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Full paper

Effect of oleoylethanolamide on diet-induced nonalcoholic fatty liver in rats

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ABSTRACT

Oleoylethanolamine (OEA), an endogenous high-affinity agonist of peroxisome proliferator-activated receptor alpha (PPAR-α), has revealed the pharmacological properties in the treatment of obesity, atherosclerosis and other diseases through the modulation of lipid metabolism. To assess whether OEA can also regulate non-alcoholic fatty liver disease (NAFLD) caused by fat accumulation, we administrated OEA or fenofibrate in Sprague Dawley (SD) rats fed with a high fat diet (HFD). After 6 or 17 weeks treatment, OEA (5 mg/kg/day, i.p.) relieved the development of NAFLD compared with control groups by regulating the levels of plasma TG, TC, ALT and AST and liver inflammatory cytokines. Gene expression analysis of liver tissue and plasma from the animal models showed that OEA and fenofibrate both promoted the lipid β-oxidation by activating PPAR-α. Detailed research revealed that OEA inhibited the mRNA expression of lipogenesis in a PPAR-α-independant manner, while fenofibrate expressed an opposite effection. In summary, our research results suggested that as a potential lead compound, OEA could improve HFD-induced NAFLD with higher efficacy and safety than fenofibrate.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease which affects 25–30% of the world's population. Onset of NAFLD manifests as large lipid droplet accumulation in liver cells and triggers the formation of obesity, type II diabetes, hyperlipidemia, insulin resistance and other metabolic syndromes (1). The important early metabolic events leading to NAFLD can be best described as a "two-hit" theory. The "first hit" is characterized by the deposition of triglycerides in hepatocyte during the pathologic process of obesity or insulin resistance. The disease is sensitive to additional cellular events as oxidative stress, lipid peroxidation, pro-inflammatory cytokines release (the "second hit"), which leads the progress of nonalcoholic steatohepatitis. Current clinical therapies for NAFLD is limited to control of associated metabolic syndromes, reduction risk of cardiovascular disease, and suggestions of lifestyle changes. So, although dietary control, exercise, insulin-sensitizing drugs, antioxidants and lipid-lowering drugs can do favor for NAFLD (2), other modalities of treatment is suggested by clinical observations.

Peroxisome proliferator-activated receptor alpha (PPAR-α) is predominantly distributed in the active metabolic tissues, such as liver, kidney, heart and skeletal muscle, in which modulates the lipid metabolism and energy balance. PPAR-α activation not only up-regulates the expression levels of carnitine palmitoyl transferase 1 (CPT1) to promote fatty acid β-oxidation and reduces triglyceride deposition *in vivo*, but also inhibits the signal transduction mediated by AP-1 and NF-κB to relieve inflammation (3). Fatty acid oxidases in the mitochondria, peroxisomes and microsomes expressed abnormally in PPAR-α knockout (KO) mice, leading to increase of free fatty acids in plasma and excessive accumulation of lipids in liver (4). In comparison to wild-type mice, PPAR-α KO mice exhibited high expression levels of many inflammatory factors, as tumor necrosis factor alpha (TNF-α) and interleukin 1β (IL-1β) in liver and adipose tissues (5). Fibrates are widespread applied as PPAR-α agonists into clinic practice to regulate plasma lipid disorders (6). Some clinic data has shown that fenofibrate plays a central role in reducing plasma and hepatic triglyceride concentrations and transaminase activities and improving insulin resistance in NAFLD patients *in vivo* (7). However,

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due to the relatively low affinity of fenofibrate to PPAR-α receptor (EC_{50} = 30 μM) (8), as well as dose-dependent side effects that have been reported (9), there is an unsatisfied clinical requirement for new PPAR-α agonists with potential specificity and safety to play regulator roles in metabolic syndromes.

Compared with fenofibrate, an endogenous lipid OEA shows a higher affinity with PPAR-α (EC_{50} = 120 nM), which has been indicated by using luciferase assay for PPRE and PPAR-α vectors-transfected HEK293 cells (10). In the prior reports, this molecular was considered as a better regulator for the treatment of obesity, atherosclerosis and other metabolic diseases (11,12). However, the effects and mechanisms about OEA applied to improve NAFLD have not been reported. OEA plays an important regulatory role in lipid metabolism and can enhance fatty acid oxidation in primary cultured skeletal muscle cells, liver cells and cardiac myocytes. But it does not affect fatty acid oxidation processes in similar cells extracted from PPAR-α KO mice, which confirms that OEA regulates fatty acid metabolism by activating PPAR-α (13). Obesity and insulin resistance are often accompanied by lipid metabolic disorders (14), which are turned out to be the main cause of NAFLD. As OEA has been demonstrated to induce weight loss in association with improvements in body lipid metabolism, we hypothesize that OEA may play a protective role against NAFLD. In present experiments, we evaluated the role and mechanism of OEA on NAFLD induced via a high fat diet (HFD) in rats. Furthermore, whether OEA can play an improved regulatory role over the traditional PPAR-α agonist, fenofibrate, has also been studied.

Materials and methods

Materials

OEA was synthesized in our lab as previously described (15); fenofibrate, dimethyl sulfoxide (DMSO) and all other chemicals were obtained from Sigma–Aldrich (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Shanghai, China). Lipofectamine® 2000 (Lipo2000) and Human PPAR-α Silencer Select siRNA (ID s10880) were purchased from Invitrogen (Shanghai, China). The chemical constituents were dissolved in saline supplemented with 5% polyethylene glycol 400 (PEG400) and 5% Tween-80 for the *in vivo* studies. For the *in vitro* studies, OEA was dissolved in DMSO to a concentration of 50 mmol/L (stock solution) and then diluted in the culture medium to a final concentration of 50 μmol/L. TNF-α and IL-6 enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems (Shanghai, China). Total cholesterol (TC), triglycerides (TG), alanine transaminase (ALT) and aspartate transaminase (AST) commercial assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Animals and diets

Male Sprague Dawley (SD) rats (250–300 g) were sourced from Shanghai Laboratory Animal Center (Shanghai, China). All experimental protocols were approved by the Committee for Animal Research at Xiamen University. The rats were housed in a room with controlled temperature (21–23 °C), humidity (55–60%) and lighting (12 h light/dark cycles) and given water ad libitum. Rats were fed a HFD diet (30% fat, 2% cholesterol, 2% sugar, 0.5% choline and 65.5% normal diet) or a normal diet (ND) for 6 weeks or 17 weeks to induce nonalcoholic fatty liver. The HFD-fed rats were randomly divided into three experimental groups (n = 8 per group). One group was treated with OEA (5 mg/kg/day; intraperitoneal injection, i.p.) and another group was treated with fenofibrate (10 mg/kg/day; i.p.). The third group was treated with the vehicle (5% Tween-80 + 5% PEG400 + 90% saline). All these compounds were treated once daily.

Liver histological studies

Fresh liver tissue was fixed in 10% neutral-buffered formalin for 3 days and then embedded in paraffin for histological examinations. Tissue sections (5 μm thick) were cut by a Leica SM2010 R Sliding microtome (Shanghai, China) and stained with hematoxylin–eosin (H&E); stained areas were viewed and imaged under microscopy (Nikon, Shanghai, China).

Plasma biochemistry assays

Plasma transaminase (ALT and AST) and triglyceride (TC and TG) concentration were measured individually by a Thermo Scientific Multiskan GO Microplate Spectrophotometer with commercial kits (Nanjing Jiancheng Bioengineering Institute, China). Plasma TNF-α, IL-6 and leptin protein levels were measured using ELISA (R&D Systems, Shanghai, China).

RNA isolation and cDNA synthesis

Liver samples were homogenized and total RNA was extracted using the TRIzol™ isolation reagent (Invitrogen) according to the manufacturer's recommendations. cDNA was synthesized from total RNA using a ReverTra Ace® qPCR RT kit (Toyobo, Shanghai, China) according to the manufacturer's instructions.

Real-time PCR

Quantification of mRNA was performed on an Applied Biosystems 7300 real-time polymerase chain reaction (PCR) system using SYBR® Premix Ex Taq™ II (Takara, Dalian, China). Primer sequences were synthesized as follows: TNF-α, 5′-GGC TCC CTC TCA TCA GTT CCA-3′ (forward), 5′-CGC TTG GTG GTT TGC TAC GA-3′ (reverse); IL-6, 5′-TGC CTT CTT GGG ACT GAT GTT G-3′ (forward), 5′-TGG TCT GTT GTG GGT GGT ATC C-3′ (reverse); C-reactive protein (CRP), 5′-TGT CTC TAT GCC CAC GCT GAT G-3′ (forward), 5′-GGC CCA CCT ACT GCA ATA CTA AAC-3′ (reverse); CPT-1, 5′-AAC TTT GTG CAG GCC ATG ATG-3′ (forward), 5′-GGC AGA AGA TGG CGG TCG-3′ (reverse); enoyl-CoA hydratase (ECHS1), 5′-ATG GCT ATG CTC TTG GTG-3′ (forward), 5′-GTG ATT TGC CGA CTG CTC-3′(reverse); PPAR-α, 5′-AAT CCA CGA AGC CTA CCT GA-3′(forward), 5′-GTC TTC TCA GCC ATG CAC AA-3′ (reverse); stearoyl-CoA desaturase-1 (SCD-1), 5′-ACA TGC TCC AAG AGA TCT CCA G-3′ (forward), 5′-GTA CTC CAG CTT GGG CGG-3′ (reverse); OB-Rb, 5′-GCA TGC AGA ATC AGT GAT ATT TGG-3′ (forward), 5′-CAA GCT GTA TCG ACA CTG ATT CTT C-3′ (reverse); glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5′-ACC ACG AGA AAT ATG ACA ACT CCC-3′ (forward), 5′-CCA AAG TTG TCA TGG ATG ACC-3′ (reverse). The levels of mRNA were normalized relative to the amount of GAPDH mRNA.

Lipid extraction and analysis

Total lipids were extracted from liver samples according to the modified methods as previously described (16). C17:0 margaric acid was added as an internal standard for the quantitation of fatty acids. An Agilent 1200 Series HPLC (Agilent Corporation, MA, USA) interfaced to an Applied Biosystems 3200 Q-Trap triple-quadrupole linear ion trap mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Canada) was operated during experiments. Fatty acids were separated using a Hypersil Gold C18 column (dimensions: 250 × 4.6 mm; particle size: 5 μm; Thermo) eluted with a constant mobile phase that was composed of 5 mM ammonium acetate in acetonitrile (solvent A, 65%) and 5 mM ammonium acetate in 2-propanol (solvent B, 35%);