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# Valproate induced hepatic steatosis by enhanced fatty acid uptake and triglyceride synthesis

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ABSTRACT

Steatosis is the characteristic type of VPA-induced hepatotoxicity and may result in life-threatening hepatic lesion. Approximately 61% of patients treated with VPA have been diagnosed with hepatic steatosis through ultrasound examination. However, the mechanisms underlying VPA-induced intracellular fat accumulation are not yet fully understood. Here we demonstrated the involvement of fatty acid uptake and lipogenesis in VPA-induced hepatic steatosis *in vitro* and *in vivo* by using quantitative real-time PCR (qRT-PCR) analysis, western blotting analysis, fatty acid uptake assays, Nile Red staining assays, and Oil Red O staining assays. Specifically, we found that the expression of cluster of differentiation 36 (CD36), an important fatty acid transport, and diacylglycerol acyltransferase 2 (DGAT2) were significantly up-regulated in HepG2 cells and livers of C57B/6J mice after treatment with VPA. Furthermore, VPA treatment remarkably enhanced the efficiency of fatty acid uptake mediated by CD36, while this effect was abolished by the interference with CD36-specific siRNA. Also, VPA treatment significantly increased DGAT2 expression as a result of the inhibition of mitogen-activated protein kinase kinase (MEK) – extracellular regulated kinase (ERK) pathway; however, DGAT2 knockdown significantly alleviated VPA-induced intracellular lipid accumulation. Additionally, we also found that sterol regulatory element binding protein-1c (SREBP-1c)-mediated fatty acid synthesis may be not involved in VPA-induced hepatic steatosis. Overall, VPA-triggered over-regulation of CD36 and DGAT2 could be helpful for a better understanding of the mechanisms underlying VPA-induced hepatic steatosis and may offer novel therapeutic strategies to combat VPA-induced hepatotoxicity.



## 1. Introduction

Valproate (VPA) is a broad-spectrum antiepileptic drug which has been widely used in the treatment of various epileptics with convulsive, bipolar, or schizoaffective disorders (Chateauvieux et al., 2010). It has been reported that VPA therapy is a long-term process and even sustaining in patient's lifetime. However, adverse drug reactions (ADR), such as hepatotoxicity, obesity, hemorrhagic pancreatitis, bone marrow suppression, and hyperammonemic encephalopathy, caused by VPA may occur during the whole antiepileptic therapy (Tsiropoulos et al., 2009). Thereinto, hepatotoxicity is one of the most frequent and severe ADR that limit the continual use of VPA. Approximately 61% of patients treated with VPA had been diagnosed with hepatic steatosis through ultrasound examination, and nearly 25% of patients developed non-alcoholic fatty liver diseases (NAFLD) which increase the risk of hepatic cirrhosis and malignancy (Luef et al., 2009; Farinelli et al., 2015). Besides, VPA was associated with hepatic fatalities at an incidence of 1/49000 in adults and 1/800 in children (Dreifuss et al., 1989). The hepatic damage was usually accompanied by elevations in serum aminotransferase and is pathologically characterized by the presence of microvesicular and macrovesicular steatosis, hepatocellular necrosis and fibrosis, and cholestatic liver injury (Zimmerman and Ishak, 1982). The National Institutes of Health has warned patients and doctors that VPA associated with an elevated risk of NAFLD and may result in life-threatening hepatic injury (Nanau and Neuman, 2013).

Despite the growing number of papers regarding VPA hepatotoxicity, the mechanisms underlying the development of this liver injury have not been fully understood. It is generally accepted that steatosis is the characteristic type of VPA-induced hepatic lesion and may result in life-threatening liver failure (Nanau and Neuman, 2013). Earlier study reported that the accumulation of liver fat could be attributed to the competitively inhibitory effect of VPA on mitochondrial fatty acid β-oxidation (Dreifuss and Langer, 1985). Also, VPA may inhibit endogenous synthesis of carnitine by suppressing the γ-butyrobetaine hydroxylase

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(Farkas et al., 1996). Wang et al. (2012) found that the mRNA expressions of sterol regulatory element-binding protein-1c (SREBP-1c), acetyl-CoA carboxylase 1 (ACC1), fatty acid synthase (FAS), and stearoyl-CoA desaturase 1 (SCD1) were elevated in primary hepatocytes after divalproex sodium treatment. By using oligonucleotide microarray analysis, Lee et al. (2008) indicated that >60 genes related to lipid transport, triglyceride (TG)/cholesterol biosynthesis, and catabolism of fatty acid in mice were changed by both acute and subchronic VPA treatment. However, their roles in the development of VPA-induced hepatic steatosis have not been elucidated yet (Avery and Bumpus, 2014).

Cluster of differentiation 36 (CD36), also known as fatty acid translocase, is an important membrane protein that was first identified on platelets as an 88 kDa thrombospondin-1 and collagen receptor (Hajri and Abumrad, 2002). It plays an important role in facilitating fatty acids uptake in the liver and adipose tissue of human (Pepino et al., 2014). The expression of CD36 had close associations with insulin resistance, metabolic syndrome, and NAFLD in human beings (Bradbury, 2006; Bokor et al., 2010; Noel et al., 2010; Love-Gregory et al., 2011). For example, humans with NAFLD had higher hepatic expression of CD36 (Greco et al., 2008). Further investigation revealed that activated CD36 directly accelerated the development of fatty liver through modulating fatty acid uptake rate of hepatocytes, whereas disruption of excess hepatic CD36 protected against hepatic steatohepatitis and insulin resistance in high-fat food-treated mice (Wilson et al., 2016b). In addition, higher CD36 expression was also observed in tetracycline-induced fatty liver (Choi et al., 2015b). However, the contribution of CD36 to VPA-induced hepatic steatosis has not been fully uncovered yet.

Diacylglycerol acyltransferase 2 (DGAT2) is a rate-limiting enzyme that catalyzes the final step of TG synthesis by introducing diacylglycerol to long chain fatty acyl-CoAs (Cases et al., 1998). DGAT2 is mainly expressed in liver and adipose tissue (Cases et al., 2001). Elevated DGAT2 expression was confirmed in chronic alcohol-induced fatty liver disease (AFLD) as a result of the suppression of transmethylation reactions (Wang et al., 2010). Up-regulated DGAT2 caused by suppressed mitogen-activated protein kinase kinase (MEK) – extracellular regulated kinase (ERK) pathway has been found to contribute to tetracycline-induced intracellular lipid accumulation *in vitro* (Choi et al., 2015b), whereas DGAT2 knockdown by antisense oligonucleotide reversed diet-induced hepatic steatosis and insulin resistance in mice (Yu et al., 2005; Choi et al., 2007). However, the role of DGAT2 in VPA-induced hepatic steatosis remains unknown.

Taken together, the objective of this study was to investigate the roles of fatty acid transport and triglyceride synthesis in VPA-induced hepatotoxicity *in vitro* and *in vivo*. We found that VPA-induced overregulation of CD36 and DGAT2 contributed to increased fatty acid uptake and esterification, respectively, leading to the development of steatosis *in vivo* and *in vitro*. In addition, we also found that SREBP-1c-mediated fatty acid synthesis may be not involved in VPA-induced fatty liver. These data could be helpful for a better understanding of the mechanisms underlying VPA-induced hepatotoxicity and may offer novel therapeutic strategies to combat VPA-induced hepatotoxicity.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Sodium valproate (VPA) (≥98% purity) and dexamethasone were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). 100× solution of insulin, ransferrin, Selenium (ITS-G), L-glutamine, and sodium pyruvate were purchased from Gibico (Grand Island, New York State, USA). Boron-dipyrromethene (BODIPY)-$C_{16}$ was purchased from Invitrogen Life Sciences (Carlsbad, California, USA). Powders of Oil Red O, Nile Red, 1× phosphate buffer saline (1× PBS), bovine serum albumin (BSA), and oleic acid (OLA) were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). Rabbit monoclonal anti-CD36 (ab133625) and anti-laminin B (ab133741) antibodies and mouse monoclonal anti-SREBP-1c (ab3259) antibody were purchased from Abcam (Cambridge, Massachusetts, USA). Mouse monoclonal anti-DGAT2 antibody (sc-293211) was from Santa Cruz Biotechnology (Santa Cruz, California, USA). Anti-rabbit (7074) and anti-mouse (7076) secondary horseradish peroxidase-conjugated antibodies and rabbit monoclonal antibodies against p-ERK1/2 (Thr202/Tyr204) (4376), ERK1/2 (5013), p-MEK1/2 (Ser217/221) (3958), MEK1/2 (8727), p-AMPK (Thr172) (4188), AMPK (5832), p-SREBP-1c (Ser372) (9874), FAS (3180), SCD1 (2794), and ACC1 (3676) were purchased from Cell Signaling Technology (Beverly, Massachusetts, USA). Mouse monoclonal anti-GAPDH antibody (D198662) was obtained from Sangon Biotech Co., Ltd. (Shanghai, China). Other chemicals were of analytical grade from commercial suppliers.

### 2.2. Animal experiments

Specific-pathogen-free male C57B/6J mice (8 weeks old, 18–22 g) were purchased from Guangdong Medical Laboratory Animal Center (No. CNASL3623; Guangzhou, China). They were kept in specific pathogen-free animal facility under controlled conditions at the temperature (24 ± 2 °C), humidity (55 ± 15%) and a 12-h light-dark cycle. All mice were allowed *ad libitum* access to water and food during adaption period. Since VPA could cause loss of appetite in rodents (Wolden-Hanson et al., 1998), all mice were receiving average amount of food that the mice of 500 mg/kg group had eaten the day before during experimental period to eliminate the interference of food intake on the study. All animal experiments have been conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Sun Yat-sen University, in compliance with the National Institute of Health and Nutrition Guidelines for the Care and Use of Laboratory Animals. The protocol was approved by the Animal Ethical and Welfare Committee of Sun Yat-sen University (Approval No. IACUC-DD-16-0321).

All mice were randomly divided into five groups after one week adaption period (6 mice per group). The VPA solution was prepared fresh in physiologic saline (pH 7.4) and four treatment groups were given VPA at 50, 100, 250, 500 mg/kg/day by intragastric administration for 14 consecutive days, respectively, while the control group received the same volume of physiologic saline by intragastric administration simultaneously. Mice were sacrificed 24 h after the last administration. Blood samples were collected for plasma biochemical assays. Livers were collected and weighed immediately. One portion of the liver was fixed in 10% neutral buffered formalin for histopathological analysis, while the remaining parts were stored for liver biochemical analysis and western blot analysis.

### 2.3. Cell culture and transient transfection assays

Human hepatoma cell line (HepG2) were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA) and maintained in Dulbecco's Modified Eagle Medium (DMEM, HyClone, Logan, Utah, USA) containing 10% fetal bovine serum (FBS, Gibco, Grand Island, New York State, USA) and 1% antibiotic-antimycotic solution (HyClone, Logan, Utah, USA). Cells were cultured at 37 °C in a humidified atmosphere of 5% $CO_2$ and were used between passages 8 and 16 in the study. Experiments were performed once the cells reached 90% confluency. The VPA and OLA solution for *in vitro* study was prepared fresh in DMEM and cells in treatment groups were treated with various concentrations of VPA (1, 2.5, and 5 mM) or 50 μM OLA, while the control group was only treated with DMEM simultaneously. The si-control and si-RNA for CD36 and DGAT2 were purchased from RiboBio Co., Ltd. (Guangzhou, China). HA-tagged CA-MEK overexpressing and mock plasmids were bought from YouBio Co., Ltd. (Hunan, China). HepG2 cells were seeded in 6-well plates at a density of $4 \times 10^5$ cells per well. Transient transfection of si-RNA or plasmid was performed using