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Sodium valproate induces mitochondrial respiration dysfunction in HepG2 *in vitro* cell model

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ABSTRACT

Sodium valproate (VPA) is a potentially hepatotoxic antiepileptic drug. Risk of VPA-induced hepatotoxicity is increased in patients with mitochondrial diseases and especially in patients with *POLG1* gene mutations. We used a HepG2 cell *in vitro* model to investigate the effect of VPA on mitochondrial activity. Cells were incubated in glucose medium and mitochondrial respiration-inducing medium supplemented with galactose and pyruvate. VPA treatments were carried out at concentrations of 0–2.0 mM for 24–72 h. In both media, VPA caused decrease in oxygen consumption rates and mitochondrial membrane potential. VPA exposure led to depleted ATP levels in HepG2 cells incubated in galactose medium suggesting dysfunction in mitochondrial ATP production. In addition, VPA exposure for 72 h increased levels of mitochondrial reactive oxygen species (ROS), but adversely decreased protein levels of mitochondrial superoxide dismutase SOD2, suggesting oxidative stress caused by impaired elimination of mitochondrial ROS and a novel pathomechanism related to VPA toxicity. Increased cell death and decrease in cell number was detected under both metabolic conditions. However, immunoblotting did not show any changes in the protein levels of the catalytic subunit A of mitochondrial DNA polymerase γ, the mitochondrial respiratory chain complexes I, II and IV, ATP synthase, E3 subunit dihydrolipoyl dehydrogenase of pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and glutathione peroxidase. Our results show that VPA inhibits mitochondrial respiration and leads to mitochondrial dysfunction, oxidative stress and increased cell death, thus suggesting an essential role of mitochondria in VPA-induced hepatotoxicity.

1. Introduction

Sodium valproate (VPA) is a widely used antiepileptic drug in various types of epileptic seizures (Perucca, 2002). In clinical practice, the therapeutic range of VPA is fairly narrow 300–700 μm/l (Silva et al., 2008; Callaghan et al., 1985). VPA is known to be a hepatotoxic drug, which can cause reversible or fatal liver injury (Schmid et al., 2013; Silva et al., 2008; Koenig et al., 2006; Bryant and Dreifuss, 1996; Cotariu and Zaidman, 1988; Scheffner et al., 1988). VPA-induced hepatic injury commonly occurs during the first 5–90 days of drug administration, but it can also cause delayed hepatic injury after several months or years (Schmid et al., 2013;

Abbreviations: DLD E3, dihydrolipoyl dehydrogenase, the E3 subunit of pyruvate dehydrogenase complex and 2-oxoglutarate dehydrogenase complex; GPX, glutathione peroxidase; MMP, mitochondrial membrane potential; OCR, oxygen consumption rate; pol γA, catalytic subunit A of mitochondrial DNA polymerase γ; ROS, reactive oxygen species; SOD2, mitochondrial superoxide dismutase 2; VPA, sodium valproate.

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Koenig et al., 2006; Lee, 2003; Bryant and Dreifuss, 1996). The potential hepatotoxicity of VPA even at therapeutic doses poses a challenge for its clinical use (Schmid et al., 2013; Koenig et al., 2006; Bryant and Dreifuss, 1996; Scheffner et al., 1988). VPA is associated with two types of hepatotoxicity: (i) dose-dependent increase in liver enzymes and decrease in fibrinogen, which can be reversed by discontinuation of the drug with full clinical recovery, and (ii) idiosyncratic, irreversible and ultimately fatal liver failure (Silva et al., 2008; Koenig et al., 2006; Cotariu and Zaidman 1988). The latter type is characterized by microvesicular steatosis and liver necrosis (Koenig et al., 2006).

The exact mechanism of valproate-induced hepatotoxicity is still unclear. As microvesicular hepatosteatosis is a typical feature for VPA-induced liver damage, it has been suggested that the hepatotoxic effect of VPA is caused by mitochondrial dysfunction, with impaired mitochondrial β-oxidation playing a central role in the intracellular accumulation of triglycerides and the development of lactic acidosis (Begriche et al., 2011; Silva et al., 2008; Lee, 2003; Pessayre et al., 1999). In addition, VPA has been shown to inhibit mitochondrial respiration in isolated rat liver mitochondria (Ponchaut et al., 1992; Haas et al., 1981), but there are only few previous studies on VPA toxicity using human liver models. Patients with metabolic diseases – particularly those affecting mitochondrial metabolism – are at high risk of developing VPA-induced hepatotoxicity (Silva et al., 2008). Mutations in the *POLG1* gene encoding the catalytic subunit A (pol γA) of mitochondrial DNA polymerase γ (Graziewicz et al., 2006; Kaguni 2004,) can cause impaired function of mitochondrial DNA polymerase leading to rearrangements of mitochondrial genome and mitochondrial dysfunction (Chan and Copeland 2009). Previous studies demonstrate that patients with mutations in the *POLG1* gene – in particular patients suffering from Alpers–Huttenlocher syndrome – are at increased risk of valproate toxicity (Saneto et al., 2010; McFarland et al., 2009; Delarue et al., 2000; Schwabe et al., 1997). Stewart et al. (2010) showed that the carrying of *POLG1* gene mutations, principally p.Q1236H, increases the risk of VPA-induced liver failure by 23.6-fold.

The aim of the study was to investigate the role of mitochondrial oxidative phosphorylation in VPA-induced hepatotoxicity by using HepG2 cells as an experimental *in vitro* cell model to screen mitochondrial drug toxicity. We examined the effect of VPA on mitochondrial respiration under different metabolic conditions by culturing the cells either in medium supplemented with glucose or glucose-free medium containing galactose and pyruvate. Our results indicate that VPA affects cellular respiration, reduces cell growth, causes ATP depletion, induces cell death and compromises ROS detoxification.

2. Materials and methods

2.1. Reagents and materials

Sodium valproate, 1× Eagle's minimum essential medium (EMEM), D-glucose, D-galactose, sodium pyruvate, L-glutamine, 100× non-essential amino acid solution, penicillin, streptomycin, phosphate buffered saline (PBS), and propidium iodine (PI) were products of Sigma–Aldrich (St. Louis, MO, U.S.A.). Fetal bovine serum (FBS) was acquired from PAA Laboratories GmbH (Cölbe, Germany). Glucose-free 1× Dulbecco's Modified Eagle Medium (DMEM) and phenol-free 1× DMEM were products of Gibco (Life Technologies, Thermo Fisher Scientific, Waltham, MA, U.S.A.). Cell culture plates and flasks were manufactured by NalceNunc (Roskilde, Denmark). MitoXpress-Xtra-HS was purchased from Luxcel Biosciences (Cork, Ireland). MitoTracker Red CMXRos, ATP Determination Kit, MitoSOX Red and Hoechst 33342 were obtained from Invitrogen (Life Technologies, Thermo Fisher Scientific). cOmplete Mini EDTA-free protease inhibitor was purchased from Roche Diagnostics (Basel, Switzerland). Pierce Protein Assay Kit was a product of Thermo Fisher Scientific (Waltham, MA, U.S.A.). Molecular grade water was purchased from Qiagen (Hilden, Germany). Antibodies used in immunoblotting (pol γA, OXPHOS complexes II and IV, ATP synthase, mitochondrial superoxide dismutase, and glutathione peroxidase) were products of Abcam (Cambridge, UK) except for E3 subunit (dihydrolipoyl dehydrogenase, DLD E3) of pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase (PDC and OGDC, respectively) enzyme complexes (Pierce Antibodies, Thermo Fisher Scientific) and 39 kDa subunit of the OXPHOS chain complex I (Molecular Probes, Life Technologies, Thermo Fisher Scientific).

2.2. Cell culture conditions

HepG2 cells (American Type Culture Collection, Rockville, MD, U.S.A) were grown in 1× EMEM with 5 mM D-glucose, 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, non-essential amino acids, 100 IU/ml penicillin, 100 μg/ml streptomycin. The cells were incubated at 37 °C in 5% CO_2. Fresh medium was changed on the cells every 2–3 days. During VPA treatments, cells were incubated in normal growth medium (referred to as glucose medium) or galactose medium designed to induce mitochondrial respiration (Palmfeldt et al., 2009; Marroquin et al., 2007). Galactose medium was prepared with glucose-free 1× DMEM supplemented with 25 mM D-galactose, 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate and 100 IU/ml penicillin. As in cell culture conditions the concentration of glucose ranges from 5 mM to 25 mM, the concentration of galactose was chosen to simulate the highest possible osmolality of glucose.

2.3. Sodium valproate treatment time course

To study the effect of VPA on mitochondrial function in HepG2 cells under different metabolic conditions, HepG2 cells were treated with VPA in glucose or galactose medium described above. In case of treatment in galactose medium, the cells were first plated down in glucose medium for 24 h, then changed to galactose medium and pre-incubated for 24 h before the drug treatment. Initially, cells were in 20–40% confluency depending on duration of valproate treatment (40% for 24 h, 30% for 48 h and 20% for 72 h). VPA was dissolved in molecular grade water and added at concentrations of 0.5 mM (a therapeutic dosage), 1.0 mM and 2.0 mM (overdose). Plain molecular grade water was added into media to prepare a 0 mM VPA control (a mock-control), which was used as a reference. Cells were treated with VPA for 24, 48 and 72 h. Fresh VPA treatment medium was added on the cells every 24 h. Following VPA treatment the drug containing media was aspirated, cells were washed with PBS and prepared for further analysis. When treatment was stopped, cells in control wells were grown on average to 80% confluency. All the experiments were repeated three times. For immunoblot analysis the cells were treated with VPA for 72 h in galactose medium, one sample was collected from each treatment group and the immunoblotting assay was performed three times from one sample.

2.4. Oxygen consumption rate assays (MitoXpress)

For oxygen consumption rate (OCR) assays cells were seeded on a black 96-well plate with clear bottom. After the drug treatment, the plate was washed with the corresponding clear respiration media consisted of phenol-free 1× DMEM, either D-glucose or D-galactose and supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin. The MitoXpress-Xtra-HS, a phosphorescent oxygen sensitive probe,