Comparative Study on Beneficial Functions of Resveratrol and Its Dimer Epsilon-Viniferin in the Adipocyte

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Contents

List of abbreviations

- ACC: acetyl CoA carboxylase
- AMPK: AMP activated protein kinase
- ATGL: adipose triglyceride lipase
- C/EBP-α: CCAAT/enhancer binding protein-α
- C/EBP-γ: CCAAT/enhancer binding protein-γ
- CRP: C reactive protein
- CS: calf serum
- CVD: cardiovascular disease
- DEX: dexamethasone
- DMEM: Dulbecco's modified Eagle's medium
- DMSO: dimethyl sulfoxide
- FABP4: fatty acid binding protein 4
- FAS: fatty acid synthase
- FGF-21: fibroblast growth factor-21
- HDL: high density lipoprotein
- HMW: high molecular weight
- HSL: hormone sensitive lipase
- IBMX: 3-isobutyl-1-methylxanthine
- IL-1: including interleukin-1
- IL-6: interleukin-6
- LKB1: liver kinase B1

- LDL: low density lipoprotein
- LMW: low molecular weight
- MCP-1: monocyte chemotactic protein-1
- MMW: middle molecular weight
- NEFA: non-esterified fatty acid
- NF-κB: nuclear factor-kappa B
- PDGF: platelet derived growth factor
- PGC-1 α: peroxisome proliferator-activated receptor γ coactivator-1 α
- PPAR-γ: peroxisome proliferator activator receptor-γ
- PVDF: polyvinylidene difluoride
- RES: resveratrol
- ROS: reactive oxygen species
- SIRT1: sirtuin 1
- SREBP-1c: sterol regulatory element-binding protein-1c
- T2DM: type 2 diabetes
- TNF-α: tumor necrosis factor-α
- VIN: ε-viniferin
- VLDL: very low density lipoprotein
- cAMP: cyclic adenosine monophosphate

Chapter 1

1. General introduction

Resveratrol (RES) and its dimer ε-viniferin (VIN) are polyphenolic compounds found in red wine. The present study compared the effects of VIN and RES on the differentiation of preadipocytes and found that low concentrations of VIN, but not RES, promoted favorable adipocyte differentiation, with enhanced expression of the beneficial hormone adiponectin and decreased lipid accumulation.

Obesity is a primary feature of metabolic disease and has become a major public health concern worldwide. As the main clinical manifestation of the disturbance of energy intake and consumption, obesity is associated with the development of various diseases, including coronary heart disease, hypertension, type 2 diabetes mellitus (T2DM), cancer, and osteoarthritis. The hyperplasia and hypertrophy of adipocytes contribute to an increase of body adipose mass, which is a hallmark of obesity. Adipose tissue plays an important role in lipid homeostasis and energy balance. Its primary role is to store energy in the form of triglycerides when energy intake exceeds energy expenditure and release it in the form of free fatty acids during starvation. Adipocyte differentiation, known as adipogenesis, is a process that is accompanied with coordinated changes in cell morphology, hormone sensitivity, and gene expression. Adipocytes also synthesize and secrete various biologically active molecules known as adipocytokines. Among them, adiponectin is regarded as beneficial because it efficiently reduces insulin resistance, exerts anti-inflammatory effects, and regulates lipid metabolism. During adipocyte differentiation, transcriptional factors, such as peroxisome proliferator-activated receptor-γ (PPAR-γ) and CCAAT/enhancer-binding protein-γ (C/EBP-γ), are involved in the sequential expression of adipocyte-specific

proteins. The regulation of adipogenesis has been studied using various cellular and animal models. The 3T3-L1 cell line is one of the best characterized and most reliable model cell for studying adipogenesis in vitro. It is generally thought that compounds suppressing adipogenesis in 3T3-L1 cells could be effective in treating and preventing obesity.

Numerous studies have revealed that dietary polyphenols can play a beneficial function in preventing obesity and obesity-related chronic diseases. RES, a natural polyphenolic compound found in grapes and red wine, is probably the most widely studied among them. Many cellular and animal studies have shown that RES exerts anti-obesity effects by reducing the viability and proliferation of preadipocytes, suppressing adipocyte differentiation with decreased triglyceride accumulation, inhibiting lipogenesis, and/or stimulating lipolysis and fatty acid β-oxidation. Comparatively less is understood about its dimer, VIN, although the VIN content in grapes and red wine is comparable to or greater than that of RES, depending on the type of red wine and the stage of the noble rot on the grapes. The objectives of this study were to investigate the effect of VIN and RES on 3T3-L1 preadipocyte differentiation, focusing on the expression of adiponectin and regulators of differentiation and lipid accumulation, and to compare the effects of the two compounds.

Chapter 2

A review of adipose tissue biology and the beneficial effects of RES and VIN

1. Adipose tissue

1.1. Adipose tissue function

Obesity is a condition in which adipocytes accumulate a large amount of fat and become enlarged. At the cellular level, it is characterized by an increase in the number and size of adipocytes differentiated from fibroblastic pre-adipocytes in adipose tissue.

Adipose tissue is composed of adipocytes and plays an important role in furnishing mechanical support, lipid homeostasis, and energy balance. The primary role of adipose tissue is to store energy in the form of triglycerides when energy intake exceeds energy expenditure and release it in the form of free fatty acid during starvation [1]. For this reason, the size of the adipose tissue decreases when energy expenditure exceeds intake, whereas stores increase during periods of positive energy balance. Adipocytes also include lipases that decompose triglycerides into glycerol and free fatty acids, which are then transported in the blood to the muscles, liver, and brown adipose tissue, where they are used in lipid oxidation [2]. Previous data have demonstrated that fatty acids and glycerol can be re-esterified in adipose tissue, thereby regulating the free fatty acid balance. The two main important functions of adipocyte tissue are to store excessive energy as triglycerides in lipid droplets and to discharge energy in the form of free fatty acids. Adipocyte tissue stores triglycerides as fuel for thermogenesis through mitochondrial “uncoupling” in oxidative phosphorylation of free fatty acids.

1.2. Adipose tissue as an endocrine organ

Mature adipocytes act as an active endocrine and paracrine organ and are capable of synthesizing beneficial adipocytokines that affect energy metabolism, such as adiponectin. When adipocytes hypertrophy, they secrete harmful adipocytokines, such

as the pro-inflammatory protein monocyte chemoattract protein-1 (MCP-1), which induce macrophages infiltration and activation in adipose tissue. Macrophages are important sources of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). Adipocytes contribute to the increased pro-inflammatory state in obesity and diabetes. Adipokines have several important functions: adipose tissue (mature adipocytes) behaves as a paracrine and endocrine organ and forms a communication network with the brain, sympathetic nervous system, and other tissues that influence immunity, energy balance, lipid metabolism, appetite, blood pressure, blood sugar, insulin sensitivity, homeostasis, angiogenesis, and diabetes [3]. They are able to synthesize pro-inflammatory and anti-inflammatory proteins. Recent research has shown that there are a diversity of adipokines [4, 5–7], including growth factors, proteins involved in vascular hemostasis, angiogenesis, classical cytokines, glucose hemostasis, and acute phase responses. Adipokines are mainly secreted by adipocytes. Adipose tissue produces various factors, primarily adiponectin and leptin (and possibly also visfatin, adipsin, and resistin), which are classified as adipokines.

1.2.1. Leptin

Leptin is secreted by adipocytes, and it is still virtually undiscoverable in the stromal vascular cells (SVC and nonadipocytes, including macrophages) on the fraction of adipose tissue. Initially, leptin [8] was thought to be a signal for the brain to reduce food intake and decrease weight [9]. This idea was partly driven by the observation that rodents and humans lacking a functional leptin protein showed signs of voracious eating and obesity. The reduction of leptin that occurs rapidly in response to fasting also produces profound changes in the hormones and energy balance controlled by the

hypothalamus. Low leptin levels cause overfeeding and inhibit energy expenditure, immunity, the functions of reproductive hormones, and thyroid function. The adaptations that are mediated by a decrease in leptin may have developed as protection against the threat of starvation, by restricting energy use and improving energy storage [10]. This indicates that the weight-decrease state is a condition of relative leptin scarcity. According to a recent study, in addition to its effects on the hypothalamus, leptin may also act on the limbic areas and the cortex, which are involved in the emotional and cognitive regulation of feeding behavior [11, 12]. However, in general obesity, leptin resistance can facilitate ectopic lipid accumulation, which can in turn further weaken insulin sensitivity [13]. Leptin therapy, such as leptin replacement treatment in congenital leptin scarcity, inhibits obesity, hypogonadism, and hyperphagia and reduces T-cell-mediated immunity [14].

Aside from completing deficiency, relative leptin deficiency is an emerging syndrome that has been noted in several clinical conditions, including acquired or congenital lipodystrophy, as well as anorexia, hypothalamic amenorrhea nervosa, and exercise-induced energy deficiency. Leptin substitution therapy could be a potential therapeutic option for patients with these disorders [15–17].

1.2.2. Adiponectin

Adiponectin, also referred to as AdipoQ, Acrp30, GBP28, or apM1, is an adipose-specific hormone produced in adipose tissue [18, 19] and, similarly to leptin, is secreted by mature white and/or brown adipocytes [20].

Adiponectin is present in the bloodstream in three main forms: trimer, a low molecular weight form; hexamer, a middle molecular weight form; and 12- to 18-mer, a

high molecular weight adiponectin [21]. Differentiating from most types of adipocytokines, circulating adiponectin is negatively correlated with the proportion of body fat and is decreased in obese subjects, in cardiovascular disease (CVD) patients, and in T2DM and metabolic disorders [22]. The mechanisms underlying suppressed adiponectin secretion may include the abnormal hormonal milieu, higher level of oxidative stress [23], and chronic inflammatory state that prevails in obesity and metabolic syndrome [24]. The major receptors of adiponectin are AdipoR1 and AdipoR2. These receptors are comprised of seven transmembrane domains, but are functionally and structurally different, with G-protein-coupled receptors. AdipoR1 is found abundantly in muscle tissue, and AdipoR2 is found in high levels in the liver [25]. AdipoR1 is more closely linked to the AMPK pathway that governs the repression of gluconeogenesis and enhances fatty acid oxidation, whereas AdipoR2 is responsible for the activation of the PPAR-α pathway, which promotes energy consumption by improving lipid oxidation and inflammation and decreasing oxidative stress [26]. The interplay of the adaptor protein containing PH domain, PTB domain, and leucine zipper motif 1 (APPL1) with AdipoR1 seems to have a central role in adiponectin signaling. In skeletal muscles, a decrease of plasma triglycerides has been attributed to enhanced very-low-density lipoprotein catabolism, and the lipid profile is also improved in adiponectin transgenic mice. Finally, adiponectin may weaken TNF-α, including interleukin-1 (IL-1), IL-6 expression, and inflammation, promoting an improvement in overall insulin sensitivity [27].

Adiponectin confers cardiovascular protection, and hypoadiponectinemia is detected in patients with angiographically indicated coronary artery disease [22]. Adiponectin exerts beneficial effects in almost all stages of atherogenesis. The molecular

mechanisms include (1) the reduction of the expression of the adhesion factors on vascular endothelial cells, (2) inhibited migration and proliferation of vascular smooth muscle cells, (3) suppression of macrophage transformation to foam cells, and (4) anti-inflammatory and antioxidant functions [28, 29]. In addition to atherosclerosis, adiponectin also has a potential role in the physiopathology of hypertension [30]. Clinical research has indicated that a low adiponectin level is a risk factor for hypertension, independent of diabetes and insulin resistance [31]. The antioxidant and anti-inflammatory effects of adiponectin may be partly responsible for its beneficial effects in cardiovascular and metabolic disorders, including insulin resistance and atherosclerosis [22].

Adiponectin suppresses TNF-α, leading to the activation of nuclear factor-kappa B (NF-κB) in endothelial cells. Adiponectin and TNF-α exert negative mutual interactions on their local production in adipose tissue, indicating that the reduced secretion of adiponectin in obesity may intensify inflammation in the adipocyte. For this reason, lower adiponectin production creates a vicious cycle [32]. In endothelial cells treated with high glucose or oxidized low-density lipoprotein (LDL), adiponectin inhibits superoxide radical generation, and oxidative stress reduces the secretion of adiponectin in adipose tissue. This could be a local protective mechanism to protect against the deleterious effects of oxidative damage, inflammatory reactions, and insulin resistance. Adiponectin has pleiotropic effects on metabolic syndrome, and a lower adiponectin level is a risk factor for nonalcoholic steatohepatitis (NASH), which is not dependent on insulin resistance [33]. The beneficial effects of adiponectin on NASH development may be regulated by the suppression of *de novo* lipogenesis, which is the acceleration of lipid oxidation. These effects may also be regulated by the anti-inflammatory,

antioxidative, and anti-fibrosis properties of adiponectin. Furthermore, as obesity is a clinical factor for several cancer types [34], adiponectin may decrease cancer risk directly by exerting anti-proliferative effects on tumor cells or indirectly by reducing insulin resistance.

1.2.3. TNF-α

TNF-α is a pro-inflammatory protein that is mainly secreted by macrophages and monocytes and has an important role in inflammation. TNF-α expression is enhanced in adipose tissue in T2DM and obesity [35]. TNF-α-promoted signaling in obesity leads to an increase in insulin sensitivity, which corresponds to an increase in insulin signaling in adipose tissue and muscles [35–37]. At the molecular level, TNF-α weakens insulin-stimulated tyrosine phosphorylation of the insulin receptor in adipose tissue and muscles, enhancing insulin resistance [36]. These data demonstrate that TNF-α functions as a pro-inflammatory protein and has a major role in obesity associated insulin resistance. TNF-α levels are enhanced in the plasma and adipose tissue in obesity, and a decrease in body weight has been observed to lead to a reduction in TNF-α expression [38, 39]. TNF-α levels are positively correlated with markers of insulin resistance [40]. Clinical studies that have tested the capability of TNF-α confrontation to increase insulin sensitivity have not supply consistent the clinical studies that have tested the capability of TNF-α confrontation to increase insulin sensitivity results. For example, short-term treatment with TNF-α blocking reagents in obesity in T2DM patients led to a decrease in systemic inflammatory markers, but did not improve insulin resistance [41, 42]. Similarly, inhibition of TNF-α in obesity with metabolic syndrome led to an improvement in muscle adiposity, but did not have an

effect on insulin sensitivity [43]. Inhibition of TNF-α in patients with severe inflammatory diseases, such as psoriasis and rheumatoid arthritis, is reported to induce insulin sensitivity [44], which indicates that suppression of TNF-α could be effective in increasing insulin resistance in certain conditions, particularly those that involve serious inflammatory states.

1.2.4. IL-6

IL-6 is an interleukin that acts as a pro-inflammatory cytokine and may also be involved in obesity associated with insulin resistance. In a clinical study, plasma IL-6 levels were positively correlated with obesity [45]. Improved IL-6 levels are predictive of the progression of T2DM, and plasma levels of IL-6 are enhanced in patients with T2DM [46]. It is thought that adipose tissues secrete about one-third of total IL-6 [45], and improving the production of IL-6 in obesity may also promote metabolic dysfunction.

IL-6 infusion in mice reduced the capacity of insulin to inhibit gluconeogenesis in the liver [47]. In contrast, IL-6 shortage accelerated hepatic inflammation and insulin resistance in high-calorie diet mice [48]. However, decreasing IL-6 in adipose tissue prevented insulin resistance through the regulation of the suppressor of cytokine signaling 3 (SOCS3) expression in the liver [49].

The distinct actions of IL-6 on insulin signaling may be due to its disparate actions depending on the source of the IL-6 (such as muscle versus fat) or its varying effects on different organs (such as liver versus muscle) [50, 51].

1.3. Visceral obesity induces metabolic syndrome

1.3.1. Overweight

Though obesity is a major risk factor for CVD and a risk index for T2DM, not all obese patients are insulin-resistant or at a high risk of CVD and T2DM. This is why obesity is an ill-defined modifiable CVD risk factor compared with other risk factors, such as smoking, hypertension, and high cholesterol, high LDL/low high-density lipoprotein (HDL) [52].

However, for any given quantity of total body lipid, the subgroup of individuals with a selective surplus of visceral or intra-abdominal adipose tissue is at a higher risk of having the characteristics of metabolic syndrome and insulin resistance [53–55]. As excessive visceral fat accumulation is associated with diabetogenic abnormalities and various atherogenic problems [53–55], the question of whether visceral fat is simply a marker of a dysmetabolic profile, or a resulting factor, has been discussed.

1.3.2. Physiopathology of visceral obesity

There is sufficient evidence that weakened non-esterified fatty acid (NEFA) metabolism may promote the insulin-resistant state observed in individuals with visceral obesity. Hypertrophied abdomen lipid accumulation is characterized by a hyper lipolytic state that is resistant to the antilipolytic effect of insulin [56]. The cause NEFA stream to the liver may weaken liver metabolism, resulting in enhanced hepatic glucose glycogenesis. Hepatic insulin resistance improves the production of triacylglycerol-rich lipoproteins and reduces apolipoprotein B degradation. Though there is a link between portal delivery and visceral lipid cumulative of NEFA to the liver, the majority of portal

NEFAs come from the systemic circulation. This indicates that other factors might be responsible for the altered metabolic profile of viscerally obese patients [57].

Recent research has shown that adipose tissue is not only involved in the mobilization and storage of lipids but is also an endocrine organ producing numerous adipokines, such as the pro-inflammatory molecules TNF-α and IL-6. Macrophage infiltration is expressed in adipose tissue in obesity [58], which could explain the inflammatory profile that has been reported in abdomen obesity [59]. In plasma, C-reactive protein (CRP) is an inflammatory marker and is a predictor of the risk of myocardial infarction either greater than that reckoned by traditional risk factors, are improved in visceral obese patients [60]. Adiponectin is plentiful in the blood and is secreted by adipose tissue. As reduced of anti-inflammatory adipokines, adiponectin levels are decreased in adiposity, particularly among patients with excess visceral obesity [61]. Adiponectin has a number of effects on atherosclerosis and improves insulin signaling [62]. The decreased adiponectin levels found in visceral obesity could be a key factor responsible for diabetogenic metabolic risk factors and atherogenesis. The plethora of visceral adipose tissue in belly fat has been shown to promote plasma CRP concentrations and increase TNF-α and IL-6 levels and is accompanied with decreased adiponectin levels [61, 63]. Low adiponectin concentrations are a major feature of the visceral obese patient. These outcomes are consistent with the important endocrine function of the expanded visceral lipid deposits that not only change NEFA metabolism but also lead to a pro-inflammatory profile that can alter glucose homeostasis and provide insulin resistance in visceral obesity [64]. Both the altered NEFA metabolism and endocrine function indicate that visceral adipose tissue is causally related to the physiopathology of metabolic syndrome, which is frequently found in visceral obesity. An insufficiency

of the capacity of subcutaneous adipose tissue to store overfull energy causes enhanced accumulation of lipids at undesirable sites, including the skeletal muscle, liver, heart, and pancreatic β-cells, a phenomenon that has been termed ectopic lipid deposition [65].

In mammals, the serious insulin-resistant state of lipodystrophy conditions is also the same with the function of subcutaneous adipose tissue as a warehouse buffering the excess energy. Dyslipidemia and the insulin-resistant state that characterizes metabolic syndrome may be partly caused by the peculiar endocrine and metabolic properties of the expanded visceral adipose tissue. [66, 67].

2. Cell experiments on adipocyte differentiation

2.1. 3T3-L1 cell line

Obesity is associated with metabolic syndrome that comprises T2DM, insulin resistance, glucose intolerance, hypertriglyceridemia, hypertension, high cholesterol, and low HDL cholesterol.

Adipokines are produced by adipose tissue and play an important role in the status [68, 69]. Adipocytes also produce pro-inflammatory cytokines, which are typically secreted from macrophages, IL-1, IL-6, TNF-α, and MCP-1, that is concerning to insulin resistance [70, 71]. These pro-inflammatory cytokines exert effects on the autocrine, endocrine, and paracrine functions and alter the metabolic status.

The 3T3-F422A and 3T3-L1 cell lines derived from disaggregated Swiss 3T3 mouse embryos are the most diffusely utilized cultures in adipocyte models [72]. Specifically, the 3T3-L1 preadipocyte is the most reliable and well-characterized model for researching the conversion of preadipocytes into adipocytes. Though frequently thought to be immortalized, the ability of the 3T3-L1 cell line to differentiate into adipocytes is known to decline with an expanding number of passages. The representation of many adipokines is detected in the status of differentiation. The 3T3-L1 preadipocyte is a useful tool for studying the molecular mechanisms of diseases related to obesity, including metabolic syndrome and diabetes, and is often used to efficiently screen and evaluate the adipogenic latent of cellular perturbations or different agents.

The 3T3-L1 cell line is useful in researching three-dimensional or co-cultures cell cultures and the high-throughput sieving of compounds [73, 74]. Nevertheless, the 3T3-L1 cell model does have some limitations, including the amount of time needed for preliminary subculture. In addition to the actuality that differentiation and adipogenic

demand to over two weeks [75]. In addition, when 3T3-L1 cells have been passaged repeatedly or become confluent, they are difficult to transfect and differentiate into adipocytes. Thus, the cell line originates from a single clone and does not have the characteristics of primary cell models [76].

2.2. Transcriptional factors and metabolic factors of adipocytes

2.2.1. PPAR-γ

PPAR-γ is a member of the ligand-activated transcription factors of nuclear hormone receptor superfamily, which is important for adipogenesis. It was confirmed to induce during preadipocyte differentiation that bound an enhancer element within the fatty acid-binding protein 4 (FABP4) promoter [77]. The importance of PPAR-γ is demonstrated by the knockout PPAR-γ gene that results in lipodystrophy, which has a significant effect on the endocrine properties and lipid accumulation of mature adipocytes [78, 79]. The characterization and identification of the target genes of preadipocyte differentiation transcription factors have aided the understanding of the role of these proteins in adipocytes. Differentiation and adipogenesis of preadipocytes occur due to a transcriptional cascade that relates to the closely regulated induction of numerous transcription factors, such as the PPAR families, C/EBP families, and sterol regulatory element-binding protein-1c (SREBP-1c). Many of these differentiation transcription factors possess the capability to adjust one another's gene expression and have been shown to induce preadipocyte differentiation *in vivo* and *in vitro*.

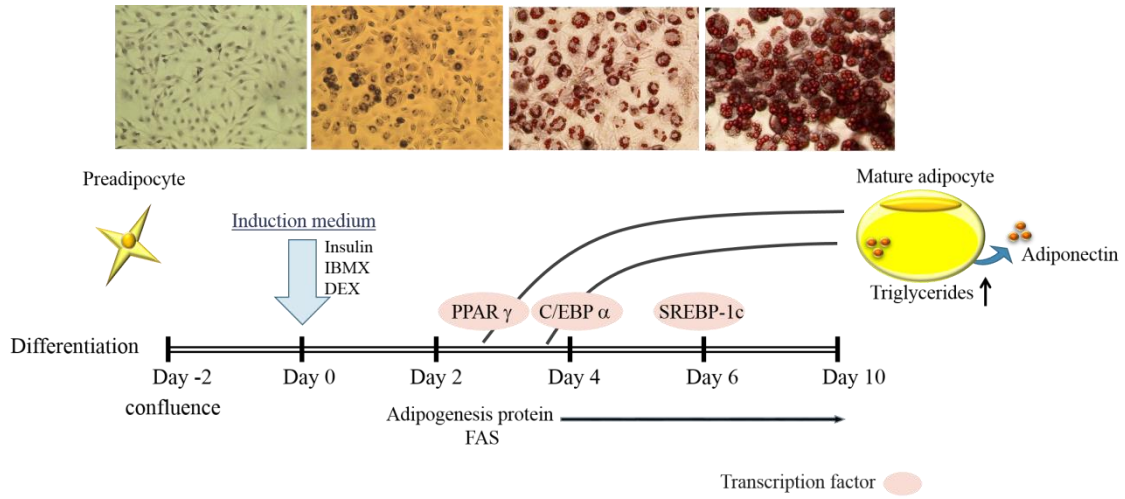

Fig. 1: 3T3-L1 preadipocyte differentiation.

The differentiation of preadipocytes is characterized by morphological changes and is accompanied with complex sequence changes in gene expression and lipid storage. The major transcriptional factors regulated by adipocyte differentiation are C/EBP-α, PPAR-γ, and SREBP-1c. These factors regulate adipocyte differentiation by modulating the expression of their target genes in a coordinated fashion. Therefore, it is presumed that adipocytes are inclined to be in differentiation progress or pause through detecting those gene expressions of transcriptional factors. In Fig. 1, the images represent adipocyte shapes in different differentiation stages. In the upper left image, the original preadipocyte is spindle-shaped. During the differentiation stage, triglycerides accumulate in cells and become round-shaped.

Mature adipocytes are responsible for energy storage and adipokine (e.g., adiponectin) secretion.

2.2.2. Adipose tissue triglyceride lipase (ATGL) and Fatty acid synthase (FAS)

ATGL, an enzyme that catalyzes the rate-limiting hydrolysis step of triglycerides [80] in the triacylglycerol lipolysis cascade, is expressed in adipocytes but is also found in lesser amounts in skeletal and cardiac muscle [81]. It initiates the breakdown of triglycerides into fatty acid monomers [82]. Individuals deficient in the ATGL enzyme are at a higher risk of cardiac dysfunction and premature death due to the increased size and accumulation of lipid droplets within cardiac myocytes.

FAS is a multi-enzyme protein that catalyzes fatty acid synthesis. Its main function is to catalyze the synthesis of palmitate from acetyl-CoA and malonyl-CoA in the presence of NADPH. Fatty acids are synthesized by a series of decarboxylative Claisen condensation reactions from acetyl-CoA and malonyl-CoA. Following each round of elongation, the beta-keto group is reduced to the fully saturated carbon chain by the sequential action of ketoreductase, dehydratase, and enoyl reductase. The homeostasis and metabolism of FAS is transcriptionally regulated by upstream stimulatory factors 1 and 2 (USF 1 and USF 2) and SREBP-1c in response to feeding in animals [83, 84].

2.2.3. AMPK, SIRT1, and FGF-21

AMPK is an important energy transducer and a major regulator of metabolic homeostasis [85]. Liver kinase B1 (LKB 1), a major regulator of AMPK activation, is a serine/threonine kinase. LKB 1 activates kinase activity by phosphorylating Thr-172 of AMPK [86]. LKB 1 acts as a crucial mediator of gluconeogenesis in the liver, and its expression is increased by exercise [87]. Recently, AMPK has been shown to play a major role in the therapeutic benefits of thiazolidinediones [88], exercise [89, 94], and metformin [85, 91], all of which are cornerstones of the regulation of metabolic

syndrome and T2DM. Activation of AMPK effects energy homeostasis by improving mitochondrial biogenesis, oxidative metabolism, and switching to catabolic pathways [85]. Recently, AMPK was shown to improve SIRT1 and NAD+-dependent type III deacetylase activity by enhancing cellular NAD+ levels, leading to the regulation of the activity of downstream SIRT1 targets [92]. SIRT1 has a significant role in longevity and metabolic function [93, 94]. Activation of SIRT1 also results in increased mitochondrial oxidative function and selective nutrient utilization to regulate energy balance. Both SIRT1 and AMPK act in concert with the master regulator of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) and mitochondrial biogenesis to regulate energy balance in response to nutritional and environmental stimuli [85, 95].

FGF-21 has been shown to be an effective metabolic regulator. Systemic administration of FGF-21 in mice and mammals with genetic or diet-induced obesity and triglyceride-lowering effects and diabetes exerts strong antihyperglycemic effects and reduces body weight [96–101]. FGF-21 is increased in fasting and is required for the activation of triglyceride clearance, hepatic lipid oxidation, and ketogenesis during fasting [100–102].

2.3. Effects of food compounds in 3T3-L1 preadipocyte

Some of the major benefits of the 3T3-L1 cell line are that it costs less and is easier to culture than primary cells, such as mature adipocytes, even though primary cells enable different comparisons, for example, the *in vitro* assessment of various *in vivo* status. Furthermore, they allow a repeated number of passages and are homogeneously called of the cell population. These cells produce a homogenous response following changes and treatments in experimental conditions [103]. 3T3-L1 preadipocytes have been

widely used over the last 10 years to assess the effects of nutrients on the adipogenesis of compounds, to study the underlying metabolism mechanisms of adipogenesis, and to appraise the potential application of compounds in the treatment of obesity and different compounds [104–106]. In particular, the natural compounds RES [107] and quercetin [108] have been shown to inhibit adipogenesis in 3T3-L1 preadipocytes.

RES is a natural polyphenolic compound and is a famous constituent of red wine, red grapes, and ground nuts [109, 110]. RES displays anti-inflammatory and antioxidant actions [109] and exhibits positive effects in preventing the development of many diseases, such as metabolic syndrome, diabetes, and obesity [112].

Quercetin is a common flavonoid compound in fruits, wine, vegetables, and tea [113]. An *in vitro* study on the potential anti-obesity function of quercetin on primary adipocytes found that quercetin prompted lipolysis of the primary adipocytes in a time- and dose-dependent manner, by improving hormone-sensitive lipase activity and cyclic adenosine monophosphate (cAMP) levels. Quercetin had also been observed to inhibit adipogenesis by decreasing the rate of incorporation of free fatty acids into adipocyte triacylglycerols in fat pads, improving acetyl-CoA carboxylase, and inhibiting the gene expression levels of FAS [114].

Quercetin restrains lipogenesis by reducing the gene expression of the key preadipocyte differentiation and lipogenic factors PPAR-γ and C/EBP-α [114]. 3T3-L1 preadipocytes have been used to depict the effects of melatonin, for example, its antioxidant effects and its ability to inhibit reactive oxygen species (ROS) and decrease adipocyte differentiation [115–117].

Certain obesogenic compounds and endocrine interference compounds have also been assessed during 3T3-L1 preadipocyte differentiation [118]. Various gene stillness

technology, such as shRNA and siRNA, along with various transfection processes, such as lentivirus transfection adenovirus and plasmid electroporation, have been used to research the effects of various genes related to lipogenesis on 3T3-L1 cells [119]. In addition, gene silencing in 3T3-L1 cells has been used to study adipokine synthesis, inflammatory pathways, and adipokine functions [116–118].

3. RES and VIN

The relationship between diet and health has been examined through research on foods that contain bioactive compounds. Wine is a mandatory ingredient and has been proposed to contribute to the health of the people in the Mediterranean.

The ‘‘French Paradox,’’ a published research ascertaining the connection between death by CVD and taking a meal, started the trend for research into the relationship between wine and health [120]. The rate of myocardial infarction in France is about 40% lower than those in other Europe countries, though meals are typically high in saturated fats.

The French population perfectly confirms the “French Paradox” model, if red wine intakes were deliberated [121]. Reports that red wine is more beneficial for health than other liquor have led to a focus on the study of phenols. Research has sought to distinguish the functions of the nonalcoholic and phenolic constituents of red wine from those from alcohol. In *in vivo* models, it has been shown that a red wine polyphenolic extract protects against CVD and cancer [122]. Contrasted to heart weight, ROS and blood pressure *in vivo* experiment whose feed had been complemented with the polyphenolic compound, both polyphenolic compound or ethanol and ethanol together. The study concluded that the polyphenolic compound was the most important supplement for decreasing CVD risk and indicated that red wine as a component of a restrict completed diet postponed tumor onset in mice. These benefits could be due to the synergistic effects of bioactive polyphenolic compounds [123]. Synergistic effects have been indicated among the three RES, catechin, caffeic acid, and other phenols. Although plasma chroma is comparatively low following wine consumption, the

synergistic effects of the compounds increase their beneficial properties, for example, their antioxidative activity. The interplay between polyphenols may also affect their molecular metabolism and kinetics [124].

VIN, a RES dimer, is a natural polyphenolic compound. Like RES, it is found in peanuts, grapes, and wine. Unlike RES, for which *in vivo* and *in vitro* experiments have demonstrated its multiple biological effects, including its antioxidant, anti-inflammatory, anticarcinogenic, and platelet antiaggregatory properties [125, 126], the biological effects of VIN have not been widely studied. VIN is known to have antioxidant activity [127], restrain human cytochrome P450 enzymes, exert hepatoprotective effects on hepatocytes, and promote leukemia B-cells apoptosis [128]. However, the capacity of VIN to mitigate metabolic disorder or exert anti-obesity activity has not been thoroughly investigated.

3.1. Effects of RES

3.1.1. Antioxidant activity

Cell metabolism produces normal reactive oxygen intermediates, including hydrogen peroxide, hydroxyl radicals, and superoxide, which are antioxidant by intracellular antioxidative enzymes, such as SOD, catalase, and glutathione. In animal experiments, RES has been shown to reduce fat peroxidation and improve plasma antioxidant activity [129], which is closely related to the risk of myocardial infarction and coronary heart disease [130]. Research *in vivo*, such as in humans, mice, and pigs, indicate that RES can inhibit increases in the lipid peroxidation of other macromolecules [131].

3.1.2. Cardioprotective capacity

RES has been shown to protect the cardiovascular system. Low doses of RES have been shown to restrain cell apoptosis, thus supply conservation from different diseases, such as ventricular arrhythmias, myocardial ischemic reperfusion injury, and atherosclerosis. However, in high concentrations, RES promotes cell death by apoptosis and acts as a CVD preventive alternative.

RES regulates the metabolism of lipoprotein and lipids and may inhibit physiological increases of lipid peroxidation. Research has found that RES reduces hepatic lipid composites, prevents the storage of triglycerides, and lowers cholesterol in the liver [132]. Platelet polymerization is one of the main factors contributing to atherosclerosis. RES prevents platelet polymerization in cell and animal experiments [133, 134]. Studies have shown that RES decreases atherosclerotic plaque formation and recovers flow-regulated expansion in rabbits fed a high cholesterol diet [135]. *In vivo*, the addition of RES to water for 16 days (14 mg/kg body weight) improved the ability of cardiac muscle to eliminate oxidants [136]. The main mechanism seems to involve an increase in nitric oxide (NO) chroma by reducing the inactivation of NO by free radicals and improving NO synthase levels.

3.1.3. Anti-diabetic activity

Research indicates that RES can play a major role in preventing T2DM and type 2 diabetic complications [137]. *In vivo* research revealed that RES regulation of normal rats at a RES concentration of 50 mg/kg (body weight) reduced blood insulin at 30 min, without changes in glycemia. This indicates that RES inhibited insulin in rats [138].

3.1.4. Anti-aging activity

RES extends the life of *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and *Caenorhabditis elegans* by activating the sirtuin pathways. Recent studies indicate that RES diverts the physiological of the middle-aged mouse on high-calorie diet change direction that of the mouse on a normal diet and significantly improves the lived. In particular, research on mice has shown that obese mice whose diets were complemented with RES survived longer and were healthier and more active on a high-calorie diet. Moreover, they had an improved number of mitochondria, decreased insulin-like growth factor 1, and enhanced motor function [139].

3.1.5. Anti-obesity activity

Long-term animal research indicates that RES exerts beneficial effects in animals fed a high-fat diet. Experiments on high-calorie diet mice found that RES (incorporated into the diet at 0.04% for 15 or 60 weeks) improved motor function and extended survival, altering the expression of a number of genes in the direction of mice fed a standard RES diet [140]. In addition, in high-fat diet mice, RES reduced the total declined depots of epididymal, retroperitoneal, and inguinal white adipose tissue and body fat content [141]. Recently, the beneficial effects of RES on rats fed a high-fat diet have been shown to include a substantial reduction in liver mass index and visceral fat index [142].

It is not the single effect of SIRT1 activation. Thus, reducing the pathological outcome of a high-calorie diet, RES would mimic calorie restraint. It has been proposed that some benefits of RES result from the phosphorylation/activation of AMPK. AMPK restrains acetyl-CoA carboxylase, improves the oxidation of fatty acids, and reduces their synthesis once activated. RES has been shown to substantially induce AMPK

activity in rats fed a high-fat diet similar to obese rats [142]. The relationship between AMPK and SIRT1 in energy metabolism and mitochondrial biogenesis has been described in detail. Some data have indicated that the central nervous system is involved in the action of RES, as intracerebroventricular injection of this compound was shown to exert numerous beneficial effects in mice fed a high-energy diet [143].

The direct impact of RES on the metabolism of white adipose tissue has also been studied. Exposure of freshly isolated rat adipocytes to RES for 90 min resulted in reduced basal and insulin-induced glucose conversion to total lipids. This effect was accompanied by a decrease in glucose oxidation and an increase in the release of lactate. Furthermore, RES attenuated the capacity of insulin to counteract lipolysis and potentiated the lipolytic response to epinephrine in adipose cells. The magnification of cAMP found in adipocytes incubated with RES is thought to be responsible for the improved lipolytic rate [143].

3.2. Effects of VIN

3.2.1. Cardioprotective capacity

As opposed to the many studies that have explored the effects of RES on the heart and vasculature, few studies have looked at the effects of VIN, a dimer of RES. VIN is like or better than RES, relying on the class of the stage of noble rot development on grapes and red wine [144, 145]. Recent research has indicated that compared with RES, VIN has enhanced antioxidant activity. VIN has been shown to be more effective than RES in inducing the vasorelaxation of isolated thoracic aortas and restraining platelet-derived growth factor (PDGF)-dependent cell migration and proliferation of cultured vascular smooth mice cells by decreasing PDGF-induced intracellular ROS

levels [146]. These data demonstrate that VIN, as well as RES, may help reduce the pathogenesis of atherosclerosis.

3.2.2. Anti-inflammatory activity

VIN demonstrates higher anti-inflammatory activity in white adipose tissue than RES *in vivo*. RT-PCR analyses revealed that a typical inflammatory marker gene, TNF-α, decreased significantly in VIN-fed mice, but not in RES-fed mice. In adipocytes, the anti-inflammatory function of VIN was superior to RES. In the RAW264.7 cell line, the anti-inflammatory function of VIN cause TNF-α expression in RAW264.7 cells were not reduced by VIN, the primary target of VIN is possibly to improve the anti-inflammatory effect in the adipocytes [147].

3.2.3. Antioxidant activity

By suppressing intracellular ROS accumulation, VIN protects VECs from H_2O_2-induced cell death. Our research indicates that VIN has antioxidant utility owing to the redox attributed to its phenolic hydroxyl groups [148]. Scavenging of H_2O_2 directly may reflect the cytoprotective effects of VIN against H_2O_2. VIN may also facilitate the expression of antioxidant enzymes in the vascular involved in scavenging H_2O_2, for example, GPx and catalase.

Our study indicated that, *in vitro* treatment with RES improved oxidative stress opposition to VECs through scavenging H_2O_2 directly and upregulating antioxidant enzymes.

Many of the effects of VIN are still unknown, and in obesity, it is necessary to clarify

the mechanisms underlying its beneficial effects.

Chapter 3

Comparative study on beneficial functions of RES and its dimer VIN in the adipocyte

1. Abstract

RES and its dimer VIN are polyphenolic compounds present in red wine. Although various beneficial functions of RES have been identified, those of VIN remain unclear. The present study, therefore, compared the effects of VIN and RES on the differentiation of 3T3-L1 preadipocytes. The results illustrated that VIN suppressed intracellular lipid accumulation more effectively than RES. VIN, but not RES, reduced the protein expression of the lipogenic enzymes PPAR-γ and fatty acid synthase and increased the expression of the lipolytic enzyme adipose triglyceride lipase. Adiponectin, which plays a crucial role in preventing T2DM and metabolic syndrome, was upregulated only by VIN. In addition, only VIN increased SIRT1 expression with increased AMPK phosphorylation. These findings suggested that VIN promotes favorable adipocyte differentiation with enhanced adiponectin expression and decreased lipid accumulation more effectively than RES. Therefore, VIN may be a candidate phytochemical in red wine for preventing obesity.

2. Introduction

Obesity has become a global public health concern because it is associated with the development of various diseases including T2DM, coronary heart disease, and hypertension [149-151]. Obesity originates from an energy imbalance caused by excess caloric intake relative to energy expenditure, and it is characterized by adipocyte hypertrophy and hyperplasia [152]. Thus, maintaining the normal state of the adipose tissue is critical for preventing the development of obesity and its related diseases.

Adipogenesis, which is the process of adipocyte differentiation, is a complex process that is accompanied with coordinated changes in morphology, hormone sensitivity, and gene expression. PPAR-γ and C/EBP-α are two key regulators of adipocyte differentiation that orchestrate the expression of adipogenic and lipogenic genes [153-155]. FAS and ATGL play central roles in lipogenesis and lipolysis, respectively [153-156]. AMPK activation leads to PPAR-γ and C/EBP-α downregulation [9, 10]. The NAD^+-dependent protein deacetylase SIRT1, a critical factor that affects obesity and lifespan, often functions coordinately with AMPK and inhibits PPAR-γ activity [157, 158].

The primary roles of the adipose tissue include storing energy in the form of triglycerides when energy intake is sufficient and releasing energy in the form of free fatty acids during starvation. The adipose tissue also acts as an endocrine organ that generates various biologically active molecules called adipokines. Among them, adiponectin, which is abundantly expressed in adipocytes, is considered beneficial because it efficiently alleviates insulin resistance, exerts anti-inflammatory effects, and regulates lipid metabolism [159]. Therefore, impaired adipocyte differentiation with

decreased adiponectin expression is believed to contribute to insulin resistance, T2DM, and other metabolic disorders [160-162].

Numerous studies have revealed the roles of dietary polyphenols in preventing obesity and obesity-related chronic diseases [155, 163]. RES, a natural polyphenolic compound present in grapes and red wine, is probably the most widely studied among them. The results of several cellular and animal studies illustrated that RES exerts anti-obesity effects by reducing the viability and proliferation of preadipocytes, suppressing adipocyte differentiation with decreased triglyceride accumulation, inhibiting lipogenesis, and/or stimulating lipolysis and fatty acid β-oxidation [155, 163-168]. Conversely, less is understood about VIN, a dimer of RES, even though its content in grapes and red wine is comparable to or greater than that of RES depending on the type of red wine and the stage of the noble rot on grapes [169, 170]. More reports have described the function of VIN over the past decade [171, 172] and most of them suggest that VIN is more effective than RES. Mi et al. [171] demonstrated that VIN more potently induces the vasorelaxation of isolated thoracic aortas than RES. The data also indicated that VIN more effectively facilitated the repair of wounded vascular endothelial cells than RES and improved blood pressure and cardiac hypertrophy in spontaneously hypertensive rats [173].

However, only two prior reports described the effects of VIN on the adipose tissue [174, 175]. Ohara et al. [174] reported that VIN, but not RES, prevented diet-induced obesity in mice at the same doses and that VIN decreased triglyceride accumulation and PPAR-γ expression more effectively than RES during 3T3-L1 adipogenesis. Lu et al. [175] also revealed that VIN more effectively suppressed diet-induced obesity in mice and reduced the activity of hydroxymethyl glutaryl CoA reductase, a key enzyme that

catalyzes the rate-limiting step of cholesterol, than RES at high concentrations (80, 120, and 160 μM) in 3T3-L1 preadipocytes. However, these studies did not apparently compare the effects of VIN and RES on important regulators of adipogenesis in 3T3-L1 preadipocytes such as FAS, ATGL, AMPK, and SIRT1. In particular, the increased expression of adiponectin during adipocyte differentiation has not been described even though adiponectin downregulation appears to result in insulin resistance, T2DM, and metabolic syndrome [160-162].

The aim of the present study was to compare the effects of RES and VIN on 3T3-L1 preadipocyte differentiation, focusing on the expression of adiponectin and regulators of differentiation and lipid accumulation. These compounds were used at low concentrations to avoid inducing apoptosis when evaluating their effects on preadipocyte differentiation.

3. Materials and methods

3.1. Chemical reagents and antibodies

RES was purchased from Wako Pure Chemical Industries (Osaka, Japan). We respectively obtained 3T3-L1 preadipocytes and newborn calf serum (CS) from the American Type Culture Collection (Manassas, VA, USA) and Gibco (Gaithersburg, MD, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium with high glucose (DMEM), 3-isobutyl-1-methylxanthine (IBMX), a mixture of penicillin and streptomycin, insulin, dexamethasone (DEX), Oil Red O, and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies for PPAR-γ (D69), adiponectin, ATGL, FAS, and anti-rabbit and -mouse secondary antibodies coupled to horseradish peroxidase were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA).Anti-FGF-21 antibody was purchased from Sigma Chemical Co. Anti-SIRT1(H-300) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

3.2. Cell culture and manipulations

The 3T3-L1 mouse embryo fibroblasts that can be induced to differentiate into an adipocyte-like phenotype were maintained in DMEM containing high glucose (4500 mg/L) supplemented with 10% CS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified 5% CO_2atmosphere, and sub-cultured when they reached approximately 70% confluence. When the 3T3-L1 preadipocyte sreached 100% confluence, the medium was changed to DMEM containing 10% FBS, 1m MDEX, 0.5 mM IBMX, and 1μg/mL insulin (≥27 IU/mg) to initiate adipogenic differentiation. After two days of initiation, the medium was replaced with DMEM containing 1μg/mL

insulin for 10 days. RES and VIN were applied throughout the period of differentiation.

3.3. Cell viability

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-5- (3-carboxymethoxy phenyl) -2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assays using Cell_Titer 96 Aqueous One Solution (Promega, Madison, WI, USA). The 3T3-L1 preadipocytes (6×10^4) were seeded in 96-well plates, cultured for 12-16 h, and then incubated with or without RES and VIN for 24 h. The cells were incubated with 20 μL of MTS solution for 4 h at 37°C and absorbance was measured at 490 nm to determine cell viability.

3.4. Triglyceride content

After differentiation for 10 days, the adipocytes were washed three times with PBS (-) and scraped into cell lysis buffer for 1 min on ice. Total triglyceride contents of the cells were determined using Lab Assay™ triglyceride kits (Wako Pure Chemical Industries, Ltd. Osaka, Japan) according to the manufacturer's instructions. Triglyceride contents obtained as μg triglyceride/μg protein were converted into arbitrary units.

3.5. Oil Red O staining and lipid quantitation

Cells differentiated into adipocytes during a period of 10 days were washed three times with PBS (-) and fixed in 10% neutral formalin for 30 min at room temperature. After three rinses with distilled water, the adipocytes were incubated with 60% isopropanol for 5 min, and stained with fresh 0.5% Oil Red O in isopropanol diluted 3:2 (v/v) with distilled water for 20 min. Stained adipocytes were washed three times and then stained with hematoxylin for 60 sec. After three washes with distilled water, the

cells were assessed by microscopy and images were collected on an Olympus microscope (Tokyo, Japan). Oil Red O stain was dissolved in Dimethyl sulfoxide (DMSO) and optical density was quantified at 520 nm.

3.6. Western blot analysis

On day 10 after initiating differentiation, adipocytes were washed three times with cold PBS and scraped into lysis buffer. The cell lysates were centrifuged at 14,000 rpm for 15 min and the supernatant was collected. Cell lysates containing equal quantities of proteins (35 μg) were separated by electrophoresis on 12% polyacrylamide gels and proteins were transferred to polyvinylidene difluoride (PVDF) membranes using a Trans-Blot® Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% BSA in TBS-T (150_mMNaCl, 0.05% Tween20, and 10 mM Tris-HCl (pH 7.4) for 60 min at room temperature and then incubated with appropriately diluted primary antibodies over night at 4°C. The primary antibodies were then detected using horseradish peroxidase-conjugated goat anti-mouse or donkey anti-rabbit secondary antibodies (1/5,000) for 60 min at room temperature. Immunocomplexes were visualized using the Chemi-Lumi One L kit according to the manufacturer's instructions (Nacalai Tesque Ltd., Kyoto, Japan).

3.7. Statistical analysis

Data are presented as means ± SD and were statistically analyzed using ANOVA with Bonferroni's multiple comparison test or Student's t-test whenever appropriate. Values with $p<0.05$ were considered statistically significant.

4. Results

4.1. RES, but not VIN, reduces the viability of 3T3-L1 preadipocyte cells

Figure 1A and B shows the structural formulae of RES and VIN, respectively. RES is believed to suppress lipid accumulation by decreasing cell viability and reducing adipogenesis [176]. Therefore, the potential effects of RES and VIN on the viability of 3T3-L1 preadipocytes were examined under our experimental conditions. Culture with RES at 20 and 30 μM for 24 h significantly decreased cell viability, whereas 5–30 μM VIN did not exert detectable effects (Fig. 2). Thus, RES and VIN were used at concentrations of 5, 10, and 15 μM for subsequent experiments to determine their effects on adipocyte differentiation.

4.2. Intracellular lipid accumulation was suppressed by VIN, but not RES, during 3T3-L1 preadipocyte differentiation

The effects of RES and VIN on total lipid accumulation in 3T3-L1 preadipocytes were next examined to clarify their effects on adipocyte differentiation. The results of Oil Red O staining indicated that 10 and 15 μM VIN decreased intracellular oil droplet levels by 22% and 27%, respectively (Figs. 3 and 4A). Conversely, RES did not exert detectable effects at these concentrations. In close agreement with these results, 10 and 15 μM VIN reduced intracellular triglyceride accumulation by 24% and 27%, respectively, whereas RES at these concentrations had no such effects (Fig. 4B). These data suggested that VIN, but not RES, suppresses adipocyte differentiation in terms of lipid accumulation.

Several reports indicated that high concentrations of RES reduce lipid accumulation during 3T3-L1 preadipocyte differentiation [155, 163]. Consistent with the findings of

these studies, 50 μM RES decreased lipid accumulation by 32% (Fig. 5A and B). This reduction may be attributable to apoptosis induced by high concentrations of RES.

4.3. Effect of RES and VIN on the protein expression of adiponectin, PPAR-γ, FAS, and ATGL during 3T3-L1 cell preadipocyte differentiation

Adiponectin plays a critical role in preventing insulin resistance, T2DM, and metabolic syndrome [160-162]. Therefore, understanding the effects of RES and VIN on adiponectin expression is extremely important. Figure 4A illustrates that 15 μM VIN, but not RES, significantly enhanced adiponectin protein expression.

The effects of the two compounds on PPAR-γ, FAS, and ATGL protein expression were further compared. PPAR-γ is a key regulator of adipocyte differentiation, and FAS and ATGL are critically involved in lipogenesis and lipolysis, respectively, in adipose tissue. The results demonstrated that treatment with VIN significantly reduced PPAR-γ and FAS expression and increased ATGL expression (Fig. 6 B-D). These results agree well with the finding that VIN suppressed lipid accumulation during adipocyte differentiation (Figs. 3 and 4). Therefore, the VIN-dependent reduction of lipid accumulation may be associated with both decreased FAS expression and increased ATGL expression. Contrarily, 5–15 μM RES did not exert any effects.

Together, these findings suggest that VIN, but not RES, exerts favorable effects with reduced lipid accumulation accompanied by enhanced adiponectin expression during adipocyte differentiation.

4.4. Expression of SIRT1, phosphorylated AMPK (p-AMPK), and FGF-21 is increased by VIN, but not RES

A number of previous reports have indicated that RES activates AMPK [177-180], a major metabolism-sensing protein, and derives several of its beneficial effects by targeting SIRT1 [180-183], which is activated by AMPK. There are also reports demonstrating that polyphenols including RES suppress adipocyte differentiation and obesity [155, 163]. Therefore, whether these compounds increase the expression of SIRT1 and p-AMPK, an active form of AMPK, during adipocyte differentiation was examined. Figure 7A and B indicates that 15 μM VIN significantly elevated the expression of these proteins, whereas 15 μM RES had no such effects. These findings indicate that VIN function is mediated by increased p-AMPK and SIRT1 expression.

Despite primarily acting as a hepatic endocrine regulator in glucose and lipid metabolism, FGF-21 is also expressed in adipocytes [184]. Several studies suggested that FGF-21 induces weight loss in mice with diet-induced obesity [184, 185]. Therefore, the effects of VIN and RES on FGF-21 expression were compared. Figure 7C shows that 15 μM VIN increased FGF-21 protein expression, which might be associated with its anti-obesity function.

4.5. Intracellular lipid accumulation is reduced by VIN *via* SIRT1 during adipocyte differentiation

To determine whether SIRT1 mediates the inhibitory effect of VIN on lipid accumulation, 3T3 preadipocytes were differentiated in the presence or absence of the SIRT1 inhibitor EX527. Lipid accumulation was suppressed by 15 μM VIN and reversed by EX527 (Fig. 8), suggesting that SIRT1 mediates this function of VIN.

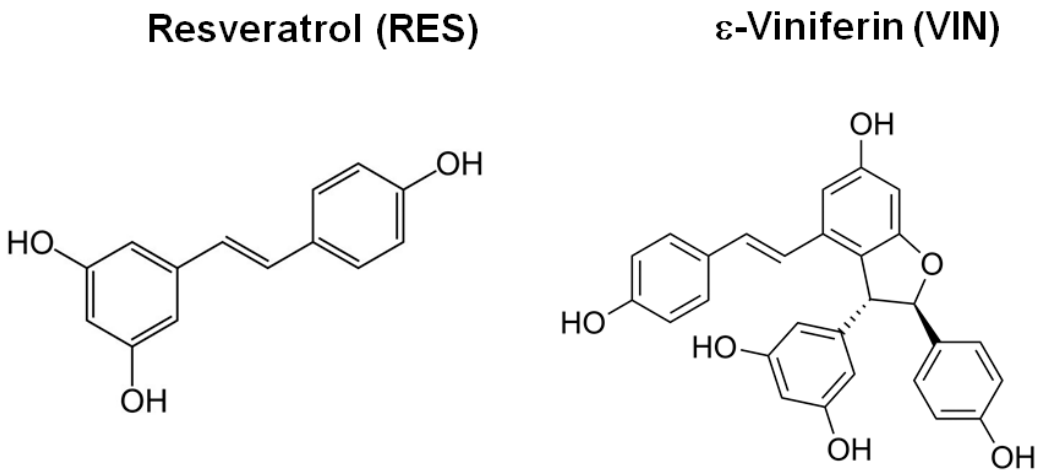

Fig. 1: Chemical structures of RES (a) and VIN (b).

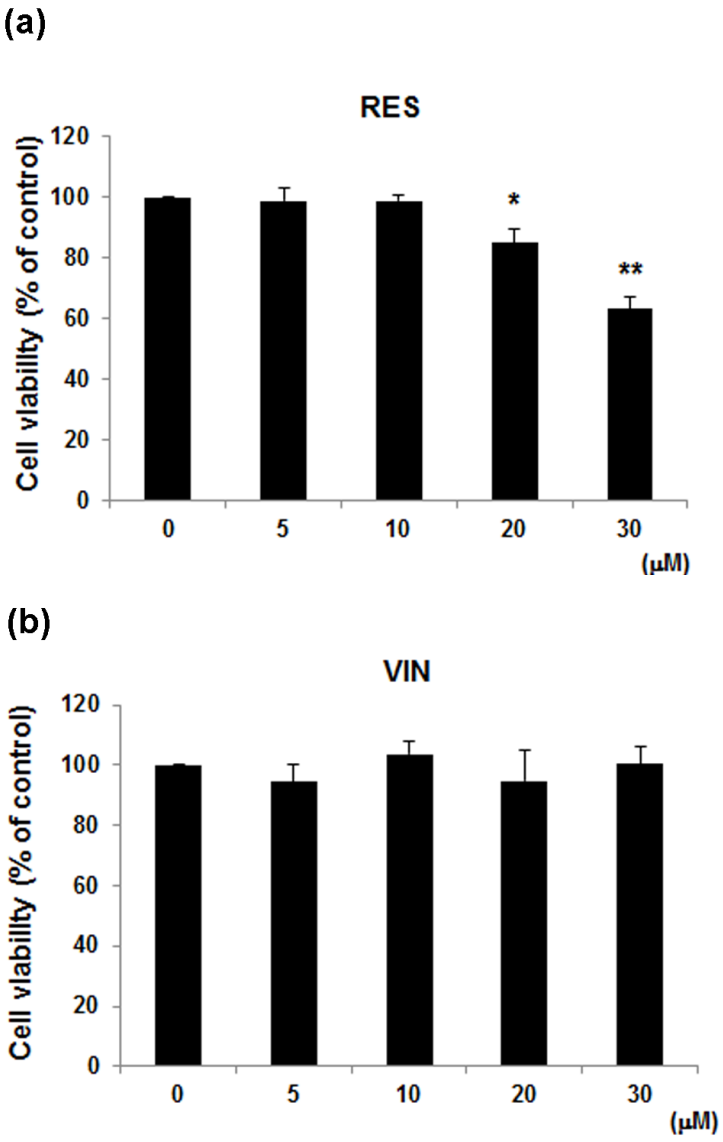

Fig. 2: Effect of RES and VIN on cell viability of 3T3-L1 preadipocytes.

3T3-L1 preadipocytes were treated with different concentrations (0, 5, 10, 20 and 30 μM) of RES (a) and VIN (b) for 24 h, and the cell viability was assessed by MTS assay. Data are presented as means ± SD (n=3). *P < 0.05 vs. Control (0 μM), **P < 0.01 vs. Control (0 μM)

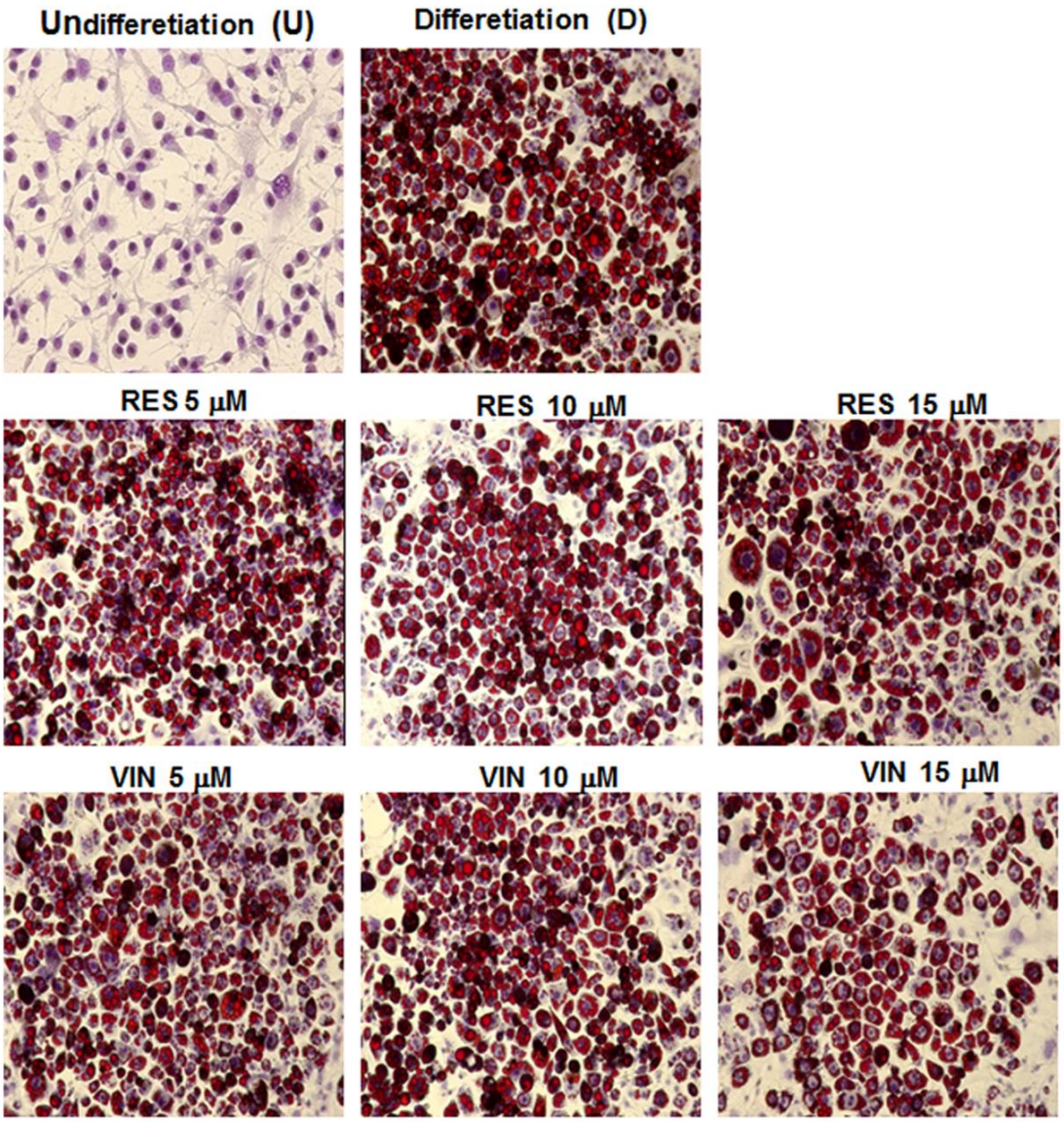

Fig. 3: Effect of RES and VIN on lipid accumulation during differentiation of 3T3-L1 preadipocytes.

3T3-L1 cells were treated with different concentrations (0, 5, 10 and 15 µM) of RES and VIN for 10 days. Intracellular lipid accumulation was visualized with Oil Red O staining.

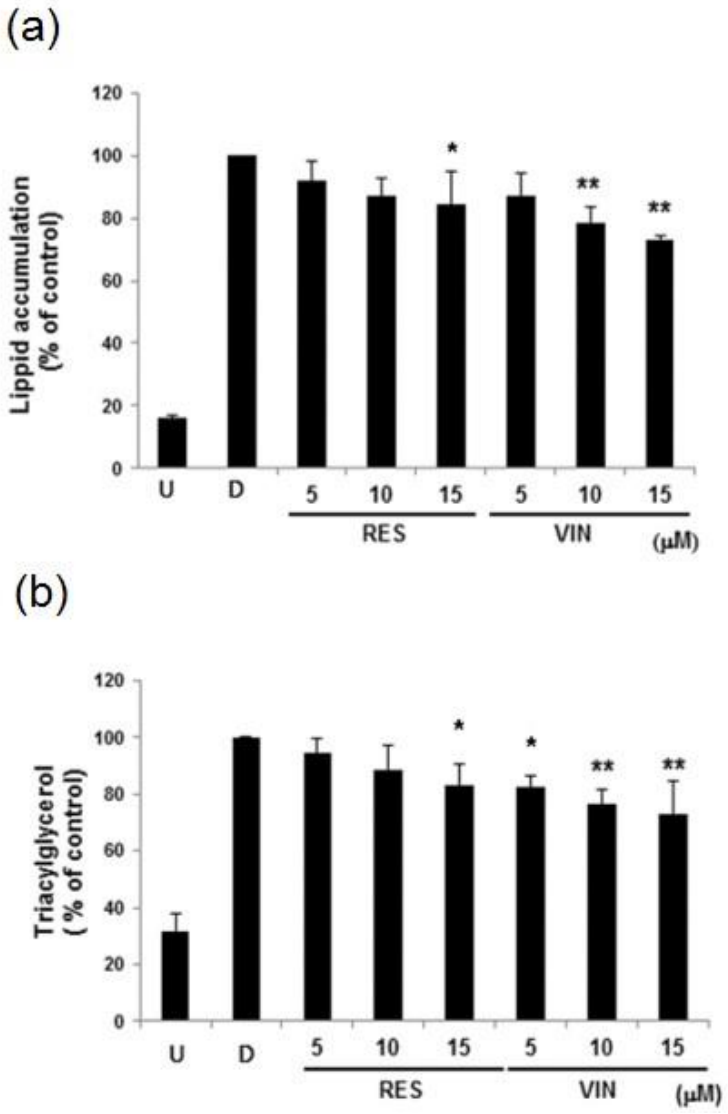

Fig. 4: Effect of RES and VIN on lipid accumulation during differentiation of 3T3-L1 preadipocytes.

3T3-L1 cells were treated with different concentrations (0, 5, 10 and 15 μM) of RES and VIN for 10 days (a, b), and subjected to spectrometric quantification after dissolved in DMSO (a, b). Intracellular triacylglycerol contents were measured as described in Materials and methods. D: differentiated, U: undifferentiated. Data are presented as means ± SD (n=3). *P < 0.05 vs. D, **P < 0.01 vs. D.

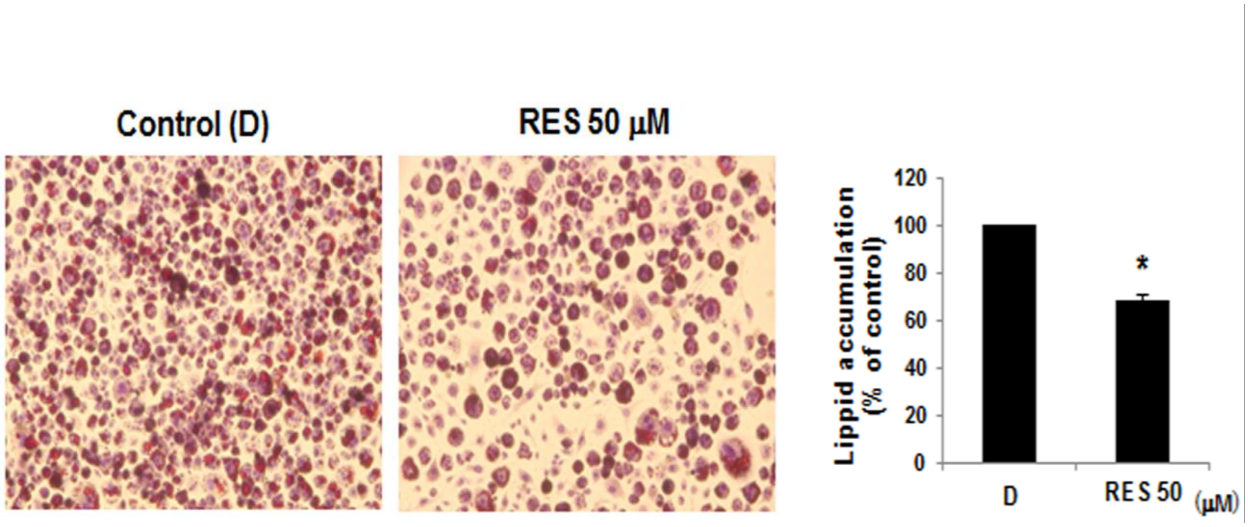

Fig. 5: Effect of RES and VIN on lipid accumulation during differentiation of 3T3-L1 preadipocytes.

3T3-L1 cells were treated with different concentrations (50 µM) of RES and VIN for 10 days. Intracellular lipid accumulation was visualized with Oil Red O staining (a) and subjected to spectrometric quantification after dissolved in DMSO (b). Intracellular lipid contents were measured as described in Materials and methods. D: differentiated, U: undifferentiated. Data are presented as means ± SD (n=3). *P < 0.05 vs. D.

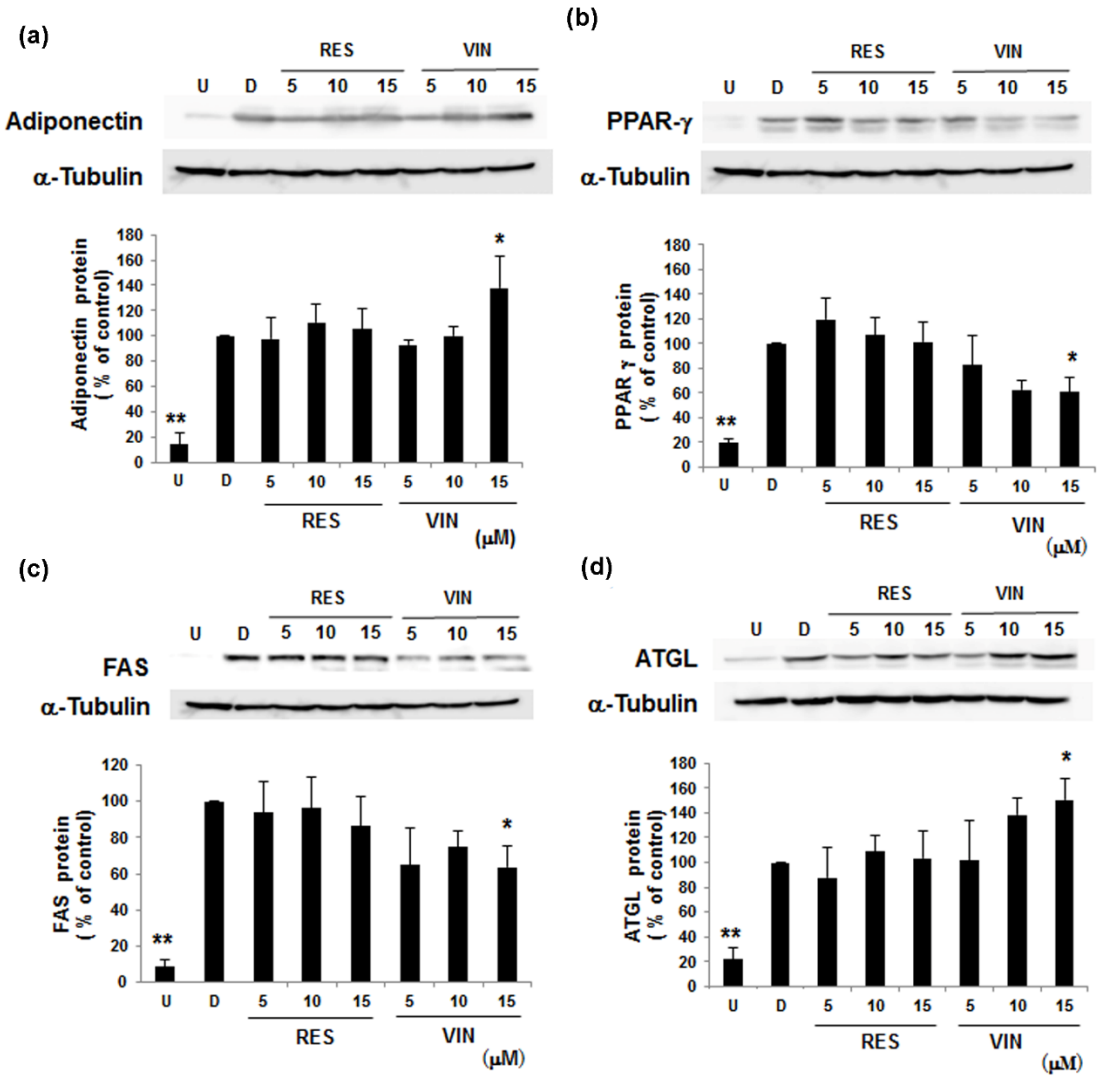

Fig. 6: Effect of RES and VIN on protein expression of adiponectin, PPAR-γ, FAS, and ATGL during differentiation of 3T3-L1 cells.

3T3-L1 cells were treated with different concentrations (0, 5, 10 and 15 μM) of RES and VIN for 10 days, and protein expression of adiponectin (a), PPAR-γ (b), FAS (c), and ATGL (d) was detected by western blotting. D: differentiated, U: undifferentiated. Data are presented as means ± SD (n=3). *P < 0.05 vs. D.

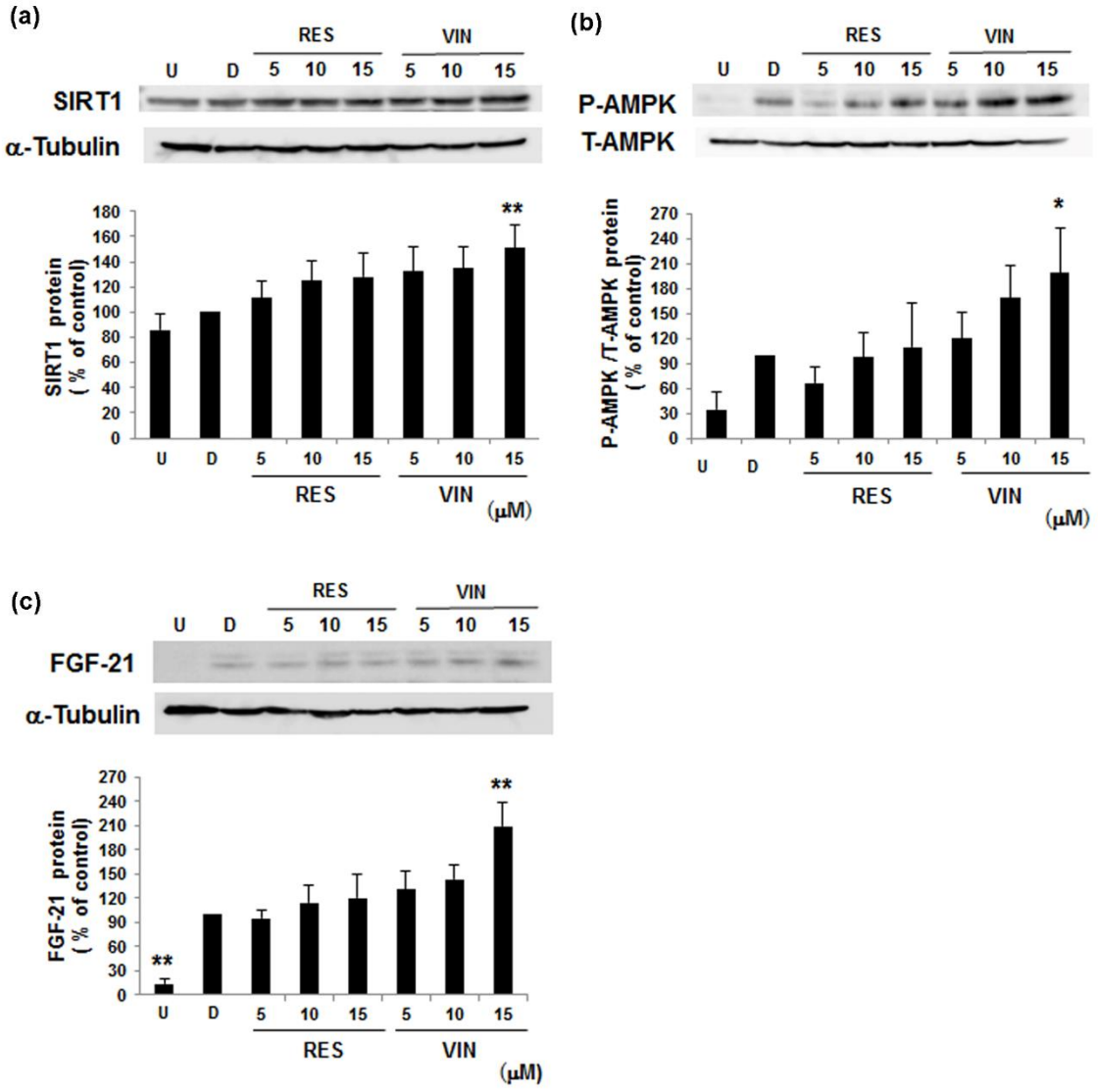

Fig. 7: Effect of RES and VIN on protein expression of on SIRT1, p-AMPK, and FGF-21 during differentiation of 3T3-L1 cells.

Differentiation of 3T3-L1 cells. 3T3-L1 cells were treated with different concentrations (0, 5, 10 and 15 μM) of RES and VIN for 10 days and protein expression of SIRT1 (a), p-AMPK (b), and FGF-21 (c) was measured by western blotting. D: differentiated, U: undifferentiated. Data are presented as means ± SD. (n=3). *P < 0.05 vs. D, **P < 0.01 vs. D.

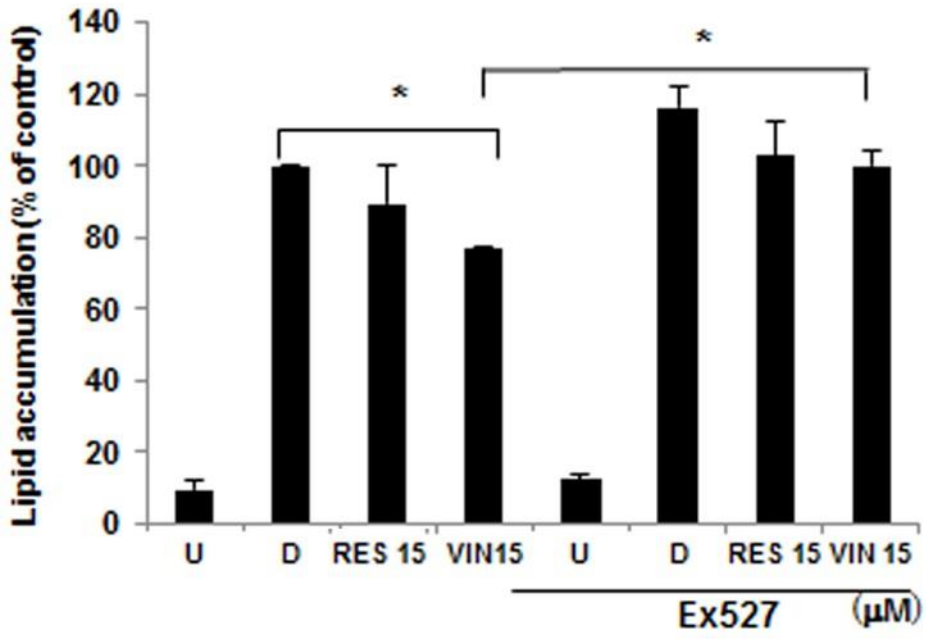

Fig. 8: Effect of SIRT1 inhibitor on lipid accumulation during 3T3-L1 preadipocyte differentiation in the presence and absence of RES and VIN.

3T3-L1 cells were treated with and without 15 μM RES or VIN in the presence and absence of 10 μM EX527 for 10 days. Intracellular lipid droplets were stained with Oil Red O and subjected to spectrometric quantification after dissolved in DMSO. D: differentiated, U: undifferentiated. Data are presented as means ± SD (n=3). *P < 0.05.

5. Discussion

The present study compared the effects of RES and its dimer VIN at non-cytotoxic concentrations (5–15 μM) on the adipogenesis of 3T3-L1 preadipocytes. The main findings of the study were as follows. First, VIN, but not RES, promoted favorable adipocyte differentiation with enhanced adiponectin expression and decreased triglyceride accumulation. Second, VIN reduced PPAR-γ and FAS expression and increased SIRT1, p-AMPK, and ATGL expression, suggesting that these proteins coordinately contribute to the induction of favorable adipocyte differentiation.

Impaired adipogenesis, in addition to obesity, is implicated in insulin resistance [186]. This is because adipogenesis contributes to increasing the numbers of insulin-sensitive adipocytes accompanied by increased generation of adiponectin, which plays a critical role in preventing insulin-resistant T2DM. Therefore, whether adiponectin expression is affected by the suppression of adipogenesis induced by various phytochemicals should be determined. Hsu et al. [175] investigated the inhibitory effect of 15 phenolic acids and 6 flavonoids on adipocyte differentiation. Most of the investigated compounds suppressed adipogenesis, and among them, *o*-coumaric acid and rutin remarkably reduced lipid accumulation and increased adiponectin expression. The data illustrated that VIN lowered lipid accumulation and upregulated adiponectin. Thus, these compounds are considered to promote favorable adipocyte differentiation.

In contrast to a number of reports indicating that phytochemicals exert anti-adipogenic effects, only three reports have described the phytochemical-dependent promotion of adipogenesis [155, 177, 187]. The phytochemicals in these three reports were phloretin in apples, 4-hydroxyderricin in *Angelica keiskei*, and 4-methoxychalcone, all of which

belong to the chalcone class of flavonoids. These three compounds stimulate the adipogenesis of 3T3-L1 preadipocytes by enhancing the expression of PPAR-γ in addition to adiponectin and some other proteins involved in adipocyte differentiation. Anti-diabetic thiazolidinedione derivatives are PPAR-γ activators that improve insulin sensitivity and lower the elevated blood glucose levels associated in T2DM [188]. These agents promote adipocyte differentiation by increasing glucose and free fatty acid uptake, triglyceride storage, and adiponectin generation *via* PPAR-γ activation. Therefore, these chalcone derivatives might function as PPAR-γ ligands. Phytochemicals can inhibit or stimulate adipogenesis even though both inhibitory and stimulatory phytochemicals can increase adiponectin expression. The relationships between chemical structures and their effects on the adipose tissue need to be clarified to facilitate the effective use of dietary compounds to prevent and improve obesity as well as T2DM and related diseases. He et al. [189] found that ursolic acid lowers lipid accumulation during 3T3-L1 preadipocyte differentiation accompanied by AMPK activation. The compound also increases SIRT1 expression and decreases PPAR-γ and FAS expression. These protein expression data are in good agreement with the findings of this study. However, the SIRT1 inhibitor nicotinamide did not reverse this anti-adipogenic effect of ursolic acid, whereas EX527, a SIRT1 inhibitor, reversed the VIN-dependent suppression of lipid accumulation in the present study. The findings of this study are consistent with those reported by Pichard et al. [157], who found that SIRT1 suppressed the adipogenesis of 3T3-L1 preadipocytes with reduced expression of PPAR-γ and that this effect was reversed by SIRT1 RNAi. Bai et al. [190] also indicated that RES suppressed pig preadipocyte differentiation with increased SIRT1 expression and reduced PPAR-γ expression and that SIRT1 might modulate the differentiation of these cells based on the effects of nicotinamide. The

involvement of SIRT1 in adipocyte differentiation might differ depending on the nature of phytochemicals. Ono et al. [191] reported that apigenin suppresses lipid accumulation during 3T3-L1 differentiation with decreased PPAR-γ expression and AMPK activation. However, ATGL expression did not change in response to 4 days of incubation with apigenin. Adipose triglyceride lipase promotes the catabolism of stored triglycerides in adipose tissues [192]. Several reports demonstrated that various phytochemicals including RES upregulate ATGL expression accompanied by reduced lipid accumulation [163]. To the best of our knowledge, this is the first study to suggest that upregulation of the lipolytic enzyme ATGL and downregulation of the lipogenic enzyme FAS are involved in the reduction of lipid accumulation induced by RES and its oligomers during adipocyte differentiation.

In summary, low concentrations of VIN promoted favorable adipocyte differentiation with enhanced adiponectin expression and decreased triglyceride accumulation, whereas RES has no such effects. Therefore, VIN may prevent and improve obesity and its related disorders more effectively than RES.

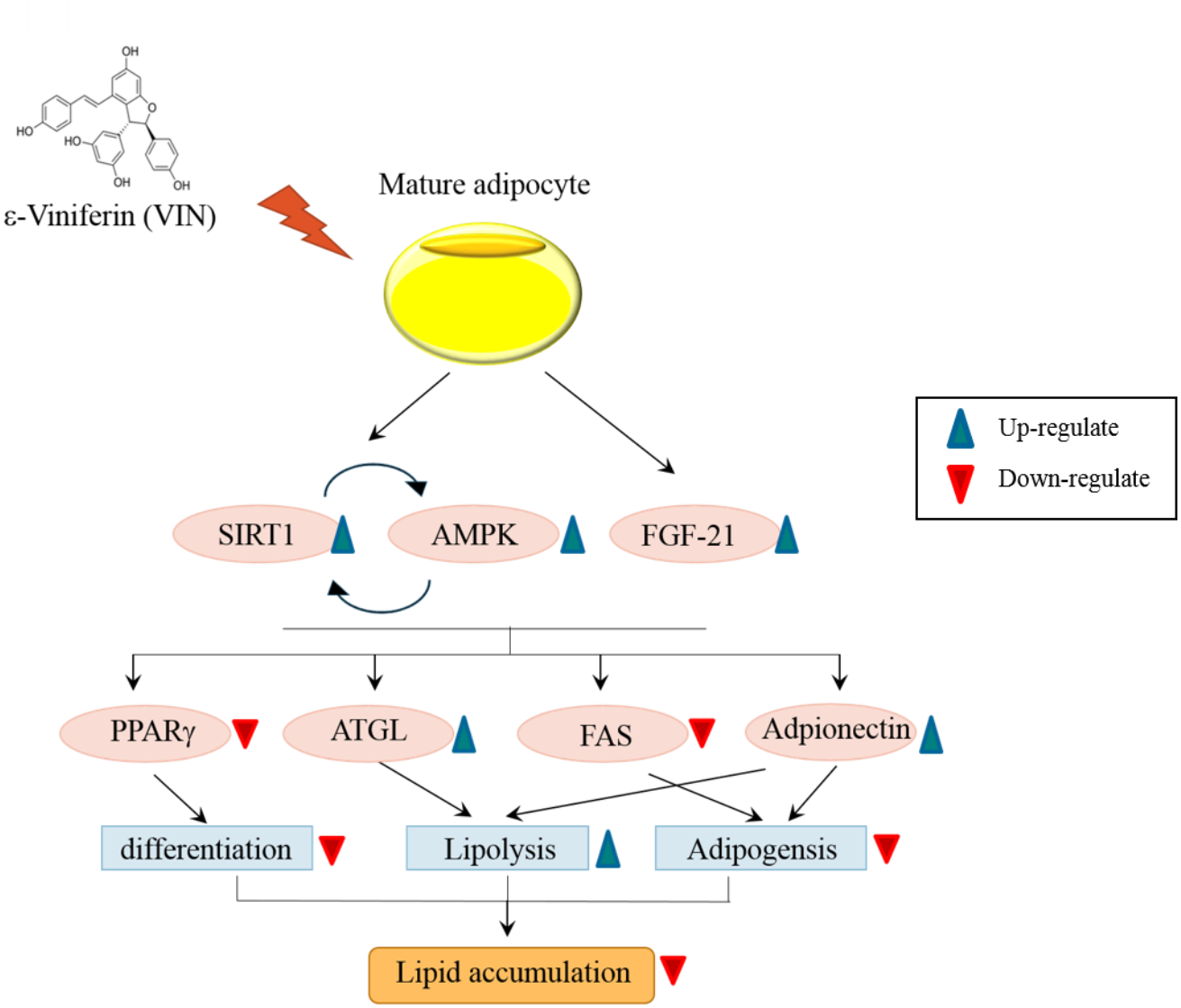

Fig. 9: The effect of VIN on 3T3-L1 cell.

Through following these experiments, I can find that VIN has the effects on anti-obesity. Furthermore, I can also speculate that the main mechanism of pathways may be: Inhibit the differentiation of preadipocytes into mature adipocytes by lessening PPAR-γ transcription factor expression, which will reduce lipid accumulation and increase lipolysis by inducing SITR 1 pathway and FGF-21 protein expression.

Chapter 4

1. General conclusion

Obesity, a lifestyle-related disease, causes T2DM, hypertension, arteriosclerosis, stroke, and myocardial infarction. In Japan, 20% of the people are obese. Recently, it has been observed that obesity is not only a risk factor for cerebrovascular and heart disease but also for dementia, cancer, osteoporosis, and reproductive disorders, among others, and the condition has become a challenging problem in society. Although many medicines have been developed for the treatment of the various diseases caused by obesity, there are some problems, such as increases in medical expenses, and the prevention thereof is regarded as important. Diet, exercise, and sleep are three factors that have been closely studied in relation to the prevention of obesity, and this study is consistent with research that aims to prevent obesity using nutritional measures.

Obesity is characterized by an increase in the number of cells that accumulate fat and the enlargement of individual fat cells. Adipocytes are produced by differentiation from fibroblasts, which are progenitor cells. It has been found that adipose tissue composed of adipocytes acts as an endocrine organ, secreting hormones called adipokines, in addition to the role it plays in accumulating surplus energy sources. Adiponectin, which is indicated as a longevity hormone, is a typical adipokine and is considered essential in preventing the onset of various diseases, including T2DM. For this reason, fat cells themselves are essential for maintaining health. On the other hand, while normal adipocytes secrete and synthesize a sufficient level of adiponectin, the level of synthesis is reduced in obese adipocytes that accumulate a large amount of lipids. In recent studies aimed at preventing obesity, inhibition of the differentiation of adipocytes by various dietary ingredients has been reported. However, even the fat accumulation is

reduced, if the food component is not healthy and the synthesis of adiponectin that increases with differentiation would also be suppressed.

This study focused on RES, a polyphenol found in red wine, and its dimer VIN, and examined whether these chemicals can have positive effects on adipocytes, such as reducing fat accumulation and increasing adiponectin. Resveratrol is attracting attention because it activates the synthesis of SIRT1, the longevity gene, though our previous study showed VIN to have a stronger effect on the cardiovascular system than RES. A comparative study on both cells was also performed simultaneously.

In this study, the results were obtained using 3T3-L1, which is frequently used for adipocyte research. Our results revealed that (1) resveratrol reduced the survival rate of preadipocytes 3T3-L1 at 20 and 30μM, whereas VIN had no effect at the same concentration. (2) VIN suppressed fat accumulation during differentiation into adipocytes at a lower concentration than resveratrol. (3) Only VIN (15 μM) enhanced the protein expression of adiponectin accompanying differentiation into adipocytes. (4) Only VIN (15 μM) promoted the expression of transcription factor PPAR-γ, which is important for adipocyte differentiation. (5) Only VIN (15 μM) suppressed the increase in the expression of fatty acid synthase (FAS) accompanied by differentiation and promoted an increase in the expression of adipose tissue triglyceride degrading enzyme ATGL. (6) Only VIN (15 μM) increased the protein expression of the longevity gene SIRT1 and activated phosphorylase AMPK, working in conjunction with SIRT1 through phosphorylation. (7) Only VIN (15 μM) enhanced the protein expression of FGF-21 accompanied by differentiation into adipocytes. (8) The suppression of fat accumulation by sputum-viniferin accompanying differentiation into adipocytes was inhibited by an SIRT1 inhibitor.

These results reveal that VIN suppresses fat accumulation accompanying fat differentiation while at the same time promoting the expression of adiponectin. Adiponectin is a longevity hormone that has a favorable fat differentiation action and promotes health. It is suggested that the suppression of fat accumulation involves both the suppression of fat synthesis and the promotion of lipolysis, as well as the upregulation of the longevity gene SIRT1. Furthermore, VIN had a stronger effect on adipocytes than RES as well as on the cardiovascular system, and resveratrol has been reported to inhibit fat differentiation at high concentrations. This was found to be caused by the induction of cell death in progenitor cells, rather than the suppression of differentiation by RES. Since VIN is contained in red wine in equal or higher amounts than RES, depending on the variety, the results of this study indicate that VIN is more likely to be responsible for the beneficial effects of red wine on health than RES. Our study reveals that VIN could exert its health-promoting actions *via* its effects on lipid metabolism.

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