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Keyword: Hepatic steatosis, purple sweet potato color, Src, CCAAT/enhancer binding protein β, high-fat diet

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Abstract

Our previous work showed that purple sweet potato color (PSPC), a class of naturally occurring anthocyanins, effectively improved hepatic glucose metabolic dysfunction in high-fat diet (HFD)-treated mice. This study investigated the effects of PSPC on HFD-induced hepatic steatosis and the signalling events associated with these effects. Mice were divided into four groups: Control group, HFD group, HFD+PSPC group and PSPC group. PSPC was administered daily for 20 weeks at oral doses of 700 mg/kg/day. Our results showed that PSPC significantly improved obesity and related metabolic parameters, as well as liver injury in HFD-treated mice. Moreover, PSPC dramatically attenuated hepatic steatosis in HFD-treated mice. PSPC markedly prevented oxidative stress-mediated Src activation in HFD-treated mouse livers. Furthermore, PSPC feeding remarkably suppressed mitogen-activated protein kinase kinase /extracellular-signal-regulated kinase (MEK/ERK) signalling and consequent CCAAT/enhancer binding protein β (C/EBPβ) activation, restored AMPK activation in HFD-treated mouse livers, which was confirmed by U0126 treatment. Ultimately, PSPC feeding dramatically reduced protein expression of FAS and CD36 and the activation of ACC, and increased the protein expression of CPT1A in the livers of HFD-treated mice, indicating decreased lipogenesis and fatty acid uptake, and enhanced fatty acid oxidation. In conclusion, PSPC exhibited beneficial effects on hepatic steatosis which were associated with blocking Src and C/EBPβ activation.

Keywords: hepatic steatosis; purple sweet potato color; oxidative stress; Src; extracellular-signal-regulated kinase; CCAAT/enhancer binding protein β; high-fat diet
Introduction

Non-alcoholic fatty liver disease (NAFLD), one of the most common liver diseases worldwide, is the hepatic manifestation of metabolic syndrome and is strongly associated with obesity, dyslipidaemia, insulin resistance and type 2 diabetes (Byrne and Targher 2015). Steatosis is the primary factor responsible for the pathogenesis of NAFLD, which can progress to more advanced stages of liver disease, such as fibrosis, cirrhosis and even hepatocellular cancer (Rinella 2015). Hepatic steatosis occurs because import or synthesis of fatty acids in hepatocytes exceeds export or catabolism (Rinella 2015). It is well established that western dietary patterns, characterised by high intakes of fat and sugar, play an important role in the development of steatosis (Byrne and Targher 2015; Koch et al. 2015). However, the mechanism contributing to high-fat diet (HFD)-induced steatosis has not been fully elucidated.

The Src family of kinases comprise nine non-receptor protein tyrosine kinases that share similar structure and function (Liu et al. 2013). Src, a ubiquitously expressed Src family kinase, is involved in many fundamental cellular processes, including proliferation, differentiation and inflammation (Liu et al. 2013). More recently, a number of studies have highlighted the role of Src activation in the pathogenesis of various metabolic syndrome features, such as atherosclerosis (Chattopadhyay et al. 2015; Tao et al. 2015) and insulin resistance (Holzer et al. 2011). However, the role of Src in hepatic steatosis and the mechanism underlying its effects remains to be investigated. Substantial evidence demonstrates that Src can be activated by oxidative stress (Pal et al. 2014; Chattopadhyay et al. 2015). Additionally, it has been well demonstrated that oxidative stress is an important
mediator of NAFLD aetiology and progression (Byrne and Targher 2015; Rinella 2015). Moreover, several studies have suggested that Fyn, an Src family kinase, is associated with the development of hepatic steatosis (Bastie et al. 2007; Fukunishi et al. 2011). Based on these studies, we speculated that oxidative stress triggers Src activation, contributing to the development of HFD-induced NAFLD.

Currently, there is no approved pharmacological treatment for NAFLD, but natural products from plants, such as anthocyanin, exert beneficial effects on hepatic steatosis (Salomone et al. 2012; Xu et al. 2015). Purple sweet potato color (PSPC), a class of naturally occurring anthocyanins, has a variety of biological activities such as antioxidant (Wu et al. 2015), anti-inflammatory (Zhang et al. 2009; Wang et al. 2014), hepato-protective (Zhang et al. 2009), antidiabetic (Lee et al. 2016), and anticancer (Lim et al. 2013) effects. Previous work by our group has demonstrated that PSPC effectively attenuates hepatic insulin resistance and glucose metabolic dysfunction in HFD-treated mice (Zhang et al. 2013).

However, whether PSPC improves HFD-induced hepatic steatosis has never been investigated. Convincing experimental data have demonstrated that PSPC has strong antioxidant properties (Chao et al. 2014; Hu et al. 2016). Therefore, we hypothesised that PSPC ameliorates HFD-induced hepatic steatosis through inhibiting oxidative stress and consequent Src activation. This study was designed to explore the effects of PSPC on HFD-induced hepatic steatosis and the signalling events associated with these effects.

Materials and methods

Animals and administration

All the experimental protocols and euthanasia procedures were approved by the
Institutional Animal Care and Use Committee of Jiangsu Normal University. Eight-week-old male ICR mice were purchased from Hua-fu-kang Biological Technology Co., Ltd (Beijing, China). Mice were housed at a temperature of 23°C±1°C and received food and drinking water ad libitum, with a relative humidity of 60% and a 12/12 h light/dark cycle (light from 8:30 a.m. to 8:30 p.m.). After one week of acclimatisation to the laboratory conditions, mice were randomly divided into four groups including Control group: HFD group, HFD+PSPC group and PSPC group. Each group received the following treatments for 20 weeks. Control group: normal diet (ND, 15.9 kJ/g, 10% of energy as fat, 70% of energy as carbohydrates and 20% of energy as protein; D12450B, Research Diets, New Brunswick, NJ, USA), daily oral administration of sterile distilled water containing the PSPC solvent [0.1% Tween-80 (Sigma-Aldrich, St. Louis, MO, USA)]; HFD group: HFD diet (21.9 kJ/g, 60% of energy as fat, 20% of energy as protein, 20% of energy as carbohydrate; D12492, Research Diets, New Brunswick, NJ, USA), daily oral administration of sterile distilled water containing the PSPC solvent; HFD+PSPC group: HFD diet, daily oral administration of PSPC at doses of 700 mg/kg/day in distilled water containing 0.1% Tween 80; PSPC group: normal diet, daily oral administration of PSPC at doses of 700 mg/kg/day in distilled water containing 0.1% Tween 80. PSPC were purchased from Qingdao Pengyuan Natural Pigment Research Institute (Qingdao, China). The major components of PSPC by HPLC analysis are cyanidin acyl glucosides and peonidin acyl glucosides (>90%, peonidin 3-O-(6-O-(E)-caffeoyl-2-O-β-D-glucopyranosyl-β-D-glucopyranoside)-5-O-β-D-glucopyranoside, peonidin 3-O-(2-O-(6-O-(E)-caffeoyl-β-D-glucopyranosyl)-6-O-(E)-caffeoyl-β-D-glucopyranoside)-5-O-β-D-glucopyranoside, Peonidin3-O-(2-O-(6-O-(E)-feruloyl-β-D-glucopyranoside)-5-O-β-D-glucopyranoside).
yranosyl)-6-O-(E)-caffeoyl-β-D-glucopyranoside)-5-O-β-D-glucopyranoside, cyanidin
3-O-(6-O-p-coumaroyl)-β-D-glucopyranoside) and the rest is other flavonoids), as described
in our previous work (Lu et al. 2012). The dosage of PSPC used in this study was based on
our previous work (Zhang et al. 2013) and our pilot study.

Eighteen weeks after HFD feeding, some mice from the HFD group were divided into two
subgroups. The ERK1/2 inhibitor U0126 (Selleck Chemicals, Houston, TX, USA), was
solubilised in phosphate buffered saline (PBS) containing 5% dimethyl sulfoxide (DMSO),
was administered to one subgroup (HFD + U0126 group) for two weeks by daily
intraperitoneal (i.p.) injections at a dosage of 20 mg/kg/day, and another subgroup
(HFD-control group) received daily i.p. injections of an equal volume of vehicle.

Food intake was measured every 3 days. The body weights of mice were measured weekly.
After 20 weeks of treatment, the mice were deeply anaesthetised and sacrificed, and the blood,
liver and epididymal fat were immediately collected for experiments or stored at -70 °C for
later use.

**Liver slice collection and histopathological analysis**

Liver slice collection and histopathological analyses were performed as described in our
previous work (Zhang et al. 2010). The mice were transcardially perfused with 100 ml of 0.9%
sterile saline. The liver tissues were fixed in a fresh solution of 4% paraformaldehyde (PH 7.4)
at 4°C for 24 h, then incubated overnight at 4°C in 100 mmol/L sodium phosphate buffers
(PH 7.4) containing 30% sucrose and embedded in optimal cutting temperature (OCT)
compound (Leica, CA, Germany). The cryosections were collected on
3-animopropyl-trimethoxysilane-coated slides (Sigma-Aldrich, St. Louis, MO, USA) and
stored at −70°C.

The liver slices were stained with haematoxylin and eosin (H&E, Sigma-Aldrich, St. Louis, MO, USA), and examined by an expert in liver pathology blinded to the type of treatment received by the animals. Oil Red O (Sigma-Aldrich, St. Louis, MO, USA) staining was performed on 8-μm sections that were cut from frozen OCT samples, mounted on slides, and allowed to air-dry for 30 to 60 minutes at room temperature. The sections were fixed in ice-cold 10% formalin for 10 minutes and stained with Oil Red O for 10 minutes. The sections were then counterstained with Mayer’s haematoxylin for 1 minute and rinsed in distilled water.

**Tissue homogenates**

Tissue homogenates were prepared for biochemical studies and western blot analysis as described in our previous work (Zhang et al. 2013). For biochemical studies, the liver was promptly dissected and homogenized 1:10 (w/v) in 50 mmol/L (pH 7.4) ice-cold Tris-buffered saline containing a protease inhibitor cocktail using a Teflon-glass Potter-Elvehjem homogenizer for 10 strokes at 1200 rpm (Kontes, Vineland, NJ, USA). Homogenates were centrifuged at 12 000 g for 10 min, and the supernatant was used to determine reactive oxygen species (ROS) levels, thiobarbituric acid reactive species (TBARS) levels, glutathione (GSH) levels and enzymes activities. For western blot analysis, the liver was homogenized 1:5 (w/v) in ice-cold RIPA lysis buffer (1 × Tris-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.004% sodium azide) supplemented with 30 μl 10 mg/ml phenylmethanesulfonyl fluoride (PMSF), 30 μl sodium orthovanadate (Na3VO4) and 30 μl protease inhibitor cocktail per gram of tissue. The
homogenates were sonicated 4 times for 30 s with 20 s intervals using a sonicator, centrifuged at 15 000 g for 10 min at 4°C, then the supernatant was collected and stored at -70 °C for western blot studies. Supernatant protein levels were determined using the bicinchoninic acid assay kit (Pierce Biotechnology, Rockford, IL, USA).

**Biochemical analysis**

The serum level of aminotransferase (ALT) was measured spectrophotometrically using kits (Jiancheng Institute of Biotechnology, Nanjing, China) according to the manufacturer’s instructions. For glucose measurements, blood samples were taken by tail venipuncture after 6 h of fasting. Fasting blood glucose levels were measured with an Ascensia Elite glucose meter (Bayer Corporation, Mishawaka, IN, USA). Serum insulin levels were measured with the appropriate enzyme-linked immunosorbent assay kits (ELISA; ALPCO Diagnostics, Windham, NH, USA) according to the manufacturer’s instructions. Hepatic lipids were extracted from approximately 200 mg frozen liver samples using chloroform : methanol (2:1 v/v) solution, and resuspended in PBS containing 5% Triton X-100. The serum sample and hepatic lipid extraction solution were used to determine triglyceride (TG), cholesterol (Chol), non-esterified fatty acid (NEFA), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) levels using the commercial kits (Wako Chemicals Richmond, VA, USA) according to the manufacturer’s instructions.

**Reactive oxygen species (ROS) assay**

ROS was measured based on the oxidation of 2’, 7’-dichlorodihydrofluorescein diacetate (H2-DCF-DA) to 2’, 7’-dichlorofluorescein (DCF) as described in our previous work (Zhang et al. 2013). Briefly, the homogenate was diluted 1:20 (v/v) with ice-cold Locke’s buffer [154...
mmol/L NaCl, 5.6 mmol/L KCl, 3.6 mmol/L NaHCO₃, 2.0 mmol/L CaCl₂, 10 mmol/L D-glucose and 5 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4] to obtain a tissue concentration of 10 mg/ml. The reaction mixture (1 ml) containing Locke’s buffer, 0.2 ml of homogenate and 10 µl of 5 mmol/L H2-DCF-DA was incubated for 15 minutes at room temperature to allow the H2-DCF-DA to be incorporated into any membrane-bound vesicles and the diacetate group to be cleaved by esterases. After 30 minutes of further incubation, the conversion of H2-DCF-DA to the fluorescent product DCF was measured using a Molecular Devices M2 plate reader (Molecular Devices Corporation, Menlo Park, CA, USA) with excitation at 484 nm and emission at 530 nm. Background fluorescence (conversion of H2-DCF-DA in the absence of homogenate) was corrected by the inclusion of parallel blanks. ROS formation was quantified from a DCF standard curve. Data are expressed as pmol DCF formed/min/mg protein.

Measurement of lipid peroxidation level

The level of TBARS in liver tissue homogenates was determined as described previously (Zhang et al. 2009). Half a millilitre of each homogenate was mixed with 3 ml of H₃PO₄ solution (1%, v/v) followed by addition of 1 ml of thiobarbituric acid solution (0.67%, w/v). The mixture was incubated at 95 °C in a water bath for 45 minutes. The coloured complex was extracted into n-butanol, and the absorption at 532 nm was measured using tetramethoxypropane as standard. The TBARS levels were expressed as nmol per milligram of protein.

Glutathione (GSH) assays

The levels of GSH in the hepatic supernatants were determined according to the protocols
of a commercially available GSH assay kit (Cayman Chemical, Ann Arbor, MI, USA). After reaction with 5, 5-dithiobis-(2-ni-trobenzoic acid) (DTNB), the GSH levels were determined at 405 nm with a spectrophotometer (Shimadzu UV-2501 PC, Shimadzu Corporation, Japan). The results are expressed as the contents (µmol GSH) per mg of protein.

**Glutathione peroxidase (GPx) activity assay**

The GPx activity assay was performed as described in our previous work (Zhang et al. 2013). Tert-butylhydroperoxide was used as the substrate. The assay measures the enzymatic reduction of H$_2$O$_2$ by GPx through consumption of reduced GSH that is restored from oxidised glutathione disulfide (GSSG) in a coupled enzymatic reaction by glutathione reductase (GR). GR reduces GSSG to GSH using NADPH as a reducing agent. The decrease in absorbance at 340 nm due to NADPH consumption was measured in a Molecular Devices M2 plate reader (Molecular Devices Corporation, Menlo Park, CA, USA). GPx activity was computed using the molar extinction coefficient of 6.22 mM$^{-1}$ cm$^{-1}$. One unit of GPx was defined as the amount of enzyme that catalysed the oxidation of 1.0 µmol of NADPH to NADP$^+$ per minute at 25 °C.

**Kinase Activity Assay**

Hepatic supernatants containing equivalent amounts of protein (500µg) were precleared using protein A-sepharose beads for 1 h at 4 °C followed by incubation with 1.5 µg anti-Src antibodies overnight at 4°C. Immune complexes were affinity-precipitated with protein A-sepharose beads and washed 3 times with 25 mmol/L HEPES buffer (pH 7.4), 10 mmol/L MgCl$_2$, 1 mmol/L NaF, 1% NP-40, and 1 mmol/L Na$_3$VO$_4$) and 2 times in Src kinase reaction buffer [100 mmol/L Tris–HCl, pH 7.2, 125 mmol/L Mg(C$_2$H$_3$O$_2$)$_2$, 25 mmol/L MnCl$_2$, 2
mmol/L EGTA, 0.25 mmol/L sodium orthovanadate, and 2 mmol/L dithiothreitol]. Assays were performed with reagents obtained from a commercially available Src kinase assay kit (Upstate Biotechnology Inc., Lake Placid, NY, USA) according to the manufacturer’s instructions. Changes in Src activity in livers were calculated against the mean value of Src activity in control liver and expressed as percent of control.

Western blot analysis

Western blot analyses were performed as described in our previous work (Zhang et al. 2013). Briefly, samples (50 µg protein) were separated on denaturing SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes (Roche Diagnostics Corporation, Indianapolis, IN, USA) by electrophoretic transfer. The membrane was blocked with 5% non-fat milk or bovine serum albumin (BSA) in 0.1% Tween-20/TBS followed by overnight incubation with primary antibodies: rabbit anti-Src, rabbit anti-p-Src (Tyr416), rabbit anti-mitogen-activated protein kinase kinase (MEK), rabbit anti-p-MEK (Ser217/221), rabbit anti-extracellular-signal-regulated kinase (ERK), rabbit anti-p-ERK (Thr202/Tyr204), rabbit anti-p- AMPKα (Thr172), rabbit anti-fatty acid synthase (FAS), rabbit anti-ACC, rabbit anti-p- acetyl-CoA carboxylase (ACC) (Ser79) and rabbit anti-β-Actin antibodies (Cell Signaling Technology, Beverly, MA, USA); rabbit anti-CCAAT/enhancer binding protein β (C/EBPβ), rabbit anti-p-C/EBPβ (T188/T235) and rabbit anti-cluster of differentiation 36 (CD36) antibodies (Abcam, Cambridge, UK); rabbit anti- carnitine palmitoyltransferase 1A (CPT1A) antibody (Proteintech Group, Rosemont, IL, USA). After washing, proteins were detected using HRP-conjugated anti-rabbit secondary antibodies (Cell Signaling Technology, Beverly, MA, USA). Immunoreactive proteins were visualised using 20 × LumiGLO®
Reagent and 20 × peroxide (Cell Signaling Technology, Beverly, MA, USA). The optical
density (OD) values of the detected bands were measured with Scion Image analysis software
(Scion Corp., Frederick, MD, USA). The OD values were normalised using appropriate
internal controls (optical density detected protein/optical density internal control).

Statistical analysis

All statistical analyses was performed using SPSS version 11.5. All the data were analysed
with a one-way analysis of variance (ANOVA) followed by Tukey’s Honestly Significant
Difference (HSD) post-hoc test. Data were expressed as means ± standard deviation (SD).
Statistical significance was set at p<0.05.

Results

PSPC attenuates obesity and related metabolic parameters as well as liver injury in
HFD-treated mice.

The food intake of mice fed an ND was higher than that of mice fed HFD due to the low
energy density of the ND (Fig.1A). Oral administration of PSPC did not markedly influence
the food intake in either ND-fed mice or HFD-fed mice (Fig.1A). After 4 weeks, an obvious
increase in body weight was found in HFD-fed mice (Fig.1B; 4 weeks: p < 0.01, 8 weeks: p <
0.001, 12 weeks: p < 0.001, 16 weeks: p < 0.001, 20 weeks: p < 0.001, versus control group).
Moreover, 20 weeks of HFD significantly elevated obesity-related metabolic parameters in
mice, including epididymal adipose tissue masses, liver index, fasting blood glucose, serum
insulin, serum TG, serum Chol, serum NEFA, serum LDL-C and HDL-C (Fig. 2; epididymal
adipose tissue: p < 0.001; liver index: p < 0.001; fasting blood glucose: p < 0.001; serum
insulin: p < 0.001; serum TG: p < 0.001; serum Chol: p < 0.001; serum NEFA: p < 0.001;
serum LDL-C: $p < 0.001$; serum HDL-C: $p < 0.01$, versus control group). HFD-fed mice had significantly higher serum ALT levels than control mice (Fig. 3A; ALT: $p < 0.001$, versus control group), indicating the occurrence of obesity-related liver injury. Obesity-related liver injury was confirmed by histological analysis, which showed that HFD treatment induced hepatocyte hypertrophy and vacuolisation and inflammatory cell infiltration in mouse livers (Fig. 3B). PSPC notably attenuated HFD-induced obesity (Fig. 1B; 4 weeks: $p < 0.01$; 8 weeks: $p < 0.001$; 12 weeks: $p < 0.001$; 16 weeks: $p < 0.001$; 20 weeks: $p < 0.001$, versus HFD group) and effectively improved these obesity-related metabolic parameters and liver injuries in HFD-treated mice (Fig. 2 and 3; epididymal adipose tissue: $p < 0.001$; Liver index: $p < 0.001$; fasting blood glucose: $p < 0.001$; serum insulin: $p < 0.001$; serum TG: $p < 0.001$; serum Chol: $p < 0.001$; serum NEFA: $p < 0.001$; serum LDL-C: $p < 0.001$; serum HDL-C: $p < 0.05$; ALT: $p < 0.001$, versus HFD group). No significant differences in obesity and related metabolic parameters, and liver injuries were found among the HFD+PSPC, PSPC and the control groups. The above results indicated that PSPC effectively suppressed HFD-induced obesity and related metabolic parameters and liver injury.

**PSPC ameliorates hepatic steatosis in HFD-treated mice.**

A striking accumulation of hepatic lipids was evidenced by a marked increase in hepatic TG, Chol and NEFA levels in HFD-treated mice (Fig. 4A-C; hepatic TG: $p < 0.001$; hepatic Chol: $p < 0.001$; hepatic NEFA: $p < 0.001$, versus control group), which was confirmed by Oil Red O staining of liver sections (Fig. 4D). In contrast, PSPC markedly lowered hepatic lipid accumulation in HFD-treated mice (Fig. 4; hepatic TG: $p < 0.001$; hepatic Chol: $p < 0.001$; hepatic NEFA: $p < 0.001$, versus HFD group). There were no significant differences in
hepatic lipid levels among the HFD + PSPC, PSPC and control groups. These results suggested that PSPC protected against hepatic steatosis in HFD-treated mice.

**PSPC abates hepatic oxidative stress in HFD-treated mice.**

HFD provoked severe oxidative stress characterised by markedly elevated hepatic ROS and TBARS (a marker of lipid peroxidation) levels in mouse livers (Fig. 5A and B; ROS: \( p < 0.001 \); TBARS: \( p < 0.001 \), versus control group). At the same time, the GSH level and antioxidant enzyme (GPx) activity were largely reduced in the livers of HFD-treated mice (Fig. 5C and D; GSH: \( p < 0.001 \); GPx: \( p < 0.001 \), versus control group). PSPC dramatically renewed the hepatic redox state in the livers of HFD-treated mice by diminishing ROS production and TBARS level, remarkably improving GSH content and GPx activity (Fig. 5; ROS: \( p < 0.001 \); TBARS: \( p < 0.001 \); GSH: \( p < 0.001 \); GPx: \( p < 0.001 \), versus HFD group). There were no significant differences in hepatic redox status among the HFD + PSPC, PSPC and control groups. These results indicated that PSPC significantly abated oxidative stress in the livers of HFD-treated mice.

**PSPC blocks Src activation in HFD-treated mouse livers.**

It is well demonstrated that oxidative stress activates Src under various pathological conditions (Pal et al. 2014; Chattopadhyay et al. 2015), which might promote the development of HFD-induced hepatic steatosis. As shown in Figure 6, HFD treatment induced the marked activation of Src by enhancing the phosphorylation of Src, as well as its activity, in mouse livers (Fig. 6; p-Src\(_{\text{protein}}\): \( p < 0.01 \); Src\(_{\text{activity}}\): \( p < 0.001 \), versus Control group). PSPC significantly lowered the activation of Src in the livers of HFD-treated mice (Fig. 6; p-Src\(_{\text{protein}}\): \( p < 0.01 \); Src\(_{\text{activity}}\): \( p < 0.001 \), versus HFD group). There were no
significant differences in Src activation among HFD + PSPC, PSPC and control groups. These results demonstrated that PSPC notably blocked Src activation in the livers of HFD-treated mice.

**PSPC suppresses ERK activation in HFD-treated mouse livers.**

The MEK/ERK signalling pathway, a downstream signalling of Src (Taniguchi et al. 2013), is involved in the regulation of hepatic steatosis (He et al. 2016). As shown in Figure 7, HFD treatment dramatically enhanced MEK and ERK activation as evidenced by increased MEK (Ser217/221) and ERK (Thr202/Tyr204) phosphorylation in mouse livers (p-MEK: \( p < 0.01 \); p-ERK: \( p < 0.01 \), versus control group). PSPC significantly restrained the activation of the MEK/ERK signalling pathway in the livers of HFD-treated mice (Fig. 7; p-MEK: \( p < 0.01 \); p-ERK: \( p < 0.01 \), versus HFD group). No significant differences in the activation of MEK/ERK signalling were found among HFD + PSPC, PSPC and control groups. These findings indicated that PSPC markedly suppressed MEK/ERK signalling in the liver of HFD-treated mice.

**PSPC depresses C/EBPβ activation-mediated lipid metabolism disorders in HFD-treated mouse livers.**

C/EBPβ has been established as an important participants in hepatic steatosis (Rahman et al. 2007; Jin et al. 2013). The C/EBPβ phosphorylation level was markedly increased in the livers of HFD-treated mice, indicating an enhancement of C/EBPβ activation (Fig. 8A; \( p < 0.01 \), versus control group).

To investigate whether the HFD-induced C/EBPβ activation was mediated by ERK signalling, we used U0126 to inhibit ERK activation in the HFD-fed mouse livers. U0126
significantly suppressed the ERK (Thr202/Tyr204) phosphorylation in the livers of HFD-treated mice (Fig. 8B; \( p < 0.01 \), versus HFD-control group). Moreover, U0126 treatment effectively attenuated C/EBPβ activation in the HFD-fed mouse livers (Fig. 8C; \( p < 0.01 \), versus HFD-control group). The above results indicated that HFD-induced C/EBPβ activation was mediated by ERK signalling in the mouse livers.

AMPK is a key regulator of energy metabolism in metabolic tissues, such as liver, muscle and adipose tissue. HFD remarkably restrained AMPK activation as evidenced by decreased p-AMPK (Thr 172) levels in the mouse livers (Fig. 8D; p-AMPK: \( P < 0.01 \), versus control group). The HFD-mediated decrease in AMPK activation was reversed by U0126 treatment (Fig. 8E; p-AMPK: \( P < 0.01 \), versus HFD-control group).

Accordingly, the protein expression and activation of two lipogenic enzymes, ACC and FAS, were largely augmented in the livers of HFD-treated mice (Fig. 8F and G; p-ACC, \( p < 0.01 \); FAS, \( p < 0.001 \), versus control group). Moreover, HFD treatment markedly increased the protein expression of CD36 in mouse livers (Fig. 8G; \( p < 0.001 \), versus control group), suggesting an enhancement of lipid uptake. Additionally, a significant down-regulation of CPT1A protein expression was found in the livers of HFD-fed mice (Fig. 8G; \( p < 0.01 \), versus control group), indicating diminished fatty acid oxidation. PSPC dramatically diminished C/EBPβ activation and restored AMPK activation, and thus significantly suppressed the protein expression of FAS and CD36 and the activation of ACC, and increased the protein expression of CPT1A in the livers of HFD-treated mice (Fig. 8; p-C/EBPβ; \( p < 0.01 \); p-AMPK; \( p < 0.01 \); p-ACC, \( p < 0.01 \); FAS, \( p < 0.001 \); CD36: \( p < 0.001 \); CPT1A: \( p < 0.01 \), versus HFD group). There were no significant differences in C/EBPβ and
AMPK activation, and the levels of these lipid metabolic enzymes among HFD + PSPC, PSPC and control groups. These results suggested that PSPC improved C/EBPβ activation-mediated lipid metabolism disorders in the livers of HFD-treated mice.

Discussion

Hepatic steatosis has been well established as a cause of endoplasmic reticulum (ER) stress, mitochondrial dysfunction and insulin resistance (IR), ultimately contributing to the development of NAFLD and related diseases including diabetes (Byrne and Targher 2015; Rinella 2015). However, the mechanisms underlying HFD-induced steatosis are complicated and have not been fully elucidated. The present study revealed that Src activation-mediated enhancement of C/EBPβ signalling was involved in the pathogenesis of hepatic steatosis. The amelioration of HFD-induced hepatic steatosis by PSPC is associated with inhibiting oxidative stress-mediated Src activation, further suppressing the downstream ERK-C/EBPβ signaling axis and consequent lipid metabolism disorders. This study provided novel mechanistic insights into hepatic steatosis and the beneficial effects of PSPC on NAFLD.

A considerable amount of evidence has indicated that oxidative stress is a response to the development of NAFLD (Rinella 2015; Byrne and Targher 2015). Oxidative stress results from an imbalance between prooxidants and antioxidants, characterised by increased level of ROS, which acts directly on essential biomolecules or indirectly activates redox-sensitive signalling cascades to induce steatosis (Rinella 2015). In the present study, HFD induced an increase in the levels of ROS and lipid peroxidation and a decline in the reducing potential in mouse livers, suggesting that oxidative stress was involved in the development of HFD-induced hepatic steatosis. Substantial evidence has demonstrated that PSPC has
stronger antioxidant activity than many other plant pigments (Chao et al. 2014). Our previous findings also suggest that PSPC attenuates HFD-induced hepatic insulin resistance via blocking oxidative stress (Zhang et al. 2013). This study showed that PSPC attenuated oxidative stress in the livers of HFD-treated mice by reducing ROS and lipid peroxidation levels and increasing GSH content and antioxidant enzyme activity. Our results consistently suggested that PSPC improved HFD-induced hepatic steatosis by abating oxidative stress.

Once activated, Src participates in many pathophysiological processes, including autophagy (Pal et al. 2014) and inflammation (Byeon et al. 2012). Saturated long chain fatty acids have been reported to induce Src activation to trigger c-Jun-N-terminal kinase (JNK) signaling giving rise to insulin resistance (Holzer et al. 2011). HFD stimulates Src activation, leading to lung vascular injury in obese mice (Shah et al. 2015). Addition, high glucose levels increase Src activation to promote epidermal growth factor receptor (EGFR) transactivation and collagen synthesis in the kidneys of diabetic mice (Taniguchi et al. 2013). These results indicate that Src is dramatically activated by high-fat or high-glucose treatment in vitro and in vivo, which contributes to the pathogenesis of diabetes and its complications. In the present study, a remarkable increase in Src phosphorylation and activity was observed in the liver of HFD-fed mice, suggesting that Src activation may play a role in the pathogenesis of hepatic steatosis. It is well demonstrated that Src is activated by oxidative stress (Martel-Gallegos et al. 2013; Pal et al. 2014; Chattopadhyay et al. 2015). Thus, HFD may promote Src activation by inducing oxidative stress in mouse livers. A large number of natural products have been evaluated as antioxidant agents against commonly occurring diseases by suppressing Src activation (Pasdois et al. 2007; Lee et al. 2010; Byun et al. 2013). For example, kaempferol...
and apigenin decrease Src activation to suppress ultraviolet radiation B (UVB)-induced inflammation in skin cancer (Lee et al. 2010; Byun et al. 2013). Additionally, ouabain protects against postischaemic cardiac dysfunction by blocking Src activation (Pasdois et al. 2007). This study revealed that PSPC consistently suppressed Src activation in the livers of HFD-treated mice, indicating that PSPC might alleviate hepatic steatosis via inhibiting oxidative stress-mediated Src activation.

Src activation results in intramolecular phosphorylation and activation of critical downstream targets, including MAPK (Holzer et al. 2011; Limami et al. 2012; Taniguchi et al. 2013), which contributes to various physiological and pathological processes. Src has been reported to activate the MAPK-signalling pathway in high-glucose-induced diabetic nephropathy (Taniguchi et al. 2013). In this study, our findings showed that ERK signalling was markedly activated in the livers of HFD-treated mice, characterised by increased phosphorylation of MEK and ERK. Consistent with the results of these studies, our results suggested that HFD increased Src activation to stimulate ERK signalling and consequently promoted steatosis in mouse livers. Recent study has indicated that caffeine blocks Src activation-mediated enhancement of MAPK signalling to inhibit liver fibrosis (Wang et al. 2014). Additionally, flavonoids are used in the treatment of cancer through inhibition of Src/ERK signalling (Huang et al. 2009; Saud et al. 2013). In this study, our results showed that PSPC significantly depressed ERK signalling in the livers of HFD-fed mice. Our findings indicated that PSPC might suppress Src activation to block its downstream ERK signalling pathway, thereby attenuating steatosis in the livers of HFD-treated mice.

C/EBPβ, which is a downstream target of ERK, is a transcription factor belonging to the
basic leucine zipper family that participates in the pathogenesis of steatosis (Farmer 2005; Jin et al. 2013). In this study, our results showed that ERK inhibitor U0126 effectively attenuated C/EBPβ activation in HFD-fed mouse livers, indicating that HFD-induced C/EBPβ activation was mediated by ERK signalling. In hepatic steatosis, C/EBPβ is an integral part of signalling causes lipogenesis, which induces the expression of lipogenic enzymes, such as ACC and FAS (Rahman et al. 2007; Yang et al. 2009). In the present study, our results showed that HFD augmented C/EBPβ activation and consequent protein expression and activation of lipogenic enzymes. Consistently, our findings suggested that HFD induced hepatic steatosis by increasing C/EBPβ activation-mediated lipogenesis. Elevated expression of CD36, a fatty acid transporter, is involved in the development of hepatic steatosis by facilitating fatty acid uptake (Miquilena-Colina et al. 2011; Pisonero-Vaquero et al. 2015). Recent evidence has suggested that C/EBPβ overexpression augments the mRNA and protein expression of CD36, which leads to diet-induced hepatic steatosis (Qiao et al. 2008; Pisonero-Vaquero et al. 2015). In this study, our data showed that the protein level of CD36 was markedly increased in the livers of HFD-fed mice, indicating that HFD might promote fatty acid uptake to facilitate hepatic steatosis via enhancing C/EBPβ-mediated CD36 expression. Accumulated evidence has demonstrated that decreased expression of CPT1A, a rate-limiting enzyme in fatty acid oxidation, abates the entry of fatty acids into mitochondria for oxidation, which contributes to steatosis (Long and Zierath 2006; Derdak et al. 2013). Enhanced ACC has been indicated to increase the production of malonyl-CoA to inhibit CPT1A, exacerbating steatosis, and this process may be stimulated by elevated C/EBPβ signalling (Long and Zierath 2006; Rodriguez et al. 2008). Our results revealed that HFD significantly diminished CPT1A levels.
in mouse livers, indicating that HFD might impair CPT1A-mediated fatty acid oxidation via augmenting C/EBPβ-mediated ACC expression. A growing body of research has shown that flavonoids effectively alleviated lipid metabolism disorders in obesity, diabetes and NAFLD by suppressing C/EBPβ signalling (Pisonero-Vaquero et al. 2015; Zhang et al. 2016).

Consistent with previous reports, our results showed that PSPC inhibited the expression and activation of lipogenic enzymes (ACC and FAS) and a fatty acid transporter (CD36), while increasing the expression of a rate-limiting enzyme in fatty acid oxidation (CPT1A) in the livers of HFD-fed mice. Our findings indicated that PSPC might exhibit its beneficial effects on hepatic steatosis via blocking C/EBPβ signalling, consequently depressing lipogenesis and fatty acid uptake and enhancing fatty acid oxidation. Substantial evidence has indicated that impaired AMPK activation contributes to the development of hepatic steatosis (Castaño et al. 2014; Woods et al. 2017). AMPK has been established to directly phosphorylate ACC to inactivate it, which abates lipogenesis and facilitates fatty acid oxidation (Fullerton et al. 2013). In the present study, our results showed that HFD remarkably restrained AMPK activation in the mouse livers, which was reversed by U0126 treatment. Recently, ERK signalling has been reported to inhibit AMPK activation in some settings, such as insulin resistance (Damm et al. 2012; Hwang et al. 2013). Thus, our findings indicated that HFD might suppress AMPK activation via activating ERK signaling in mouse livers. Accumulating evidence has suggested that natural flavonoids improve the features of NAFLD, including steatosis, via activating AMPK (Salomone et al. 2016; Song et al. 2016). Our findings indicated that PSPC might ameliorate hepatic steatosis by restoring AMPK activation in the livers of HFD-fed mice.
Obesity is closely associated with the features of metabolic syndrome, such as steatosis, dyslipidaemia, hyperglycaemia, hypertension, and insulin resistance. Excessive accumulation of adipose tissue aberrantly secretes adipokines and cytokines during obesity, which causes abnormalities of glucose and lipid metabolism in the liver, contributing to the development of NAFLD (Duval du et al. 2010; Plessis et al. 2015). In obesity, the intestinal mucosal integrity is impaired and the intestinal permeability is increased, which elevate circulating bacterial endotoxins, leading to the development and progression of NAFLD (Brun et al. 2007). In the present study, PSPC significantly reduced the body weight and epididymal adipose tissue masses without affecting food intake in HFD-fed mice. Natural flavonoids are reported to improve aberrant release of adipokines/cytokines by adipose tissue and ameliorate obesity-associated intestinal alterations (Liu et al. 2007; Suzuki and Hara 2011). Thus, our findings indicated that PSPC might improve the dysregulation of hepatic lipid metabolism by ameliorating obesity-associated aberrant release of adipokines/cytokines and intestinal alterations, although this requires further study.

In conclusion, our data showed that PSPC exhibited a remarkable protective effect against HFD-induced hepatic steatosis, which were associated with inhibiting oxidative stress-mediated Src activation, further blocking the ERK signal pathway and its downstream C/EBPβ signalling and restoring AMPK activation, ultimately suppressing lipogenesis and fatty acid uptake and enhancing fatty acid oxidation. These ameliorative effects of PSPC on hepatic steatosis might be related to its anti-obesity effect. Therefore, this study provides novel mechanistic insights into NAFLD pathogenesis and a theoretical basis for PSPC as a therapeutic agent in NAFLD and diabetes.
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**Figure Captions**

Fig.1. Purple sweet potato color (PSPC) decreases body weight in high-fat-diet (HFD)-treated mice (n=8). (A) Food intake in all treated groups. (B) Total body weight in all treated groups. All of the values are expressed as the mean ± standard deviation (SD). ** p < 0.01, *** p < 0.001 vs. the control group; ## p < 0.01, ### p < 0.001 vs. the HFD group.

Fig.2. Purple sweet potato color (PSPC) attenuates obesity-related metabolic parameters in high-fat-diet (HFD)-treated mice (n=5). (A) Epididymal adipose tissue masses in all treated groups. (B) Liver indices in all treated groups. (C) Fasting blood glucose levels in all treated groups. (D) Serum insulin levels in all treated groups. (E) Serum triglyceride (TG) levels in all treated groups. (F) Serum cholesterol (Chol) levels in all treated groups. (G) Serum non-esterified fatty acid (NEFA) levels in all treated groups. (H) Serum low density lipoprotein cholesterol (LDL-C) levels in all treated groups. (I) Serum high-density lipoprotein cholesterol (HDL-C) levels in all treated groups. All of the values are expressed as the mean ± standard deviation (SD). ** p < 0.01, *** p < 0.001 vs. the control group; # p < 0.05, ## p < 0.01, ### p < 0.001 vs. the HFD group.
Fig. 3. Purple sweet potato color (PSPC) improves liver injury in high-fat-diet (HFD)-treated mice (n=5). (A) Serum aminotransferase (ALT) activities in all treated groups. (B) Haematoxylin and eosin (H&E) staining of liver sections. Scale bar: 50 µm. Inflammatory cells are indicated by black arrows. All of the values are expressed as the mean ± standard deviation (SD). *** p < 0.001 vs. the control group; ### p < 0.001 vs. the HFD group.

Fig. 4. Purple sweet potato color (PSPC) ameliorates hepatic steatosis in high-fat-diet (HFD)-treated mice (n=5). (A) Hepatic triglyceride (TG) levels in all treated groups. (B) Hepatic cholesterol (Chol) levels in all treated groups. (C) Hepatic non-esterified fatty acid (NEFA) levels in all treated groups. (D) Oil red O staining of liver sections. Scale bar: 50 µm. All of the values are expressed as the mean ± standard deviation (SD). *** p < 0.001 vs. the control group; ### p < 0.001 vs. the HFD group.

Fig. 5. Purple sweet potato color (PSPC) abates hepatic oxidative stress in high-fat-diet (HFD)-treated mice (n=5). (A) Reactive oxygen species (ROS) production in mouse livers. (B) Thiobarbituric acid reactive species (TBARS) levels in mouse livers. (C) Glutathione (GSH) content in mouse livers. (D) Glutathione peroxidase (GPx) activities in mouse livers. All of the values are expressed as the mean ± standard deviation (SD). *** p < 0.001 vs. the control group; ### p < 0.001 vs. the HFD group. *** p < 0.001 vs. the control group; ### p < 0.001 vs. the HFD group.

Fig. 6. Purple sweet potato color (PSPC) blocks Src activation in high-fat-diet (HFD)-treated mouse livers (n=3). (A) Immunoblotting and densitometry analysis of p-Src in mouse livers. (B) Src kinase activities in mouse livers. All of the values are expressed as the mean ± standard deviation (SD). ** p < 0.01 vs. the control group; ## p < 0.01 vs. the HFD group.
Fig. 7. Purple sweet potato color (PSPC) suppresses extracellular-signal-regulated kinase (ERK) activation in high-fat-diet (HFD)-treated mouse livers (n=3). (A) Immunoblotting and densitometry analysis of p-mitogen-activated protein kinase kinase (p-MEK) in mouse livers. (B) Immunoblotting and densitometry analysis of p-ERK in mouse livers. All of the values are expressed as the mean ± standard deviation (SD). ** p < 0.01 vs. the control group; ## p < vs. the HFD group.

Fig. 8. Purple sweet potato color (PSPC) depresses CCAAT/enhancer binding protein β (C/EBPβ)-mediated lipid metabolism disorders in high-fat-diet (HFD)-treated mouse livers (n=3). (A) and (C) Immunoblotting and densitometry analysis of p-C/EBPβ in mouse livers. (B) Immunoblotting and densitometry analysis of p-ERK in mouse livers. (D) and (E) Immunoblotting and densitometry analysis of p-AMPK in mouse livers. (F) Immunoblotting and densitometry analysis of p-acetyl-CoA carboxylase (p-ACC) in mouse livers. (G) Immunoblotting and densitometry analysis of fatty acid synthase (FAS), cluster of differentiation 36 (CD36) and carnitine palmitoyltransferase 1A (CPT1A) in mouse livers. All of the values are expressed as the mean ± standard deviation (SD). ** p < 0.01, *** p < 0.001 vs. the control group; ## p < 0.01, ### p < 0.001 vs. the HFD group.
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