Abstract: Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disorder. Glaucocalyxin A (GLA) is a diterpenoid compound that possesses various activities. In the study, we explored the effect of GLA on high fat diet (HFD)-induced fatty liver and fibrosis. We showed that GLA significantly inhibited hepatic steatosis and decreased the hepatic level of triglyceride and total cholesterol and serum level of alanine aminotransferase and aspartate aminotransferase in HFD-fed mice. GLA significantly inhibited the increase of hydroxyproline, mRNA expression of α-smooth muscle actin (α-SMA) and transforming growth factor-beta 1 (TGFβ1) induced by HFD in vivo and PA in vitro. Moreover, GLA decreased the level of proinflammatory cytokines in livers of HFD-fed mice and PA-treated hepatocytes. Decrease of sirtuin 1 (SIRT1) expression induced by HFD and PA was inhibited by GLA. Furthermore, knockdown of SIRT1 suppressed the inhibitory effect of GLA on lipid accumulation, inflammation and fibrosis-biomarkers. The data in our study showed that GLA protected against fatty liver, inflammation and fibrosis. Upregulation of SIRT1 was responsible for GLA-induced inhibition of lipid accumulation, inflammation and fibrogenesis. Overall, our study demonstrated that GLA may be a promising choice for the therapy of fatty liver and fibrosis.

Keywords: Glaucocalyxin A, fatty liver, liver injury, sirtuin 1

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disorder in Western countries [1], which has been rapidly increasing in the last decades [2, 3]. At the earliest stages, it is only manifested with steatosis, a form of fatty infiltration of the liver. Some individuals of NAFLD develop nonalcoholic steatohepatitis (NASH) characterized by inflammatory foci and ballooning degeneration of hepatocytes [4]. Ensuing cycles of hepatocellular injury and regeneration lead to fibrosis, cirrhosis and, eventually the development of primary liver cancer [5-7]. Dietary caloric restriction is usually suggested for NAFLD prevention. However, its efficacy is hampered by poor compliance and gut microbial alterations [8]. Thus, pharmaceutical interventions are important for the prevention of NAFLD and subsequent outcomes.

Glaucocalyxin A (GLA), a diterpenoid compound derived from Rabdosia japonica (Burm. f.) var. glaucocalyx (Maxim.) Hara, has been shown to exhibit therapeutic effects in cancer, inflammation and thrombotic disorders [9]. GLA inhibits the growth of liver cancer cells by inducing G2/M stage cell-cycle arrest and cell apoptosis [10]. In human HL-60 leukemia cells, GLA was shown to induce apoptosis through ROS-dependent mitochondrial dysfunction pathway [11]. In human breast cancer cells, GLA was found to inhibit the proliferation through the JNK pathway [12]. Liu et al. showed that GLA ameliorated myocardial ischemia-reperfusion injury in mice by suppression of microvascular thrombosis [13]. Li et al. reported that GLA inhibited platelet p-selectin secretion and integrin activation through GPVI signaling pathway [14]. Inhibition of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway and cellular immune system was involved in the anti-inflammatory activity of GLA [15].

In the study, we investigated the effect of GLA on high fat diet (HFD)-induced fatty liver and fibrosis through regulation of sirtuin 1.

Jia Yuan1,2*, Shuixiang He1*, Liangshan Lv1, Weizhi Li1, Peijie Li1, Hui Xue1

1Department of Gastroenterology, The First Affiliated Hospital of Xi’an Jiaotong University, Xi’an, Shaanxi, China; 2School of Medicine, Xi’an Jiaotong University, Xi’an, Shaanxi, China. *Equal contributors.

Received December 23, 2016; Accepted April 28, 2017; Epub October 15, 2017; Published October 30, 2017
fibrosis in mice and examined the possible molecular mechanisms in palmitic acid (PA)-treated hepatocytes. We showed that GLA protected against hepatic lipid accumulation and fibrosis in vivo and in vitro. Upregulation of Siru1 (SIRT1) was involved in the protective effects of GLA.

Materials and methods

Animals and treatment

C57 mice (6-8 weeks old) were purchased from Animal Center of Xi’an Jiaotong University. Animal treatment was approved by Animal Care and Use Committee of the First Affiliated Hospital of Xi’an Jiaotong University and abided the guidelines for the care and use of laboratory animals published by the National Institute of Health. Mice were housed in a room with controlled temperature (22 ± 2°C), humidity (40-60%) and light cycle (12/12 h light/dark).

Forty mice were randomly divided into four groups: Control: normal diet (10% of total calories from fat); HFD: high fat diet (45% of total calories from fat); 10 mg/kg group: HFD plus 10 mg/kg GLA injection; 20 mg/kg group: HFD plus 20 mg/kg GLA injection. Mice were fed chow or HFD diet for 4 months. In the last month, mice in GLA group were given 10 mg/kg/day or 20 mg/kg/day GLA injection intraperitoneally. GLA was prepared as described previously [14]. Mice in control and HFD group were intraperitoneally injected with equal amount of vehicle (10% DMSO in phosphate buffer saline (PBS)). After the treatment, blood and liver tissues were obtained. Part of liver tissues were fixed in 10% formalin and embedded in paraffin. Paraffin-embedded sections (4 μm) were stained with hematoxylin and eosin.

Measurement of hepatic triglycerides (TG), total cholesterol (TC), and hydroxyproline, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST)

TG and TC content in liver homogenates were measured using assay kits obtained from Nanjing Jiancheng Company (Nanjing, China). Hydroxyproline content was detected using a Hydroxyproline Colorimetric Assay Kit (BioVision, Milpitas, CA, USA). Serum levels of ALT and AST were measured using commercial kits (Nanjing Jiancheng Company, Nanjing, China) according to the manufacture’s protocols. Serum levels of TNFα, IL-1β, IL-6, and MCP-1 were determined using commercial ELISA kits (Cayman Chemical, USA) according to the manufacturer’s instructions.

Cell culture and treatment

Mouse normal liver cell line AML-12 was obtained from Cell Bank of the Chinese Academy of Medical Sciences. Cells were cultured in DME/F-12 containing 4500 mg/L glucose, 100 mg/L penicillin, 100 mg/L streptomycin, and 2 mM L-glutamine and supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO2 incubator. Cells were treated by 200 μM PA in the presence or absence of 10 or 20 μM GLA for 24 h. 20 mM GLA was dissolved in DMSO as stock solution. PA was dissolved at 70°C in a solution of 0.1 M NaOH and 70% ethanol to produce PA stock solution (50 mM). The final concentration of PA was 200 μM.

Cell transfection

AML-12 cells were transfected with lentivirus (LV) vector or LV-shSIRT1 (Santa Cruz Biotechnology, CA, USA). AML-12 cells were infected with LV to generate cell lines with stable decreased expression of SIRT1. AML-12 cells were incubated with infection medium containing LV vectors at a multiplicity of infection of 20 for 16 hours, and then the medium was replaced with fresh complete medium. Empty vector was used as a negative control. The transfected AML-12 cells were cultured under puromycin condition and verified by Real-time PCR and western blot assay.

RNA isolation and Real-time PCR

Liver tissues and cells were lysed using Trizol reagent (Life Technologies, Carlsbad, CA, USA) and total RNA was extracted. The concentration of RNA was measured. The reverse transcription reaction was conducted using 1 μg RNA in a final volume of 20 μL by a PrimeScript RT Reagent Kit (Takara, Dalian, China). 1 μl cDNA was used for the performance of real-time PCR with SYBR Green PCR kit (Thermo Scientific, IL, USA). The PCR reaction conditions were as follows: initial denaturation at 95°C for 10 min followed by 30 cycles at 95°C for 1 min, annealing at 53°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 5 min.
Glaucocalyxin A inhibits steatosis and fibrosis

The PCR was conducted using a CFX96 real-time PCR system (BioRad, USA). The expression of target gene transcripts was related to internal control β-actin. Results were expressed as folds of control. Primers were shown in Table 1.

Western blot

Liver tissues and cells were lysed with ice-cold RIPA buffer (TIANGEN, China) and then centrifuged at 13,200 rpm for 1 hr at 4°C to isolate total protein. Protein content was measured using BCA method (Thermo Scientific, IL, USA). Equal volume of protein extraction and loading buffer were mixed. Samples equal to 20 μg protein was separated on a 10% SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane. The membrane was then blocked with 8% non-fat milk in TBS for 1 h at room temperature, and then incubated with primary antibodies (β-actin: Santa, 1:500; SIRT1: CST, 1:1000) overnight at 4°C. The blots were washed with TBST buffer for four times, and then incubated with horseradish peroxidase-linked IgG secondary antibodies (Thermo Scientific, IL, USA) for 30 min at 37°C. The bands were visualized using enhanced chemiluminescence (Thermo Scientific, IL, USA). Finally, bands were captured with an image analysis system (BioRad) and quantified with Quantity One software (BioRad).

Measurement of lipid accumulation

Lipid accumulation in cells was measured by Bodipy staining. After the treatment, cells were fixed in 4% paraformaldehyde and stained with 1 μg/ml Bodipy (3922, Life Technologies, Carlsbad, CA, USA). Fluorescence was observed using a confocal microscopy (Olympus, Japan).

Table 1. Primers used in the study

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>5'-AGGCCAACCGTGAAAGATG-3'</td>
<td>5'-TGGCGGTAGGGAGAGCAT-3'</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5'-GTCAGAGCAAAGTGGAG-3'</td>
<td>5'-CCAGCCCATACTT4AGA-3'</td>
</tr>
<tr>
<td>Collagen-1</td>
<td>5'-CAGCTGTTGATGAGGTT-3'</td>
<td>5'-GACCCGGGGACACCTT4A-3'</td>
</tr>
<tr>
<td>TNFα</td>
<td>5'-TCAACCTCCTCTGCGG-3'</td>
<td>5'-CCTAGACCGCTGCCG-3'</td>
</tr>
<tr>
<td>α-SMA</td>
<td>5'-GGCCATCCAGAACCACCTA-3'</td>
<td>5'-TGGAAGTACACAGGAAAG-3'</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>5'-CTCCGTGCTTTGATCGC-3'</td>
<td>5'-GCTTTATGTGGGACAGATTG-3'</td>
</tr>
<tr>
<td>IL-6</td>
<td>5'-TCACATGCTCTCTTAAC-3'</td>
<td>5'-TGACACACAGTGAGAAATG-3'</td>
</tr>
<tr>
<td>MCP-1</td>
<td>5'-GTGGAAAATCAACGGGATCAG-3'</td>
<td>5'-ACTTCCACCCAGGTCTTC-3'</td>
</tr>
</tbody>
</table>

Statistical analysis

Data were expressed as mean ± S.E.M. The level of statistical significance was determined by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison tests using the GraphPad Prism-6, Graphpad software Inc, (La Jolla, California, USA). Statistical differences were considered significant at ‘p’ value less than 0.05.

Results

GLA reduces hepatic fatty liver in mice and lipid accumulation in hepatocytes

Mice were fed HFD to induce fatty liver and metabolic disorder. The administration of GLA significantly reduced hepatic steatosis induced by HFD, which was in a dose-dependent manner (Figure 1A). Hepatic contents of TG and TC were detected and the results showed that GLA markedly inhibited the increase of TG and TC contents in livers of HFD-fed mice (Figure 1B and 1C). Moreover, HFD-induced increase of ALT and AST levels were inhibited by GLA (Figure 1D and 1E). Furthermore, we evaluated the effect of GLA on lipid accumulation in AML-12 hepatocytes in vitro. As shown in Figure 1F, PA-induced increase of Bodipy staining was significantly inhibited by GLA. Together, these data suggested that GLA protected against hepatic steatosis and liver injury in mice and reduced lipid accumulation in hepatocytes.

GLA reduces fibrosis in livers of mice and hepatocytes

In HFD-fed mice, ensuing cycles of hepatocellular injury and regeneration occur which can result in fibrosis. In the study, we examined the effect of GLA on fibrosis in livers and hepatocytes. We showed that GLA significantly inhibited the increase of hydroxyproline levels in livers of mice fed with HFD (Figure 2A). Moreover, GLA markedly reduced the mRNA level of collagen-1 (Figure 2B and 2E), α-smooth muscle actin (α-SMA) (Figure 2C and 2F) and transforming growth factor-beta 1 (TGFβ1) (Figure 2D and 2G) in livers of HFD-fed mice (Figure...
Glaucocalyxin A inhibits steatosis and fibrosis

Figure 1. GLA reduces hepatic fatty liver in mice and lipid accumulation in hepatocytes. Mice were fed with HFD and administered with 10 mg/kg or 20 mg/kg GLA. A: HE staining of livers in mice. B and C: Hepatic levels of TG and TC. D and E: Serum levels of AST and ALT. AML-12 hepatocytes were treated by 200 μM PA for 24 h with or without 10 or 20 μM GLA. F: Bodipy staining of hepatocytes. #P<0.05, vs. control. ##P<0.05, vs. HFD.

GLA inhibits inflammation in mice and hepatocytes

The effect of GLA on inflammation in mice and hepatocytes was examined by the determination of several proinflammatory cytokines. We showed that serum levels of tumor necrosis factor α (TNFα) (Figure 3A), interleukin-1β (IL-1β) (Figure 3B), IL-6 (Figure 3C), and monocyte chemoattractant protein (MCP-1) (Figure 3D) in HFD-fed mice were significantly reduced by GLA administration. In hepatocytes, PA-induced increase of mRNA levels of TNFα (Figure 3E), IL-1β (Figure 3F), IL-6 (Figure 3G), and MCP-1 (Figure 3H) was markedly inhibited by GLA treatment. The results suggested that GLA exhibited potent anti-inflammatory activities in
Glaucocalyxin A inhibits steatosis and fibrosis

HFD-fed mice and PA-treated hepatocytes, which may be involved in the amelioration of hepatic steatosis and fibrosis.

Upregulation of SIRT1 is involved in protective effects of GLA

To examine the molecular mechanism of the protective effects of GLA against lipid accumulation-associated fibrosis and inflammation, we tested the expression of SIRT1. The protein expression of SIRT1 in livers of HFD-fed mice and PA-treated hepatocytes was significantly reduced (Figure 4A and 4B). In addition, this reduction of SIRT1 expression was significantly reversed by GLA (Figure 4A and 4B). To further test whether SIRT1 mediated the protective effect of GLA, we transfected the cells with LV-shSIRT1 or LV-Ctrl. SIRT1 expression was efficiently silenced by LV-mediated knockdown (Figure 4C). We found that down-regulation of SIRT1 markedly reversed the inhibitory effect of GLA on Bodipy staining in PA-treated hepatocytes, indicating that GLA-resulted decrease of lipid droplet formation was mediated by SIRT1 (Figure 4D). Moreover, in PA-treated hepatocytes, GLA-induced decrease of the mRNA level of collagen-1 (Figure 4E), α-SMA (Figure 4F) and TGFβ1 (Figure 4H) was reversed by knockdown of SIRT1. Furthermore, knockdown of SIRT1 suppressed GLA-induced decrease of mRNA expression of TNFα (Figure 4H), IL-1β (Figure 4I), and MCP-1 (Figure 4J) in PA-treated hepatocytes. The results indicated that SIRT1 mediated the protective effects of GLA against inflammation and fibrosis.

Discussion

Previous literature reported that GLA exhibits anti-inflammatory activities in nervous system and lung tissue [15, 16]. GLA functions as an anti-fibrotic agent in bleomycin-induced pulmonary fibrosis in mice [16]. Whether GLA plays a
Glaucocalyxin A inhibits steatosis and fibrosis

Figure 3. GLA inhibits inflammation in mice and hepatocytes. Mice were fed with HFD and administered with 10 mg/kg or 20 mg/kg GLA. (A-D) Serum levels of collagen-1 TNFα (A), IL-1β (B), IL-6 (C), and MCP-1 (D). AML-12 hepatocytes were treated by 200 μM PA for 24 h with or without 10 or 20 μM GLA. (E-H) Serum levels of collagen-1 TNFα (E), IL-1β (F), IL-6 (G), and MCP-1 (H). 

protective role against hepatic lipid accumulation-associated inflammation and fibrosis is not known.

NAFLD can progress to NASH, fibrosis, cirrhosis and eventually the development of hepatic carcinomas. The stage from hepatic steatosis to fibrosis is important for the clinical intervention. NAFLD is characterized by disorder of lipid metabolism that plays a fundamental role in the development of a series of detrimental events [17, 18]. In the study, we found that GLA could attenuate liver injury and reduce lipid accumulation in liver and hepatocytes.

Liver fibrosis is a complicated process that is regulated by cell-cell interactions and a battery of key regulators [19]. Collagen-1 and α-SMA are commonly used as markers of liver fibrogenesis [20, 21]. The disturbance of TGFβ signaling pathway plays an important role in fibrosis as well as in liver and gastrointestinal cancers [22]. Activated TGFβ activates Smads, specifically Smad2 and Smad3, which then form a complex with Smad4 and translocate into the nucleus. In combination with various transcriptional co-activators and co-repressors, the activated Smad complexes regulate a multitude of target genes, which could result in a series of outcomes, including connective tissue deposition, immune suppression, cell cycle arrest in G1/S phase, induction of apoptosis, as well as tumorigenesis [23, 24]. Collagen and α-SMA have been found to be regulated by TGFβ [25]. In our study, we showed that GLA inhibited the expression of collagen-1, α-SMA and TGFβ1 both in vivo and in vitro. The finding suggested that GLA inhibited the development from fatty liver to hepatic fibrosis.

SIRT1 is a NAD+-dependent class III histone deacetylase that plays important roles in vari-
Glaucocalyxin A inhibits steatosis and fibrosis

Inhibition of SIRT1 is involved in the development of fatty liver and dyslipidemia [27]. SIRT1 plays a role in the regulation of hepatocellular proliferation, circadian rhythms, and lipid metabolism during liver regeneration in mice [28]. SIRT1 has been shown to deacetylate Smad3 that contributes to kidney fibrosis [29]. Considering the important role of SIRT1 in the regulation of fibrosis, we evaluated the possible role of SIRT1 in GLA-induced protective effects. As expected, knockdown of SIRT1 suppressed the inhibitory effect of GLA on lipid accumulation, inflammation and fibrosis. Moreover, the effects of GLA on collagen-1, α-SMA and TGFβ expression were inhibited by SIRT1 knockdown, indicating a role of SIRT1 in the regulation of upstream of TGFβ signal. Since GLA-induced decrease of lipid accumulation was suppressed by SIRT1, it was indicated that SIRT1 mediated the inhibitory effect of GLA on hepatic steatosis, which is the initial stage of liver injury induced by HFD.

In conclusion, the data in our study showed that GLA protected against fatty liver, inflamm-
Glaucocalyxin A inhibits steatosis and fibrosis. Upregulation of SIRT1 was responsible for GLA-induced inhibition of lipid accumulation, inflammation and fibrogenesis. Overall, our study demonstrated that GLA may be a promising choice for the therapy of fatty liver and fibrosis.

Disclosure of conflict of interest

None.

Address correspondence to: Hui Xue, Department of Gastroenterology, The First Affiliated Hospital of Xi’an Jiaotong University, Xi’an 710061, Shaanxi, China. Tel: 029-85323925; E-mail: xuehui1321@163.com

References

Glaucocalyxin A inhibits steatosis and fibrosis


