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Valproic Acid-induced PPAR-alpha and FGF21 Expression Involves Survival Response in Hepatocytes

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Hepatocyte damage caused by medications or herbal products is one of the important problem when these compounds are chronically administrated. Thus, improving hepatocyte survival during treatment offers a wide range of opportunities. Valproic acid (VPA), a branched short-chain fatty acid derived from naturally occurring valeric acid, is commonly used to treat epilepsy and seizures. Although VPA exerts numerous effects in cancer, HIV therapy, and neurodegenerative disease, its effects on the liver and its mechanism of action have not been fully elucidated. Here, we demonstrated that VPA caused moderate liver cell toxicity and apoptosis. Interestingly, VPA treatment increased transcription levels of PPAR alpha (PPAR-α) and fibroblast growth factor 21 (FGF21) in murine (Hepa1c1c7) hepatoma cells in a time and concentration dependent manner. VPA-induced FGF21 expression was significantly weaker under PPAR-α silencing condition than in cells transfected with non-targeting control siRNA. Subsequent experiments showed that cell viability was significantly lowered when the FGF21 signaling pathway was blocked by FGF receptor antagonist. Finally, we further determined that AMPK phosphorylation was not responsible for VPA-induced FGF21 expression and PPAR-α increments. These results indicate that increases of FGF21 expression alleviate VPA-induced hepatic toxicity, thereby making FGF21 a potential biomarker for predicting liver damage during VPA treatments.

Key words : Cell viability, fibroblast growth factor 21, liver toxicity, PPAR-alpha, valproic acid

Introduction

Liver is the crucial organ that is responsible for supporting various functions, such as metabolism, immunity, digestion, and detoxification of alcohol. Liver toxicity, also known as toxic hepatitis or hepatotoxicity, is a condition where the liver becomes inflamed or damaged due to exposure to harmful substances [13]. Various substances like alcohol, drugs, chemicals, or nutritional supplements are leading cause of liver failure. One of the most important causes of liver dysfunction is drug-induced liver injury (DILI). DILI is a term that refers to liver damage caused by exposure to certain medications, herbal products, or dietary supplements [2, 19]. Some of the factors that influence the risk and severity of DILI include the type, dose, and duration of the drug, the genetic makeup of patient, and the interaction with other drugs or substances [5].

Valproic acid (VPA) is a medication that is primarily used to treat epilepsy and seizures, but is also used in bipolar, anxiety, and psychiatric disorders [8]. It is a chemical compound consisting of a branched-chain carboxylic acid, and its chemical name is 2-propylpentanoic acid. Mechanistically, it increases the concentration of gamma-aminobutyric acid (GABA) in the brain, which is an inhibitory neurotransmitter to control seizure activity and stabilize mood in bipolar disorder [15]. Recent study has shown that VPA acts as an HDAC inhibitor by preventing the removal of acetyl groups from histones. This inhibition results in increased histone acetylation, leading to a more open chromatin structure that allows for enhanced gene transcription [9, 10]. This modulation of gene expression can have various effects on cellular processes, such as cell cycle control, cellular differentiation, and apoptosis [28]. Thus, beyond its established uses in epilepsy and bipolar disorder, numerous researches has explored the potential use of VPA as an adjunct treatment in various con-

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ditions, including certain cancers and neurodegenerative disorders [14, 16, 25, 35]. However, VPA in high doses leads to hepatotoxicity, which can contribute to severe liver failure [7]. Although the exact mechanism of how VPA works is not fully understood, impairing mitochondrial function, depleting coenzyme A and carnitine by VPA is thought to contribute to liver toxicity [18, 21].

Fibroblast Growth Factor 21 (FGF21) is a protein that belongs to the fibroblast growth factor (FGF) family. It plays a crucial role in various metabolic processes, particularly those related to energy homeostasis and glucose and lipid metabolism [17]. FGF21 is produced by several tissues, including the liver, adipose tissue, and pancreas, in response to various physiological conditions [37]. Activation of PPAR-α has been shown to induce the expression of FGF21. This occurs as part of the adaptive response to fasting or conditions where increased fatty acid oxidation is required for energy production [32]. Because FGF21 promotes the breakdown of fats (lipolysis) and increases the utilization of fatty acids for energy, it has been implicated in the regulation of energy expenditure and body weight [23]. While FGF21 is primarily known for its roles in metabolic regulation, particularly in glucose and lipid metabolism, there is some evidence to suggest that FGF21 may have an impact on cell proliferation [22, 24]. Interestingly, a recent study shows that VPA can upregulate FGF21 gene expression and promote process elongation in glia by inhibiting HDAC 2 and 3. This suggests that VPA may have a new mechanism for promoting the survival of brain cells [20]. Although FGF21 can protect hepatocytes against drug-induced acute liver injury [12], potential effects of FGF21 on VPA-induced liver damage remain unclear. This study therefore evaluated whether FGF21 affects VPA-induced hepatotoxicity, and the cellular mechanism by which VPA increases FGF21 in murine hepatocytes.

Materials and Methods

Cell culture and material treatment

Murine hepatoma (Hepa1c1c7) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The Hepa1c1c7 cells were maintained in Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were maintained at 37°C with 5% CO_2 in a humidified chamber. Valproic acid (cat. no. P4543) and Compound C (cat. no. 171260) were purchased from Sigma–Aldrich (Sigma-Aldrich, St. Louis, MO, USA). PD173074 were obtained from SelleckChem (cat. no. S1264).

WST1 assay

For the WST1 assay, Hepa1c1c7 cells were seeded in 96-well culture plates (2×10^4 cells/well) and cultured for 24 hr and cells were exposed to the given dose of valproic acid. After discarding incubation medium, cells were treated with WST1 reagent (cat. no. 5015944001, Sigma-Aldrich, St. Louis, MO, USA) dissolved in growing media for 1 hr in a 37°C CO_2 incubator. After discarding the solution, 100 ml DMSO was added to each well, and the plate was vortexed for 10 min. The absorbance of the assay solution was measured at a wavelength of 540 nm.

Fluorescence-activated cell sorting (FACS) assay

For apoptosis measurement, Hepa1c1c7 cells were maintained in 6-well culture dish and incubated with an indicated concentration of valproic acid for 24 hr. After trypsinization, the cells were detached and collected by centrifugation (1500 rpm, 5 min). Harvested cells were incubated with FITC-Annexin V (BD Biosciences, cat. no. 556419) and 7-ADD (BD Biosciences, cat. no. 559925) in the dark for 20 min. Each population for living and apoptotic cells were analyzed using flow cytometry (FACS Aria; BD Bioscience, Franklin Lakes, USA). 5-Fluorouracil (cat. no. F6627-1G, Sigma-Aldrich, St. Louis, MO, USA) was used for positive control.

RNA*i* experiment

Scrambled siRNA, siRNAs against mouse PPAR-α were purchased from Santa Cruz (cat. no. sc-36308, Santa Cruz Biotechnology, Santa Cruz, CA). According to the protocol of the manufacturer for siRNA transfection, 12.5 μl of Opti-MEM containing 10 μM siRNA was added to 12.5 μl of Opti-MEM containing 2 μl of Lipofectamine 2000 (Invitrogen, Waltham, MA) after 5-min incubation at room temperature. After gentle mixing, the siRNA-Lipofectamine mixture was incubated at room temperature for 15 min and treated to the Hepa1c1c7 cells. Final concentration of siRNA for the silencing experiment was 100 nM.

Immunoblotting

Following antibodies were used for immunoblotting: Phospho-AMPKα (Thr172) (Cell signaling # 2535S), AMPKα (Cell signaling # 2532S), Phospho-p44/42 MAPK (Thr202/Tyr204) (Cell signaling #4370S), Actin (Cell signaling # 4967S). Immunoblotting was achieved by harvesting the treated cells and isolating the total proteins. To prepare total

cell lysates, the cells were washed with cold PBS and then lysed in cold lysis buffer (in mmol/liter: 40 HEPES, pH 7.5, 120 NaCl, 1 EDTA, 10 pyrophosphate, 10 glycerophosphate, 50 NaF, 1.5 Na3VO4, 1 PMSF, 5 MgCl2, 0.5% Triton X-100, and protease inhibitor mixture). The lysates were sonicated briefly, denatured by heating for 5 min at 95°C, then, separated on SDS–PAGE (9%) gels. After transfer, nitrocellulose membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T, pH 7.6). Each primary antibody was incubated overnight at 4°C. After washing membranes three times with TBS-T, blots were incubated with HRP-conjugated secondary antibody, washed three times with TBS-T, and detected by enhanced chemiluminescence (ECL system; GE healthcare, Amersham U.K.).

Quantitative RT-PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen, Waltham, MA), and cDNA was reverse-transcribed from 3 μg of total RNA using High-Capacity cDNA Reverse Transcription Kit (cat. no. 4368813, Applied Biosystems, Foster City, USA). A total volume of 20 μl of PCR amplification mixtures were prepared by mixing 10 μl of 2× SYBR™ Green PCR Master Mix (cat. no. 4364344, Applied Biosystems, Foster City, USA), 2 μl of the primer mix (1 μM forward and 1 μM reverse primers), and 8 μl of the diluted cDNA templates. Real-time quantitative PCR was performed on the QuantStudio Real-Time PCR system (Applied Biosystems, Foster City, USA) using the following conditions: 95°C for 1 min, followed by 40 amplification cycles comprising 95°C for 15 s, 60°C for 15 s (annealing), and 72°C for 30 s. After amplification, melting curve analysis was performed according to manufacturer's instructions. Oligo sequences for this study were as follows: *Ppar-α*, forward 5'- ACAAGGCC TCAGGGTACCA -3', reverse 5'- GCCGAAAGAAGCCCTT ACAG -3'; *Atf4*, forward 5'- ACTCTAATCCCTCCATGTGT AAAGG -3', reverse 5'- CAGGTAGGACTCTGGGCTCAT -3'; *Fgf21*, forward 5'- CCTCTAGGTTTCTTTGCCAACAG -3', reverse 5'- AAGCTGCAGGCCTCAGGAT -3'; *Gdf15*, forward 5'- GAGAGGACTCGAACTCAGAAC -3', reverse 5'- GACCCCAATCTCACCTCTG -3'; *Cd36*, forward 5'- GG AACTGTGGGCTCATTGC -3', reverse 5'- CATGAGAATG CCTCCAAACAC -3'; *Gapdh*, forward 5'- CAAGGTCATCC ATGACAACTTTG-3', reverse 5'-GGCCATCCACAGTCTT CTGG-3'. Expression levels were measured using the $\Delta\Delta^{CT}$ analysis.

Statistical analysis

All data were evaluated using the GraphPad software (San Diego, CA, USA) and are expressed as mean ± S.E.M. Data for multiple variable comparisons were analyzed by a one-way ANOVA with Tukey post hoc test, and comparison between two groups was analyzed by unpaired 2-tailed Student's *t*-test, with a $p<0.05$ considered significant.

Results

Effect of Valproic acid on cellular viability

To confirm whether VPA has cytotoxic effects in hepatocytes, we performed the WST1 assay to evaluate hepatoma cells viability. Contrast to previous report, which confirmed non-toxicity of VPA using C2C12 myotubes [30], there was significant decreases in the hepatic cell number (Fig. 1A) and viability (Fig. 1B) after VPA treatment. In addition, cell necrosis and apoptosis were assessed using 7-AAD and Annexin V dyes following VPA treatment by flow cytometry. As shown at Fig. 1C, the percentage of apoptotic cells from VPA-treated group was slightly, but significantly, increased (Vehicle: 2.8±0.21%, VPA 5 mM: 4.57±0.20%). However, necrotic cell death was not detected (Vehicle: 0.2±0.07%, VPA 5 mM: 0.1±0.03%).

Valproic acid increases transcript levels of fibroblast growth factor 21

Previous studies have shown that FGF21 affects cell survival rates in various cell types [22, 36]. To elucidate the effects of VPA on FGF21 expression, Hepa1c1c7 cells were treated with various concentration of VPA for 24 hr. As a results, we confirmed that VPA treatment induced a significant increase in the FGF21 mRNA levels in a dose-dependent manner (Fig. 2A). Based on kinetic experiment, VPA appeared to increase FGF21 transcripts after 6 hr (Fig. 2B). Meanwhile, Growth/differentiation factor 15 (GDF15) levels, which is another important hepatocyte-derived hormone, was significantly suppressed by VPA treatment (Fig. 2A).

Next we checked what kind of transcription factor is responsible for VPA-induced FGF21 expression. It has been demonstrated that FGF21 expression is regulated by peroxisome proliferator-activated receptor alpha (PPAR-α) or activating transcription factor 4 (ATF4) under various stress conditions [6]. Interestingly, VPA treatment increased mRNA expression of PPAR-α in a dose- and time-dependent manner (Fig. 3A, 3B), whereas ATF4 levels remained similar (Fig. 3A). Finally, the *in vitro* experiments demonstrated that si-

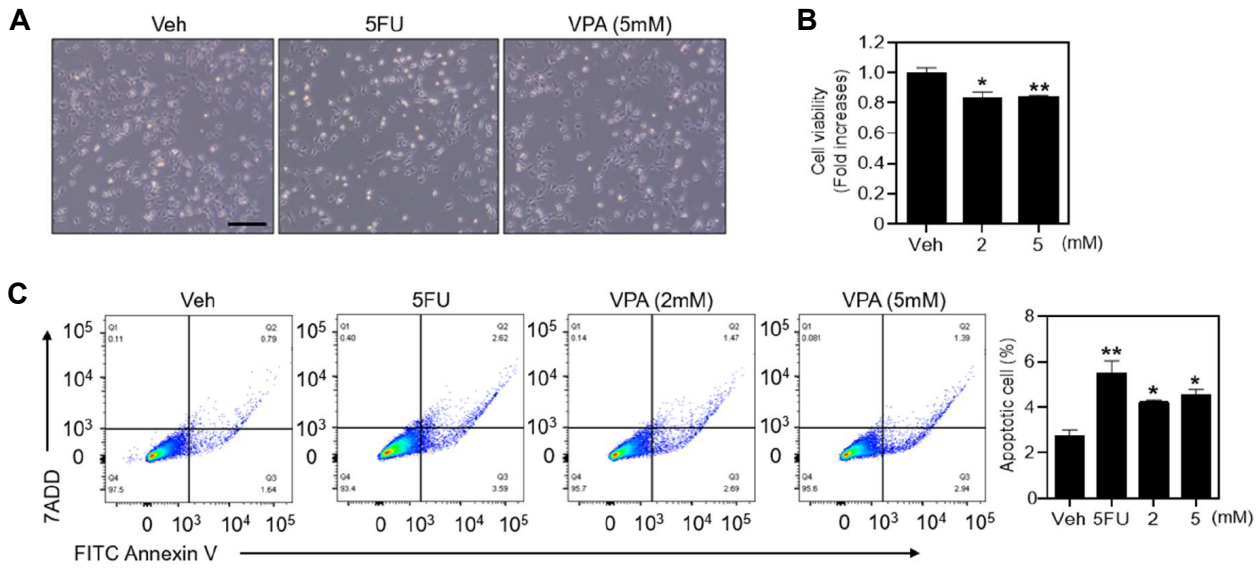

Fig. 1. Cytotoxicity of valproic acid in hepatocytes. (A) The cell images were obtained after 5 mM VPA treatment for 24 hr using light microscopy. (B) The survival rate was evaluated via WST1 assay after treatments with indicated concentrations of VPA in Hepa1c1c7 cells. (C) Following VPA treatment for 24 hr, apoptosis was determined by FACS assay using Annexin V/7-ADD double staining. Values are shown as ± S.E.M *p<0.05 and **p<0.01.

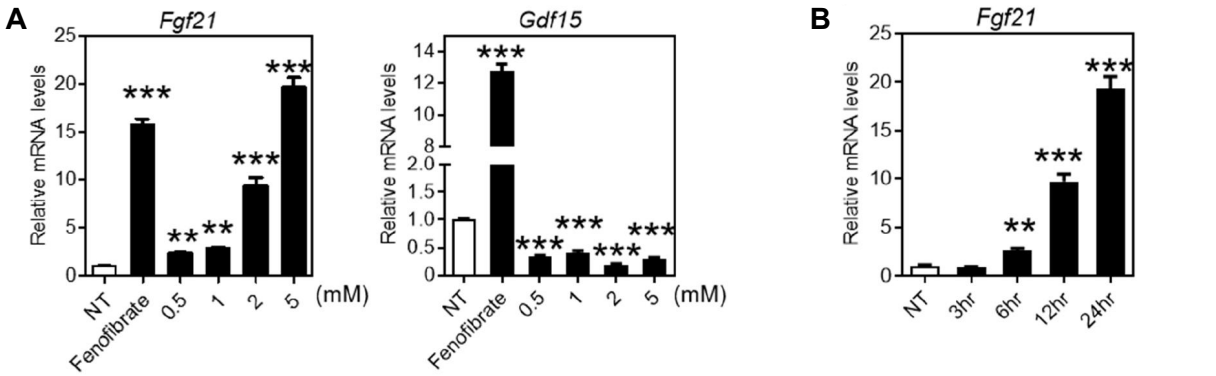

Fig. 2. Valproic acid increased Fgf21 gene expression. (A) Hepa1c1c7 cells were treated with indicated concentration of VPA for 24 hr. Relative hepatic *Fgf21* and *Gdf15* mRNA levels were determined by real-time qPCR. Fenofibrate was used as a positive control. (B) The cells were treated with 5 mM VPA at different time points. Values are shown as ± S.E.M **p<0.01 and ***p<0.001.

lencing of PPAR-α partially inhibited the FGF21 expression (Fig. 3C).

PPAR-α and FGF21 expression by Valproic acid occurs in AMPK-independent manner

Recent study reported that VPA stimulates AMP-activated protein kinase (AMPK) activity in human hepatocytes [3]. Similar to this study, VPA also increased phosphorylation of AMPK in mouse hepatoma cells (Fig. 4A). It is noteworthy that AMPK activates the PPAR-α expression. To determine whether AMPK activity affects VPA-induced PPAR-α and FGF21 expression, pharmacological AMPK inhibitor, Compound C was treated with VPA. Importantly, the transcript levels of Cd36, which is a downstream target of AMPK, were largely suppressed when AMPK activity was blocked (Fig. 4B). However, the induction of PPAR-α and FGF21 by VPA remained similar between control and Compound C-treated group (Fig 4B). Taken together, these results indicate that AMPK activation is not responsible for VPA-induced PPAR-α and FGF21 expression.

Inhibition of FGF21 signaling enhances the effect of valproic acid in reducing cell viability

Next, we wanted to investigate whether FGF21 signaling

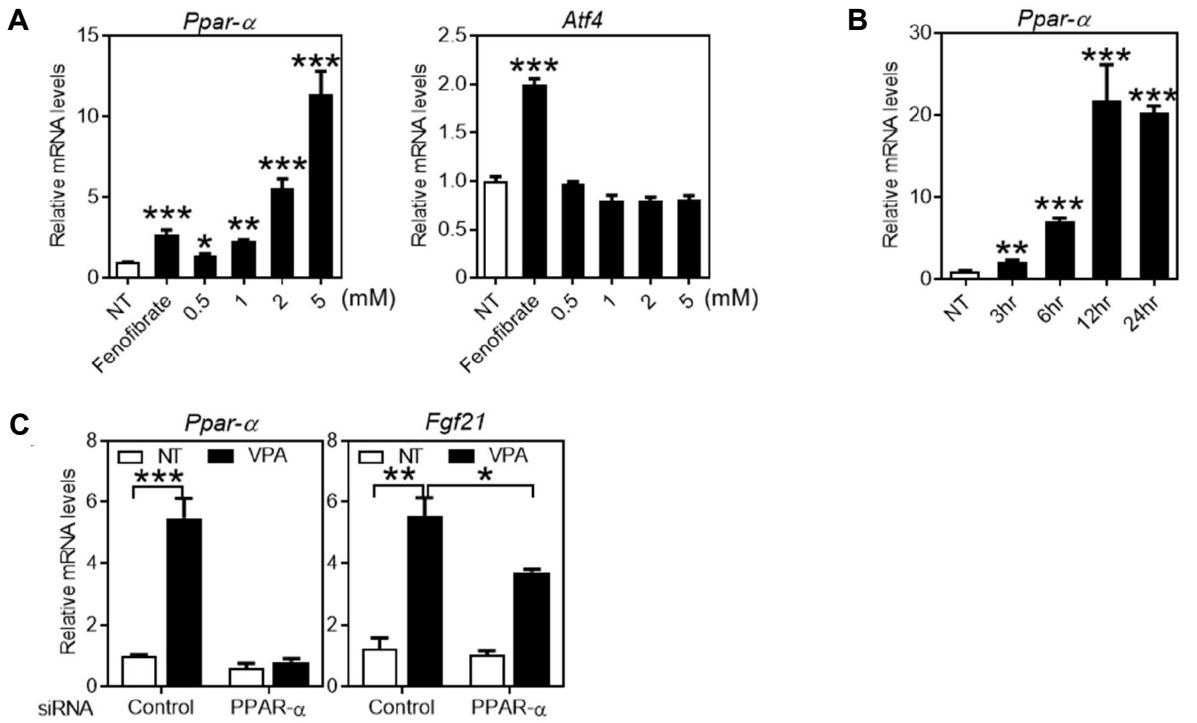

Fig. 3. Valproic acid regulated Fgf21 gene expression through PPAR-α in hepatocytes. (A) Hepa1c1c7 cells were treated with indicated concentration of VPA for 24 hr. Relative hepatic *Ppar*-α and *Atf4* mRNA levels were determined by real-time qPCR. (B) The cells were treated with 5 mM VPA at different time points to confirm *Ppar*-α mRNA levels. (C) Inhibition of PPAR-α expression in Hepa1c1c7 cells lowered *Fgf21* transcript levels. The cells were transfected with PPAR-α siRNA as indicated (final 100 nM). After 12 hr, VPA was incubated for 24 hr Values are shown as ± S.E.M *p<0.05, **p<0.01, and ***p<0.001.

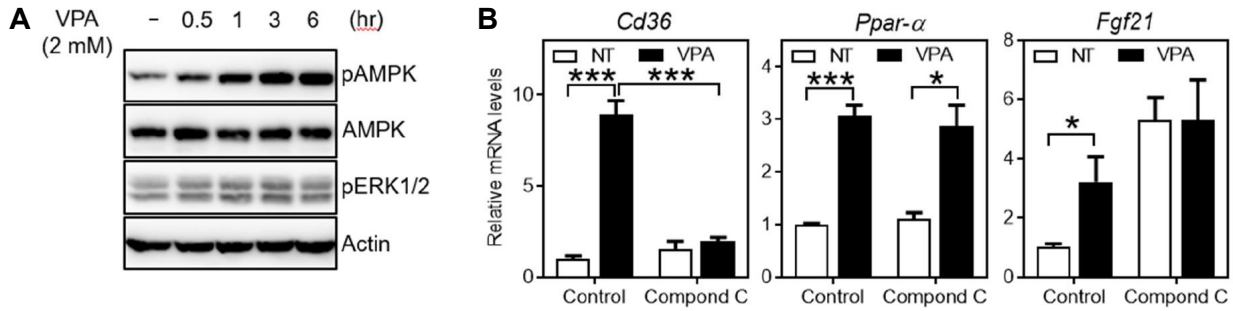

Fig. 4. Effects of AMPK inhibition on valproic acid-induced FGF21 and PPAR-α expression. (A) Hepa1c1c7 cells were treated with VPA (2 mM). The lysates were subjected to immunoblot assay using indicated antibodies. (B) qPCR analysis for checking mRNA expression of CD36, PPAR-α, and FGF21 using AMPK inhibitor. VPA was treated with either vehicle or Compound C (10 μM) for 12 hr. Values are shown as ± S.E.M *p<0.05 and ***p<0.001.

could affect AMPK activation. PD173074, which is a potent kinase inhibitor targeting FGFR1 and 3, is known to inhibit FGF21 signaling [1]. As shown in Fig. 5A, AMPK phosphorylation did not change upon FGF receptor antagonist, PD173074 treatment. Numerous studies have shown that FGF21 protects against cellular damage under various stress conditions [11, 34]. To evaluate whether VPA-induced FGF21 expression is a natural protective mechanism in hepatocytes, cells viability under VPA treatment was determined with FGF receptor antagonist. As expected, blockade of FGF21 signaling using PD173074 further deteriorated VPA-induced cell toxicity. Interestingly, AMPK inhibition by Compound C showed similar effects (Fig. 5B). Taken together, our findings suggest that both FGF21 expression and AMPK activation

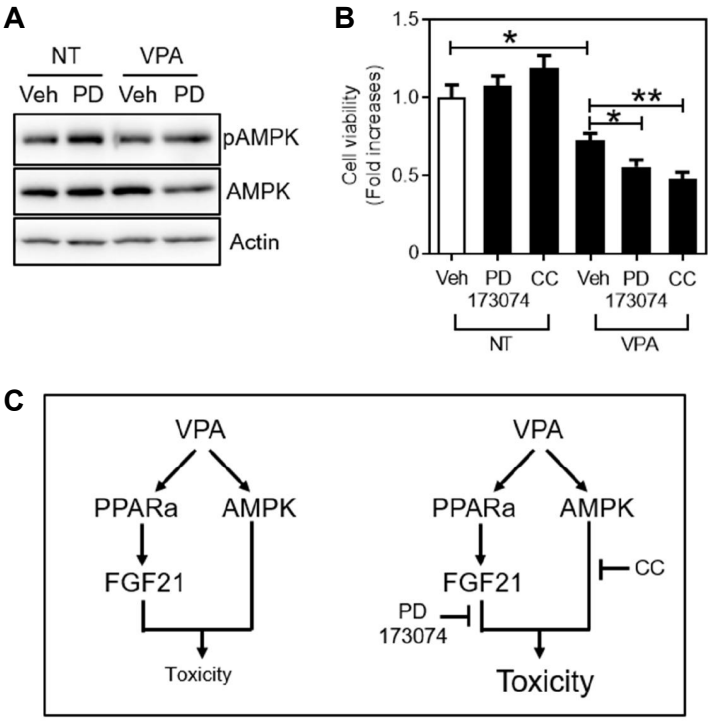

Fig. 5. FGF21 and AMPK regulated cell viability independently. (A) Hepa1c1c7 cells were treated with a combination of VPA (2 mM) and PD173074 (100 nM) for 30 minutes. The lysates were subjected to immunoblot assay using indicated antibodies. (B) Cell viability was measured after inhibiting FGF21 or AMPK signaling pathway. Either PD173074 (100 nM) or Compound C (3 μM) was treated with VPA for 24 hr. Values are shown as ± S.E.M *p<0.05 and **p<0.01. (C) Working mechanisms illustrating the protective role of FGF21 and AMPK against VPA-induced cell toxicity.

might function to protect the hepatocytes against VPA-induced cellular damage and apoptosis (Fig. 5C).

Discussion

In the present study, we have proved that valproic acid (VPA), which known to have anti-epileptic and anti-convulsant activity shows mild hepatotoxicity. Importantly, hepatocytes has intrinsic defense mechanism against the damages by increasing FGF21 expression and AMPK activity. Thus, this study suggests that measuring FGF21 levels or AMPK activity can be good parameters to evaluate potential liver damage during VPA treatment in patients.

Our results suggest that inhibition of PPAR-α expression partly decreases VPA-induced FGF21 mRNA levels (Fig. 3C). Although the knock-down effects of PPAR-αwas quite significant, FGF21 transcript levels from VPA treated group were still higher than NT under PPAR-α silencing condition. These results imply that various types of transcriptional regulation would exist in VPA-induced PPAR-α and FGF21 regulation. In eukaryotes, genes can also be regulated by chemical modifications of DNA and histones, which are proteins that package DNA into chromatin. For example, DNA methylation and histone acetylation are two common epigenetic modifications that are associated with gene silencing and activation, respectively [27]. As an epigenetic modulator, VPA can inhibit the activity of histone deacetylases (HDACs), which are enzymes that remove acetyl groups from histone proteins and affect gene expression [29]. Because some studies already have shown that HDAC inhibitors can upregulate FGF21 expression in hepatocytes [33, 38], VPA can modulate FGF21 expression through histone modification, in addition to PPAR-α pathway. To prove this possibility, histone acetylation near FGF21 promoter region after VPA treatment should be determined.

Mechanisms by which FGF21 decreases VPA-induced cell toxicity is not clear yet. Several studies reported that VPA treatment induced reactive oxidative species (ROS) generation that cause disruption of cell viability [26, 31]. Interestingly, recent study from Chen et al. demonstrated that FGF21 modulates expression of antioxidant enzymes and activate the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway to protect cells from oxidative stress-induced damage in various tissues, including heart and kidneys [4]. Given that FGF21 has been shown to upregulate the expression of antioxidant enzymes in the liver, such as superoxide dismutase (SOD) and catalase, determining whether the FGF21 treatment reduces VPA-induced oxidative species levels to affect cell viability, is also a worthwhile study.

In summary, we demonstrated that valproic acid largely increases PPAR-αand FGF21 mRNA levels, which are an important factors for inhibiting cell toxicity induced by VPA.

Since VPA-induced metabolic syndrome, such as weight gain, dyslipidemia, and diabetes [39] is also associated with abnormal regulation of liver physiology, FGF21 co-treatment may provide new strategy for preventing adverse drug reaction (ADR) of valproic acid.

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : Valproic acid에 의해 증가하는 PPAR-alpha 및 FGF21의 발현이 간세포 생존에 미치는 영향

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약물이나 허브 제품으로 인한 간세포 손상은 이러한 화합물을 만성적으로 투여할 때 일어날 수 있는 중요한 문제 중 하나이다. 따라서 여러 가지의 치료 과정 중 간세포 생존율을 향상시키는 것은, 약물 적용에 있어 광범위한 기회를 제공할 수 있다. Valproic acid (VPA)는 자연적으로 발생하는 발레르산에서 파생된 분지형 단사슬 지방산으로 뇌전증과 발작을 치료하는 데 일반적으로 사용된다. 뿐만 아니라 VPA는 암, HIV 치료, 신경 퇴행성 질환에 수많은 효과를 발휘하지만, 간에 대한 VPA의 잠재적인 영향과 그 작용 메커니즘은 완전히 설명되지 않았다. 여기서, 우리는 VPA의 처리가 쥐 간세포(Hepa1c1c7)에서 PPAR 알파(PPAR-α)와 섬유아세포 성장 인자 21(FGF21)의 전사 수준을 증가시킨다는 것을 입증했다. VPA에 의해 유도된 FGF21 발현은 PPAR-α 결손 조건에서 상당히 억제되었다. 후속 실험에서 FGF21 신호 경로가 FGF 수용체 억제제에 의해 차단되었을 때, 간세포 생존력이 크게 억제되었음을 보여주었다. 마지막으로, 우리는 AMPK 인산화가 VPA에 의해 유도된 PPAR-α 증가에 작용하지 않는다는 것을 추가로 확인했다. 이러한 결과는 FGF21 발현의 증가가 VPA에 의해 유도된 간 독성을 완화시킬 수 있다는 것을 제시하며, 이와 같은 결과는 FGF21의 증감 여부가 VPA 치료 중 나타날 수 있는 간 손상을 예측하는 잠재적인 바이오마커로 사용될 수 있음을 제시한다.