Thesis for the Degree of Master of Science

# Ginsenoside Rb1 Induces Beta 3 Adrenergic Receptor-dependent Lipolysis and Thermogenesis in 3T3-L1 Adipocytes and *db/db* Mice

Seona Lim

Department of Science in Korean Medicine
Graduate School
Kyung Hee University
Seoul, Korea

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by

Seona Lim

Advised by

Prof. Jae-Young Um, Ph.D.

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Dissertation Committee:

Chairman Hyeung-Jin Jang, Ph.D.

Seong-Kyu Choe, Ph.D.

Jae-Young Um, Ph.D.

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# Abbreviations

ACC, acetyl-CoA carboxylase

ATGL, adipose triglyceride lipase

AMPK$\alpha$, AMP-activated protein kinase alpha

β3AR, beta 3 adrenergic receptor activator;

BAT, brown adipose tissue

CGI58, Comparative gene identification 58

DXA, dual-energy X-ray absorptiometry

eWAT, epididymal WAT

GAPDH, glyceraldehyde-3-phosphate dehydrogenase

H&E, hematoxylin and eosin

HSL, hormone sensitive lipase

IF, Immunofluorescence

iWAT, inguinal WAT

LKB1, liver kinase B1

PGC1α, peroxisome proliferator activated receptor gamma-coactivator 1 alpha

PPARα, peroxisome proliferator activated receptor alpha

Rb1, ginsenoside Rb1

SIRT1, silent information regulator T1

SIRT3, silent information regulator T3

UCP1, uncoupling protein 1

WT, wild type

# ABSTRACT

Ginsenoside Rb1 Induces Beta 3 Adrenergic Receptor-dependent Lipolysis and Thermogenesis in 3T3-L1 Adipocytes and *db/db* Mice

By Seona Lim
Master of science
Graduate School of Kyung Hee University
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Obesity is constantly rising into a major health threat worldwide. Activation of brown-like transdifferentiation of white adipocytes (browning) has been proposed as a promising molecular target for obesity treatment. In this study, we investigated the effect of ginsenoside Rb1 (Rb1), a saponin derived from Panax ginseng Meyer, on browning using 3T3-L1 murine adipocytes and leptin receptor mutated *db/db* mice. Rb1 treatment suppressed lipid accumulation and decreased lipid droplet size in 3T3-L1 adipocytes. Rb1 also induced phosphorylations of AMP-activated protein kinase alpha (AMPKα) pathway and sirtuins. Moreover, lipases and thermogenic factors such as uncoupling protein 1 were increased by Rb1 treatment, suggesting activated signaling of non-shivering thermogenesis in 3T3-L1 adipocytes. In *db/db* mice, 6-week-injection of Rb1 resulted in decreased inguinal white adipose tissue (iWAT) weight associated with shrinked lipid droplets and increased lipolysis and thermogenesis. The thermogenic effect of Rb1 was possibly due to beta 3 adrenergic receptor activation (β3AR), as a β3AR inhibitor (L748337) pre-treatment abolished the effect of Rb1. In conclusion, we suggest Rb1 as a

potential lipolytic and thermogenic therapeutic agent which can be used for obesity treatment.

**Keywords:** ginsenoside Rb1, obesity, non-shivering thermogenesis, lipolysis, beta 3 adrenergic receptor

# I. INTRODUCTION

Obesity is rising as a major health issue worldwide, especially in developed countries. As it is a risk factor for type 2 diabetes mellitus (T2DM) and other chronic metabolic disorders, the interest on managing obesity is constantly growing [1]. There are currently five different drugs approved by the United States Food and Drug Administration; orlistat, lorcaserin, phentermine/topiramate, naltrexone/bupropion, and liraglutide [2]. These drugs indeed help people lose weight, but serious side effects, such as steatorrhea, headache, nausea, vomiting and etc., encourage the search for alternative treatments [3].

Obesity is a condition defined where excess/abnormal fat accumulation impact health. As they are the key players in energy homeostasis, the importance of adipose tissues cannot be neglected in the strategy for obesity treatment. Adipose tissues can be categorized into two subsets based on function and morphology: white adipose tissue (WAT) and brown adipose tissue (BAT). Functions of WAT store the energy in the form of lipids [4] while BAT dissipate energy as heat by a process called non-shivering thermogenesis [5]. Non-shivering thermogenesis is a defense mechanism to fight against cold through enhanced mitochondrial content and uncoupling protein 1 (UCP1). Since its identification in the 16th century, brown adipose tissue (BAT) has been well-noticed as the main organ for non-shivering thermogenesis [6].

Recently, apart from classical BAT, recruitable brown fat has risen into another potential strategy for obesity management [7]. These brown-like white adipocytes, also called ‘beige’ or ‘brite’ adipocytes, mimic the role of brown adipocytes by expressing abundant mitochondria associated with high expression levels of UCP1 [8]. Beige and brown adipocytes share numerous characteristics, from the UCP1-mediated thermogenesis to several differentiation factors such as PPARγ coactivator 1 alpha (PGC1α) and RD1-BF1-RIZ1 homologous domain containing 16 (PRDM16) [9]. As

various stimuli such as cold exposure, endogenous signals as well as dietary factors and pharmacological agents can induce the trans-differentiation of white adipocytes into beige adipocytes [3, 10], the search for relatively safe natural products which can induce 'browning' of white adipocytes is a promising, attractive target for obesity treatment.

Ginsenoside Rb1 ($C_{54}H_{92}O_{23}$, Rb1) is a triterpenoid saponin derived from the highly valued herb, *Panax ginseng* Meyer (*P. ginseng*). This herb has been used as a powerful tonic for qi and blood over 50 centuries in traditional Chinese and Korean medicine, and is also appreciated by the western countries as well [11]. Among the known saponins of *P. ginseng*, Rb1 is considered as one of the most abundant ingredient which may be responsible for the various biological functions of *P. ginseng* [12]. Several studies report the anti-adipogenic effect of Rb1 [13-17]. In contrast, an early work by Shang et al. reported that Rb1 promotes adipogenesis by enhancing two major adipogenic factors, CCAAT/enhancer binding protein alpha (C/EBPα) and peroxisome proliferator-activated receptor gamma (PPARγ) [18]. Furthermore, this was later supported by Mu's study, as it showed that Rb1 induced increase of PPARγ and C/EBPα could have been a result of its browning effect in adipocytes. Mu's team reported Rb1 significantly increased the levels of UCP1, PGC1α and PRDM16, thus leading to increased thermogenic capacity of 3T3-L1 adipocytes [19]. However, although the browning effect of Rb1 has already been reported, its detailed mechanism still remains unknown up to date. We hereby show that Rb1 treatment indeed resulted in browning of 3T3-L1 adipocytes, and this effect was due to regulation of beta 3 adrenergic receptor (β3AR)-mediated lipolysis induced by Rb1.

# II. MATERIALS AND METHODS

## 1. Chemical reagents and antibodies

Rb1 was purchased from Abcam (Cambridge, UK). 3-Isobutyl-1-methylxanthine (IBMX), dexamethasone (Dex), insulin, and Oil Red O powder were purchased from Sigma (St. Louis, MO, United States). L748337 was from Tocris Bioscience (Bristol, UK). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) were purchased from Corning (NY, United States). Antibodies for liver kinase B1 (LKB) (3047S), pLKB1 (Ser428) (3482S), AMP-activated protein kinase alpha AMPKα (2532S), pAMPKα (Thr172) (2535S), acetyl-CoA carboxylase (ACC) (3676S), pACC (Ser79) (3661S), silent information regulator T1 (SIRT1) (8469S), SIRT3 (5490S), phospho-hormone sensitive lipase (pHSL) (Ser563) (4139S), UCP1 (14670S) and β-actin (3700S) were purchased from Cell Signaling Technology (Beverly, MA, United States); the antibody for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sc-32233) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, United States); antibodies for PGC1α (ab54481), Comparative gene identification 58 (CGI58) (ab59488), adipose triglyceride lipase (ATGL) (ab207799), HSL (ab45422) and β3AR (ab94506) were purchased from Abcam (Cambridge, UK); the antibody for peroxisome proliferator activated receptor alpha (PPARα) (PA1-822A) was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

## 2. Ethical statement

All animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Review Board of Kyung Hee University (confirmation number: KHUASP (SE)-13-012).

### 3. Cell culture and differentiation

3T3-L1 adipocytes mouse embryo fibroblasts cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA) cultured and differentiated as previously described [20]. Briefly, cells were grown in DMEM containing 10% FBS and 100 units/ml of penicillin streptomycin solution at 37 °C, in 5% $CO_2$, at 95% humidity until confluence. After 2 days from full confluence (day 0), the cells were differentiated by a 48 h incubation in differentiation medium consisting of DMEM plus 10% FBS containing 0.5 mM IBMX, 1 µM Dex, and 1 µg/ml insulin. At day 2, the cells were cultured in DMEM plus 10% FBS supplemented with 1 µg/ml insulin and various concentrations of Rb1 (1, 5, 10 and 20 µM) for another 48 h followed by fresh DMEM culture medium containing 10% FBS and1 µg/ml insulin.

### 4. Cell cytotoxicity assay

Cell viability was measured with a Cell Proliferation MTS kit (Promega Co., Maddison, WI, USA) as previously described [21]. Briefly, Cells were seeded ($2 \times 10^4$ cells per well) on 96-well plates and incubated for 24 h, followed by incubation with various concentrations (1, 5, 10, 20, 40 and 100 µM in 3T3-L1 cells) of Rb1 in culture medium for an additional 48 h. The absorbance was measured at 490 nm in a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

## 5. Oil Red O staining

Intracellular triglyceride (TG) accumulation was measured using the Oil Red-O staining method as previously described [20]. Photomicrograph images were obtained from a regular light microscope (Olympus, Tokyo, Japan), and absorbance was measured at 500 nm using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

## 6. Protein extraction and western blot analysis

Western blot analyses were performed as previously described [20]. In brief, homogenized tissues and harvested cells were lysed in lysis buffer (Cell Signaling Technology, Beverly, MA, United States), and protein concentration was determined using a protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of total protein were resolved by 8–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membranes were incubated with the primary antibody at 4 °C overnight, and then incubated with a 1:10000 dilution of the proper horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson Immuno Research, West Grove, PA, USA) for 1 h at RT.

## 7. Immunofluorescence (IF) assay

IF analyses were performed as previously described [22]. BODIPY 558/568 $C_{12}$ (D3835) for staining lipid droplets was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Images were acquired using a fluorescence microscope (Logos Biosystems, Anyang, Korea).

## 8. Animal experiment

Male 6-week-old *db/db* mice and age matched wild-type (WT) C57BL6/J mice were purchased from Daehan Biolink Co. (Eumsung, Korea) and maintained for 1 week prior to the experiments, provided with a laboratory diet and water ad libitum. Healthy mice were randomly divided into three groups as follows ($n$ = 5 per group): a WT group, a *db/db* group, and a *db/db* group administered with Rb1. The control groups (WT group, *db/db* group) were administered distilled water (i.p.), while the experiment group was administered Rb1 prepared in distilled water (10 mg/kg of body weight, i.p.) five times per week for 6 weeks. Body weight and food intake were measured three times per week. At the end of the experiment, the animals were anesthetized, tissues were collected and placed in a tube, then stored at -80 °C.

## 9. Hematoxylin and Eosin (H&E) staining

Hematoxylin and eosin (H&E) staining was done as previously described [21]. Photographs were taken under a microscope EVOS M7000 (Thermo Fisher Scientific, Waltham, MA, USA).

## 10. Measurement of body fat by DXA scan

Body fat scan was performed using a dual-energy X-ray absorptiometry with an InAlyzer instrument (Medikors, Seongnam, Korea) based on the manufacturer's instructions as a previous report [23].

**11. Statistical analysis**

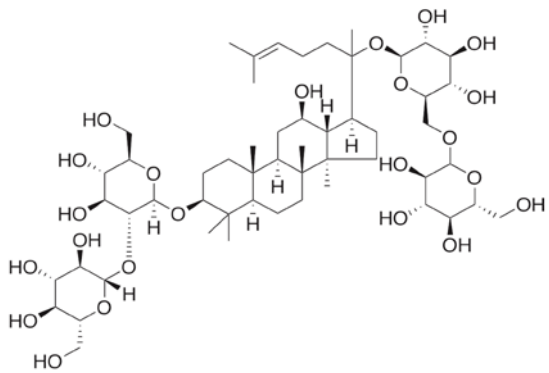
Data were expressed as the means ± standard error mean (S.E.M.). Significant differences ($p < 0.05$) between groups were determined with the ANOVA test. All statistical analyzes were performed using an SPSS statistical analysis software version 11.5 (SPSS Inc., Chicago, IL, USA).

# III. RESULTS

## 1. Effect of Rb1 on cell viability

Figure 1A shows the chemical structure of Rb1. To evaluate the cytotoxicity of Rb1, 3T3-L1 cells were treated with Rb1 at various concentrations (1-100 μM) and the cell viability measured by the MTS assay. As in Fig. 1B, the cell viability results show that Rb1 had no significant cytotoxicity up to a concentration of 20 μM. However, concentrations of 40, 100 μM had significant cytotoxic effect on 3T3-L1 cells. Due to this result, further treatments proceeded at the concentrations of 1, 5, 10 and 20 μM.

**(A)**



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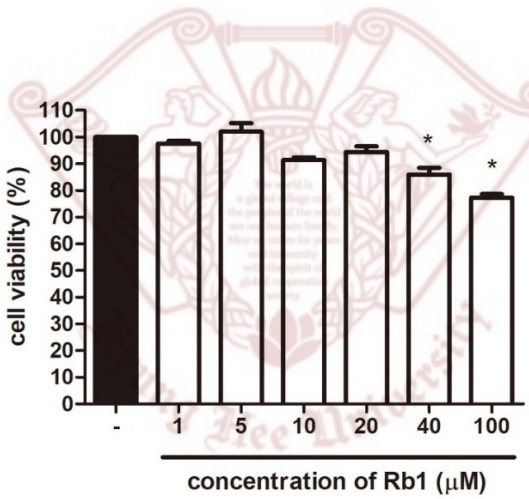


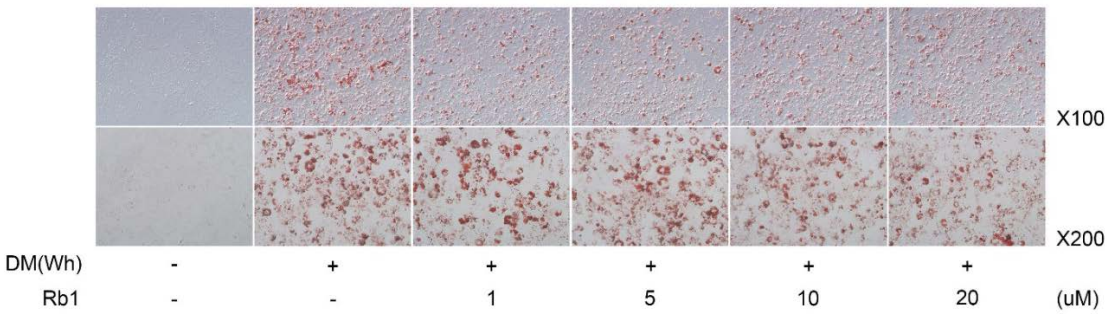

**Figure 1. Chemical structure of Rb1 and cytotoxicity of Rb1 in 3T3-L1 adipocytes.**

(A) Chemical structure of Rb1 is shown. (B) An MTS assay was performed in order to evaluate the cytotoxicity of Rb1 on 3T3-L1 adipocytes. Data are expressed as mean ± S.E.M. of three or more experiments. $^{*}p < 0.05$ vs. untreated cells. Rb1, ginsenoside Rb1.

**2. Rb1 decreases the size of lipid droplets in 3T3-L1 adipocytes**

To investigate the effects of Rb1 on 3T3-L1 adipocyte differentiation, the lipid accumulation was measured by and Oil Red o staining assay (Fig. 2A). Rb1 treatment did not suppress nor increase triglyceride (TG) accumulation in 3T3-L1 adipocytes (Fig. 2B), however, the size of lipid droplets was significantly reduced in Rb1-treated cells when compared to differentiated adipocytes (Fig. 2C). The change in lipid droplet morphology suggests that Rb1 may have induced browning in 3T3-L1 adipocytes.

**(A)**

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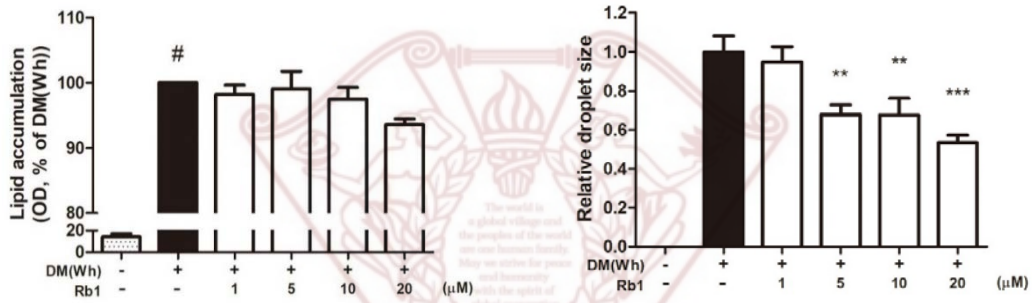



**Figure 2. Effect of Rb1 on lipid accumulation in 3T3-L1 adipocytes**

(A) Representative photomicrographs of Oil Red O staining in differentiated 3T3-L1 adipocytes. (B) Relative TG level was quantified by measuring the absorbance. (C) Relative lipid droplet sizes of each group was measured. Data are expressed as mean ± S.E.M. of three or more experiments. $^{\#}p < 0.05$ vs. un-differentiated pre-adipocytes; $^{**}p < 0.01$ vs. vehicle-treated mature adipocytes; $^{***}p < 0.001$ vs. vehicle-treated mature adipocytes. DM (Wh), white adipocyte differentiation media; Rb1, ginsenoside Rb1.

### 3. Rb1 increased phosphorylation of LKB-AMPK-ACC pathway and expression of sirtuin proteins in 3T3-L1 adipocytes

AMPK is a well-known sensor and regulator of energy metabolism [24]. As shown in Fig. 3A-D, Rb1 treatment up-regulated the phosphorylation level of AMPK in a concentration-dependent manner, showing that Rb1 can increase energy metabolism in 3T3-L1 adipocytes. Similar results were shown in the upstream and downstream factors of AMPK, LKB and ACC, respectively.

After observing the effect of Rb1 on the AMPK pathway, we then performed further experiments to verify whether Rb1 affects the levels of sirtuins. The sirtuins, consisted of seven homologs in mammals, is a family of NAD+-dependent histone/protein deacetylases [25]. Among the seven sirtuins, silent information regulator T1 (SIRT1) and SIRT3 are closely related to the AMPK function in lipid homeostasis [26, 27]. Our results showed that Rb1 also increased the levels of both sirtuins, but without statistical significance in SIRT3 (Fig. 4A-C).

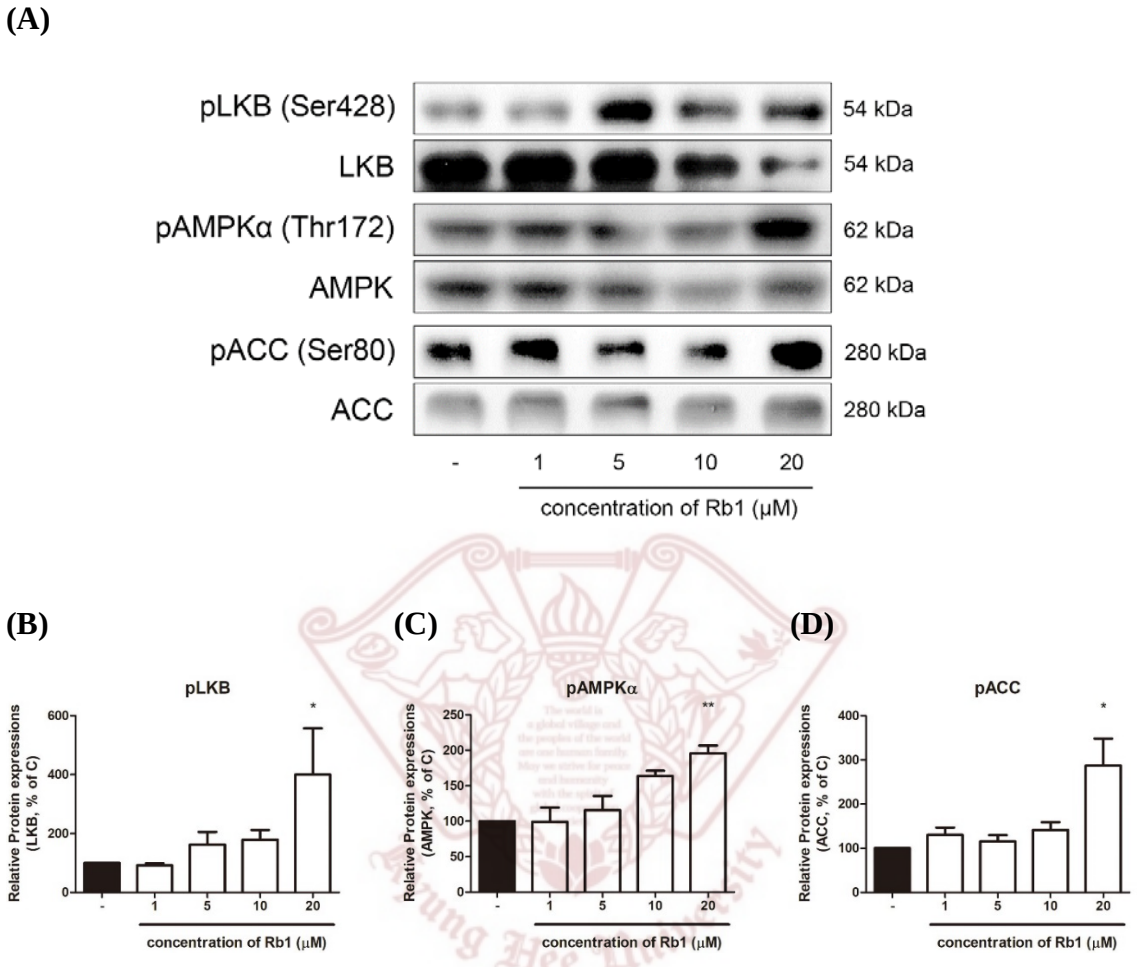
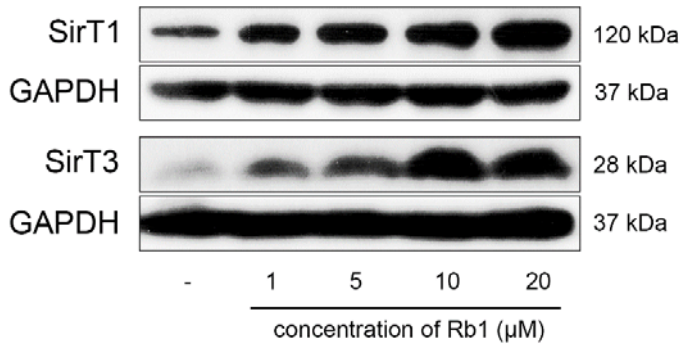



**Figure 3. Effect of Rb1 on AMPK pathway in mature 3T3-L1 adipocytes.**

(A) Western blot assays were performed to measure the changes on pLKB, LKB, pAMPKα, AMPK, pACC and ACC. Relative expression levels of (B) pLKB, (C) pAMPKα and (D) pACC were quantified. Expressions of pLKB, pAMPKα and pACC were normalized against expressions of LKB, AMPK and ACC respectively. Data are expressed as mean ± S.E.M. of three or more experiments. $^{*}p < 0.05$ vs. vehicle-treated mature adipocytes; $^{**}p < 0.01$ vs. vehicle-treated mature adipocytes. Rb1, ginsenoside Rb1.

**(A)**




**(B)** **(C)**

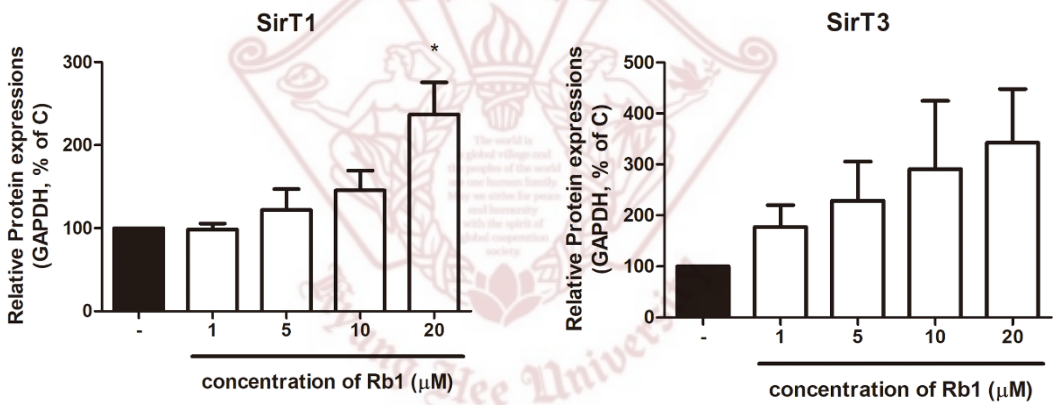


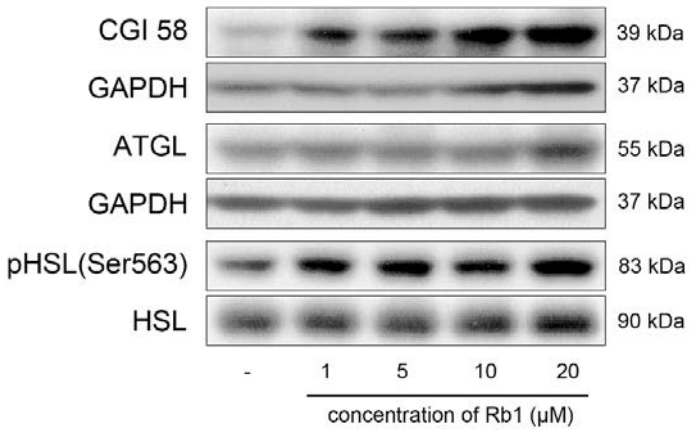

**Figure 4. Effect of Rb1 on sirtuin protein in mature 3T3-L1 adipocytes.**

(A) Western blot assays were performed to measure changes on SIRT1 and SIRT3. Relative expression levels of (B) SIRT1 and (C) SIRT3 were quantified. Expressions of SIRT1 and SIRT3 were normalized against GAPDH. Data are expressed as mean ± S.E.M. of three or more experiments. $^{*}p < 0.05$ vs. vehicle-treated mature adipocytes; Rb1, ginsenoside Rb1.

**4. Rb1 induces lipolysis in 3T3-L1 adipocytes**

AMPK, the energy metabolism sensor, also acts as a regulator of lipolysis by phosphorylating ATGL and HSL, the two major lipases working in the lipolytic process [28]. Since we verified the effect of Rb1 on AMPK pathway and sirtuins, our next objective was to investigate its effect on lipolysis. CGI58, a critical regulator of ATGL, showed an increasing tendency in Rb1-treated adipocytes (Fig. 5A and B). In addition, protein levels of ATGL and pHSL were significantly increased by Rb1 treatment (Fig. 5A, C and D). The lipolytic activation induced by Rb1 was re-confirmed when we performed an IF staining assay of ATGL (Fig. 6).

**(A)**




**(B)** **(C)** **(D)**

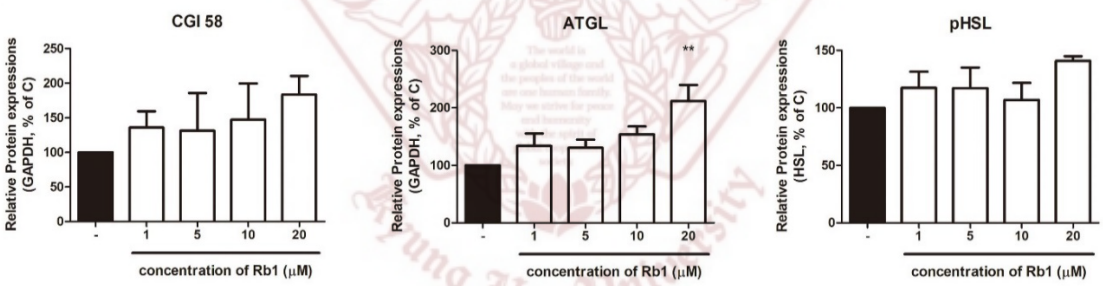



**Figure 5. Effect of Rb1 on lipolysis in mature 3T3-L1 adipocytes.**

(A) Western blot assays were performed to measure the changes on CGI58, ATGL and HSL. Relative expression levels of (B) CGI58, (C) ATGL and (D) pHSL were quantified. Expressions of CGI58 and ATGL were normalized against GAPDH; expression of pHSL was normalized against HSL. Data are expressed as mean ± S.E.M. of three or more experiments. $^{**}p < 0.01$ vs. vehicle-treated mature adipocytes. Rb1, ginsenoside Rb1.

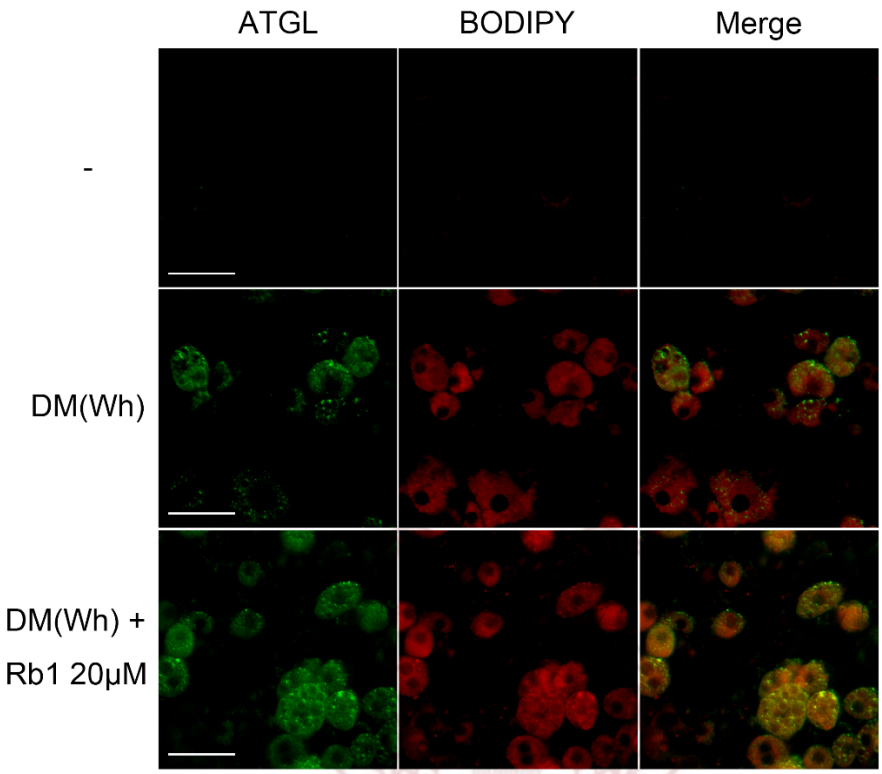


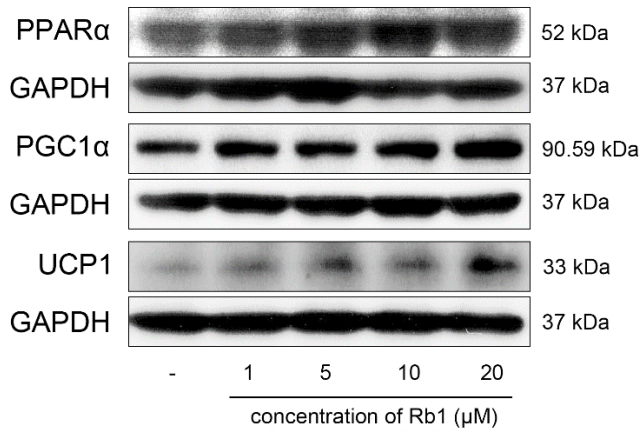

**Figure 6. Effect of Rb1 on expressions of ATGL in mature 3T3-L1 adipocytes.**

IF staining was performed to evaluate the expression level and pattern of ATGL and BODIPY (Scale bar: 50 μm). DM(Wh), white adipocyte differentiation media; Rb1, ginsenoside Rb1.

**5. Rb1 increases thermogenic capacity in 3T3-L1 adipocytes**

Lipolysis is known as the pre-step of brown and beige adipocyte thermogenesis. Importantly, lipolysis plays a central role in the catabolic activity of BAT and WAT. Fatty acids (FAs) induce oxidative phosphorylation and provide fuel that supports UCP1-mediated respiration [29]. Lipolysis also provides ligands for PPARα, which acts crucially in the browning of WAT [30]. As shown in Fig. 7A and B, Rb1-treated adipocytes showed increased levels of PPARα, implying the thermogenic triggering by Rb1. PGC1α, the major transcription factor of both brown and beige adipocytes [31], were also increased by Rb1 treatment (Fig. 7A and C). The thermogenic capacity was determined by assessing the changes in UCP1. Rb1 treatment induced a 2.06-fold change in the protein expression of UCP1 when compared to vehicle-treated adipocytes (Fig. 7D).

**(A)**




**(B)** **(C)** **(D)**

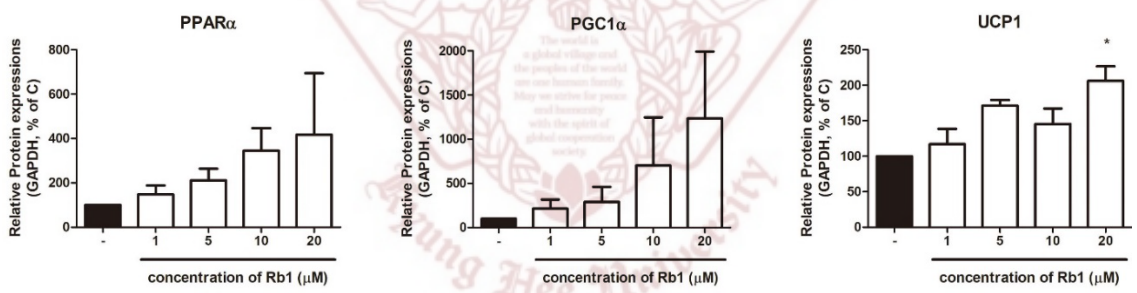



**Figure 7. Effect of Rb1 on browning in mature 3T3-L1 adipocytes.**

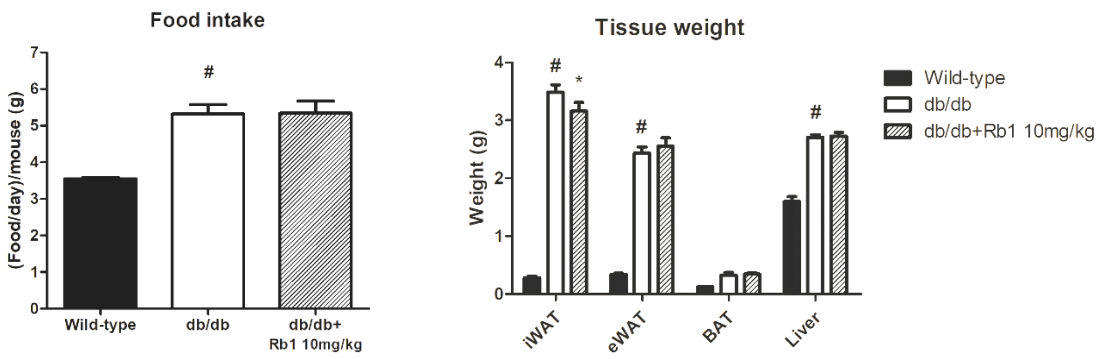
(A) Western blot assays were performed to measure the changes of PPARα, PGC1α and UCP1. Relative expression levels of (B) PPARα, (C) PGC1α and (D) UCP1 were quantified. Expressions of PPARα, PGC1α and UCP1 were normalized against GAPDH. Data are expressed as mean ± S.E.M. of three or more experiments. $^{*}p < 0.05$ vs. vehicle-treated mature adipocytes; Rb1, ginsenoside Rb1.

### 6. Rb1 induces browning in inguinal white adipose tissue (iWAT) of *db/db* mice

We then conducted an animal study to evaluate the effect of Rb1 *in vivo*. After feeding obese *db/db* mice with Rb1 (10 mg/kg/day) for 6 weeks, we observed unchanged food intake between vehicle-fed and Rb-fed *db/db* mice (Fig. 8A). Surprisingly, not in accordance with the *in vitro* results, Rb1 did not affect body weight change in *db/db* mice (data not shown). However, a significant reduce in iWAT was shown in Rb1-treated mice, while epididymal white adipose tissue (eWAT), BAT and liver tissue weight was not affected (Fig. 8B). A DXA scan analysis showed decreased fat body mass in Rb1-fed group compared to vehicle-treated *db/db* group (Fig. 8C). Next, we evaluated the Rb1-induced histological changes in iWAT. As a result, average size of lipid droplets was increased by 4.68-fold in *db/db* mice than WT mice, and this was decreased down to 61% by Rb1 administration (Fig. 9A and B).

Further western blot assays were performed to evaluate the effect of Rb1 on thermogenesis-related factors. As shown in Fig. 10A and B, Rb1 treatment increased the protein expressions of pAMPK, ATGL, and UCP1 in iWAT of mice, suggesting a browning effect was induced by Rb1.

**(A)** **(B)**

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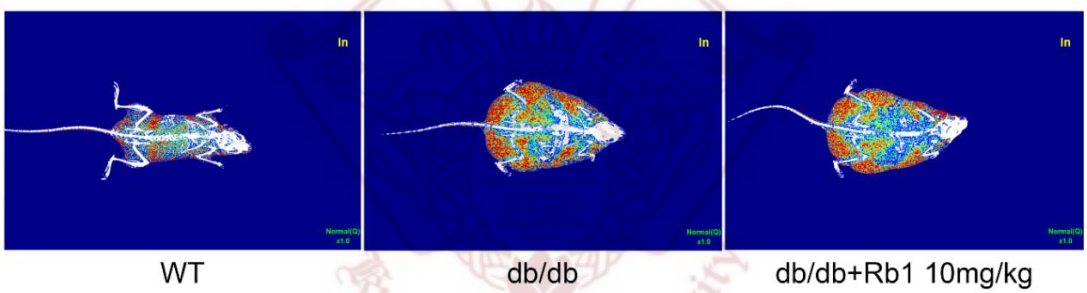
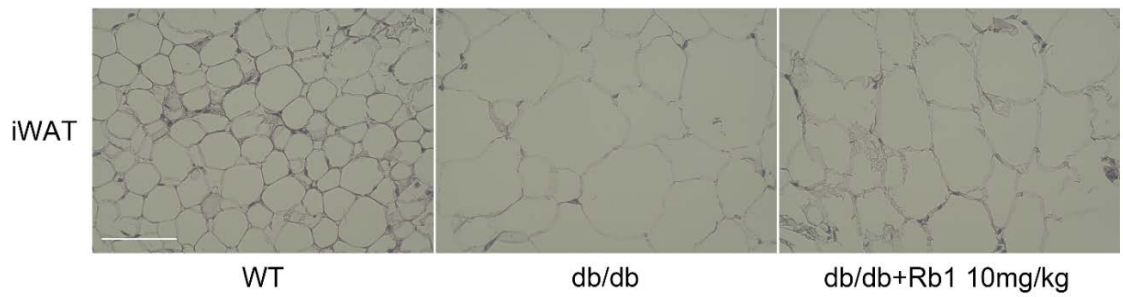



**Figure 8. Effect of Rb1 on tissues weight and fat mass in *db/db* mice.**

(A) Food intake of each group was measured. (B) Tissue weight of inguinal white adipose tissue (iWAT), epididymal white adipose tissue (eWAT), brown adipose tissue (BAT) and liver of each group was measured. (C) Body fat was measured using DXA scan. Data are expressed as mean ± S.E.M. of three or more experiments. $^{\#}p < 0.05$ vs. vehicle-treated wild-type C57BL6/J mice; $^{*}p < 0.05$ vs. vehicle-treated *db/db* mice

**(A)**




**(B)**

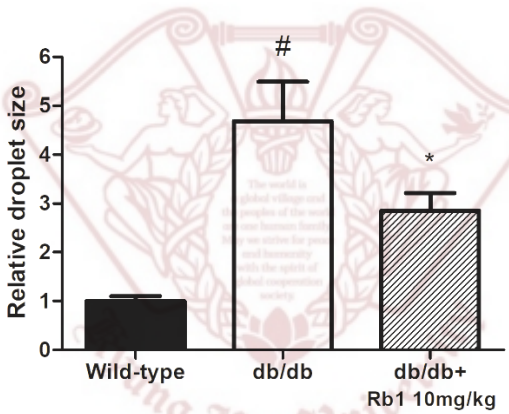
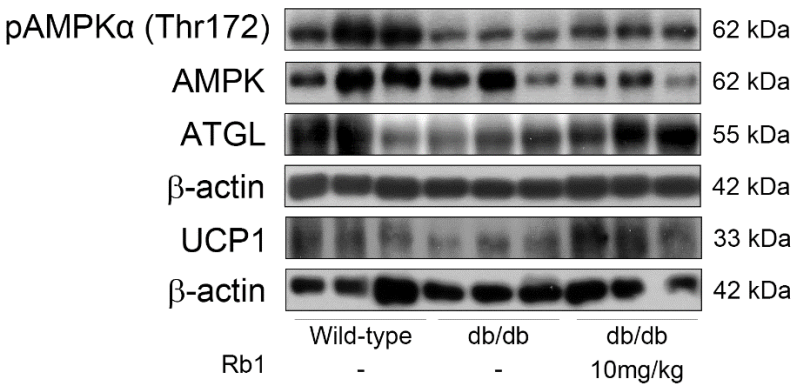



**Figure 9. Effect of Rb1 on histological structure of inguinal white adipose tissue in *db/db* mice.**

(A) H&E staining was performed to evaluate histological changes in iWAT of *db/db* mice (Scale bar: 100 μm). (B) Relative lipid droplet sizes in iWAT of each group was measured. Data are expressed as mean ± S.E.M. of three or more experiments. $^{\#}p < 0.05$ vs. vehicle-treated wild-type C57BL6/J mice; $^{*}p < 0.05$ vs. vehicle-treated *db/db* mice

**(A)**




**(B)**

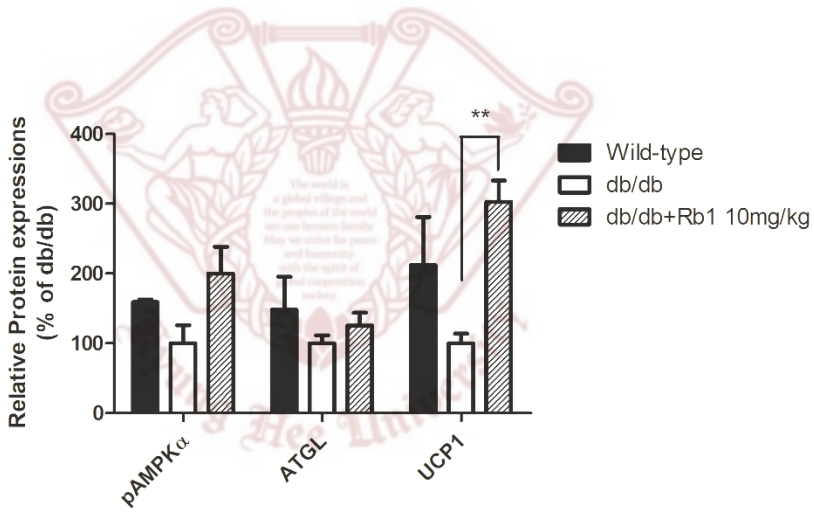


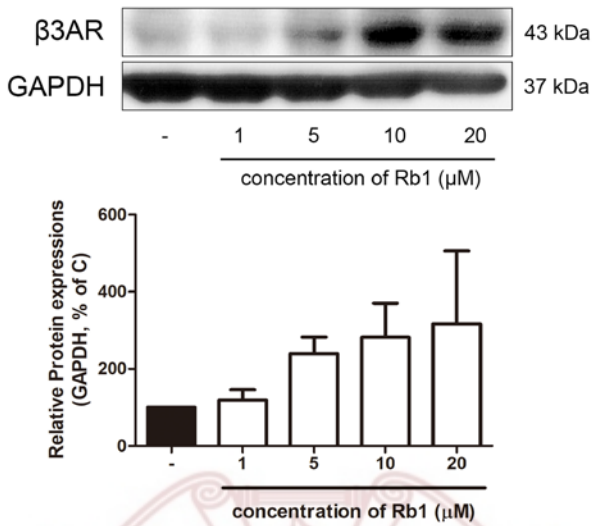

**Figure 10. Effect of Rb1 on lipolysis and thermogenesis in *db/db* mice.**

(A) Western blot assays were performed to measure the changes of pAMPKα, AMPK, ATGL and UCP1. (B) Relative expression levels of pAMPKα, AMPK, ATGL and UCP1 were quantified. Expression of pAMPKα was normalized against AMPK; expressions of ATGL and UCP1 were normalized against β-actin. Data are expressed as mean ± S.E.M. of three or more experiments. $^{**}p < 0.01$ vs. vehicle-treated *db/db* mice.

### 7. Thermogenic effect of Rb1 is dependent on β3AR pathway

As β3AR is considered as one of the highest upstream signals of non-shivering thermogenesis [32, 33], we attempted to investigate whether Rb1 could regulate the expression of this receptor. As expected, Rb1 treatment in 3T3-L1 adipocytes resulted in a dose-dependent increase of β3AR expression (Fig. 11A). Furthermore, when L748337, a selective β3AR antagonist was pre-treated in adipocytes, the effect of Rb1 on UCP1 induction was abolished, down to nearly 67% (Fig. 11B), suggesting the thermogenic effect of Rb1 is dependent on the β3AR signaling pathway, at least partially.

**(A)**




**(B)**

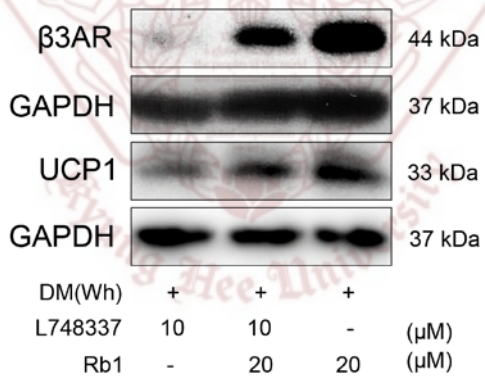



**Figure 11. Effect of Rb1 on β3AR in mature 3T3-L1 adipocytes.**

(A) A western blot assay was performed to measure the change β3AR. Expression of β3AR was normalized against GAPDH. (B) Changes in β3AR and UCP1 after β3AR inhibition were mesured by western blot assays. Data are expressed as mean ± S.E.M. of three or more experiments. Rb1, ginsenoside Rb1.

# IV. DISCUSSION

The negative impact of obesity on mankind health forces clinicians and researchers to seek a promising anti-obese strategy. In 2016, the World Health Organization (WHO) reported that around 2 billion adults were overweight, and 650 million obese [34]. However, currently available strategies, mostly medications, display unwelcome side effects [2]. Thus, the task of safe and effective anti-obese agents is still an ongoing challenge. In this context, the potential of natural products, also proved by the steady growth of its market size [35], may give an advantage for the next promising strategy for obesity care.

There are some obvious clues of the anti-obese action of Rb1 from previously published literature. Park et al. reported Rb1 and another saponin of P. ginseng, ginsenoside Rg1 suppressed TG accumulation *in vitro* [13], while Xiong et al. showed the anti-obese effect of Rb1 was effective *in vivo* as well [14]. Shen et al. suggested AMP-activated protein kinase (AMPK) was responsible for this effect [15], Lin and colleagues explained it was resulted by decreased appetite by Rb1-regulated neuropeptide Y (NPY) and peptide YY (PYY) [16], and Yu et al. reported perilipin expression was the clue [17]. However, up to date, the exact cascade mechanism of the thermogenic effect of Rb1 still remains to be elucidated. Therefore, in this study, we aimed to investigate the detailed action mechanism of Rb1 on beige adipocyte recruitment and thermogenesis induction using *db/db* mice and 3T3-L1 adipocytes.

Non-shivering thermogenesis in BAT and WAT is a promising molecular target for obesity management. The recently discovered beige adipocytes within WAT differ from classic white adipocytes both by morphology and ability. By certain stimulation, such as cold exposure [36, 37] or pharmacological activation [38, 39], these beige adipocytes, which was normally acting as a storage unit for lipid, become capable of

producing heat through mitochondrial UCP1 activation. Once activated either in BAT or WAT, the thermogenic action of mitochondria requires fuel: FAs. In order to supply FAs, the lipolysis signaling is activated within the adipocyte, and the lipases ATGL, HSL and monoacylglycerol lipase (MG) subsequently process TG into FAs for β-oxidation [40]. In this study, we observed Rb1 can increase ATGL, suggesting a possible role of Rb1 on induction of the lipolysis signaling for thermogenic actions.

β3ARs have a critical role in activation of non-shivering thermogenesis via PKA signaling, both in BAT and 'beiged' WAT [41, 42]. Jimenez et al. showed that β3AR knocked-out mice displayed low mRNA level of *Ucp1* and fail to induce thermogenesis by cold stimuli [43]. In accordance, β3AR activation by pharmacological agonists CL316,243 and isoproterenol results in enhanced UCP1 expression in BAT [44], and higher thermogenic capacity in WAT [45]. Nature-derived nutritional agents are also candidates for β3AR-activated thermogenesis as well. Ephedrine, an active compound of *Ephedra* genus, is shown to induce β3AR expression and glycerol release, which was potentiated with β3AR agonist BRL37344 and inhibited by β3AR antagonist SR59230A co-treatment [46]. Another team reported that cinnamon extract induces browning in 3T3-L1 adipocytes and WAT of high fat-diet induced obese mice by activating β3AR [47]. Our results suggest another natural product which can induce browning by β3AR activation. Rb1 administration increased expressions of lipases and thermogenic factors including UCP1, and these effects were suppressed when a β3AR antagonist, L748337, was pre-treated.

AMPK is a metabolism regulator protein consisted of three subunits: catalytic α, regulatory β and γ [48]. As its name, the elevation of AMP/ADP associated with reduced ATP triggers the activating phosphorylation of AMPK specifically at Thr172 of the α subunit [49]. Once phosphorylated, AMPK regulates activation of metabolic proteins and transcription factors leading to an energy production. Numerous nature-derived materials such as berberine [50], quercetin [51] and resveratrol [52] are shown to

induce AMPK activation and thus ameliorate metabolic diseases. Our results also suggest the possible use of Rb1 as an AMPK-activating anti-obesity agent. Rb1 increased phosphorylation of not only AMPK, but also its upstream kinase LKB and downstream factor ACC. In addition, Rb1 increased the levels of SIRT1, which is known to facilitate with metabolic AMPK action [26].

In our study, we have shown that Rb1 can decrease lipid accumulation *in vivo* and *in vitro* by inducing the lipolysis-thermogenesis cascade. This effect was probably due to activation of β3ARs, as β3AR inhibitor treatment decreased the thermogenic effect of Rb1. However, further studies are required to understand the whole body significance of Rb1-activated β3AR, as our study mainly focused on the beige adipocyte recruitment in WAT. Because the mechanism of WAT browning and BAT activation are both related in the β3AR-dependent thermogenesis, thus to investigate the whole precise mechanism of anti-obese effect of Rb1, relevant studies dealing on the role of BAT in the effect of Rb1 is necessary. Furthermore, as the non-adrenergic pathway is also capable of progressing lipolysis and thermogenesis [53], related investigation on Rb1 action has to be carried out as well.

Overall, our results demonstrate the anti-obesity effect of Rb1 by regulating β3AR-dependent lipolysis and thermogenesis. Regarding the well-known clinically beneficial features of *P. ginseng* in traditional Korean medicine, we suggest Rb1 as a potentially safe and effective therapeutic agent for obesity treatment.

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# 국문 초록

## 진세노사이드 Rb1 의 베타 3 아드레날린 수용체 활성화를 통한 항 비만 효과에 대한 연구

임선아
기초한의과학과
경희대학교 대학원
지도교수: 엄재영

비만이 전 세계 국가들의 심각한 건강문제로 대두됨에 따라 많은 비만치료제들이 상용화되고 있다. 그러나 현 시판 중인 비만치료제는 부작용 등의 한계가 존재하며, 따라서 신규 항비만 기전 및 상대적으로 부작용이 적은 천연물을 이용한 항비만 소재 개발에 대한 연구가 각광 받고 있다.

포유류에는 백색지방세포와 갈색지방세포가 존재하며, 이 두 종류의 세포는 에너지 저장에 관련하여 반대의 기전을 가진다. 백색지방세포는 신체 내에 남아있는 에너지를 저장하는 것이 대표적인 기능이며 이에 반해 갈색지방세포는 저장되어 있는 에너지를 열 발생을 통해 방출하는 역할을

수행한다. 또한 '베이지지방세포화'란 특정 자극에 의해 백색지방세포가 갈색지방세포와 유사한 형질을 띄는 현상으로서, 이러한 베이지지방세포로의 전환분화 및 활성 유도는 열 발생을 일으켜 항비만 효과로 이어지게 된다. 본 연구는 인삼의 대표적 활성 성분인 진세노사이드 Rb1 (Rb1) 의 베이지지방세포 활성화를 통한 항비만효과를 확인하고 이에 대한 상세 조절 기전을 규명하기 위해 진행하였다.

3T3-L1 지방전구세포에 Rb1 을 처리한 결과 지방 방울 크기가 유의적으로 감소하였다. 또한 에너지 대사에 관련된 AMP-activated protein kinase (AMPK) 경로 인자들과 silent information regulator (SIRT) 1, 3 기전이 Rb1 처리에 의해 농도의존적으로 활성화 되었으며, 지방 분해 관련 인자인 comparative gene identification 58 (CGI 58), adipose triglyceride lipase (ATGL) 그리고 hormone sensitive lipase (HSL) 이 증가하는 한 편, 비떨림 열생성 기전에 관한 인자인 peroxisome proliferator activated receptor alpha (PPAR$\alpha$), peroxisome proliferator activated receptor gamma-coactivator 1 alpha (PGC1$\alpha$) 그리고 uncoupling protein 1 (UCP1) 의 발현을 증가시킴에 따라 Rb1 이 베이지지방세포 활성화를 유도함을 확인하였다. 이와 같은 Rb1 의 효과는 렙틴 수용체의 점돌연변이 (point mutation) 로 인해 비만 형질을 나타내는 *db/db* 마우스를 활용한 동물모델에서도 같은 양상으로 확인되었다. 또한 Rb1 의 열발생효과의 상위 인자를 규명하기 위해 베타 3 아드레날린 수용체 길항제인 L748337 을 Rb1 과 동시에 3T3-L1 에 처리한 결과, Rb1 단독군에서는 베타 3 아드레날린 수용체가 활성화된 반면, L748337 의 전 처리로 인해 베타 3 아드레날린 수용체가 차단된 환경에서는 Rb1 의 효과가 감소한 것을 확인하였다.

결론적으로, 본 연구는 Rb1 이 베타 3 아드레날린 수용체의 활성화를 통해 에너지 대사와 지방분해 경로를 조절하며 백색지방세포가 열 발생 작용이 증가되는 베이지지방세포로의 전환분화 유도 및 활성화 효능을 가짐을 보여주었으며, 따라서 이 같은 결과는 진세노사이드 Rb1 이 새로운 기전의 항비만 소재로 활용될 수 있는 가능성을 시사한다.



핵심어: 진세노사이드 Rb1, 비만, 비 떨림 열 발생, 지방분해, 베타 3 아드레날린 수용체