Biomedicine & Pharmacotherapy 88 (2017) 625–634

Available online at
ScienceDirect
www.sciencedirect.com

Elsevier Masson France
EM|consulte
www.em-consulte.com/en

Ginkgolide A ameliorates non-alcoholic fatty liver diseases on high fat diet mice

Hyeon-Soo Jeong[a,b,1], Kang-Hoon Kim[a,b,1], In-Seung Lee[a,b], Ji Young Park[a,b], Yumi Kim[a,b], Ki-Suk Kim[a], Hyeung-Jin Jang[a,b,*]

[a] Department of Biochemistry, Graduate School, Kyung Hee University, Heogi-dong, Dongdaemun-gu, Seoul 02447, Republic of Korea
[b] Department of Science in Korean Medicine, Graduate School, Kyung Hee University, Republic of Korea

ARTICLE INFO

Article history:
Received 6 December 2016
Received in revised form 17 January 2017
Accepted 18 January 2017

Keywords:
Ginkgolide A
NAFLD
Lipogenesis
Inflammation
Therapeutic agent

ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is one of the most common diseases worldwide and has continuously increased. NAFLD refers to a spectrum of diseases ranging from fatty liver to steatohepatitis, cirrhosis, and even to hepatocyte carcinoma. Excessive fatty acid enters the cell and the mitochondria undergo stress and unremoved ROS can trigger a form of cell apoptosis known as 'lipoapoptosis'. NASH arises from damaged liver hepatocytes due to lipotoxicity. NASH not only involves lipid accumulation and apoptosis but also inflammation. *Ginkgo biloba* has been tested clinical trials as a traditional medicine for asthma, bronchitis and cardiovascular disease. The effects of Ginkgolide A (GA), derived from the ginkgo biloba leaf, are still unknown in NAFLD. To determine the protective effects of GA in NAFLD, we examined the fatty liver disease condition in the non-esterified fatty acid (NEFA)-induced HepG2 cell line and in a high fat diet mouse model. The findings of this study suggest that GA is non-toxic at high concentrations in hepatocytes. Moreover, GA was found to inhibit cellular lipogenesis and lipid accumulation by causing mitochondrial oxidative stress. GA showed hepatoprotective efficacy by inducing cellular lipoapoptosis and by inhibiting cellular inflammation. The results demonstrated that GA may be feasible as a therapeutic agent for NAFLD patients.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is one of the most common diseases worldwide that has been continuously increasing [1]. As the development of civilization, a change of diet results in decreased physical activity and increased fat intake [2]. NAFLD is wide spectrum that includes fatty liver, steatohepatitis, cirrhosis, and hepatocyte carcinoma. Initial phase of non-alcoholic fatty liver (NAFL) disease is hard to notice due to its painless symptoms. NAFL is potentially develop to non-alcoholic steatohepatitis (NASH) or liver fibrosis. Among NASH or liver fibrosis cause to liver failure or hepatocellular carcinoma [3]. The histologic features of NASH reported lipid accumulation in liver, along with fibrosis, apoptosis, inflammation, and steatosis. The pathological states of NASH increased inflammation and apoptosis relate factors in liver tissue. And, the increased levels of aspartate transaminase (AST) and alanine transaminase (ALT) concentration in blood are observed in patients with NASH [4]. When non-esterified fatty acid (NEFA) enters hepatocyte, hepatocyte start to not only oxidize fatty acid in mitochondria (β-oxidation) but also synthesize triglyceride (TG) for using fatty acid and storage TG. However, an influx of excessive and continuous fatty acid into cell causes mitochondria is getting stress and secretes reactive oxygen species (ROS). ROS acts as the trigger that regulates cell apoptosis called 'lipoapoptosis'. Jun-*n*-terminal kinase (JNK) and caspases are also well-known cell apoptosis trigger [5]. Mitochondria oxidizes fatty acid to acetyl coenzyme A (acetyl coA). Oxidized acetyl coA is used in TCA cycle or secreted to cell plasma. Acetyl coA (ACC), a biotin-dependent enzyme, converts acetyl coA to malonyl coA via its two catalytic activities: biotin carboxylase (BC) and carboxyltransferase (CT). Fatty acid synthase (FASN) catalyzes the synthesis of palmitate from acetyl coA and malonyl coA in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) [6]. Synthesized palmitates change into TG via glycerol bond. Combined TG is stored in cell vacuole [7].

* Corresponding author at: Department of Biochemistry, Graduate School, Kyung Hee University, Heogi-dong, Dongdaemun-gu, Seoul 02447, Republic of Korea.
E-mail address: hjjang@khu.ac.kr (H.-J. Jang).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.biopha.2017.01.114
0753-3322/

To treat NAFLD associated with steatosis linked to obesity, insulin resistance, and dyslipidemia, the treatments of NAFLD have approached pharmacologic therapies such as Thiazolidinediones, Pioglitazone, Rosiglitazone, Metformin, Statins, and Fibric acid derivatives. Because drugs using pharmacologic therapies have effects such as reduction in hepatic fibrosis by increasing in insulin sensitivity (Thiazolidinediones), improvement of ALT level and insulin sensitivity in clinical study of steatosis (Pioglitazone, Rosiglitazone, and Fibric acid derivatives), stimulation of β-oxidation by suppressing lipogenic enzymes (Metformin), and reduction of fat content (Statins) [8]. However, these drugs have reported side effects from pharmacologic therapies of NAFLD such as risk of cardiovascular disease, edema, nausea, and selenium deficiency [9–12].

Herbal medicines have been used in clinical patients without side effects as a traditional medicine in Eastern culture[13]. Because herbal medicines have pharmacologic effects of ingredients as a complementary and alternative medications and roles of nutritional supplements. Usage of herbal medicines prevailed worldwide because they are considered to be natural resources and guarantee safety of medicinal reactions [14]. It is approximately calculated that up to 42% of Americans consume in complementary and alternative medications annually, while one-third of patients estimated in US liver clinics report the use of herbal medicines or extracts[15].

Ginkgo biloba has been used as an herbal medicine for treatment of asthma, bronchitis and cardiovascular disease [16,17]. Previous study of ginkgolide A (GA), derived from ginkgo biloba leaf, suggested that GA was an attenuative effects on coronary artery endothelial dysfunction, an induction of cyp3a on hepatocytes, and protection against liver inflammation [17–19] However, the effects of GA on NAFLD are unknown. In this study, we studied anti-steatosis effects of GA on NEFA-induced NAFLD HepG2 cells and high fat diet fed mice. Because, HepG2 hepatocyte cell line has been used broadly as in vitro liver cell model at fatty liver and liver cancer [20]. To determine anti-steatosis effects of GA on steatosis conditioned HepG2 cells, western blotting analysis, Oil red O and Nile red staining were performed whether GA decreases apoptosis or inflammation-related proteins expression, and lipid accumulation respectively. Moreover, defining anti-steatosis effects of GA in high fat diet-induced fatty liver mice model, liver weight, liver TG, and plasma AST and ALT were measured. In conclusion, this study demonstrated that GA had possibility of used as NAFLD treatment. GA was non-toxicity at high concentration in hepatocytes. As the result of this study, we suggested that GA had effects of inhibit liver cell lipogenesis, lipid accumulation, and reduction of mitochondria oxidative stress through in vitro and in vivo study.

2. Material and methods

2.1. Chemicals

GA and Neutral buffered formalin (NBF) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Gaithersburg, MD, USA). Phosphate buffer saline (PBS) was purchased from Corning (Corning, NY, USA). Bovine serum albumin (BSA) was purchased from Affymetrix (Cleveland, OH, USA). Oil red O, oleic and palmitic acids, Nile red, and the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) were purchased from Invitrogen (Eugene, OR, USA). B-cell lymphoma-2 (Bcl-2) and Bcl-2 associated X protein (Bax) primary antibodies and liver TG quantification kit were purchased from Abcam (Cambridge, UK). JNK, *p*-JNK, caspase-9, cleaved caspase-9, caspase-3, and cleaved caspase-3 primary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Beta-actin and FASN primary antibodies and anti-rabbit, anti-mouse secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The enhanced chemiluminescence (ECL) solution (LuminataTM crescendo western horseradish peroxidase, HRP substrate) was purchased from Merck Millipore (Jeffrey, NH, USA). Bradford protein assay reagent was purchased from Bio-Rad (Hercules, CA, USA).

2.1.1. Cell culture

Hepatocyte HepG2 cells were purchased from Korea Cell line Bank (Seoul, Korea). HepG2 cells were incubated with DMEM containing 10% FBS and 1% anti-biotic and anti-mycotic (ABAM) in 37 °C, 5% CO_2 condition [21]. Before the experiment, fully grown cells were seeded cell culture plate then incubated for 8 h. After incubation, media was changed into low-glucose DMEM containing only 1% ABAM for 16 h for serum starvation [22].

2.1.2. Cell viability assay

HepG2 cells were seeded 1×10^4 cells/well in 96-well cell culture plate then incubated for 8 h after incubate, media was replaced to DMEM that contained different concentration (0, 10, 50, 100, or 500 μM) of GA for 24 h. After GA treatment, media was discarded and cells were incubated with MTT solution (0.5 mg/mL dissolved in PBS) for 1 h. After incubation, MTT solution in each well was removed and dimethyl sulfoxide (DMSO) was added. The plate was shaking incubated for 5 min at room temperature for dissolving precipitated formazan [23]. When formazan was completely dissolved, measured absorbance of 570 nm with a Bio-Rad model 680 microplate reader (Bio-Rad, Hercules, CA) to determine cell viability [24].

2.1.3. Oil red O staining

HepG2 cells were seeded 1×10^6 cells/well in 6-well cell culture plate then incubated for 8 h. After incubation, cells were treated with 1 mM NEFA (dissolved in DMEM with 1% BSA)for 24 h, then GA (10, 50 and 100 μM) were treated for 24 h. Incubated cells were washed with ice-cold PBS and fixed with ice-cold 10% NBF at least 1 h. After then, washed with PBS and 60% isopropanol sequentially. Then stained with 0.3% Oil red O solution (in 60% isopropanol) for 1 h. Stained cell were washed with running tap water for 1 min then stained with Harris's hematoxylin for 2 min. After staining, destained with 1% acid alcohol (HCl in 70% ethanol), blued with 0.3% ammonia water. After then, washed with PBS and observed and take photograph with Olympus IX71 microscope (Olympus, Tokyo, Japan).

2.1.4. Nile red staining

HepG2 cells were seeded 5×10^5 cells/well in 12-well cell culture plate then incubated for 8 h. Cells were pretreated 1 mM NEFA (dissolved in DMEM with 1% BSA) for 24 h, then GA (10, 50 and 100 μM) were treated for 24 h. Cells were washed with ice-cold PBS, fixed with 10% NBF for 1 h. After then, cells were stained with 0.3 μM nile red solution (in PBS) for 30 min protected from light. After staining, cells were washed with PBS then collected into flow cytometry sample tube using cell scraper. Collected cells were well suspended and measured fluorescence using Facscalibur flow cytometer (excitation: 488 nm and emission: 585 nm).

2.1.5. Animals

Four weeks old C57BL/6 male mice were purchased from Daehan-Biolink (DBL, Chungcheongbuk-do, Korea). Mice were housed in animal room that maintaining room temperature (21–23 °C) and humidity (55–60%). All mice were offered food and water *ad libitum*. After 2 weeks adaptation period, high fat chow (60% fat calorie) was offered for 7 weeks except normal chow