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Valproate inhibits glucose-stimulated insulin secretion in beta cells

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Abstract

Valproate (VPA), an FDA approved anti-epileptic drug with a half-life of 12–18h in humans, has been shown to perturb the vacuolar proton pump (vH^+ -ATPase) function in yeasts by inhibiting myo-inositol phosphate synthase, the first and rate-limiting enzyme in inositol biosynthesis, thereby resulting in inositol depletion. vH^+ -ATPase transfers protons (H^+) across cell membranes, which helps maintain pH gradients within cells necessary for various cellular functions including secretion. This proton pump has a membrane (V_0) and a soluble cytosolic (V_1) domain, with C-subunit associated with V_1 . In secretory cells such as neurons and insulin secreting beta cells, vH^+ -ATPase acidifies vesicles essential for secretion. In this study, we demonstrate that exposure of insulin-secreting Min6 cells to a clinical dose of VPA results in inositol depletion and loss of co-localization of subunit C of vH^+ -ATPase with insulin secreting granules. Consequently, a reduction of glucose-stimulated insulin secretion is observed following VPA exposure. These results merit caution and the reassessment of the clinical use of VPA.

Keywords

Valproate; vH^+ ATPase; subunit C; insulin secretion; Min6 cells

Introduction

Valproate (VPA), an FDA approved drug with unknown mechanism of action, has been clinically used for the past four decades in treating migraines, bipolar disorders and epileptic seizures. Owing to its structural resemblance to gamma amino butyric acid (GABA), a

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master inhibitory neurotransmitter, VPA was initially thought to diminish neuro-excitability and regulate various neuronal pathways (Kumamaru et al. 2014; Nalivaeva et al. 2009; Bertelsen et al. 2017). Known effects of VPA include histone deacetylase (HDAC) inhibition, which alters neuronal gene expression (Ganai et al. 2015; Dozawa et al. 2014). Inositol depletion has recently been demonstrated in cells treated with VPA, similar to the action of lithium, a mood stabilizing predecessor of VPA (Lubrich et al. 1997; Williams et al. 2002). Inositol depletion is attributed to inhibition of myo-inositol phosphate synthase (MIPS), the rate-limiting enzyme in the inositol biosynthesis pathway (Shaltiel et al. 2004; Teo et al. 2009). Inositol is a precursor to secondary signaling messengers including inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Berridge and Irvine 1989; Liu and Bankaitis 2010; Berridge and Irvine 1984). A major consequence of VPA-induced inositol loss, is its inhibitory effects on vH^+ -ATPase as demonstrated in *Saccharomyces cerevisiae* (Deranieh et al. 2015).

vH^+ -ATPase is a large multi-subunit complex consisting of a membrane (V_0) and cytosolic domain (V_1), 260 kDa and 650 kDa respectively. V_0 , the proton translocator, consists of six subunits whereas V_1 , which hydrolyses ATP, consists of eight subunits (Forgac 2007). vH^+ -ATPase is present on the insulin secretory granule (ISG) membrane and functions similarly to its suggested role in neuronal synaptic vesicles (Brunner et al. 2007). It also capacitates ISGs, making them competent to secrete insulin, and similar to synaptic vesicles, ISG acidification is required for its maturation (Barg et al. 2001; Louagie et al. 2008). The vH^+ -ATPase functions specifically to pump H^+ into ISGs with a simultaneous influx of Cl^- ions by $ClC-3$ channel, leading to an ATP-dependent priming of ISGs, facilitating insulin secretion (Barg et al. 2001). Moreover, granule acidification is necessary for enzymatic function of PC1/3 and PC2 (pro-protein convertases) residing within ISGs, which produce mature insulin from its pro-hormone peptide (Louagie et al. 2008). Studies suggest vH^+ -ATPase to be important for membrane fusion events involving SNARE proteins (Wang et al. 2014; El Far and Seagar 2011). Therefore, prevention of ISG acidification interrupts ISG maturation and its capability to fuse at the cell plasma membrane, leading to inhibited insulin release. Hence, we hypothesized that loss of vH^+ -ATPase function in pancreatic β cells, specifically in ISGs due to VPA-induced inositol depletion, would lead to decreased glucose-stimulated insulin secretion. In the current study, using mouse insulinoma (Min6) cells, we demonstrated that VPA exposure indeed causes intracellular inositol depletion. This resulted in the inability of vH^+ -ATPase cytosolic subunit C to assemble at the ISG membrane, precluding vH^+ -ATPase activity, and the consequent loss of glucose-stimulated insulin secretion.

Materials and Methods

Glucose-stimulated insulin secretion from Min6 cells in culture

Min6 cells were grown to confluence using sterile 100 × 13-mm plastic Petri dishes according to published procedure (Naik et al. 2016). Cells were cultured in 25 mM glucose Dulbecco's Modified Eagle Medium (Invitrogen) containing 10% fetal calf serum, penicillin, streptomycin, and 50 μ M β -mercaptoethanol. Cells were stimulated using 35 mM glucose, and insulin secreted into the medium was collected at 10 and 30 min. Stimulation

assays were performed following 0.5 h, 2 h, 5 h, 16 h, and 24 h of exposure to 1mM VPA. All secretion assays were carried out at room temperature (RT), and cells were washed in phosphate buffered saline (PBS) pH 7.4 prior to stimulation. Following glucose stimulation, 200 μ L aliquots of the PBS incubation medium were collected at 10 and 30 minutes post stimulation. Aliquots were centrifuged at $4,000 \times g$ to remove any aspirated cells, and 160 μ L of the supernatant was mixed with 40 μ L of 5X Laemmli reducing sample preparation buffer (Laemmli 1970) for Western blot assay. To obtain the total amount of insulin in cells, Min6 cells were solubilized in 100 μ L of homogenization buffer (2 mM EDTA, 2 mM ATP, 0.02% Triton X-100, 1:500 protease inhibitor cocktail, pH 7.4) following secretion assays, and protein concentrations (Bradford 1976) were determined prior to Western blot analysis.

Estimation of Inositol in Min6 Cells

Intracellular inositol levels were determined as described previously with modification (Yu et al. 2016). Briefly, confluent Min6 cell plates (3 per time point) were incubated with 1 mM VPA for 30 minutes, 2 hours, 5 hours, 16 hours, and 24 hours respectively. After incubation, cells were washed twice with ice-cold PBS and lysed in ice-cold water containing protease inhibitor. Cells were centrifuged at $16,000 \times g$ for 10 minutes to remove cellular debris, and protein concentration of the supernatants were determined (Bradford 1976). Supernatant protein (400 μ g) was mixed with 7.5% perchloric acid and stored on ice for 20 minutes. This mix was centrifuged at $10,000 \times g$ for 10 minutes at 4 °C to remove protein precipitates. Supernatants containing cytosol were used for inositol measurement. Samples were centrifuged and loaded onto columns with 1 mL of AG 1-X8 resin/H₂O (1:1) mixture. Inositol was eluted with 5 mL of H₂O. Eluates were dried in an oven at 70 °C, stored at -80 °C Prior to assay, samples were dissolved in dH₂O and inositol levels were measured using the Maslanski and Busa Method (Maslanski and Busa 1990).

Western Blot Analysis

Min6 cell lysates (10 μ g) in Laemmli buffer were resolved on 12.5% SDS-PAGE and electro-transferred to 0.2 mm nitrocellulose membrane. The membrane was incubated at RT for 1 hour in blocking buffer (5% non-fat milk in PBS-0.1% Tween pH 7.4), washed thrice with PBS-0.1% Tween, and immunoblotted at room temperature (RT) for 1 hour with rabbit polyclonal anti-insulin (SC 9168), rabbit polyclonal anti-vH-ATPase-C1 (SC 20944), or rabbit polyclonal anti-GAPDH (SC 25778) (Santa Cruz Biotechnology Inc, Santa Cruz, CA). Prior to incubation for 1 hour at RT with secondary antibodies (Donkey anti-rabbit, SC 2313, Mouse anti-rabbit, SC 2357 and Bovine anti-goat SC 2350), nitrocellulose membranes were washed in PBS-0.1% Tween pH 7.4, thrice. Immunoblots were processed for enhanced chemiluminescence, exposed to X-Omat-AR film, developed and analyzed using ImageJ.

Details of Antibody Used

Primary antibodies utilized in the study include rabbit polyclonal anti-insulin (SC 9168) from Santa Cruz Biotechnology Inc. raised against amino acids 25–110 of insulin, which was used at 1:1000 dilution for Western blotting and 1:500 dilution for immunocytochemistry. Rabbit polyclonal anti-vH-ATPase-C1 (SC 20944) from Santa Cruz Biotechnology Inc. was used at 1:1000 dilution for Western blotting and 1:500 dilution for immunocytochemistry. Rabbit polyclonal anti-GAPDH (SC 25778) from Santa Cruz

Biotechnology Inc. was used at 1:1000 dilution, and mouse monoclonal anti-insulin (SC 8033), from Santa Cruz Biotechnology Inc. at 1:1000 dilution. Secondary antibodies utilized include donkey anti-rabbit SC-2313 from Santa Cruz Biotechnology Inc., used at 1:5000 dilution, mouse anti-rabbit SC-2357 from Santa Cruz Biotechnology Inc. at 1:5000 dilution, bovine anti-goat SC-2350 from Santa Cruz Biotechnology Inc. at 1:5000 dilution, donkey anti-rabbit Alexa Fluor 594 from ThermoFisher Scientific at 1:1000 dilution, and donkey anti-mouse Alexa Fluor 488 from ThermoFisher Scientific at 1:1000 dilution.

Immunocytochemistry

Min6 cells were grown on 35 mm glass bottom Petri dishes for immunocytochemistry (Naik et al. 2016). The distribution of vH^+ -ATPase-C1 subunit and ISGs in 1 mM VPA-treated (5 hours at 37 °C) Min6 cells were compared with vehicle-treated control (PBS) Min6 cells. Primary antibodies, rabbit polyclonal anti- vH^+ -ATPase-C1 (SC 20944) and mouse monoclonal anti-insulin (SC 8033), and secondary antibodies, donkey anti-rabbit AF 594 and donkey anti-mouse AF 488 (Life Technologies), were used in the study. Cells were exposed to DAPI nuclear stain for nucleus localization. An immunofluorescence FSX100 Olympus microscope was used to acquire immuno-fluorescent images through a 63X objective lens (numerical aperture, 1.40) with illumination at 405, 488, or 647 nm. Insulin and vH^+ -ATPase localization and cellular distribution were obtained through merging fluorescent images using ImageJ.

Results and Discussion

VPA first came into medical use in 1962 and although its molecular mechanism of action is far from fully understood, it is listed in the World Health Organization as a safe and essential medicine (Scott 1993). Recent advances in understanding its mechanism of action have revealed perturbation of yeast vH^+ -ATPase function as a consequence of VPA-induced inositol depletion (Shaltiel et al. 2004). The current study confirms this finding in a mammalian cell line, demonstrating that clinical levels of VPA exposure decrease inositol levels in Min6 cells. VPA-treated Min6 cells from various incubation time points (Fig 1a) demonstrated a significant decline in inositol after 5h (Fig 1b). VPA has previously (Deranieh et al. 2013) been shown to deplete inositol by inhibiting MIPS, the first and rate-limiting enzyme of inositol biosynthesis.

In agreement, VPA-induced inositol depletion disrupts vacuolar morphology and function in wild-type yeast cells (Deranieh et al. 2015), and reduces vH^+ -ATPase subunit C1 localization to ISG. vH^+ -ATPase is localized to the ISG membrane in β cells of pancreatic islets (Brunner et al. 2007; Louagie et al. 2008; Sun-Wada et al. 2006). Similar to neurons, granule acidification is important for insulin release. Glucose stimulation lowers intraluminal ISG pH, and pharmacological inhibitors of vH^+ -ATPase block this effect (Barg et al. 2001; Louagie et al. 2008; Sun-Wada et al. 2006; Tompkins et al. 2002). Further, studies have demonstrated reduced plasma insulin levels in oc/oc mice carrying a mutation for the $\alpha 3$ isoform of the V_0 domain (Sun-Wada et al. 2006). The assembly of the various vH^+ -ATPase subunits is a highly orchestrated process and depends on the cell's functional requirements (Morel and Poëa-Guyon 2015). For example, V_1 association with V_0 is

glucose dependent in yeast as well as in porcine (HK-2) and mammalian kidney cells (LLC-PK1), while phosphoinositide 3-kinase (PI3K) inhibition is known to impede this outcome (Poea-Guyon et al. 2013). Therefore, in the current study, we wanted to determine the consequences of VPA-induced inositol depletion on Min6 vH^+ -ATPase organization. Since the 5-hour time point following VPA exposure shows a significant drop in inositol in the cell cytosol (Fig 1), the distribution of insulin-containing granules and the subunit C of vH^+ -ATPase was examined in Min6 cells. Immunofluorescence labeling of Min6 cells (Fig 2a–2e) demonstrated co-localization of the vH^+ -ATPase subunit C (red) with insulin (green) containing ISGs. In contrast, double immunofluorescence labeling of VPA-treated Min6 cells (Fig 2f–2j) demonstrated substantially diminished co-localization of the vH^+ -ATPase subunit C (red) with insulin (green) containing ISGs. Additionally, ISGs appear to localize to the center of the cell following VPA treatment and fail to traffic to the plasma membrane unlike normal competent ISGs observed in control cells. These results are noteworthy because subunit C, together with the other cytosolic subunits, needs to be assembled in the cellular membrane for a fully functional vH^+ -ATPase. Relative absence of subunit C from ISG membrane of VPA-induced inositol-depleted cells compromises vH^+ -ATPase assembly and activity, and would therefore negatively impact cell secretion.

Several studies attest to the knowledge that vH^+ -ATPase provides the electrochemical proton gradient for neurotransmitter uptake, storage, and ultimately release by a synaptic vesicle (Bodzeta et al. 2017; Morel 2003; Di Giovanni et al. 2010; Sautin et al. 2005; Kane 2006). Therefore, we wanted to study the influence of VPA on insulin secretion from β cells. Glucose-stimulated insulin secretion from Min6 cells measured at various time points post-VPA treatment demonstrated loss in insulin release compared to untreated control cells (Fig 3). Additionally, a significantly lower rate of insulin release was observed in VPA-treated cells compared to controls (Fig 3b). The percent release of total cell insulin was substantially lower at both 10 min and 30 min following glucose stimulation of VPA-treated cells compared to controls (0h) (Fig 3a–3c). In summary, these studies demonstrate the detrimental effect of VPA on cell secretion. VPA exposure significantly lowered glucose-stimulated insulin release from Min6 cells as observed using Western blot analysis. Conventionally, ELISA assays are performed to estimate insulin, however in the current study Western blots were used to estimate insulin release since ELISA assays will be unable to differentiate insulin from non-secretory proinsulin that may be released into the medium as a possible consequence of cell lysis. Our study further demonstrated that VPA treatment resulted in the accumulation of insulin in Min6 cells. We analyzed total cellular insulin content in Min6 cells post 1 mM VPA treatment to understand whether loss in secretion was due to fusion incompetence of the ISGs or reduced insulin biosynthesis. Western blot analysis shows a significant increase in insulin content in total Min6 cell lysate after 5 h of 1 mM VPA treatment compared to untreated control cells (Fig 4a, b). Immuno-GAPDH signal is used as a loading control, demonstrating equal loading of cell lysate. Hence, insulin synthesis in VPA-treated cells appears to be normal; however, ISGs are unable to optimally secrete their intra-granular contents possibly due to poor acidification of ISG (Fig 4c), leading to loss of insulin secretion and their consequent insulin accumulation in cells.

To our knowledge, this is the first demonstration of inositol depletion and the loss of insulin function in pancreatic β cells treated with a clinical dosage of VPA. A fully assembled vH^+ -

ATPase leading to ISG granule acidification is required for insulin secretion (Morel 2003). Dissociation of the soluble C subunit from the vH^+ -ATPase complex at the ISG in valproate-treated Min6 cells observed using immunocytochemistry, further supports the requirement of a fully assembled vH^+ -ATPase at the ISG in insulin secretion from β cells. We chose to examine subunit C of the proton pump as an indicator of assembly/disassembly, since it is a soluble subunit present in the peripheral stalk of the eukaryotic proton pump. Furthermore, when vH^+ -ATPase disassembles, it is known to separate into a V0 domain, V1 domain (without subunit C), and subunit C of V1 domain (Forgac 2007; Iwata et al. 2004). Thus, incorporation of subunit C is the final step in assembly of a fully assembled and functional vH^+ -ATPase pump.

Our demonstration of a loss in insulin secretion from Min6 cells upon VPA exposure suggests that the VPA effect is not due to a decrease in insulin synthesis, since an increase in total cellular insulin was observed in these cells as a consequence of a loss in its ability to secrete. Close examination of immunostained cells shows that majority of insulin is localized centrally and at the perinuclear region, suggesting their presence in the Golgi, and their inability to be appropriately packaged into secretory vesicles in VPA-treated cells. Additionally, it could mean that the granules are somehow incompetent to traffic away from the Golgi toward the plasma membrane for fusion and secretion. Further studies using mouse and human islets are in progress to confirm these observations, including the use of expansion microscopy for obtaining nanometer scale distribution of various components of the vH^+ -ATPase pump. In summary, we demonstrate that a clinical dose of VPA leads to a loss in glucose-stimulated insulin secretion from Min6 cells. This loss is due in part to the incomplete assembly of vH^+ -ATPase at the ISG membrane as a consequence of VPA treatment. It is likely that inhibition of vH^+ -ATPase by VPA was due to inositol depletion, as shown in yeast. Since vH^+ -ATPase provides the electrochemical proton gradient for neurotransmitter uptake, storage, and ultimately release by a synaptic vesicle, we speculate that VPA action may occur by reducing neurotransmitter release by altering vH^+ -ATPase activity as a consequence of inositol depletion, thereby abrogating seizures in epileptic patients (Bodzeta et al. 2017; Morel 2003; Di Giovanni et al. 2010; Sautin et al. 2005; Kane 2006). It is to be noted, that since VPA has a half-life of nearly 16 h and in some cases administered daily to both adults and children, sometimes for a period of two weeks, its detrimental effects observed at very early time points in our study are alarming. Results from the current findings therefore merit caution and the careful reassessment of the clinical use of VPA.

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Figure 1: Inositol depletion in Min6 cells is observed following 5 hours of VPA exposure.

a: Schematic diagram showing Min6 cells growing in culture dishes for different periods of VPA exposure. Inositol is determined in the extracted cell cytosol. **b:** Inositol concentrations in Min6 cell cytosol show significantly decreased intracellular inositol levels first after 5h of 1mM VPA treatment (n=3, p< 0.05)

Figure 2: Decreased co-localization of vH^{+} -ATPase subunit C with insulin in VPA-treated Min6 cells.

a-c: Immunofluorescent images of control Min6 cells labeled with the nuclear stain DAPI (blue) and antibodies against vH^{+} -ATPase C-subunit (red) and insulin (green). **d:** Composite image of Min6 cells labeled with DAPI, vH^{+} -ATPase, and insulin. Note the co-localization of insulin and the C-subunit of the vH^{+} -ATPase in insulin granules. **e:** Digitally zoomed inset from D; white arrows indicate increased vH^{+} -ATPase subunit C and insulin co-localization. **f-h:** Immunofluorescent images of 5 h 1 mM VPA-treated Min6 cells labeled

with DAPI and antibodies against vH⁺-ATPase C-subunit and insulin. **i**: Composite image of Min6 cell labeled with DAPI, vH⁺-ATPase, and insulin. **j**: Digitally zoomed inset from I; yellow arrows indicate individual vH⁺-ATPase and insulin puncta. Note there is little co-localization of vH⁺-ATPase and insulin. *Scale bar = 15 μm*.

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Figure 3: VPA treatment significantly reduces glucose-stimulated insulin secretion in Min6 cells.
a: Representative western blots of Min6 cell total homogenate (TH) and insulin secretions collected at 10 and 30 minutes after glucose stimulation in control and 1 mM VPA-treated cells. **b-c:** Note the significant decrease in percent release of total cellular insulin at 10 and 30 minutes post-glucose stimulation following 30 min, 2 h, 5 h, 16 h, and 24 h of exposure to VPA (n=3, p< 0.05)

Figure 4: VPA treatment increases total intracellular insulin content in Min6 cells.

a: Representative Western blots of Min6 cell homogenate from control and VPA-treated Min6 cells at various time points. **b:** Total intracellular insulin significantly increases after 5 h of VPA treatment, suggesting the cellular accumulation of insulin is due to a loss in the ability of the cell to secrete ($n=3$, $p<0.05$). **c:** Schematic drawing showing valproate preventing the C subunit of v-H-ATPase from associating with the rest of the complex to activate it, rendering the transporter inactive, and consequently preventing ISG acidification and therefore a loss in insulin secretion and its accumulation in ISG.