Artemisinin Reduces Lipid Accumulation in Hepatocytes by Inhibition of CD36 Expression

Jian Wang, Song Wang, Xiaonan Zhang, Linlin Hu, Ping Shi*

State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, P.R. CHINA.

ABSTRACT
Background: Artemisinin, a kind of sesquiterpene lactone endoperoxide, is isolated from Artemisia annua, has been used to effectively treat different forms of malaria. Recently, some researchers showed that artemisinin decreased serum triacylglycerol in rats. However, the molecular mechanism remains unclear. Aim: In this study, we investigated the effect of artemisinin on lipid accumulation in an in vitro model of NAFLD. Methods: The cytotoxicity of OA and artemisinin on SMMC-7721 cells was measured by using the WST-8 based Colorimetric Assay Cell Counting kit8. To establish an in vitro cellular model of NAFLD, the human hepatocarcinoma SMMC-7721 cells were treated with OA. Lipid and TAG contents were measured by Oil Red O staining and enzymatic assay. Effects of artemisinin on expression of lipogenic and lipolytic genes in SMMC-7721 cells were analyzed by RT-PCR, real time PCR and Western Blotting. Results: The in vitro model of NAFLD was constructed in SMMC-7721 cells with the addition of 1.0 mM OA. Artemisinin reduced intracellular lipid accumulation and TAG concentration in SMMC-7721 cells induced by OA. The addition of artemisinin induced a decrease in the mRNA and protein level of CD36, while it had no effect on both mRNA and protein level of PPARα and SREBP1. Conclusion: Artemisinin can reduce hepatocellular lipid accumulation and triacylglycerol content via the inhibition of CD36 expression.

Key words: Artemisinin, CD36, Hepatic lipid accumulation, NAFLD, SMMC-7721 Cells.

INTRODUCTION
With the increasing numbers of adults and children who are obese or overweight, non-alcoholic fatty liver disease (NAFLD) is a highly prevalent chronic liver disease.1,2 It encompasses a wide spectrum of liver disorders and damages characterized by hepatic steatosis without significant alcohol consumption, including simple steatosis, inflammatory non-alcoholic steatohepatitis (NASH), liver cirrhosis and hepatocellular carcinoma.3,4 So far, the validated therapy of NAFLD remains uncertain.

Traditional Chinese medicines have long been used to treat NAFLD with few adverse effects.5 Artemisinin, or qinghaosu, a sesquiterpene lactone endoperoxide, is isolated from Artemisia annua. (sweet or annual wormwood) that has been used as a herbal remedy for fever and malaria.6,7 It is exciting that Tu Youyou was awarded half of the 2015 Nobel Prize in Medicine because of her discovery concerning a novel therapy of artemisinin against malaria. Recently, artemisinin and its derivatives have been shown to induce growth arrest and apoptosis,8-10 as well as inhibit angiogenesis by down-regulation of the vascular endothelial growth factor and its cellular receptor KDR/flk-1.11,12 Furthermore, artemisinin inhibits rat cardiac hypertrophy,13 prevents in vitro and in vivo invasion and metastasis of human hepatocellular carcinoma cells14 and has function of anti-inflammatory and immunomodulatory on contact hypersensitivity.15 Aniefiok et al. (2010) found that artemisinin increased total serum cholesterol level, decreased serum triacylglycerol (TAG) level and reversed the ratio of Low Density Lipoprotein to High Density Lipoprotein from...
1:2 to 2:1 in rats. However, its molecular mechanism involved in lipid metabolism is unclear. Although the pathogenesis of NAFLD is not completely understood, hepatic lipid metabolic disorders are likely to play important roles in its initiation and development. Previous studies have shown that CD36, PPARα, and SREBP1 are important molecules in regulating fatty acid transport, lipolysis, and lipogenesis, respectively. They play important roles in hepatic and myocardial accumulation of triglyceride. Additionally, in vitro models of NAFLD have been constructed and used to study the hepatic lipid metabolic disorders in hepatic cells and screen drug candidates for suitability for the treatment of NAFLD. These models include hepatocyte cell lines and primary hepatocytes treated in culture with monounsaturated and saturated fatty acids, which simulate the key characteristics of NAFLD in human liver. Therefore, in order to understand the probable molecular mechanism of artemisinin involved in hepatic lipid metabolism, it is essential to investigate the effect of artemisinin on CD36, PPARα, and SREBP1 in the in vitro model.

In the present study, we explored the alleviation of lipid accumulation effect of artemisinin on an in vitro model of NAFLD. Our results demonstrated that artemisinin attenuated lipid accumulation via the downregulation of CD36, PPARα, and SREBP1. It is expected that artemisinin may be a therapeutic candidate of the treatment of NAFLD in the near future.

MATERIALS AND METHODS

Antibodies and reagents

CD36, PPARα and SREBP1 rabbit polyclonal antibody were purchased from Santa Cruz Biotechnology Inc. (Texas, USA). β-actin rabbit monoclonal antibody was purchased from Cell Signaling Technology Inc. (MA, USA). The secondary antibody HRP-labeled Goat Anti-Rabbit IgG (H+L) was purchased from Novo protein Scientific Inc. (Shanghai, China). Reagents used in SDS-polyacrylamide gel electrophoresis were purchased from Shenghong Company (Shanghai, China). Other reagents used in our study were purchased from Solarbio life science, Beijing, China. Other reagents used in our study were purchased from Solarbio life science, Beijing, China. Artemisinin was dissolved in dimethylsulfoxide (DMSO) to a concentration of 200 μM (final concentration of DMSO was less than 0.1% by volume) and was added at the indicated concentrations in SMMC-7721 cells.

Cell viability assay

Cell viability was evaluated by Cell Counting Kit-8 (Dojindo, Tokyo, Japan) according to the manufacturer's instruction. Cells were plated in 96-well micro-well plate in 0.1 ml volume of RPMI-1640 medium with 10% FBS, at a density of 0.1×10⁴ cells/well. Absorbance was measured at 450 nm with an iMark Microplate reader (Bio-Rad, California, USA).

Oil Red O staining

The SMMC-7721 cells at 0.5×10⁵ cells/well were seeded in 48-well plates and pre-incubated with RPMI 1640 containing 10% of FBS for 12 h. The cells were then exposed to different concentration of artemisinin for 12 h and then treated with 0.5 mM or 1.0 mM oleic acid (OA) for 24 h or 48 h, fixed with 4% paraformaldehyde and stained with a freshly prepared working solution of Oil Red O (three parts of 1% stock in isopropanol and two parts double distilled H₂O) for 30 min at room temperature. The stained cells were observed under a fluorescence microscope (Olympus BX51, Tokyo, Japan). To quantify the Oil Red O content, isopropanol was added to each sample, which was shaken at room temperature for 5 min, and the optical density of each 100 μl isopropanol extracted sample was read with a spectrophotometer (Bio Tek Synergy2, Vermont, American) at 510 nm.

Measurement of cellular triglyceride concentration in SMMC-7721 cells

The concentration of triglyceride (TAG) in cells was measured with enzymatic assays from Nanjing Jiangcheng Bioengineering Institute (Nanjing, China), according to the manufacturer's instructions. Absorbance was measured with a microplate spectrophotometric system at 546 nm. The result of TAG was expressed as mmol TAG per g protein (mmol/g protein).
Reverse Transcription-Polymerase Chain Reaction and real time PCR

The expression of CD36, PPARα and SREBP1 mRNA in the cells was determined by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and real-time PCR. The cells (5×10^5 cells/well) in 6-well plates were pre-treated with artemisinin at indicated concentration for 12 h and then treated with 1.0 mM OA for 24 h. Total RNA was prepared from cells using cold Trizol (Takara, Japan) according to the manufacturer’s protocol. The cDNA was synthesized by reverse transcription with PrimeScript RT regent kits (Takara, Japan) according to the manufacturer’s protocol and then subjected to PCR and real-time PCR detection with Emerald Amp PCR Master Mix and SYBR Premix Ex Taq (Tli RNAaseH Plus) kit (Takara, Japan). The primer sequences were as follows: 5'-GAGAAGCTTGATGGGGCTAT-3' (sense), 5'-TTCAACTGGAGAGGGAAGAGCAGAAAGG-3' (antisense) for CD36, 5'-GGAGATCTAGGATGCTTAGAG-3' (sense), 5'-AGCTGGAATTACCGCGGCT-3' (antisense) for PPARα, 5'-CTGAGAGCCC GTTATC-3' (sense), 5'-GCCAAAGGCTTCCAGAATTACCTCC-3' (antisense) for SREBP1 and 5'-GCCAAAGGCTTCCAGAATTACCTCC-3' (antisense) for 18s RNA.

Western Blotting

The cell pellets were collected by centrifugation and washed two times with cold PBS, then dissolved in lysis buffer and lysis for 30 min. Insoluble materials were removed by centrifugation, the supernatant was saved as the total cell proteins. Proteins were detected using a chemiluminescence-based kit (Tian Gen Biotech Co., Ltd., China). Signal density was detected using the MF-Chemi BIS Family including gel capture software (DNR Bio-Image system, Ltd., Jerusalem, Israel) and quantitatively analyzed by densitometry using the Image J software.

Statistical Analysis

Values were expressed as means ± standard deviations. Statistical analysis was performed using the Student’s t-test. Values of p<0.05 were considered to be statistically significant.

RESULTS

Cytotoxic effect of artemisinin and OA on SMMC-7721 cells

Using the WST-8 based Colorimetric Assay Cell Counting kit 8, the cytotoxicity of OA and artemisinin on SMMC-7721 cells was measured. After treatment for 24, 48 and 72 h, 0.5 mM and 1.0 mM OA had no obvious effect on the viability of SMMC-7721 cells, however 1.5 mM and 2.0 mM OA caused 10% and 81% cell death compared with control separately after treating for 24 h (Fig 1 B). At 24 h and 48 h, 50 μM and 100 μM artemisinin had no effect on cell viability of SMMC-7721 cells. However, when treated cells with 100 μM artemisinin for 72 h, 17% cells were dead and treatment of 200 μM artemisinin caused more than 50% cells death (Figure 1 C).

Construction an in vitro model of NAFLD by OA

To establish an in vitro cellular model of NAFLD, SMMC-7721 cells were treated with different concentrations of OA. As shown in Figure 2A, 0.5 mM and 1.0 mM OA increased numbers of lipid droplets compared with control cells and 1.0 mM OA caused more lipid droplets than 0.5 mM OA. Furthermore, as shown in Figure 2B, lipid content was significantly elevated in a concentration-dependent manner. After treated for 24 h and 48 h, the lipid content in cells treated with 1.0 mM OA was 1.7 and 1.4 times than those treated with 0.5 mM OA. According to these data, we chose 1.0 mM OA to construct the in vitro model of NAFLD.

Artemisinin reduced intracellular lipid accumulation and TAG concentration in SMMC-7721 cells induced by OA

Next, 50 μM and 100 μM artemisinin was chosen to treat model cells of NAFLD. As shown in Figure 3A, there was significant difference between cells treated with or without 100 μM artemisinin. The numbers and sizes of the lipid droplets were significantly reduced after the treatment of 100 μM artemisinin with the observation of fluorescence microscope and 50 μM artemisinin had no effect. Furthermore, lipid content was significantly elevated after treated with 1.0 mM OA for 24 h, compared with the control cells (p<0.01), while 100 μM artemisinin decreased lipid content induced by 1.0 mM OA (p<0.05) in SMMC-7721 cells (Figure 3B). Compared to 0.272 mmol/g protein in the control group, the intracellular TAG content increased significantly in the OA treated cells and reached 0.596 mmol/g protein (p<0.01 versus the control), and decreased significantly after treated with 100 μM artemisinin and it was 0.378 mmol/g protein (p<0.01 versus the OA treated cells) (Figure 3C). The results showed that artemisinin reduced...
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Figure 1: Chemical structure of artemisinin (A) and the effects of OA or artemisinin on SMMC-7721 cells viability (B and C). Various concentrations of OA (0-2.0 mM) or artemisinin (0–200 μM) were used to treat human SMMC-7721 hepatoma cells for 24, 48 and 72 h. Cell viability was measured by CCK-8 assay. The experiments were repeated three times and the data were presented as means±SD for triplicate experiments.

Figure 2: Construction of an in vitro model of NAFLD. The SMMC-7721 cells were treated with OA (0-1.0 mM) for 24 h, then were stained by Oil Red O (A). Lipid concentration in cells treated with OA was measured by Oil Red O staining (B). Data are presented as means±SD for triplicate experiments.

Figure 3: Artemisinin inhibits OA induced intracellular lipid accumulation. The cells were pre-treated with artemisinin (100 μM) for 12 h, and then were treated with OA (1.0 mM) for 24 h. In the indicate time, cells were stained by Oil Red O (A). In the indicate time, the lipid content in SMMC-7721 cells was measured by Oil Red O colorimetric assay (B) and intracellular TAG content was measured by TAG kit (C). Results are expressed as means±S.D for triplicate experiments. *p<0.01 compared to the control group; ^p<0.05, §§<0.01 compared to OA group.
intracellular lipid accumulation and TAG concentration in the cellular model of NAFLD.

**Effects of artemisinin on expression of lipogenic and lipolytic genes in SMMC-7721 cells**

The mRNA levels of CD36, PPARα, SREBP1 in different treated cells were detected by RT-PCR and real-time PCR, respectively. The results were shown in Figure 4, the mRNA expression of CD36 was significantly up-regulated about 2-fold in the OA group compared with that in the control group \((p<0.01)\). However, the up-regulated effect of OA was decreased to 0.23 fold of control group by 100 μM artemisinin \((p<0.01)\). The mRNA of SREBP1 was both decreased in OA and OA with artemisinin groups compared with control group \((p<0.01)\), however artemisinin had no effect on mRNA expression of SREBP1 compared to OA group. In contrast, the mRNA expression of PPARα had no change in both OA and OA with artemisinin groups and artemisinin had no effect on it.

The proteins of CD36, PPARα, SREBP1 were detected by western blot, respectively. The results were presented in Figure 5. The level of CD36 in OA group was elevated and decreased by artemisinin, similar as the result of mRNA expression. PPARα and SREBP1 protein levels were different from their mRNA expressions, which were up-regulated by OA while artemisinin did not change their high levels.

**DISCUSSION**

According to our previous studies, OA was chosen to treat SMMC-7721 cells to establish an *in vitro* model of NAFLD.\(^{27,28}\) The present results showed that intracellular lipid droplets increased significantly in the OA-treated cells, indicating that the *in vitro* NAFLD model had been successfully established. In this cellular model, we observed that intracellular lipid accumulation and TAG concentration were significantly reduced by 100 μM artemisinin. Additionally, artemisinin was found to down-regulate both mRNA and protein expressions of CD36 and have no effect on those of PPARα and SREBP1. As very important molecules in regulating lipid metabolism, PPARα is a nuclear receptor expressed primarily
in the liver and regulates the transcription of genes involved in hepatic fatty acid uptake and oxidation. While SREBP1 is the most important transcription factor regulation gene involved in de novo lipogenesis. From our results, it suggests that artemisinin had no impact on hepatocellular lipid synthesis and fatty acid oxidation.

CD36/FAT resides in the plasma membrane of hepatocytes and is known to translocate to the outer mitochondrial membrane to facilitate uptake and transport of fatty acids across the mitochondrial membrane. Previoulsy, Koonen et al. found that high-fat diet increased expression of hepatic CD36 protein and hepatic triglyceride storage. In CD36 knockout mice, uptake and utilization of long chain fatty acids in muscle and adipose tissues was reduced. Krammer et al. once reported that overexpression of CD36 in Huh 7 and HepG2 cells increased uptake of OA. Podsuzn et al. indicated that orally administered atorvastatin and RRR-α-tocopherol (αT) individually, but not synergistically, prevent diet-induced lipid accumulation in the liver of guinea pigs by down-regulation of hepatic CD36 protein. In our work, it showed that OA induced up-regulation of CD36 and increase of TAG, while artemisinin reduced the expression of CD36 and lipid accumulation. It suggests that artemisinin reduced hepatic lipid accumulation via down-regulation of CD36 protein.

CONCLUSION

In conclusion, we demonstrated that artemisinin attenuated lipid accumulation via the downregulation of CD36 in the cellular model of NAFLD (Figure 6). The results obtained here suggest that artemisinin may be a therapeutic candidate of the treatment of NAFLD in the near future.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBRIEVIATION USED

NAFLD: Nonalcoholic fatty liver disease; TAG: Triglyceride; OA: Oleic acid; RT-PCR: Reverse transcription-polymerase chain reaction; CD36: Cluster of differentiation 36; PPARα: Peroxisome proliferator-activated receptor; SREBP1: Sterol regulatory element-binding transcription factor 1; FBS: Fetal bovine serum; DMSO: Dimethylsulfoxide.

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Artemisinin attenuated lipid accumulation via the downregulation of CD36 in the cellular model of NAFLD.

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About Authors

**Prof. Ping Shi:** Working as a teacher at State Key Laboratory of Bioreactor Engineering in East China University of Science and Technology. Her research area includes pharmacological studies on various bioactive compounds. She has over 70 scientific research papers published in international journals.

**Mr. Jian Wang:** Presently in a Ph.D student at State Key Laboratory of Bioreactor Engineering in East China University of Science and Technology. His research areas include pharmacological screening and active principles of traditional Chinese medicinal plants, biotechnological tools and techniques.