Antioesity Activity of Vigna nakashimae Extract in High-Fat Diet-Induced Obesity

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In this study, we evaluated the antioesity effects of Vigna nakashimae (VN) extract and elucidated the underlying mechanisms. VN extract suppressed adipocyte differentiation and significantly attenuated the expression of adipogenic genes in 3T3-L1 cells. It decreased the expression of peroxisome proliferator-activated receptor γ (PPARγ) and its target genes in fully differentiated 3T3-L1 cells. Moreover, it enhanced the phosphorylation of AMP-activated protein kinase (AMPK) and acetyl CoA carboxylase (ACC), and increased the expression of fatty acid oxidation genes. In high-fat diet (HFD) fed mice, VN extract suppressed HFD-induced increases in body weight, epididymal fat tissue weight, and hepatic lipid levels, and decreased the plasma levels of triacylglycerols, fatty acid, total cholesterol, and inflammatory cytokines. Consistently with in vitro study results, VN extract prevented HFD-induced increases in the expression of PPARγ and its target genes, and restored the decrease in the phosphorylation of AMPK and ACC in epididymal fat and liver tissues. These findings suggest that Vigna nakashimae prevents obesity through suppression of PPARγ expression and activation of AMPK, and that it might be a useful dietary supplement for the prevention of obesity.

Key words: Vigna nakashimae; antioesity activity; adipocyte differentiation; peroxisome proliferator-activated receptor γ (PPARγ); AMP-activated protein kinase (AMPK)

Adipocytes have dramatically increased in most developing nations and is a prevalent condition related to metabolic disorders worldwide. It is associated with an imbalance between energy intake and expenditure and a subsequent excess accumulation of adipose tissue. The increase in adipose tissue mass is caused by enlargement of adipocytes induced by the lipid accumulation and an increase in the total number of adipocytes due to differentiation of pre-adipocytes into mature adipocytes.1) Obesity is involved in metabolic diseases such as type2 diabetes, atherosclerosis, hypertension, and fatty liver.2) Therefore, prevention of the process of obesity is critical in many clinical fields. Inhibition of adipocyte differentiation and proliferation could be used as a strategy for the treatment and prevention of metabolic diseases. Adipocyte differentiation from fibroblastic pre-adipocytes is regulated by a highly organized cascade of transcriptional factors.3) The most important transcription factors are the peroxisome proliferator-activated receptor (PPAR) and CCAAT/enhancer binding protein (C/EBP) families. These transcription factors activate the expression of adipocyte markers such as fatty acid synthase (FAS), fatty acid binding protein (aP2), and lipoprotein lipase (LPL). Suppression of this process is critical to achieve an antioesity effect, and an extensive search for agents that suppress this process is underway.

Adenosine monophosphate (AMP)-activated protein kinase (AMPK), a metabolic sensor that acts as a cellular fuel gauge in eukaryotes, is involved in cellular energy homeostasis.4) AMPK stimulates pathways that increase energy production, such as fatty acid oxidation, and switches off pathways that consume energy, such as lipogenesis. In addition to controlling energy homeostasis, it enhances insulin sensitivity by increasing glucose uptake and lipid oxidation in skeletal muscle and inhibiting glucose and lipid synthesis in the liver.4) Thus it is a key molecule in the control of metabolic diseases such as type 2 diabetes, obesity, and cancer. The discovery and development of a natural AMPK activator should provide a novel strategy to overcome these human diseases.

Vigna species are an important source of protein for people, particularly in tropical Africa and Asia, and several Vigna species have been domesticated in Asia. Among these, the cultigens of mungbean (V. radiata (L.); Wilczek), black gram (V. mungo (L.); Hepper), and azuki bean (V. angularis (Willd.); Ohwi & Ohashi) are the most important economically. In addition, the rice bean (V. unballate (Thunb.); Ohwi & Ohashi) is occasionally cultivated in parts of south-east and east Asia.5) Vigna mungo has antihyperglycemic effects in diabetic rats.6) Extracts of V. angularis improve blood glucose and cholesterol in mice fed a high-fat diet. In addition, the hypoglycemic effect of extracts of V. angularis have been confirmed in type2 diabetes mellitus model

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Abbreviations: ACC, acetyl-CoA carboxylase; aP2, adipocyte fatty acid binding protein; AMPK, AMP-activated protein kinase; C/EBPs, CCAAT/enhancer binding proteins; FAS, fatty acid synthase; HFD, high-fat diet; LPL, lipoprotein lipase; NAFLD, non-alcoholic fatty liver disease; PPARγ, peroxisome proliferator-activated receptor γ
KK-A(y) mice and a streptozotocin-induced type 1 diabetes model. Recently, we reported that *V. nakashimae* (VN), another species of *Vigna* widely cultivated in Korea, has antidiabetic effects in *db/db* mice via inhibition of α-glucosidase activity, but its antiobesity effect has not been explored.

Therefore, in this study, we investigated the potential beneficial antiobesity effects of VN extract using the adipocyte 3T3-L1 cell system and an animal model in which VN extract inhibits adipocyte differentiation in mouse adipocytic 3T3-L1 cells through suppression of PPARγ expression and activation of AMPK, and suppresses weight gain in HFD-treated mice.

### Materials and Methods

#### Cell culture

3T3-L1 pre-adipocytes, purchased from the American Type Culture Collection (ATCC, CL-173TM; Manassas, VA), were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM; HyClone, Logan, UT) containing 10% fetal bovine serum, 5 mM insulin, 0.5 mM 3-isobutylmethylxanthine (IBMX), and 1 µM dexamethasone (DEX) for 3 days with M1 (DMEM containing 10% fetal bovine serum, 5 mM insulin, 0.5 mM IBMX, and 1 µM dexamethasone (DEX)) and for 8 days with M2 (DMEM containing 10% FBS and 5 µM insulin).

#### Reagents

Antibodies against AMPK and phospho-AMPK (Thr172) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and antibodies against adenosine acetyl-CoA carboxylase (ACC) and phospho-ACC (Ser79) were from Cell Signaling Technology (Beverly, MA). Insulin, IBMX, and DEX were from Sigma-Aldrich (St. Louis, MO).

#### Preparation of VN extract

VN (IT178464) germplasm was provided by the Genetic Resources Division of National Institute of Agricultural Biotechnology, Rural Development Administration (RDA), South Korea, and seeds were grown in an experimental field (St. Louis, MO). Insulin, IBMX, and DEX were from Sigma-Aldrich (St. Louis, MO).

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#### Statistical analysis

All data are presented as means ± SD. The data were evaluated by one-way ANOVA, and differences between means were determined by Tukey-Kramer post-hoc test. Values were considered statistically significant at p < 0.05.

### Results

**VN extract inhibited the differentiation of 3T3-L1 cells**

To study the effects of VN on adipocyte differentiation, 3T3-L1 pre-adipocytes were differentiated into mature adipocytes for 8 d in the presence of various concentrations of VN extract, and the accumulated intracellular lipids were detected by Oil-red O staining. As shown in Fig. 1A, VN extract significantly suppressed lipid drop formation in a dose-dependent manner as compared with untreated differentiated 3T3-L1 cells. Next we measured intracellular TG levels. Intracellular TG was also reduced in the VN-treated cells (Fig. 1B). To confirm inhibition of adipocyte
differentiation, we measured the expression of adipogenic marker genes such as PPARγ and its target genes (aP2 and FAS) by RT-PCR. Expression of PPARγ, FAS, and aP2 was decreased in the presence of VN extract (Fig. 1C). These results indicate that VN extract inhibits the differentiation of 3T3-L1 cells.

To exclude the possibility that VN extract has toxicity, cell viability was measured by MTT assay. No significant decrease in cell viability was observed in the cells treated with VN extract at the given concentrations (data not shown).

**VN extract inhibited the expression of PPARγ and activated AMPK in 3T3-L1 adipocytes**

Next we investigated the effects of VN extract on the expression of PPARγ, the master regulator of adipocyte differentiation, and its target genes in differentiated 3T3-L1 cells. As shown in Fig. 2A, VN extract decreased the expression of PPARγ, FAS, and aP2 in a dose-dependent manner (Fig. 2A). To elucidate the molecular mechanism of the suppressive effect of VN on adipogenesis further, we investigated the possibility that VN acts as an activator of AMPK. AMPK is known to be involved in the regulation of adipogenesis, and activation of AMPK suppresses adipogenesis.10) AMPK stimulates energy-generating pathways such as fatty acid oxidation and inhibits energy-consuming pathways such as lipogenesis by directly phosphorylating key metabolic enzymes and thereby regulating gene expression. Hence we investigated AMPK activation in fully differentiated 3T3-L1 cells treated with various concentrations of VN extract. The extract increased phosphorylation of AMPK and phospho-ACC in a concentration-dependent manner without affecting the protein level of AMPK (Fig. 2B, left), suggesting that it activates AMPK in 3T3-L1 adipocytes. Then we assessed molecular changes in fatty acid oxidation genes in differentiated 3T3-L1 cells treated with VN extracts for 24 h by RT-PCR. The extract significantly increased fatty acid oxidation genes such as CPT-1 and ACO, in a dose-dependent manner (Fig. 2B, right).

**VN extract ameliorated HFD-induced obesity**

To investigate further whether VN extract would modulate obesity in an animal model, C57BL/6J mice were fed a HFD and administered VN extracts (300 mg/Kg/d, 500 mg/Kg/d) orally over 40 d. Body weight was measured every 5 d. The weight gain in the regular diet and in the HFD mice over the 40 d period was about 20 g (a 117% increase over initial body weight) and about 24.5 g (a 144% increase over initial body weight), respectively (Fig. 3A). However, the weight gain in the VN-treated HFD mice was significantly less than in the HFD control mice. Weight gain decreased by about 126% in the mice treated with 300 mg/Kg VN extract and about 129% in the mice treated with 500 mg/Kg VN extract. The width of the mice was also reduced in the mice treated with 500 mg/Kg VN extract (Fig. 3B). No significant difference in food intake was observed between the vehicle and VN extract-treated mice (data not shown), indicating that the reduction in body weight
gain in the VN-treated HFD mice was not due to reduced caloric intake.

To determine whether reduced body weight gain was related to decreased fat accumulation, epididymal fat tissue was dissected and weighed. The weight of epididymal fat tissue was increased in the HFD mice compared to control, but VN extract reduced the increment of epididymal fat tissue weight, whereas no difference was observed in liver tissue weight (Fig. 3C), indicating that ameliorated obesity in VN extract-treated mice was due to reduced adiposity in white adipose tissue. Histological analysis of the epididymal fat tissue revealed smaller adipocytes in the mice treated with VN extract than in the HFD control mice, further demonstrating that reduced body weight gain was due to decreased fat accumulation in the adipocytes.

**VN extract improved obesity-related plasma markers in HFD-induced obese mice**

To determine whether the alleviation of adiposity by VN extract correlated with changes in the plasma levels of obesity-related markers, blood samples were obtained from each group of mice. As shown in Fig. 4, administration of VN extract reduced the HFD-induced levels of TG, NEFA, and total cholesterol. Furthermore, inflammatory cytokines such as IL-6 and TNF-α, which are induced by HFD and are positively correlated with insulin sensitivity, were also decreased in the mice treated with VN extract. In contrast, the levels of adiponectin and HDL/cholesterol ratio (H/C ratio) were increased by administration of VN extract. These data indicate that VN extract improves obesity-related blood markers.

**VN extract suppressed the expression of PPARγ and activated AMPK in HFD-induced obese mice**

To determine whether reduced adiposity was associated with reduced PPARγ expression and AMPK activation, we analyzed the expression of PPARγ and the phosphorylation of AMPK and ACC in epididymal adipose tissue isolated from HFD control mice and from VN extract-treated mice. As shown in Fig. 5A, the expression of PPARγ and its target genes, C/EBPα, FAS, and aP2, was increased in the HFD control mice but reduced in the mice treated with VN extract. Furthermore, phosphorylation of AMPK and its target ACC was decreased in the HFD control mice, whereas VN extract increased the phosphorylation of AMPK and ACC in the mice. In accordance with AMPK activation, expression of the genes associated with fatty acid oxidation, including CPT-1 and ACO were increased in the mice treated with VN extract (Fig. 5B). These data are consistent with our results for 3T3-L1 adipocytes, and suggest that the antiobesity effect of VN extract is based on inhibition of PPARγ and activation of AMPK, which inhibits adipogenesis in vivo and in vitro.

**VN extract suppressed fatty liver in the HFD obese mice**

Obesity is known to induce non-alcoholic fatty liver. AMPK has been implicated in the development of fatty livers...
liver in an HFD fed animal. Hence we determined whether VN extract affects the development of fatty liver in HFD obese mice. To this end, we examined lipid accumulation in the liver by measuring TG content and Oil-red O staining. As shown in Fig. 6, TG contents of the liver were increased in the HFD control mice, but reduced in the VN-treated HFD mice (Fig. 6A). Oil-red O staining of liver sections also revealed reduced lipid accumulation in the VN-treated HFD mice (Fig. 6B), suggesting that VN extract improves fatty liver in HFD obese mice. To determine whether the reduction in fatty liver in the VN-treated mice was associated with AMPK activation and molecular changes in the target genes involved in lipid metabolism, we analyzed phosphorylated AMPK and the expression of genes related to lipid metabolism in the mouse livers. In accordance with the results for epididymal adipose tissue, VN extract restored AMPK activity (Fig. 6C), reduced the expression of lipogenic genes, including PPAR\(\gamma\), C/EBP\(\alpha\), aP2, and FAS (Fig. 6D), and increased the expression of fatty acid oxidation genes including ACO and CPT-1 (Fig. 6E).

**Discussion**

We have reported that VN, which showed high-performance liquid chromatography (HPLC) chromatogram profiles different from \textit{V. angularis} in our preliminary study, had anti-diabetic effects in \textit{db/db} mice through inhibition of \(\alpha\)-glucosidase activity\(^9\). The extract potently inhibited \(\alpha\)-glucosidase \textit{in vitro} and \textit{in vivo}, and significantly decreased both postprandial and fasting blood glucose levels in \textit{db/db} mice. Nevertheless, the antiobesity effects of VN has not been explored. Hence in the present study we investigated the antiobesity effects of VN extracts using 3T3-L1 adipocyte cells and HFD obese mice. In the \textit{in vitro} investigations, maturation and lipid accumulation in 3T3-L1 cells were inhibited in the presence of VN extract, suggesting that VN extract might be useful for the treatment and prevention of obesity. Our results showed marked increases in body weight in the HFD mice and significant reductions of body weight in the VN-treated HFD mice. Whereas the weight of epididymal adipose tissue was decreased by VN treatment, the liver was not affected. The VN extract also reduced plasma obesity-related markers, including TG, free fatty acid, total cholesterol, and inflammatory cytokines such as TNF-\(\alpha\) and IL-6, but increased adiponectin and the H/C ratio as compared with those in the HFD control. These results indicate that VN extract exerts antiobesity effects in \textit{in vitro} and \textit{in vivo} models.

We also elucidated the molecular mechanisms of VN-mediated antiobesity. PPAR\(\gamma\) is a critical transcription factor in adipocyte differentiation that stimulates expression of the genes necessary for adipogenesis, including aP2 and FAS. VN extract repressed the expression of PPAR\(\gamma\) and its target genes such as aP2.
and FAS in pre-adipocytes and in fully differentiated adipocytes. Decreased expression of PPARγ led to suppression of 3T3-L1 differentiation, which may have prevented weight gain in the HFD mice. Reduced expression of PPARγ and its target genes was also observed in the epididymal adipose tissue of the VN extract-treated mice. Therefore, the antiobesity effect of VN can be ascribed partially to downregulation of PPARγ. To elucidate the antiobesity mechanism of VN further, we examined AMPK activation, because AMPK is known to be involved in the regulation of adipogenesis and activation of AMPK suppresses adipogenesis.10) AMPK might play a beneficial role in the prevention of metabolic diseases, including type 2 diabetes, obesity, and cancer. It is also involved in the maintenance of lipid and cholesterol homeostasis, and it stimulates β-oxidation of fatty acids in the mitochondria for lipid utilization.4) It inhibits the activity of ACC through phosphorylation. Under normal conditions, inhibition of ACC by AMPK through phosphorylation leads to a decrease in malonyl-CoA and a subsequent decrease in fatty acid synthesis and an increase in mitochondrial fatty acid oxidation via allosteric regulation of CPT-1, which catalyzes the entry of long-chain fatty acyl-CoA into the mitochondria. Inactivation of ACC by AMPK helps to promote fatty acid utilization, leading to fat burning in fat and muscle. In the present study, VN extract stimulated AMPK and ACC phosphorylation in a dose-dependent manner in 3T3-L1 cells and in the adipose tissue of VN extract-treated mice. Thus inactivation of ACC by VN-mediated AMPK activation might be involved in the suppression of adipogenesis. Furthermore, AMPK is known to attenuate adipogenesis by augmenting fatty acid oxidation. Consistently with AMPK activation, VN increased the expression of fatty acid oxidation genes such as CPT-1 and ACO in 3T3-L1 cells and in the adipose tissue of the VN extract-treated mice. AMPK activation also suppresses the expression of PPARγ, FAS, aP2 in 3T3-L1 adipocytes, and inhibits the adipogenesis of 3T3-L1 cells.12) Here we found that VN extract suppressed the expression of PPARγ and its target genes. Therefore, suppression of PPARγ expression by treatment with VN extract might be due to AMPK activation rather than to direct inhibition of PPARγ by VN extract. Together, these results suggest that activation of AMPK is also involved in the VN-mediated antiobesity effect. Flavonoids and plant polyphenols found in abundance in fruits and vegetables have been reported to suppress adipogenesis through AMPK activation. Quercetin and

Fig. 6. VN Extract Suppressed Fatty Liver in the HFD-Treated Obese Mice.
Liver tissues from the mice were analyzed. A, TG contents were measured. **p < 0.01 as compared to lean group. ##p < 0.01 as compared to the HFD group. B, Photomicrographs of Oil-red O-stained liver tissue sections. C, AMPK activation was analyzed by immunoblotting. The genes involved in lipogenesis (D) and fatty acid oxidation (E) were analyzed by RT-PCR.
epigallocatechin, flavonoids found in most diets, suppress adipogenesis through activation of AMPK and a mitogen-activated protein kinase pathway. Curcumin, a major polyphenol in turmeric spice, genistein, a plant flavonoid, also suppress adipocyte differentiation through activation of AMPK. Furthermore, Foenum-graecum, an herbal plant used as a traditional oriental medicine, has been reported to inhibit adipocyte differentiation by activating AMPK and regulating the expression of genes involved in lipid metabolism. Even though the component that exerts antiobesity effect has not yet been characterized, the flavonoids in VN extract may contribute to the antiobesity effect of V. nakashimae. Further study is needed to characterize the component that has the antiobesity effect of V. nakashimae extract.

HFD is known to induce non-alcoholic fatty liver disease (NAFLD) in animal models and humans by causing ectopic fat deposition in the liver, and NAFLD is closely associated with obesity, diabetes, and insulin resistance. Since AMPK has been implicated in the development of NAFLD in animal models, we investigated the effect of VN extract on fatty liver in HFD fed mice. No difference was observed between the liver weights of the VN extract-treated mice and of HFD control mice. However, TG accumulation was significantly reduced in the liver of the VN extract-treated mice. Histological analysis of the liver showed an accumulation of lipid droplets in the HFD control mice, but fewer lipid droplets were observed in VN extract-treated mice. Taken together, these results suggest that VN extract works against fatty liver in HFD obese mice. Next, we determined whether alleviation of fatty liver is associated with activation of AMPK and with lipogenesis. Phosphorylation of AMPK and ACC was enhanced in the livers of the VN extract-treated mice, and in accordance with AMPK activation, its target genes were increased in the liver. The expression of lipogenesis genes was also increased in the livers of the VN extract-treated mice as compared to HFD control mice. These results indicate that VN extract works against fatty liver in HFD mice through activation of AMPK and reduction of lipogenesis. Since the components that exert the antiobesity effect have not yet been characterized, further study is needed to characterize the antiobesity component of V. nakashimae extract. According to our previous and current results, VN extract has diverse bioactivities that improve metabolic disturbances including type2 diabetes and obese and non-alcoholic fatty liver as compared with another phytochemicals.

In conclusion, V. nakashimae extract had antiobesity effects by decreasing PPARγ expression and activating AMPK. Hence it might prove a useful natural antiobesity agent reducing the risk of metabolic disorders.

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References