A Thesis for the Degree of Master of Science

Anti-adipogenic effect of erucin in early stage of adipogenesis by regulating Ras activity in 3T3-L1 preadicpocytes

Erucin 의 Ras 활성 조절을 통한 3T3-L1 에서의 지방세포 분화억제 효능

February, 2014

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Abstract

Obesity is a serious health problem since it causes various metabolic diseases such as cancer, type 2 diabetes, and cardiovascular disease. Erucin is a natural isothiocyanate in rocket salad, broccoli, and so on. Anti-cancer effect of erucin has been intensively studied so far, but anti-obesity effect of erucin has not been reported yet. In this study, I investigated anti-adipogenic effect of erucin in 3T3-L1 preadipocytes and its underlying molecular mechanism. Erucin inhibited adipogenesis of 3T3-L1 preadipocytes and reduced protein expression level of PPARγ, CEBP/α, FAS, and SREBP-1c dose-dependently. Early stage of adipogenesis including mitotic clonal expansion was shown to be crucial in the adipogenesis inhibition by Erucin. FACS analysis and trypan blue assay results showed that erucin induced G1/S phase arrest and inhibited cell proliferation at mitotic clonal expansion stage. Western blot analysis results showed that eruin reduced GTP-bound Ras protein and subsequent phosphorylation of downstream signal, Raf1, MEK, ERK, and $p90^{RSK}$. Taken together, these results indicated that erucin inhibited adipogenesis of 3T3-L1 preadipocytes by blocking Ras activity at mitotic clonal expansion.

Key Word : Erucin; Adipogenesis; Mitotic Clonal Exapnsion; Ras

Student ID : 2012-21187

Contents

Ⅰ. Introduction

Obesity rate has been increasing worldwide, , and the prevalence of obesity has become a global health issue, including in Asia (*1*). Since 1997, World Health Organization indicated that obesity is serious disease throughout the world, since its link to other various metabolic diseases, such as type 2 diabetes, coronary heart disease, and certain type of cancer, has been reported (*2*, *3*).

Obesity is caused by from energy imbalance between energy intake and energy expenditure. Since excess energy is stored at adipocytes as a form of triacylglycerol, continuous energy surplus causes hyperplasia and hypertrophy of adipocytes (*4*), which means increased adipocyte number and increased adipocyte size, respectively. Both processes have been recognized as important factors for the adipocyte expansion.

Increase in adipocyte number is achieved by adipogenesis, a differentiation process of preadipocyte into mature adipocytes. Adipogenesis has been well-characteriszed using 3T3-L1 preadiocytes. It consists of two stage, early phase differentiation involving mitotic clonal expansion (MCE) and terminal differentiation (TD) (*5*, *6*). In MCE stage, CCAAT/enhancer-binding β (C/EBPβ) protein is expressed and continuously phosphorylated by extracellular signal-regulated kinase (ERK)

and glycogen synthase kinase 3β (GSK 3β) to acquire DNA binding activity. It acts as transcription factor to induce expression of adipogenic master regulators, PPARγ and C/EBPα, and other proteins such as sterol regulatory element-binding protein-1c (SREBP-1c), fatty acid synthesis (FAS) (*7, 8*). Meanwhile, one or two round of cell cycle is progressed for cell proliferation (*9*), through extracellular-signal-regulated kinase (ERK) and Protein kinase B (Akt) signaling cascades (*10*). Studies have shown that blocking MCE resulted in the inhibition of adipogenesis, indicating the importance of MCE process in adipogenesis. When MCE was blocked, the cell number was not increased, and terminal differentiation did not occur (*11*).

Ras protein belongs to a group of protein called small GTPase. It is activated when it binds with GTP, and inactivated when the bound GTP is hydrolyzed to GDP. Many previous studies have reported that Ras is involved in transmitting cell signals through its downstream signaling molecules such as Raf/MEK/ERK/$p90^{RSK}$ (*12-15*). Also, inhibition of ERK signaling pathway has been reported to be related to inhibition of adipogenesis of 3T3-L1 preadipocytes at early phase of adipogenesis (*16, 17*). Min *et al*. reported that Akt and ERK is involved in cell cycle progression at early phase of adipogenesis and blocking of cell cycle is critical to inhibition of adipogenesis (*17*). Kortum *et al*. also reported that ERK signaling is required for adipogenesis (*18*). They found out that proper

level of kinase suppressor of Ras 1 (KSR1), which is a scaffold protein for activation of Raf/MEK/ERK kinase cascade, promotes adipogenesis at early phase of adipogenesis, suggesting activation of Ras is required for adipogenesis (*18*).

Cruciferous vegetables contain various bioactive phytochemicals, and particularly, they are rich in isothiocyanates. Isothiocyanates have been reported to have multiple health promotion effects (*19*-*22*). Erucin (structure is depicted at Figure 1), one of the isothiocyanates, were found in rocket salad and broccoli sprouts (*23*). Erucin have been well known for anti-carcinogenic effect (*23*-*26*), but there have been no study about anti-obesity or anti-adipogenic effect of erucin.

In this study, I investigated anti-adipogenic effect of erucin and its underlying molecular mechanisms in 3T3-L1 preadipocytes. Erucin was found to block adipogenesis at MCE stage. Erucin induced cell cycle arrest at G1/S phase to block cell number increase. Erucin also inhibited Ras activity and subsequent downstream signaling pathway. Collectively, these results suggest that erucin inhibit adipogenesis of 3T3-L1 preadipocytes at MCE stage by inhibiting Ras activity.

Ⅱ. MATERIALS AND METHODS

2.1. Reagents

Erucin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), Dulbecco's modified Eagle's medium (DMEM) were purchased from WELGENE, INC (Daegu, South Korea), Fetal Bovine Serum (FBS), Bovine Calf Serum (BCS) were purchased from GIBCO (Grand Island, NY), 3-isobutyl-1-methylxanthine (IBMX), insulin, dexamethasone, oil red O powder were purchased from Sigma Aldrich (St. Louis, MO). Antibodies against PPARγ, CEBP/α, FAS, SREBP-1c, p-c-Raf (ser338), p-MEK1/2, total MEK1/2, p-ERK, total ERK, p-$p90^{RSK}$, total $p90^{RSK}$, p-Akt (ser478), total Akt, p-$p70^{S6K}$, total $p70^{S6K}$, p-IRS1 (tyr895), total IRS-1 were purchased from Cell signaling Biotechnology (Beverly, MA). p27 antibody was obtained from BD bioscience (San Jose, CA). Antibodies against Raf1, Cyclin D1, Cyclin A were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against β-actin was purchased from Sigma Aldrich (St. Louis, Mo). Trypan blue solution (0.4%) was purchased from Sigma Aldrich (St. Louis, MO). Ras assay reagent (Raf-1 RBD, Agarose) was purchased from Millipore (Billerica, MA).

2.2. Cell culture and preadipocytes differentiation

3T3-L1 cell line was obtained from ATCC (Manassas, VA), cell

culture media and serum were purchased from WELGENE, INC and GIBCO respectively. 3T3-L1 preadipocytes were cultivated in DMEM supplemented with 10% BCS at 5% CO_2 and 37℃ until confluence. After confluence, media were changed to MDI media (DMEM supplemented with 10% FBS, 0.5 mM IBMX, 1 μM dexamethasone and 5 μg/ml insulin) and cells were cultured for 2 days. Then media were changed to insulin media (DMEM supplemented with 10% FBS and 5 μg/ml insulin) and cells were cultured for 2 days. After that, media were changed to FBS media (DMEM supplemented with 10% FBS) and cells were cultured for 2 days.

2.3. MTS assay

Chemical cytotoxicity is evaluated by MTS assay. 3T3-L1 preadipocytes were cultured in 48 well plate. After confluence, chemicals were treated at concentration of 5, 10, 20, 40 and 80 μM for 72 hours. Cell viability was evaluated by CellTiter 96 AQUEOUS Non-Radioactive Cell Proliferation Assay (Promega corporation, Fitchburg, WI), which is composed of 2-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazinemethosulfate (PMS). After 72 hours treatment, The cells were treated with MTS/PMS mixture (20:1) and incubated for 1 hour at 5% CO_2 and 37℃. The media were transferred into 96 well plate and then absorbance was measured with microplate reader at 490 nm.

2.4. Oil Red O staining

3T3-L1 preadipocytes were seeded in the 24 well plate. Adipogenesis was induced as described at 'cell culture and preadipocytes differentiation' section for 6 days. Then, cells were fixed with 4% formaldehyde solution for 20 minutes. Then, 500 μl of oil red O (0.2 g of oil Red O powder in 40 ml of 60 % isopropyl alcohol) solution was added each well and incubated for 15 minutes. After 15 minutes, cells were washed with PBS 2 times. Then, 500μl of isopropyl alcohol was added each well and then absorbance was measured microplate reader at 515 nm.

2.5. Trypan blue Assay

3T3-L1 preadipocytes were cultured in 12 well plate. After confluence, cells were treated with MDI media, and incubated for indicated time points. Then, cells were trypsinized and stained with 0.4 % trypan blue solution. The stained cells were placed into a hematocytometer, and the living cells (unstained cells) were counted.

2.6. Fluorescence-activated cell sorter (FACS)

3T3-L1 preadipocytes were maintained in 6 cm dishes until confluence. And then, cells were treated with MDI media for indicated time points. After that, cells were trypsinized and centrifuged at 4℃, 1000 rpm.

The pellets were washed with PBS 2 times then fixed with 70 % ethanol overnight at 4℃. Then, the cells were stained with PI propidium iodide solution (Sigma Aldrich) containing 0.2 mg/ml RNase (AMRESCO LLC, Solon, OH). Stained cells were measured using guava easyCyteTM Flow Cytometer (Millipore, Billerica, MA). Five thousand cells per each sample were analyzed.

2.7. Western blot assay

3T3-L1 preadipocytes were cultured in 6 cm dish until confluence. After confluence, each dish was treated with MDI media with or without 5, 10, 20 μM erucin. The cell were harvested with cell lysis buffer and cell lysates were centrifuged at 10 min, 14,000 rpm, 4℃. Protein concentration in supernatant was measured using DC protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA) as described at manufacture's instruction. Same amount of protein was loaded into sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. The loaded proteins were transferred to BioTrace polyvinylidenefluoride (PVDF) transfer membrane (PALL Corporation, Port Washing ton, NY). The membrane was blocked with 5% skim milk for an hour and incubated with specific primary antibody overnight at 4℃. And then, membrane was incubated with HRP conjugated secondary antibody for an hour. The protein bands were visualized with

chemiluminescence detection kit (GE healthcare Life Science, Little Chalfont, UK).

2.8. Ras activity assay

The Ras activity assay was carried out according to the manufacturer's instruction. In short, supernatant (prepared as described at 'western blot assay' section) containing 2 mg of protein was incubated with beads coated with a fusion protein consisting of GST fused to the Ras binding domain of Raf-1 at 4℃ overnight. Next day, they were centrifuged at 4℃, 12500 rpm, then supernatant was removed. After that, beads were washed with MG-containing lysis buffer (MLB) 3 times. Then the beads were mixed with 2x Laemmli sample buffer and heated for 5 minutes at 95℃. Supernatant was loaded into SDS-PAGE gel and immunoblotted with anti-pan Ras Antibody as described at 'western blot assay' section.

2.9. Statistical analaysis

Each data were expressed as means ± standard deviation (S.D.) value. Student's *t*-test was used for statistical analysis. A probability value of $p < 0.05$ and $p < 0.01$ was used as the criterion for statistical significance.

Ⅲ. Results

3.1. Erucin inhibits MDI-induced adipogenesis in 3T3-L1 preadipocytes

To investigate anti-adipogeneic effect of erucin, 3T3-L1 preadipocytes were differentiated with or without erucin, and cells were stained with oil red O solution. Oil red O staining result showed that erucin dose-dependently inhibits adipogenesis of 3T3-L1 preadipocytes (Fig. 2A, 2B). Intracellular lipid accumulation was reduced by 70% at 20 μM of erucin, compared to MDI-treated group (Fig. 2B). Erucin also reduced expression of various adipocyte marker proteins, such as PPARγ, C/EBPα, FAS, and SREBP-1c (*5*) (Fig. 2C). To confirm whether this anti-adipogenic effect of erucin is due to its cytotoxicity or not, MTS assay was carried out. MTS result showed that erucin did not have cytotoxicity up to 40 μM (Fig. 2D). All these results together suggested that eurcin has anti-adipogenic effect in 3T3-L1 preadipocytes.

3.2. Erucin inhibits the early stage of adipogenesis

Adipogenesis consists of two stage, early phase differentiation involving mitotic clonal expansion and terminal differentiation in 3T3-L1 (*5*). To find which stage is more important in anti-adipogenic activity of

erucin, oil red O staining was performed with erucin treatment at different period (Fig. 3A). Results showed that erucin effectively inihibits adipogenesis when it is treated during 0-2 days (Fig 3B, 3C). Though erucin inhibited adipogenesis of 3T3-L1 preadipocytes during 2-6 days, its activity was significantly weaker. These results showed that anti-adipogenic activity of erucin is majorly confined to early stage of adipogenesis, that is, mitotic clonal expansion.

3.3. Erucin inhibits MDI-induced cell proliferation and cell cycle progression during mitotic clonal expansion

To investigate mechanism of anti-adipogenic effect of erucin during mitotic clonal expansion, trypan blue assay and FACS analysis were performed. Trypan blue assay result showed that cell number of MDI-treated group was increased about 2 times compared to control, but co-treatment of erucin 20 μM with MDI maintained the cell number at the control level (Fig. 4A). Next, cell cycle profile was analyzed by FACS and the results showed that erucin induces cell cycle arrest from 16 hours to 20 hours (Fig. 4B, 4C). Proportion of cells in S or G2/M phase was significantly increased by MDI between 16 hours and 24 hours. However, erucin significantly decreased the cells in S or G2/M phase (Fig. 4D). Next, I examined the protein expression of p27, cyclin D1, and cyclin A1, which have been reported to be related to cell cycle progression (*27*, *28*).

Consistent with FACS analysis result, erucin restored MDI-decreased p27 protein expression and decreased MDI-induced cyclin D1 and cyclin A1 protein expression (Fig. 4E). These results showed that erucin inhibits MDI-induced cell cycle progression and consequent cell proliferation at mitotic clonal expansion stage.

3.4. Erucin regulates MDI-induced ERK signaling pathway, but not Akt signaling pathway

Previous studies have reported that insulin stimulates cell cycle progression and consequent adipogenesis in 3T3-L1 preadipocytes (*10, 29, 30*). Also, ERK and Akt signaling pathway have been reported to be important in MDI-induced cell cycle progression (*17, 31-34*). Therefore, the effect of erucin on ERK and Akt signaling pathway in 3T3-L1 preadipocytes was examined. The results showed that erucin modulates ERK signaling pathway, but not Akt signaling pathway (Fig. 5A, 5B). Erucin effectively decreased phosphorylation of Raf/MEK/ERK/$p90^{RSK}$. However, Akt/$p70^{S6K}$ phosphorylations were not affected by erucin (Fig. 5A, 5B). These results demonstrated that erucin suppressed MDI-induced ERK signaling pathway, without affecting Akt signaling pathway.

3.5. Erucin decreases Ras Activity

To investigate how erucin regulates ERK siganaling pathway, Ras activity assay was performed. Since previous studies have reported that Ras exerts their activity when it binds to GTP (*35*), amount of GTP-bound Ras was measured by immunoprecipitaion. Results showed that erucin strongly decreased GTP-bound Ras protein, without altering Ras protein expression (Fig 6A). Next, the effect of erucin on phosphorylation of IRS-1, upstream of Ras (*17*), was evaluated. Results showed that erucin decreased neither phosphorylation nor expression of IRS-1 protein (Fig. 6B). These results suggest that erucin decreases Ras activity, without affecting upstream kinase.

Figure 1

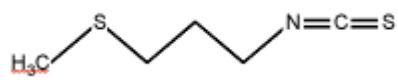


Figure 1. Structure of erucin

Structure of erucin.

Figure 2

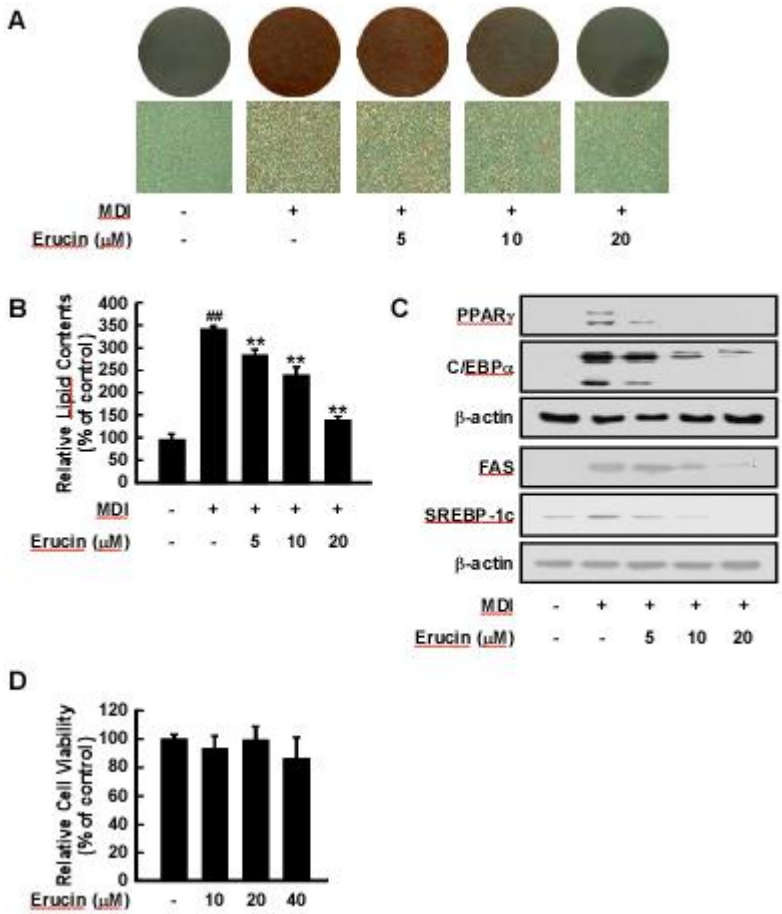

A
MDI - + + + +
Erucin (μM) - - 5 10 20
B
Relative Lipid Contents (% of control)
400
350
300
250
200
150
100
50
0
##
**
**
**
MDI - + + + +
Erucin (μM) - - 5 10 20
C
PPARγ
C/EBPα
β-actin
FAS
SREBP-1c
β-actin
MDI - + + + +
Erucin (μM) - - 5 10 20
D
Relative Cell Viability (% of control)
120
100
80
60
40
20
0
Erucin (μM) - 10 20 40

Figure 2. Effect of erucin on MDI-induced adipogenesis and cytotoxicity of erucin in 3T3-L1 preadipocytes

(A, B) Effect of erucin on MDI-induced adipogenesis of 3T3-L1 preadipocytes. (A) Adipogenesis of 3T3-L1 preadipocytes was induced with or without erucin at indicated concentrations. Intracelllular lipid accumulations were stained by oil red O solution as described at Materials and Methods section. Then they were visualized by microscope (upper) and photograph (below). (B) Adipogenesis of 3T3-L1 preadipocytes was induced with or without erucin at indicated concentrations. Intracelllular lipid accumulations were stained by oil red O solution as described at Materials and Methods section. The intracellular lipid accumulations were expressed as a percentage of control values. Data were presented as means ± S.D (n=3). ##: compared with control ($p < 0.01$). **: compared with MDI ($p < 0.01$). (C) Effect of erucin on MDI-induced adipogenic protein expression in 3T3-L1 preadipocytes. Adipogenesis of 3T3-L1 preadipocytes was induced with or without erucin at indicated concentrations. Then cells were lysed as described at Materials and Methods section. Expression of indicated proteins was determined by Western blotting as described at Materials and Methods section. β-actin was used as loading control. Data are representative of three independent experiments that yield similar result. (D) Cytotoxicity of erucin on 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were cultured until confluence. Then, cells were treated with erucin at

indicated concentirations for 72 hours. Cell viability was measured by MTS assay as described at Materials and Methods section. The cell viabilities were expressed as a percentage of control values. Data were presented as means ± S.D (n=3).

Figure 3

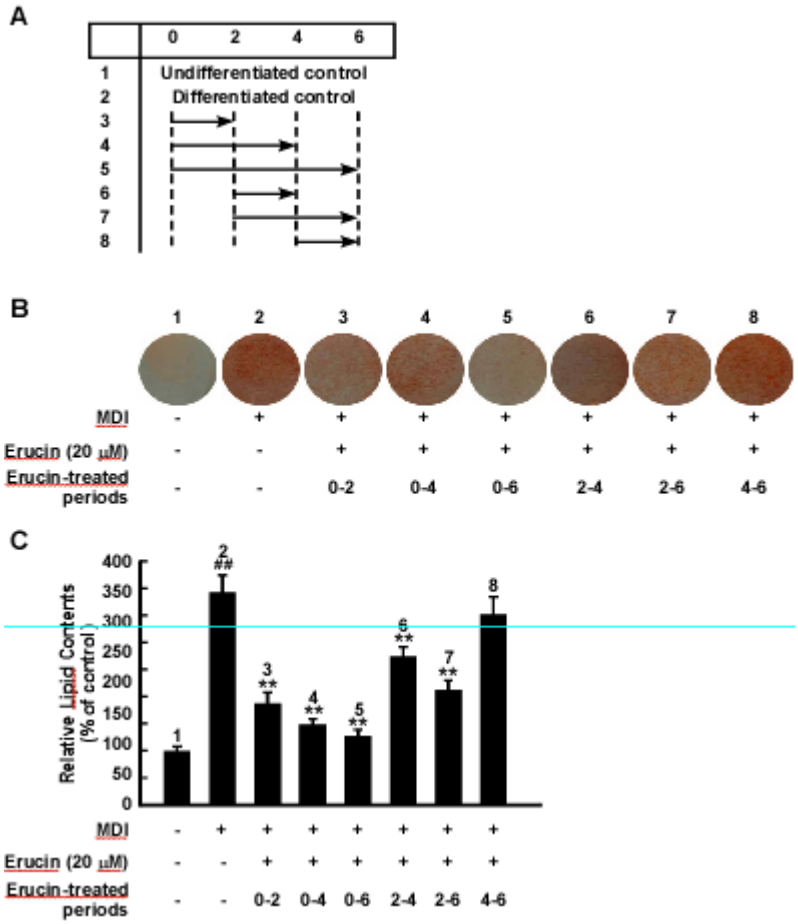

A
0
2
4
6
1
Undifferentiated control
2
Differentiated control
3
4
5
6
7
8
B
1
2
3
4
5
6
7
8
MDI
- + + + + + + +
Erucin (20 μM)
- - + + + + + +
Erucin-treated periods
- - 0-2 0-4 0-6 2-4 2-6 4-6
C
Relative Lipid Contents (% of control)
400
350
300
250
200
150
100
50
0
1
2 ##
3 **
4 **
5 **
6 **
7 **
8
MDI
- + + + + + + +
Erucin (20 μM)
- - + + + + + +
Erucin-treated periods
- - 0-2 0-4 0-6 2-4 2-6 4-6

Figure 3. Time-dependent effect of erucin on MDI-induced adipogenesis of 3T3-L1 preadipocytes

(A) Schematic experimental schedule (B, C) Time-dependent effect of erucin on MDI-induced adipogenesis of 3T3-L1 preadipocytes. (B) Adipogenesis of 3T3-L1 preadipocytes was induced with or without erucin at 20 μM at indicated time periods. Intracelllular lipid accumulations were stained by oil red O solution as described at Materials and Methods section. Then they were visualized by photograph. (C) Adipogenesis of 3T3-L1 preadipocytes was induced with or without erucin at 20 μM at indicated time periods. Intracelllular lipid accumulations were stained by oil red O solution as described at Materials and Methods section. The intracellular lipid accumulations were expressed as a percentage of control values. Data were presented as means ± S.D (n=3). ##: compared with control ($p < 0.01$). **: compared with MDI ($p < 0.01$).

Figure 4

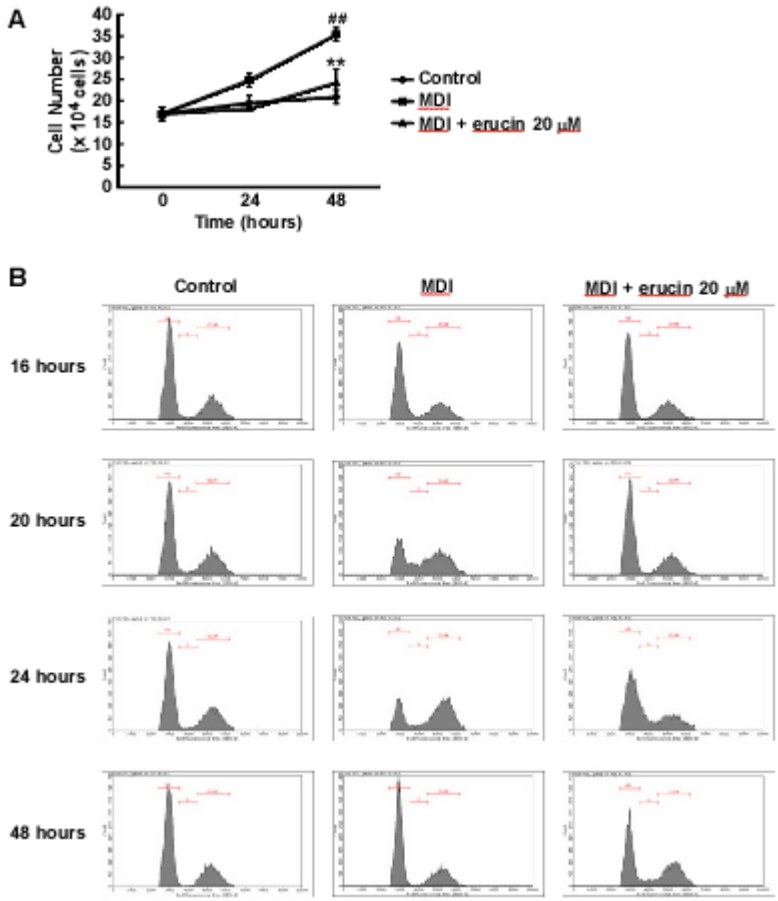
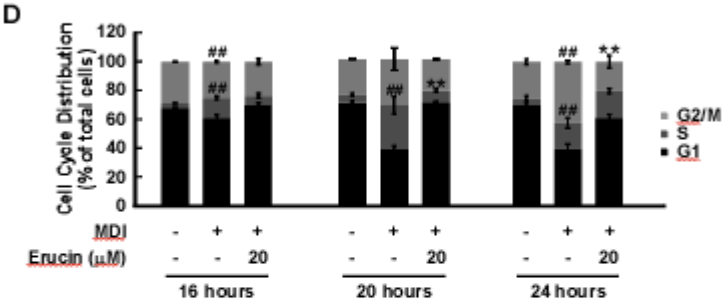

A
Cell Number (x 10⁴ cells)
40
35
30
25
20
15
10
5
0
0
24
48
Time (hours)
##
**
Control
MDI
MDI + erucin 20 μM
B
Control
MDI
MDI + erucin 20 μM
16 hours
20 hours
24 hours
48 hours

Figure 4

C

	Control			MDI			MDI + erucin 20 μM		
	G1	S	G2/M	G1	S	G2/M	G1	S	G2/M
16 hours	65.9	3.1	30.2	63.0	6.9	29.3	67.6	4.0	28.1
20 hours	62.7	4.2	32.1	36.0	16.7	46.1	67.0	3.4	29.1
24 hours	62.3	3.3	33.2	29.3	8.2	60.5	54.4	16.5	27.4
48 hours	67.3	2.0	29.9	67.6	2.5	29.9	51.5	7.5	40.0

D



E

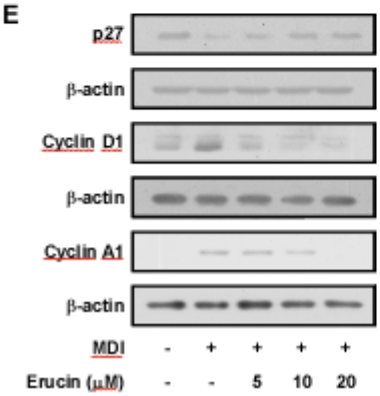


Figure 4. Effect of erucin on MDI-induced cell number-increase and cell cycle progression of 3T3-L1 preadipocytes

(A) Effect of erucin on MDI-induced cell number-increase of 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were cultured until confluence. Then, cells were treated as indicated. At indicated time points, trypan blue assay was conducted as described at Materials and Methods section. Data were presented as means ± S.D (n=3). ##: compared with control ($p < 0.01$). **: compared with MDI ($p < 0.01$). (B-D) Effect of erucin on MDI-induced cell cycle progression of 3T3-L1 preadipocytes. (B) 3T3-L1 preadipocytes were cultured until confluence. Then, cells were treated as indicated. At indicated time points, FACS analysis was conducted as described at Materials and Methods section. Data are representative of three independent experiments that yield similar result. (C) Percentage of cells at each cell cycle were presented. (D) 3T3-L1 preadipocytes were cultured until confluence. Then, cells were treated as indicated. At indicated time points, FACS analysis was conducted as described at Materials and Methods section. The percentage of cells in each cell cycle were presented as means ± S.D (n=3). ##: compared with control ($p < 0.01$). **: compared with MDI ($p < 0.01$). (E) Effect of erucin on MDI-induced cell cycle-related protein expression in 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were cultured until confluence. Then, cells were treated as indicated. Then cells were lysed as described at Materials and Methods section. Expression of indicated proteins was

determined by Western blotting as described at Materials and Methods section. β-actin was used as loading control. Data are representative of three independent experiments that yield similar result.

Figure 5

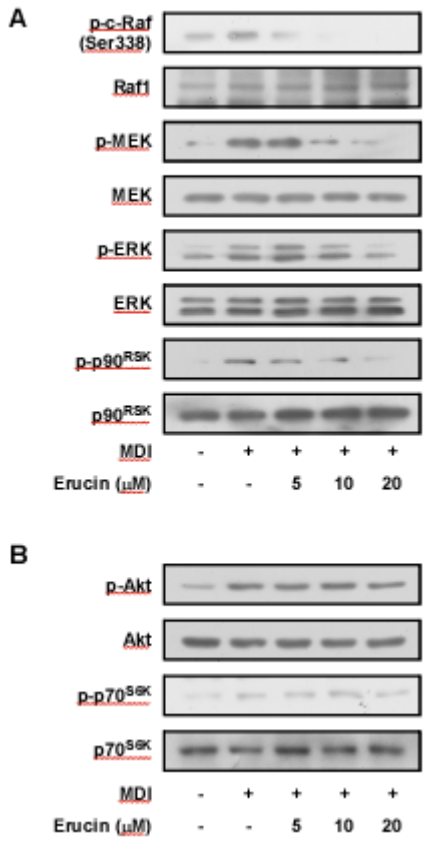

A
p-c-Raf (Ser338)
Raf1
p-MEK
MEK
p-ERK
ERK
p-p90RSK
p90RSK
MDI - + + + +
Erucin (μM) - - 5 10 20
B
p-Akt
Akt
p-p70S6K
p70S6K
MDI - + + + +
Erucin (μM) - - 5 10 20

Figure 5. Effect of erucin on MDI-induced ERK and Akt signaling pathway in 3T3-L1 preadipocytes

(A) Effect of erucin on MDI-induced ERK signaling pathway in 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were cultured until confluence. Then, cells were treated as indicated. Then cells were lysed as described at Materials and Methods section. Phosphorylation or expression of indicated proteins was determined by Western blotting as described at Materials and Methods section. Total form of each protein were used as loading control. Data are representative of three independent experiments that yield similar result. (B) Effect of erucin on MDI-induced Akt signaling pathway in 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were cultured until confluence. Then, cells were treated as indicated. Then cells were lysed as described at Materials and Methods section. Phosphorylation or expression of indicated proteins was determined by Western blotting as described at Materials and Methods section. Total form of each protein were used as loading control. Data are representative of three independent experiments that yield similar result.

Figure 6

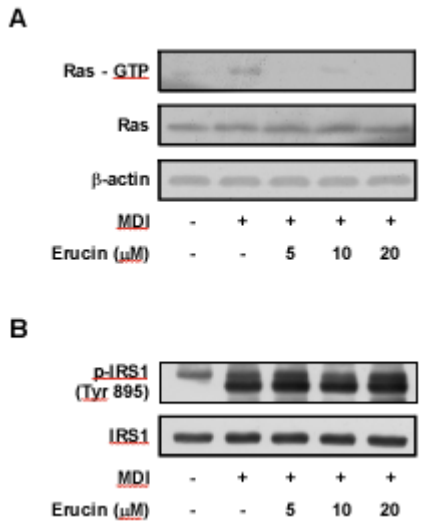

A
Ras - GTP
Ras
β-actin
MDI - + + + +
Erucin (μM) - - 5 10 20
B
p-IRS1 (Tyr 895)
IRS1
MDI - + + + +
Erucin (μM) - - 5 10 20

Figure 6. Effect of erucin on MDI-induced Ras activity and phosphorylation of IRS-1(Y895) in 3T3-L1 preadipocytes

(A) Effect of erucin on MDI-induced Ras activity in 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were cultured until confluence. Then, cells were treated as indicated. Then Ras activity assay was conducted as described at Materials and Methods section. β-actin was used as loading control. Data are representative of three independent experiments that yield similar result. (B) Effect of erucin on MDI-induced IRS-1 phosphorylation at tyrosin 895 in 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were cultured until confluence. Then, cells were treated as indicated. Then cells were lysed as described at Materials and Methods section. Phosphorylation or expression of indicated proteins was determined by Western blotting as described at Materials and Methods section. IRS1 was used as loading control. Data are representative of three independent experiments that yield similar result.

Figure 7

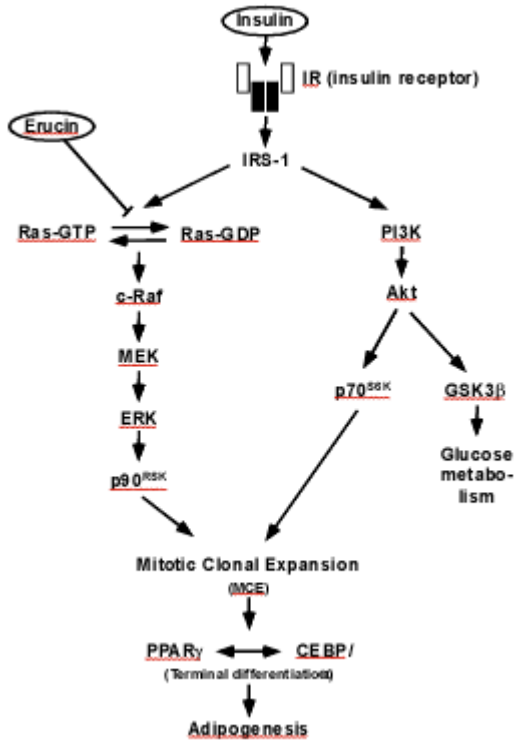

Insulin
IR (insulin receptor)
Erucin
IRS-1
Ras-GTP
Ras-GDP
PI3K
c-Raf
Akt
MEK
p70S6K
GSK3β
ERK
Glucose metabo-lism
p90RSK
Mitotic Clonal Expansion
(MCE)
PPARγ
C/EBPα
(Terminal differentiation)
Adipogenesis

Figure 7. Schematic figure on molecular mechanism of anti-adipogenic effect of erucin in 3T3-L1 preadipocytes

Schematic figure on molecular mechanism of anti-adipogenic effect of erucin in 3T3-L1 preadipocytes.

Ⅳ. Discussion

In the present study, I investigated anti-adipogenic effect of erucin and its underlying molecular mechanisms in 3T3-L1 preadipocytes. Erucin effectively inhibited adipogenesis of 3T3-L1 preadipocytes at mitotic clonal expansion stage by decreasing GTP-bound Ras protein. However, erucin did not alter phosphorylation of IRS1, upstream kinase of Ras. Taken together, these results suggest that erucin inhibits adipogenesis of 3T3-L1 preadipocytes at mitotic clonal expansion stage by targeting Ras activity.

Ras/Raf/MEK is one of the major signal transduction pathways regulating cell proliferation and differentiation (*36*). Here, I showed that erucin decreases the amount of GTP-bound Ras protein, however, the detailed molecular mechanisms how erucin decreases GTP-bound Ras remained elusive. There are several possibilities on this. The first is that erucin might inhibit binding between Ras and GTP by direct binding to Ras. If erucin bind Ras at GTP binding site, erucin might decrease GTP-bound Ras. The second is that erucin might inhibit activity of SOS (Son of Sevenless), that could activate Ras-GTPases throughout releases guanine nucleotide from Ras (*37*). Ras possesses intrinsic GTPase activity, so Ras-bound GTP readily is hydrolyzed to GDP. SOS acts by binding to Ras and releasing its bound guanine nucleotide (usually GDP) from Ras to bind other guanine nucleotide (usually GTP). So, if erucin might inhibit SOS,

Ras-bound guanine nucleotide (usually GDP) cannot be released from Ras, so GTP-bound Ras protein could be decreased.

Various previous reports have indicated that MCE stage is an essential step for adipogenesis, and some phytochemicals exert anti-adipogenic effect at MCE stage (*17*, *31*-*33*). Especially, Min *et al*. have reported that cocoa polyphenol extract (CPE) suppress adipogenesis *in vitro* and obesity *in vivo*, suggesting that inhibition of adipogenesis *in vitro* can be a good strategy of anti-obesogenic effect *in vivo* (*17*). Though those previous studies suggested the relationship of anti-adipogenic effect *in vitro* to anti-obesogenic effect *in vivo*, animal study is further being needed to ensure anti-obesity effect of erucin.

Collectively, these data suggests that erucin inhibited adipogenesis of 3T3-L1 preadipocytes at MCE stage by decreasing GTP-bound Ras protein. Though these results provide basic insight of anti-obesity effect of erucin, further studies, such as animal study and mechanism study on details how erucin inhibit GTP-bound Ras, are needed. Those further studies could lead erucin to be developed as anti-obesity agent.

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Ⅵ. 국문초록

비만은 암, 당뇨, 심혈관계 질환 등 여러 성인병의 원인이 되기 때문에 전 세계적으로 심각한 문제가 되고 있다. 이루신(erucin)은 로켓셀러드(rocket salad), 브로콜리 등에 많이 함유되어있는 천연물로, 이루신의 항암 활성에 대해서는 많은 연구가 진행되어 왔으나, 비만 예방 및 치료에 대한 연구는 제대로 진행되어 있지 않다. 본 연구에서는 3T3-L1 지방 전구 세포를 이용하여, 이루신의 지방 세포 분화 억제 효능을 확인하고 관련 메커니즘을 밝혔다. 이루신은 3T3-L1 지방 전구 세포가 지방 세포로 분화하여 지방이 축적되는 과정을 농도 의존적으로 저해 하였을 뿐 아니라, PPARγ, CEBP/α, FAS, SREBP1c 와 같은 지방 분화에 관련된 단백질의 발현 감소시켰다. 이루신은 유사 분열성 세포 증식(mitotic clonal expansion)이라 불리는 지방 세포 분화 초기 단계에서 지방 세포 분화 억제 효능을 효과적으로 나타내었다. 이루신은 유사 분열성 세포 증식 단계에서 세포 주기가 G1기(G1 phase)에서 S기(S phase)로 진행되는 것을 억제하였으며, 세포 수의 증가를 억제하였다. 또한 이루신은 GTP에 결합한 Ras의 양을 감소시키는 기작을 통해 하위 신호 전달인자인 Raf1,

MEK, ERK, 그리고 $p90^{RSK}$의 인산화를 억제하였다. 이를 통하여 이루신의 지방 세포 분화 억제 효과는 유사 분열성 세포 증식 단계에서 효과적으로 나타나며, 이루신은 Ras의 활성을 감소시켜 세포 분열을 억제함으로서, 3T3-L1 지방 전구 세포의 분화를 억제한다는 것을 밝혀 내었다.

주요어 : 이루신, 지방세포분화, 유사 분열성 세포 증식, Ras

학번 : 2012-21187