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Ramalin inhibits differentiation of 3T3-L1 preadipocytes and suppresses adiposity and body weight in a high-fat diet-fed C57BL/6J mice

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ABSTRACT

Obesity is a serious global health problem. Natural substances that could be effective remedies for treatment of obesity, and which are relatively safe, are desired. The aim of this study was to examine the anti-obesity effect and the mechanism of ramalin in 3T3-L1 preadipocytes and high fat diet (HFD)-induced obese mice. In this study, 3T3-L1 cells were treated with various concentrations of ramalin (1, 5, and 10 μg/ml). Ramalin reduced the accumulation of intracellular lipid droplets in 3T3-L1 cells. In addition, ramalin inhibited 3T3-L1 adipocyte differentiation by blocking adipogenic gene expression including CCAAT enhancer binding proteins (C/EBPs), peroxisome proliferator-activated receptors γ (PPARγ), adipocyte fatty acid-binding protein (aP2), and leptin. The suppression of adipogenesis by ramalin was mediated through the inhibition of MAPK pathways. Ramalin also reduced the secretion of TNF-α and IL-6 in 3T3-L1 adipocytes. Oral administration of ramalin (50 and 100 mg/kg) to HFD-fed mice reduced body weight gain and abdominal fat accumulation without changes in food intake. Ramalin also attenuated organ weight and basal serum level by inhibiting liver X receptors (LXRs), sterol regulatory element-binding protein-1c (SREBP-1c), and lipoprotein lipase (LPL) mRNA expression in HFD-fed mice. Taken together, these results indicate that ramalin inhibits adipogenesis in 3T3-L1 preadipocytes and prevents HFD-induced obesity. The present study also provides insight into the mechanisms underlying the anti-obesity activity of ramalin and suggests that ramalin has the potential to prevent obesity.

1. Introduction

Obesity is a major risk factor for several metabolic disorders including type 2 diabetes, hypertension, cardiovascular diseases and osteoarthritis [1]. Obesity is increasing in prevalence globally and obesity has become a significant global health threat. Cellular and animal studies on the development of obesity have revealed that dietary factors can change the number of adipocytes and adipocyte size [2]. Adipose tissue stores excess energy and is an endocrine organ [3].

In response to an adipogenic cocktail (MDI) consisting of 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, and insulin, postconfluent 3T3-L1 preadipocytes undergo a series of transcriptional activations [4]. The process begins with CCAAT/enhancer binding protein beta (C/EBPβ) expression, followed by C/EBP δ, peroxisome proliferator-activated receptor gamma (PPARγ), and C/EBPα, which play an important role in adipocyte phenotype [5,6]. The process of adipocyte differentiation is divided into the early, intermediate, and late stages [7]. The early stage of adipogenesis is mainly controlled by C/EBPδ and C/EBPβ, which are rapidly increased by inducers of cell division and hormonal stimulation [8]. Increased levels of C/EBPδ and C/EBPβ trigger changes in the expressions of PPARγ and C/EBPα, which are involved in the

Abbreviations: CCAAT/enhancer-binding protein alpha, C/EBPα; CCAAT/enhancer-binding protein beta, C/EBPβ; Peroxisome proliferator-activated receptors gamma, PPARγ; Adipocyte fatty acid-binding protein, aP2; Liver X receptor alpha, LXRα; Liver X receptor beta, LXRβ; Lipoprotein lipase, LPL; Sterol regulatory element-binding protein-1c, SREBP-1c; Mitogen-activated protein kinase, MAPK; Interleukin-6, IL-6; Tumor necrosis factor-alpha, TNF-α; Normal fat diet, NFD; High fat diet, HFD; 3-isobutyl-1-methylxanthine, IBMX.

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regulation of the late stage of adipogenesis [9]. The activation of PPARγ and C/EBPα regulates the expression of multiple adipogenic genes necessary for fat accumulation.

Intracellular mitogen-activated protein kinase (MAPK) signaling pathways play a critical role in the regulation of cell proliferation and differentiation [10]. MAPK pathways are involved in the early stage of adipocyte differentiation and regulate the expression of C/EBPα and PPARγ, contributing to the expression and phosphorylation of downstream proteins related to adipocyte differentiation in 3T3-L1 cells [8,10]. Therefore, it is plausible that MAPK pathways are a potential target for the treatment of obesity. Understanding the mechanism by which preadipocytes differentiate into adipocytes would aid in developing therapeutic strategies to prevent obesity.

Various food materials or natural compounds isolated from edible plants that show an anti-obesity effect have been screened for use as functional foods or dietary supplements. Metabolites extracted from Antarctic lichen reportedly have a variety of bioactivities that include antibiotic, anti-mycobacterial, anti-viral, anti-inflammatory, analgesic, anti-pyretic, anti-proliferative, and cytotoxic effects [11]. In addition, they have been applied in natural cosmetics and medicines, and have demonstrated fewer side effects compared to industrial products. However, there is still no adequate information pertaining to the health-promoting properties of the bioactive compounds in lichen and their pharmaceutical potential.

Ramalin, isolated from the Antarctic lichen, *Ramalina terebrata* (Ramalinaceae) has anti-oxidant and anti-inflammatory activities [12,13]. There is increasing evidence that inflammation plays a central role in the metabolic consequence of obesity [14]. Recent reports also showed that the plasma levels of inflammatory mediators, such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), are increased in the insulin resistant states of obesity and type 2 diabetes [15]. In preliminary experiments, we have shown that the exposure of 3T3-L1 preadipocytes to ramalin induces significant changes in the expression pattern of adipogenesis-related genes [16]. These findings lead us to explore the effect of ramalin on the regulation of lipid accumulation *in vitro* and *in vivo*. In the present study, we examined the effects of ramalin on adipogenesis in 3T3-L1 cells *in vitro* and whether ramalin can decrease obesity in high fat diet (HFD)-fed C57BL/6J mice *in vivo*. The results demonstrate that ramalin inhibits adipogenesis and reduces obesity in HFD-fed mice.

2. Materials and methods

2.1. Reagents

Unless otherwise indicated, all chemicals used in this study were purchased from Sigma-Aldrich Co. (St Louis, MO). Orlistat was were purchased from Cayman Chemical (Ann Arbor, MI). Bio-Rad DC protein assay was obtained from Bio-Rad (Hercules, CA). Antibodies against extracellular signal-regulated kinase (ERK), phospho-ERK, p38, phospho-p38, c-Jun N-terminal kinase (JNK), phospho-JNK, phospho-C/EBPβ, and PPARγ were obtained from Cell Signaling Technology (Beverly, MA). Antibodies against C/EBPβ, cyclin A, CDK2, p27, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence (ECL) kit was obtained from Amersham (Arlington Hights, IL). Trizol Reagent and SuperScript II cDNA synthesis kit were purchased from Invitrogen (Carlsbad, CA). Mouse TNF-α and IL-6 Enzyme-linked immunoassay (ELISA) MAX™ Standards were obtained from Biolegend, Inc. (San Diego, CA).

2.2. Synthesis of ramalin

Ramalin was synthesized as described previously and the synthesis of ramalin was already patented (Fig. 1) [17]. Briefly, 1.2-protected L-glutamic acid and 1-benzyl 2-aminophonol were the starting materials. Total synthesis of ramalin required four steps. The first step was 1′ carboxyl acid and 2′ amino site protected glutamic acid activation step for coupling reaction. Another coupling agent, phenyl hydrazine, was synthesized from 1-benzyl 2-aminophonol using $SnCl_2$ reagent. Coupling reaction with GPH-09 and GPH-04 produced GPH-11. Final deprotection reaction with palladium (on carbon) and H_2 gas produced ramalin.

2.3. Measurement of cell viability

3T3-L1 preadipocytes were seeded at a concentration of 8×10^3 cells/well in 96-well tissue culture plates and treated with the indicated concentrations of ramalin for 48 h. Cell viability was measured by a quantitative colorimetric assay with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], which indicates the mitochondrial activity of living cells. The extent of reduction of MTT to formazan within cells was quantified by measuring the optical density at 540 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

2.4. Determination of lipid accumulation by Oil Red O staining

Oil Red O staining was performed 8 days following ramalin exposure to stain the accumulated lipid droplets in the differentiated adipocytes. The cells were washed with phosphate buffered saline (PBS) and fixed with 10% formalin for 1 h at room temperature. The cells were rinsed with 60% isopropanol and stained with filtered Oil Red O solution for 20 min at room temperature. After removing the staining solution, the stained cells were washed with distilled water and dried. The stained lipid droplets were microscopically examined and photographed. To quantify intracellular lipid accumulation, the stained lipid droplets were dissolved with 100% isopropanol for 10 min. The optical density was measured at 490 nm using the aforementioned microplate reader.

2.5. Western blot analysis

After cell lysis, lysates were clarified by centrifugation at 13,000 rpm for 15 min at 4°C. Protein concentration was determined using the Bio-Rad DC protein assay with bovine serum albumin (BSA) as the standard. The whole cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The fractionated proteins were electrophoretically transferred to PVDF membranes (Amersham, Arlington Hights, IL) and probed with antibodies to extracellular signal-regulated kinase (ERK), phospho-ERK, p38, phospho-p38, c-Jun N-terminal kinase (JNK), phospho-JNK, phospho-C/EBPβ, PPARγ, C/EBPβ, cyclin A, CDK2, p27, and β-actin. The blots were developed using an enhanced chemiluminescence (ECL) kit. In all immunoblotting experiments, the blots were reprobed with an anti-β-actin antibody as a control for protein loading.

2.6. Cell cycle progression analysis

Cell cycle progression was measured by flow cytometric analysis after propidium iodide (PI) staining. Post-confluence preadipocytes were treated with 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 10 μg/ml insulin in the presence or absence of 1, 5, and 10 μg/ml ramalin and 100 μg/ml orlistat for 24 h. A suspension of the cells was fixed overnight with 70% ethanol at 4°C, and then incubated with 10 μg/ml of RNase A and 50 μg/ml of propidium iodide staining buffer for 30 min at room temperature in the dark. Then, 10,000 cells per experiment were analyzed using a

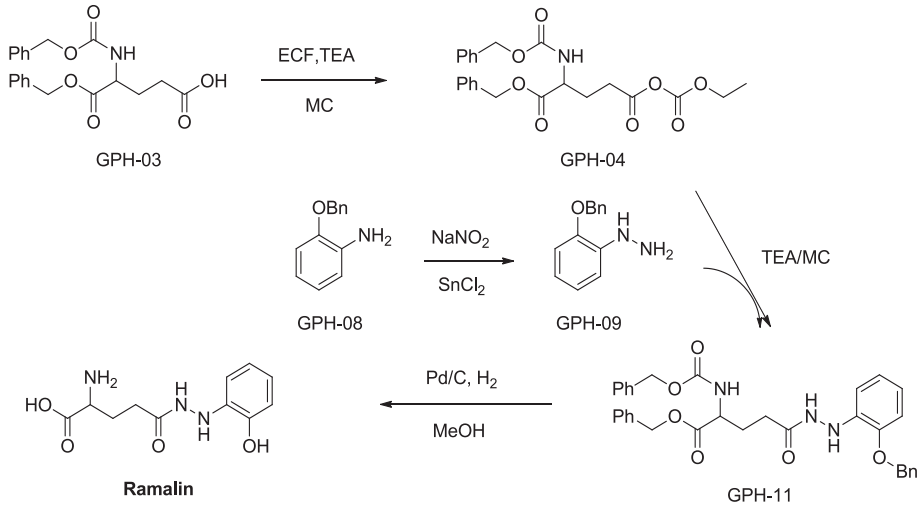

Fig. 1. Schematic representation of the synthesis route of ramalin. (U.S. Patent 8,865,934).

FACS Canto™ II apparatus (BD Biosciences, San Jose, CA) and cell cycle progression was measured with FCS Express 4 Flow Cytometry software (De Novo Software, Los Angeles, CA).

2.7. Isolation of total RNA and quantitative real-time PCR

Following the manufacturer's protocol, total RNA was extracted from liver tissue isolated from HFD-fed mice orally administered ramalin, and from 3T3-L1 cells using Trizol Reagent. Isolated RNA (1 μg/μl) was reverse transcribed using a SuperScript II kit for cDNA synthesis. The cDNA was subjected to quantitative real-time (qRT)-PCR using thermocyclers from Applied Biosystems (Franklin Lakes, NJ). The sequences of the primers corresponding to the mouse adipogenic genes analyzed in this study are shown in Table 1.

2.8. Animals and diets

Male, 4-week-old C57BL/6J mice were purchased from SLC Inc. (Hamamatsu, Shizuoka). Upon arrival, mice were fed a normal-fat diet (NFD) for 1 week. The animals were housed in individual cages in a temperature-controlled room with a 12 h light/dark cycle. Mice were divided into five groups (n = 8 in all groups) at 5 weeks of age. Mice in a group were fed the NFD and orally administered 200μl PBS every 3 days. The other four groups were fed the HFD containing 60% fat (Research Diets Inc., New Brunswick, NJ). One group was orally administered 200μl PBS every 3 days as a negative control. Another group of mice was orally administered 200μl orlistat (50 mg/kg) every 3 days as a positive control. The remaining two groups were orally administered 200μl containing 50 mg/kg or 100 mg/kg ramalin every 3 days. Food intake and body weight were monitored every 3 days for 8 weeks. Blood was drawn from the abdominal aorta into an ethylenediamine tetra-acetic acid (EDTA)-coated tube, and the serum was obtained by centrifuging the blood at 2000 rpm for 15 min at 4°C. Livers, kidneys, testis, and abdominal fat-pads were removed, rinsed with PBS, and weighed. The serum, livers, kidneys, testis, and abdominal fat-pad samples were stored at −70°C until they were analyzed. This study was approved by the animal care and use committee of Sungkyunkwan University.

2.9. Statistical analysis

Each result is reported as mean ± S.E.M. One-way analysis of variance (ANOVA) was used to determine significance among the groups, after which the modified *t*−test and two-way ANOVA were used for comparison between individual groups. Significant values ($p < 0.05$) are represented by an asterisk.

3. Results

3.1. Effect of ramalin on lipid accumulation and expression of adipocyte differentiation-related genes in MDI-stimulated 3T3-L1 preadipocytes

We investigated the effect of ramalin on cytotoxicity in 3T3-L1 preadipocytes using the MTT assay at the indicated concentration of ramalin for 48 h. Ramalin was cytotoxic at concentrations exceeding 50 μg/ml (Fig. S1). In subsequent experiments, cells were treated with 1, 5, and 10 μg/ml ramalin. Next, we examined the effect of ramalin on intracellular lipid accumulation during adipocyte differentiation using Oil red O staining. As shown in Fig. 2A, treatment of confluent 3T3-L1 with MDI resulted in a 3.3-fold

Table 1
Quantitative real-time-PCR primer sequences.

Gene	Forward primer (5′→3′)	Reversed primer (5′→3′)
PPARγ	CGC TGA TGC ACT GCC TAT GA	AGA GGT CCA CAG AGC TGA TTC C
C/EBPα	GCG AGC ACG AGA CGT CTA TAG A	GCC AGG AAC TCG TCG TTG AA
aP2	CCG CAG ACG ACA GGA AGG T	AGG GCC CCG CCA TCT
Leptin	TCG GTA TCC GCC AAG CA	GGT GAA GCC CAG CAA TGA AG
LXRα	GAG TGT CGA CTT CGC AAA TGC	CCT CTT CTT GCC GCT TCA GT
LXRβ	CAG GCT TGC AGG TGG AAT TC	ATG GCG ATAAGC AAG GCA TACT
LPL	ATC GGA GAA CTG CTC ATG ATGA	CGG ATC CTC TCG ATG ACG AA
SREBP-1c	GGC TAT TCC GTG AAC ATC TCC TA	ATC CAA GGG CAT CTG AGA ACT C
GAPDH	TGC ATC CTG CAC CAC CAA	TCC ACG ATG CCA AAG TTG TC

increase in lipid accumulation compared to untreated cells. In contrast, treatment of the cells with ramalin (1–10 μg/ml) for 8 days remarkably decreased intracellular lipid accumulation in a concentration-dependent manner. Orlistat (100 μg/ml), which is a prescription medicine for obesity, inhibited lipid accumulation. These results correlated exactly with the absorbance values of eluted Oil Red O staining solution in differentiation-induced adipocytes. PPARγ and C/EBPα are the two critical transcription factors, regulating the terminal process of adipocyte differentiation. To investigate whether ramalin inhibits the expression of PPARγ and C/EBPα in MDI-induced 3T3-L1 cells, we treated 3T3-L1 cells with 1, 5, and 10 μg/ml of ramalin and the positive control orlistat. Protein and mRNA expression of PPARγ and C/EBPα were significantly inhibited by ramalin in a concentration-dependent manner (Fig. 2B and C). In addition, quantitative real-time PCR analysis showed that the level of aP2 and leptin mRNA expression was significantly reduced by ramalin during adipocyte differentiation in a concentration-dependent manner, compared to the MDI-induced control (Fig. 2D). These results indicated that ramalin inhibits adipocyte differentiation in 3T3-L1 preadipocytes through the down-regulation of PPARγ, C/EBPα, aP2, and leptin.

3.2. Ramalin suppresses the early stage of adipogenesis

Adipocyte differentiation begins at the preadipocyte stage and progresses through early, intermediate, and late stages [7]. We investigated whether ramalin affects stage-dependent inhibition during adipocyte differentiation. The lipid accumulation in adipocytes was completely inhibited when cells were treated with ramalin (10 μg/ml) during the early stage (Fig. 3A). The decreased formation of lipid droplets in the early stage (0–2 days) after ramalin treatment was similar to that seen during the entire period of adipocyte differentiation (0–6 days). Relative lipid contents in adipocytes during the entire differentiation period (0–6 days) or the early (0–2 days) stages were decreased by ~45.6% and 43.4%, respectively. These results indicated that the effect of ramalin on the inhibition of adipocyte differentiation occurs during the early stage. C/EBPβ expression is crucial for the induction of mitotic clonal expansion (MCE) [18]. In addition, the phosphorylation of C/EBPβ at Thr^{188} by MAPK is necessary for the induction of genes involved in adipogenesis [8]. Therefore, we examined the effects of ramalin on C/EBPβ expression and the phosphorylation of C/EBPβ. Ramalin did not affect the protein expression of C/EBPβ, whereas phosphorylation of C/EBPβ at Thr^{188} was significantly attenuated by ramalin in a concentration-dependent manner (Fig. 3B). When growth-arrested preadipocytes were treated with adipogenic inducer, the number of adipocytes increased 4–5-fold during the early stage [19]. DNA replication and the changes in chromatin structure occur during the mitotic clonal expansion of adipogensis [20]. We investigated whether ramalin affects the inhibition of cell cycle progression in MCE. Flow cytometric assay revealed that ramalin inhibited adipocyte proliferation during the early stage of adipocyte differentiation; the percentage of G_0/G_1 arrested cells was increased in ramalin-treated adipocytes compared to MDI-treated cells (Fig. 3C). However, orlistat did not affect the process of MCE. In addition, the expression of the two cell cycle progression proteins, Cyclin A and CDK2, were inhibited by ramalin at the highest concentration, whereas the expression of the CDK inhibitor, p27, was significantly recovered (Fig. 3D). The results suggested that ramalin treatment could suppress the cells from re-entering the cell cycle by increasing p27 expression, which inhibited MCE, during the early stage of adipocyte differentiation.

3.3. Ramalin inhibits the phosphorylation of MAPK in MDI-induced 3T3-L1 cells

The observations that ramalin affected the early stage of adipocyte differentiation, MCE, and C/EBPβ phosphorylation

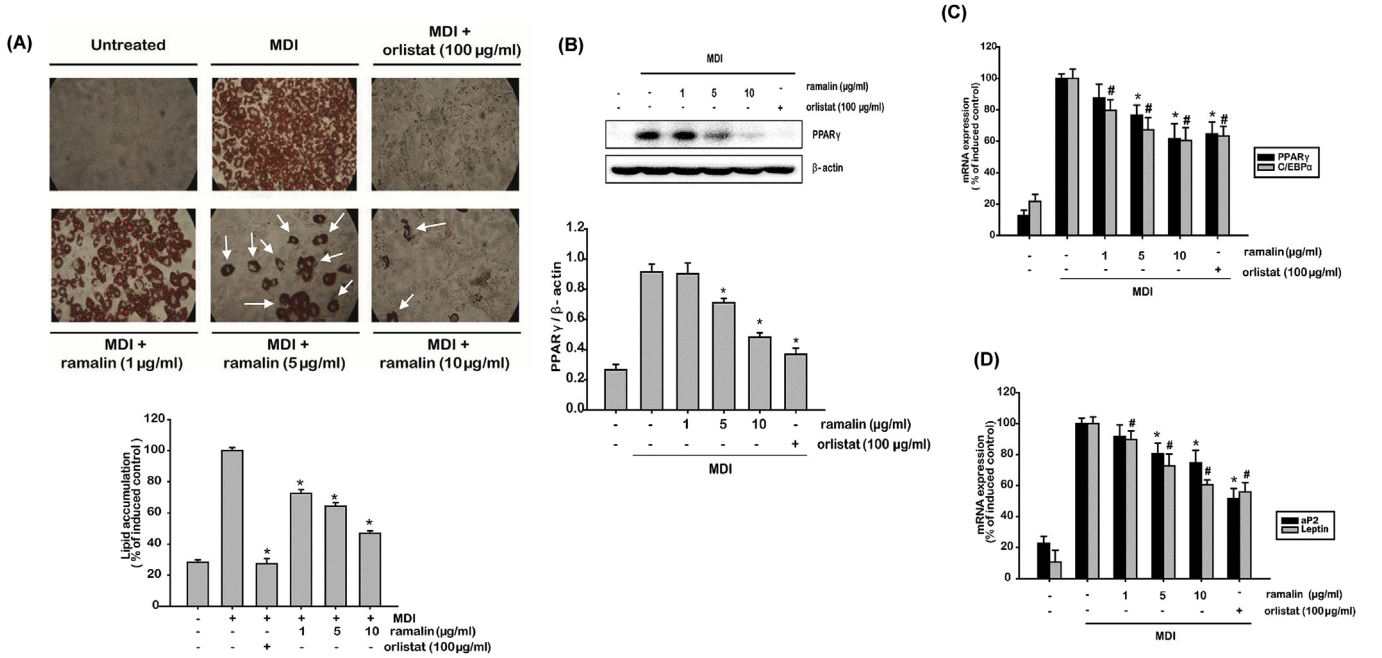

Fig. 2. Effect of ramalin on lipid accumulation and expression of adipocyte differentiation-related genes in MDI-induced 3T3-L1 preadipocytes. (A) Differentiated 3T3-L1 cells with visible lipid accumulations were observed by Oil red O staining at 8 days. Lipid accumulation was quantified by a microreader at 490 nm. (B) Expression of PPARγ in MDI-induced 3T3-L1 cells with the indicated concentration of ramalin for 6 days. Densitometry analysis showed as relative ration of β-actin considered an internal control. (C, D) mRNA expression of PPARγ, C/EBPα, aP2, and leptin in MDI-induced 3T3-L1 with the indicated concentration of ramalin for 6 days. Data are means ± S.E.M of three independent experiments. *, #$P < 0.05$, significantly different from MDI-treated cells.

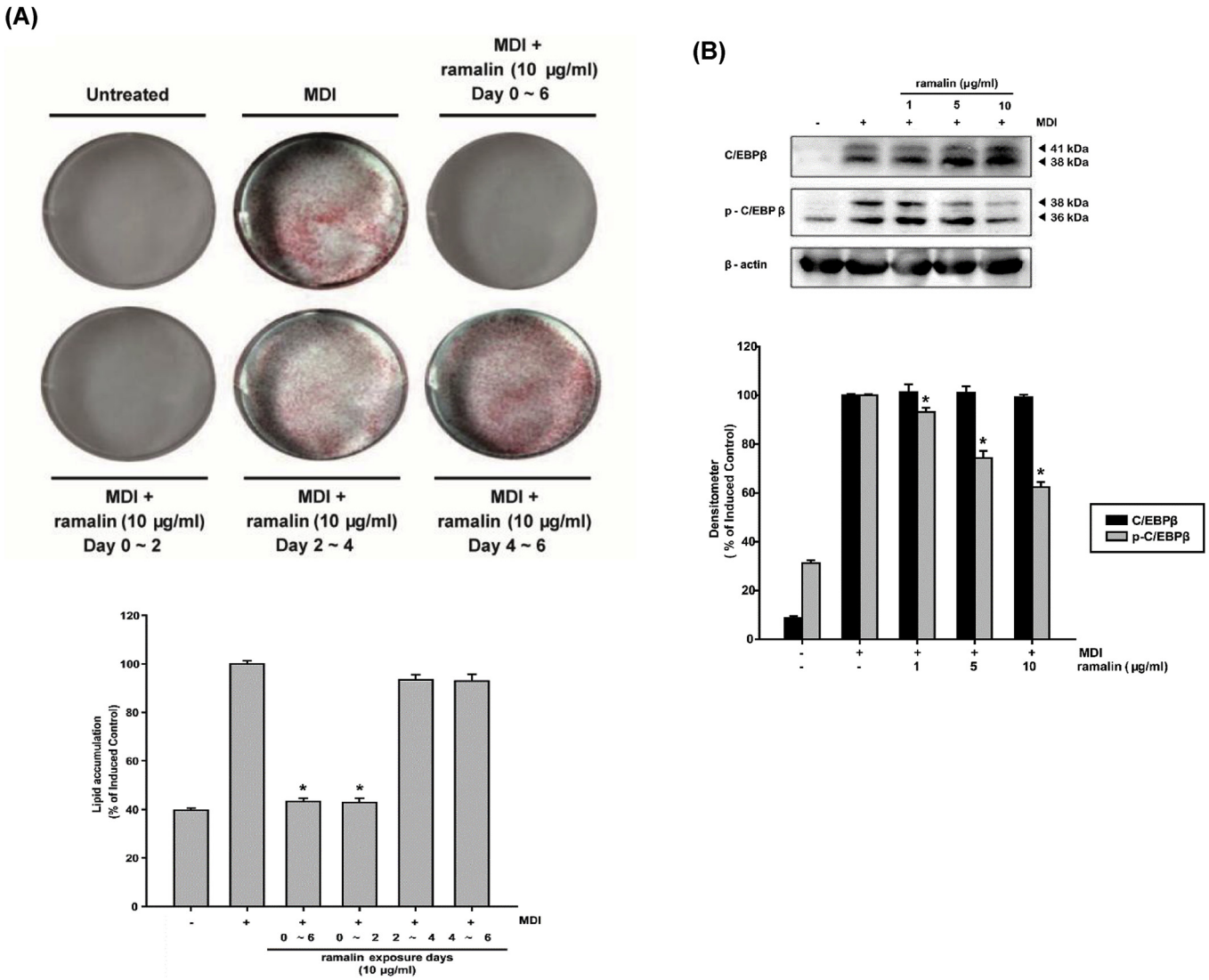

Fig. 3. Effect of ramalin on the phosphorylation of C/EBPβ and cell proliferation during mitotic clonal expansion. (A) Lipid accumulation in 3T3-L1 cells after co-incubation with ramalin (10 μg/ml) and MDI for 6 days was visualized by Oil red O staining. Lipid accumulation was quantified by a microreader at 490 nm. (B) Post-confluent 3T3-L1 cells were treated with MDI for 6 h with co-treatment of ramalin at 1, 5, and 10 μg/ml. Expression of C/EBPβ and phospho-C/EBPβ was analyzed by Western blot analysis and quantified by scanning densitometry. (C) Cell cycle progression was subjected to the differentiation medium containing 1, 5, and 10 μg/ml of ramalin for 24 h by flow cytometry. (D) Expression of cyclin A, CDK2, and p27 in MDI-induced 3T3-L1 with ramalin (10 μg/ml) for 24 h was subjected to Western blot assay. The β-actin protein level was considered an internal control. Data are means ± S.E.M of three independent experiments. *$P < 0.05$, significantly different from MDI-treated cells.

prompted us to examine whether ramalin inhibited the early stage of adipogenesis by regulating MAPK pathways. Cells were treated with MDI for various times. ERK, p38, and JNK were rapidly and transiently phosphorylated within 1 or 2 h by MDI treatment (Fig. 4A). However, the activation of MAPK was decreased gradually within 8 h. When treated with ramalin for 2 h after the induction with MDI, the phosphorylation of MAPK was significantly reduced in a concentration-dependent manner (Fig. 4B). These results suggested that ramalin suppresses adipocyte differentiation via the inhibition of MCE and C/EBPβ phosphorylation through the regulation of MAPK pathways.

3.4. Ramalin inhibits the production of proinflammatory cytokines in MDI-induced 3T3-L1 adipocytes

It is widely accepted that the levels of TNF-α and IL-6 are elevated in obesity, leading to regulate glucose homeostasis and lipid metabolism during adipogenesis [15]. We examined whether ramalin inhibits the secretion of proinflammatory cytokines using ELISA. As shown in Fig. 5, ramalin reduced the production of TNF-α and IL-6 in a concentration-dependent manner, suggesting the potential efficacy of ramalin on glucose homeostasis and lipid metabolism.

3.5. Effect of ramalin on body weight, fat weight, serum levels, and lipid metabolism-related genes in HFD fed C57BL/6J mice

We investigated the inhibitory effects of ramalin *in vivo* using HFD fed C57BL/6J mice. Body weight and food intake were monitored every 3 days. HFD-induced weight was significantly decreased with oral ramalin administration starting from 9 days (Fig. 6A), with no association with the amount of food intake (Fig. 6B). Abdominal fat weight was also remarkably inhibited by orlistat and ramalin at 50 or 100 mg/kg (Fig. 6C). An increase in abdominal fat weight was reduced by 31% in mice receiving 100 mg/kg ramalin. Changes in organ weight and serum

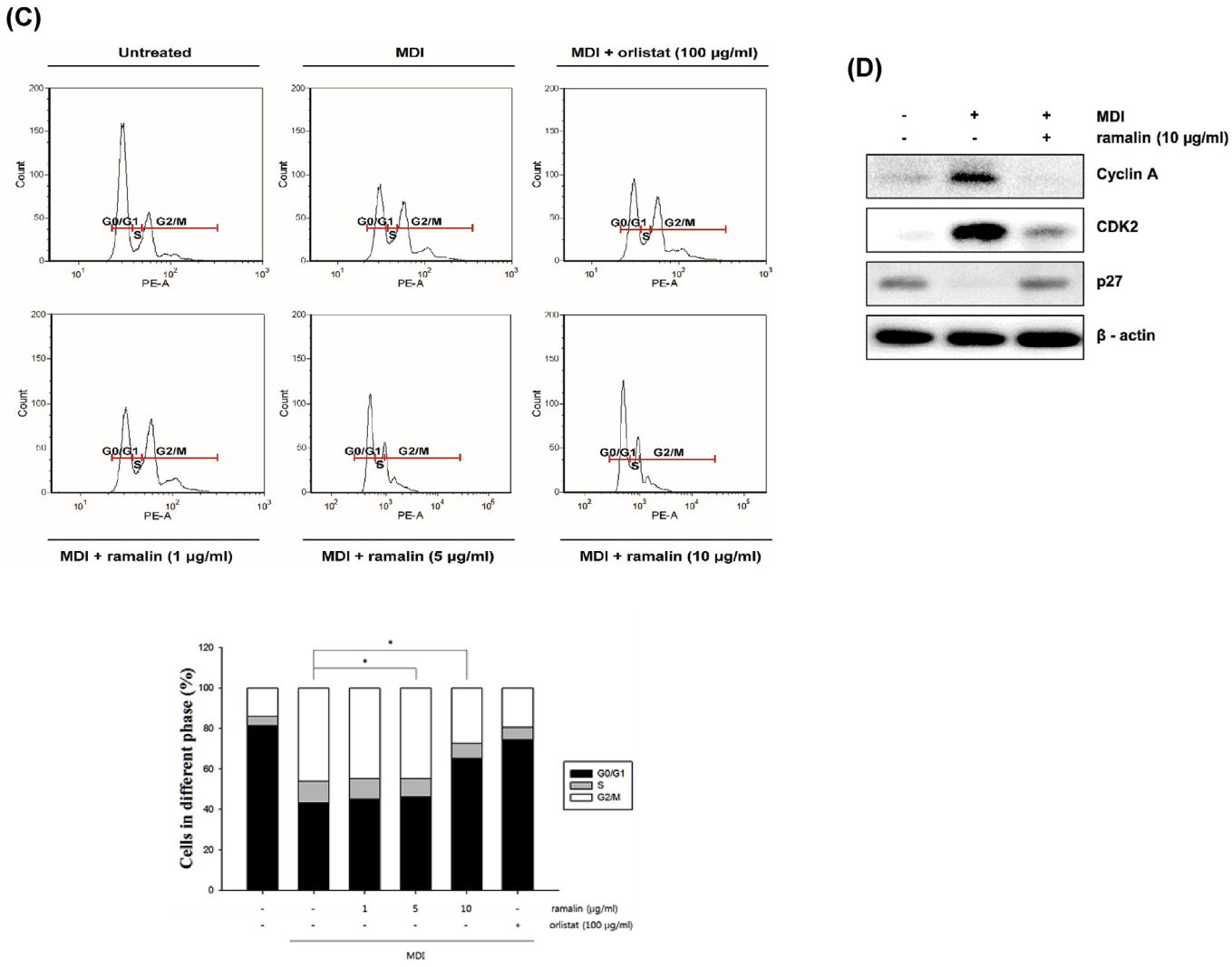

Fig. 3. (*continued*).

parameters among the five groups are shown in Tables 2 and 3. Significant differences in liver, kidney, and testis weights but not heart weights in orlistat or ramalin oral-administered groups compared with the HFD group (Table 2). Oral administration of ramalin at 50 or 100 mg/kg significantly suppressed the concentration of glucose compared to the HFD group. In addition, the levels of triglyceride (TG), total cholesterol (TC), and low density lipoprotein (LDL)-cholesterol were significantly increased and high density lipoprotein (HDL)-cholesterol level was decreased in the HFD group compared with the NFD group. These changes associated with HFD were suppressed by ramalin and the results were similar to the effects of orlistat (Table 3). Taken together, these findings suggest that ramalin inhibits liver, kidney, and testis weight gain by suppressing the levels of glucose, TG, TC, and LDL-cholesterol, but improves the level of HDL-cholesterol.

We next examined the effect of ramalin on the mRNA level of Liver X receptors (LXRs) and LXR-related gene expression in liver tissues isolated from the HFD fed and ramalin-administered mice groups. LXRs play a crucial role in the regulation of metabolism of cholesterol, fatty acid, and glucose by activating the expression of target genes including lipoprotein lipase (LPL) which are regulated by sterol regulatory element-binding protein-1c (SREBP-1c) [21]. The levels of LXRα, LXRβ, LPL, and SREBP-1c were remarkably suppressed in the ramalin and orlistat-administered mice (Fig. 6D and E), indicating that oral administration with ramalin was effective in preventing HFD-induced body weight gain and adiposity through the regulation of cholesterol, fatty acid, and glucose homeostasis.

4. Discussion

In this study, we evaluated the *in vitro* and *in vivo* anti-obesity properties of ramalin in 3T3-L1 adipocytes and HFD-fed mice. The results indicate that inhibition of lipid accumulation by ramalin could be useful in the treatment of obesity. *In vitro*, ramalin reduced lipid accumulation in a concentration-dependent manner in MDI-stimulated 3T3-L1 cells during the early stage of adipocyte differentiation by regulating the phosphorylation of C/EBPβ and expression of CDK2, cyclin A, and p27. *In vivo*, ramalin attenuated obesity by inhibiting body, abdominal fat, and organ weights, obesity-associated serum markers, and lipid synthesis-related factors in HFD-fed C57BL/6J mice.

Adipocyte differentiation is separated into early, intermediate, and terminal stages. Each stage of adipogenesis in 3T3-L1 cell line model is accompanied by a complex network of key transcription factors that include PPARγ and members of the C/EBP family [9].

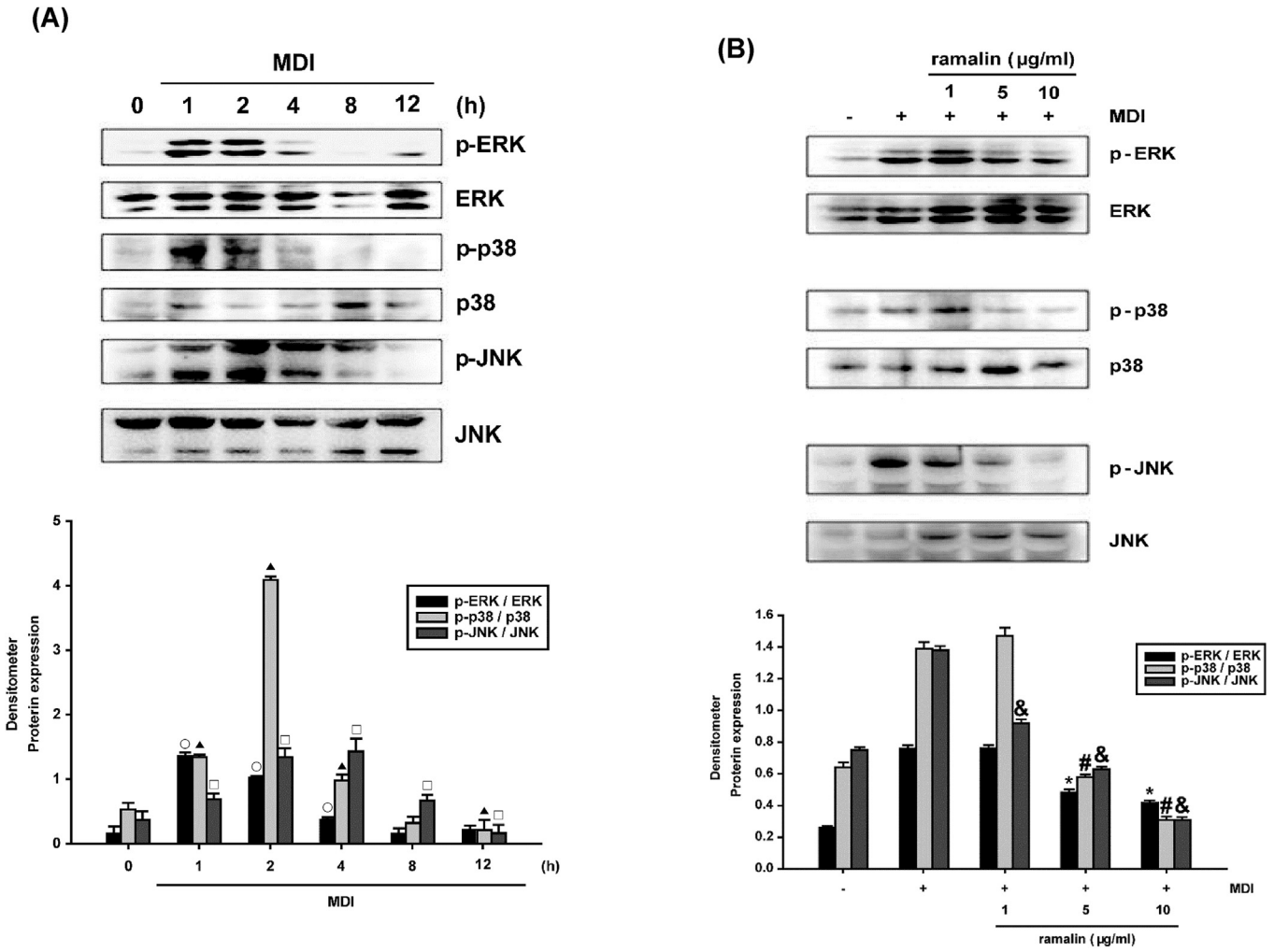

Fig. 4. Effect of ramalin on the phosphorylation of MAPKs in MDI-induced 3T3-L1 preadipocytes. (A) Post-confluent 3T3-L1 cells were treated with MDI for the indicated times (0–12 h). (B) Post-confluent 3T3-L1 cells were subjected to the differentiation medium containing 1, 5, and 10 μg/ml of ramalin for 2 h. The whole cell lysates were analyzed by western blot analysis using phospho-ERK, ERK, phospho-p38, p38, phospho-JNK, and JNK specific antibodies. Data are mean ± S.E.M of three independent experiments. ○, ▲, □ $P < 0.05$, significantly different from untreated cells. *, #, & $P < 0.05$, significantly different from MDI-treated cells.

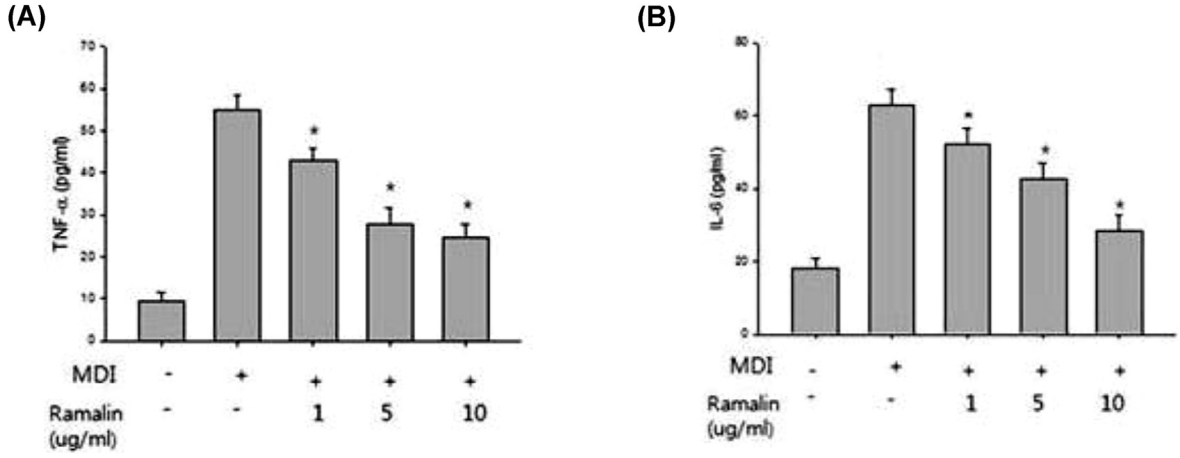

Fig. 5. Effect of ramalin on the secretion of proinflammatory cytokines in MDI-induced 3T3-L1 adipocytes. 3T3–L1 preadipocytes were cultured in differentiation medium containing 1, 5, and 10 μg/ml of ramalin for 7 days. The supernatants of the cells were then measured for TNF-α (A) and IL-6 (B) by ELISA. Data are means ± S.E.M of three independent experiments. *$p < 0.05$, significantly different from MDI-treated cells.

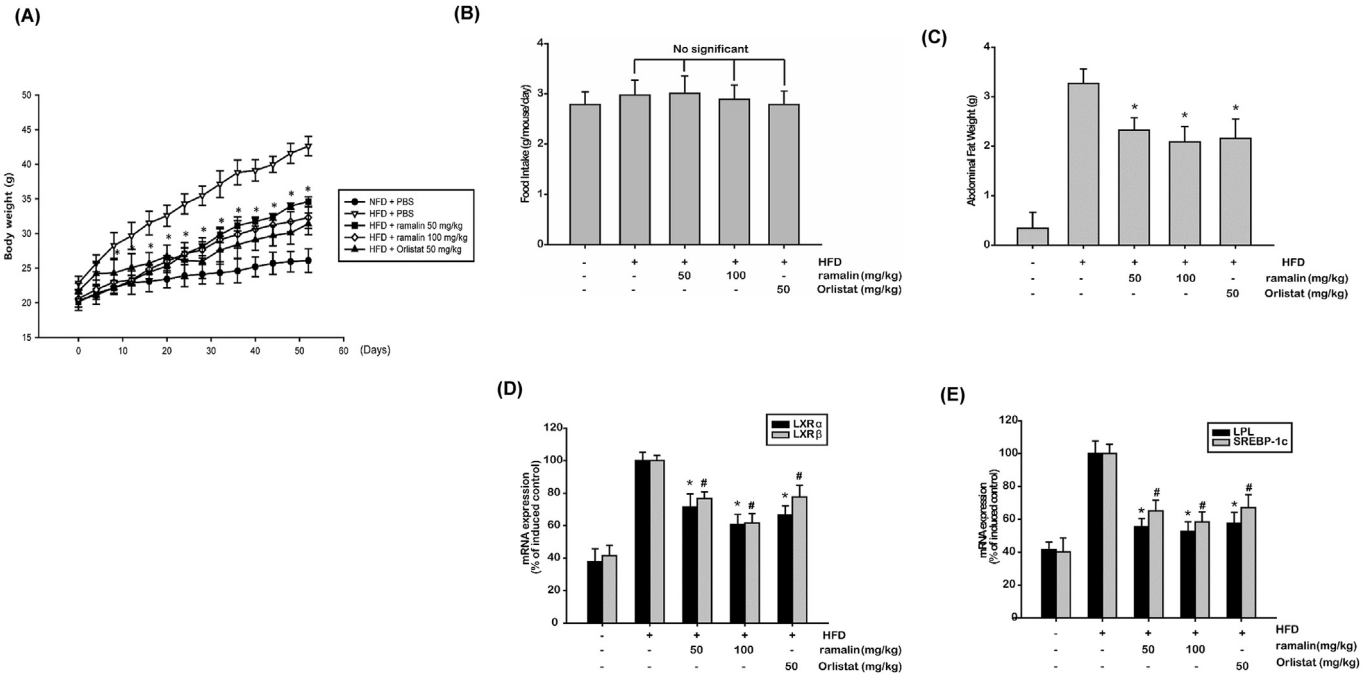

Fig. 6. Effect of ramalin on increased body weight, fat weight, and lipid metabolism-related genes in HFD fed C57BL/6J mice. (A) The body weight of the mice with oral administration of ramalin and orlistat was measured every 3 days. (B) Food intake measurements. No significant difference in amount of food intake was noted between the four groups. (C) Abdominal fat pads which were isolated at the conclusion of the study were weighed. Values are given as means ± S.E.M. n = 8 for all groups. (D, E) mRNA expression of LXRα, LXRβ, LPL, and SREBP-1c in HFD-fed mice with oral administration of ramalin and orlistat. Data are the means ± S.E.M from three independent experiments. *, #$P < 0.05$, significantly different from HFD-fed mice group. N.S, no statistical significance.

Table 2
Selected organ weights in C57BL/6J mice fed a HFD and oral-administered with ramalin or orlistat for 8 weeks.

Weight (g)	Dietary groups				
	NFD + PBS	HFD + PBS	HFD + ramalin 50 mg/kg	HFD + ramalin 100 mg/kg	HFD + orlistat 50 mg/kg
Liver	1.07 ± 0.13	2.12 ± 0.21	1.22 ± 0.1*	1.15 ± 0.03*	1.11 ± 0.03*
Kidney	0.28 ± 0.44	0.75 ± 0.04	0.34 ± 0.01*	0.32 ± 0.01*	0.45 ± 0.02*
Heart	0.12 ± 0.02	0.16 ± 0.02	0.15 ± 0.01	0.15 ± 0.01	0.16 ± 0.02
Testis	0.17 ± 0.04	0.22 ± 0.01	0.18 ± 0.03*	0.17 ± 0.01*	0.17 ± 0.01*

All values are given as means ± S.E.M. n = 8 for all groups. One-way analysis of variance (ANOVA) was used to analyze among the groups. *P < 0.05, significantly different from HFD-fed mice group.

Table 3
Plasma glucose, TG, total cholesterol, HDL and LDL-cholesterol levels in C57BL/6J mice fed a HFD and oral-administrated with ramalin or orlistat for 8 weeks.

Measurements (mg/dL)	Dietary groups				
	NFD + PBS	HFD + PBS	HFD + ramalin 50 mg/kg	HFD + ramalin 100 mg/kg	HFD + orlistat50 mg/kg
Glucose	219.4 ± 16.4	391.0 ± 10.2	295.0 ± 4.3*	232.9 ± 10.3*	240.8 ± 5.6*
Triglyceride	84.2 ± 5.7	289.0 ± 7.6	165.3 ± 11.9*	136.0 ± 4.7*	139.3 ± 5.2*
Total cholesterol	75.2 ± 8.9	191.0 ± 9.7	144.0 ± 8.1*	125.3 ± 3.4*	123.9 ± 4.8*
HDL-cholesterol	17.2 ± 4.1	122.8 ± 7.1	132.5 ± 1.2*	147.5 ± 2.9*	128.6 ± 7.3*
LDL-cholesterol	14.6 ± 2.3	42.6 ± 5.5	30.8 ± 4.4*	27.4 ± 1.3*	32.7 ± 6.9*

All values are given as means ± S.E.M. n = 8 for all groups. One-way analysis of variance (ANOVA) was used to analyze among the groups. *P < 0.05, significantly different from HFD-fed mice group.

Particularly, the role of C/EBPβ is important during the early stage of adipocyte differentiation. C/EBPβ is sequentially phosphorylated by MAPK and GSK-3β binds to the C/EBP regulatory element. After that, C/EBPβ and C/EBPδ, the major transcription factors, activate the expression of C/EBPα and PPARγ induced during intermediate stage [18]. In our study, ramalin displayed an inhibitory effect on the phosphorylation of C/EBPβ, whereas C/EBPβ expression in 3T3-L1 cells treated with ramalin was not different from the control cells. These results suggest that ramalin inhibits lipid accumulation by inhibiting the phosphorylation of C/EBPβ during the early stage of adipocyte differentiation.

During the early stage of adipogenesis, the cell population is increased by two progressions of the cell cycle in the MCE process [20]. Cell cycle progression during MCE in adipocyte differentiation is affected by cyclin D-CDK4/6 complexes, which are essential for progression through the early G1 phase, as well as by cyclin A- or cyclin E-CDK2 complexes required for late G1 phase and S phase entry. p21 and p27, which bind to cyclins/CDKs, can inhibit the

activation of CDKs [19,22]. In addition, the ERK/MAPK pathway plays a pivotal role in cell cycle progression and ERK acts as a mitogenic signaling molecule in 3T3-L1 preadipocytes [10]. The present results demonstrated that ramalin inhibited cell proliferation and the expression of cell cycle progression proteins, cyclin A and CDK2, by up-regulating p27 during MCE. Moreover, ramalin suppressed the phospholyation of ERK1/2, p38, and JNK. Based on these findings, it is plausible that the inhibitory effect of ramalin on the MAPK pathway could contribute to the regulation of cell proliferation during MCE. Taken together, our data suggest that the MCE is a target of ramalin for preventing adipogenesis in 3T3-L1 cells during the early stage.

Up-regulated PPARγ and C/EBPα alone or in cooperation with each other induce the transcription of many adipogenesis-related genes involved in creating and maintaining the adipocyte phenotype in the late stage [23]. PPARγ and C/EBPα, which combine in a transcriptional complex, regulate the transcription of target genes including aP2, LPL, leptin, and fatty acid synthase (FAS) [24]. Leptin is an adipocyte-secreted cytokine that plays a critical role in energy homeostasis as well as controls the fat mass through reducing food intake and increasing energy expenditure [25]. Adipocyte fatty acid binding protein, ap2, functions as a key mediator of intracellular transport and metabolism of fatty acids [26]. In the present study, ramalin remarkably inhibited adipocyte differentiation through the down-regulation of PPARγ, C/EBPα, aP2, and leptin mRNA levels in differentiation-induced 3T3-L1 preadipocytes.

Recent studies have shown that obesity induces chronic and low-grade inflammation in adipose tissue. As an active endocrine organ, adipose tissue synthesizes and secretes various adipokines into circulation, including TNF-α, IL-6, angiogenic proteins, and metabolic regulators in obesity [27]. The levels of IL-6 and TNF-α secreted by adipose tissues are associated with insulin resistance which causes the derangements in whole body glucose and lipid metabolism [15,28]. Thus, targeting proinflammatory cytokines has been suggested as a promising treatment for insulin resistance and obestiy. Our data showed that ramalin treatment resulted in decreased TNF-α and IL-6 secretion, suggesting the inhibitory effects of ramalin on obesity-associated inflammation and insulin resistance.

Development of obesity is related with the increased serum levels of total TG, TC, and LDL-cholesterol [29]. The level of TC is the main determinant of vascular disease risk [30]. Decreasing the level of LDL-cholesterol reduces the risk of symptomatic vascular disease [31]. Moreover, abdominal obesity has been reported to be associated with dyslipidemia, characterized by increasing TG and decreasing HDL-cholesterol levels [32]. Additionally, a previous study reported that decreased serum TG level is critical in weight loss [33]. Based on these findings, a possible mechanism for the anti-obesity effects of ramalin could be the inhibition of pathophysiological factors related to the development of obesity. In this study, ramalin significantly reduced the level of total and LDL-cholesterol as well as TG in HFD-fed C57BL/6J mice. Moreover, oral administration of ramalin resulted in a significant decrease in body and abdominal fat weights, which is not associated with the amount of food intake. Based on these findings, it is plausible that the significant reduction in body weight and body fat by ramalin is not due to a decrease of appetite.

Many lines of evidence indicate that obesity induced by high fat food can increase the secretion of several biologically active adipokines including leptin, TNF-α, and IL-6 [15]. These cytokines play an important role in obesity-related inflammation and metabolic diseases. The levels of cytokines are markedly elevated in obese individuals and a reduction of adipose mass leads to a decrease in cytokine levels [28]. It also was reported that TNF-α could regulate leptin production by increasing its gene expression and plasma levels [34]. In addition, previous results suggest that a reduction in body weight could lead to a decrease in the levels of leptin [35]. The data in the present study show that ramalin significantly reduced the secretion of TNF-α and the mRNA level of leptin in a concentration-dependent manner during adipocyte differentiation. Taken together, these findings suggest that ramalin reduces body weight and fat mass by attenuating the adipose tissue inflammation leading to potential novel therapy for reducing the risk of obesity and hyperlipidemia.

In the present study, oral administration with ramalin significantly inhibited the HFD-induced LXRα, LXRβ, and LPL as well as SREBP-1c mRNA level in liver tissue, indicating that ramalin is able to regulate cholesterol, fatty acid, and glucose homeostasis. In addition, ramalin (100 mg/kg) had significant anti-obesity effects, compared to orlistat suggesting that ramalin treatment could have more favorable effect than orlistat in the treatment of obesity.

In summary, our results suggest that ramalin acts as a negative regulator of adipogenesis in 3T3-L1 cells through the inhibition of C/EBPβ phosphorylation and C/EBPα and PPARγ expression. This inhibitory effect could be mediated by the suppression of MAPK signaling pathways during the early stages of adipogenesis. *In vivo*, ramalin remarkably reduced body and abdominal fat weight in HFD-fed mice through the attenuation of glucose, total-cholesterol and LDL-cholesterol.

The present data provide insight into the mechanisms of the inhibitory effects of ramalin and suggest that the inhibitory activity of ramalin indicates a potential pharmacological intervention specifically directed toward metabolic disorders including obesity.

Conflict of interest

The authors have no conflict of interest to declare.

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Transparency document

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Appendix A. Supplementary data

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