NASH, in order to reverse the accumulation of triglycerides (TG) in alcoholic steatohepatitis (NASH). NASH is the most common cause of liver steatosis with liver in diabetes, obesity and atherosclerotic cardiovascular disease [2]. NAFLD resistance and a variety of metabolic diseases, including type 2 diabetes, which could result in cirrhosis and subsequently lead to the development of hepatocellular carcinoma [3–5].

Weight loss is highly recommended as a prevention for NAFLD and NASH, in order to reverse the accumulation of triglycerides (TG) in hepatocytes. Moreover, suppression of hepatic inflammation can also prevent NAFLD progression [6]. In fact, there are no targeted treatment regimens currently available for NAFLD. However, some studies have suggested that pioglitazone, a type 2 diabetes medicine, could improve NASH, but further exploration are still required to evaluate its toxicity for long-term use [7].

Sodium tanshinone IIA sulfonate (STS) is a water-soluble compound derived from tanshinone IIA, extracted from the dried roots of a traditional Chinese medicine called Salvia miltiorrhiza (also known as danshen). STS was reported to show neuroprotective activity towards additional Chinese medicine called Danshen). STS was demonstrated to attenuate myocardial inamy and lipid accumulation in cells treated with OA-PA, shown by decreased transcriptional levels of tumor necrosis factor (TNF), transforming growth factor beta 1 (TGFβ1) and interleukin 1 beta (IL1B). Consistently, protective effects on hepatic steatosis in db/db mice were observed after STS administration, demonstrated by decreased lipid accumulation in mouse hepatocytes. This protective effect might be associated with STS induced activation of sirtuin 1 (SIRT1)/protein kinase AMP-activated catalytic subunit alpha 1 (PRKAA1) pathways. Our findings suggest a potential therapeutic role for STS in the treatment of NAFLD.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is becoming an epidemic disease in adults and children worldwide. Importantl, there are currently no approved treatments available for NAFLD. This study aims to investigate the potential applications of sodium tanshinone IIA sulfonate (STS) on improving the NAFLD condition using both in vitro and in vivo approaches. The results showed that STS markedly inhibited lipid accumulation in oleic acid (OA) and palmitic acid (PA) treated HepG2 and primary immortalized human hepatic (PHH) cells. STS suppressed lipogenesis by inhibiting expression of sterol regulatory element binding transcription factor 1 (SREBF1), fatty acid synthase (FASN) and stearoyl-CoA desaturase (SCD). In addition, STS reduced inflammation in cells treated with OA-PA, shown by decreased transcriptional levels of tumor necrosis factor (TNF), transforming growth factor beta 1 (TGFβ1) and interleukin 1 beta (IL1B). Consistently, protective effects on hepatic steatosis in db/db mice were observed after STS administration, demonstrated by decreased lipid accumulation in mouse hepatocytes. This protective effect might be associated with STS induced activation of sirtuin 1 (SIRT1)/protein kinase AMP-activated catalytic subunit alpha 1 (PRKAA1) pathways. Our findings suggest a potential therapeutic role for STS in the treatment of NAFLD.

**Keywords:** NAFLD, Sodium tanshinone IIA sulfonate, SIRT1, PRKAA1

**ABSTRACT**

Non-alcoholic fatty liver disease (NAFLD) is becoming an epidemic disease in adults and children worldwide. Importantly, there are currently no approved treatments available for NAFLD. This study aims to investigate the potential applications of sodium tanshinone IIA sulfonate (STS) on improving the NAFLD condition using both in vitro and in vivo approaches. The results showed that STS markedly inhibited lipid accumulation in oleic acid (OA) and palmitic acid (PA) treated HepG2 and primary immortalized human hepatic (PHH) cells. STS suppressed lipogenesis by inhibiting expression of sterol regulatory element binding transcription factor 1 (SREBF1), fatty acid synthase (FASN) and stearoyl-CoA desaturase (SCD). In addition, STS reduced inflammation in cells treated with OA-PA, shown by decreased transcriptional levels of tumor necrosis factor (TNF), transforming growth factor beta 1 (TGFβ1) and interleukin 1 beta (IL1B). Consistently, protective effects on hepatic steatosis in db/db mice were observed after STS administration, demonstrated by decreased lipid accumulation in mouse hepatocytes. This protective effect might be associated with STS induced activation of sirtuin 1 (SIRT1)/protein kinase AMP-activated catalytic subunit alpha 1 (PRKAA1) pathways. Our findings suggest a potential therapeutic role for STS in the treatment of NAFLD.

https://doi.org/10.1016/j.biopha.2018.12.019

Received 26 October 2018; Received in revised form 5 December 2018; Accepted 5 December 2018

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STSS could reduce lipid accumulation in both in vitro and in vivo models. The protective effects of STS were associated with the suppression of lipogenesis and inflammation due to the activation of SIRT1/PRKAA1 pathways.

2. Materials and methods

2.1. Cell culture

HepG2 and PIH cells were cultured in Minimum Essential Medium (Thermo Fisher Scientific, Massachusetts, USA), supplemented with 10% fetal bovine serum (Thermo Fisher Scientific), 1X antibiotic-
antimycotic (Thermo Fisher Scientific) and maintained under standard conditions of 37 °C with 5% CO₂ in a humidified incubator (Thermo Fisher Scientific).

2.2. Cell viability

HepG2 and PIH cells were seeded in 96-well plates at a concentration of 6000 cells per well and allowed for attachment overnight. These cells were treated with STS at concentrations of 1 μM, 10 μM and 100 μM for 24 h. Cell viability was measured by CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) (Promega, Wisconsin, USA) and analysed using the Opera Phenix high content imaging system (PerkinElmer, Massachusetts, USA).

2.3. Nile red staining

In order to establish the NAFLD cellular model, cells were treated with medium containing fatty acid free bovine serum albumin (BSA) (Sigma-Aldrich, Darmstadt, Germany) conjugated oleic acid (OA) (Sigma-Aldrich) and palmitic acid (PA) (Sigma-Aldrich) at a ratio of 2:1 (0.4 mM and 0.2 mM, respectively) for 24 h. Meanwhile corresponding BSA containing medium without OA-PA was used as control. This medium was then replaced with varying dosages of STS for an additional 24 h. After treatment, cells were washed twice with phosphate buffered saline (PBS) and fixed in cold 4% paraformaldehyde (PFA) for 15 min at room temperature (RT). Cells were washed with PBS and stained with nile red (Sigma-Aldrich) at a concentration of 3 μM in PBS for 15 min. After staining, cells were washed thoroughly three times with PBS. Fluorescence intensity (FI) was detected using the CLARIOstar plate reader (BMG Labtech).

2.4. Neutral lipid droplet staining

Cell treatment procedures were as described in the nile red staining method, except for the following changes. After STS treatment, cells were stained with 2 μM BODIPY 493/503 (Thermo Fisher Scientific) in

Fig. 2. STS inhibited lipogenesis in OA-PA treated HepG2 and PIH cells. Cells were exposed with different concentrations of STS for 24 h. (A) Western blot analyses were performed to detect protein levels of SREBF1, FASN and SCD. (B and C) Quantitative analyses of target proteins relative to ACTB in HepG2 and PIH cells, respectively. Values were expressed as mean ± SD of three independent blots. *P < 0.05; **P < 0.01, ***P < 0.001, versus control.
PBS for 10 min, followed by washing with PBS and fixation in cold 4% PFA for 15 min at RT. Cells were washed three times with PBS before imaging under confocal microscope (Leica TCS SPE Confocal Microscope, University Life Sciences, The Hong Kong Polytechnic University).

2.5. Western blot analyses

Protein was extracted and processed using the Qproteome Mammalian Protein Prep Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. Concentrations of protein were determined using Bradford Protein Assay kit (Bio-Rad, California, USA). Protein was separated on a 8% or 10% SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Massachusetts, USA). Membranes were first blocked in 5% (v/v) non-fat milk, followed by incubation with specific primary antibodies at 4 °C overnight. Information of antibodies used were as follows: SREBF1 (sc-366) (C-20), Santa Cruz Biotechnology, Texas, USA), FASN (C20G5) (Cell Signaling Technology, Massachusetts, USA), SCD (Abcam, Cambridge, United Kingdom), PCK1 (Abcam), PCK2 (Cell Signaling Technology), SIRT1 (E104) (Abcam), phospho-PRKAA1 (Thr172) (40H9) (Cell Signaling Technology), ACTB (13E5) (Cell Signaling Technology). Corresponding secondary antibodies were incubated at RT for 1.5 h. Targeted proteins were detected using a horseradish peroxidase-conjugated chemiluminescent kit (Millipore).

2.6. Quantitative RT-PCR

RNA was extracted using TRizol reagent (Invitrogen Thermo Fisher Scientific) following the manufacturer’s instructions. Complementary DNA (cDNA) was synthesized using the PrimeScript RT Master Mix (Takara Bio Inc., Kusatsu, Japan) following the manufacturer’s instructions. Quantitative RT-PCR was conducted using GoTaq qPCR Master Mix (Promega). Actin beta (ACTB) was used as a reference gene to normalize the mRNA expression of TNF, IL1B and TGFB1. Primers used were as follows: ACTB-For, 5′-AGAGCTACGAGCTGCCTGAC-3′ and ACTB-Rev, 5′-AGCCTGTTGGCGT1ACAG-3′; TNF-For, 5′-GGGAGAAGGTTACCGACTCA-3′ and TNF-Rev, 5′-TCGCCAGACGCACTGG CAA-3′; IL1B-For, 5′-TGCCCGCAATGATGCT-3′ and IL1B-Rev, 5′-TGAAAGAACACCTCAGGTT-3′; TGFB1-For, 5′-GGAAATTGAGG GCTTTGCC-3′ and TGFB1-Rev, 5′-CGGTTAGTAACCGCT-3′.

2.7. Animal experiments

The db/db and wild-type (WT) C57BL/6J-db/m mice at 7 weeks old were obtained from Nanjing Biomedical Research Institute of Nanjing University, Nanjing, China. Mice were randomly divided into four groups (eight mice per group): WT group (db/m, 0.9% saline), db/db model group (db/db, 0.9% saline), low dosage STS treatment group (db/db & STS-L, 10 mg/kg/day), and high dosage STS treatment group (db/db & STS-H, 20 mg/kg/day). Saline and STS were delivered daily to mice by oral gavage. Mice were housed in a 12 h light-dark cycle with water and standard mouse chow ad libitum. STS treatment started at 16 weeks of age and lasted for 10 weeks. STS was purchased from Shanghai No.1 Biochemical & Pharmaceutical Co., Ltd, China.

2.8. Hematoxylin and eosin (HE) staining

Paraffin embedded tissues from mouse livers were sectioned at 5 μm using a standard microtome (Leica Biosystems, Wetzlar, Germany), mounted and heat-fixed onto glass slides. Tissue section slides were processed, stained with hematoxylin (Leica Biosystems) and eosin (Leica Biosystems) using standard protocols.

2.9. Oil red O staining

Frozen sections of 10 μm thickness were mounted onto slides and air dried for 60 min at RT. Oil red O staining was performed as suggested by the manufacturer’s instructions (Nanjing Jiancheng Institute of Biotechnology, China).
2.10. Detection of biochemical markers

Heparin-containing blood was centrifuged to obtain plasma. Aspartate aminotransferase (AST), alanine aminotransferase (ALT) activities, free fatty acid (FFA), triglyceride (TG) and total cholesterol (TC) levels in plasma were detected using commercial kits (Nanjing Jiancheng Institute of Biotechnology).

2.11. Statistical analyses

Raw data was analysed using GraphPad Prism (Version 6, California, USA) and expressed as mean ± standard deviation. Student’s t-test and ANOVA in Prism were used for statistical analyses. Value of \( P < 0.05 \) was considered as statistically significant.

3. Results

3.1. STS treatment ameliorated lipid accumulation in OA-PA treated HepG2 cells and PIH

The structure of STS was shown in Fig. 1A. The toxicity of STS on HepG2 and PIH cells were first evaluated. Cells treated with 0, 1, 10, 100 \( \mu \)M STS for 24 h did not affect cell viability by MTS analyses (Fig. 1B). Effects of STS on lipid accumulation were assessed using nile red and BODIPY493/503 staining. STS significantly reduced the lipid amount in OA-PA treated HepG2 and PIH cells by nile red staining, as shown by decreased FI values (Fig. 1C). Similar trends were also observed using the BODIPY493/503 staining assay (Fig. 1D).

3.2. STS inhibited lipogenesis and inflammation in HepG2 and PIH cells

In order to evaluate the effects of STS on lipogenesis, several related transcriptional factors were analyzed using Western blot. STS inhibited protein levels of SREBF1, and its downstream FASN and SCD in HepG2 and PIH cells in a dose-dependent manner (Fig. 2A). Semi-quantitative analyses of these blots suggested that STS treatment could significantly inhibit SREBF1, FASN and SCD especially at a concentration of 100 \( \mu \)M (Fig. 2B and C).

Inflammation has been implicated in the development of NAFLD, and its progression to further liver damage. Therefore, important inflammatory elements were also examined in this study. STS treatment significantly reversed levels of TNF, TGF\( \beta \)1 and IL1\( \beta \) in both OA-PA treated HepG2 and PIH cells (Fig. 3A and B, respectively), indicating that STS could be used as a potential regimen for inhibiting lipogenesis and inflammation in NAFLD.

3.3. STS affects the SIRT1/PRKAA1 pathway

To further explore the potential mechanisms of STS in suppression of lipogenesis and inflammation, we hypothesized that STS could...
inhibit lipogenesis through the SIRT1/PRKAA1 cellular metabolic processing pathway. Indeed, STS significantly increased protein levels of SIRT1 and phosphorylated PRKAA1 (p-PRKAA1) in both HepG2 and PIH cells (Fig. 4).

3.4. STS ameliorated hepatic steatosis in db/db mice

To investigate whether STS could improve hepatic steatosis in vivo, db/db mice were administered with saline or STS by oral gavage for 10 weeks. Histological analyses using HE staining of liver sections showed excessive lipid droplets in saline-treated db/db model mice, but was strikingly alleviated by STS treatment (Fig. 5A). Consistent with the HE staining results, oil red O staining displayed decreased lipid accumulation in db/db mice treated with STS in a dose-dependent manner (Fig. 5B). In addition, STS treatment was shown to decrease plasma free fatty acid (FFA), total cholesterol (TC) and triglycerides (TG) levels (Fig. 5C). Hepatic injury markers alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were also reduced by STS treatment (Fig. 5D).

Fig. 5. STS treatment ameliorates hepatic steatosis in db/db mice. (A) Representative HE staining images of liver sections from four groups of mice. Scale bar, 50 μm. (B) Representative oil red O staining images of liver sections from four groups of mice. Scale bar, 100 μm. (C) Plasma FFA, TC and TG levels from four groups of mice. (D) Effects of STS on plasma ALT and AST levels from four groups of mice. Values were presented as mean ± SD (n = 8). *p < 0.05; **p < 0.01; ***p < 0.001, versus WT; #p < 0.05; ###p < 0.01, ####p < 0.001, versus vehicle treated db/db mice.

4. Discussion

Previous studies about STS have long been focused on its protective effects on cardiomyocytes [14–16]. Interestingly, there was a study showing STS ameliorated myocardial inflammation and lipid accumulation in Beagle dogs [11]. These studies led to the hypothesis that STS might also show protective effects on NAFLD development and progression, where lipid accumulation and inflammation are the main pathological factors [6]. In this study, we have demonstrated that STS treatment elicited a protective effect against lipid accumulation both in vitro and in vivo. Activation of the SIRT1/PRKAA1 pathway induced by STS treatment was associated with its inhibition on lipogenesis and inflammation.

OA-PA exposure was used to establish the in vitro cellular model of NAFLD. STS could significantly reduce lipid content in OA-PA treated HepG2 and PIH cells using Nile red and BODIPY493/503 staining (Fig. 4D).
TNF is an important factor in human NAFLD and NASH [30]. TGFB1 has been inhibited by STS treatment, as STS treatment could significantly reduce lipid accumulation in db/db mouse hepatocytes, as demonstrated by HE and oil red O staining images (Fig. 5A and B). Moreover, plasma FFA, TC and TG were significantly reduced in db/db mice treated with STS, compared with vehicle treated db/db mice (Fig. 5C). Liver damage was also evaluated by detecting ALT and AST content. STS treatment improved liver damage compared with that from vehicle treated db/db group (Fig. 5D). These results indicated that STS could improve lipid accumulation in hepatocytes and ameliorate hepatic steatosis in mice.

Lipogenesis involves lipid synthesis in hepatocytes and adipocytes [17]. However, the excess accumulation of lipids in hepatocytes result in NAFLD. NAFLD has been correlated with increased hepatic expression of several transcription factors involved in lipogenesis, such as SREBF1, MLXIPL, FASN and SCD [18,19]. SREBF1 is the pivotal regulatory transcription factor for TG synthesis in hepatocytes, and dysregulation of SREBF1 has been implicated in the pathogenesis of hepatic steatosis by activating lipogenic genes and inducing higher TG content [20–22]. In our study, STS significantly inhibited SREBF1 and subsequent FAS and SCD expression levels in HepG2 and PIH cells (Fig. 2). This effect was also supported by decreased FFA, TC and TG content in STS treated db/db mice (Fig. 5C). PRKAA1 activation was demonstrated to suppress SREBF1-dependent lipogenesis and attenuated hepatic steatosis in mice [23]. As expected, STS activated PRKAA1 by increasing its phosphorylation level (Fig. 4), suggesting that PRKAA1 as an upstream mediator involved in the regulation of STS inhibited SREBF1 expression.

Glucagon signaling pathway has been reported to play a role in the contribution of NAFLD development [24,25]. This process is regulated by PEPCK, which has two isoforms, cytosolic PCK1 and mitochondrial PCK2, both of which are essential in glucose homeostasis [26,27]. In addition, PCK2 can potentiate function of PCK1 in liver glucogenesis [28]. However, STS displayed no effect on PCK1 and PCK2 expression levels (data not shown), indicating that STS does not affect the glucogenesis process.

Hepatic steatosis is thought to be a prerequisite for NASH and a risk factor for liver fibrosis. Inflammation is considered to be one of the most important contributing factors for NAFLD and NASH progression [6]. TNF, TGFβ1 and IL1β involvement with inflammation have been well documented and proven to be closely related with NAFLD occurrence [29]. TNF is an inflammatory element, which has been proven to be a key factor in human NAFLD and NASH [30]. TGFβ1 has been suggested to be involved in hepatic fibrosis, and its upregulation detected in experimental models and patients with chronic liver diseases [31]. Similarly, increased IL1β expression has also been identified to be risk factor for NAFLD [32]. Therefore, anti-inflammatory treatment is one of the promising approaches for NAFLD therapy. Administration of OA accelerated the inflammatory phase via increase in TNF and activation of NFκB [33], while PA exerted proinflammatory effects via interleukin-8 in hepatocytes [34]. More importantly, our data demonstrated that STS could dramatically reversed OA-PA induced inflammation in HepG2 and PIH cells (Fig. 3). These results suggest that STS has the potential to prevent the transformation from NAFLD to NASH and fibrosis.

SIRT1 has been demonstrated to mediate hepatic lipid metabolism via activation of PRKAA1 [35]. SIRT1/PRKAA1 signalling pathway has been well studied in sensing and mediating hepatic fatty acid metabolism [36]. Hepatocyte-specific deletion of SIRT1 alters fatty acid metabolism and results in hepatic steatosis and inflammation [37]. SIRT1/PRKAA1 have been considered to play many similar regulatory roles in response to stress and nutritional status, including regulation of lipogenesis, glucose homeostasis and mitochondrial biogenesis [38]. A complicated interaction exists between SIRT1 and PRKAA1, PRKAA1 was shown to activate SIRT1 via the increase of cellular NAD⁺ content [39]. While, SIRT1 can activate PRKAA1 via deacetylation on PRKAA1 kinase serine/threonine kinase LB1 [40]. Therefore, the SIRT1/PRKAA1 may act as the central communication hub for cell energy balance and response. In this study, STS increased expression of both SIRT1 and PRKAA1 in a dose-dependent manner, supporting the beneficial effects of STS on the improvement of NAFLD (Fig. 4). In addition, SIRT4, another member from the Sirtuins family, has been shown to dampen fatty acid oxidation in liver and muscle cells [41]. From our preliminary data, STS does not affect the β-oxidation process (data not shown).

In conclusion, STS treatment suppresses lipogenesis and inflammation by activating the SIRT1/PRKAA1 signaling pathway (Fig. 6). Importantly, our current study provides new insights into the effects of STS on NAFLD, providing further evidence to support STS as a potential therapeutic option in the treatment of NAFLD and NASH.

Availability of data and materials

The datasets in the current study are available from the corresponding authors on reasonable request.

Conflict of interest disclosure statement

The authors have no conflict of interest.

Acknowledgments

This study was supported by The Shenzhen Science and Technology Innovation Commission (JCYJ20170413154748190); Research Grants Council Collaborative Research Fund Scheme (C5012-15E), Hong Kong SAR Government; and the Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hong Kong SAR (G-YBTA).

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