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Tetrandrine induces lipid accumulation through blockade of autophagy in a hepatic stellate cell line

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ABSTRACT

Macroautophagy, or autophagy, is a cellular response in which unnecessary cytoplasmic components, including lipids and organelles, are self-degraded. Recent studies closely related autophagy to activation of hepatic stellate cells (HSCs), a process critical in the pathogenesis of liver fibrosis. During HSC activation, cytoplasmic lipid droplets (LDs) are degraded as autophagic cargo, and then cells express fibrogenic genes. Thus, inhibition of autophagy in HSCs is a potential therapeutic approach for attenuating liver fibrosis. We found that tetrandrine, a bisbenzylisoquinoline alkaloid isolated from *Stephania tetrandra*, induced lipid accumulation, a phenotype associated with quiescent HSCs, through blockade of autophagy in the rat-derived HSC line HSC-T6. Tetrandrine inhibited autophagic flux without affecting lysosomal function. A phenotypic comparison using siRNA knockdown suggested that tetrandrine may target regulators, involved in fusion between autophagosomes and lysosomes (e.g., syntaxin 17). Moreover, perilipin 1, an LD-coated protein, co-localized specifically with LC3, a marker protein for autophagosomes, in tetrandrine-treated HSC-T6 cells. This suggests a potential role for perilipin 1 in autophagy-mediated LD degradation in HSCs. Our results identified tetrandrine as a potential tool for prevention and treatment of HSC activation.

1. Introduction

Macroautophagy (hereafter referred as to autophagy) is an intracellular degradation system that plays an important role in maintenance of cellular homeostasis [1]. In response to nutrient starvation or hormone stimulation, unnecessary cytoplasmic components, including unfolded proteins, lipids and organelles, are self-degraded and recycled as energy sources [2]. The initial step in autophagy involves generation of a double-membrane particle called an autophagosome, which surrounds the targeted cytoplasmic components. The completed autophagosomes are fused with lysosomes, and then the cytoplasmic contents are degraded by lysosomal hydrolases. Autophagy occurs in various types of cells and tissues and its dysfunction is closely involved in a broad array of mammalian diseases [3], including cancer, neurodegenerative diseases and type-2 diabetes.

In addition, autophagy is believed to closely correlate with activation of hepatic stellate cells (HSCs), a central event in liver fibrosis [4]. In normal liver, HSCs exhibit a quiescent phenotype, characterized by accumulation of cytoplasmic LDs containing retinyl esters and triacylglycerols [5]. When the liver is exposed to injurious stimuli, cytoplasmic LDs in HSCs become recognized through the process of autophagy and are thereby subjected to lysosomal degradation [6]. Free fatty acids released by LD degradation are thought to serve as energy sources for transdifferentiation of HSCs into myofibroblast-like cells to produce fiber including smooth muscle α-actin (α-SMA) and collagen [7], suggesting that LD degradation closely correlates to HSC activation. In fact, recent studies demonstrated that inhibition of autophagy resulted in lipid accumulation in HSCs, indicating a quiescent phenotype, and attenuated liver fibrosis *in vivo* [8]. This suggested that blockade of autophagy in HSCs is a potential therapeutic approach to inhibiting their activation and, therefore, mitigating liver fibrosis.

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Tetrandrine is a bisbenzylisoquinoline alkaloid isolated from the Chinese medicinal herb *Stephania tetranda* [9,10]. This compound has been reported to possess various bioactivities such as hypotensive and anti-inflammatory activities [11]. In addition, it was reported that administration of tetrandrine decreased α-SMA positive cells in the fibrotic livers of rats [12] and attenuated expression of α-SMA in rat-derived primary HSCs via up-regulation of Smad7 [13]. However, the detailed molecular mechanism of this anti-fibrotic activity, including whether tetrandrine can affect lipid accumulation to induce a quiescent phenotype in stellate cells, remains unclear. Our study focused on the effects of tetrandrine on LD metabolism in a cultured HSC cell line and elucidated the potential mechanism by which tetrandrine can affect LD degradation via blockade of autophagic flux.

2. Materials and methods

2.1. Plasmids and antibodies

EGFP-LC3, mRFP-LC3 and mRFP-EGFP-LC3 plasmids were a gift from Professor Tamotsu Yoshimori through the Addgene repository (www.addgene.org). Antibodies used in this study were as follows: anti-LC3 (Medical & Biological Laboratories, Nagoya, Japan), anti-perilipin 1 (Cell Signaling Technology, Danvers, MA, USA), anti-ADRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p62 (Medical & Biological Laboratories), anti-Atg7 (Cell Signaling Technology) and anti-GAPDH (Santa Cruz Biotechnology).

2.2. Cell lines and cell culture

HSC-T6 cells were a gift from Professor Scott L. Friedman, the Mount Sinai School of Medicine (New York, NY). HepG2 cells (RCB1886) were provided by the RIKEN BRC through the National Bio-resource project of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Tokyo, Japan. All cells used in this study were maintained in Dulbecco's modified Eagle's medium (Wako) supplemented with 10% fetal bovine serum, 100 μg/mL penicillin and 100 units/mL streptomycin (Gibco, Carsbad, CA, USA) at 37 °C in a humidified 95% air, 5% CO_2 atmosphere.

2.3. siRNA transfection

Transfection of HSC-T6 cells with Stx17 siRNA or control siRNA (Integrated DNA Technologies, Coralvile, IA, USA) was performed using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. NC-1 (#51-01-14-03, Integrated DNA Technologies) was used as the control siRNA. The sequence of rat Stx17 siRNA was: sense 5′-GCAGAAUCUGGGACAAGUUGCAUGA -3'.

2.4. Statistical analyses

Data are expressed as means ± standard deviation (SD). Significant differences were assessed using the Student's t-test.

3. Results

3.1. Tetrandrine induced lipid accumulation in a PPARγ-independent manner

We initially investigated whether tetrandrine caused lipid accumulation, a typical characteristic of quiescent or non-fibrotic HSC, in HSC-T6 cells, stellate cell line derived from rats. HSC-T6 cells were incubated with tetrandrine for 24 h and then observed for intracellular lipid accumulation by Oil Red-O staining. As shown in Fig. 1A, treatment with tetrandrine dramatically increased LDs in HSC-T6 cells. We also confirmed that tetrandrine inhibited transforming growth factor-β (TGF-β)-mediated collagen production in HSC-T6 cells using Sirius Red staining, a well-characterized indicator of fibrosis (Fig. 1B). Peroxisome proliferator-activated receptor γ (PPARγ), a member of nuclear receptor super family, is believed to play a role in HSC quiescence and PPARγ receptor agonists inhibited activation of HSCs [14]. This led us to hypothesize that tetrandrine induces lipid accumulation through PPARγ activation. Effects of tetrandrine on PPARγ transactivation was evaluated by GAL4 DNA-binding domain/PPARγ LBD chimera protein using the plasmid pGAL4- PPARγ LBD and a luciferase reporter plasmid pUAS-tk-Luc containing the target sequence of GAL4 [15]. Troglitazone, a well-known PPARγ full agonist, significantly activated PPARγ based on activity in the luciferase reporter assay. In contrast, tetrandrine did not activate PPARγ (Fig. 1C), suggesting that tetrandrine stimulates lipid accumulation in stellate cells in a PPARγ-independent manner.

3.2. Tetrandrine induced lipid accumulation in HSC-T6 cells via blockade of autophagy

Based on previous reports that blockade of autophagy attenuates HSC activation and causes accumulation of LDs [6,8], we evaluated whether tetrandrine stimulates LD accumulation via blockade of the autophagy pathway. First, we assessed the cellular levels of the microtubule-associated protein 1 light chain 3 (LC3)-II, a phophatidylethanolamine-conjugated LC3 form and a marker for autophagosomes [16]. When HSC-T6 cells were treated with tetrandrine, conversion from LC3-I to LC3-II was increased in time- and dose-dependent manners (Fig. 2A and B), suggesting that tetrandrine modulated the autophagy pathway. Increases in LC3 protein levels should be interpreted carefully [2] because they can be caused by one of two independent mechanisms, activation of upstream autophagic pathways or inhibition at a later stage. Then, to examine the effects of tetrandrine on autophagic flux, we performed an LC3 turnover assay [2]. HSC-T6 cells were treated with tetrandrine in the presence or absence of E64d/Pepstatin A or bafilomycin A_1 ($BafA_1$), a lysosomal protease inhibitor or a specific inhibitor for vacuolar-type H^+ -ATPase (V-ATPase), respectively. As shown in Fig. 2C, co-treatment with E64d/Pepstatin A or $BafA_1$ did not affect LC3-II levels in HSC-T6 cells treated with tetrandrine, suggesting that tetrandrine inhibited autophagy. Also, the expression levels of SQSTM1/p62 (p62), a well-known autophagic substrate [2], were increased by treatment with tetrandrine along with $BafA_1$ in HSC-T6 cells (Fig. 2D). To provide further evidence that tetrandrine blocked autophagic flux, we established an HSC-T6 cell line stably expressing a tandem fluorescent-tagged LC3 (tfLC3) [17]. This protein was tagged with monomeric red fluorescent protein (mRFP) and enhanced green fluorescent protein (EGFP). After treatment with $BafA_1$, a positive control, both mRFP and EGFP puncta were remarkably increased and co-localized (Fig. 2E). Similarly, both punctate fluorescence were increased by treatment with tetrandrine. Taken together, these results strongly suggest that tetrandrine blocks autophagic flux, resulting in accumulation of autophagosomes in the cytosol.

Next, to examine whether tetrandrine regulates lipid accumulation through autophagy pathway, we established HSC-T6 cells stably expressing EGFP-fused LC3 (EGFP-LC3). After 24 h treatment with tetrandrine, EGFP-LC3 puncta were remarkably increased and co-localized with LDs stained by Nile Red, a specific fluorescent dye for LDs, and perilipin 1, a protein specific for LD surface [18] (Fig. 2F and G). These indicated that LC3-positive autophagosomes coexisted with accumulated LDs in cells treated with tetrandrine. In contrast, levels of adipose differentiation-related protein (ADRP) [18], another LD-associated protein, were not substantially changed nor co-localized with LC3 fluorescent puncta in cells treated with

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