Valproate activates ERK signaling pathway in primary human hepatocytes

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Aim. Valproic acid (VPA) is a widely-used anticonvulsant and mood-stabilizing agent. VPA is also known to inhibit histone deacetylases (HDACs) affecting the expression of numerous genes.

Methods. In the present study, we examined the effect of VPA on the extracellular signal-related kinase (ERK, p42/p44) pathway (Ras-Raf-MEK-ERK) belonging to the mitogen-activated protein kinases (MAPKs) pathways in primary human hepatocytes. In the liver, the pathway is associated with progression of hepatocellular carcinoma.

Results. We found that VPA in a therapeutically relevant concentration (500 μ M) activates the ERK pathway, as indicated by increased ERK Thr202/Tyr204 phosphorylation. Interestingly, a prototype HDAC inhibitor, trichostatin A, also activated ERK phosphorylation in primary human hepatocytes. These data suggest that HDAC inhibition might be the primary stimulus for ERK pathway activation in primary human hepatocytes. Notably, U0126, a MEK1 inhibitor, was ineffective in inhibiting ERK pathway activation, likely due to its metabolic deactivation in metabolically competent primary human hepatocytes.

Conclusion. We conclude that VPA activates the ERK pathway in primary human hepatocytes.

Key words: valproic acid, ERK pathway, cellular signaling

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INTRODUCTION

Valproic acid (VPA) has been used in the treatment of epilepsy for almost 40 years¹. Currently, it is used for its anti-seizure activity, as well as in the treatment of migraine and bipolar disorders^{2,3}. Valproate is also used off-label for the treatment of other psychiatric diseases. In the treatment of epilepsy, VPA is an effective broad-spectrum anticonvulsant used in the treatment of primary generalized tonic-clonic, absence, and partial seizures^{4,5}. In the human brain, valproic acid primarily affects the function of the neurotransmitter γ -aminobutyric acid (GABA) by potentiating the inhibitory activity of GABA through several pathways, including the inhibition of GABA degradation, increased synthesis of GABA, and decreased GABA turnover⁶.

VPA is commercially available, for instance as Convulex, Depakine, or Stavzor. Although VPA is a well-tolerated drug, hepatotoxicity⁷⁻⁹, bone marrow suppression¹⁰, decreased bone cell production, and osteomalacia are some of its adverse effects¹¹. Even though it is extremely rare, the potential hepatotoxicity resulting in fatal liver failure is of major concern in treating patients with valproate^{12,13}. The mechanism of the hepatotoxicity is not clear¹². In addition to the hepatotoxicity, VPA is a human teratogen¹⁴.

It is well-known that VPA activates the mitogen-activated protein kinases (MAPKs) pathways in different cell lines¹⁵⁻¹⁸. In addition, it has been demonstrated that valproic acid interferes with multiple cellular regulatory

mechanisms, including histone deacetylases, GSK3 α and β , Akt, the phosphoinositol pathway, the tricarboxylic acid cycle, GABA, and the OXPHOS system¹⁵. Valproate has been recognized as a histone deacetylases (HDAC) inhibitor (class I and IIa) in micromolar concentrations¹⁹. Therefore, VPA treatment promotes histone acetylation allowing the chromatin to adopt a relaxed structure which facilitates binding of various transcription factors. Thus, VPA is believed to induce global transcriptional changes. Concordantly, recently obtained data have shown that valproic acid affects cell growth, differentiation, apoptosis, and immunogenicity of cultured cancer cells and tumors¹⁵. Recently, we have identified VPA as the activator of pregnane X receptor (PXR) and inducer of CYP3A4 gene in primary human hepatocytes²⁰.

The MAPKs (from the family of serine/threonine kinases) regulate diverse cellular programs by transferring extracellular signals to intracellular responses via downstream transcription factors. In mammals, there are more than a dozen MAPK enzymes that coordinately regulate cell proliferation, differentiation, motility, and survival. The best known MAPKs are the extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun amino-terminal kinases 1 to 3 (JNK1- to 3), p38 (α , β , γ , and δ), and ERK5 (ref.²¹). MAP kinases are activated after cell stimulation by a variety of growth factors and hormones and as well as during cell response to stress.

The extracellular signal-related kinase (ERK, p42/p44) pathway (Ras-Raf-MEK-ERK) one of the MAPKs pathways is known to be crucial in many biological pro-

cesses, such as cell proliferation, motility, survival, and carcinogenesis. Importantly, Ras-driven liver tumorigenesis by targeting its downstream effectors, including the Raf-MEK-ERK and PI3K-AKT-mTOR pathways, is a well-known factor²². Several *in vitro* studies showed that valproic acid specifically triggers phosphorylation of ERK, the upstream modulator of AP-1(ref.^{16,17}). Interestingly, it has been proposed that VPA activates pERK2 through attenuation of the PKA signalling pathway and this might be the common mechanism for VPA and lithium in the treatment of bipolar disorder²³. In addition, although some reports suggest that HDAC inhibition mediated by VPA causes ERK pathway activation, there are also contradictory data observed in other tissues^{16,24,25}.

In an earlier paper, we demonstrated that VPA activated ERK but not c-Jun-N-terminal kinase (JNK) nor p38 MAPKs in HEK cells²⁶.

The effect of valproate on MAPKs activation in quiescent non-tumour primary human hepatocytes has however, not been studied.

For this reason, in the current paper, we concentrated on the effect of valproate on ERK pathway activation in three primary human hepatocyte cultures by Western blotting using phospho-specific antibodies. We also used

trichostatin A (TSA), a model HDAC inhibitor, to elucidate whether HDAC inhibition leads to ERK pathway activation in primary human hepatocytes.

METHODS

Chemicals

VPA and trichostatin A (TSA) were purchased from Sigma-Aldrich (St. Louis, USA). TSA, an antifungal antibiotic produced by *Streptomyces hygroscopicus*, is a potent and specific inhibitor of HDACs from classes I, IIa and IIb leading to an increase in histone acetylation^{27,28}. TSA is active at nanomolar concentration and causes accumulation of highly acetylated histone molecules in mammalian cells.

U0126 (purchased from Cell Signaling Technology, Inc., Boston, MA) is a chemically synthesized organic compound that inhibits Mitogen activated protein kinase Kinase 1 and 2 (also known as MEK1 and MEK2, with IC₅₀ in nanomolar concentrations). U0126 inhibits both active and inactive MEK1 and MEK2 kinases²⁹.

Fig. 1. The effects of VPA (500 μM), TSA (100 nM) and MEK1 inhibitor U0126 (10 μM) on ERK pathway activation were examined in three independent primary human hepatocyte cultures LH18, LH19 and LH21. After isolation, the cells were plated on collagen-coated culture dishes at the density of 1.4×10^5 cells/cm². The medium was replaced with serum-free medium the following day, and the cells were allowed to get stabilized for an additional 48 to 72 h before the treatment with VPA (500 μM), TSA (10 nM) or U0126 (10 μM) or their combinations, carried out for another 24 h. We analyzed total cellular lysates by Western blotting with a phospho-specific Thr202/Tyr204 ERK antibody and β-actin was used as a loading control.

Primary human hepatocyte cultures

Human liver samples used in this study were obtained from several donors: (LH18) - a woman, 69 years old, tumor metastasis; and (LH19) - a woman, 46 years old, Caroli syndrome. LH21 hepatocyte preparation was described in our previous paper³⁰. Hepatocytes were isolated as described previously³¹. After isolation, the cells were plated on collagen-coated culture dishes at the density of 1.4×10^5 cells/cm². The culture medium was enriched for plating with 2% FCS (v/v). The medium was replaced with serum-free medium the following day, and the cells were allowed to get stabilized for an additional 48 to 72 h before the treatment with VPA (500 μ M), TSA (10 nM) or U0126 (10 μ M) or their combinations, carried out for another 24 h.

Western blotting detection of proteins

The total protein extracts from cultures were prepared as we have described elsewhere^{32,33}. SDS-PAGE gels were run on a Hoefer apparatus according to the general procedure and protein transfer onto a polyvinylidene difluoride membrane was carried out. The membrane was stained with Ponceau S for control of transfer and then saturated with 8% non-fat dried milk in Tris-buffered saline (TBS) for 2 h at the room temperature. Blots were probed with primary antibodies against p42/44 MAPK phospho Thr202/Tyr204 (rabbit polyclonal antibody, ref 9101S) purchased from Cell Signaling Technology Inc. (Danvers, USA). β -actin was used as a loading control using goat polyclonal antipody (I-19; Santa Cruz Biotechnology). Chemiluminescence detection was performed using horseradish peroxidase conjugated secondary antibodies and an Amersham (GE Healthcare, Piscataway, USA) ECL kit. Films were scanned and the intensity of the bands was evaluated by densitometry.

RESULTS

In three independent primary human hepatocyte cultures, we examined the effects of VPA (500 μ M), TSA (100 nM) and MEK1 inhibitor U0126 (10 μ M) on ERK pathway activation. We analyzed total cellular lysates by Western blotting with a phospho-specific Thr202/Tyr204 ERK antibody.

We found that VPA activates phosphorylation of ERK in all three primary human hepatocyte cultures (Fig. 1).

In order to eliminate the potential effect of dimethyl sulfoxide (DMSO) used as a vehicle for VPA, TSA, and U0126, we also tested the effect of VPA in the absence of DMSO. In this case, PBS buffer was used as a vehicle for VPA. We observed a consistent effect of VPA on ERK phosphorylation irrespective of vehicle used (Fig. 1). Notably, DMSO itself activated ERK phosphorylation. Consistently, the effect of VPA dissolved in DMSO was weaker than the effect of VPA dissolved in PBS as a vehicle.

TSA also activated ERK phosphorylation, however, the effect was modest (Fig. 1). U0126, an ERK cascade

inhibitor, had no effect on the ERK activation by VPA, which may be the consequence of U0126 metabolic deactivation in primary human hepatocytes.

Collectively, these data suggest that VPA activates the ERK pathway in therapeutically relevant concentrations in primary human hepatocytes.

DISCUSSION

VPA, an effective broad-spectrum anticonvulsant, has proven to affect the expression of a large number of genes³⁴. Some of these alterations in gene expression have been attributed to the HDAC inhibitory activity of VPA. However, it is also well-known that VPA activates different MAP kinases and other signaling pathways, such as GSK3 α and β , Akt, the phosphoinositol pathway, and the tricarboxylic acid cycle in different cells¹⁵.

Recently, we found that VPA slightly activated the extracellular signal-regulated kinase (ERK) and weakly inhibited c-Jun N-terminal kinase (JNK), while it had no effect on p38 kinase in HEK293 cells²⁶.

However, a completely different pattern of MAPKs activation in other cell lines and tissues has been demonstrated in previous research. For example, VPA induced the phosphorylation of p38 but neither JNK nor extracellular signal-regulated kinase (ERK1/2) in lymphocytes³⁵. In microglial cells, VPA increased the level of phospho-p38 mitogen-activated protein kinase (MAPK), but had no effect on phospho-ERK and phospho-JNK MAPKs (ref.³⁶). These data suggest the need to study the effect of VPA in different cell lines and tissues. In addition, there is lack of information about the effect of VPA on cell signaling cascade activation in primary human cells derived from normal non-transformed tissues.

Hepatocellular carcinoma (HCC) is the most common and malignant type of liver cancer, which causes approximately 0.6 million deaths each year³⁷. Importantly, activation of the Raf-MEK-ERK and PI3K-AKT-mTOR pathways has been reported in human HCCs (ref.^{22,38-41}) and pharmacological inhibition of these pathways has exhibited significant anti-tumoral effects in clinical trials⁴².

Therefore, it is of importance to learn about the impact of VPA on MAPKs activation in normal hepatocytes. Our current data suggest that VPA activates the Ras-Raf-ERK cascade in primary human hepatocyte cultures in therapeutically relevant concentrations (500 μ mol/L). The therapeutic steady state C_{ss} level of VPA is about 100 μ g/mL (corresponding to 600 μ mol/L) (ref.⁴³). Serious intoxication is likely to occur at levels greater than 450 μ g/mL.

CONCLUSION

In conclusion, VPA activates the extracellular signal-related kinase (ERK) pathway in primary human hepatocytes, which may be the consequence of its HDAC inhibitory activity. These data encourage further study

of the effects of VPA on cellular signaling and gene expression profile in normal primary human hepatocytes with the aim of reducing the risk of HCC progression in patients treated with VPA.

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