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The anti-adipogenic effect of vitexin is via ERK 1/2 MAPK signaling in 3T3-L1 adipocytes

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Abstract

Vitexin, identified as apigenin-8-C-D-glucopyranoside, a natural flavonoid compound found in certain herbs such as hawthorn herb. The aim of this study is to investigate the regulation of glycolysis underlying vitexin-induced anti-adipogenesis in 3T3-L1 adipocytes. Vitexin inhibited adipose accumulation, glucose consumption and triglyceride synthesis. The expression of ERK 1/2 MAPK greatly induced, whereas the expression of adipogenic markers Akt and peroxisome proliferator-activated receptor γ (PPARγ) diminished. ERK 1/2 MAPK inhibitor PD98059 (10 μM) significantly enhanced lipid accumulation, triglyceride synthesis and the expression of adipogenic markers. However, PD98059 had no effect on glucose consumption. In regulation of glycolysis, vitexin induced the expression of glycerol-3-phosphate dehydrogenase (GPDH) at higher dosage (50 and 100 μM) and without any effect on glucose-6-phosphate dehydrogenase (G6PDH) expression. PD98059 had an opposing effect that it significantly increased the expression of G6PDH, but decreased the expression of GPDH. Vitexin increased the lactic acid synthesis to about 3.8 folds in concentration dependent, whereas, PD98059 decreased the level of lactic acid in media to about 72% when compared with controls. Moreover, PD98059 abolished the anti-adipogenic effect of vitexin. Vitexin influence the expansion of adipose tissue through its ability to inhibit preadipocyte migration to about 80% via decreased the activity of active MMP-2. We demonstrated evidences for the first time that using of vitexin to against adipose accumulation, at least in part, was regulation of glycolysis via activation of ERK 1/2 MAPK signaling. Moreover, blockade pentose phosphate pathway may be a novel strategy for obesity prevention and therapy.

Keywords: vitexin, 3T3-L1 adipocyte, migration, adipogenesis, ERK 1/2 MAPK, pentose phosphate pathway

Introduction

Glucose is the major carbohydrate available to most animal cells. Most of the carbon for fatty acid synthesis is derived from glucose. Glycolytic intermediates fuel several biosynthetic pathways that are essential for duplication of biomass during cellular proliferation. After cellular uptake through glucose transporters, glucose must be phosphorylated by hexokinase (HK), which produced glucose-6-phosphate (G6P), to prevent its transport out of the cell and to prime it for metabolism in subsequent reactions.

The glycolysis can be divided into four master shunts after G6P (Figure. 1). (1) Pentose phosphate pathway (PPP, also called the phosphogluconate pathway and the hexose monophosphate shunt). G6P can either proceed into glycolysis through conversion into fructose-6-phosphate (F6P) by glucose-6-phosphate isomerase (GPI), or it can be shunted into the oxidative branch of the PPP by glucose-6-phosphate dehydrogenase (G6PDH) [1]. G6PDH, the rate-limiting enzyme of PPP, produces cellular NADPH which is required for the biosynthesis of fatty acids and cholesterol [2]. (2) Glycerol-3-phosphate (G3P) shunt, the interconversion of G3P and dihydroxyacetone phosphate (DHAP) by glycerol-3-phosphate dehydrogenase (GPDH). G3P is important as a precursor for glycerol and glycerolipid synthesis [3]. GPDH occupies a key position in metabolism linking glycolysis to phospholipid and triacylglycerol synthesis. It catalyzes the conversion of DHAP and NADH to G3P and NAD^+ [4]. The activity of GPDH is also increased during preadipocyte differentiation, presumably playing an important role in the conversion process and it is thus used as a classical marker for adipose cell differentiation [5]. (3) Oxidative phosphorylation (OXPHOS). Before the introduction of free oxygen into the atmosphere, life on earth depended on glycolysis for energy production. With the rise of atmospheric oxygen, cells evolved the ability to use OXPHOS to produce more energy per metabolite than the more ancient anaerobic pathway [6]. About 95% of the energy that cells need to live is produced in the mitochondria through OXPHOS such as in normal differentiated cells [7]. (4) Anaerobic glycolysis, the transformation of pyruvate to lactate when limited amounts of oxygen are available. In most cancer cells instead rely on

aerobic glycolysis to produce lactic acid, a phenomenon termed "Warburg effect" [8]. Most tumors in vivo synthesize some ATP by oxidative metabolism, and some by glycolytic metabolism to lactate (aerobic glycolysis). Clearly, if the oxygen supply is removed (acute hypoxia), the tumor cells switch to anaerobic glycolysis, just as would normal tissue [9]. Oncogenic signaling drives glucose uptake and metabolism in excess of cellular needs. Because most tumor cells cannot store carbon as glycogen or triglyceride, the excess carbon from glycolysis must be secreted as lactate [6].

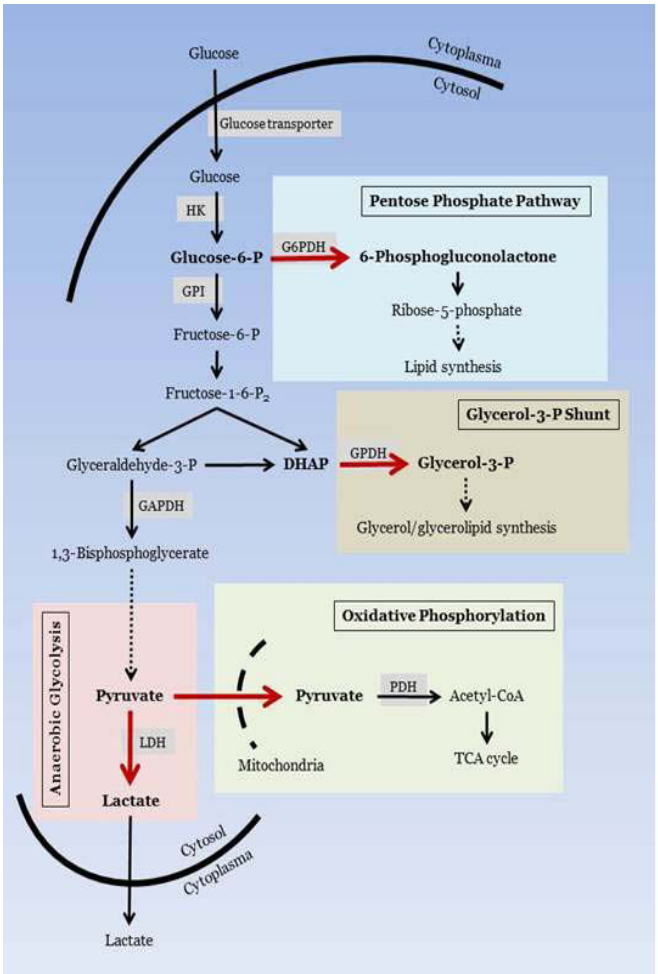

Figure 1. Schematic diagram showing master shunts of glycolysis. The glycolytic pathway can be divided into four master shunts: (1) Pentose phosphate pathway (PPP, also called the phosphogluconate pathway and the hexose monophosphate shunt) branched off glycolysis and it shunted by glucose-6-phosphate dehydrogenase (G6PDH). (2) Glycerol-3-phosphate (G3P) shunt, the interconversion of G3P and dihydroxyacetone phosphate (DHAP) by glycerol-3-phosphate dehydrogenase (GPDH). (3) Oxidative phosphorylation (OXPHOS), by which most ATPs (~34-36) are produced in cellular respiration (4) Anaerobic glycolysis, in which glucose broken down to produce energy and lactate.

Vitexin, identified as apigenin-8-C-D-glucopyranoside, a natural flavonoid compound found in certain herbs such as hawthorn herb [10]. It showed remarkable promise for a wide range of pharmacological uses, including anti-inflammatory [11], anti-oxidant [12] and anti-turmor [13]. Here, we demonstrated evidences for the first time that using of vitexin to against adipose accumulation, at least in part, was regulation of glycolysis via activation of ERK 1/2 MAPK signaling. Moreover, blockade pentose phosphate pathway may be a novel strategy for obesity prevention and therapy.

Materials and Methods

Cell culture

Mouse 3T3-L1 preadipocytes were cultured in maintained medium , DMEM (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Gibco BRL) , streptomycin (10000 U/ ml) and penicillin (10000 U /ml), at 37 ˚C in 5 % CO_2. In 3T3-L1 cell line, growth arrest is required before initiation of differentiation and growth-arrested postconfluent cells can be converted into adipocytes by the presence of the adipogenic hormones dexamethasone, 3-isobutyl-1-methylxanthine and insulin [14]. To induce adipocyte differentiation, cells were grown in plates to full confluence and then the medium was changed to adipogenic medium, maintained medium containing 10 μg/ml insulin (Sigma Chemical Co., St. Louis, MO), 0.5 μM dexamethasone (Sigma), and 0.5 mM isobutylmethyl xanthine (IBMX) (Sigma). Concurrently, the adipogenic medium supplemented with various concentrations (0, 12.5, 25, 50 and 100 μM, respectively) of vitexin (Sigma) for 8 days. The media was changed every two days in cultivation. Vitexin was dissolved in DMSO (Sigma) and stored at 20 C. The volume of DMSO was equalized to 0.1 or 0.2% in all culture dishes.

Cell viability assay

Cell viability was measured by alamar blue assay (AbD Serotec, Oxford, UK) as the manufacturer recommended. Alamar blue assay was quantified the reducing environment of the cells. The reducing environment of the cells in the alamar blue assay is measured through the conversion of resazurin (oxidised form) to resorufin (reduced form). This results in colorimetric (absorbance) and fluorescence changes. Resazurin is blue and non-fluorescent whereas resorufin is red and highly fluorescent. In short, cells were seeded in a 24 wellplate as described in "Cell culture". At the end of incubation, add alamar blue reagent in an amount equal to 10% of the volume in the well. Incubated cultures for 4 hours then measured cytotoxicity by using spectrophotometry at 570 and 600 nm.

In vitro migration assay

Cell migration assay was identified by the ability to migrate through Transwell inserts (Millipore Co., Billerica, MA) with 8.0 μM pore size polyethylene terephthalate (PET) membrane. Briefly, placed 10000 isolated cells in the upper chamber and filled both the upper and lower compartments of the migration chamber with DMEM medium containing 10% FBS and various concentrations of vitexin. After incubation for 2 hours at 37 ˚C, fixed with methanol and stained with 0.5% crystal violate. Non-migrated cells were removed from the upper chamber with a cotton swab to aid visualization of migrated cells. After acquired pictures, air dried the Transwell

insert membranes followed by dissolving in 33% glacial acetic acid (Sigma). The absorbance was determined at 570 nm.

Gelatinase zymography

Gelatinase activity was determined by zymography. It was performed in 10% SDS polyacrylamide gel in the presence of 0.2% gelatin (Sigma) under non-reducing conditions. Briefly, the cultured media (10 μg/lane) was mixed with sample buffer and applied to non-reduced SDS-polyacrylamide gel electrophoresis. Following electrophoresis the gels were renatured by exchanging SDS with 2.5% Triton X-100 for 30 min twice at room temperature and then incubated at 37˚C overnight in substrate buffer containing 40 mM Tris-HCl and 10 mM CaCl2 at pH 8.0. Stained with 0.5% Coomassie Blue R250 in 50% methanol and 10% glacial acetic acid for 30 min and destained. Gels were scanned and images of band patterns were analyzed with NIH image software (National Institutes of Health, Bethesda, MD).

Glucose and triglyceride assay

Glucose and triglyceride were measured by using assay kit, (HUMAN GmbH, Wiesbaden, Germany), as the manufacturer recommended. Briefly, the medium was collected and centrifuged to remove the cells, and incubated for 5 min at 37 ˚C with assay reagent. The concentration can be measured by the absorbance at 500 nm.

Lactic acid biosynthesis

Lactic acid synthesis was measured by using lactate assay kit (Sigma) as the manufacturer recommended. Briefly, the medium was collected and centrifuged to remove the cells, and lactate concentration is determined by an enzymatic assay, which results in a colorimetric/fluorometric product, proportional to the lactate present.

Oil Red O staining

Oil Red O dye (Sigma) was dissolved in isopropanol (Sigma) and then diluted with distilled water. Cell monolayer was rinsed with PBS and fixed with 10% formalin at room temperature for 1 hour. After fixation, cells were washed with 60% isopropanol for 5 min then let the cells dry completely at room temperature. Stained for 10 min at room temperature by immersion with Oil Red O solution followed by washed with distilled water several times. After acquired images under microscope, dried the cells completely again and then eluted Oil Red O dye by 100% isopropanol and measured the absorption at 500 nm.

Immunoblotting analysis

3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation. The cells were washed twice with cold PBS before being extracted with cell lysis reagent (Fermentas Inc., Hanover, MD). The protein quantity was determined with Bio-Rad protein assay (Bio-Rad Laboratories Inc., Hercules, CA) using bovine serum albumin as a standard. The expression level of p53 (Sigma) and a set of regulatory proteins, including PPARγ, PCNA, MMP-2, G6PDH, β-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA), Akt, ERK 1/2 (Cell Signalling Technology, Beverly, MA) and GPDH (Abcam, Cambridge, UH) were analyzed by western blot. The tumor suppressor p53 inhibitor pifithrin- and ERK 1/2 MAPK inhibitor PD98059 were purchased from Sigma. The blotted PVDF transfer membrane (Millipore Co.) was exposed to X-ray films or FluorChem HD Imaging System (Alpha Innotech Co., San Leandro, CA), and images of blotted patterns were analyzed with NIH image software (National Institutes of Health, Bethesda, MD). Blots were routinely re-probed with anti-actin to ensure equivalence of loading. If necessary, membranes were stripped by western blot stripping reagent (T-Pro Biotechnology, Taipei, Taiwan) at room temperature for 3 min.

Statistical analysis

All data were analyzed by ANOVA (analysis of variance) and expressed as mean ± standard deviation. A p-value of less than 0 05 was considered statistically significant.

Results

The anti-adipogenic effect and adipogenic genes expression of vitexin –To determine the anti-adipogenic effect of vitexin, 3T3-L1 adipocytes were cultured in 6 wellplates for 8 days as described in “Materials and Methods”. Our result showed that there is no difference in cell viability after treated with vitexin for 8 days (Figure. 2A). Glucose consumption, triglyceride level in media and adipose accumulation were inhibited by vitexin in a dose-dependent manner (Figure. 2B and 2C). 3T3-L1 adipocyte treated with vitexin up-regulated ERK 1/2 MAPKs expression, whereas it down-regulated the expression of adipogenic marker PPARγ and PI3K/Akt (Fig. 2D). Yu and colleagues indicated that inhibitor of phosphatidylinositol 3-kinase (PI3K) LY294002 severely suppressed lipid accumulation, as well as the expression of two master adipogenic transcription factors PPARγ_2 and C/EBP [15]. The result suggested that vitexin inhibited adipogenesis through classic adipogenic markers PPARγ and PI3K/Akt as previous studies.

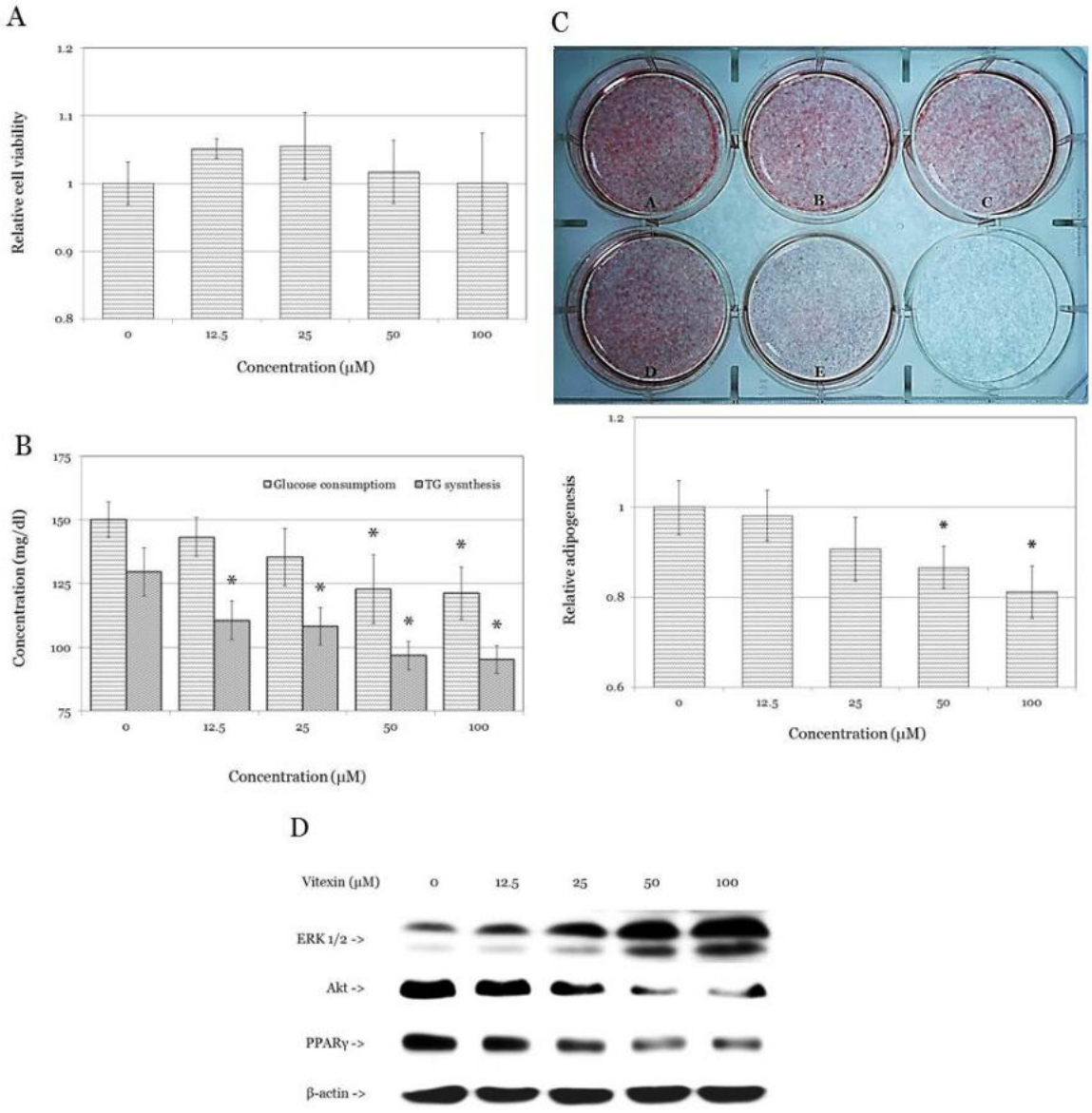

Figure 2. The anti-adipogenic effect and adipogenic genes expression of vitexin.

(A) Vitexin had no effect on cell viability. (B) The cultured media was collected and centrifuged to remove cells. The amount of glucose consumption and triglyceride synthesis decreased in dose-dependent. (C) Vitexin significantly diminished adipose accumulation at 50 and 100 M. (A:0, B:12.5, C:25, D:50 and E:100 M.) (D) The expression of ERK 1/2 MAPK were up-regulated by vitexin, whereas vitexin down-regulated the expression of adipogenic markers PPAR and PI3K/Akt. The values are expressed as mean±standard deviation from three independent experiments, and representative photos are shown ($p<0.05$).

ERK 1/2 MAPK played a negative role in adipogenesis

It has been reported that ERK 1/2 MAPK, PPARγ and PI3K/Akt were involved in regulation of glucose metabolism. In general, polypeptides that stimulate cell growth block fat cell differentiation. Activation of ERK 1/2 MAPK signaling significantly inhibited the transcriptional activity and ability to promote adipogenesis of PPARγ through phosphorylation of PPARγ in adipocytes, thereby promoting lipolysis [16]. When 3T3-L1 preadipocyte in culture reach a confluent state, their growth rate decreases greatly and many of the cells undergo differentiation [17]. ERK 1/2 MAPK would be necessary to initiate the preadipocyte into the differentiation process and, thereafter, this signal transduction pathway needs to be shut-off to proceed with adipocyte maturation [18]. To investigate the role of ERK 1/2 MAPK played in adipose accumulation, we used PD98059 as specific inhibitor to abolish the expression of ERK 1/2 MAPK. The result showed that PD98059

(10μM) induced the expression of adipogenic genes PPARγ and PI3K/Akt (Fig. 3A). However, it had no effect on cell viability (data not shown) and glucose consumption (Fig. 3B). On the contrary, PD98059 significantly induced triglyceride synthesis (Fig. 3B) and adipose accumulation (Fig. 3C). Moreover, PD98059 abolished the ability to decrease triglyceride synthesis and adipogenic effect of vitexin (Fig. 3B and 3C). The result indicated that ERK 1/2 MAPK played a negative role in adipogenesis. Interestingly, triglyceride synthesis and adipose accumulation, but not glucose consumption, were significantly induced by PD98059. It implied that cells may regulate glucose flux, rather than increase glucose uptake, to supply abundant glucose for adipogenesis.

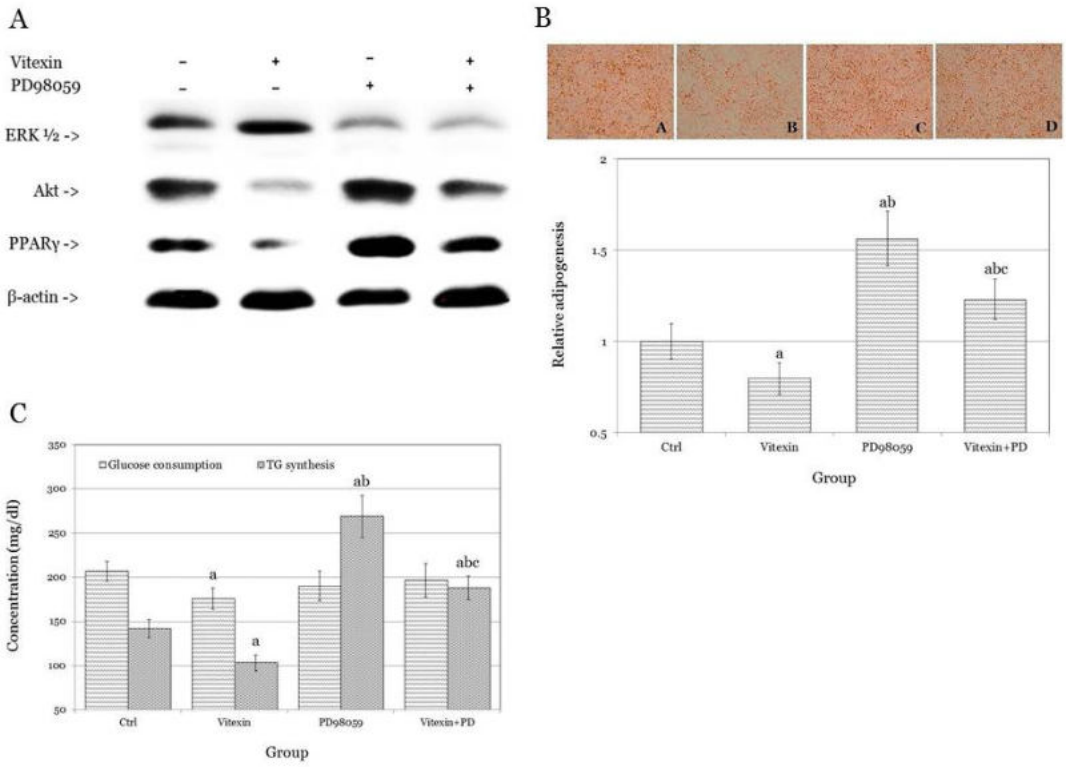

Figure 3. Down-regulation ERK 1/2 MAPK activity promoted adipogenesis.

(A) By using PD98059 (10 μM) as ERK 1/2 MAPK inhibitor, the expression of adipogenic markers Akt and PPARγ were induced. (B, C) Knockdown the expression of ERK 1/2 MAPK increased triglyceride synthesis and adipose accumulation significantly, but without any effect on glucose uptake. (A:control, B:100 μM vitexin, C:10 μM PD98059 and D: 100 μM vitexin +10 μM PD98059). The values are expressed as mean±standard deviation from three independent experiments, and representative photos are shown ($p<0.05$).

Adipogenesis is associated with regulation of glycolysis

Glucose is traditionally viewed as the main precursor of the glycerol backbone and thus, enhanced glucose uptake would be expected to result in increased triacylglycerol synthesis and contribute to obesity [19]. During adipose cell differentiation, both differentiation marker GPDH and G6PDH were greatly induced [2, 5]. In untreated 3T3-L1 cells, glucose uptake and triglyceride synthesis were significantly increased at day 6 and day 8 (Figure. 4A). The expression of ERK 1/2 MAPK were significantly decreased accompanied with time. However, the expression of G6PDH and GPDH significantly induced and maintained stable from day 2 to day 6 after starting differentiation. At day 8, the expression of G6PDH increased about 35% and GPDH expression decreased to about 55% when compared with day 6 (Figure. 4B). The excess carbon from glycolysis may be secreted as lactate (Fig 4C). Previous studies showed that fat cells from obese or diabetic rats (or humans) can metabolize to lactate as much as 50-70% of the glucose taken up. Thus, in obesity, with increased adipose cell size and cell number, a considerable amount of lactate can be produced [20]. Actually, cells greatly accumulated of adipose droplets mostly occurred at day 8 in cultivation (data not shown). The result suggested that overexpression of G6PDH and GPDH were associated with onset of adipocytes differentiation, whereas the mature adipocyte diverted glucose flux away from glycerol-3-p shunt for lipid biosynthesis.

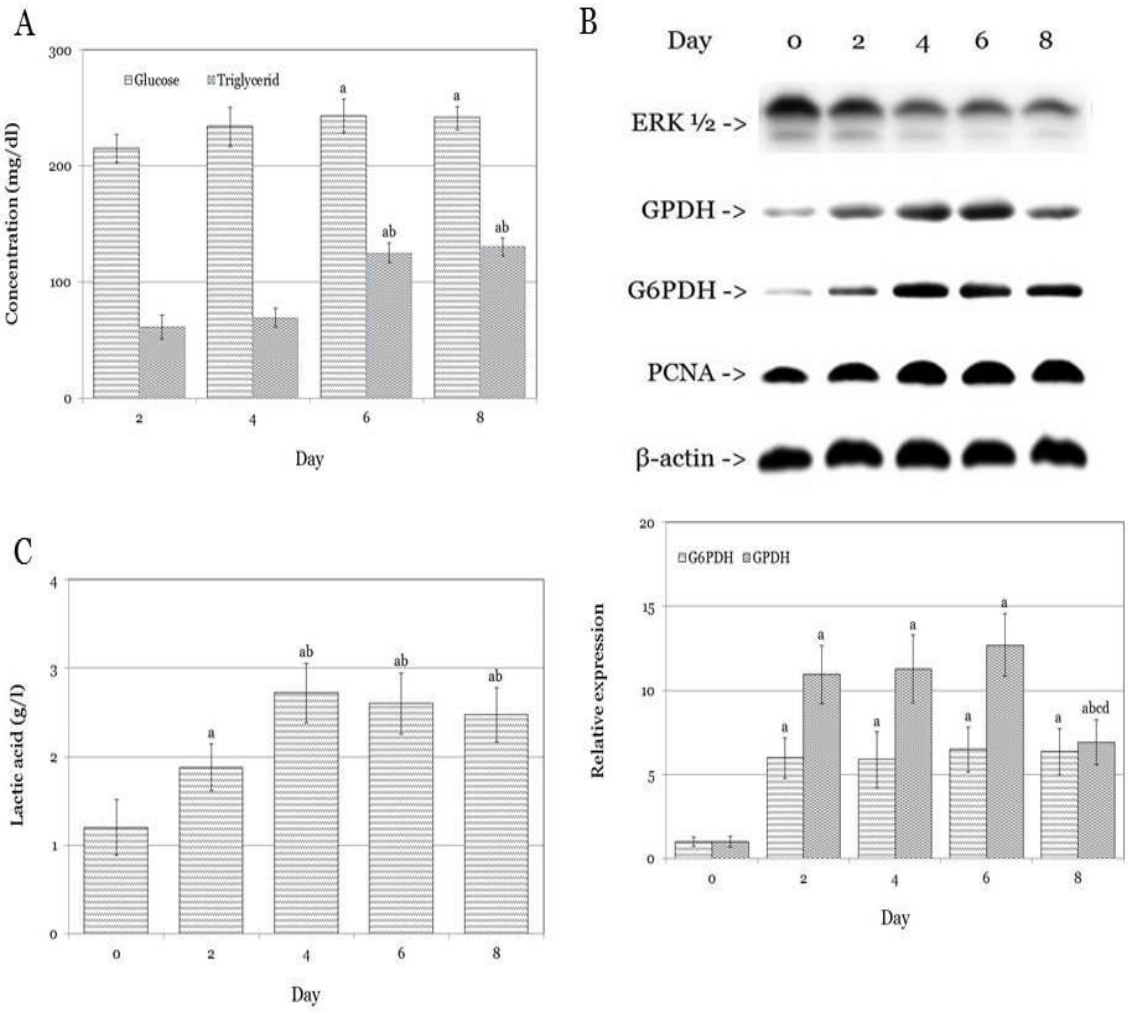

Figure 4. Alteration of the glycolysis is associated with adipose accumulation.

(A) In untreated 3T3-L1 cells, glucose uptake and triglyceride synthesis were significantly increased at day 6 and day 8. (B) The expression of G6PDH and GPDH of untreated 3T3-L1 adipocyte. Day 0: cells were full confluence and untreated with adipogenic medium. (C) The excess carbon from glycolysis secreted as lactate, it increased after differentiation and maintained stable from day 4 to day 8 in untreated cells. The values are expressed as mean±standard deviation from three independent experiments, and representative photos are shown ($p<0.05$).

The effect of vitexin on regulation of glycolysis

Previous study indicated that G6PDH overexpression stimulated the expression of most adipocyte marker genes such as PPARγ, C/EBP and adipocyte protein 2 (aP2, also called fatty acid binding protein 4), and elevated the levels of cellular free fatty acids, triglyceride, and free fatty acids release in 3T3-L1 cells. In our result showed that vitexin greatly induced the expression of GPDH at higher concentration (50 and 100 μM), however, it had no effect on the expression of G6PDH (Fig 5A). PD98059 greatly induced the expression of G6PDH, and down-regulated the expression of GPDH (Figure. 5B). In lactic acid synthesis, it increased to about 2.5 g/l after differentiation and maintained stable from day 4 to day 8 in untreated cells (Figure. 4C). Vitexin increased the lactic acid synthesis to maximum 9.4 g/l in concentration dependent significantly (Figure 5C). PD98059 decreased the level of lactic acid in media to about 1.8 g/l (Figure. 5D). Our results evidenced that diverted glucose metabolism to pentose phosphate pathway is associated with adipogenesis.

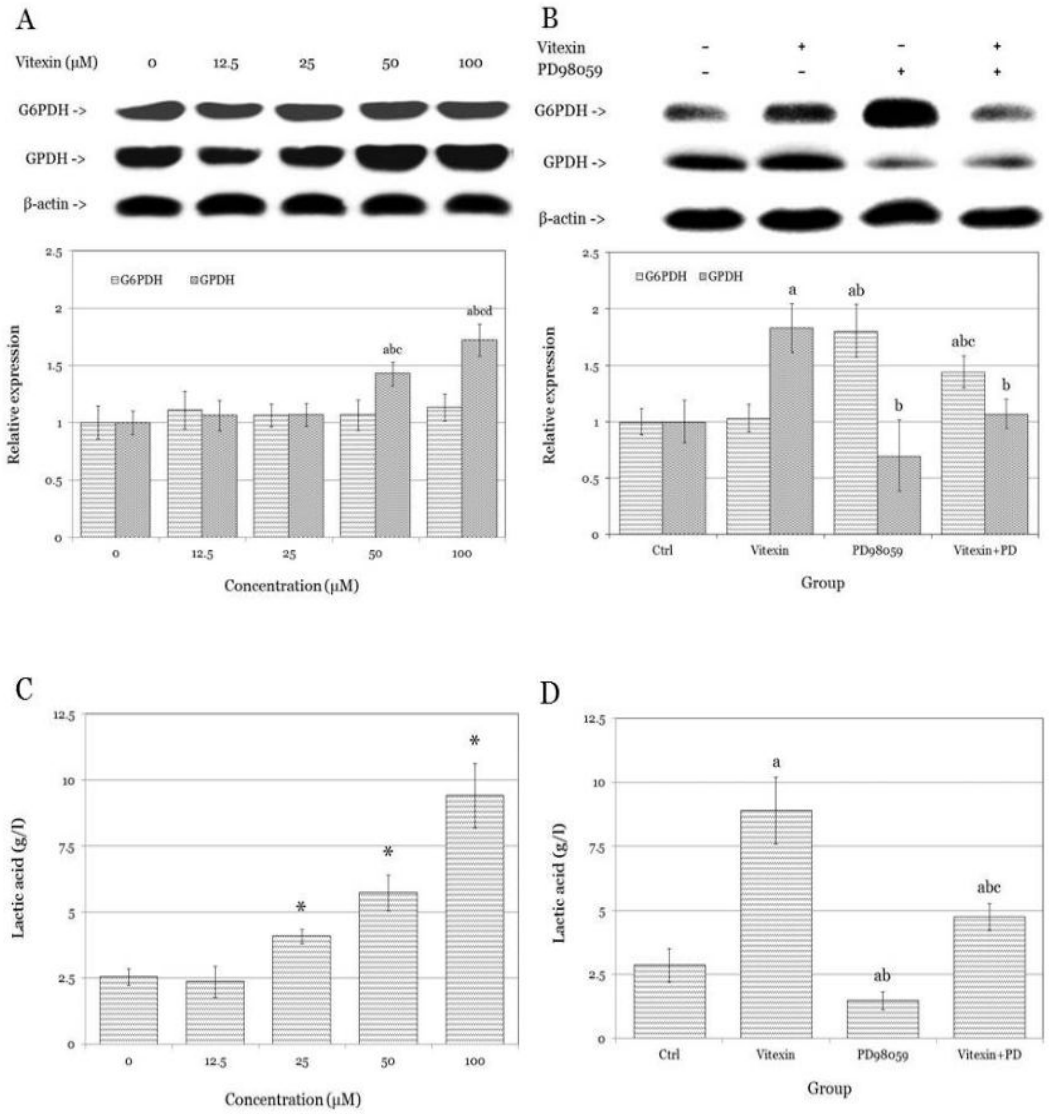

Figure 5. The effect of vitexin on regulation of glycolysis.

(A) Vitexin greatly induced the expression of GPDH at higher concentration (50 and 100 μM), however, it had no effect on the expression of G6PDH. (B) PD98059 greatly induced the expression of G6PDH, and down-regulated the expression of GPDH. (C) Vitexin increased the lactic acid synthesis in concentration dependent. (D) PD98059 decreased the level of lactic acid in media and abolished the effect of vitexin. The values are expressed as mean±standard deviation from three independent experiments, and representative photos are shown ($p<0.05$).

The anti-metastatic effect of vitexin

Enhancement of preadipocyte migration into fat cell clusters is one of the essential processes of adipose tissue development *in vivo* that induces adipocyte hyperplasia [14]. The mRNA level for MMP-2 strongly induced in obese adipose tissues compared with lean tissues [21]. To investigate the effect of vitexin on preadipocyte migration, we performed migration assays as described in "Materials and Methods". The result showed that vitexin inhibited preadipocyte migration to about 80% at 25 to 100 μM after 2 hours incubation (Figure. 6A). Previously studies showed that vitexin has anti-metastatic potential in rat pheochromacytoma [22] and human oral cancer cells [13]. In the meanwhile, 3T3-L1 cells were cultured in 24 wellplates for 2 hours followed by harvesting the cultured media to analysis the activity of MMP-2. The MMP-2 gelatinase

activity profile was examined by gelatin zymography. The activity of MMP-2 decreased to about 50% in concentration dependent (FigURE. 6B). We demonstrated that vitexin influence the expansion of adipose tissue through its ability to inhibit preadipocyte migration.

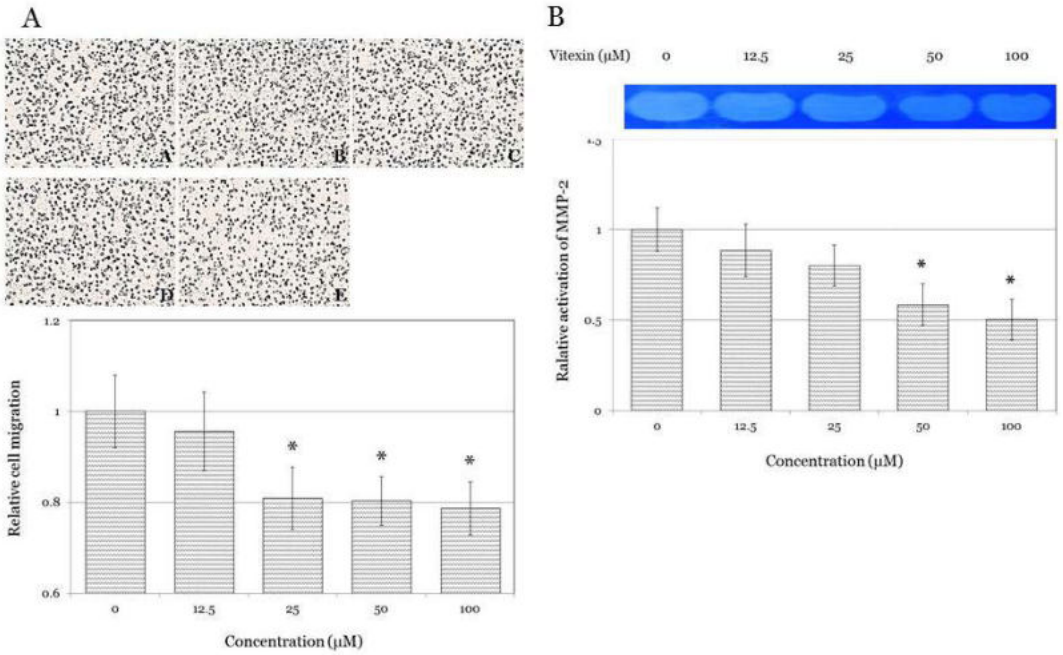

Figure 6. The anti-metastatic effect of vitexin.

(A) Vitexin inhibited cell migration to about 80%. (A:0, B:12.5, C:25, D:50 and E:100 μM). (B) Vitexin decreased the activity of 62 kDa active MMP-2 in concentration dependent (10 μg/lane). The values are expressed as mean±standard deviation from three independent experiments, and representative photos are shown ($p<0.05$).

Discussion

The conclusions of ERK 1/2 MAPK played in regulating adipogenesis are somewhat controversial. Previous study showed that activation of MAPK by various effectors inhibited adipogenesis [23], whereas others suggest that it promote preadipocyte differentiation [24]. It is quite possible that both claims are correct. The distinguishing factor might involve the precise time of MAPK activation during the initial stages of the differentiation process. For instance, effectors that activate the MEK/ERK pathway at late stages of adipogenesis are likely to block adipogenic gene expression due to a MAPK-dependent phosphorylation of PPARγ. Activation of the pathway early during adipogenesis prior to PPARγ expression might, on the other hand, promote differentiation by activating transcription factors operating to initiate PPARγ and C/EBP expression [24].

The intracellular redox potential (also known as reduction potential, oxidation / reduction potential) plays an important role in cell survival. G6PDH is expressed in all tissues and controls the flow of carbon through the pentose phosphate pathway and also produces the principal intracellular reductant NADPH [25]. G6PDH is important for cellular antioxidant defense to against oxidative stress. Adipose cells are highly sensitive to oxidative stress, overproduction of oxidative stress in adipose tissue may up-regulate inflammation, cellular proliferation, dysregulation of adipokines and insulin resistance [26, 27]. The reactive oxygen species (ROS) production and G6PDH activity exponentially increased as differentiation progressed. ROS from accumulated fat in obesity leads to elevated systemic oxidative stress and contributes to the development of obesity-linked chronic disorders [28].

Lactic acid, a major waste product, is mainly produced from glucose metabolism but can also be produced in small amounts from glutamine [29]. Previous study showed that resting thymocytes meet their ATP demand mainly by oxidative glucose breakdown (88%), whereas proliferating thymocytes produce 86% by glycolytic degradation of glucose to lactate and only 14% by oxidation to CO2 and water. Aerobic glycolysis by proliferating cells being a means to minimize oxidative stress and supply the enhanced energy demand during the phases of the cell cycle where maximally enhanced biosynthesis and cell division do occur [30]. Moreover, low extracellular pH benefits tumor cells because it promotes invasiveness, whereas a high intracellular pH gives them a competitive advantage over normal cells for growth [9]. The acidifying extracellular medium generated by decreasing pH or increasing lactate promoted the retention of adipogenic

differentiation potential of rabbit mesenchymal stem cells (rMSCs) during in vitro expansion [31].

One of the initial stages of adipogenesis is migration of preadipocytes into cell clusters to form primitive fat organs. Several proteases and their specific inhibitors modulate the interdependent processes of cell migration and matrix proteolysis. The most widely distributed cell-associated proteolytic enzymes are the plasminogen activators, including urokinase plasminogen activator (uPA) and its inhibitor PAI-1. The PAI-1 is synthesized and released from human adipose tissue and plays an important role through inhibits the effect of uPA on the conversion of inactive zymogen plasminogen to active plasmin, which activated the proenzyme forms of the matrix metalloproteinases (MMPs), such as MT1-MMP [32] and MMP-2 [33], MMP-9 [34] to prevent preadipocyte migration [35].

Conclusions

We demonstrated evidences for the first time that using of vitexin to against adipose accumulation, at least in part, was regulation of glycolysis via activation of ERK 1/2 MAPK signaling. Moreover, blockade pentose phosphate pathway may be a novel strategy for obesity prevention and therapy.

Authors' contribution

Yu-Hsien Lee, Shiow-Ling Chen Shih-Huang Yang and Ya-Fang Pan carried out the molecular genetic studies, immunoassays, zymography and drafted the manuscript. Chia-Ming Liu and Meng-Wei Li carried out the biosynthesis assays. Shih-Shen Chou carried out the in vitro migration assay and participated in the design of the study. Ming-Yung Chou and Su-Chung Youn conceived of the study, and participated in its design and coordination and helped to draft the manuscript.

Acknowledgement

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