科技部補助專題研究計畫成果報告
期末報告

糖解作用、脂肪生成作用與細胞凋亡間相互作用探討(第 2 年)

計 畫 類 別 ：個別型計畫
計 畫 編 號 ：NSC 102-2314-B-040-009-MY2
執 行 期 間 ：103 年 08 月 01 日至 104 年 07 月 31 日
執 行 單 位 ：中山醫學大學牙醫學系（所）

計 畫 主 持 人：周明勇

計畫參與人員：學士級-專任助理人員：林鳴峰

處 理 方 式 ：
1.公開資訊：本計畫可公開查詢
2.「本研究」是否已有嚴重損及公共利益之發現：否
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中 華 民 國 104 年 07 月 21 日

中 文 摘 要 ： 腫瘤細胞於能量代謝上最大的特點就是不論含氧量是否充足，腫瘤細胞都優先利用 glycolysis 來降解葡萄糖以產生 ATP 此即所謂的 Warburg effect。因此針對改變腫瘤能量代謝機轉成為抗癌的策略之一。在我們執行計畫的過程中我們發現具有抑制脂肪生成作用(adipogenesis)且能誘發腫瘤細胞凋亡(apoptosis)的藥物如 aspirin 與 vitexin 皆能抑制脂肪細胞 glycolysis 的速率限制酵素 Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)表現。進一步先期實驗亦發現 vitexin 能抑制口腔腫瘤細胞的 GAPDH 表現。另外，此兩種藥物皆藉由 tumor suppressor p53 訊息傳導路徑來調控
腫瘤細胞凋亡與抑制脂肪細胞的脂肪生成作用。我們可預期 glycolysis、apoptosis 與 adipogenesis 三者間存在著某種相互依存與調控的關係。
感謝科技部提供的研究經費，讓我們能延續上一計畫的成果。這兩年裡我們持續完成未盡完善之處並發表成果於相關的國際期刊。對於此一課題我們已完成階段性任務。除此，我們於計畫執行期亦對口腔癌進行相關研究並於相關的國際期刊發表了兩篇研究論文，以做為下一研究計畫的基石。我們於 2014-2015 年發表相關的研究報告如下：
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pentose phosphate pathway. European Journal of Pharmacology 2014 Sep 5；738:101-10. (Co-corresponding author)

中文關鍵詞： 阿斯匹靈、p53腫瘤抑制基因、ERK 1/2 MAPK、五碳糖磷酸路徑、脂肪生成作用

英文摘要： Obesity has become a major public health problem of global significance. Today, aspirin remains the most commonly used medication for the treatment of pyrexia, pain, inflammation and antiplatelet. The present study aims at evaluating the possible existence of a putative p53-dependent pathway underlying the aspirin-induced inhibition of adipogenesis. Cell migration assay was identified by the ability to migrate through Transwell insert. Oil Red O staining was employed to quantify adipose accumulation. The concentration of glucose and triglyceride were measured by using assay kits. The expression levels of several master regulatory molecules controlling various signal pathways were monitored using the immunoblotting techniques. Aspirin significantly inhibited preadipocyte migration and adipose accumulation. The p53–p21 signaling and the expression of differentiation marker glycerol-3-phosphate dehydrogenase were increased in a dose-dependent manner. It indicated that aspirin induced adipocyte differentiation through p53–p21 pathway. The oncogenic ERK 1/2 MAPK signaling was induced, whereas, the expression of adipogenic markers peroxisome proliferator-activated receptor γ (PPARγ), adipocyte fatty acid-binding protein (A-FABP) and inflammatory factors cyclooxygenase-2 (Cox-2), tumor necrosis factor α (TNFα) and inducible nitric oxide synthase (iNOS) were inhibited. Aspirin negatively regulated the pentose phosphate pathway (PPP) by inhibiting the expression of rate-limiting enzyme glucose-6-phosphate dehydrogenase. Knockdown the expression of oncogenic ERK 1/2 MAPK by using 10 μM PD98059 significantly increased triglyceride synthesis, adipose accumulation and activated PPP, however, decreased glucose uptake. Diverted the glucose flux

to PPP, rather than increased glucose uptake, was associated with adipogenesis. Down-regulated the expression of tumor suppressor p53 by 10 μM pifithrin-α (PFTα) alone had no effect on adipose accumulation. However, administration of aspirin accompanied with PFTα abolished aspirin-induced inhibition of adipogenesis. We demonstrated that aspirin-induced inhibition of adipogenesis was p53-dependent and associated with inactivation of PPP. Blockade PPP may be a novel strategy for obesity prevention and therapy. Moreover, when use aspirin in therapeutic strategy, the p53 status should be considered.

英文關鍵詞： Aspirin； Aspirin resistance； Tumor suppressor p53； ERK 1/2 MAPK； Pentosephosphate pathway； Adipogenesis

科技部補助專題研究計畫成果報告

（□期中進度報告/■期末報告）

糖解作用、脂肪生成作用與細胞凋亡間相互作用探討

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計畫主持人：周明勇 教授
共同主持人：
計畫參與人員：林鳴峰 研究助理

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□執行國際合作與移地研究心得報告
□出席國際學術會議心得報告

期末報告處理方式：

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中　華　民　國　104 年　07　月　22

腫瘤細胞於能量代謝上最大的特點就是不論含氧量是否充足，腫瘤細胞都優先利用glycolysis 來降解葡萄糖以產生ATP 此即所謂的Warburg effect。因此針對改變腫瘤能量代謝機轉成為抗癌的策略之一。在我們執行計畫的過程中我們發現具有抑制脂肪生成作用(adipogenesis)且能誘發腫瘤細胞凋亡(apoptosis)的藥物如 aspirin 與vitexin 皆能抑制脂肪細胞glycolysis 的速率限制酵素Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)表現。進一步先期實驗亦發現vitexin 能抑制口腔腫瘤細胞的GAPDH 表現。另外，此兩種藥物皆藉由tumor suppressor p53 訊息傳導路徑來調控
腫瘤細胞凋亡與抑制脂肪細胞的脂肪生成作用。我們可預期glycolysis、apoptosis 與adipogenesis 三者間存在著某種相互依存與調控的關係。

感謝科技部提供的研究經費，讓我們能延續上一計畫的成果。這兩年裡我們持續完成未盡完善之處並發表成果於相關的國際期刊。對於此一課題我們已完成階段性任務。除此，我們於計畫執行期亦對口腔癌進行相關研究並於相關的國際期刊發表了兩篇研究論文，以做為下一研究計畫的基石。我們於2014-2015 年發表相關的研究報告如下：

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Abstract

Obesity has become a major public health problem of global significance. Today, aspirin remains the most commonly used medication for the treatment of pyrexia, pain, inflammation and antiplatelet. The present study aims at evaluating the possible existence of a putative p53-dependent pathway underlying the aspirin-induced inhibition of adipogenesis. Cell migration assay was identified by the ability to migrate through Transwell insert. Oil Red O staining was employed to quantify adipose accumulation. The concentration of glucose and triglyceride were measured by using assay kits. The expression levels of several master regulatory molecules controlling various signal pathways were monitored using the immunoblotting techniques. Aspirin significantly inhibited preadipocyte migration and adipose accumulation. The p53–p21 signaling and the expression of differentiation marker glycerol-3-phosphate dehydrogenase were increased in a dose-dependent manner. It indicated that aspirin induced adipocyte differentiation through p53–p21 pathway. The oncogenic ERK 1/2 MAPK signaling was induced, whereas, the expression of adipogenic markers peroxisome proliferator-activated receptor γ (*PPARγ)*, adipocyte fatty acid-binding protein (A-FABP) and inflammatory factors cyclooxygenase-2 (Cox-2), tumor necrosis factor α (TNFα) and inducible nitric oxide *synthase* (iNOS) were inhibited. Aspirin negatively regulated the pentose phosphate pathway (PPP) by inhibiting the expression of rate-limiting enzyme glucose-6-phosphate dehydrogenase. Knockdown the expression of oncogenic ERK 1/2 MAPK by using 10 μM PD98059 significantly increased triglyceride synthesis, adipose accumulation and activated PPP, however, decreased glucose uptake. Diverted the glucose flux to PPP, rather than increased glucose uptake, was associated with adipogenesis. Down-regulated the expression of tumor suppressor p53 by 10 μM pifithrin-α (PFTα) alone had no effect on adipose accumulation. However, administration of aspirin accompanied with PFTα abolished aspirin-induced inhibition of adipogenesis. We demonstrated that aspirin-induced inhibition of adipogenesis was p53-dependent and associated with inactivation of PPP. Blockade PPP may be a novel strategy for obesity prevention and therapy. Moreover, when use aspirin in therapeutic strategy, the p53 status should be considered.

Keywords

Aspirin; Aspirin resistance; Tumor suppressor p53;
ERK 1/2 MAPK; Pentosephosphate pathway; Adipogenesis

1. Introduction

Early descriptions of a medical role for acetylsalicylic acid, known as aspirin, occurred over 100 years ago when it was discovered to have anti-inflammatory property. Today, aspirin remains the most commonly used medication for the treatment of pyrexia, pain, inflammation and antiplatelet. Through subsequent years, previous studies paid more attention to anti-inflammatory (Morris et al., 2009), anti-thrombotic (Kaber et al., 2011), and anti-tumor (Ho et al., 2003) effects of aspirin. Comparatively little is known about the molecular mechanism of aspirin regulates adipocyte migration and adipogenesis. The present study aims at evaluating the possible existence of a putative p53-dependent pathway underlying the aspirin-induced inhibition of adipogenesis.

It is well known that p53 is a key tumor suppressor protein that acts as an important general regulator that controls various cellular gene networks (Vogelstein et al., 2000). Its primary mode of action has generally been ascribed to the induction of cell-cyclearrest and apoptosis. Furthermore, tumor suppressor p53 was involved in adipogenesis. The activation of p53 and its target genes are highly induced in adipocytes of ob/ob mice might constitute a negative feedback loop against excess fat accumulation in adipocytes (Yahagi et al., 2003). The p21, as p53 downstream gene, is involved in both adipocyte differentiation and in protecting hypertrophied adipocytes against apoptosis (Inoue et al., 2008). Recent evidences demonstrated that tumor suppressor p53 plays a critical role in regulation of pentose phosphate pathway (PPP), mitochondrial oxidative phosphorylation (OXPHOS) and glycolysis. p53 inhibits lipid accumulation by directly binding to glucose-6-phosphate dehydrogenase (G6PDH) and thus inhibiting PPP (Jiang et al., 2011), Wild-type p53 expression activates pyruvate dehydrogenase (PDH) through decreased levels of pyruvate dehydrogenase kinase-2 (PDK2), which inhibits PDH by phosphorylation of PDHA1, in turn promoted conversion of pyruvate into acetyl-CoA instead of lactate (Contractor and Harris, 2012).

It is now clear that obesity is associated with a state of chronic, low-level inflammation (Wellen and Hotamisligil, 2005). TNFα, the first molecular link between inflammation and obesity, overexpressed in the adipose tissues of obese human (Hotamisligi et al., 1995).Pro-inflammatory cytokine TNFα inhibits OXPHOS through phosphorylation ofcytochrome c oxidase (Samavati et al., 2008). Adipose cells are highly sensitive tooxidative stress. Over-production of oxidative stress in adipose tissue may up-regulateinflammation, cellular proliferation, dysregulation of adipokines and insulin resistance (Soares et al., 2005). Hyperglycemia caused inhibition of the PPP via decreased expression and increased phosphorylation of G6PDH, which therefore decreased the principal intracellular reductant NADPH production and led to increase oxidative stress (Xu et al., 2005). Excess energy intake or decreased expenditure results in excess triacylglyceride accumulation in adipose tissue which led to accumulate of oxidative stress and increase the expression of tumor suppressor p53 (Minamino et al., 2009).

2. Materials and methods

2.1. 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 (10,000 U/ml) and penicillin (10,000 U/ml), at 37 ℃ 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 (Omatsu-Kanbe et al., 2006). 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, 0.0001, 0.001, 0.01, 0.1 and 1 mM, respectively) of aspirin (acetylsalicylic acid) (Sigma) for 8 days. The media was changed every two days in cultivation. Aspirin was dissolved in DMSO (Sigma) and stored at −20 ℃. The volume of DMSO was equalized to 0.1 or 0.2% in all culture dishes.

2.2. Cell viability assay

To examine the cytotoxic effect, 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 (oxidized 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 h then measured cytotoxicity by usingspectrophotometry at 570 and 600 nm.

2.3. 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 10,000 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 aspirin. After incubation for 2 h at 37 ℃, 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.

2.4. Glucose consumption assay

Glucose consumption was measured by using glucose assay kit, glucose liquicolor (HUMAN GmbH, Wiesbaden, Germany), as the manufacturer recommended. The glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The formed hydrogen peroxide reacts under catalysis of peroxidase with phenol and 4-aminophenazone to a red-violet quinoneimine dye as indicator. Briefly, the medium was collected and centrifuged to remove the cells, and incubated for 5 min at 37 ℃ with enzyme reagent. The glucose concentration can be measured by the absorbance at 500 nm.

2.5. Triglyceride synthesis

Triglyceride synthesis was measured by using triglyceride assay kit, triglycerides liquicolor^{mono} (HUMAN GmbH), as the manufacturer recommended. The triglyceride determined after enzyme hydrolysis with lipases. Indicator is quinoneimine formed from hydrogen peroxide, 4-aminoantipyrin and 4-chlorophenol under

the catalytic influence of peroxidase. Briefly, the medium was collected and centrifuged to remove the cells, and incubated for 5 min at 37 ℃ with triglyceride assay buffer. The absorbance was determined at 500 nm.

2.6. 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 h. 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.

2.7. 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 signaling (Sigma) and a set of regulatory proteins, including PPARγ, G6PDH, PDH, LDH, TPI, PCNA, caspase 3 and β-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA), Akt, ERK 1/2 (Cell Signaling Technology, Beverly, MA) and GPDH (Abcam, Cambridge, UH) were analyzed bywestern blot. The tumor suppressor p53 inhibitor pifithrin-α and ERK 1/2 MAPK inhibitor PD98059 were purchased from Sigma. Briefly, samples were heated at 95 ℃ for 5 min in Laemmli buffer and then chilled on ice. Subsequently, after electrophoresis (30 μg/lane), the proteins were electro-blotted to PVDF transfer membrane (Millipore Co.). Nonspecific binding on the PVDF transfer membrane was blocked with 5% nonfat dry milk in 20 mM of Tris and 150 mM of NaCl before incubating with primary antibodiesagainst specific antigens. After incubation with the conjugated second antibody, the proteins of interest were detected with an ECL western blotting detection reagent (Amersham Pharmacia Biotech, Buckinghamshire, UK) and the blotted PVDF transfer membrane was exposed to 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.

2.8. 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.

3. Results

3.1. Aspirin induced preadipocytes differentiation through p53–p21 pathway

When 3T3-L1 preadipocyte in culture reach a confluent state, their growth rate decreases greatly and many of the cells undergo differentiation (Green and Kehinde, 1974). Cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$, a downstream gene of tumor suppressor p53, is induced during adipocyte differentiation (de la Torre et al., 2013). Anti-obesity and anti-diabetes properties acquired by up-regulating the p53–p21 pathway, resulting in G0/G1 cell cycle arrest and the inhibition of cellular hypertrophy of adipocytes(Nakatsuka et al.,

2012). Pairault and Green (1979) indicated that only resting cells seem susceptible to the reprogramming of their differentiated state necessary for the adiposeconversion. The absence of p21 in 3T3-L1 fibroblasts by RNA-mediated interference knockdown or in embryonic fibroblasts from p21−/− mice impaired adipocyte differentiation, resulting in smaller adipocytes (Inoue et al., 2008). Our result showed that the expression of tumor suppressor p53 and its downstream genes p21 and Bax were up-regulated (Fig. 1A), as well as the differentiation marker GPDH expression (Fig. 1B). It indicated that aspirin induced preadipocytes differentiation through p53–p21 pathway.

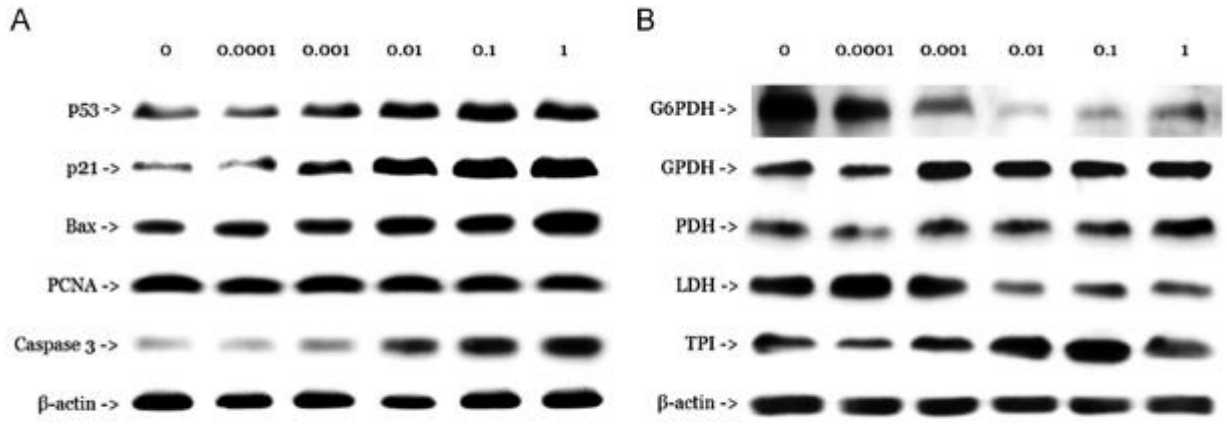

Fig. 1. The effect of aspirin on the expression of p53 signaling and rate-limiting enzymes of glycolysis. 3T3-L1 adipocytes were treated with aspirin as indicated concentrations. Cells were harvested and equal amount of protein (30 μg/lane) was processed for western blot analysis. Blots were stripped and reprobed with β-actin antibody to confirm equal loading. (A) The p53–p21 signaling and the expression of apoptotic markers caspase 3 and bax were increased in a dose-dependent manner. (B) Aspirin induced adipocyte differentiation by increasing the expression of GPDH. The activation of pentose phosphate pathway, oxidative phosphorylation and anaerobic glycolysis were inhibited by down-regulation the rate-limiting enzymes G6PDH, PDH and LDH respectively. These results are representative of three independent experiments.

3.2. Aspirin inhibited preadipocyte migration and adipose accumulation

The formation of primitive adipose tissue is the initial process in adipose tissue development followed by the migration of preadipocytes into adipocyte clusters (Torii et al., 2010). To investigate the effect of aspirin on preadipocyte migration, cell migration assay was identified by the ability to migrate through Transwell inserts with 8.0 μM pore size polyethylene terephthalate (PET) membrane. The result demonstrated that treated the 3T3-L1 preadipocyte with various concentrations (0, 0.0001, 0.001, 0.001, 0.1, 1 mM) of aspirin for 2 h greatly decreased cell migration to about 65–70% (Fig. 2A). By using Oil Red O stain, aspirin showed the ability to decrease lipid accumulation at each concentration (Fig. 2B). Previous studies showed that aspirin induced apoptosis in oral cancer cells (Ho et al., 2003) and Madin-Darby canine kidney cells (Nishimura et al., 2002). However, aspirin had no effect on cell viability (Fig. 2C) and the expression ofPCNA (Fig. 1A) in 3T3-L1 adipocytes. The cell multiplication is not essential for adipose conversion, 3T3-L1 preadipocytes could differentiate into adipocytes without DNA synthesis and mitotic clonal expansion (Qiu et al., 2001). Aspirin administration decreased the serum glucose, total cholesterol, C-reactive protein, triglycerides, insulinclearance, low-density lipoprotein cholesterol (LDL-C) and VLDL-C (Yuan et al., 2001,Hundal et al., 2002, van Diepen et al., 2011 and Sethi et al., 2011). In adipocytes,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 (Muñoz et al., 2010). Our result showed that glucose consumption and triglyceride synthesis decreased at high-dose aspirin, 0.1 and 1 mM (Fig. 2D and E).

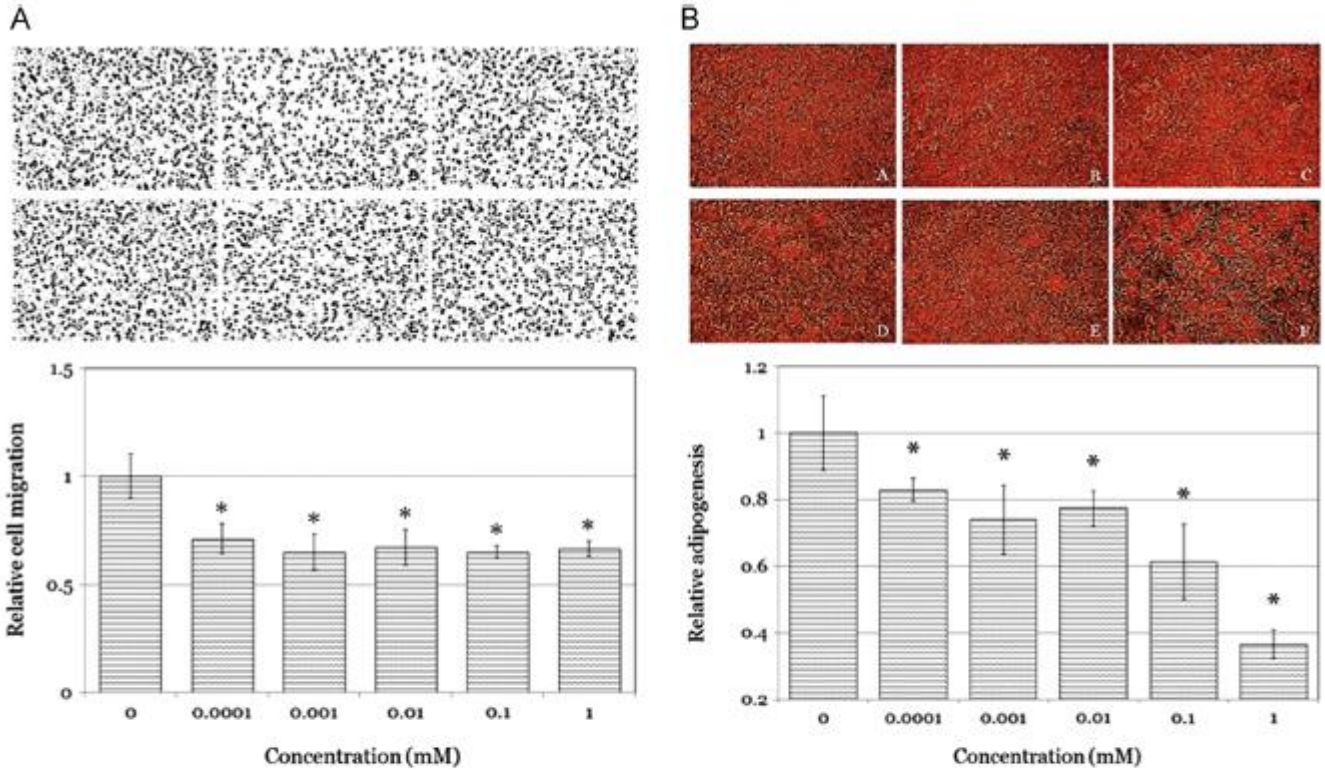

Fig. 2. Aspirin inhibited preadipocyte migration and adipogenesis. (A) Cell migration assay was identified by the ability to migrate through Transwell inserts with 8.0 μM pore size polyethylene terephthalate (PET) membrane. Placed cells in the upper chamber and filled both the upper and lower compartments of the migration chamber with medium and various concentrations of aspirin, followed by incubating for 2 h. 3T3-L1 preadipocyte treated with indicated concentrations of aspirin greatly decreased cell migration to about 65–70%. (B) Oil Red O staining was employed to quantify adipose accumulation. After acquired images under microscope, dried the cells completely and then eluted Oil Red O dye by 100% isopropanol and measured the absorption at 500 nm. Aspirin inhibited adipose accumulation in dose-dependent. (C) The cell viability was measured by alamar blue assay. After incubated for 4 h, cytotoxicity was measured by using spectrophotometry at 570 and 600 nm. (D, E) The concentration of glucose and triglyceride were measured by using assay kits. Aspirin inhibited glucose uptake and triglyceride synthesis at high-dose. The value are expressed as mean ± standard deviation from three independent experiments, and representative photos are shown ($P < 0.05$).

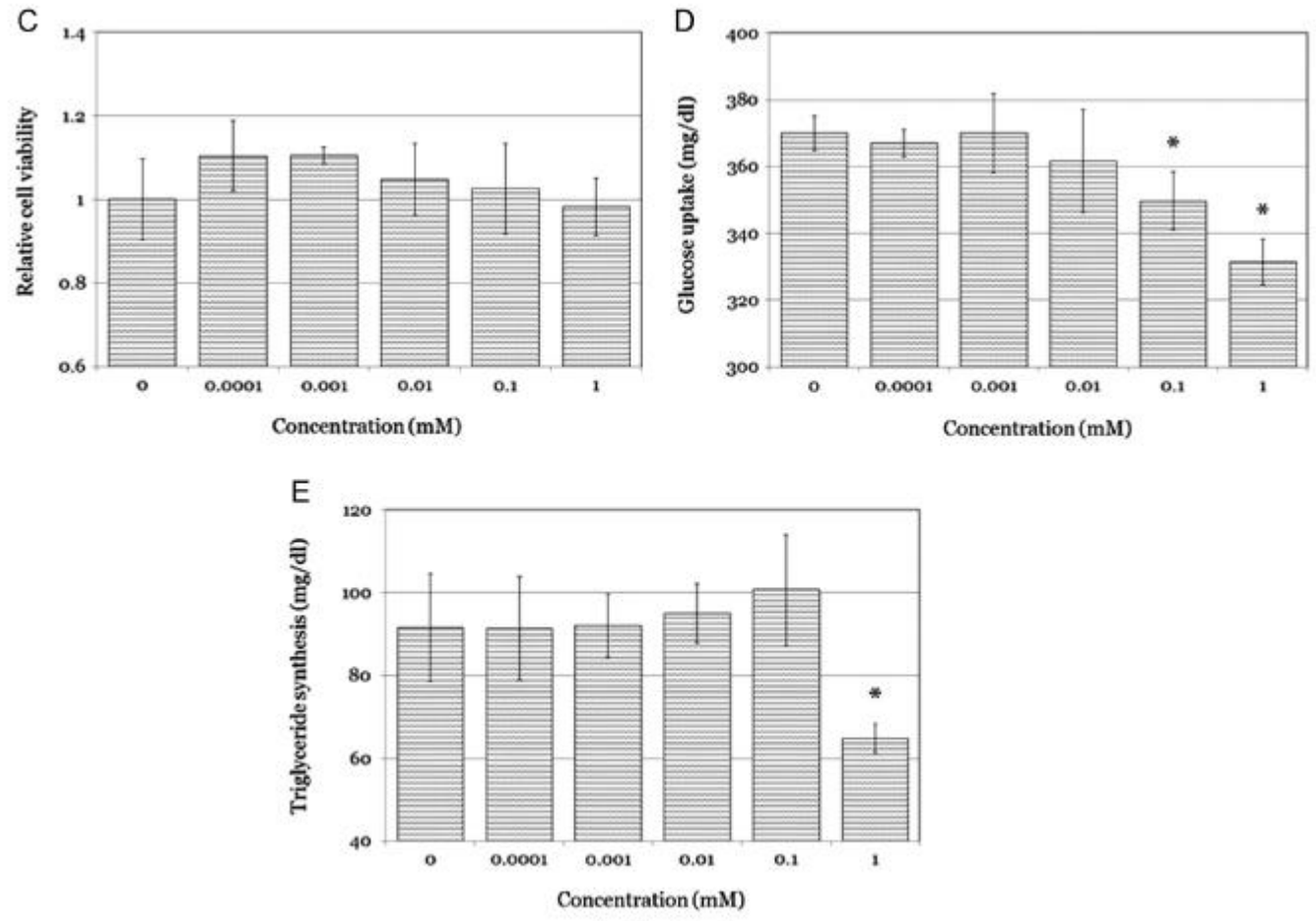

Fig. 2. (*continued*)

Previous study reported that aspirin was shown to inhibit pentose phosphate pathway (PPP) in proportion to the amount present. At any concentration of this substance there was greater inhibition of the PPP in G6PD-deficient than in normal red cells (Worathumrong and Grimes, 1975). However, others showed that acetylsalicylic acid had no effect on glutathione level (Na et al., 1999) and the RBC count or the hemoglobin levels (Glader, 1976). McDonagha et al. (2012) indicated that the effect of aspirin on G6PD-deficient individuals may be dependent on the variant type, dosage and pre-existing clinical conditions (such as an infection or inflammatory disease). To investigate the effect of aspirin on glucose metabolism, we determined the expression of key enzymes which played as the rate-limiting enzyme in master shunts of glycolysis. Aspirin decreased the expression of G6PDH, PDH and LDH (Fig. 1B). The result indicated that aspirin negatively regulated the pentose phosphate pathway, oxidative phosphorylation and anaerobic glycolysis by inhibiting the expression of G6PDH, PDH and LDH respectively.

3.3. The regulation of adipogenic markers and inflammatory factors by aspirin

The ERK 1/2 MAPK and PI3K/Akt pathways are major intracellular signaling modules, which are known to regulate diverse cellular processes including cell proliferation, survival and malignant transformation. PI3K/Akt signaling pathways are essential for adipogenesis of human mesenchymal stem cells (MSCs). LY294002, a specific inhibitor of PI3K severely suppressed lipid accumulation, as well as the expression of PPARγ and C/EBPα, two master adipogenic transcription factors (Yu et al., 2008). Several growth factors that inhibit fat cell differentiation caused activation of the MEK/ERK signaling pathway and ERK pathway negatively modulates the PI3K/Akt pathway in response to growth factor stimulation (Prusty et al., 2002). Circulating concentrations of A-FABP were significantly higher in overweight/obese than in lean persons (Xu et al., 2006) and positively associated with the risk of diabetes (Djoussé and Gaziano, 2012). A-FABP interact directly with PPARγ and that they do so in a receptor- and ligand-selective manner, thereby enabling PPARγ to exert their biological functions (Tan et al., 2002). To investigate the effect of aspirin on the expression of adipogenic markers, we examined lipid accumulation and its involved signaling molecules expression such as Akt, ERK 1/2 MAPK, PPARγ and A-FABP. The result showed that the protein expression of ERK 1/2 MAP kinase greatly induced by aspirin. On the contrary, aspirin inhibited the expression of adipogenic markers, PPARγ, A-FABP, and Akt (Fig. 3A). It indicated that aspirin-induced inhibition of adipogenic markers is through ERK 1/2 MAPK signaling.

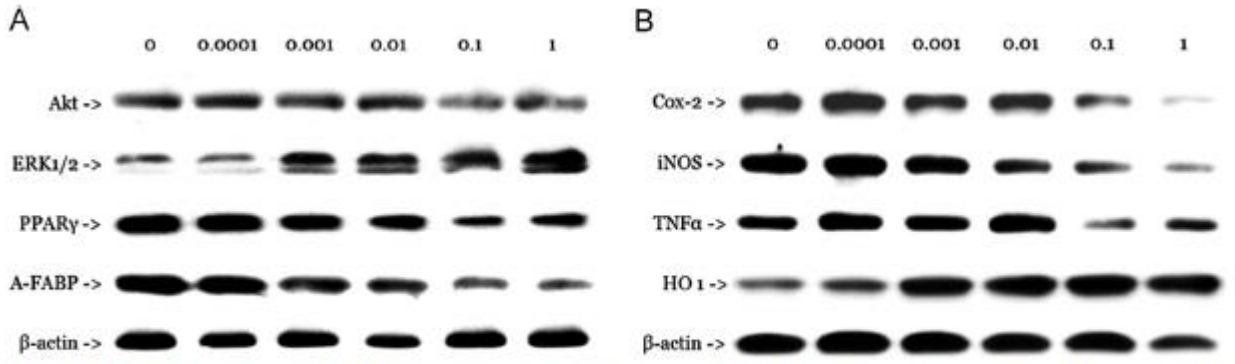

Fig. 3. The expression of adipogenic markers and inflammatory factors. Cells were harvested and equal amount of protein (30 μg/lane) was processed for western blot analysis. Blots were stripped and reprobed with β-actin antibody to confirm equal loading. (A) The oncogenic ERK 1/2 MAPK expression was induced, whereas, the expression of adipogenic markers PPARγ and A-FABP were inhibited. (B) Aspirin up-regulated anti-inflammatory factor HO-1 expression and down-regulated the expression of inflammatory factors Cox-2, iNOS and TNFα. These results are representative of three independent experiments.

An increasing evidence is linking adipogenesis and inflammation. Obesity, alone or as a part of the metabolic syndrome, is characterized by a state of chronic low-level inflammation as revealed by raised plasma levels of

inflammatory cytokines and acute-phase proteins including NF-κB (Berg et al., 2004), Interleukin 6 (IL-6), iNOS (Hemmrich et al., 2007 and Qiu et al., 2013), Cox-2 (Chu et al., 2010), prostaglandins (Lu et al., 2004) and TNFα (Hotamisligi et al., 1995). To investigate the effect of aspirin on oxidative stress and inflammatory factors during adipogenesis, we determined the expression of heme oxygenase 1 (HO-1), Cox-2, TNFα and iNOS. Our result showed that aspirin reduced the expression of Cox-2, TNFα and iNOS except HO-1 (Fig. 3B). Aspirin is known to exert antioxidant effects. It (30–300 μM) increased HO-1 protein levels in a concentration-dependent fashion (Grosser et al., 2003). Induction of HO-1 resulted in a reduction in C/EBPα, PPARγ, Peg-1/Mest, aP2, CD36, TNFα and IL-6 expression and lipid accumulation (Kim et al., 2008 and Vanella et al., 2013). The expression of Cox-2 was induced transiently in a biphasic manner upon the triggering of the differentiation and maturation phases while Cox-1 was constitutive (Lu et al., 2004). The genetic deficiency of Cox-2 resulted in a significant reduction in total body weight and percent body fat (Ghoshal et al., 2011). On the contrary, Nishimura et al. (2004) showed that SC-560, a specific Cox-1 inhibitor, suppressed adipogenesis dose dependently. The Cox-2 inhibitor NS-398 had little influence on the maturation processes. Stable transfection of preadipocytes with each of Cox-1 and Cox-2 similarly blocks adipogenesis program. Aspirin or other Cox inhibitors at different phases of life cycle of adipocytes failed to reverse the reduced storage of fats (Chu et al., 2009). Overexpression of Cox-2 increased systemic energy expenditure and protected mice against high-fat diet-induced obesity. Prostaglandin (PG) pathway regulates systemic energy homeostasis (Vegiopoulos et al., 2010). Production of PGD_2 was significantly reduced in Cox-2-deficient adipose tissue (Ghoshal et al., 2011). The treatment of the mature adipocytes with exogenous PGD_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2 and PGE_2, in the presence of aspirin, enhanced the adipogenesis (Lu et al., 2004). However, previous study showed that PG release, including PGE_2, 6-keto $PGF_{1\alpha}$, PGD_2 and 15d-PGJ_2, significantly decreased following differentiation in 3T3-L1 cells (Xie et al., 2006). The up-regulation of fat storage was appreciably prevented by anti-adipogenic prostanoids, such as PGE_2 and $PGF_{2\alpha}$, during the maturation phase (Chu et al., 2010). PGE_2 and $PGF_2\alpha$ strongly suppress the early phase of adipocyte differentiation. In contrast, PGD_2 and its non-enzymatic metabolite, Δ12-PGJ_2, activate the middle-late phase of adipocyte differentiation (Fujimori, 2012).

3.4. Knockdown the expression of oncogenic ERK 1/2 MAPK increased adipose accumulation and activated pentose phosphate pathway

Blockage of ERK phosphorylation in osteo-induced human adipose-derived stem cells (hASCs) by PD98059 supplemented with dexamethasone led to adipogenic differentiation (Liu et al., 2009). By using PD98059 as ERK 1/2 MAPK inhibitor, knockdown the expression of ERK 1/2 MAPK significantly induced adipose accumulation (Fig. 4A) and triglyceride synthesis (Fig. 4C), however, decreased glucose consumption (Fig. 4B). To investigate the effect of ERK 1/2 MAPK on glycolysis, we performed western bolt to analyze the expression of the rate-limiting enzyme in master shunts of glycolysis. The result showed that knockdown the expression of ERK 1/2 MAPK significantly induced the expression of G6PDH (Fig. 5A). PD98059 had no significant effect on the expression of PDH, LDH and TPI (Fig. 5A). It indicated that knockdown the ERK 1/2 MAPK signaling inhibited preadipocytes differentiation. When adipocyte treated with aspirin and 10 μM PD9059 simultaneously, PD98059 abolished the effect of aspirin-induced inhibition of adipose accumulation (Fig. 4A), triglyceride synthesis (Fig. 4C) and the expression of key enzymes in glycolysis (Fig. 5A). ERK 1/2 MAPK played a negative role in aspirin-induced inhibition of adipogenesis. Our result demonstrated that

diverted the glucose flux to pentose phosphate pathway, rather than increased glucose uptake, was associated with adipogenesis.

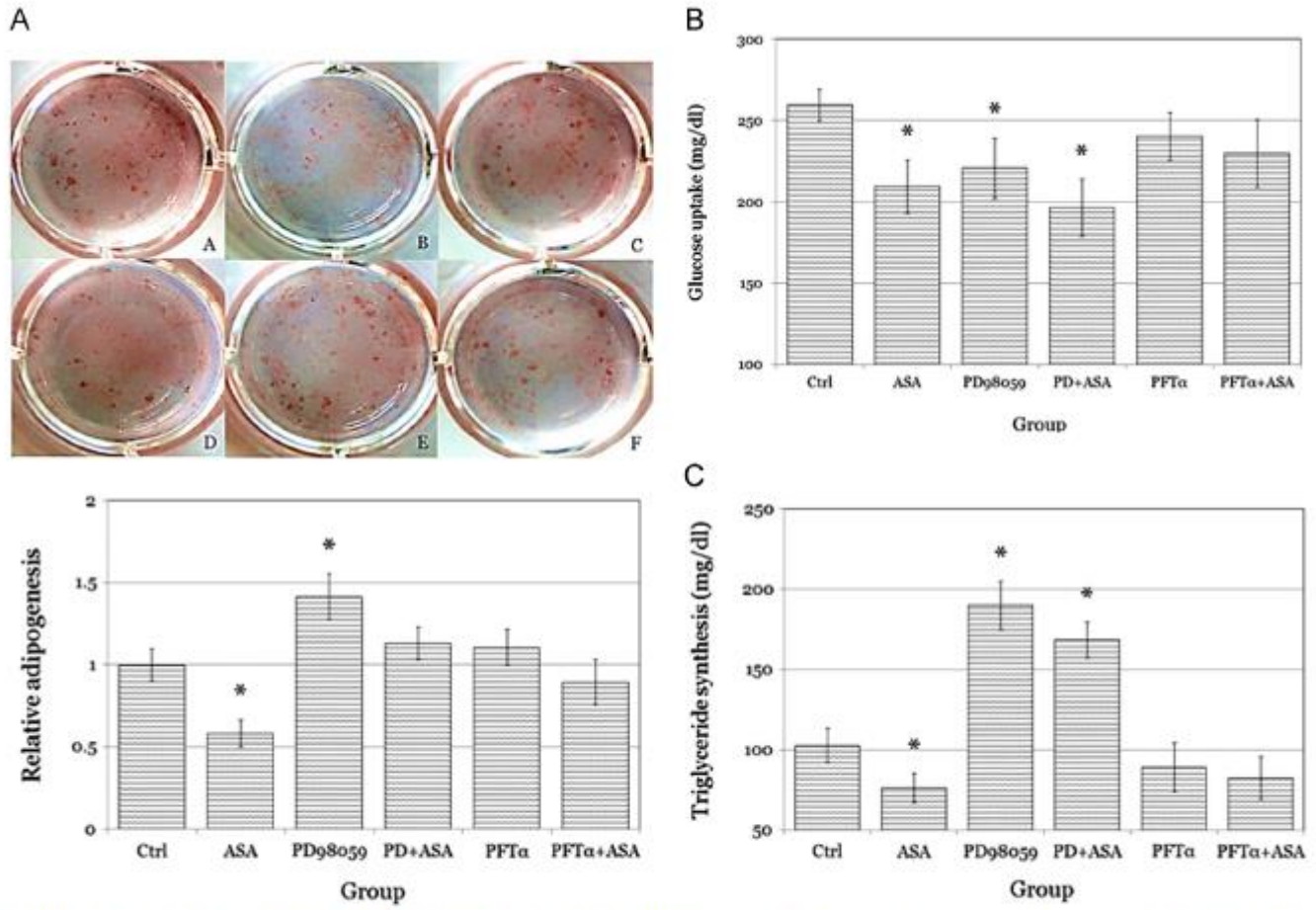

Fig. 4. The effect of p53 and ERK 1/2 MAPK inhibitors on adipogenesis. (A) Oil Red O staining was employed to quantify adipose accumulation. The ERK 1/2 MAPK inhibitor PD98059 induced adipose accumulation. (B, C) The concentration of glucose and triglyceride were measured by using assay kits. PD98059 decreased glucose uptake, however, increased triglyceride synthesis. Diverted the glucose flux to pentose phosphate pathway, rather than increased glucose uptake, was associated with adipogenesis. Using PFTα as a p53 inhibitor, it had no effect on glucose uptake, triglyceride synthesis and adipose accumulation. However, administration of aspirin accompanied with PFTα abolished aspirin-induced inhibition of adipogenesis. Aspirin-induced inhibition of adipogenesis was p53-dependent. The value are expressed as mean ± standard deviation from three independent experiments, and representative photos are shown ($P < 0.05$).

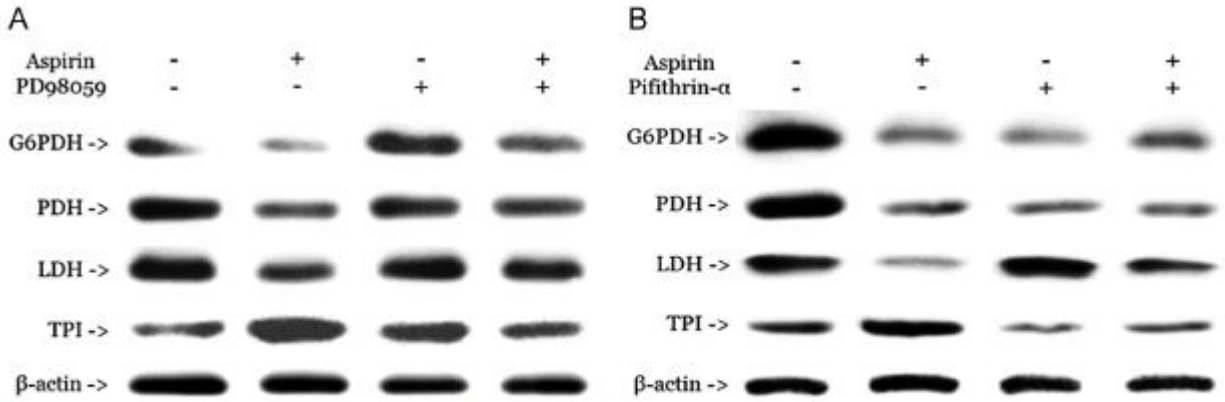

Fig. 5. The effect of PFTα and PD98059 on glycolysis regulation. Cells were harvested and equal amount of protein (30 μg/lane) was processed for western blot analysis. Blots were stripped and reprobed with β-actin antibody to confirm equal loading. (A) Using PD98059 to knockdown the expression of ERK 1/2 MAPK lead to activate pentose phosphate pathway by inducing the expression of G6PDH. (B) The p53 inhibitor PFTα inhibited the pentose phosphate pathway and oxidative phosphorylation by inhibiting the expression of G6PDH and PDH respectively. On the contrary, PFTα activated anaerobic glycolysis by inducing the expression of LDH. These results are representative of three independent experiments.

3.5. Aspirin-induced inhibition of adipose accumulation was p53-dependent

The tumor suppressor p53, one of the most frequently mutated genes in cancers, modulates the balance between the utilization of mitochondrial oxidative phosphorylation and glycolytic pathways. Previous study indicated that the activation of p53 might constitute a negative feedback loop against excess fat accumulation in adipocytes (Yahagi et al., 2003). Wild-type p53 expression activates PDH through its transcriptional

repression of pyruvate dehydrogenase kinase-2 (PDK2), which inhibits PDH by phosphorylation of PDHA1. Decreased the level of PDK2, in turn, promoted conversion of pyruvate into acetyl-CoA instead of lactate (Contractor and Harris, 2012). To investigate the link between tumor suppressor p53, glycolysis and adiogenesis, we used 10 μM PFTα as tumor suppressor p53 inhibitor to abolish the expression of p53. The result demonstrated that p53 inhibitor alone had no effect on adipose accumulation. However, administration of aspirin accompanied with PFTα abolished the anti-lipogenic effect of aspirin (Fig. 4A). Knockdown the expression of p53 had no effect on glucose uptake and triglyceride synthesis (Fig. 4B and C). Western blot was performed to analysis the expression of the rate-limiting enzyme in master shunts of glycolysis. Our result showed that attenuated the expression of p53 induced the expression of LDH, however, inhibited the expression of G6PDH, PDH and TPI (Fig. 5B). The result was consistent with previous study that p53-deficient cells recapitulated the metabolic switch toward glycolysis, the Warburg effect (Matoba et al., 2006). Our result suggested that aspirin-induced inhibition of adipose accumulation was p53-dependent.

4. Discussion

Despite the development of newer antiplatelet drugs in the last decade, aspirin is still the most widely used antiplatelet agent across the world to prevent cardiovascular diseases (Mansour et al., 2009). However, a significant number of particular patients do not benefit from aspirin therapy, a concept known as "aspirin resistance". Aspirin resistance may reflect treatment failure rather than resistance to aspirin. The incidence of aspirin resistance is unknown, but it may approach 20–30% (Knoepp and Laposata, 2005). The mechanism for aspirin resistance remains uncertain and is also probably multifactorial. Diabetic patients had significantly higher aspirin resistance. Glycemic control, obesity, and the dose of aspirin have influence on aspirin resistance in diabetic subjects (Ertugrul et al., 2010). Obese insulin-resistant subjects have a blunted response to platelet-inhibitory effect of aspirin. It could contribute to the increased risk of atherothrombosis in insulin-resistant individuals (Tamminen et al., 2003). The centration range (10^{-4}–10^{-1} mM) used in this study is consistent with the plasma salicylate concentrations in individuals taking therapeutic doses of aspirin or sodium salicylate (Xu et al., 1999). In this study, blockade the expression of tumor suppressor p53 impaired the aspirin-induced inhibition of adipose accumulation. The effect of aspirin on adipogenesis was p53-dependent. We suggest that when use aspirin in therapeutic strategy p53 status should be considered. MAPKs are activated by a large variety of stimuli and one of their major functions is to connect cell surface receptors to transcription factors in the nucleus, which consequently triggers long-term cellular responses. 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 (Bost et al., 2005). The conclusions of ERK 1/2 MAPK played in regulating adipogenesis are somewhat controversial. Some studies showed that activation of MAPK by various effectors inhibited adipogenesis (Hu et al., 1996, Dang and Lowik, 2004, Constant et al., 2008, Lii et al., 2012 and Lim et al., 2012), whereas others suggest that it promote preadipocyte differentiation (Prusty et al., 2002 and Chuang et al., 2008). 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/EBPα expression (Prusty et al., 2002). PD98059 and U0126 are considered as specific inhibitors of the p42/44 MAPK pathway, which affects adipogenesis. PD98059 dose-dependently stimulated

adipogenesis. In contrast, U0126 had no effects on adipogenesis, although it inhibited p42/44 MAPK more potently than PD98059 (Dang and Lowik, 2004).

Over the last decade, an abundance of evidence has emerged demonstrating a close link between metabolism and immunity. Metabolic and immune pathways have evolved to be closely linked and interdependent. Many hormones, cytokines, signaling proteins, transcription factors, and bioactive lipids can function in both metabolic and immune roles. In addition to using some of the same cellular machinery, metabolic and immune systems also regulate each other. It is now clear that obesity is associated with a state of chronic low-level inflammation (Wellen and Hotamisligil, 2005). Adipocytes and diverse types of immune cells such as T cells and macrophages possess similar roles in pathways such as complement activation and inflammatory cytokine production (Wellen and Hotamisligil, 2003). Preadipocyte and macrophage phenotypes are very similar and that preadipocytes have the potential to be very efficiently and rapidly converted into macrophages in response to appropriate stimuli (Charrière et al., 2003). TNFα is the first molecular link between inflammation and obesity. It is well known that TNFα plays an important role in both acute and chronic inflammation. TNFα is a multifunctional cytokine which exerts a myriad of biological actions in different tissues and species. In adipose tissue, in particular, TNFα has been demonstrated to regulate or interfere with adipocyte metabolism at numerous sites including transcriptional regulation, glucose and fatty acid metabolism and hormone receptor signaling (Sethi and Hotamisligil, 1999). Obese individuals express 2.5-fold more TNFα mRNA in fat tissue relative to the lean controls (Hotamisligi et al., 1995).

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, 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 shunts after G6P. (1) Pentose phosphate pathway (PPP, also called the phosphogluconate pathway and the hexose monophosphate shunt). Glucose-6-phosphate dehydrogenase (G6PDH) is a rate-limiting enzyme of pentose phosphate pathway. All cell types contain G6PDH activity, however, regulation of the enzyme only occurs in liver and adipose tissue (Stabile et al., 1998). The enzymatic activity and expression levels of G6PDH were significantly elevated in white adipose tissues of obese models. In 3T3-L1 cells, G6PDH overexpression stimulated the expression of most adipocyte marker genes, oxidative stress and inflammatory responses and elevated the levels of cellular free fatty acids (FFA), triglyceride, and FFA release (Park et al., 2005). Treatment of 3T3-L1 cells with dehydroepiandrosterone (DHEA) and its analogues results in intracellular inhibition of G6PDH, which is associated with the block of differentiation (Shantz et al., 1989). Carbohydrate overfeeding markedly increased net de novo lipogenesis in adipose tissue. The pentose-phosphate pathway is active in adipose tissue of healthy humans, consistent with an active role of this tissue in de novo lipogenesis (Minehira et al., 2003). Tumor suppressor p53, the most frequently mutated gene in human tumors, inhibits the pentose phosphate pathway by binding to G6PDH and prevents the formation of the active dimer. Through the pentose phosphate pathway, p53 suppresses glucose consumption, NADPH production and biosynthesis (Jiang et al., 2011). The reaction catalyzed by G6PDH provides 50–75% of NADPH needed for fatty acid biosynthesis (Rognstad and Katz, 1979). (2) Glycerol-3-phosphate (G3P) shunt. Cytosolic GPDH is a key enzyme providing G3P for triacylglycerol synthesis in adipose tissue and is regarded as a marker for adipocyte differentiation. The cellular GPDH is a main marker enzyme for the differentiation. The effect of biological factors on lipogenesis and adipogenesis can be estimated by determining GPDH activity and cellular fatty acid accumulation in the differentiating adipocytes (He et al.,

2009). In mammals, the synthesis of glycerolipids, including triacylglycerol, diacylglycerol and phospholipids, occurs predominantly by the G3P pathway in most cell types (Alves-Bezerra and Gondim, 2012). Elevated GPDH might contribute to the increase of triacylglycerol synthesis in obese subjects (Swierczynski et al., 2003). (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 (Bui and Thompson, 2006). An efficient mitochondrial OXPHOS system is essential since it provides most of the adenosine triphosphate (ATP), the chemical energy required for a cell′s metabolism (Shoffner, 2000). Pyruvate dehydrogenase complex (PDC) catalyzes the oxidative decarboxylation of pyruvate and links glycolysis to the tricarboxylic acid (TCA) cycle. Mammalian PDC activity is inactivated by phosphorylation by the pyruvate dehydrogenase kinases (PDKs) (Holness and Sugden, 2003). Different tissues display distinct sensitivities to defective mitochondrial OXPHOS. Tissues highly dependent on oxygen such as the cardiac muscle, skeletal and smooth muscle, the central and peripheral nervous system, the kidney, and the insulin-producing pancreatic β-cell are especially susceptible to defective OXPHOS (Fosslien, 2001). Reductions in OXPHOS can induce a state of insulin sensitivity and resistance to metabolic disease (Pospisilik et al., 2007). The p53 regulates aerobic respiration at the glycolytic and OXPHOS steps. The p53 negatively regulates glycolysis through activation of TP53-induced glycolysis regulator (TIGAR) (an inhibitor of the fructose-2,6-bisphosphate). On the contrary p53 positively regulates OXPHOS through up-regulation of synthesis of cytochrome c oxidase (SCO2) (Madan et al., 2011). (4) Anaerobic glycolysis. Adipose tissue uses glucose to produce lactate and pyruvate, in addition to CO_2 and triglycerides. Lactate as an active metabolite, capable of moving between cells, tissues and organs, where it may be oxidized as a fuel or reconverted to form pyruvate or glucose (Philp et al., 2005). Lactate metabolism is altered in obesity. Increasing obesity is associated with increased blood lactate levels (Lovejoy et al., 1992 and King and DiGirolumo, 1998). Diabetes is also associated with markedly increased lactate production in adipocytes. Fat cells from obese or diabetic rats (or humans) can metabolize to lactate as much as 50–70% of the glucose taken up (Digirolamo et al., 1992). Acidifying extracellular medium and lactate ion promoted the retention of adipogenic differentiation potential of MSCs during in vitro expansion (Chen et al., 2009).

科技部補助計畫衍生研發成果推廣資料表

日期:2015/07/17

科技部補助計畫	計畫名稱: 糖解作用、脂肪生成作用與細胞凋亡間相互作用探討
	計畫主持人: 周明勇
	計畫編號: 102-2314-B-040-009-MY2　　學門領域: 牙醫學

無研發成果推廣資料

102 年度專題研究計畫研究成果彙整表

計畫主持人：周明勇	計畫編號：102-2314-B-040-009-MY2
計畫名稱：糖解作用、脂肪生成作用與細胞凋亡間相互作用探討	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	4	2	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	1	1	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	無

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

科技部補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：■已發表 □未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：（以 100 字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

感謝國科會提供的研究經費，讓我們能延續上一計畫的成果。這一年裡我們持續完成上一年未盡完善之處並發表成果於相關的國際期刊。對於此一課題我們已完成階段性任務。除此，我們於計畫執行期亦對口腔癌進行相關研究並於相關的國際期刊發表了兩篇研究論文，以做為下一研究計畫的基石。我們於 2014-2015 年發表相關的研究報告如下：

1.Chou MY, Hu FW, Yu CH, Yu CC*. Sox2 expression involvement in the oncogenicity and radiochemoresistance of oral cancer stem cells. Oral Oncol. 2015,51:31-39.

2.Yu CC, Hu FW, Yu CH, Chou MY. Targeting CD133 in the enhancement of chemosensitivity in oral squamous cell carcinoma-derived side population cancer stem cells. Head Neck, 2015. doi: 10.1002/hed.23975.

3.Yu-Hsien Lee1,2,*, Shih-Huang Yang1*, Shiow-Ling Chen3,*, Ya-Fang Pan3, Chia-Ming Liu1,2, Meng-Wei Li3, Shih-Shen Chou1,2, Ming-Yung Chou1,2,†, Su-Chung Youn4, † The anti-adipogenic effect of vitexin is via ERK 1/2 MAPK signaling in 3T3-L1 adipocytes. International Journal of Phytomedicine

6(2):206-215. 2014.
4.Ying-Fang Su1,2,4,*, Shih-Huang Yang1,*, Yu-Hsien Lee1,2, Buor-Chang Wu1,2, Shu-Ching Huang1,2, Chia-Ming Liu1,2, Shiow-Ling Chen1, Ya-Fang Pan3, Shih - Shen Chou1,2, Ming-Yung Chou1,2,†, Hui-Wen Yang 1,2, †. Aspirin-induced inhibition of adipogenesis was p53-dependent and associated with inactivation of pentose phosphate pathway. European Journal of Pharmacology 2014 Sep 5；738:101-10.