Ginkgolide C reduced oleic acid-induced lipid accumulation in HepG2 cells

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1. Introduction

Obesity causes many chronic diseases, including diabetes mellitus, hyperlipidemia, and cancer (Forte et al., 2012). Many studies have also shown that excessive lipid accumulation in the liver of obese subjects induces nonalcoholic fatty liver disease (NAFLD) (Neuschwander-Tetri, 2017; Patil and Sood, 2017). NAFLD is defined as abnormal lipid accumulation in hepatocytes, and this interferes with the normal metabolism of carbohydrates and lipids for reduced glycogen synthesis and increased lipid synthesis, leading to lipid accumulation in the liver (Reccia et al., 2017). NAFLD can be divided into simple fat accumulation (hepatic steatosis) and deteriorated steatohepatitis. When the affected hepatocytes are not repaired, sustained inflammatory and oxidative damage occurs and nonalcoholic steatohepatitis (NASH) develops (Benedict and Zhang, 2017). If NASH patients do not maintain a healthy lifestyle with moderate rest and regular exercise,
irreversible liver fibrosis, cirrhosis, and even liver failure and liver cancer may develop (Reccia et al., 2017). The development of NAFLD is closely associated with obesity and diabetes. Therefore, improving obesity and reducing liver lipid accumulation may attenuate its development.

The most promising treatments for NAFLD are regulated diet, moderate exercise, weight loss, and possibly bariatric surgery (Brouwers et al., 2016). Regulating food intake with excess triglycerides is especially important since free fatty acids in the digestive tract can be transported through the blood to the liver for metabolism and be converted to simple lipids or cholesterol (Romero-Gómez et al., 2017). Transcription factors regulating hepatic lipogenesis are important for liver lipid synthesis, and can activate fatty acid chain synthesis, causing excessive triglyceride synthesis and lipid accumulation in the liver (Guo et al., 2015). Thus, blocking the expression of lipogenesis transcription factors will attenuate the synthesis of triglycerides in the liver.

AMP-activated protein kinase (AMPK) is a source of energy, and some studies have found that AMPK activity can regulate lipogenesis transcription factors in hepatic steatosis (Smith et al., 2016). The excessive storage of energy in cells leads to AMPK phosphorylation, followed by phosphorylation of its substrate acetyl-CoA carboxylase (ACC) (Lim et al., 2010), which plays an essential role in regulating fatty acid synthesis (Smith et al., 2016). Interestingly, ACC phosphorylation reduces the synthesis of malonyl-CoA and the extension and synthesis of the fatty acid chain (Hou et al., 2008).

Recent studies have found that many plant extracts and flavonoids can improve liver steatosis and NAFLD (Feng et al., 2017; Tian et al., 2016). Ginkgo biloba is a herbal medicine that has long been used in Eastern and Western medicine to improve cardiovascular disease (Yin et al., 2014). Western medicine has used the G. biloba extract EGB-761 to treat cardiovascular disease and dementia, and the ginkgo fruit is used in Chinese medicine to improve asthma (Babayigit et al., 2009; Stein et al., 2015). In recent years, several diterpene lactones and flavonoids were isolated from G. biloba (Zeng et al., 2013). Ginkgolide A, B, and C are diterpene lactones that can improve atherosclerosis and attenuate platelet activating factor (Huang et al., 2014; Zeng et al., 2013). A previous study found that ginkgolide A can improve NAFLD in high fat diet-induced obese mice (Jeong et al., 2017). Another study found that ginkgolide C could reduce transcription factors of adipogenesis and increase lipolysis by enhancing Sirt1/AMPK activity in 3T3-L1 differentiated adipocytes (Liou et al., 2015). In this study, we investigated whether ginkgolide C reduced lipid accumulation and regulated the molecular mechanism of lipogenesis in oleic acid-induced HepG2 hepatocytes.

2. Materials and methods

2.1. Chemical reagent

Ginkgolide C (purity ≥ 96% by HPLC) was purchased from Sigma (St. Louis, MO, USA) and was dissolved in DMSO (< 0.1% in all cell experiments).

2.2. Cell culture and induced fatty liver cells

The HepG2 cell line was obtained from the Bioresource Collection and Research Center (BCRC, Taiwan). HepG2 cells were grown in a humidified atmosphere of 5% CO₂ at 37°C in DMEM medium containing 10% fetal bovine serum (FBS) and 100 mg/L penicillin and streptomycin. Hepatocytes were treated with 0.5 mM oleic acid for 48 h and then 3–100 μM ginkgolide C and were incubated in cell culture plates for 24 h.

2.3. Cell viability assay

HepG2 cells were seeded on culture plates and incubated with various concentrations of ginkgolide C for 24 h. The culture plates were treated with 5 mg/ml MTT solution (Sigma) as previously described (Huang et al., 2017), and purple formazan crystals were dissolved in isopropanol. Cell viability was determined via the absorbance at 570 nm using a spectrophotometer (Multiskan FC, Thermo, Waltham, MA, USA).

2.4. Oil Red O staining

HepG2 cells were seeded on culture plates and incubated with 0.5 mM oleic acid for 48 h. Then, cells were treated with ginkgolide C for 24 h. Next, cells were fixed with formalin, and Oil Red O staining was performed (Liou et al., 2015). Oil droplets were observed using microscopy (Olympus). Next, cells were treated with isopropanol and lipid accumulation was measured using a microplate reader (Multiskan FC, Thermo Fisher Scientific) and recording the absorbance at 490 nm.

2.5. Hepatic lipid accumulation

HepG2 cells were seeded on culture plates and incubated with oleic acid (0.5 mM) for 48 h. Then, cells were treated with ginkgolide C for 24 h. Cells were fixed with 10% formalin, and lipid accumulation was evaluated using BODIPY 493/503 (Invitrogen, Carlsbad, CA, USA) as previously described (Chang et al., 2018). Lipid accumulation was observed using fluorescence microscopy (Olympus, Tokyo, Japan) in cells with DAPI stained nuclei.

2.6. Western blot analysis

Equal amounts of protein were separated on 8–10% SDS–polyacrylamide gels, and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) using a previously described method (Liou and Huang, 2017). The PVDF membranes were blocked with TBST buffer (150 mM NaCl, 10 mM Tris-HCl pH 8.0, 0.1% Tween 20) containing 5% FBS for 1 h. Then, the membranes were incubated overnight at 4°C with primary antibodies, including SREBP-1c, carnitine palmitoyltransferase 1 (CPT-1) and 2 (CPT-2) (Cell Signaling Technology, MA, USA); phosphorylated-AMPKα (pAMPKα), AMPKα, fatty acid synthase (FAS) (Santa Cruz, CA, USA); sirtuin 1 (Sirt1) (Millipore); hormone-sensitive lipase (HSL), phosphorylated HSL (pHSL), adipose triglyceride lipase (ATGL), phosphorylated-acetyl CoA carboxylase-1 (pACC-1), ACC-1, peroxisome proliferator-activated receptor α (PPAR-α) and γ (PPAR-γ) (Epitomics, Burlingame, CA, USA), and β-actin (Sigma). The membranes were washed with TBST and incubated at room temperature with secondary antibodies for 1 h. All specific proteins were detected with an enhanced chemiluminescence reagent (Millipore), and protein signals were detected and quantified using the BioSpectrum 600 system (UVP, Upland, CA, USA).

2.7. Statistical analysis

Statistical analyses were performed using one-way ANOVA and Dunnett’s post-hoc test. The results were expressed as the mean ± standard deviation, and p < 0.05 was considered statistically significant.
3. Results

3.1. Cell viability of HepG2 cells treated with ginkgolide C

Cell viability was evaluated using the MTT method, and oleic acid did not have a significant effect on cell viability at concentrations ≤ 0.5 mM in HepG2 cells (Fig. 1A). Therefore, 0.5 mM oleic acid was used in all experiments. Moreover, ginkgolide C did not have a significant effect on cell viability at concentrations ≤ 100 μM (Fig. 1B) in HepG2 cells. Therefore, 3–100 μM ginkgolide C was used in all experiments.

3.2. Effect of ginkgolide C on lipid accumulation in oleic acid-induced hepatic steatosis

Oleic acid was used to induce hepatic steatosis and cells were then treated with ginkgolide C for 24 h to evaluate lipid accumulation by Oil Red O staining. Oleic acid was able to induce lipid accumulation, which could be reduced by ginkgolide C (Fig. 1C). Hepatocytes were treated with isopropanol to release oil droplets, and we found that cells treated with ginkgolide C had significantly decreased lipid accumulation compared with cells treated only with oleic acid (Fig. 1D). The fluorescent dye BODIPY 493/503 was also used to detect lipid accumulation, and fluorescence images demonstrated that ginkgolide C markedly attenuated lipid accumulation compared with the oleic acid–induced hepatic steatosis cells (Fig. 1E and F).

3.3. The effect of ginkgolide C on transcription factors of lipogenesis

Ginkgolide C significantly suppressed transcription factors of lipogenesis, such as PPAR-γ and SREBP-1c expression, compared with oleic acid–induced HepG2 cells. Ginkgolide C also reduced FAS expression for lipogenesis in fatty liver cells (Fig. 2).

3.4. The effect of ginkgolide C on lipolysis in hepatocytes

Ginkgolide C significantly increased ATGL and pHSL expression in HepG2 cells compared with cells treated with only oleic acid (Fig. 3). Ginkgolide C also significantly promoted CPT-1, CD36, and PPAR-α expression for fatty acid β-oxidation, but CPT-2 was not increased compared with the oleic acid-induced HepG2 cells (Fig. 4).

Fig. 1. Ginkgolide C reduced lipid accumulation in HepG2 cells. (A) Cell viability of oleic acid (OA) in HepG2 cells. (B) Cell viability of ginkgolide C (GC) in HepG2 cells. Data represent the mean ± SD; **P < 0.01 compared with HepG2 cells not treated with OA or GC. Next, HepG2 cells were treated with 0.5 mM oleic acid (OA) at 37 °C for 48 h to induce lipid accumulation in hepatocytes, followed by ginkgolide C (3–100 μM) for 24 h. (C) Oil Red O staining showed lipid accumulation that was observed with a microscope. (D) HepG2 cells were treated with isopropanol and lipid accumulation was measured using the absorbance at OD 490 nm. (E) Staining with the fluorescent dye BODIPY 493/503 (green) to detect hepatic lipid droplets. Nuclei were stained with DAPI (blue). (F) Fluorescent images were quantified, data represent the mean ± SD; *P < 0.05, **P < 0.01 compared with OA group. Three independent experiments were analyzed.
3.5. Ginkgolide C activated sirt1/AMPK in HepG2 cells

Western blotting was used to evaluate whether ginkgolide C regulated sirt1 and AMPK in oleic acid-induced HepG2 cells. We found that ginkgolide C significantly promoted the expression of sirt1 and phosphorylation of ACC-1 and AMPKα compared with oleic acid–induced hepatocytes (Fig. 5).
and cause hepatic steatosis (Kang et al., 2015). Our experiments showed that ginkgolide C had the ability to reduce PPAR hepatocytes, followed by ginkgolide C (3–100 μM) for 24 h. (A) The AMPK/Sirt-1 pathway proteins were detected by Western blot. (B) Three independent experiments were analyzed, and the fold expression levels were measured relative to the expression of β-actin (internal control).

4. Discussion

This study demonstrated that the diterpene lactone ginkgolide C could stimulate AMPK, leading to suppressed ACC activity and the reduction of fatty acid chains, and CPT-1 for enhanced fatty acid β-oxidation. Ginkgolide C also decreased lipogenesis-related transcription factors for down-regulated FAS expression, and promoted expression of lipolysis-related enzymes to accelerate the decomposition of triglycerides. Hence, ginkgolide C significantly reduced lipid accumulation for improved hepatic steatosis in vitro.

Hepatocytes that take up excessive free fatty acids would activate enzymes associated with lipogenesis, leading to the synthesis of triglycerides and more energy accumulation in the liver (Cao et al., 2016). The expression of lipid synthesis enzymes requires lipid transcription factors to bind to the promoter of the FAS and switch on the lipid synthesis genes (Angeles and Hudkins, 2016). PPARα is the main transcription factor for lipid synthesis (Liss and Finck, 2017). Many studies confirmed that overexpression of PPARγ contributed to the differentiation of adipocytes and increased lipid accumulation in adipocytes and hepatocytes (Janani and Ranjitha Kumari, 2015). In the liver cells, oleic acid could activate PPARγ expression to accelerate lipid accumulation and cause hepatic steatosis (Kang et al., 2015). Our experiments showed that ginkgolide C had the ability to reduce PPARγ production and the accumulation of oil droplets; thus, it was confirmed that ginkgolide C could reduce excessive lipid accumulation by reducing PPARγ in liver cells. In addition, Srebp-1c could also bind to the promoter of FAS to switch on triglyceride synthesis (Wang et al., 2015). We found that oleic acid stimulated Srebp-1c expression and enhanced downstream FAS expression to initiate fat synthesis. Oil Red O and fluorescent staining demonstrated that oleic acid-stimulated hepatocytes had significantly increased oil droplet accumulation compared with normal hepatocytes. However, oleic acid-induced liver cells treated with ginkgolide C had reduced Srebp-1c expression, and ginkgolide C also significantly inhibited FAS expression to block lipid accumulation in hepatocytes. Thus, we believe that ginkgolide C can improve lipid accumulation in fatty liver cells by modulating the transcription of lipid synthesis and FAS.

Another strategy to improve the lipid accumulation in liver cells is to accelerate the decomposition of triglycerides (Romero-Gomez et al., 2017; Smith et al., 2016). The main enzymes that regulate this are ATGL and HSL. ATGL can break down triglycerides to produce diacylglycerol, a molecule of free fatty acid, and the active HSL can also break down diacylglycerol to produce monoacylglycerol and a molecule of free fatty acid (Frühbeck et al., 2014). Many studies found that some flavonoids could enhance lipolysis and inhibit lipid accumulation in hepatocytes (Chang et al., 2013; Lasa et al., 2012). Quercetin could reduce lipid accumulation by enhancing ATGL expression in high glucose-induced fatty hepatocytes (Liu et al., 2015). Caffeic acid also promoted lipolysis via activated ATGL and HSL in oleic acid-induced hepatic steatosis (Liao et al., 2014). Our results demonstrated that ginkgolide C could significantly promote ATGL and phosphorylation of HSL for accelerated decomposition of triglycerides to glycerol and free fatty acids. Hence, ginkgolide C can accelerate lipolysis to improve lipid accumulation in fatty liver cells.

The excessive triglycerides broken down by the liver would release more free fatty acids to stimulate vascular epithelial cells and macrophages leading to an inflammatory response (Ducharme and Bickel, 2008; Young and Zechner, 2013). These macrophages will release pre-inflammatory cytokines to induce inflammation and interfere with the metabolism of carbohydrates and lipids, leading to insulin resistance in liver cells or adipocytes (Hazlehurst et al., 2016; Morrison and Kleemann, 2015). In recent years, it was determined that excessive fatty acids could be broken down by fatty acid β-oxidation to produce energy and reduce the damage to cells (Nguyen et al., 2007; Smith and Minson, 2012). Our findings suggest that ginkgolide C has the ability to enhance β-oxidation-associated enzyme expression, including CPT-1, CD36, and PPARα, while not significantly enhancing CPT-2 production. CD36 is a fatty acid translocase that can transfer free fatty acids or long chain fatty acids from the circulatory system into adipocytes, muscle cells, and liver cells (Pardina et al., 2017; Xie et al., 2017). Some studies showed that CD36 overexpression caused excessive free fatty acid uptake and hepatic steatosis, but reduced free fatty acid damage in hepatocytes, vascular epithelial cells, and adipocytes (Choi et al., 2017). Interestingly, CD36 can activate the β-oxidation break down of fatty acids in hepatocytes (Zingg et al., 2017). Previous studies found that a CD36 gene deletion blocked β-oxidation and increased lipid accumulation in hepatocytes; thus, CD36 contributed to β-oxidation expression and the decomposition of free fatty acids (Xie et al., 2017). The CPT enzyme is the most important for regulating β-oxidation, and can carry the free fatty acids from the cytoplasm into mitochondria (Pucci et al., 2016). CPT1 is located on the outer mitochondrial membrane and can convert the long-chain acyl-CoA to acylcarnitine, and assist carnitine translocase to carry acylcarnitine into the inner mitochondria for β-oxidation.
lipogenesis. Ginkgolide C also promoted lipolysis and Our result demonstrated that ginkgolide C significantly increased insulin resistance by promoting AMPK activity (Wu et al., 2016). trol also regulated the oral glucose tolerance test and improved ing to decreased synthesis of the fatty acid chain (Smith et al., 2010). When cells took up decreased free fatty acids, inflammation, and insulin resistance in hepatocytes. Recent studies found that sirt1/AMPK activation could regulate the intracellular energy flow (Lim et al., 2010). When cells took up more energy, they increased AMPK activity to reduce lipid synthesis in liver cells or adipocytes (Forbes-Hernandez et al., 2017). AMPK phosphorylation also induced phosphorylation of ACC leading to decreased synthesis of the fatty acid chain (Smith et al., 2016). Resveratrol is a sirt1 inducer, and some studies showed that it could reduce lipid accumulation and improve NAFLD via the activated sirt1/AMPK pathway in HFD-induced obese mice (Elgebaly et al., 2017; Lasa et al., 2012). In a diabetic mouse model, resvera trol also regulated the oral glucose tolerance test and improved insulin resistance by promoting AMPK activity (Wu et al., 2016). Our result demonstrated that ginkgolide C significantly increased sirt1 expression and AMPK phosphorylation. Ginkgolide C also promoted ACC phosphorylation to block the synthesis of the fatty acid chain in oleic acid–induced hepatocytes. In conclusion, ginkgolide C can inhibit hepatic accumulation by blocking transcription factors regulating lipid synthesis and FAS for lipogenesis. Ginkgolide C also promoted lipolysis and β-oxidation in hepatocytes, and increased sirt1/AMPK activity to suppress synthesis of the long fatty acid chain. Therefore, ginkgolide C has potential for improving hepatic steatosis.

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Conflict of interest statement

The authors have declared no conflict of interest.

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