Thesis for the Degree of Master of Science

AP1 inhibits adipogenesis through down-regulation of PPARγ and modulation of PI3K/Akt and FoxO pathways in 3T3-L1 cells

by

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February 2013

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(전복에서 분리된 AP1 펩타이드에 의한 3T3-L1 지방세포에서의 PPARγ의 억제와 PI3K/Akt, FoxOs 신호전달을 통한 항비만 효과)

Advisor: Prof. Gun Do Kim

by

Soon Jin Kim

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

In Department of Microbiology, The Graduate School,
Pukyong National University

February 2013

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February 22, 2013

CONTENTS

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ABSTRACT

Abalone has long been used as a valuable food source in East Asian countries. Although the nutritional importance of abalone has been reported through *in vitro* and *in vivo* studies, there is little evidence about the potential anti-obesity effects of abalone. The present study investigated the inhibitory effects of AP1 on the differentiation of 3T3-L1 preadipocytes. AP1 potently and dose-dependently suppressed accumulation of lipid droplets in adipocyte. Western blot analysis revealed that AP1 markedly down-regulated the protein levels of adipogenesis regulator peroxisome proliferator-activated receptor (PPAR)γ, CCAAT/enhancer binding protein (C/EBP)α, sterol regulatory element-binding protein (SREBP)1 as well as a key enzyme involved in adipogenesis. In addition, AP1 diminished the insulin-stimulated phsphoinositide 3-kinase (PI3K), phosphorylarion of Akt, glycogen synthase kinase-3 beta (GSK3β) and forkhead transcription factors, which may reduce glucose uptake in response to insulin and lipid accumulation. These results indicate that AP1 suppresses adipocyte differentiation and lipid accumulation through multiple mechanisms and may have applications for the treatment of obesity.

INTRODUCTION

Adipose tissue is crucial for energy storage and lipid homeostasis, but an imbalance between energy intake and expenditure leads to obesity, which is a major risk factor for many chronic diseases and metabolic disorders such as type 2 diabetes, hypertension, hyperlipidemia, and arteriosclerosis (Kopelman PG., 2000). Obesity is caused by an increase in adipose tissue mass, which results from the multiplication of fat cells through adipogenesis and the increased deposition of cytoplasmic triglycerides (Lefterova *et al.*, 2009).

Adipogenesis is the process by which an undifferentiated preadipocyte is converted to a fully differentiated adipocyte (Otto *et al.*, 2005). The programmed differentiation of preadipocytes is accompanied by an increase in the expression of various transcription factors and adipocyte-specific genes. Lipid accumulation increases throughout the adipogenic process, and it is regulated by genetic and growth factors (Cristancho *et al.*, 2011). The 3T3-L1 cell line is one of the most reliable models for the study of the conversion of preadipocytes into adipocytes (Green *et al.*, 1976). The 3T3-L1 mouse preadipocyte cell line has the capacity to differentiate into adipocytes when treated with a mixture of dexamethasone, 3-iso-butyl-1-methylaxanthine, and insulin (DMI). After initiation of differentiation, 3T3-L1 cells pass through a transient cell proliferation stage, known as clonal expansion, before acquiring the functions of a mature adipocyte, including lipid droplet accumulation and the secretion of adipocytokines.

Previous studies reveal that many transcription factors are associated with adipocyte differentiation. The ligand-dependent nuclear receptor type transcription factor PPARγ and the leucine zipper type transcription factor C/EBPβ, a member of the C/EBP family, rises 2-4 h after the induction of

differentiation and gradually accumulates, after 14 h C/EBPβ and C/EBPδ induces the expression of C/EBPα and PPARɣ, and the differentiation phase C/EBPα and PPARɣ induce adipocyte-specific gene expression and mutual expression, and this leads to the development of the mature adipocyte with insulin sensitivity and the capacity to form lipid droplets (Rosen *et al*. 1999). SREBP1 may regulate adipocyte differentiation by increasing the transcriptional activity of PPARɣ, and SREBP1 expression is significantly enhanced in response to insulin (Kim *et al*., 1998). It has already been reported that the transcriptional network of adipocyte differentiation is dominated by three pathways (Insulin, Glucocorticoid and cAMP) (Farmer *et al*., 2006).

The intracellular signaling cascade involving phosphoinositide 3-kinase (PI3K) and Akt is involved in the regulation of many cellular processes. In particular, several lines of evidences have implicated the PI3K/Akt pathway as a positive regulator of terminal adipocyte differentiation in murine preadipocytes. Disruption of PI3K function by pharmacological inhibitors or dominant negative mutations abolishes adipocyte differentiation. Expression of constitutively active form of Akt, the main downstream effector of PI3K, in 3T3-L1 has been shown to cause spontaneous adipocyte differentiation (Magun *et al*., 1996).

At least two downstream branches of Akt signaling have been implicated in the regulation of PPARɣ expression and adipocyte differentiation. The forkhead box O (FOXO) family of transcription factors are phosphorylated and inhibited by Akt. The forkhead-type transcription factor family forkhead box class O (Foxo) is also thought to play a role in adipocyte differentiation (Nakae *et al*., 2003). Another major downstream signaling branch downstream of Akt results in activation of the mammaliam target of rapamycin (mTOR),

a critical regulator of mRNA translation and cell growth (Wullschleger *et al.*, 2006). Primarily through use of the mTOR-specific inhibitor rapamycin, several studies have concluded that mTOR activitiy is required for proper differentiation of preadipoyte cell lines (Yeh *et al.*, 1995).

Abalones are medium to very large-sized edible sea snails, marine gastropod mollusks in the family Haliotidae and the genus Haliotis (Geiger *et al.*, 1999). Abalones are largely cultivated and used as valuable food resources in East Asian countries. In Korea, not only the protein-rich body part of abalone but also the viscera portion is taken in the form of sashimi or pickle and used as stamina food from ancient times. Although the nutritional composition of abalone visceral extract have not yet been identified in depth, since abalone live on brown algae like Ecklonia, Laminaria and Undaria for cultivation (Hwang *et al.*, 2009). It is regarded that the visceral portion of abalone may contain concentrated nutritional components derived from sea weed. Besides, it is well known that polysaccharides and glycoproteins of the brown algae possess potential immune-stimulant, anti-tumoral and anti-viral activity (Smit *et al.*, 2004). However, there is still not much information about the anti-obesity effects of abalone viscera itself.

In the present study, the effect of AP1 in adipocyte differentiation in 3T3-L1 cells was investigated by measuring lipid accumulation and evaluating the expression levels of adipocyte marker proteins. Moreover, we examined whether PI3K/Akt, FoxOs pathway related protein activation is critical for the anti-adipogenic functions of AP1 to better understand the specific mechanisms mediating AP1 function.

MATERIALS AND METHODS

Design of peptide

Analogue peptide was designed from the primary structure of a novel antimicrobial peptide purified from the Gill of the abalone, *Haliotis discus discus*, as a parent peptide (Seo et al., 2012, unpublished data). To design the AP1 peptide, three basical points of views including αα-helical structure having the optimal amphipathic character, C-terminal amidation increasing net positive charge and stability, and small size reducing cost were firstly considered.

To select the optimal αα-helical region in the parent peptide, the secondary structure was predicted by the GOR method. In addition, to optimize the amphipathcity and adjust the sequence length, a Schiffer Edmunson plot was generated by the DNASIS v2.5 demo program (Hitachi Software Engineering Co. Ltd. Japan). The theoretical isoelectric point and molecular mass were estimated with ExPASy. Hydrophobicity and hydrophobic moment were obtained from the DNASIS v2.5 demo program (Hitachi Software Engineering Co. Ltd. Japan).

Physical parameter of the designed peptide

The physical properties of the designed AP1 peptide were measured by several different methods as mentioned above. The peptide shows an αα-helical amphipatic structure consisting of 14 amino acid residues with 4 hydophobic residues and 5 hydrophilic residues containing 3 basic residues and contained a Trp residue for fluorescence work.

Peptide synthesis

The designed AP1 peptides were synthesized by the solid-phase synthesis method on an ASP48S Pepsynthesizer (Peptron Inc, Korea) using 9-flurorenylmethoxycarbonyl (Fmoc)-polypeptide active ester chemistry. Two forms for the peptides, one amidated (NH_2) and the other with a free carboxy terminus (COOH), were synthesized with Fmoc-NH-SAL resin or Fmoc-resin. The synthetic peptides were purified on a Vydac Everest C_{18} column (10 μμm, 300 Å, 22 mm × 250 mm) with a water-acetonitrile (ACN) linear gradient of 3-40% ACN in 0.1% TFA. Molecular weights and purities (>95%) of the synthetic peptides were confirmed using an LC/MS (Agilent HP1100 series) and the reverse phase HPLC using a CapCell-Pak C_{18} reversed-phase column (5 μm, 300 Å, 4.6 × 250 mm, Shiseido, Japan).

Cell culture

3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA) were grown to confluence in DMEM (Cellgro, Mediatech, Inc., Manassas, VA, USA) supplemented with 10% fetal calf serum (Gibco®, Grand Island, NY, USA) and penicillin (100 U/ml)/ streptomycin (100μg/ml) (PAA Laboratories GmbH, Pasching, Austria). The cells were cultured in humidified atmosphere of 95% air and 5% CO2 at 37℃. To induce adipogenesis, 3T3-L1 cells were cultured until confluent, and 1 day after reaching confluence (day 0), the culture medium was exchanged with a differentiation medium (DMI) containing 1 ug/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone (Sigma-Aldrich, Tokyo, Japan) and 10% fetal bovine serum in DMEM (Cellgro).

On day 3, the initiation media was replaced with progression media (1μg/ml

insulin in DMEM supplemented with 10% fetal bovine serum). On day 6, the progression media was replaced with maintenance media (DMEM supplemented with 10% fetal bovine serum). From days 0 to 6, the cells were treated to the AP1.

Oil-red O staining

Cells were treated either with AP1 in differentiation medium from days 0-6 of adipogenesis. On day 6, cells were stained with Oil-red O. 3T3-L1 cells were fixed in fresh 10% formalin (Junsei Chemical Co., Ltd, Tokyo, Japan) and stained with stained with filtered Oil-red O solution (in 60% isopropanol and 40% water) (Sigma-Aldrich, Tokyo, Japan) for 30 min. After staining the lipid droplets, the Oil-red O staining solution was removed, and the plates were rinsed with water and dried. The stained lipid droplets were visualized by an inverted microscope (Magnification, ×200).

Measurement of triglyceride content

Cellular triglyceride contents were measured using a commercially available triglyceride quantification kit (BioVision, Inc., San Francisco, CA, USA) according to the manufacturer's instructions. Adipocytes differentiated for 6 days were treated with AP1 at concentrations of 0, 5, 10 and 15μM in 6-well plates. To analyze the cellular triglyceride content, cells were washed with PBS and then scraped into 200 μl PBS and homogenized by sonication for 1 min. The total triglycerides in the lysates were measured using the assay kit.

Western blot analysis

For Western blotting, 3T3-L1 cells were cultured and treated with 5μM and 15 μM of the AP1 during adipocyte differentiation. The treated cells were harvested by centrifugation, lysed in ice-cold lysis buffer [(50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM DTT, 0.5% NP-40, 1% Triton X-100, 1% Deoxycholate, 0.1% SDS and cocktail of proteinase inhibitors (PMSF, EDTA, aprotinin, leupeptin, prostatin A; Intron biotechnology, Gyeonggi, Korea)]. After incubation on ice for 30 min, the insoluble materials were removed by centrifugation at 14,000 rpm for 20 min. The protein content of the cell lysates were determined by a Protein Quantification Kit (CBB solution®) (Dojindo Molecular Technologies, Rockvile, MD, USA) with bovine serum albumen (BSA) as standard. An aliquot from each sample was boiled for 5 min and then resolved by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were electrotransferred to a nitrocellulose membrane (PALL Life Sciences, Pensacola, MI, USA) and blocked in PBST buffer (135 μM NaCl, 2.7 mM KCl, 4.3 mM NaPO4, 1.4 mM KH2PO4 and 0.5% Tween-20) containing 5% skim milk. After blocking, the membrane was probed with primary antibodies (anti-SREBP1, anti-C/EBPα, anti-PPARγ, anti-mTOR, anti-phosphor-mTOR (ser2481), anti-PI3K p110α, anti-PI3K p110β, anti-PI3K p110γ, anti-GSK3β, anti-phosphor-GSK3β (ser9), anti-Akt, anti-phosphor-Akt (ser473), anti-phosphor-p70s6k (ser371), anti-FoxO1, anti-phosphor-FoxO1 (ser256), anti-FoxO3a, anti-phosphor-FoxO3a (Thr32), anti-β-actin; Cell Signaling Technology Inc., Beverly, MA, USA) and washed three times with PBST buffer followed by incubation for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG as second antibodies (Cell Signaling Technology Inc.). The blots were then washed with PBST buffer and were visualized by an enhanced chemiluminescent (ECL) detection solution (Pierce, Rockford, IL, USA).

Immunofluorescence of C/EBPα protein

Cell grown on coverglass-bottom dishes (SPL Lifesciences, Gyeonggi, Korea) were incubated with 5, 15 μM of the AP1 during adipocyte differentiation. For this, cells were pretreated with DAPI solution for 30 min at 37℃ and then and then fixed with 4% formaldehyde (Sigma, St. Louis, Mo, USA) for 15 min at room temperature followed by blocking for 1 h in 5% mouse and rabbit normal serum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The fixed and blocked cells were incubated with primary antibody (C/EBPα) (Cell Signaling Technology Inc.) for 2 h, and then with 0.1 μg/ml of anti-rabbit IgG (H+L), F (ab') 2 Fragment (Alexa Fluor® 488 Conjugate) (Cell Signaling Technology Inc.) for another 1 h. The stained cells on the slides were mounted with Prolong Antifade Reagent (Invitrogen, Eugene, OR, USA) and were observed in a fluorescent Nikon ECLIPS 50i microscope equipped with charged-coupled device (CDD) camera (Nikon, Tokyo, Japan). Images were captured and processed with a High-Content Analysis Software (Cambridge Healthtech Institute, Needham, MA, USA).

Statistical analysis

The GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA) for window was used to determine the statistical significance of differences between values for various experimental and control group. Determinations were performed in triplicates and the results are presented as mean±SD. In cases where no error bar is seen in the graph, the variation is small and thus, the bar is hidden behind the bar. ANOVA post hoc test and subsequently Dunnett's multiple comparison tests were used for statistical analysis.

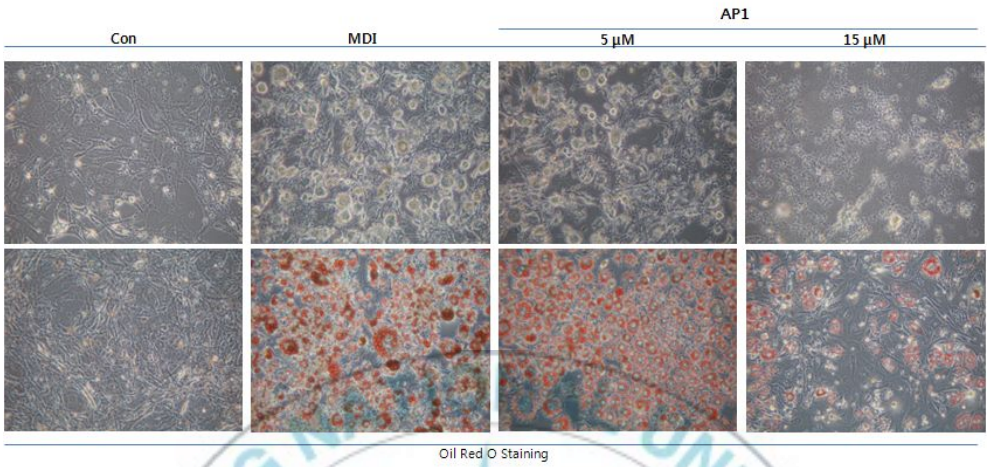
RESULTS

AP1 inhibits 3T3-L1 adipocyte differentiation

To examine the effects of AP1 on adipocyte differentiation, 3T3-L1 preadipocytes were treated with 0, 5 or 15 μM of AP1 in the presence of DMI (0.5 mM 3-isobutyl-1-methylazanthine, 0.5μM dexamethasone and 1μg/ml insulin) or DMI alone. The differentiation medium was exchanged with medium containing the insulin every two days. Ten days after the initiation of differentiation, lipid accumulation was measured by Oil Red O staining. Fig. 1A shows the result of Oil Red O staining under an inverted microscope. Oil droplets were not visible in the medium of undifferentiated 3T3-L1 cells, but many lipid droplets were visible in that of the fully differentiated cells treated with DMI. The presence of lipid droplets was used as a marker of lipid accumulation. Microscopic observations of the Oil-red O staining revealed a reduction in the amount of lipid droplets with increasing concentrations of AP1 in a dose-dependent manner. The highest concentration 15μM AP1 strongly inhibited the differentiation of 3T3-L1 preadipocytes into adipocytes and prevented lipid accumulation.

To explore the effect of AP1 in the inhibition of intracellular triglyceride accumulation, 3T3-L1 preadipocytes were differentiated with AP1 for ten days. Lipid accumulation, which was used as a major marker of adipogenesis, was quantified on day 6 of the differentiation period. The results showed that treatment of AP1 reduces triglyceride content and the inhibition of intracellular triglyceride accumulation in 3T3-L1 adipocytes occurs in a dose-dependent manner (Fig. 1B). The inhibitory effects of AP1 in triglyceride accumulation during adipogenesis were also dose-dependent. These results demonstrate that AP1 strongly blocks adipocyte differentiation in 3T3-L1 cells.

(A)



(B)

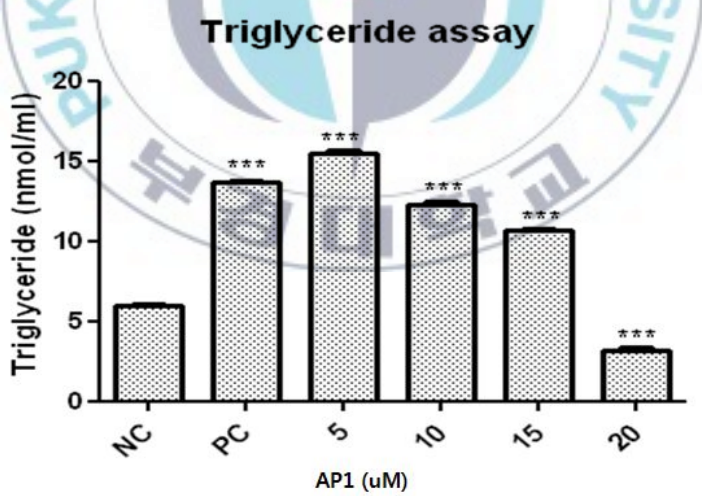


Figure 1. The Effect of AP1 on lipid accumulation of 3T3-L1 adipocytes. Confluent 3T3-L1 preadipocytes differentiated into adipocytes in medium

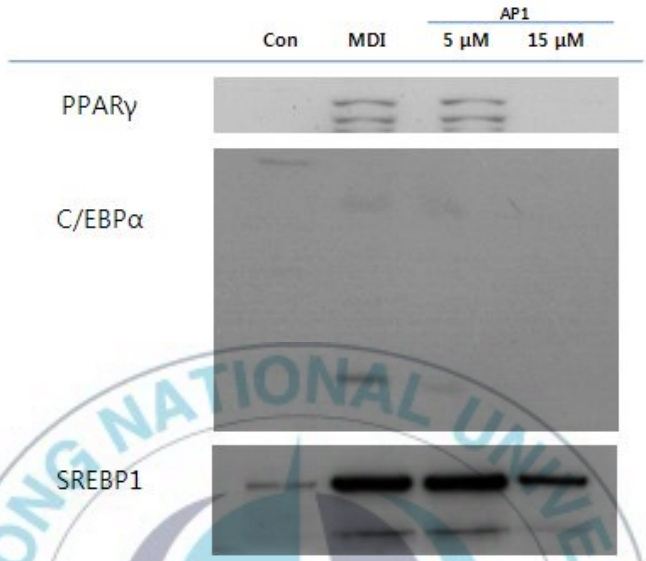
containig different concentrations of AP1. Con, 3T3-L1 preadipocytes; MDI, fully differentiated adipocytes; 5μM, fully differentiated adipocytes (DMI + 5μM AP1); 15μM, fully differentiated adipocytes (DMI + 15μM AP1). Inhibitory effects of AP1 in lipid accumulation in 3T3-L1 adipocytes. The intracellular lipid accumulation stained by Oil-red O and obseved by an inverted microscope (Magnification, X200) (A). Treatment of AP1 reduces triglyceride content and that the inhibition of intracellular triglyceride accumulation in 3T3-L1 adipocytes during adipogenesis were dose-dependent. Bars represent mean±SD of three experiments. ***$P < 0.001$ (B).

The effect of AP1 on the expression of adipocyte-specific regulators during adipocyte differentiation

PPARγ and C/EBPα are master transcription factors in the regulation of adipogenesis in adipocytes. And SREBP1 has been reported to play a role as central hubs in lipid metabolism. Hence, we next investigated the expression of PPARγ, C/EBPα and SREBP1 to characterize the effect of AP1 on 3T3-L1 adipocyte differentiation. As shown in Fig. 2A, Western blot analysis revealed that AP1 markedly reduced the protein expression levels of PPARγ. The protein expression of C/EBPα and SREBP1 were increased in differentiated adipocytes (PC) compared to undifferentiated cells(NC). In addition, the immunofluorescence experiment showed decreased expression of C/EBPα in AP1 treated cells compared to differentiated cells. (Fig. 2B)

These results indicated that AP1 suppressed adipocyte differentiation through inhibition of SREBP-1, PPARγ and C/EBPα.

(A)



(B)

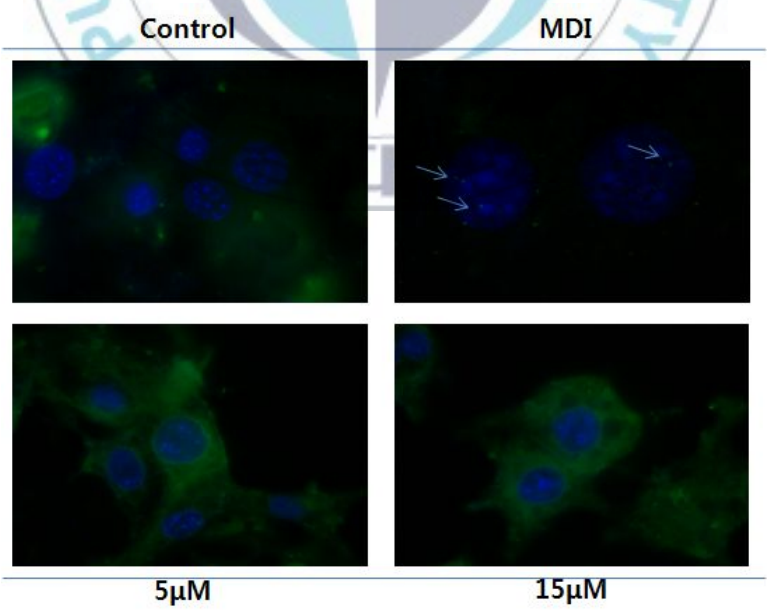


Figure 2. Effect of AP1 in the expression of adipogenesis related proteins in 3T3-L1 adipocyte. The differentiation of 3T3-L1 preadipocytes was induced by DMI medium in the absence or presence of 5 and 15μM AP1. The expression levels of PPARγ, C/EBPα and SREBP-1 were determined by western blot analysis (A). The expression levels of C/EBPα in the treated cells were detected by immunofluorescence staining (B). Cells were stained with DAPI (nuclei, blue) and immunofluorescence antibody (C/EBPα, green) and visualized under fluorescence microscopy (Margnification, X1,000).

The effect of AP1 in the regulation of PI3K/Akt, FoxO pathways during adipocyte differentiation

To determine whether AP1 changed the phosphorylation levels of molecules that were downstream of PI3K/Akt, FoxOs signaling, 3T3-L1 adipocytes were treated with DMI alone or with DMI and AP1. (Fig. 3)

The protein levels of PI3K p110 isoforms (α,β,ɣ) were decreased in 3T3-L1 adipocytes with DMI and AP1. The protein levels of mTOR, phosphor-mTOR and p70S6K were decreased in 3T3-L1 adipocytes. In the DMI and DMI+AP1 samples, wild type Akt and GSK3β were expressed at similar levels. DMI stimulation of 3T3-L1 adipocytes resulted in a significant increase in phosphor-Akt and phosphor-GSKβ. In the presence of AP1, the expression of phospho-Akt was dramatically decreased in 3T3-L1 adipocytes. Similarly, the expression of phosphor-GSK3β during 3T3-L1 differentiation was also decreased by AP1 treatment. These results demonstrate that AP1 treatment inhibits the phosphorylation of Akt, which suppresses the phosphorylation of its substrate kinase GSK3β.

The activity of FoxOs is negatively regulated by insulin and growth factor stimulation through Akt-dependent phosphorylation and nuclear exclusion. To determine whether AP1 can modulate FoxOs signaling pathways, the potential involvements of FoxO1 and FoxO3a were evaluated by using anti-phospho specific antibody in Western blot analysis. In differentiated adipocytes with AP1 treatment, both FoxO1 and FoxO3a were decreased compared to differentiated with DMI alone. Also both phosphorylated FoxO1 and FoxO3a were dramatically decreased. These results of immunoblot analysis suggested that AP1 inhibited activation of FoxOs, and suppressed 3T3-L1 adipocyte differentiation. Altogether these results demonstrated the effect of AP1 on PI3K/Akt-dependent FoxOs signaling in 3T3-L1 adipocytes.

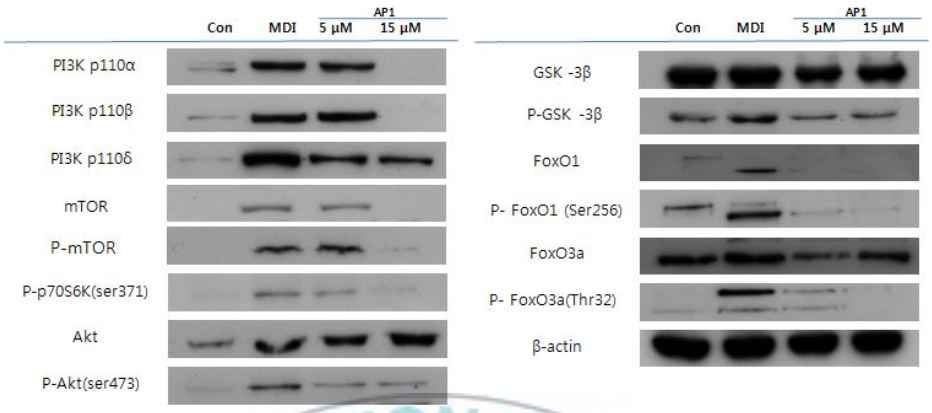


Figure 3. The effect of AP1 on the regulation of PI3K/Akt pathway during adipocyte differentiation. The differentiation of 3T3-L1 preadipocytes was induced by DMI medium in the absence or presence of 5 and 15μM AP1. The expression levels of PI3K/Akt, FoxOs pathway related proteins were determined by western blot analysis.

DISCUSSION

Numerous studies have documented that adipocytes are a potential target for anti-obesity strategy, and many dietary natural products are considered to be attractive anti-obesity drugs through inhibition of differentiation, regulation of lipid and glucose metabolism, and induction of apoptosis and cell cycle arrest in adipocyte (Rayalam *et al.*, 2008).

AP1 purified from the Gill of the abalone, *Haliotis discus*. There are some previous reports that the body and visceral portion of *Haliotis discus* showed *in vitro* antioxidant activity (Zhu *et al.*, 2008). Antioxidants are present abundantly in the dietary substances and their potent chemopreventive effects against many type of cancers have been reported. However, mechanisms involved in the anti-obesity effects of peptide from abalone have not been fully clarified (Khan *et al.*, 2008).

The aim of the present study was to determine the effect of AP1 in the adipogenesis in 3T3-L1 adipocytes. We demonstrated that AP1 inhibited lipid accumulation and differentiated 3T3-L1 adipocytes through modulation of multiple molecular pathways. AP1 treatment decreased the expression of key adipocyte differentiation regulators, including PPARγ, C/EBPα and SREBP1, and down-regulated PI3K/Akt, FoxOs pathways.

In the present study, AP1 treatment remarkably reduced the level of Oil-red O staining in a dose-dependent manner, and microscopic inspection revealed a significant decrease in the level of accumulated intracellular triglyceride. The triglyceride level in cells treated with 15μM AP1 displayed a marked reduction in adipogenesis. Adipocyte differentiation and fat accumulation are associated with the occurrence and development of obesity (Fève *et al.*, 2005). The development of obesity is characterized by both

increased adipocyte number (hyperplasia) and size (hypertrophy), which is regulated by genetic, endocrine, metabolic and nutritional factors (Wolfram *et al*., 2005). These results indicate that AP1 inhibited the differentiation of 3T3-L1 preadipocytes into adipocytes and also inhibited the accumulation of lipid droplets in the cyroplasm.

Our results show that AP1 treatment significantly down-regulated PPARγ, C/EBPα and SREBP1 at the protein levels, compared to those in fully differentiated adipocytes. The differentiation of preadipocytes into adipocytes is regulated by a complex network of transcription factors. And they induced prior to the transcriptional activation of most adipocyte-specific genes (Farmer *et al*., 2006). Moreover, PPARγ deficient cells fail to differentiate into adipocytes, and the overexpression of PPARγ and C/EBPα accelerates adipgenesis (Akune *et al*., 2004). Recent investigations suggested that SREBP1 is the earliest transcription factors involved in adipocyte differentiation and SREBP1 is required to induce PPARγ downstream of C/EBP family (Payne *et al*., 2009). Therefore, these results suggests that AP1 suppresses adipocyte differentiation through PPARγ, C/EBPα and SREBP1 during adipocyte differentiation.

Many studies using natural substances and herbal compounds focus on the PI3K/Akt signaling pathway in preventing obesity; hormones and growth factors that are specific to adipogenesis act via their receptors to transduce external differentiation signals through a cascade of intracellular events in the PI3K/Akt signaling pathway (Kim et al., 2001). Thus, Akt activation has been identified as a major target for the control of obesity and diabetes (Xu et al., 2004). Akt plays a critical role in the insulin signaling pathway, and the insulin-stimulated phosphorylation of Akt via PI3K is an important indicator of proper insulin function (Green *et al*., 2008). Constitutively active Akt causes the spontaneous differentiation of 3T3-L1 cells in the absence of

insulin stimulation. The Akt signal cascade is important for adipogenesis, and it activates PPARγ and C/EBPα during 3T3-L1 adipocyte differentiation. Moreover, Akt regulates adipogenesis via the phosphorylation and inactivation of substrates such as Foxo1 and GSK3β, which directly regulate PPARγ, C/EBPα (Grimes *et al.*, 2001).

The serine/threonine kinase Akt plays an essential role in adipocyte differentiation. Mouse embryonic fibroblasts (MEFs) lacking Akt failed to differentiate into mature adipocyte (Baudry *et al.*, 2006), and an RNAi-mediated decrease in Akt was found to block the differentiation of 3T3-L1 cells . Moreover, the overepression of active Akt in 3T-L1 adipocytes was found to promote glucose uptake and adipocyte differentiation (Xu *et al.*, 2004). Akt phosphorylates and regulates a large number of substrates that are involved in a diverse array of biological processes (Green *et al.*, 2008), many of which could contribute to the role of Akt in driving adipocyte differentiation. GSK3β is a critical downstream signaling protein in the PI3K/Akt pathway. Insulin signaling activates Akt through PI3K and induces the serine/threonine phosphorylation of down-stream targets, such as GSK3β (Summers *et al.*, 1999). GSK3β is a critically important protein kinase in adipocyte differentiation because it phosphorylates a number of substrates, including the transcription factor beta-catenin, CCAAT enhancer binding protein beta (C/EBPβ), and C/EBPα, which regulates their function (Grimes *et al.*, 2006). Therefore, to investigate the molecular mechanism underlying the anti-adipogenesis stimulated by AP1, we studied the effects of AP1 in the activation of PI3K/Akt related proteins. Our results demonstrate that AP1 caused a marked and dose-dependent attenuation of phospho-Akt, GSK3β, PI3K p110 isoforms induced by insulin. However, DMI induction of the 3T3-L1 cells increased PI3K/Akt signaling.

At least two downstream branches of Akt signaling have been implicated

in the regulation of PPAR-γ expression and adipocyte differentiation. The forkhead box O (FOXO) family of transcription factors are phosphorylated and inhibited by Akt. The forkhead-type transcription factor family forkhead box class O (FoxO) is also thought to play a role in adipocyte differentiation (Nakae *et al.* 2003). The known FoxO subfamily consists of Foxo1, Foxo3a, Foxo4, and Foxo6 (Reagan-Shaw *et al.*, 2007). FoxO protein have highly conserved phosphorylation site (Thr-24, ser-256, and Ser-319) and are phosphorylated by Akt and SFK through the insulin-dependent PI3K/Akt cascade (Czech *et al.*, 2003). Adipocyte differentiation was most severely inhibited by exposing cells to Foxo1-siRNA before induction of differentiation. The down regulation of Foxo1 decreased the expression of the transcription factors, PPARγ and C/EBPα. So Foxo1 plays an essential role in adipocyte differentiation (Munekata *et al.*, 2009). Our results demonstrate that AP1 inhibits the FoxOs signaling in 3T3-L1 aipocytes.

Another major signaling branch downstream of Akt results in activation of the mammaliam target of rapamycin (mTOR), a critical regulator of mRNA translation and cell growth (Wullschleger *et al.*, 2006). Primarily through use of the mTOR-specific inhibitor rapamycin, several studies have concluded that mTOR activitiy is required for proper differentiation of preadipoyte cell lines (Yeh *et al.*, 1995). The effects of rapamycin were found to correlated with a reduction in PPARγ mRNA and protein levels (Kim *et al.*, 2004). Interestingly, mouse knockouts affecting specific mTORC1 targets suggest that both S6K1 activation and 4E-BP1/2 inhibition might contribute to a pro-adipogenic role for mTORC1 activation (Le Bacquer *et al.*, 2007). Previous studies reveal that activation of mTORC1 signaling is a critical step in adipocyte differentiation and identifies TSC2 as a primary target of Akt driving this process. The TSC1-TSC2 complex regulates the differentiation of mesenchymal cell lineages, at least in part, through its

control of mTORC1 activity and PPARγ expression (Zhang *et al.*, 2009). mTOR activates p70S6 kinase (p70S6K) by either direct phosphorylation or inhibition of a phosphatase p70S6K in turn regulates the transcriptional activity of cAMP response element binding protein (CREB), which has been shown to be important for the adipocyte differentiation of 3T3-L1 (Um *et al.*, 2004). Our results revealed that mTOR, phospho-mTOR, p70S6K were inhibited by AP1, indicating that AP1 may promote inhibition of PI3K/Akt signaling that caused inactivation of FOXOs. However, the detailed mechanism remains to be elucidated.

In conclusion, in the present study we demonstrated that the AP1 significantly suppressed 3T3-L1 adipocyte differentiation and lipid accumulation. AP1 exerts an antiadipogeneic effect through down-regulation of SREBP1, PPARγ and C/EBPα, modulation of multiple signaling pathways including PI3K/Akt-dependent FoxOs signaling (Fig. 4). These results provided the molecular mechanism of AP1 on suppression of adipocyte differentiation. On the basis of these findings, we suggest AP1 have great potential as a novel agent for the treatment of obesity.

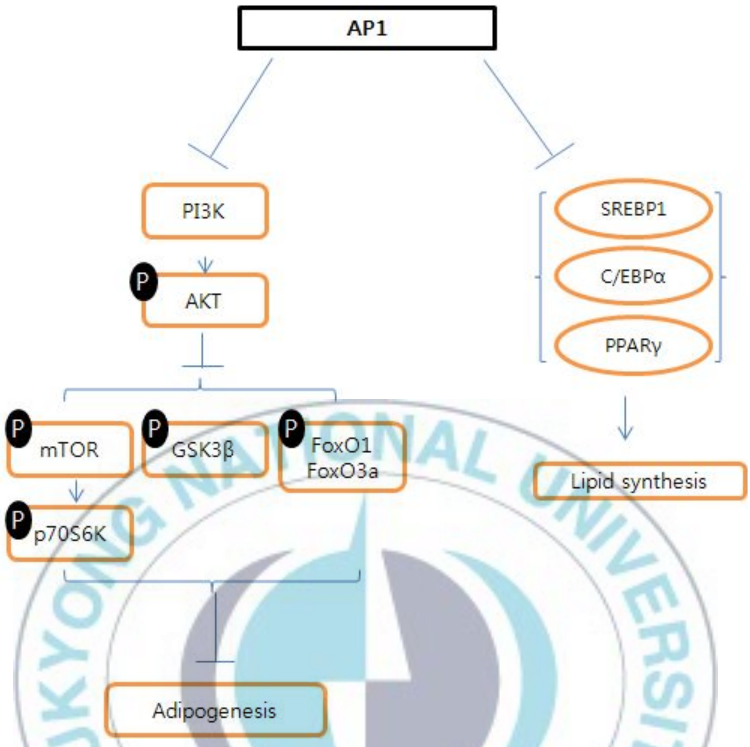


Figure 5. Possible mechanism of AP1 on the suppression of 3T3-L1 adipocytes differentiation.

국문초록

전복은 동아시아에서 주로 먹는 수산물로 특히 전복의 내장 추출물을 이용하여 항암효과가 있다고 많이 연구 되어 있다. 하지만 항 비만에 관한 연구는 미비한 실정이다. 본 연구는 전복에서 분리된 펩타이드를 이용하여 3T3-L1 전지방세포를 지방세포로의 분화를 억제하는 기작을 확인하는 실험을 진행하였다. 전복 펩타이드를 처리한 세포 내 지방 축적에 대한 효과를 확인하기 위해 3T3-L1 adipocyte를 분화 후 지방만 특이적으로 염색하는 oil red O 염색을 하였다. 물질를 첨가하지 않은 대조군의 지방구는 크고 둥근 형태인데 비해 물질을 처리한 지방구는 크기가 작아짐을 확인하였다. 지방 축적을 돕는 지방 생성 관련 단백질 발현을 western blot으로 분석한 결과 LPL(lipoproteinlipase)과 FAS (fatty acid synthase) 등의 지방산합성 관련 유전자 발현을 유도하고 지방산 합성 (FAS)과 유입(uptake)을 증가시키며 지방 조직 내로의 지질 축적을 증진시키는 것으로 보고된 SREBP1, PPARɣ, C/EBPα가 전복 펩타이드에 의해 발현이 감소하고 이는 지질 축적을 억제하는 효과가 있음을 알 수 있다. 다음은 많은 비만 연구에서 중점적으로 연구되고 있는 PI3K/Akt, FoxO signal의 활성을 Western blot으로 확인하였다. 호르몬이나 성장 인자들은 adipogenesis에서 특이적으로 반응하여 분화 signal을 통해서 PI3K Akt signal까지 작용하고 있다. 물질 농도가 증가함에 따라 PI3K, Akt의 발현이 줄어들었고 GSK -3β의 발현도 억제되었음을 확인하였다. 또 FoxO1, FoxO3a, mTOR, p70s6k 의 관련 단백질들의 발현도 억제되었다. 이로써 전복펩타이드가 3T3-L1 adipocyte의 Glucose metabolism에 관여하는 PI3K/Akt, FoxO pathway에 관여하여 지방대사를 억제함을 확인하였다. 이로써 물질이 3T3-L1adipocyte의 전 지방 세포에서 지방세포로 분화하는 과정에서 지방 조직 내로의 지질 축적과 지방 분화를 억제하여 지질대사 개선에 효과가 있다고 생각된다.

Acknowledgement

부족하고 서툰 저를 올바른 지도로 이끌어 주신 김군도 교수님 정말 감사드립니다. 그리고 바쁘신 와중에도 미흡한 논문에 아낌없는 조언과 가르침을 주신 송영환 교수님, 김영태 교수님 진심으로 감사드립니다. 언제나 학과를 걱정하고 좋은 말씀 해주시던 이명숙 교수님, 항상 살갑게 대해주시고 따뜻한 관심과 많은 가르침을 주셨던 최태진 교수님, 김경호 교수님께 감사드립니다. 또 좋은 물질로 논문 작성할 수 있게 해주신 박남규 교수님 및 연구원들에게도 감사의 마음을 전합니다.

3년 넘는 시간 동안 거의 하루의 절반을 매일 함께 보낸 실험실 선,후배들 감사합니다. 항상 챙겨주시는 성영애 선생님, 늘 웃으며 인사해주는 Kasin, 실험 잘 가르쳐 준 진수선배, 항상 공부하며 앞으로 잘 나아가고 있는 우석선배, 멀리 떨어져 있지만 같은 졸업동기 은수선배, 다방면으로 열심히 하는 스티치 사랑 덕현, 얼굴 보기 힘든 Coy, 열정적인 석사생활 보내길 바라는 민석& 종용이, 앞으로 갈 길이 바쁜 창원이, 몇 명 없는 학부생 늘 열심히 도와준 보은이, 지현이, 민재, 영익이, 함께 실험실 시작하고 함께 끝내는 초원이, 난희 너무 수고했어. 나 졸업한다고 울어준 고마운 친구들 지혜랑 쪼미! 너무너무 고마워!! 모두에게 감사의 말을 전합니다. 세포신호전달 연구실을 통해서 즐거운 인연 맺은 오목계 감사합니다. 또 학과 생활에 있어 많은 애정을 주신 선배, 후배님들에게도 감사의 말씀을 전하며 모두 좋은 결과 있으시길 원합니다.

언제 어떻게 만나도 변치 않는 우리들 수현, 정하, 지원, 혜리(절대!!생일순임) 너무 사랑하고 고마워!! 모두 성공하자!

늘 기도로 도와주는 사랑하는 청 C1셀, 신애언니, 똥박, 군대에서 고생하는 건희, 지호 감사합니다. 2013년도 찬란한 해가 되길 기도합니다.

여기까지 인도해 주시고 늘 바른 길로 인도해주셔서 하나님 감사합니다.

부족하지만 늘 물심양면으로 도와주시고 함께 해주시는 부모님, 언니 잘 데리고 놀아주는 동생 순홍, 감사 합니다 사랑합니다. 미안하고 고마운 마음 다 표현할 수는 없지만 진심으로 감사하며 이 작은 결실을 바칩니다.

I can do everything through him who gives me strength. (Philippians 4:13)

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