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Zoledronic acid, an FPPS inhibitor, ameliorates liver steatosis through inhibiting hepatic *de novo lipogenesis*

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ABSTRACT

Currently, there is no standard therapy for non-alcoholic fatty liver disease (NAFLD), and statins have been developed as a first-line pharmaceutical therapeutic option for NAFLD-associated dyslipidemia. However, prolonged statins therapy has side effects, as statins inhibit HMG-CoA reductase, an enzyme at the very beginning of the mevalonate pathway. Here, we found that zoledronic acid (ZA), an inhibitor of farnesyl diphosphate synthase in the downstream mevalonate pathway, could attenuate hepatic lipid accumulation and improve liver injury in both high-fat diet-induced *C57BL/6J* mice and *ob/ob* mice. Moreover, the hepatic lipid metabolism was largely inhibited after ZA administration in high-fat diet-induced obese mice. Mechanically, ZA inhibited SREBP-1c-mediated *de novo lipogenesis* through suppressing RhoA activation via decreasing farnesyl diphosphate and geranylgeranyl diphosphate levels. In conclusion, our data provide a novel application of ZA in improving hepatic steatosis.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD), a globally distributed clinical disease, is commonly caused by unhealthy lifestyle and junk food consumption (Rinella and Charlton, 2016). It ranges from non-alcoholic simple steatosis, non-alcoholic hepatitis, cirrhosis, to hepatocellular carcinoma (Cohen et al., 2011). Among these, hepatic steatosis status can be reversed via effective treatment; otherwise, it will progress to the more severe stages (Browning and Horton, 2004). Thus, it is necessary to explore therapeutic strategies to ameliorate hepatic steatosis and prevent it from progressing to a more severe status (Schwenger and Allard, 2014b).

Currently, the clinical treatment for NAFLD is either insufficient or has severe side effects. Specifically, the management of liver steatosis includes lifestyle intervention (diet control, weight loss and physical exercise) and pharmaceutical treatments (Schwenger and Allard, 2014a). Although lifestyle intervention is the first and best approach for NAFLD (Schwenger and Allard, 2014a), long-term prevention is insufficient to achieve the desired effects (Katsiki et al., 2016). Fibrates, agonists for PPAR-α, are widely used for clinical treatment of NAFLD-associated hyperlipidemia. However, several safety concerns have been reported for fibrates, such as lithogenic gastrointestinal complaints, an increase of transaminase, creatinine, and homocysteine, and even rhabdomyolysis (Rubenstrunk et al., 2007). Alternatively, statins have also been used to treat NAFLD-associated hypercholesterolemia and dyslipidemia (Remick et al., 2008; Tziomalos et al., 2015). Statins inhibit HMG-CoA reductase, the rate limiting enzyme in the mevalonate pathway, which lowers the plasma cholesterol level to treat hypercholesterolemia and atherosclerosis (Katsiki et al., 2016). However, prolonged statins therapy has side effects such as myopathy (El-Ganainy et al., 2016; Tomaszewski et al., 2011) and liver injury (Chalasani, 2005). This might be due to statins' suppression of mevalonate pathway at the very beginning, which leads to depletion of not only cholesterol but also intermediate isoprenoids (Beltowski et al., 2009; El-Ganainy et al., 2016). Among these intermediate isoprenoids, farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) are essential for prenylation and subsequent membrane-association and activation of some proteins, such as small G proteins and the G-γ subunit of GTPase proteins. Interestingly, previous studies have indicated that some small G proteins, like Rab (Rasineni et al., 2014; Wu et al., 2014) and RhoA

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(Kitamura et al., 2007) participated in hepatic lipid accumulation and hepatic steatosis.

Zoledronic acid (ZA) is a potent inhibitor of FPPS, which has been widely used to treat osteoporosis and metastatic bone disease and has recently been proposed for cancer applications (Nakamura et al., 2016; Wang et al., 2015). Similar to statins, ZA depletes both FPP and GGPP in cells, leading to the abrogation of protein prenylation (Reszka and Rodan, 2004).

In the present study, we hypothesized that ZA, a clinically utilized inhibitor of FPPS (Wiemer et al., 2007), has a protective effect on liver steatosis through alternating G protein prenylation via modulating hepatocellular FPP and GGPP levels. Interestingly, we found that ZA could reduce lipid accumulation both in vitro and in vivo without significant side effects. Our results indicated that ZA could be a prospective pharmaceutical therapeutic treatment for liver steatosis.

2. Materials and methods

2.1. Mice and zoledronic acid administration

15 male *ob/ob* (OB) mice and 24 *C57BL/6J* (B6) mice were purchased from the Model Animal Research Center of Nanjing University. 10-week-old OB mice received regular chow (RC) (4 kcal% fat; Xietong, Jiangsu, China). And B6 mice were either fed with RC or high-fat diet (HFD) (60 kcal% fat; Research Diets, NJ, USA) for 10 weeks to induce liver steatosis model, which is also called diet induced obesity (DIO) mice. All mice were maintained on 12 h/12 h light/dark cycle and fed ad libitum. Animal care and handling were in accordance with the Animal Care and Use Committee at the Model Animal Research Center. DIO mice were randomly and equally divided into three groups (six mice per group): DIO-PBS, DIO-50ZA (50 μg/kg) (Zoledronic acid Injection, Novartis), and DIO-200ZA (200 μg/kg) groups. PBS or ZA was administrated through in-vein injection and the injection was performed every two days for 30 days (a 50 μg/kg dose was comparable to the dose treatment of patients with osteoporosis, while the 200 μg/kg dose was used in accordance to previous publications (Heino et al., 2016; Raikkonen et al., 2009)). Similarly, OB mice were also randomly and equally divided into three groups (five mice per group): OB-PBS, OB-50ZA and OB-200ZA (200 μg/kg. All of the mice were killed seven days after the last injection.

2.2. Cell culture and cell viability assay

The AML-12 cell line (CRL-2254) was bought from ATCC (without further authentication) and cultured in DMEM/F12 medium supplemented with 10% fetal bovine saline (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. All of these cells were incubated at 37 °C in an atmosphere of 95% air/5% CO_2 incubator. Adherent hepatocytes were incubated with 1% BSA or 500 mM free fatty acids (FFA, oleic acid: palmitic acid (Sigma-Aldrich, St. Louis, MO) = 2:1) for 48 h and ZA was administered for 24 h at indicated concentrations. The final concentrations of ZA were 20 μM and 50 μM (Moriceau et al., 2010; Qiao et al., 2016; Raikkonen et al., 2009; Wiemer et al., 2007). Cell viability was measured with the Cell Counting Kit-8 (CCK-8) Kit (Nanjing Jiancheng Bioengineering Institute, NJ, China) according to the manufacturers' instructions. We have repeated each experiment at least three times.

2.3. Liver lipid content analysis and TG and TC measurement

Liver lipid content of DIO mice before ZA administration was examined via Discovery Computer Tomography (Discovery CT 750 HD, GE, USA) and the number of DIO mice was three. The hepatic/hepatocytes triglyceride (TG) and total cholesterol (TC) were measured by using Triglyceride or Total Cholesterol Quantification Kits (APPLYGEN, Beijing, China), respectively, according to the manufacturer's instructions.

2.4. Morphology analysis

Livers were washed with cold PBS and then fixed in 4% paraformaldehyde (PFA) at 4 °C for 24–48h, followed by paraffin embedding and sectioning to 5 μm. Then, hematoxylin & eosin (H & E) staining was performed according to the standard protocol. For the oil-red O staining, livers were embedded in Tissue Freezing Medium (Leica Microsystems, Heidelberg, Germany) and sectioned to 8 μm using a Leica Cryostat and the sections were stained according to the standard protocol.

2.5. Serum data analysis

Blood samples were collected from orbital venous plexus after anesthesia by intraperitoneal injection of pentobarbital (70 mg/kg body weight). After centrifugation, serum was obtained and then serum total cholesterol, triglyceride, LDL-c, HDL-c, total bilirubin, urea, creatinine, and creatine kinase were detected in a Clinical Laboratory of the Affiliated Drum Tower Hospital of the Medical School of Nanjing University.

2.6. RNA isolation, reverse transcription PCR, and Real-time PCR

RNA was extracted with the TRIzol reagent (Takara Bio, Japan), and then reversed transcribed with PrimeScriptTM RT reagent (Takara Bio, Japan) according to the manufacturer's instructions. Subsequent Real-Time PCR was conducted on the ABI-Viia 7. All quantitation was performed in triplicate and normalized to β-actin. A complete list of PCR primers is shown in Table S2 (See Supplementary material). The primers used are shown in Table S1 (See Supplementary material).

2.7. Western blot analysis

Hepatic/hepatocytes protein extracts were prepared according to the previously reported standard protocols and were separated via SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (Bio-Rad, Richmond, CA). The membranes were incubated overnight with primary antibodies, and then bound antibodies were visualized. Primary antibodies against ROCK, RhoA (sc-418, A2315), and SREBP-1c (sc-366, E3122) were purchased from Santa Cruz. Primary antibodies against GAPDH (MB001H), α-Tublin (BS1482MH), and the secondary antibody were purchased from Bioworld.

2.8. Protein prenylation examination

The FPP and GGPP levels were determined in the AML-12 cell line as previously described using the HPLC-MS/MS (Wang et al., 2013). To assess RhoA prenylation, subcellular fractionation of the hepatocytes was performed using the Triton X-114 partition method (Goalstone et al., 1999). Briefly, adherent hepatocytes were lysed with 2% Triton X-114 for 30 min on ice. Then 500 μg protein were partitioned with same volume of 0.1% Triton X-114 for 10 min at 37 °C to solubilize and fractionate the lipid-rich cell membrane. Then, two subcellular phases were immunoprecipitated with the RhoA antibody, and subjected to western blot analysis.

2.9. Statistical analysis

Results are presented as means ± standard error of the mean (S.E.M.). For comparisons between two groups, statistical significance was determined using an unpaired two-tailed Student's *t*-test. A value of $P < 0.05$ (* or #) was considered statistically significant and $P < 0.01$ (** or ##) was statistically very significant.