



## ASARONE INHIBITS ADIPOGENESIS AND STIMULATES LIPOLYSIS IN 3T3-L1 ADIPOCYTES

S. H. LEE<sup>1</sup>, K-Y KIM<sup>1</sup>, S. Y. RYU<sup>2</sup>, Y. YOON<sup>3</sup>, D-H HAHM<sup>4</sup>, S. A. KANG<sup>5</sup>, S. H. CHO<sup>6</sup>, J-S LIM<sup>1</sup>,  
E-Y MOON<sup>7</sup>, S. R. YOON<sup>8</sup>, H. G. LEE<sup>8</sup>, D. Y. YOON<sup>9</sup> AND Y. YANG<sup>1,✉</sup>

<sup>1</sup> Department of Biological Science, Sookmyung Women's University, Seoul 140-742, Korea.

<sup>2</sup> Korea Research Institute of Chemical Technology, Taejeon 305-343, Korea.

<sup>3</sup> Department of Microbiology, College of Medicine, Chung-Ang University, Seoul 140-757, Korea.

<sup>4</sup> Acupuncture & Meridian Science Research Center, Kyung Hee University.

<sup>5</sup> Department of Fermented Food Science, Seoul University of Venture & Information, Seoul 135-731, Korea.

<sup>6</sup> Department of Family Medicine, Yongsan Hospital, Chung-Ang University, Seoul 140-757, Korea.

<sup>7</sup> Department of Bioscience and Biotechnology, Sejong University, 98 Kunja-dong Kwangjin-Gu, Seoul 143-747, Korea.

<sup>8</sup> Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-343, Korea.

<sup>9</sup> Department of Bioscience and Biotechnology, Konkuk University, Seoul 143-701.

✉ Young Yang, Department of Life Science, Sookmyung Women's University, Seoul 140-742, Korea. Tel: 82-2-710-9590;  
E-mail: yyang@sookmyung.ac.kr

Received March 8<sup>th</sup>, 2009; Accepted January 19<sup>th</sup>, 2010; Published January 24<sup>th</sup>, 2010

**Abstract** – Asarone is a molecule found in certain plants such as *Acorus calamus*, the root of which is used in traditional medicine to treat diabetes. We determined the molecular mechanism underlying the anti-diabetic activity of asarone. Treatment of asarone significantly inhibited the differentiation of 3T3-L1 preadipocytes through suppression of expression of the transcription factors, CCAAT/enhancer binding protein- $\alpha$  and peroxisome proliferator activated receptor- $\gamma$ , which activate adipogenesis. Intracellular triglyceride levels were reduced by asarone in a dose-dependent manner and asarone treatment stimulated the phosphorylation of hormone-sensitive lipase. Together, the present findings indicate that asarone inhibits adipogenesis by down-regulation of PPAR $\gamma$  and C/EBP $\alpha$  and reduces lipid accumulation by stimulation of lipolysis through an increase in hormone-sensitive lipase activity.

**Key words:** C/EBP $\alpha$ ; PPAR $\gamma$ ; Adiponectin; Asarone; Hormone-sensitive lipase.

### INTRODUCTION

The molecules that regulate adipogenesis represent important pharmacological targets because obesity caused by enhanced differentiation of adipocytes is an established risk factor for Type 2 diabetes and cardiovascular diseases. Accordingly, the development of drugs inhibiting adipogenesis or stimulating lipolysis is one of the strategies for regulating obesity. Early in the adipogenic differentiation program, preadipocytes undergo mitotic clonal expansion (16), which is accompanied by the induction of expression of the transcription factors, CCAAT/enhancer binding protein  $\beta$  and  $\delta$  (C/EBP $\beta$  and  $\delta$ ). These factors in turn induce expression of the transcription factors, C/EBP $\alpha$  and peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) through binding to C/EBP regulatory elements in their promoters. Together, C/EBP $\alpha$

and PPAR $\gamma$  promote adipocyte-specific gene expression, which results in adipocyte formation. Several natural compounds including resveratrol and quercetin show anti-obesity effects by inhibiting the expression of C/EBP $\alpha$  and PPAR $\gamma$  (13, 19). Hormone-sensitive lipase (HSL) is the key enzyme responsible for stimulation of lipolysis in adipocytes. It is the rate-limiting enzyme in the breakdown of triacylglycerol (TG) to diacylglycerol (DAG) and free fatty acid (FFA). Lipolytic hormones such as catecholamines and glucagon stimulate hydrolysis of TG through phosphorylation and the associated activation of HSL by cyclic AMP (cAMP)-activated protein kinase A (PKA). In addition to these hormones, several natural compounds including phytoestrogen, genistein and procyanidin show anti-obesity effects by stimulating lipolysis through the activation of HSL (3, 15).

Asarone is a molecule found in certain plants such as *Acorus calamus*, a native plant of India, and drug preparations including asarone are used for the treatment of diphtheria, typhoid, and tuberculosis. The root of *Acorus calamus* is used in traditional medicine by the Dakotas and Indonesians to treat diabetes (1, 5). The major and non-toxic metabolite of alpha-asarone (2,4,5-trimethoxy-1-propenyl benzene) is 2,4,5-trimethoxycinnamic acid (TMC), which retains most of the pharmacological properties of alpha-asarone. When TMC or alpha-asarone is administered to hypercholesterolemic rats, total serum cholesterol and LDL-cholesterol levels are lowered (2). In the present study, we examined the effect of asarone on adipogenesis and lipolysis in murine 3T3-L1 preadipocytes and adipocytes. Co-treatment with asarone significantly inhibited the differentiation of 3T3-L1 preadipocytes and post-treatment with asarone increased lipolysis in fully differentiated 3T3-L1 adipocytes.

## MATERIALS AND METHODS

### Chemicals & Reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from HyClone (Logan, UT). Dexamethasone, 3-isobutyl-1-methylxanthine, and insulin were purchased from Sigma-Aldrich (St. Louis, MO). The PKA inhibitor, H-89, was purchased from Stressgen (Ann Arbor, MI). C/EBP $\alpha$ , C/EBP $\beta$ , PPAR $\gamma$ , and adiponectin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and phospho-HSL, phospho-ERK and ERK antibodies were purchased from Cell Signaling Technology (Danvers, MA).

### Isolation of asarone from the rhizome extracts of *Acorus gramineus* (Araceae)

The dried rhizome of *Acorus gramineus* (1 kg) was cut into small pieces and soaked in 3 L of methanol (MeOH) at room temperature for two weeks. The resulting MeOH extract was filtered through a cotton ball and concentrated *in vacuo* to yield 82 g of dark syrupy extract, which was resuspended in 4 L of distilled water and extracted twice with an equal volume of dichloromethane. The extracts were combined and concentrated to dryness to yield 32 g of the dichloromethane fraction. This was chromatographed on silica gel (230–400 mesh, 300 g) and 5 fractions (Fr.A – Fr.E) were eluted with hexane-ethylacetate (15:1~1:1). Fr. C (5 g) was purified with repeated silica gel column chromatography and eluted with hexane-ethylacetate (10:1~5:1). The eluate was 1.2 g of yellowish oil, which was identified as asarone (ca 1:2 mixture of  $\alpha$  and  $\beta$  isomers) based on NMR spectral analyses. Asarone : EI-MS  $m/z$  208.0 [M]<sup>+</sup>; <sup>1</sup>H-NMR (300MHz, CDCl<sub>3</sub>)  $\delta$  6.85 (1H, s, H-6), 6.54 (1H, s, H-3), 6.49 (1H, dq,  $J$  = 11.5, 1.9 Hz, H-1'), 5.77 (1H, dq,  $J$  = 11.5, 7.1 Hz, H-2'), 3.90, 3.85, 3.81 (each 3H, s, -OMe), 1.85 (3H, dd,  $J$  = 7.1, 1.9 Hz, H-3'); <sup>13</sup>C-NMR (125 MHz, CDCl<sub>3</sub>)  $\delta$  151.4 (C-4 $\beta$ ), 150.5 (C-4 $\alpha$ ), 148.6, 148.5 (C-2 $\alpha$ , 2 $\beta$ ), 143.3 (C-1 $\alpha$ ), 142.3 (C-1 $\beta$ ), 125.7 (C-1'  $\beta$ ), 124.9 (C-1'  $\alpha$ ), 124.7 (C-2'  $\beta$ ), 124.3 (C-2'  $\alpha$ ), 118.9 (C-

5 $\alpha$ ), 118.0 (C-5 $\beta$ ), 114.0 (C-6 $\beta$ ), 109.7 (C-6 $\alpha$ ), 97.8, 97.4 (C-3 $\alpha$ , 3 $\beta$ ), 56.6, 56.5, 56.4, 56.0 (-OMe), 18.7 (C-3'  $\alpha$ ), 14.6 (C-3'  $\beta$ )

### Differentiation of 3T3-L1 cells

The murine 3T3-L1 preadipocyte cell line was obtained from the ATCC and the cells were cultured at 37°C in an atmosphere of 95% air/5% CO<sub>2</sub> in DMEM containing 10% fetal bovine serum (HyClone, UT). When 3T3-L1 cells became growth-arrested at confluence, differentiation was induced by the addition of DMEM medium supplemented with 0.5 M 3-isobutyl-1-methylxanthine, 2  $\mu$ M dexamethasone, and 1.7  $\mu$ M insulin (differentiation medium). This medium was removed after incubation for 2 days and replaced with DMEM supplemented with 10% FBS and 1.7  $\mu$ M of insulin for 2 days, whereupon the medium was changed every other day with DMEM supplemented with 10% FBS. At the time the experiments were performed on differentiated adipocytes, more than 95% of the 3T3-L1 cells were filled with multiple lipid droplets.

### Oil-red O staining

The fully differentiated 3T3-L1 adipocytes were washed with phosphate-buffered saline (PBS) and fixed with 4% formaldehyde in PBS for 10 min. The cells were incubated with oil-red O dye, which is maximally dissolved in 60% isopropanol, for 15 min at room temperature. The stained lipid droplets were dissolved in isopropanol and quantified by measuring the absorbance at 490 nm.

### Measurement of triglyceride level and non-esterified fatty acids

The fully differentiated 3T3-L1 adipocytes were incubated with either 62.5  $\mu$ M or 125  $\mu$ M asarone for the indicated time intervals. The cells were washed and harvested in 300  $\mu$ l of PBS whereupon they were sonicated. The triglyceride levels in the sonicated lysates were measured using the Cleantech TG-S reaction kit (Asan Pharm, Co., Seoul, Korea) according to the manufacturer's instructions. Non-esterified fatty acids released from mature adipocytes were measured using the Cultured Adipocyte Lipolysis Assay Kit from Zen Bio (Research Triangle Park, NC).

### Cell viability assay

Preadipocyte 3T3-L1 cells were seeded in a 96-well plate at a density of 5000 cells/well. Cells were treated with various concentrations of asarone for 48 h and then cell viability was measured using EZ-CyTox cell viability assay kit (Daeil Lab. Service, Seoul, Korea).

### Reverse transcriptional Polymerase Chain Reaction (PCR)

Total RNA was isolated using TRI reagent (Molecular Research Center, Inc., Seoul, Korea) from differentiated 3T3-L1 adipocytes after the treatment of asarone. Five micrograms of RNA was transcribed with 0.5  $\mu$ g of random primer using reverse transcriptase (Promega, Madison, WI) at 37°C for 1 h. The followings primer sequences were used for the amplification of adipose triglyceride lipase (ATGL) C/EBP $\beta$  and C/EBP $\delta$ : ATGL forward primer (5'-cagttccacctgctcagaca-3') and ATGL reverse primer (5'-ctccgagagagatgtgcaaca-3'); C/EBP $\beta$  forward primer (5'-ctttcgggacttgatgcaatc-3') and C/EBP $\beta$  reverse primer (5'-aacaaccccgaggaacat-3'); C/EBP $\delta$  forward primer (5'-ctccgcacacaacatactg-3') and C/EBP $\delta$  reverse primer (5'-ggtaagcccgcaaacatta-3'). PCR was performed with 30

cycles of amplification using a PTC-100 thermal cycler (MJ Research Inc., MA). Each amplification cycle consisted of 30 sec of denaturation at 94°C, 30 sec of annealing at 55°C, 30 sec of extension at 72°C. Amplification of  $\beta$ -actin was used as the control. PCR products were separated by 1% agarose gel electrophoresis and photographed.

#### *Western blot analysis*

Differentiated 3T3-L1 adipocytes treated with various concentrations of asarone were washed twice with PBS and cell lysates were prepared using 100  $\mu$ l of 1 $\times$  sodium dodecyl sulfate (SDS) denaturation buffer containing phosphatase inhibitor (Sigma-Aldrich, Mo), and then sonicated for 15–20 sec. The sonicated samples were heated at 95°C for 5 min and separated electrophoretically on a 10% SDS-polyacrylamide gel. Subsequently, proteins were transferred onto a 0.45  $\mu$ m pore size nitrocellulose membrane (Whatman, Dassel, Germany) for 2 h. The membrane was then incubated with PPAR $\gamma$  or C/EBP $\alpha$  antibody (Santa Cruz, CA) or phospho-ERK and ERK antibody or phospho-HSL antibody (Cell Signaling Technology, MA) or  $\beta$ -actin antibody (Bethyl Laboratories, Inc., TX) antibody at 1:1000 dilution overnight at 4°C. The membrane was then incubated with the anti-rabbit IgG or anti-mouse IgG secondary antibody conjugated with horseradish peroxidase (Stressgen, MI) at 1:5000 dilution for 2 h at 25°C. The proteins were visualized with an enhanced chemiluminescent substrate (Thermo, IL), and analyzed by LAS3000 luminescent image analyzer (Fuji Film, Tokyo, Japan).

## RESULTS

### *Asarone inhibits adipogenic differentiation of 3T3-L1 preadipocytes*

Differentiation of the murine preadipocyte cell line 3T3-L1 into adipocytes was initiated by the incubation of cells with a differentiation cocktail in the presence or absence of asarone for 48 h. The degree of adipogenesis was determined by microscopic observation of intracellular lipid droplets formation. The asarone treatment significantly suppressed adipogenesis of 3T3-L1 cells (Fig. 1A). The asarone-induced inhibition level of lipid droplet formation in adipocytes was evaluated by oil-red O staining. The amount of oil-red O dye incorporated into cells was quantified by measuring the absorbance of isopropanol-extracts of the cells. The treatment with asarone inhibited the formation of lipid droplets in a dose-dependent manner (Fig. 1B). To verify that asarone-mediated suppression of adipogenesis was not due to a cytotoxic effect of asarone, the viability of the cells was measured by EZ-CyTox cell viability assay kit. No statistically significant cytotoxicity was found after treatment with 125  $\mu$ M asarone for 48 h (Fig. 1C). These results indicate that co-treatment of asarone with a differentiation cocktail suppresses the differentiation of 3T3-L1

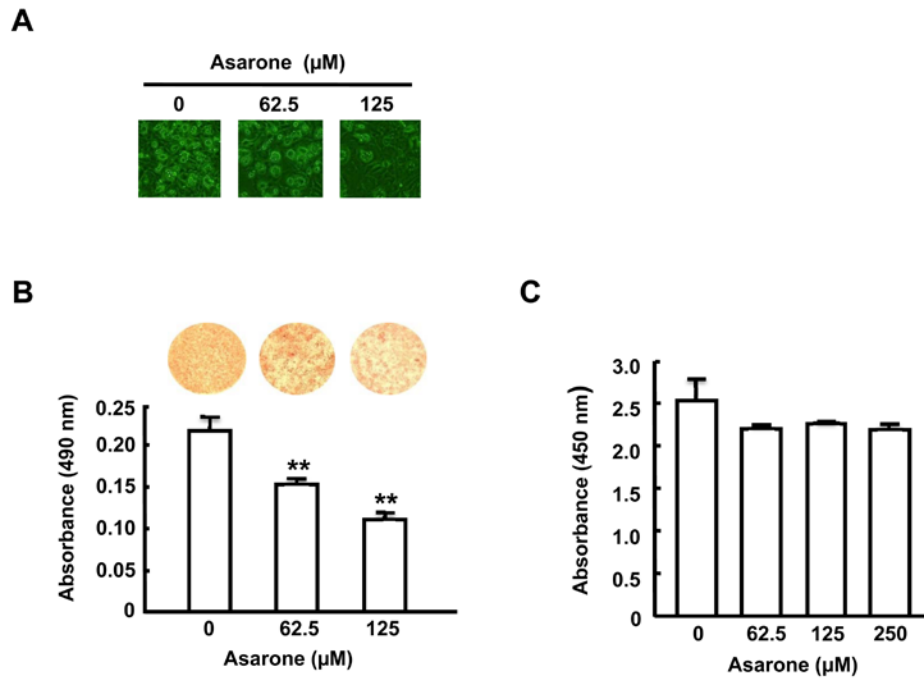
preadipocytes, and that the inhibitory effect of asarone on lipid droplet formation was not due to its cytotoxicity to 3T3-L1 cells.

### *Asarone inhibits expression of C/EBP $\alpha$ and PPAR $\gamma$*

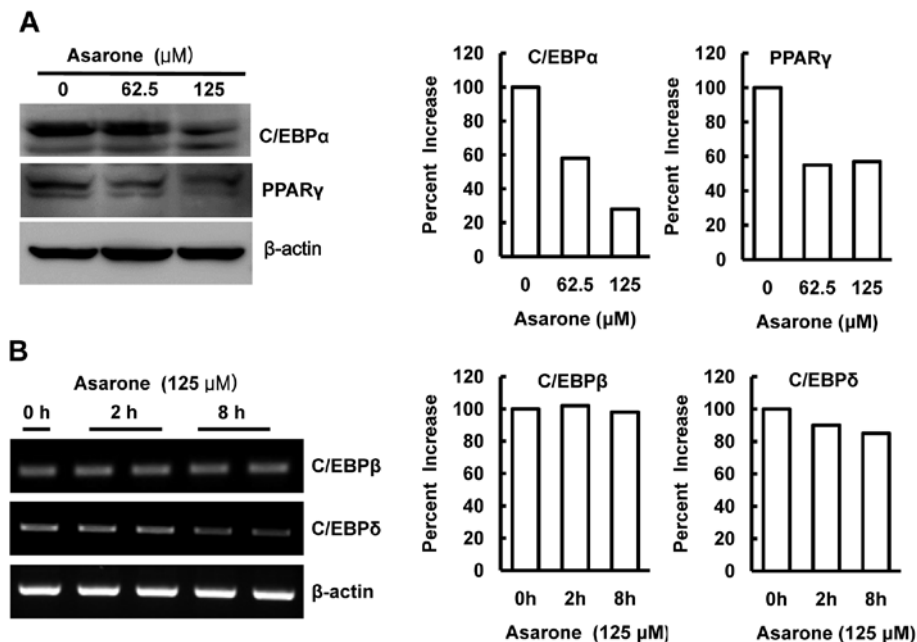
To elucidate the molecular mechanism underlying the anti-adipogenic effect of asarone, the differentiation of 3T3-L1 preadipocytes into adipocytes was initiated by incubating the cells with a differentiation cocktail in the presence or absence of asarone for 48 h. The C/EBP $\alpha$  and PPAR $\gamma$  transcription factors are responsible for the progression of adipogenesis; hence the expression levels of C/EBP $\alpha$  and PPAR $\gamma$  were assayed after an additional 8 days of culture in the absence of asarone. The protein levels of both transcription factors were dose-dependently decreased (Fig. 2A), indicating that asarone exerts its inhibitory effect on adipogenesis through down-regulation of C/EBP $\alpha$  and PPAR $\gamma$  expression. C/EBP $\beta$  and C/EBP $\delta$ , which are increased in early adipogenic process, transcriptionally activate C/EBP $\alpha$  and PPAR $\gamma$  to induce full differentiation of adipocytes (20). Therefore, we examined the effect of asarone on expression of C/EBP $\beta$  and C/EBP $\delta$ . Preadipocyte 3T3-L1 cells were treated with a differentiation cocktail in the presence of asarone for 8 h and the levels of C/EBP $\beta$  and C/EBP $\delta$  mRNAs were evaluated using RT-PCR. Asarone significantly inhibited C/EBP $\delta$  expression but did not affect expression of C/EBP $\beta$  (Fig. 2B). These data indicate that asarone-mediated inhibition of C/EBP $\delta$  in early adipogenesis may be involved in down-regulation of C/EBP $\alpha$  and PPAR $\gamma$  at later stages of adipogenesis.

### *Asarone enhances lipolysis through activation of hormone sensitive lipase*

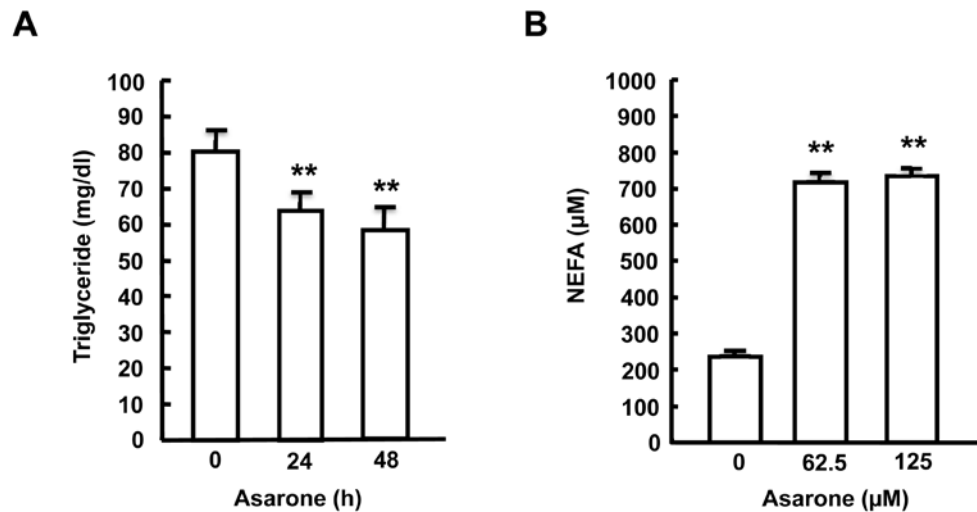
We also examined the effect of asarone on fully differentiated adipocytes. To determine whether asarone affects lipolysis in differentiated adipocytes, the intracellular level of triglycerides was measured in differentiated 3T3-L1 adipocytes treated with 125  $\mu$ M asarone. The level of triglycerides was found to decrease in a time-dependent manner with asarone treatment (Fig. 3A). To establish whether asarone increases lipolysis, the release of free fatty acids from cells was measured. Asarone treatment significantly increased the release of free fatty acid (Fig. 3B). Both HSL and ATGL play an important role in lipolysis in isolated mature fat cells (14). Therefore, the expression level of ATGL mRNA



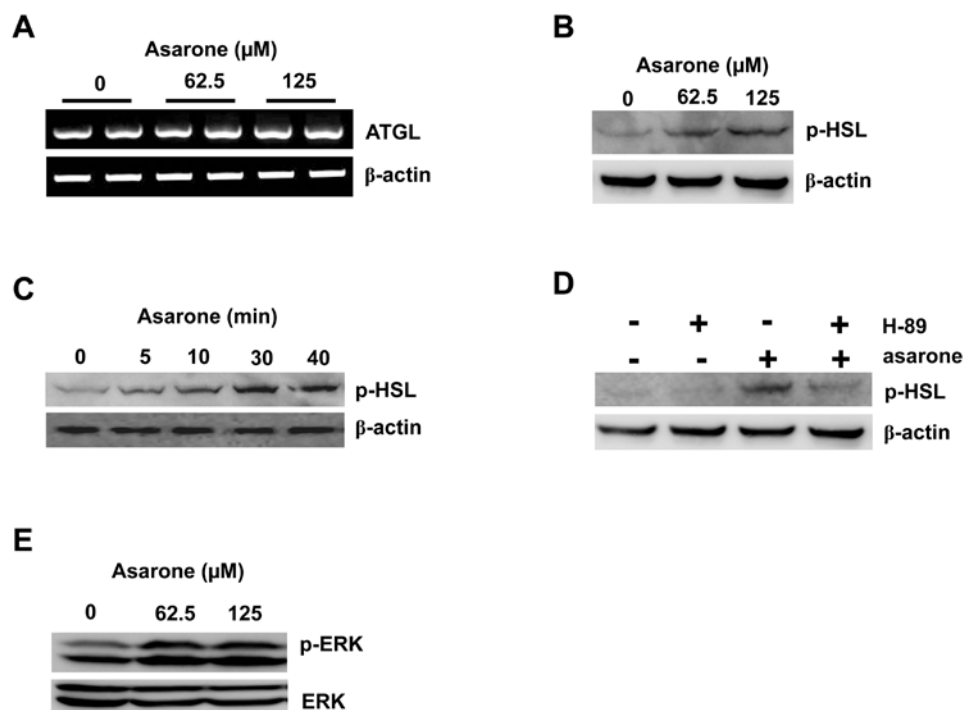
**Figure 1.** Co-treatment of asarone with differentiation cocktail inhibits adipogenesis. (A) Differentiation of preadipocyte 3T3-L1 cells into adipocytes was induced by incubation with a differentiation cocktail containing 62.5  $\mu\text{M}$  and 125  $\mu\text{M}$  asarone. On day 8 of differentiation, the morphology of the cells was examined to determine the level of adipogenesis. (B) Adipogenesis was performed in the presence of asarone. On day 8 of differentiation, differentiated cells were stained using oil red O and the level of oil red O incorporation was quantified by measuring the absorbance at 490 nm. Data indicate mean  $\pm$  SEM from triplicate experiments. \*\*,  $P < 0.01$ , compared with untreated control cells. (C) Preadipocyte 3T3-L1 cells were treated with various concentrations of asarone for 48 h and then cell viability was measured. Data indicate mean  $\pm$  SEM from triplicate experiments.



**Figure 2.** Asarone inhibits expression of C/EBP $\alpha$  and PPAR $\gamma$ . (A) Differentiation of preadipocyte 3T3-L1 cells into adipocytes was induced by incubation with a differentiation cocktail containing 62.5  $\mu\text{M}$  and 125  $\mu\text{M}$  asarone. On day 8 of differentiation, the levels of C/EBP $\alpha$  and PPAR $\gamma$  were measured using Western blot analysis. (B) Preadipocyte 3T3-L1 cells were treated with 125  $\mu\text{M}$  asarone in the presence of differentiation cocktail for the indicated time intervals. RT-PCR analysis was carried out to measure levels of C/EBP $\beta$  and C/EBP $\delta$ . The intensity of bands was measured using DNR Bio-imaging system.



**Figure 3.** Asarone stimulates lipolysis in differentiated adipocytes. (A) Preadipocyte 3T3-L1 cells were differentiated by incubation with a differentiation cocktail. On day 8 of differentiation, differentiated adipocytes were treated with 125  $\mu$ M asarone for the indicated time intervals. Intracellular triglyceride levels were measured. Data indicate mean  $\pm$  SEM from triplicate experiments. \*\*,  $P < 0.01$ , compared with untreated control cells. (B) On day 8 of differentiation, differentiated adipocytes were treated with the indicated concentrations of asarone. Levels of non-esterified fatty acids were measured 48 h after the treatment of asarone. Data indicate mean  $\pm$  SEM from triplicate experiments. \*\*,  $P < 0.01$ , compared with untreated control cells.



**Figure 4.** Asarone stimulates phosphorylation of HSL through activation of PKA and ERK. (A) Differentiated 3T3-L1 adipocytes were treated with the indicated concentrations of asarone for 24 h. RT-PCR analysis was carried out to measure the level of ATGL mRNA. (B) Differentiated 3T3-L1 mature adipocytes were treated with the indicated concentrations of asarone for 30 min. Cell lysates were subjected to Western blot analysis and the level of phospho-HSL was measured. (C) Differentiated 3T3-L1 adipocytes were treated with 125  $\mu$ M asarone for various time intervals and the level of phospho-HSL was measured using Western blot analysis. (D) Differentiated 3T3-L1 adipocytes were treated with 10  $\mu$ M H-89 for 1 h and then incubated with 125  $\mu$ M asarone for 30 min. Total protein was extracted from differentiated adipocytes and the level of phospho-HSL was measured using Western blot analysis. (E) Differentiated 3T3-L1 mature adipocytes were treated with the indicated concentrations of asarone for 30 min. Cell lysates were subjected to Western blot analysis and levels of phospho-ERK and ERK were measured.



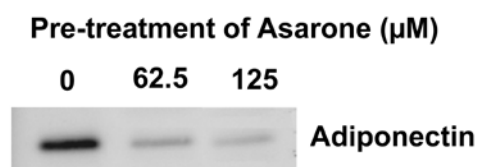
was analyzed using RT-PCR analysis; we observed that asarone treatment did not significantly affect ATGL expression (Fig. 4A). Next, we examined whether asarone affects the activity of HSL to hydrolyze triglyceride. Lipolysis in adipocytes is stimulated predominantly through PKA-mediated phosphorylation and activation of HSL. To examine whether HSL is activated, the level of phosphorylated HSL was measured by Western blot analysis using anti-phospho-HSL antibody. Differentiated 3T3-L1 adipocytes were treated with 125  $\mu\text{M}$  asarone for 30 min and total cell lysates were obtained by sonication with 1 $\times$  SDS denaturation buffer. The phosphorylation of HSL was increased dose-dependently by asarone treatment (Fig. 4B). To establish the time kinetics of HSL phosphorylation, differentiated 3T3-L1 cells were treated with 125  $\mu\text{M}$  asarone and total cell lysates were prepared at the indicated time intervals. The phosphorylation of HSL induced by asarone increased in a time-dependent fashion (Fig. 4C). Since PKA phosphorylates HSL, we tested the possibility that asarone may stimulate the PKA-dependent phosphorylation of HSL. Differentiated 3T3-L1 adipocytes were pretreated with a selective and potent cAMP-dependent PKA inhibitor, H-89, for 1 h and incubated for 30 min after the addition of 125  $\mu\text{M}$  asarone. The asarone-induced HSL phosphorylation was blocked in the presence of PKA inhibitor (Fig. 4D), indicating that asarone does indeed stimulate phosphorylation of HSL through PKA activation. It is also known that HSL, a rate-limiting enzyme in lipolysis, can be activated by extracellular signal-regulated kinase (ERK). To examine the effect of asarone on ERK activation, differentiated 3T3-L1 adipocytes were treated with the indicated concentrations of asarone and cell lysates were prepared 15 min later. The level of ERK phosphorylation was increased at 62.5  $\mu\text{M}$  asarone (Fig. 4E), indicating that both PKA and ERK activation by asarone are responsible for HSL activation.

#### *Asarone increases production of adiponectin*

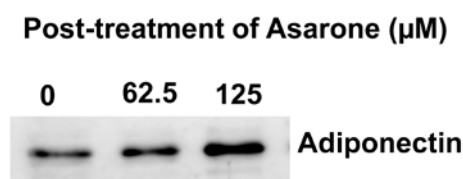
A reduced level of adiponectin is a risk factor for diabetes and various cancers (9). To investigate this effect, we determined whether asarone treatment of adipocytes affects production of adiponectin. The differentiation of preadipocyte 3T3-L1 cells into adipocytes was initiated by the incubation of cells with a

differentiation cocktail in the presence or absence of asarone for 48 h. The process of differentiation was allowed to proceed by changing with fresh medium every other day. On day 8 from the beginning of adipocyte formation, the culture medium was subjected to Western blot analysis to determine the level of secreted adiponectin. Pre-treatment with asarone significantly decreased the secretion of adiponectin (Fig. 5A). Differentiated 3T3-L1 adipocytes were treated with asarone for 48 h and the level of secreted adiponectin was determined. Interestingly, asarone markedly enhanced the secretion of adiponectin (Fig. 5B). Co-treatment of asarone with the differentiation cocktail inhibits the differentiation of 3T3-L1 preadipocytes into adipocytes. On the other hand, adiponectin secretion was increased when fully differentiated adipocytes were treated with asarone.

**A**



**B**



**Figure 5.** Asarone stimulates production of adiponectin in differentiated 3T3-L1 adipocytes. (A) Differentiation of preadipocyte 3T3-L1 cells into adipocytes was induced by incubation with differentiation cocktail containing asarone. On day 8 of differentiation, the level of adiponectin in the culture medium was measured using Western blot analysis. (B) 3T3-L1 cells were differentiated by incubation with differentiation cocktail. On day 8 of differentiation, the cells were treated with either 62.5  $\mu\text{M}$  or 125  $\mu\text{M}$  asarone for 48 h and then the level of adiponectin in the culture medium was measured using Western blot analysis.

## DISCUSSION

The root of the *Acorus calamus* plant contains asarone and has been used in traditional

medicine to treat diabetes. We have investigated the anti-diabetic effect of asarone through an examination of its effect on adipogenesis and lipolysis in murine 3T3-L1 preadipocytes and differentiated adipocytes. Asarone significantly blocked the adipogenic differentiation of 3T3-L1 preadipocytes in a dose dependent manner through down-regulation of the expression of genes associated with adipogenesis. At the molecular level, adipogenesis is driven by a complex transcriptional cascade that includes induction of the transcription factors, C/EBP $\beta$  and C/EBP $\delta$ . These transcription factors sequentially stimulate the expression of C/EBP $\alpha$  and PPAR $\gamma$  genes, which play key roles in late stage adipocyte differentiation (20).

We found that asarone decreases C/EBP $\delta$  mRNA levels during the early stage of adipogenic differentiation in 3T3-L1 cells. As a result, there is a subsequent block in the expression of C/EBP $\alpha$  and PPAR $\gamma$ , which are responsible for late stage adipogenesis. These data suggest that asarone-mediated inhibition of C/EBP $\delta$  may be the cause of the suppression of C/EBP $\alpha$  and PPAR $\gamma$  expression in the late stage of adipogenesis.

ATGL and HSL are two enzymes that are required for the lipolysis of triglycerides (11). Although asarone did not affect ATGL expression, HSL activation was markedly increased by asarone treatment. As a result of HSL activation by asarone, we observed that the level of triglycerides was decreased. These data indicate that asarone effectively reduces the level of intracellular triglycerides in two ways: firstly, by inhibiting triglyceride accumulation through inhibition of adipogenesis, and secondly by stimulating HSL lipolytic activity.

HSL is the key enzyme in the mediation of triglyceride lipolysis to non-esterified fatty acids and glycerol, so that the overexpression of HSL prevents triglyceride accumulation in adipocytes. The lipolysis of triglycerides tissue by HSL is hormonally regulated such that catecholamine acts to induce lipolysis via PKA-mediated phosphorylation of HSL in adipocytes (7). Our data show that asarone stimulates phosphorylation of HSL, resulting in increased lipolysis of triglycerides in differentiated 3T3-L1 adipocytes. Asarone-induced HSL phosphorylation was clearly blocked by the potent cAMP-dependent PKA inhibitor, H-89. It is also known that catecholamine activates ERK as well as PKA and that catecholamine-activated ERK enhances lipolysis (6). In addition, plant

extracts showing anti-oxidant activity such as *Asparagus racemosus* root and *Ginkgo Biloba* extract (12, 17) are possible to regulates lipolysis through activation of ERK pathway, since reactive oxidative stress is regulates ERK signaling pathway (10). Our data additionally show that the phosphorylation of ERK was significantly increased by asarone treatment, indicating that asarone stimulates lipolysis through HSL and ERK activation.

Adiponectin is a protein hormone that modulates many metabolic processes involving glucose regulation and free fatty metabolism (8, 18, 21). Levels of adiponectin are inversely correlated with body fat mass in adults and recovery of adiponectin-alleviated symptoms of type 2 diabetes and atherosclerosis (4). Our data show that pre-treatment with asarone significantly decreases production of adiponectin, as a result of asarone-mediated repression of adipogenesis. On the other hand, when differentiated adipocytes were treated with asarone, the secretion of adiponectin was remarkably increased. This suggests that asarone could have two beneficial effects the alleviation of the symptoms of type 2 diabetes through enhanced production of adiponectin as well as the suppression of adipogenesis.

In summary, we have shown that asarone extracted from the plant *Acorus calamus* inhibits adipogenesis, increases lipolysis, and stimulates production of adiponectin. These findings indicate a mechanism for asarone as an anti-diabetic agent. In further studies, we aim to identify the specific roles played by alpha-asarone and beta-asarone in this process.

**Acknowledgements** – This work was supported by the Biogreen 21 project (20070301034031) of Rural Development Administration, and a Korea Research Foundation Grant KRF-2008-313-C00708 and the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MEST) (R01-2008-000-20127-0).

## REFERENCES

1. Acuna U.M., Atha D.E., Ma J., Nee M.H. and Kennelly E.J., Antioxidant capacities of ten edible North American plants. *Phytother. Res.* 2002, **16**: 63-65.
2. Antunez-Solis J., Hernandez-Derramadero F., Aquino-Vega M., Ibarra-Ramirez S., Rodriguez-Paez L., Baeza I. and Wong C., 2,4,5-trimethoxycinnamic acid: the major metabolite of alpha-asarone, retains most of the pharmacological properties of alpha-asarone. *J. Enzyme. Inhib. Med. Chem.* 2009, **24**: 903-909.
3. Ardevol A., Blade C., Salvado M.J. and Arola L., Changes in lipolysis and hormone-sensitive lipase

- expression caused by procyanidins in 3T3-L1 adipocytes. *Int. J. Obes. Relat. Metab. Disord.* 2000, **24**: 319-324.
4. Berg A.H., Combs T.P., Du X., Brownlee M. and Scherer P.E., The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. *Nat. Med.* 2001, **7**: 947-953.
  5. Dennehy C.E., Tsourounis C. and Miller A.E., Evaluation of herbal dietary supplements marketed on the internet for recreational use. *Ann. Pharmacother.* 2005, **39**: 1634-1639.
  6. Greenberg A.S., Shen W.J., Muliro K., Patel S., Souza S.C., Roth R.A. and Kraemer F.B., Stimulation of lipolysis and hormone-sensitive lipase via the extracellular signal-regulated kinase pathway. *J. Biol. Chem.* 2001, **276**: 45456-45461.
  7. Holm C. Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. *Biochem. Soc. Trans.* 2003, **31**: 1120-1124.
  8. Hu E., Liang P. and Spiegelman B.M., AdipoQ is a novel adipose-specific gene dysregulated in obesity. *J. Biol. Chem.* 1996, **271**: 10697-10703.
  9. Kim J.H., Kim K.Y., Jeon J.H., Lee S.H., Hwang J.E., Lee J.H., Kim K.K., Lim J.S., Kim K.I., Moon E.Y., Lee H.G., Ryu J.H. and Yang Y., Adipocyte culture medium stimulates production of macrophage inhibitory cytokine 1 in MDA-MB-231 cells. *Cancer Lett.* 2008, **261**: 253-262.
  10. Kim J. and Wong P.K., Oxidative stress is linked to ERK1/2-p16 signaling-mediated growth defect in ATM-deficient astrocytes. *J. Biol. Chem.* 2009, **284**: 14396-14404.
  11. Miyoshi H., Perfield J.W. 2nd, Obin M.S. and Greenberg A.S., Adipose triglyceride lipase regulates basal lipolysis and lipid droplet size in adipocytes. *J. Cell. Biochem.* 2008, **105**: 1430-1436.
  12. Pierre S.V., Lesnik P., Moreau M., Bonello L., Droy-Lefaix M-T., Sennoune S., Duran M-J., Pressley T.A., Sampol J., Chapman J. and Maxient J-M., The standardized Ginkgo Biloba extract EGB-761 protects vascular endothelium exposed to oxidized low density lipoproteins. *Cell. Mol. Biol.* 2008, **54**: 1032-1042.
  13. Rayalam S., Yang J.Y., Ambati S., Della-Fera M.A. and Baile C.A., Resveratrol induces apoptosis and inhibits adipogenesis in 3T3-L1 adipocytes. *Phytother. Res.* 2008, **22**: 1367-1371.
  14. Ryden M., Jocken J., van Harmelen V., Dicker A., Hoffstedt J., Wren M., Blomqvist L., Mairal A., Langin D., Blaak E. and Arner P., Comparative studies of the role of hormone-sensitive lipase and adipose triglyceride lipase in human fat cell lipolysis. *Am. J. Physiol. Endocrinol. Metab.* 2007, **292**: E1847-1855.
  15. Szkudelska K., Nogowski L. and Szkudelski T., Genistein affects lipogenesis and lipolysis in isolated rat adipocytes. *J. Steroid Biochem. Mol. Biol.* 2000, **75**: 265-271.
  16. Tang Q.Q., Otto T.C. and Lane M.D., Mitotic clonal expansion: a synchronous process required for adipogenesis. *Proc. Natl. Acad. Sci. U. S. A.* 2003, **100**: 44-49.
  17. Visavadiya N.P., Soni B. and Madamwar D., Suppression of reactive oxygen species and nitric oxide by Asparagus racemosus root extract using in vitro studies. *Cell. Mol. Biol.* 2009, **55**: 1083-1095.
  18. Yamauchi T., Kamon J., Minokoshi Y., Ito Y., Waki H., Uchida S., Yamashita S., Noda M., Kita S., Ueki K., Eto K., Akanuma Y., Froguel P., Foufelle F., Ferre P., Carling D., Kimura S., Nagai R., Kahn B.B. and Kadowaki T., Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat. Med.* 2002, **8**: 1288-1295.
  19. Yang J.Y., Della-Fera M.A., Rayalam S., Ambati S., Hartzell D.L., Park H.J. and Baile C.A., Enhanced inhibition of adipogenesis and induction of apoptosis in 3T3-L1 adipocytes with combinations of resveratrol and quercetin. *Life Sci.* 2008, **82**: 1032-1039.
  20. Yeh W.C., Cao Z., Classon M. and McKnight S.L., Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. *Genes Dev.* 1995, **9**: 168-181.
  21. Yoon M.J., Lee G.Y., Chung J.J., Ahn Y.H., Hong S.H. and Kim J.B., Adiponectin increases fatty acid oxidation in skeletal muscle cells by sequential activation of AMP-activated protein kinase, p38 mitogen-activated protein kinase, and peroxisome proliferator-activated receptor alpha. *Diabetes* 2006, **55**: 2562-2570.