Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 27 September 2019 doi:10.20944/preprints201909.0309.v1

Hypolipidemic effects and mechanisms of Val-Phe-Val-Arg-Asn in C57BL/6J mice and 3T3-L1 cell models

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Abstract:

Val-Phe-Val-Arg-Asn (VFVRN) has been identified and screened from lipid-lowering chickpea peptides (ChPs) by using a pharmacokinetic model in our previous experiment. The present study was conducted to investigate its effects and mechanisms on lipid metabolism. A high-fat diet C57BL/6J mice model and 3T3-L1 preadipocyte cell model were used. VFVRN was found to significantly decrease the levels of some blood lipids. The expressions of LDL receptor (LDLR), peroxisome proliferator-activated receptors (PPAR)α, liver X receptor (LXR)α, cholesterol 7α-hydroxylase (CYP7A1) and AMP-activated protein kinase (p-AMPK) in liver were up-regulated by VFVRN treatment. The expressions of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), fatty acid synthetase (FAS), 1-aminocyclopropane-1-carboxylate synthetase (ACC), sterol regulatory element-binding protein (SREBP)-1c and SREBP-2 in liver were significantly ($P<0.05$) down-regulated. Additionally, the expressions of PPARα and PPARγ in adipose tissues were up-regulated by VFVRN significantly ($P<0.05$). VFVRN might also contribute to transintestinal cholesterol efflux (TICE) by up-regulating the expressions of LXRα and ATP binding cassette G5/8 transporters (ABGC5/8). Moreover, VFVRN promoted 3T3-L1 preadipocyte apoptosis by up-regulating the expressions of BaX, cleaved Caspase-3 and down-regulating Bcl-2. VFVRN had potent effects in reversing metabolic disorders of blood and liver in a high-fat diet mice model, as well as to promote the apoptosis of 3T3-L1 preadipocytes.

Keywords:

Val-Phe-Val-Arg-Asn (VFVRN); hypolipidemic effects; transintestinal cholesterol efflux (TICE); 3T3-L1 preadipocyte; apoptosis

1. Introduction

Hyperlipidemia has been a major risk factor for cardiovascular disease and premature atherosclerosis [1], and cardiovascular disease has been one of the leading causes of morbidity and mortality worldwide [2]. High correlations between lipid metabolism disorder, nonalcoholic fatty liver and obesity have been confirmed [3].

Many studies showed that the enzymatic hydrolysis of food protein sources or bioactive peptides have effects on the regulation of endogenous lipid balance [4]. Ile-Ile-Ala-Glu-Lys (IIAEK) isolated and purified from bovine milk protein hydrolysate had a strong ability to combine bile acid [5]. The cholesterol-lowering activities of IIAEK were better than β-sitosterol, which has been considered lipid-lowering drug [5]. The expression of the cholesterol 7a-hydroxylase gene (CYP7A1) was up-regulated by β-lactoalbumin to decrease the blood lipid levels [6]. The gene transcript of LDL receptor (LDLR) was significantly enhanced and the excess low density lipoprotein cholesterol (LDL-C) in serum were effectively cleared up by Phe-Val-Val-Asn-Ala-Thr-Ser-Asn (FVVNATSN), which was isolated from soybean 7s β-globulin [7]. The hydrophobic soybean peptide Trp-Gly-Ala-Pro-Ser-Leu (WGAPSL) was found to lower micellar solubility of cholesterol by 81.3% and decrease the levels of low density lipoprotein (LDL) and very low density lipoprotein (VLDL) in serum by 34% and 45%, respectively [8]. Lys-Glu-Pro-Ile (KEPI), His-Ile-Pro-Leu (HIPL) and Leu-Glu-Tyr (LEY) isolated from vitellogenin hydrolysates were also found to decrease total cholesterol (TC) and total triglyceride (TG) in serum of high-fat-diet mice by more than 50% [9].

The homeostatic balance of cholesterol influences blood fat levels [10]. Liver plays a particular role in lipid metabolism for the synthesis and catabolism of lipid and

lipoprotein [11]. In addition to the liver, intestine and adipose tissue are also important organs for lipid metabolism. Lipids are digested and absorbed in intestine and then stored in adipose tissue. Peroxisome proliferator-activated receptors (PPAR)α are enriched in tissues such as liver, adipose tissue, with high fatty acid oxidation (FAO) rates. PPARα agonists are useful in management of Non Alcoholic Fatty Liver Disease (NAFLD) and complications such as cardiovascular disease (CVD) [12]. In liver, the AMP-activated protein kinase (AMPK) is a central regulator of multiple metabolic pathways and has therapeutic importance for treating obesity, NAFLD and the disorder of lipid metabolism [13]. Reverse cholesterol transport (RCT) is a classics pathway for cholesterol to be transported from the periphery to the liver [14]. High-density lipoprotein (HDL) in serum carried the excess cholesterol in organism back to the liver, where cholesterol could be processed and excreted out of the body [15]. However, recent studies showed that the metabolism change of cholesterol in liver and the contents of cholesterol in the feces have not certain relationship [16]. Cholesterol can be secreted directly by enterocytes in small intestine, which seems to be more important than the pathway that cholesterol is excreted through bile duct [17].

We previously reported that Val-Phe-Val-Arg-Asn (VFVRN) exhibited a hypocholesterolemic effect in HepG2 cells [18]. The synthesis of TC and the expression of HMG-CoA reductase in HepG2 cells significantly reduced when the HpeG2 cells were incubated in DMEM medium with VFVRN for 24 h. Additionally, VFVRN and ChPs have a potent effect in reversing metabolism disorders of blood and decreasing the adipose tissues size in the ovariectomized rat model. Thus, in this study, we further study the lipid-lowering activity of VFVRN in a high-fat diet mice model and 3T3-L1 preadipocyte model. More importantly, the lipid-lowering mechanisms of VFVRN, especially in intestine and adipose tissues were explored.

2. Materials and Methods

2.1 Sample

VFVRN was identified from chickpea peptides by using a pharmacophore model [19] and synthesized by Fmoc (Fluorenyl methoxy carbonylsolid-phase synthesis) in Guotai biotech company (Anhui, China) with over 98% purity.

2.2 Animals

All experimental procedures were approved by the Hubei Laboratory Animal Research Center. All procedures were conducted according to the National Institute of Health Guidelines for the Care and Use of Animals and the animal ethical approval certificate number was MU-2016-001. The male C57BL/6J mice (20±2g) were maintained on a 12/12h light/dark cycle at room temperature (22±2 °C), with free access to water. After three days' acclimation, the mice were divided into 5 groups with 10 mice in each group: basal diet group (Control group); high-fat diet group (Model group); high-fat diet+10 mg/kg bw VFVRN group (L-VFVRN group); high-fat diet+50 mg/kg bw VFVRN group (H-VFVRN group); high-fat diet+10 mg/kg bw lovastatin group (Lovastain group). The composition of the basic diet and high fat diet are in Table 1. Lovastain can reduce the synthesis of cholesterol and increase the synthesis of LDL receptor, mainly in the liver. Since it has the preventive and therapeutic effects on atherosclerosis and coronary heart disease, it was used as a positive control in this study. VFVRN and lovastatin were given by gavage. At the end of the tenth week, the mice were housed individually for one day (free food and water), the feces were collected and stored at -80 °C for further analysis. After the ten weeks' experiment, all the mice were fasted for 12h and the blood was collected. Then the livers were quickly removed, weighted and stored at -80°C for further analysis.

2.3 Intestine perfusion procedures

Mice were anesthetized by intramuscular injection with 0.1 mL Zoletil and placed on a heating plate to maintain body temperature. Zoletil has no impacts on energetic metabolism and lipid metabolism. In selected sets of experiments, mice were intravenously injected with 10μg NBD-cholesterol (Invitrogen Life Technologies, Carlsbad, CA, USA) dissolved in 100 μL Intralipid, via tail vein. This fluorescent analog, NBD cholesterol (22-(N-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl) Amino)-23,24-Bisnor-5-Cholen-3β-Ol), is an environment-sensitive probe and is useful for investigating lipid transport processes as well as lipid-protein interactions. The intestine perfusion was started 30 minutes after injecting the NBD-cholesterol and bile was collected after 15 minutes to measure biliary cholesterol secretion by multiscan spectrum (Tecan, Swiss) at λEx =469 nm, λEm =538 nm [16]. The fluorescence data in control group was set as 1.0 and the other groups relative to control group was set as the biliary cholesterol secretion level in each group. Each group was tested with three mice. After the intestine perfusion procedure, the blood and livers of those mice were collected.

2.4 Serological analysis

Blood samples were collected in non-heparinized tubes and centrifuged at 1200 g for 10 min at 4°C and serum was collected. The levels of triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) were determined by using commercial kits purchased from Nanjing Jiancheng bio-engineering institute (Nanjing, China).

2.5 Liver analysis

Frozen mice livers in all groups were homogenized in saline solution. The levels of TG, TC, LDL-C, HDL-C and protein concentration were determined by using commercial kits purchased from Nanjing Jiancheng bioengineering institute (Nanjing, China).

2.6 Feces analysis

Frozen mice feces in all groups were lyophilized and homogenized in saline solution. The levels of TC and TG were determined by using commercial kits purchased from Nanjing Jiancheng bioengineering institute (Nanjing, China).

2.7 Cell culture and treatment

The 3T3-L1 cell line obtained from Cell Resource Center (School of Basic Medicine Peking Union Medical College) was routinely cultured in DMEM supplemented with 10 % heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were grown in the 75 mm^2 culture flasks at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 before treatment. (1) Cells were plated out in 96-well plates and incubated in media containing 0.05, 0.2, 0.4, 0.75, 1.5 and 3 mmol/L VFVRN for 24 h or 48 h to analyze the effect of VFVRN on cell proliferation in 3T3-L1 preadipocyte. (2) Cells were plated out in 6-well plates and incubated in media containing 1, 2, 3 mmol/L VFVRN for 24 h to analyze the apoptosis effect of VFVRN in 3T3-L1 preadipocyte. (3) Cells were plated out in 96-well plates and incubated in media containing 0.05, 1, 2, 4, 6 and 8 mmol/L VFVR, VFV, FVRN and VRN, respectively, for 48 h. It was aimed to analyze the effect of VFVRN 's derivatives on cell proliferation in 3T3-L1 preadipocyte. (4) Cells were plated out in 6-well plates and incubated in media containing 4 mmol/L VFVR for 48 h to analyze the apoptosis effect of VFVR in 3T3-L1 preadipocyte. PBS was used in

the control experiment. Each treatment was carried out at least in triplicate. The cell proliferation was determined by MTT method. The cell apoptosis was determined with flow cytometer by using commercial kits purchased from Nanjing Jiancheng bioengineering institute (Nanjing, China).

2.8 MTT analysis of cell proliferation

Briefly, after exposure of cells to samples, culture media was changed by free serum culture media. MTT dissolved (0.5mg/mL) in free serum culture media was added to each well and incubated for 3 h. After this interval, MTT was discarded and 150 μL DMSO was added to each well dissolving the precipitate. The optical densities were measured at 560 nm spectral wavelength.

2.9 Annexin V-FITC/PI double staining and flow cytometry analysis of cell apoptosis

After treatment of tryptic digestion, cells were scraped off and washed with ice-cold PBS and centrifuged for 5 min (1000 g) at 4°C. Cells ($1\text{-}5\times10^5$) were resuspended by PBS and bonding solution was added. Then, 5 μl Annexin V-FITC and 5μl propidium iodide were added and mixed thoroughly in turn. The reaction occurred at room temperature against light for 20 minutes and flow cytometry was performed immediately. The apoptosis rate was calculated from the flow cytometer by FlowJo software (Treestar). Scatter plot of the double staining (V-FITC/PI) of apoptosis : up right (UR): the late stage apoptotic cells; low right (LR): the early stage of apoptosis cells; low left (LL): the normal cells; up left (UL): the dead cells.

2.10 Western blot analysis

Protein expression levels in liver and cells were tested by western blot method. RIPA lysis buffer and PMSF were used to collected cell samples. The concentration of

protein was tested using BCA kit. Proteins were separated by 12% SDS-PAGE and then transferred to a PVDF membrane (1h, 200mA). The membrane was blocked in 3% defatted milk for 1h at room temperature and then incubated at 4℃ overnight with primary antibody against target proteins in blocking buffer. After being washed with TBST buffer under gentle agitation, membranes were incubated for 1h at room temperature with second antibody. After further washing, blots were treated with ECL detection reagents. Densitometry analysis of specific bands was performed by Scion Image software (Scion Corporation, Frederick, MD, USA). Immunoblotting was performed using the following antibodies: anti-pAMPK, anti-PPARa, anti-SREBP-1c, anti-SREBP-2, anti-LXRα, anti-LDLR, anti-HMGR, anti-ACC, anti-FAS, anti-CYP7A1, anti-ABGC5/8, anti-Bax, anti-Bcl-2, anti-cleaved-caspase 3 and anti-β-actin from Santa Cruz Biotechnology (Santa Cruz, TX, USA).

2.11 Statistical analysis

Experimental data were presented as mean values ± SD. The mean values were compared by ANOVA with $p < 0,05$ and Duncan's multiple range test at $P < 0.05$ using SAS software ver.8.1.

3. Results

3.1 Effects of VFVRN on TC, TG, HDL-C, LDL-C in serum, liver and feces

As shown in Fig. 1A, serum TG, TC and LDL-C levels in Model group were significantly ($P<0.05$) higher while serum HDL-C was significantly lower than those in the control group ($P<0.05$). After treatments with VFVRN and lovastatin, the levels of TG, TC and LDL-C were significantly lower ($P<0.05$) than those in the Model group. Additionally, the level of HDL-C was significantly elevated after the treatments with

VFVRN and lovastatin compared to the Model group. There was no significant difference of the variables mentioned above among VFVRN-treatment groups and lovastatin-treatment group.

In the liver, the levels of TG, TC, LDL-C and HDL-C in Model group were significantly higher ($P<0.05$, Fig. 1B) than those in the control group. The levels of TG, TC, HDL-C and LDL-C in VFVRN-treatment groups and lovastatin-treatment group were significantly lower than those in the Model group. As shown in Fig. 1 C, after the treatment with VFVRN, the levels of TG and TC in feces were significantly higher than those in the Model group ($P<0.05$, Fig. 1C), which indicated that the lipid excretion increased after the treatments with VFVRN. There was no significant difference ($P>0.05$) of TC and TG in feces between Model group and lovastatin-treatment group.

3.2 Effect of VFVRN on lipid metabolism in liver

3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) is the rate controlling enzyme of the mevalonate pathway which produces cholesterol and other isoprenoids [16]. The acetyl-CoA carboxylase enzymes (ACC) and fatty acid synthase (FAS) regulate the synthesis of fatty acids [20]. SREBPs are considered to play a vital role in the process of lipid metabolism [21]. As shown in Fig. 2, the expressions of HMGR (EC:1.1.1.345), FAS (EC 2.3.1.85), ACC (EC 6.4.1.2), SREBP-1c and SREBP-2 in the Model group were markedly higher ($P<0.05$) than those in the control group. Compared with the Model group, the expressions of these proteins in VFVRN-treatment groups were markedly reduced ($P<0.05$). As for lavastatin group, these proteins expressions also significantly decreased ($P<0.05$) compared to the Model group. Interesting, the effects of VFVRN, in some parameters such as HMGR, was better than lavastatin.

Liver X receptors (LXRs) are nuclear receptors that play central roles in the transcriptional control of lipid metabolism and are important to convert cholesterol to bile acid in liver for organisms to eliminate cholesterol [22]. Peroxisome proliferator-activated receptors (PPAR)α is one nuclear receptor, which allows adaptation of the rates of fatty acid (FA) catabolism [23]. AMP-activated protein kinase (AMPK) signaling system has many implications for animal and human health [24], with roles in diverse functions such as energy metabolism, glucose and lipid metabolism [25, 26]. LDL receptors (LDLR) provides an efficient mechanism for delivery of cholesterol from blood to liver [27]. The CYP7A1 plays an important role in regulation of bile acid biosynthesis and cholesterol homeostasis [28]. The expressions of p-AMPK, LXRα and PPARα were decreased and LDLR was increased in Model group compared to control group. Compared with the Model group, the expressions of LDLR, PPARα, LXRα, CYP7A1 (EC 1.14.13.17) and p-AMPK in VFVRN-treatment groups were significantly increased ($P<0.05$, Fig. 2).

3.3 Effect of VFVRN on lipid metabolism in adipose tissue

As shown in Fig. 3A, the body weight in VFVRN-treatment groups were decreased by 11.11% and 14.81% than that in the Model group, but not significant. The adipose tissue weight in the Model group was significantly higher than that in the control group (Fig. 3B). VFVRN-treatment group showed 66.5% and 69.9% decreases ($P<0.05$) in the adipose tissue weight respectively compared to the Model group. There was no significant difference between lovastatin-treatment group and control group in the weight of adipose tissue and body weight ($P>0.05$). Compared with Model group, the expressions of LXRα, PPARα and PPARγ in adipose tissues in VFVRN-treatment groups were significantly increased ($P<0.05$, Fig. 2).

3.4 Effect of VFVRN on lipid metabolism in intestinal tract

NBD-cholesterol is a fluorescent analogue of cholesterol and commonly used to investigate lipid transport processes. As shown in Fig. 3C, the level of NBD-cholesterol in high dosage VFVRN-treatment group was significantly ($P<0.05$) elevated than that in the Model and lovastatin groups. ABCG8 and ABCG5 are transmembrane transport proteins and accelerated the elimination of cholesterol in small intestine cells [29]. Compared with the Model group, the expression of LXRα, ABCG5 and ABCG8 were significantly increased in intestine ($P<0.05$, Fig. 2) after the VFVRN treatments.

3.5 Effect of VFVRN and derivatives on cell viability in 3T3-L1 preadipocytes

As shown in Fig.4A, VFVRN-treatment (≥0.2 mmol/L) could markedly ($P<0.05$) inhibit the growth of 3T3-L1 preadipocytes. VFVRN-treatment (3 mmol/L) could also induce 3T3-L1 preadipocytes apoptosis by 58.75% (Fig. 6).

The Caspase-3 protease is a predominant player in the execution of apoptosis [30]. The Bcl-2-associated X protein (Bax) protein is a member of the B-cell lymphoma 2 (Bcl-2) family and is pro-apoptotic [31]. The Bcl-2 protein is an inhibitor of apoptosis [32]. As shown in Fig. 4B, the expressions of Bax and cleaved Caspase 3 were up-regulated and Bcl-2 was down-regulated by VFVRN-treatment.

As shown in Fig.5C, the derivative VFVR (≥4 mmol/L) could markedly ($P<0.05$) inhibit the growth of 3T3-L1 preadipocytes, while VFV, FVR and FVRN had no inhibitory effect on viability of 3T3-L1 preadipocytes (≤8 mmol/L, Fig.5). Additionally, VFVR-treatment (4 mmol/L) could also induce 3T3-L1 preadipocytes apoptosis by 27.54% (Fig. 6).

4. Discussion

4.1 Effect of VFVRN on lipid metabolism in liver

The activated AMPK in the liver can effectively inhibit the non-alcoholic hepatic fatty induced by high-fat diet [24]. AMPK switches off anabolic pathways such as cholesterol, fatty acid, and triglyceride biosynthesis [33]. Treatment with VFVRN increased the expression of p-AMPK in the high-fat diet mice and high expression of p-AMPK could effectively inhibit the activities of FAS and ACC [25, 26]. Recent studies demonstrated that hepatic lipogenic transcription factors (SREBP-1c, LXRα and PPARα) could be coordinated by activated AMPK [34]. Moreover, AMPK-independent pathways are important for regulating fatty acid uptake and oxidation [34]. Treatment with lovastatin increased the expression of p-AMPK in the present study. It was reported that the AMPK signaling pathways could be activated by lovastatin to play its varieties of biological activities [35, 36].

Our results showed that the expression of SREBP-1c was down-regulated and the expression of LXRα and PPARα were up-regulated in VFVRN-treatment groups. The SREBP-1c isoform are involved in fatty acid and glucose metabolism, whereas the SREBP-2 isoform primarily regulated the cholesterol biosynthesis and contributed to the maintain cholesterol homeostasis [37]. Activated AMPK not only inhibited the hydrolysis of SREBP-1c and limited SREBP-1c getting into the nucleolus, but also inhibited SREBP-1c in nuclear and the expression of its target genes to alleviate hyperlipidemia and atherosclerosis in mice [34]. The expression of SREBP-2 and HMGR could be decreased by lovastatin to inhibit cholesterol synthesis in HepG2 cel l [38]. LXRα is highly expressed in hepatocytes and induced the expressions of a range of genes involved in cholesterol efflux and clearance [39]. CYP7A1 was the rate-limiting enzyme for the conversion of cholesterol to bile acids and CYP7A1 is the first

LXRα target gene identified [40, 41]. Another major function of LXRs in the liver is the promotion of de novo lipogenesis and the biosynthesis of fatty acids [42]. In vivo studies have revealed the role for LXRα in the control of SREBP-1c expression and its lipogenic target genes [21]. SREBP-2 regulates cholesterol homeostasis through the related target genes and HMGR is a prime target of SREBP-2 [43]. A major target of the SREBP-2 pathway was LDLR that mediates clearance of LDL particles from the circulation [44]. SREBP-mediated regulation of LDL receptors was essential for the action of statin drugs in lowering plasma LDL-cholesterol levels in individuals at risk for coronary heart disease [45]. The studies found that lovastatin could decrease de novo cholesterol synthesis and LDL Apo B-100 production rats [46]. LDLR activity in circulating monocytes was also increased by lovastatin to contribute to the lipid lowering action [12].

PPARα is a nuclear receptor, which allows adaptation of the rates of fatty acid (FA) catabolism [34]. The expression of PPARα increased by lovastatin might due to the activation of AMPK signaling pathways [47]. In patients with atherogenic dyslipidemia, fibrates acting as synthetic PPARα agonists, lower plasma TG and LDL-C and raise HDL-C levels [48]. Hepatic TG can be hydrolyzed and the released fatty acids oxidized by the mitochondrial β oxidation [49]. PPARα controls gene expression levels of the rate-limiting enzymes of peroxisomal β-oxidation and FA transport [29]. The reduction of plasma TG-rich lipoprotein upon PPARα activation was related to the enhanced FA uptake, conversion into acyl-CoA derivatives, and further catabolism via the β-oxidation pathways [50]. The plasma HDL-C was increased by stimulating the synthesis of its major apolipoprotein apoA [51].

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Overall, our results showed that VFVRN could activate the expression of AMPK and improve the metabolism of lipids in liver by AMPK/SREBPs/PPARα signaling pathway.

4.2 Effect of VFVRN on lipid metabolism in adipose tissue

Treatment with VFVRN increased the expression of PPARα and PPARγ in adipose tissue in high-fat diet mice. The PPARα anti-obesity and insulin sensibility effect were attributed to increase lipid oxidation in tissues to promote lipid depletion [34]. Activation of PPARα in adipose tissue increased the rates of fatty acid (FA) catabolism and reduced the synthesis of FA and TG [52]. PPARγ also existed in adipose tissue and regulated the expression of genes involved in lipid metabolism. Several transcription factors are involved in adipogenesis, of which PPARγ was known to be a prerequisite [53]. PPARγ is more powerful to regulate the differentiation and growth of adipocytes than PPARα in adipose tissue [54]. PPARγ was involved in differentiation and maturation of adipocytes [55]. Activation of PPARγ leaded to the differentiation of preadipocytes into adipocytes and contributed to the apoptosis of mature adipocytes [56]. When the PPARγ agonist thiazolidinedione was administered to Zucker rats, the number of new adipocytes and insulin sensitivity were found to be increased [56]. In peripheral organs, activated PPARγ augmented insulin sensitivity and inhibited gluconeogenesis to lower blood sugar [57]. Obese diabetic subjects showed significantly larger adipocytes and decreased PPARγ expression compared to the control group in human study [58]. White fat stores calories and brown fat burns energy to generate heat. Activation of PPARγ in adipose tissue could induce the switch from white to brown fat [59]. The activation of PPARγ stimulated apoptosis in variety of cell types, including 3T3-L1 adipocyte, cancer cells, endothelial and T-lymphocytes [60-

63]. PPARγ is a regulator of adipogenesis, and was found to be increased in the present study, but it also leaded to the apoptosis of mature adipocyte. Those activities could reduce the proliferation of preadipocytes and less lipid was accumulated in mature adipocyte. The mature adipocyte with large lipid droplets were dead induced by apoptotic. Our results showed that VFVRN may suppress the growth of fat tissue by activating the expression of PPARs.

4.3 Effect of VFVRN on lipid metabolism in intestinal tract

Transintestinal cholesterol efflux (TICE) is an important pathway for cholesterol excretion [64]. When serum HDL-C level was very low, the excretion of cholesterol and bile acid in feces was still normal [16]. Lovastatin could up-regulate the expression of ABCG8 and ABCG5 to reduce the cholesterol absorption [65]. Treatment with VFVRN increased the expression of LXRα, ABCG8 and ABCG5 in a dose-dependent manner. Activated LXRα could promote the process of TICE and increase the excretion of cholesterol in intestinal canal [66]. ABCG8 and ABCG5 are transmembrane transport proteins and accelerated the elimination of cholesterol in small intestine cells [29]. It was reported that there was a negative correlation between the efficiency of cholesterol absorption and the abundance of ABCG5 and ABCG8 in the jejunum and ileum [67]. Overexpressing ABCG5 and ABCG8 in mice exhibited a 50% decrease in the fractional absorption of dietary cholesterol [68]. Our results illustrated that VFVRN could promote transintestinal cholesterol efflux and reduce the absorption of cholesterol in small intestine.

V and F in VFVRN are hydrophobic amino acids. R and N are hydrophilic amino acid. These three continual hydrophobic amino acids were beneficial to inhibit HMGR activity and decrease the absorption of cholesterol in intestinal tract [69]. The

relationship between lipid-lowering activity and structure of peptides were not clear now. It was reported that IIAEK and its fragment IAEK, AEK, EK all could increase the expression of CYP7A1 [70].

Two dosages of VFVRN (10 mg/kg bw and 50 mg/kg bw) were used in the present animal experiment. Even though no significant difference was detected between these two levels, both doses of VFVRN were shown good lipid-lowering activities. Higher dosage of VFVRN may show better lipid-lowering activity.

4.4 Effect of VFVRN on cell proliferation in 3T3-L1 preadipocytes

It was proved that apoptosis is a significant factor in adipocyte depletion during weight reduction in vivo and in vitro studies [71]. It is a potential target for treatment of obesity and its related disease. Bcl-2 and Bax, members of the Bcl-2 family of proteins, are important components of apoptosis. High expression of Bcl-2 was able to inhibit apoptosis, while high expressions of Bax could stimulate apoptosis. A change in the expression ratio of these two factors determines whether apoptosis occurs [72]. Additionally, Caspase family members have major roles in cell apoptosis and Caspase-3 is an effector caspase that mediates the cleavage of many proteins [71]. VFVRN exhibited a marked inhibitory effect on cell viability on 3T3-L1 preadipocytes in vitro. Moreover, treatment with VFVRN decreased the expression of Bcl-2 and increased the levels of Bax and clevaed-caspases-3 in a dose-dependent manner. Only VFVR, which was one of four fragment peptides of VFVRN (VFV, VFVR, FVRN and VRN), could also exhibit a significant inhibitory effect on cell viability on 3T3-L1 preadipocytes in vitro. It suggested that three continual hydrophobic amino acids VFV and hydrophilic amino acid R were indispensable for the inhibitory effect of proliferation. Those results

indicated that VFVRN may be a potential high efficiency anti-obesity agent by regulating the Bax/Bcl-2/Caspase-3 signaling pathway.

5. Conclusion

VFVRN has potent effects in reversing metabolism disorders of blood and liver in high-fat diet mice model. The hypolipidemic effect of VFVRN was due to the significant up-regulation of AMPK expression in liver to regulate its target genes associated with lipid metabolism expression, such as PPARs, SREBPs, LXRα. PPARs in adipose tissue. Additionally, LXRα, ABGC5 and ABGC8 in small intestine were up-regulated by VFVRN. Moreover, VFVRN could up-regulate the expressions of BAX, caspase 3 and down-regulate Bcl-2 to induce 3T3-L1 preadipocytes apoptosis. Overall, VFVRN could effectively regulate lipid metabolism disorder in the body.

Author Contributions: Funding acquisition, Ruge Cao; Investigation, Wen Shi; Project administration, Tao Hou; Resources, Hui He; Supervision, Tao Hou; Writing – original draft, Wen Shi; Writing – review & editing, Hui He, Ruge Cao and Tao Hou.

Acknowledgments: This work was financially supported by the Open Project Program of State Key Laboratory of Food Nutrition and Safety (SKLFNS-KF-201810), Tianjin University of Science & Technology

Conflicts of Interest: The authors declare no conflict of interest.

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Table 1. The composition of basic and high-fat diet composition (g/kg)

Basic diet composition (g/kg)				High-fat diet diet composition (g/kg)			
corn flour	500	wheat bran	90	basic diet	789	lard oil	100
wheat flour	90	bean dregs	220	cholesterol	10	yolk powder	50
fish flour	70	bone meal	20	cholate	1	whole milk powder	50
sodium chloride	5	trace elements	1.6				
multivitamins	1						

Figure.1 The effect of VFVRN on (A) serum profile, (B) hepatic lipid profile and (C) fecal profile in high fat-diet C57BL/6 mice. (n=10). Control group: the group of basic diet food without VFVRN treatment. Model group: the group of high-fat diet mice without VFVRN treatment. L-VFVRN group: the group of high-fat diet mice treated with 10 mg/kg bw VFVRN. H-VFVRN group: the group of high-fat diet mice treated with 50 mg/kg bw VFVRN. Lovastatin group: the group of high-fat diet treated with 10 mg/kg bw lovastatin. [a,b,c] within column means without a common letter are significantly different, $p < 0.05$ (Duncan's multiple range test at $p < 0.05$).

Figure.2 The effect of VFVRN on the expressions of proteins in high fat-diet C57BL/6 mice (n=3). The effect of VFVRN on (A) HMGR, (B) CYP7A1, (C) FAS, (D) ACC, (E) pAMPK, (F) PPARα，(H) LXRα, (I) LDLR，(J) SREBP-1C, (K) SREBP-2 in the liver; (L) PPARα, (M) PPARγ in the adipose tissue; (N) ABGC5, (O) ABGC8, (P) LXRα in the small intestine; (Q) the WB band results of proteins in liver, adipose tissue and small intestine. [a, b, c] within column means without a common letter are significantly different, $p < 0.05$ (Duncan's multiple range test at $p < 0.05$).

Figure.3 The effect of VFVRN on (A) body weight (n=10), (B) the weight of abdominal fat (n=10), (C) the relative output levels of NBD-cholesterol by intestinal tract (n=3). [a, b, c] within column means without a common letter are significantly different, $p < 0.05$ (Duncan's multiple range test at $p < 0.05$).

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Figure.4 (A) The effect of VFVRN on cell viability in 3T3-L1 preadipocytes. (B)The effect of VFVRN on the expression of Bax, Bcl-2 and cleaved-caspases-3. The relative protein levels were the target protein to β-actin. [a, b, c] within column means without a common letter are significantly different, $p < 0.05$ (Duncan's multiple range test at $p < 0.05$).

Figure.5 The effect of FVRN、VRN、VFVR and VFV on cell viability in 3T3-L1 preadipocytes. (A) FVRN, (B) VRN, (C) VFVR, (D) VFV. [a,b,c,d] within column means without a common letter are significantly different, $p < 0.05$ (Duncan's multiple range test at $p < 0.05$, n=3).

Figure.6 The effect of VFVRN and VFVR on cell apoptosis in 3T3-L1 preadipocytes. (A) control group, (B) 3 mmol/L VFVRN treatment group, (C) control group, (D) 4 mmol/L VFVR treatment group, (E) the apoptosis rates of VFVRN and VFVR in 3T3-L1 preadipocytes. Scatter plot of apoptosis : up right (UR): the late stage apoptotic cells; low right (LR): the early stage of apoptosis cells; low left (LL): the normal cells; up left (UL): the dead cells.

Figure 1:

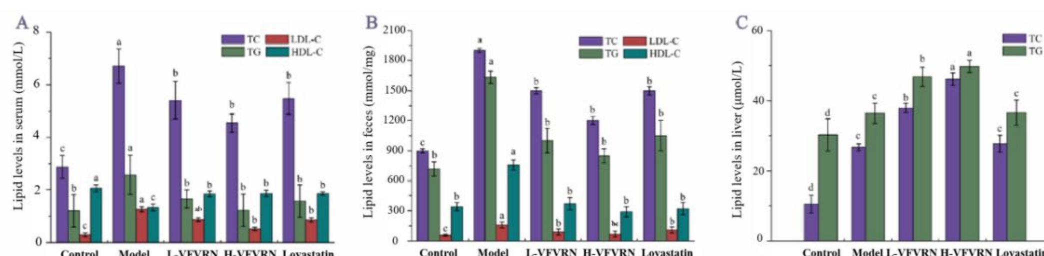

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Figure 2:

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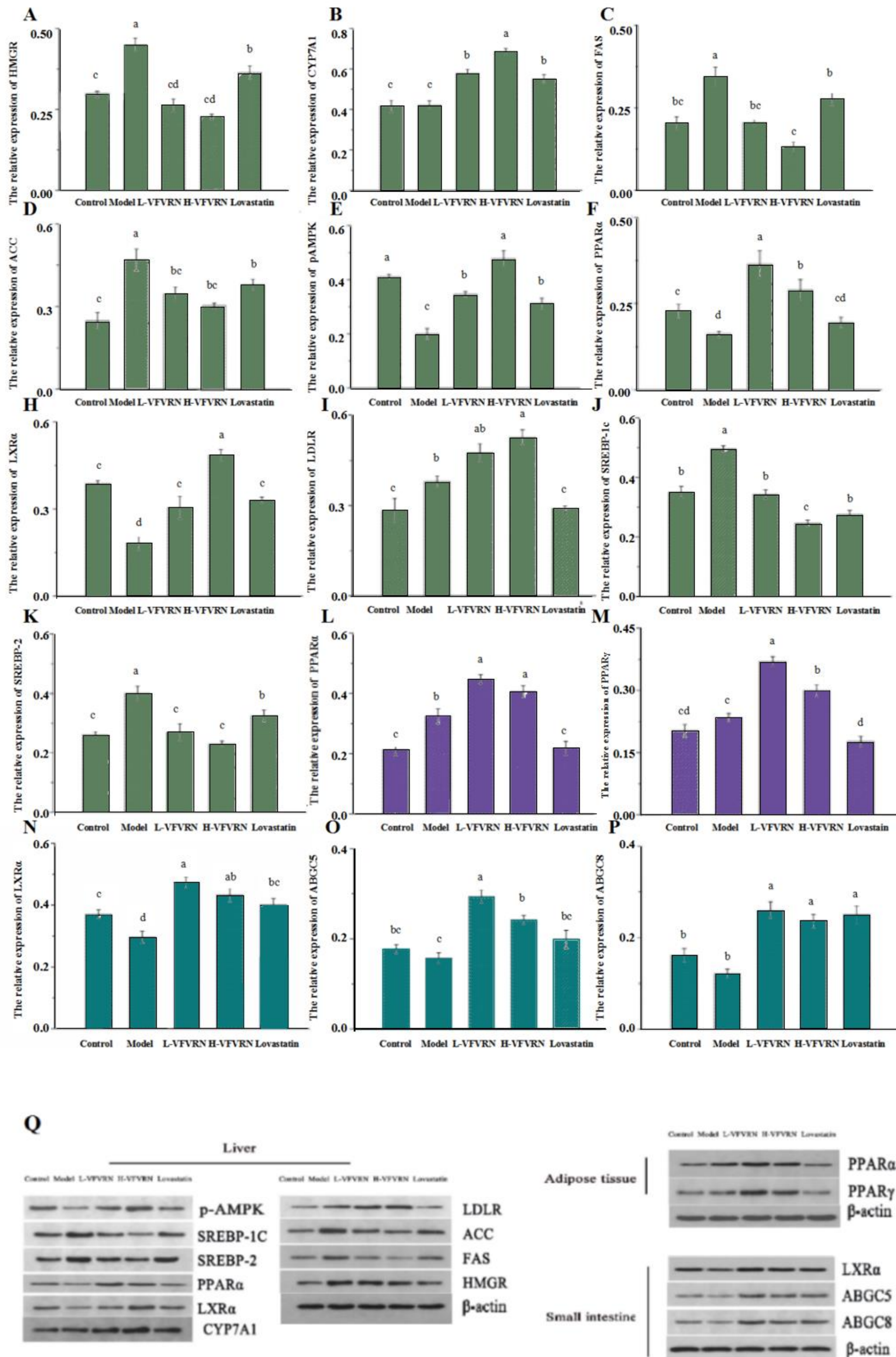

Figure 3:

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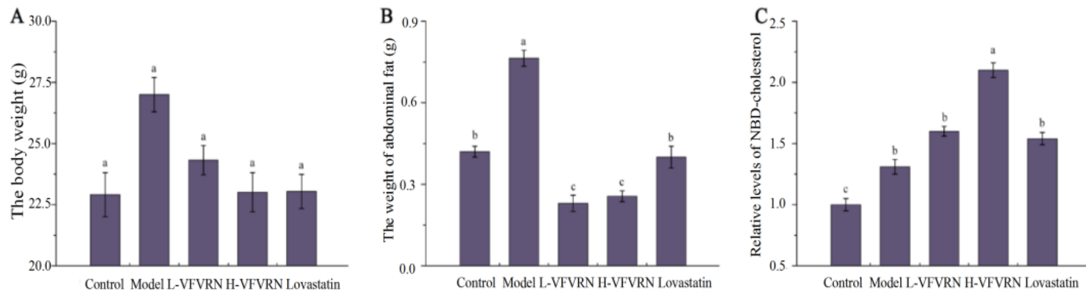

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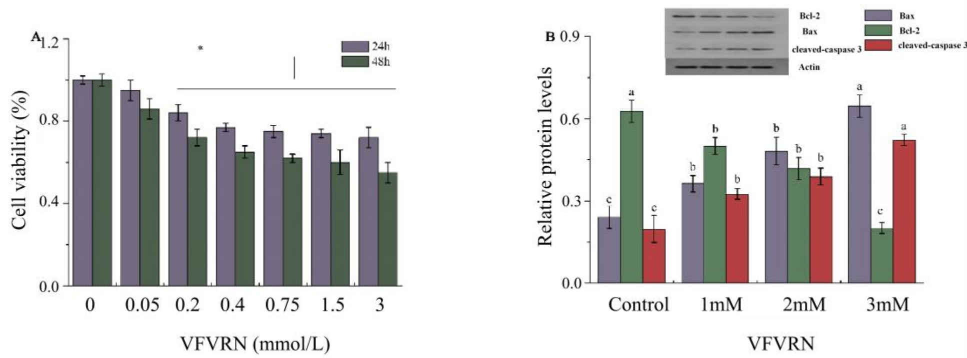

Figure 5

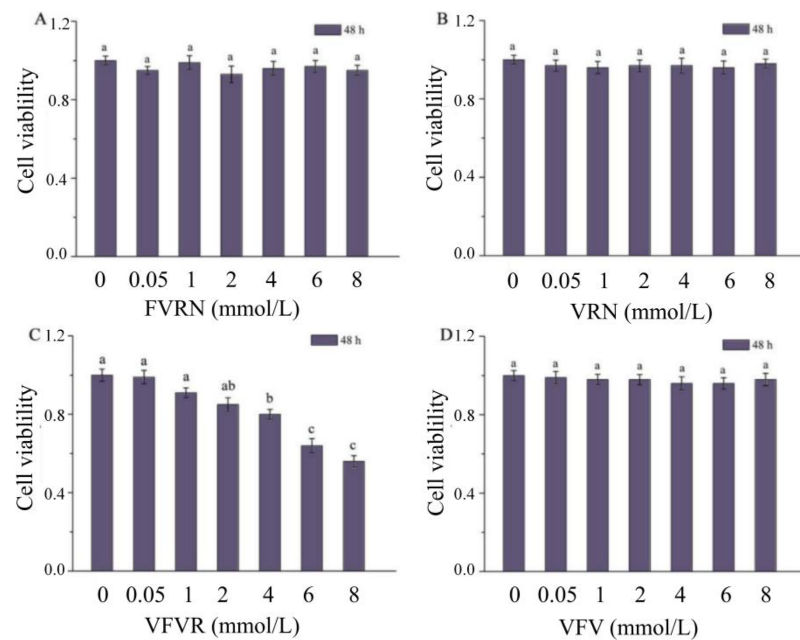

Figure 6

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