTTTGACAGCTGGGAC-3'); PPAR α (sense 5'-TCTTCA CGATGCTGTCTCTCT-3' antisense 5'-CTATGTTTAGAAG GCCAGGC-3'); PPAR δ (sense 5'-GCTGCTGCAGAAGT GCCA-3' antisense 5'-CACTGCATCATGTGGGCATG-3'); GAPDH (sense 5'-TGCACCACCAACTGCTTAG-3' antisense 5'-GGATGCAGGGATGATGTTCC-3'). Each value was normalized to that of GAPDH.

Transient transfection and luciferase assay

HEK 293T cells (ATCC) were maintained in DMEM supplemented with 10% FBS at 37°C with 5% CO₂. Cells were grown to 70% confluency, and transfected with pTK-LUC-DR-1 (50 ng) and pCMV-β-Gal (50 ng) together with either pcDNA3 (200 ng) or pSV-SPORT1-PPAR-γ (100 ng) and pCMX-RXRα (100 ng) using Lipofectamine 2000 reagent (Invitrogen). Six hours after transfection, cells were treated with rosiglitazone (1 μM), BADGE (100 μM), or LFE (10 μg/ml) and incubated for 24 h. Cell extract was prepared using lysis buffer (25 mM Tris-phosphate pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid, 10% glycerol, 1% Triton X-100). Luciferase and β-galactosidase activity was measured using Luciferase assay system and β-galactosidase enzyme assay system (Promega). Transfection efficiency was normalized by β-galactosidase activity.

Western blot analysis

3T3-L1 adipocytes and epididymal adipose tissues were lysed with TGN buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% Tween-20, 0.2% NP-40, 1 mM PMSF, 100 mM NaF, 1 mM Na₃VO₄, 10 μg/ml aprotinin, 2 μg/ml pepstatin A, and 10 μg/ml leupeptin). Equal amount of protein (100 μg) was separated on SDS-PAGE gel and transferred to nitrocellulose membrane (GE Healthcare). Blots were blocked with 5% BSA in Tris-buffered saline containing 0.1% Tween-20 (TBST) at room temperature for 30 min, followed by overnight incubation with primary antibodies at 4°C. Antibodies against phospho-AMPKα (Thr172, 1:1,000), AMPK (1:1,000), phospho-ACC (Ser79, 1:1,000), and ACC (1:1,000) were purchased from Upstate Biotechnology (Lake Placid, NY), C/EBPα (1:1,000) and PPARγ (1:1,000) were from Santa Cruz Biotechnology (Santa Cruz, CA), and β-Actin antibody (1:5,000) was from Sigma Aldrich. Blots were washed with TBST, incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000, Bio-Rad) for 2 h, and washed with TBST. Luminescent signal was detected using SuperSignal West Dura Extended Duration substrate (Thermo Scientific) with LAS-3000 image analyzer (FujiFilm, Tokyo, Japan).

Fatty acid oxidation assay

Fatty acid oxidation assays were performed as previously described, with modifications (Kim *et al.*, 2009). Briefly, cells were incubated in α-minimal essential medium (α-MEM; Hyclone) containing 0.1 mM of 9,10-³H] palmitate (5 μCi/ml, Perkin Elmer) and 2% bovine serum albumin for 24 h. After incubation, the medium was precipitated with an equal volume of 10% trichloroacetic acid (Sigma). Samples were centrifuged and supernatants were transferred to 1.5 ml tubes placed in a scintillation vial containing 0.5 ml of water, and incubated at 55°C for 12 h. The tubes were removed and the ³H₂O content was measured in a scintillation counter.

Animal treatment

All experimental procedures were approved by the Seoul National University Animal Experiment Ethics Committee. Forty C57BL/6 mice (7-week-old males) were obtained from Central Laboratory Animals (Seoul, Korea). All mice were housed (four mice/cage) and given water *ad libitum*, with a 12 h light-dark cycle beginning at 7:00 a.m. After acclimation for one week, 8 mice were randomly assigned to one of the five treatment groups with equal mean body weight between groups. Mice in a normal diet group (ND + Vehicle) were fed a standard chow diet and mice in high-fat diet (HFD) groups were fed a HFD (60% of calories derived from fat; Research Diets Inc, New Brunswick, NJ). LFE was dissolved in 0.5% methylcellulose, and administered to HFD + LFE groups daily at doses of 30, 100, or 300 mg/kg/day by oral gavage for 6 weeks. Mice in ND + Vehicle and HFD + Vehicle groups were given an equal volume of 0.5% methylcellulose. Food intake was monitored weekly and body weight was measured twice per week. After 6 weeks, the mice were fasted for 16 h, and euthanized using CO₂. Blood was collected from abdominal vena cava, immediately mixed with EDTA, and stored on ice. Plasma was isolated by centrifugation at 3,000 rpm for 10 minutes. Plasma levels of glucose and triglycerides were analyzed on an Olympus AU400 Chemistry Auto Analyzer (Olympus, Japan) using Infinity Glucose Liquid Stable Reagent and Infinity Triglycerides Liquid Stable Reagent (Thermo Electron). The concentration of adipocytokines in the plasma was analyzed by ELISA (R&D systems, Minneapolis, MN). Organs were harvested and frozen immediately in liquid nitrogen and stored at -80°C.

Histological analysis

The epididymal adipose tissues were fixed with 4% paraformaldehyde, embedded in optimal cutting temperature (OCT) compound (Bright Instruments, Huntingdon, UK), and frozen with cryospray (MEDITE, Germany). Fifty μm sections were obtained with a cryostat microtome and stained with hematoxylin and eosin.

Statistical analysis

Data are expressed as mean ± SD for at least two independent experiments in triplicates. Variances in different groups were calculated by Student-Newman-Keuls post-hoc ANOVA analysis. *P* < 0.05 was interpreted as statistically significant.

Supplemental data

Supplemental Data include a figure and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-43-5-5.pdf.

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