Published in final edited form as:

Exp Eye Res. 2014 April ; 121: 26–34. doi:10.1016/j.exer.2014.01.021.

Valproic acid suppresses Nrf2/Keap1 dependent antioxidant protection through induction of endoplasmic reticulum stress and *Keap1* promoter DNA demethylation in human lens epithelial cells

Periyasamy Palsamy¹, Keshore R. Bidasee², and Toshimichi Shinohara^{1,*}

¹ Department of Ophthalmology and Visual Sciences, University of Nebraska Medical Center, Omaha, NE 68198, USA

² Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, NE 68198, USA

Abstract

Recent epidemiological studies confirm the prevalence of cataract in epileptic patients. Similarly, the drugs used to treat epilepsy also show the connection with increased cataract formation. In this present study, we investigated the suppression of Nrf2/Keap1 dependent antioxidant protection through induction of endoplasmic (ER) stress and *Keap1* promoter DNA demethylation in human lens epithelial cells (HLECs) treated with valproic acid (VPA), an antiepileptic drug. 20 mM VPA induces ER stress and activates the unfolded protein response (UPR) within 4 h by phosphorylating the ER stress sensor proteins, such as PERK, IRE1 α , and ATF6 in HLECs. Consequently, the integrated ER stress signals, such as eIF2 α , ATF4, BiP, and CHOP are altered accordingly to induce ER-Ca²⁺ release, reactive oxygen species (ROS) overproduction, and cell death in HLECs treated with VPA. VPA also suppresses the Nrf2, catalase, and glutathione reductase expressions with significant increases in Keap1 protein. Bisulphite genomic DNA sequencing reveals the promoter DNA demethylation in the *Keap1* promoter, which results in the overexpression of *Keap1* mRNA and protein in HLECs treated with 20 mM VPA. VPA also alters the expression profiles of passive DNA demethylation pathway enzymes such Dnmt1, Dnmt3a, Dnmt3b, and active DNA demethylation pathway enzyme, TET1 leading to DNA demethylation in the *Keap1* promoter of HLECs. Overexpressed Keap1 decreases the Nrf2 level, thereby abolishing the Nrf2 dependent antioxidant protection. This might be responsible for lenticular proteins oxidation and cataract formation.

© 2014 Elsevier Ltd. All rights reserved.

* Corresponding author: Department of Ophthalmology and Visual Sciences, University of Nebraska Medical Center, Omaha, NE 68198, USA. Tel.: +1 402 559 4205; fax: +1 402 559 3869. tshinohara@unmc.edu (T. Shinohara).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of interest

The authors do not declare any conflict of interest relevant to this manuscript.

Keywords

cataracts; valproic acid; ER stress; DNA methylation; unfolded protein response; Nrf2; *Keap1* promoter demethylation

1. Introduction

Epilepsy is a most common neurological disorder in the United States. Various epidemiological studies indicate the associations between cataract prevalence and epilepsy is equivalent to association of cataract with aging, diabetes, arterial hypertension and smoking (Hanhart et al., 2010; Isaac et al., 1991; Kato et al., 1990; Kinoshita et al., 2004; Kuwabara et al., 2009; Mathers et al., 1987). The onset of epilepsy is due to over-synchronous firing of brain neurons (Kerr et al., 2013) and these firing patterns are modulated by a major excitatory neurotransmitter, glutamate, through the family of glutamate receptors (Kim et al., 2004).

The interaction of glutamate with postsynaptic membrane glutamate receptors and its overstimulation increases intracellular Ca^{2+} by direct opening of postsynaptic ion channels and secondarily affecting calcium homeostatic mechanisms (Chen et al., 2000). Since, glutamate receptors, especially GluR2 directly open the postsynaptic ion channels; they are eventually considered as a drug target of epilepsy (Rogawski, 2011; Stephen and Brodie, 2011). However, drugs used to treat epilepsy also showed associations with cataract formation (Isaac et al., 1991; Kinoshita et al., 2004; Mathers et al., 1987), because of the existence and overexpression of GluR2, RNA editing, and membrane localization in the lens, which results in Ca^{2+} influx in the lens leading to cataract formation (Barbon et al., 2006; Farooq et al., 2012; Sawada et al., 2009).

There are several drugs used for epilepsy treatment, such as carbamazepine, lamotrigine, phenobarbital, phenytoin and valproic acid (VPA), with alone or multidrug therapy. VPA is widely used anticonvulsant agent for suppressing epileptic seizures, depression and migraine headaches and also a mood stabilizer for the treatment of manic depression or bipolar disorder (Singh et al., 2012; Singh et al., 2005; Tunnicliff, 1999). Carbamazepine, phenobarbital and phenytoin are reported to induce cataract in small numbers of epileptic patients (Bar et al., 1983; Hanhart et al., 2010; Mathers et al., 1987). However, *in utero* exposure of VPA induced the formation of bilateral congenital cataracts in a child out of 37 children (Glover et al., 2002), and these congenital cataracts have little to do with age-related cataracts (ARCs). VPA is never prescribed during pregnancy in the United States and Canada, because of its teratogenic effects known as fetal valproate syndrome, which includes multiple birth defects, dysmorphic facies, developmental delay, learning difficulties and behavioural problems (Włodarczyk et al., 2012).

VPA is known to induce rapid dose-dependent hyperacetylation of the histones H3 and H4, and can cause growth arrest and induce differentiation of transformed cells in culture (Gurvich et al., 2004). VPA also down-regulates numerous structural maintenance of chromatin proteins, DNA methyltransferases (Dnmts), and heterochromatins leading to chromatin decondensation (Marchion et al., 2005). Since the inhibitors of histone

deacetylation cause epigenetic DNA modifications (Dong et al., 2010; Milutinovic et al., 2007; Vire et al., 2006), VPA also induces DNA demethylation in the non-replicating cells by a dose dependent manner (Detich et al., 2003; Dong et al., 2008). In addition, VPA stimulates intracellular reactive oxygen species (ROS) (Defoort et al., 2006; Na et al., 2003) leading to the activation of redox-sensitive transcription factors that interact with the antioxidant response element-driven genes (Achachi et al., 2005; Kawai and Arinze, 2006; Sands et al., 2000).

We found the cataractogenic stressors such as hypoxia with low glucose (Elanchezhian et al., 2012b) or high glucose (Ikesugi et al., 2006), homocysteine (Elanchezhian et al., 2012a), and galactose (Ikesugi et al., 2006), induce endoplasmic reticulum (ER) stress mediated activation of unfolded protein response (UPR) and overproduction of ROS and cell death in lens epithelial cells (LECs). We also reported the suppression of nuclear factor erythroid-2-related factor 2 (Nrf2; also known as NFE2L2) dependent antioxidant protection due to ROS overproduction in LECs (Elanchezhian et al., 2012a; Elanchezhian et al., 2012b). Recently, we found the promoter DNA demethylation of *Kelch like ECH-associated protein 1* (*Keap1*), which is a negative regulator of Nrf2, in human lens epithelial cells (HLECs) as well as in diabetic cataractous lenses (Palsamy et al., 2012). These sequential events might be responsible for ARC formation in humans. Here, we hypothesized that VPA also induces suppression of Nrf2/Keap1 dependent stress protection through induction of ER stress and *Keap1* promoter DNA demethylation in HLECs. To experiment this hypothesis, we have studied the UPR activation, Ca^{2+} release from ER, ROS production, and suppression of Nrf2/Keap1 dependent stress protection in HLECs. In addition, we also analyzed the promoter DNA demethylation status of *Keap1* gene along with expression profiles of DNA methylation and demethylation pathway enzymes. Overall, this work reveals the VPA mediated ER stress activation, Ca^{2+} release from ER, ROS overproduction, and loss of *Keap1* promoter methylation as a result of altered DNA demethylation pathway enzymes that leads to the failure of Nrf2 dependent antioxidant system in HLECs.

2. Materials and methods

2.1. Cell culture

HLECs (SRA 01/04) were cultured overnight in DMEM, High glucose (Life Technologies) supplemented with 10% fetal bovine serum (Gemini Bio-Products) under 20% atmospheric oxygen at 37°C. Cells were plated 24 h prior to experiment in DMEM, Low glucose (Life Technologies) under 4% atmospheric oxygen and cultured with 20 mM VPA (Sigma) and 1 $\mu\text{g}/1 \mu\text{L}$ tunicamycin (Alexis Biochemicals) for the indicated time. At the end of the experiment, the cells were harvested and used for Western blotting, intracellular ROS production and cell death assays, real-time quantitative PCR (RT-qPCR), and bisulfite genomic DNA sequencing.

2.2. Western blotting

At the end of experimental treatments, HLECs were gently washed with ice-cold phosphate buffered saline (PBS) and were lysed with RIPA buffer (Cell Signaling Technology). Western blotting was performed with antibodies specific to immunoglobulin heavy-chain

binding protein (BiP; BD Biosciences), C/EBP-homologous protein (CHOP), PKR-like endoplasmic reticulum kinase (PERK), p-PERK, eukaryotic translation initiation factor 2 α (eIF2 α), p-eIF2 α , inositol-requiring enzyme 1 α (IRE1 α), activating transcription factor 4 (ATF4), ATF6, ER oxidoreductin 1 (Ero1)-L α , Ero1-L β , Nrf2, Keap1, glutathione reductase (GR), catalase, Dnmt1, Dnmt3a, Dnmt3b, ten-eleven translocation 1 protein (TET1), protein disulfide isomerase (PDI), sarcoplasmic reticulum calcium-transporting ATPase (SERCA; Santa Cruz Biotechnology), and GAPDH (Novus Biologicals) as described elsewhere (Elanchezhian et al., 2012a; Palsamy et al., 2012). The intensity of each band was normalized to that of GAPDH, and the data were presented as a relative intensity using the ImageJ analysis software (Abramoff et al., 2004).

2.3. Calcium imaging

HLECs were loaded with 5 μ M Fluo-3, AM with Pluronic® F-127 (Invitrogen) in DMEM containing 1.8 mM CaCl₂ for 30 min at 37°C in glass bottom Petridish. Cells were then washed twice with Ca²⁺-free DMEM before imaging. 20 mM VPA was added to the cells in Ca²⁺-free DMEM and time-lapse confocal live imaging was conducted to assess the intracellular Ca²⁺ changes. A second bolus of 20 mM VPA was also added to these cells to check, if any, the intracellular Ca²⁺ changes for an additional 3 min. Cells earlier exposed to VPA were then challenged with 10 mM caffeine to assess the intracellular Ca²⁺ changes, if any, for 2 min. Experiments were performed in line-scan mode by using a Zeiss 410 confocal microscope (Carl Zeiss Inc.) and field-stimulating cells at 0.25 Hz (10 V for 10 ms), as described previously (Shao et al., 2009).

2.4. ROS staining and cell death

ROS production and cell death were quantified by the methods described previously (Elanchezhian et al., 2012a; Palsamy et al., 2012). Briefly, the HLECs treated with 20 mM VPA was stained for ROS production by 2',7'-dichlorodihydrofluorescein diacetate (H₂-DCFH-AD) (Invitrogen) for 30 min at 20°C. The cells were washed twice with PBS and subjected to fluorescent microscopic imaging (Nikon, Eclipse TE2000-U) with a green filter. The cell death in HLECs treated with 20 mM VPA was also determined by using viability/cytotoxicity assay kits (Biotium Inc.) for 30 min, and were washed twice with PBS and subjected to fluorescent microscopic imaging (Nikon, Eclipse TE2000-U) with a red filter.

2.5. Bisulfite genomic DNA sequencing

The genomic DNA from HLECs treated with 20 mM VPA for 3 days and 1 μ g/1 μ L tunicamycin for 1 day were subjected to bisulfite conversion by EZ DNA Methylation-Direct™ kit (Zymo Research). The bisulfite-modified DNA was amplified by bisulfite sequencing PCR using Platinum® PCR SuperMix High Fidelity (Invitrogen) with primers specific to human *Keap1* promoter (Table 1; Supplement Figure. 1). Then, the amplified PCR products were cleaned by gel extraction with Zymoclean™ Gel DNA recovery kit (Zymo Research), then cloned into pCR®4-TOPO vectors using TOPO TA Cloning® kit (Invitrogen). The recombinant plasmids were transformed into One Shot® TOP10 chemically competent *E. coli* (Invitrogen) using the regular chemical transformation method. Plasmid DNA were isolated from about 10 independent clones of each amplicon

with PureLink™ Quick Plasmid Miniprep kit (Invitrogen) and then sequenced (High-Throughput DNA Sequencing and Genotyping Core Facility, University of Nebraska Medical Center, Omaha, NE) to determine the status of CpG methylation. Clones with an insert with >99.5% bisulfite conversion, i.e., non-methylated cytosine residues to thymine were included in this study, and the remaining was excluded. Then the sequenced data of each clone was analyzed for DNA methylation in the *Keap1* promoter by BISMA software (<http://biochem.jacobs-university.de/BDPC/BISMA/>) using default filtering threshold settings (Rohde et al., 2010).

2.6. Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from the HLECs with Quick-RNA™ MicroPrep solution (Zymo Research). The column purified total RNA was reverse transcribed by iScript™ Reverse Transcription Supermix for real-time PCR (Bio-Rad) and was analyzed by MiniOpticon™ Real-Time PCR Detection System (Bio-Rad) using the SsoFast™ EvaGreen® supermix (Bio-Rad) as described elsewhere (Elanchezhian et al., 2012a; Palsamy et al., 2012). The primer sequences were designed using the ProbeFinder software (Roche) and were synthesized commercially. Primers used for RT-qPCR are listed in Table 2. The specificity of the listed primers were verified by using the *in silico* PCR (<http://insilico.ehu.es/>) and the National Center for Biotechnology Information primer blast tool. The PCR products of those primers also visualized by 2% agarose gel electrophoresis and verified by DNA sequencing. Briefly, each reaction was carried out in triplicate and three independent experiments were run. Standard curves were prepared to determine individual PCR amplification efficiencies by using a serial dilution of a reference sample and was included in each real-time run to correct for possible variations in product amplification. The relative copy numbers were obtained from the standard curve and were normalized to the values obtained for *Gapdh*, the internal control. Data acquisition, analysis and PCR efficiencies were done using Bio-Rad CFX manager software (version 3.1).

2.7. Statistical analysis

The results were expressed as mean $\pm$ SD, and statistical significance was determined by student's *t* test or one-way ANOVA followed by the *post hoc* test, least significant difference using the SPSS (version 15.0) software (SPSS Inc.). Values were considered statistically significant when $p < 0.05$.

3. Results

3.1. VPA induces ER stress in HLECs

In order to understand the initial events of ER stress cascade, we focused our experiments to study the VPA mediated induction of ER stress with concomitant activation of UPR in HLECs. Cultured HLECs treated with 20 mM VPA for varying time shows the initiation of ER stress by phosphorylating PERK, where the ratio between p-PERK/PERK is notably elevated after 4 h (Fig. 1). However, ratio between the p-eIF2 α /eIF2 α is significantly increased within 2 h, after that it is decreased (Fig. 1). The expression of ATF6 and IRE1 α are also increased significantly at 4-12 h in HLECs treated with VPA (Fig. 1). The ER chaperone protein, BiP, and the downstream-integrated stress signal of ER stress, such as

ATF4 are increased whereas the proapoptotic factor, CHOP is significantly decreased in HLECs treated with VPA (Fig. 1). Based on the amount and exposure of time of ER stressors, the adaptive or the proapoptotic pathways of UPR are activated. Adaptive UPR declines the protein load acutely through reversible translational attenuation and mRNA decay, whereas terminal UPR fails to restore cellular homeostasis, eventually triggering apoptosis (Papa, 2012). These results validate the activation of protective UPR in HLECs by VPA.

3.2. VPA releases ER-Ca²⁺ in HLECs

Next, we investigated the VPA induced ER-Ca²⁺ release in HLECs by time-lapse confocal laser scanning microscopy, because ER is a major Ca²⁺-storage organelle in the cell as well as both protein-folding reactions and protein chaperone functions necessitate higher levels of ER-Ca²⁺. If there is any perturbations in the ER-Ca²⁺ distribution and regulation, that eventually alters the vital cellular functions leading to apoptosis through terminal UPR (Hetz et al., 2013). We found spontaneous Ca²⁺ release in the HLECs at random sites and is notably increased 30 sec after the addition of 20 mM VPA (Fig. 2A). This spontaneous Ca²⁺ release is elevated around 65 sec and is continued to decline for about 175 sec. However, a second dose of 20 mM VPA after the addition of first dose does not have impact on ER-Ca²⁺ release (Fig. 2B). Similarly, we did not find any noticeable difference in the VPA induced spontaneous Ca²⁺ release by using Ca²⁺ free culture medium. We also found decreased protein expression level of SERCA in HLECs treated with 20 mM VPA for 12 h (Fig. 2C). Similarly, the well-known ER stressor, tunicamycin also decreased the SERCA protein expression in HLECs (Fig. 2C). These results suggest that VPA induces alterations of intracellular Ca²⁺ homeostasis by releasing ER-Ca²⁺ and declining the SERCA level, which results in the ER stress.

3.3. VPA stimulates ROS overproduction and cell death in HLECs

We next measured the ROS production and cell death in HLECs treated with 20 mM VPA and found that 20 mM VPA significantly increases the ROS production in HLECs within 12 h (Fig. 2D). As an outcome, the HLECs treated with 20 mM VPA results in significant cell death (Fig. 2E). These results confirm that VPA stimulates ROS production and cell death in addition with inducing ER stress and ER-Ca²⁺ release in HLECs.

3.4. VPA suppresses ER localized oxidative enzymes

Chronic activation of UPR results in the overproduction of ROS and cell death through UPR-regulated oxidative protein folding machinery in the ER (Tu and Weissman, 2004). Disulphide bond formation during oxidative protein folding is reliant on the redox state of the ER and is catalyzed by PDI and recycled by Ero1. Further, the oxidized PDI then oxidize the substrate polypeptides, thereby leading to ROS production (Pagani et al., 2000; Tu and Weissman, 2002). However, the protein expression levels of Ero1-L α , Ero1-L β , and PDI are significantly decreased in HLECs treated with 20 mM VPA for 12 h (Fig. 3). These results suggested the VPA mediated chronic activation of UPR and suppression oxidative protein folding machinery enzymes in HLECs.

3.5. VPA suppresses Nrf2 dependent antioxidant protection

We next studied the protein expression profiles of Nrf2/Keap1 dependent antioxidant protection system in HLECs treated with 20 mM VPA for 3 days, because Nrf2 is a nuclear transcriptional factor, which controls more than 200 stress-associated genes, and Keap1 is a negative regulator of Nrf2 protein. Interestingly, the protein levels of Nrf2 and its downstream enzymes, such as GR, and catalase are significantly decreased in HLECs treated with 20 mM VPA for 3 days, with concomitant increase in the level of Keap1 (Fig. 4). Similar results were obtained in the HLECs treated with tunicamycin (1 µg/1 µL), a well-known ER stressor for 1 day (Fig. 4). These results confirm the suppression of Nrf2/Keap1 dependent antioxidant protection through ER stress mediated ROS production.

3.6. VPA induces Keap1 promoter DNA demethylation in HLECs

Next, we studied the *Nrf2* and *Keap1* promoter methylation status of HLECs treated with VPA, because VPA is known to induce epigenetic changes by dose dependent manner (Detich et al., 2003; Dong et al., 2008). We could not find any notable methylation/demethylation patterns in the *Nrf2* promoter of HLECs treated with VPA and tunicamycin (Results not shown). However, we found a predominant CpG island in the *Keap1* promoter (between -460 and +341) with a total number of 68 CpG dinucleotides (Palsamy et al., 2012). Being a negative regulator of Nrf2, any epigenetic changes in the *Keap1* gene overexpression directly influences the Nrf2 levels. It has been previously shown that the CpG dinucleotides found in the *Keap1* promoter region (20 CpGs; between -433 and -96) have primarily undergone loss of methylation by epigenetic means (Palsamy et al., 2012).

We performed bisulphite genomic DNA sequencing of *Keap1* promoter region (20 CpGs; between -433 and -96) in HLECs treated with VPA and tunicamycin. We already found significant loss of promoter methylation in the *Keap1* gene of diabetic cataractous lenses (Palsamy et al., 2012). Interestingly, HLECs treated with 20 mM VPA for 5 days shows nearly 90% loss of 5-methylcytosine (5mC) in the *Keap1* promoter region between -433 and -96 than that of control HLECs (Figs. 5A, 5B and 5D). Similarly, HLECs treated with tunicamycin (1 µg/1 µL) for 1 day shows 22% loss of 5-mC in the same region (Figs. 5C and 5D).

The loss of *Keap1* promoter DNA methylation eventually leads to overexpression of *Keap1* resulting in Nrf2 suppression, because Keap1 is a negative regulator of Nrf2. To test this, we studied the mRNA expression profiles of *Keap1* and *Nrf2* in HLECs treated with VPA and tunicamycin by RT-qPCR. The mRNA levels of *Keap1* and *Nrf2* are significantly increased in the HLECs treated with 20 mM VPA for 24 h (Fig. 5E). Similarly, tunicamycin (1 µg/1 µL; 1 day), treated HLECs also showed notable increase in the mRNA levels of *Keap1* and *Nrf2* (Fig. 5E). These results confirm that VPA mediated *Keap1* promoter DNA demethylation significantly increases the *Keap1* transcription.

3.7. VPA alters DNA demethylation pathway enzymes in HLECs

Since VPA induced the *Keap1* promoter DNA demethylation, we next determined the expression profiles of active and passive DNA demethylation pathway enzymes in HLECs. The mRNA expression profiles of passive DNA demethylation pathway enzymes such as

Dnmt1, *Dnmt3a*, and *Dnmt3b* as well as the active DNA demethylation pathway enzyme, *Tet1* are significantly increased in HLECs treated with 20 mM VPA for 3 days (Fig. 6A). Similar results were obtained with the HLECs treated with tunicamycin (1 µg/1 µL) for 1 day (Fig. 6A). However, the protein blot experiments shows significant decrease in the passive DNA demethylation pathway enzymes, such as *Dnmt1*, *Dnmt3a*, and *Dnmt3b* with concomitant increase in the active DNA demethylation pathway enzyme, TET1 (Fig. 6B). Tunicamycin (1 µg/1 µL; for 1 day) treated HLECs also show similar results (Fig. 6B). These results confirm that the loss of *Keap1* promoter DNA methylation in HLECs treated with 20 mM VPA is induced by increased expressions of active DNA demethylation pathway enzymes and decreased expressions of passive DNA demethylation pathway enzymes.

4. Discussion

This present investigation shows that VPA induces ER stress mediated UPR activation, ROS overproduction, ER-Ca²⁺ release, and suppression of Nrf2/Keap1 dependent antioxidant protection by *Keap1* promoter demethylation by altering the expression levels of active and passive DNA demethylation pathway enzymes, and finally cell death in HLECs. These sequential events may be one of the mechanisms for cataract formation. Previously, we have reported the induction of ER stress mediated UPR activation by various ER stressors such as homocysteine (Elanchezhian et al., 2012a), tunicamycin (Ikesugi et al., 2006), galactose (Ikesugi et al., 2006), hypoxia with low glucose or high glucose (Elanchezhian et al., 2012b; Ikesugi et al., 2006), which eventually results in UPR activation, ROS overproduction and cell death of LECs. Further, we reported notable promoter demethylation in the *Keap1* gene of diabetic cataractous lenses than that of clear lenses (Palsamy et al., 2012).

The lens researchers have common agreement that ARCs are strongly coupled with lens oxidation and aging (Baynes and Thorpe, 1999; Brennan and Kantorow, 2009; Lou, 2003). Our results also concurrent with them, because the chronic UPR induced by VPA establishes a common source for ER-Ca²⁺ release, ROS overproduction, and suppresses the Nrf2/Keap1 dependent stress protection by inducing DNA demethylation in the *Keap1* promoter through the altered expressions of active and passive DNA demethylation pathway enzymes. The promoter DNA demethylation of *Keap1* gene results in the overexpression of Keap1 protein as an outcome the Nrf2 levels is ultimately decreased through proteasomal degradation. Thereby, Nrf2/Keap1 dependent antioxidant protection is deteriorated and the cellular redox balance in the HLECs is shifted towards lens oxidation leading to cataract formation. Further, aging is associated with a gradual progression of epigenetic promoter DNA demethylation of *Keap1* gene in human clear lenses, whereas, ER stressors are associated with rapid, radical and severe loss of *Keap1* promoter demethylation leading to ARC formation (Palsamy et al., 2012). Thus, unrelated etiological stresses can induce the same basic mechanism, resulted in the lens oxidation, and ARCs formation.

In addition, we found that 20 mM VPA activates the ER stress with 2-8 h in HLECs by phosphorylating the ER stress transmembrane sensor protein, PERK into p-PERK. Then, p-PERK phosphorylates the eIF2α to p-eIF2α as well as phosphorylating Nrf2 to p-Nrf2 (Cullinan and Diehl, 2004, 2006). The p-eIF2α then inhibits the protein synthesis by

quickly diminishing the misfolded protein load in ER thereby it enhances the prosurvival effects of the cells (Harding et al., 2000). The p-Nrf2 is then dissociated from the Keap1 and translocated into the nucleus, where it binds with antioxidant response elements of promoter and activates more than 200 stress-associated genes (Rushmore et al., 1991). However, if the ER stress is chronic, the Nrf2 level is decreased and resulting in redox imbalance in HLECs. VPA also activates other ER stress sensing proteins, such as IRE1 α within 8 h, and ATF6 within 4h in HLECs. These signaling molecules are responsible for expression of genes involved in apoptosis. VPA also induces ROS overproduction and cell death by suppressing the expressions of ER localized oxidative enzymes such as, PDI, Ero1-L α , and Ero1-L β in HLECs. Due to these sequential events, the expressions levels of Nrf2, and its downstream enzymes such as, GR and catalase were diminished notably. VPA also reported to induce ROS production in various cell lines (Defoort et al., 2006; Na et al., 2003), leading to induce the activation of redox-sensitive transcription factors that interact with the antioxidant responsive elements (Kawai and Arinze, 2006). However, our study evidently provides a link between VPA mediated ER stress, ROS overproduction, Nrf2 suppression due to *Keap1* promoter DNA demethylation and cell death.

Activation of ER stress proteins have been reported to deplete the ER-Ca²⁺, because ER-Ca²⁺ level together with ER localized oxidative machinery, is crucial for the proper folding of proteins (Kiviluoto et al., 2013). Therefore, low levels of ER-Ca²⁺ significantly diminishes the ER chaperone proteins leading to ER stress (Coe and Michalak, 2009). The elevated cytosolic Ca²⁺ also responsible for up-regulating the m-calpain activity (Morishima et al., 2002; Scorrano et al., 2003). These alterations subsequently activate various caspases including caspase-3 and caspase-1. Along with m-calpain, these caspases are known to cleave crystallin proteins (Azuma et al., 1997; Elanchezhian et al., 2012a), which ultimately results in the cataract formation (Morishima et al., 2002; Scorrano et al., 2003; Shih et al., 2001). Our study also suggest that the ER stress induced by variety of cataractogenic stresses activates the terminal UPR thereby depletes the ER-Ca²⁺, which is sufficient to activate m-calpain to cleave the lenticular proteins and the vicious changes in the ER-Ca²⁺ homeostasis further results lenticular protein degradations.

The *Keap1* gene has completely methylated CpG island in its promoter region between -433 bp and -96 bp in the HLECs (SRA 01/04) as well as in the 17-year-old clear human lens (Palsamy et al., 2012). VPA induces *Keap1* promoter DNA demethylation in HLECs, because VPA is a well-known DNA demethylating agent and an inhibitor of histone deacetylase. VPA can be initially induced the passive DNA demethylation by suppressing the Dnmt1, Dnmt3a, and Dnmt3b in the replicating HLECs. VPA also reported to induce DNA demethylation by replication independent, active DNA demethylation pathway (Detich et al., 2003). As an outcome, the expression profile of TET1 is increased notably. This leads to the loss of *Keap1* promoter DNA methylation in the lens fiber cells and differentiated LECs resulting in the suppression of Nrf2 protein. These results further support an idea that loss of *Keap1* promoter DNA methylation appears to be a primary event and then either translational arrest through UPR or proteasomal degradation can occur to lower the Nrf2 level. We also reported the abrupt changes in the loss of *Keap1* promoter DNA methylation, i.e. an average loss of 90% 5-mC in human diabetic cataractous lenses

regardless of age (Palsamy et al., 2012), which results in aberrant gene modifications (Richardson, 2002).

Even though, the mRNA expressions of *Nrf2*, *Dnmt1*, *Dnmt3a*, and *Dnmt3b* are significantly elevated, because VPA is a well-known DNA demethylating agent, which generally activates the transcription. By contrast, protein expressions of those are significantly decreased in the HLECs treated with 20 mM VPA for 3 days. This must be due to degradation by ubiquitin-mediated proteasomal degradation, and m-calpain or other proteinases mediated degradation (Elanchezhian et al., 2012a). Recent reports also showed that the expression of *Nrf2* is regulated by miRNA-28 (Yang et al., 2011), miRNA 93 (Singh et al., 2013), iRNA153/miR27a/miR142-5p/miR144 (Narasimhan et al., 2012), in addition, *Nrf2* regulates the expression of miRNA in various cell types (Shah et al., 2013). These reports suggest that post-transcriptional and translational regulations by miRNAs are also crucial in addition to proteolytic cleavages.

In summary, the present investigation has established that the ER stress mediated activation of UPR, ER- Ca^{2+} release, ROS overproduction, cell death, and suppression of *Nrf2*/*Keap1* dependent stress protection in HLECs treated with 20 mM VPA. Further, we have found loss of promoter DNA methylation in the *Keap1* promoter by the altered expressions of active and passive DNA demethylation pathway enzymes in HLECs treated with 20 mM VPA. These results are comparable with our previous findings of *Keap1* promoter DNA demethylation and suppression of *Nrf2*/*Keap1* dependent stress protection in diabetic cataractous lenses. As an outcome, redox imbalance is resulted in lens oxidation and finally cataract formation. Overall, we have potentially demonstrated a possible mechanism by which VPA suppresses the *Nrf2*/*Keap1* dependent stress protection in HLECs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported in part by the RPB and EY0180172. We thank Janice A. Taylor and James R. Talaska of the Confocal Laser Scanning Microscope Core Facility at the University of Nebraska Medical Center for providing assistance with confocal microscopy and the Nebraska Research Initiative and the Eppley Cancer Center for their support of the Core Facility, and the UNMC DNA Sequencing Core Facility (supported by P20 RR016469 from the INBRE Program of the National Center for Research Resources) for sequencing analysis.

Abbreviations

ARCs	age-related cataracts
ATF4	activating transcription factor 4
BiP	immunoglobulin heavy-chain binding protein
CHOP	C/EBP-homologous protein
Dnmts	DNA methyltransferases
eIF2α	eukaryotic translation initiation factor 2 α

ER stress	endoplasmic reticulum stress
Ero1	ER oxidoreductin 1
GR	glutathione reductase
H₂-DCFH-AD	2',7'-dichlorodihydrofluorescein diacetate
HLECs	human lens epithelial cells
IRE1α	inositol-requiring enzyme 1 α
Keap1	Kelch like ECH-associated protein 1
Nrf2	nuclear factor erythroid-2-related factor 2
PBS	phosphate buffered saline
PDI	protein disulfide isomerase
PERK	PKR-like endoplasmic reticulum kinase
ROS	reactive oxygen species
RT-qPCR	real-time quantitative PCR
SERCA	sarcoplasmic reticulum calcium-transporting ATPase
TET1	ten-eleven translocation 1 protein
UPR	unfolded protein response
VPA	valproic acid

References

- Abramoff MD, Magelhaes PJ, Ram SJ. Image processing with ImageJ. *Biophotonics Int.* 2004;36–42.
- Achachi A, Florins A, Gillet N, Debacq C, Urbain P, Foutsop GM, Vandermeers F, Jasik A, Reichert M, Kerkhofs P, Lagneaux L, Burny A, Kettmann R, Willems L. Valproate activates bovine leukemia virus gene expression, triggers apoptosis, and induces leukemia/lymphoma regression in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 2005; 102:10309–10314. [PubMed: 16006517]
- Azuma M, Fukiage C, David LL, Shearer TR. Activation of calpain in lens: a review and proposed mechanism. *Exp. Eye Res.* 1997; 64:529–538. [PubMed: 9227270]
- Bar S, Feller N, Savir H. Presenile cataracts in phenytoin-treated epileptic patients. *Arch. Ophthalmol.* 1983; 101:422–425. [PubMed: 6830496]
- Barbon A, Popoli M, La Via L, Moraschi S, Vallini I, Tardito D, Tiraboschi E, Musazzi L, Giambelli R, Gennarelli M, Racagni G, Barlati S. Regulation of editing and expression of glutamate alpha-amino-propionic-acid (AMPA)/kainate receptors by antidepressant drugs. *Biol. Psychiatry.* 2006; 59:713–720. [PubMed: 16460696]
- Baynes JW, Thorpe SR. Role of oxidative stress in diabetic complications: a new perspective on an old paradigm. *Diabetes.* 1999; 48:1–9. [PubMed: 9892215]
- Brennan LA, Kantorow M. Mitochondrial function and redox control in the aging eye: role of MsrA and other repair systems in cataract and macular degenerations. *Exp. Eye Res.* 2009; 88:195–203. [PubMed: 18588875]
- Chen CJ, Liao SL, Kuo JS. Gliotoxic action of glutamate on cultured astrocytes. *J. Neurochem.* 2000; 75:1557–1565. [PubMed: 10987836]
- Coe H, Michalak M. Calcium binding chaperones of the endoplasmic reticulum. *Gen. Physiol. Biophys.* 2009; 28:F96–F103. Spec No Focus. [PubMed: 20093733]

- Cullinan SB, Diehl JA. PERK-dependent activation of Nrf2 contributes to redox homeostasis and cell survival following endoplasmic reticulum stress. *J. Biol. Chem.* 2004; 279:20108–20117. [PubMed: 14978030]
- Cullinan SB, Diehl JA. Coordination of ER and oxidative stress signaling: the PERK/Nrf2 signaling pathway. *Int. J. Biochem. Cell Biol.* 2006; 38:317–332. [PubMed: 16290097]
- Defoort EN, Kim PM, Winn LM. Valproic acid increases conservative homologous recombination frequency and reactive oxygen species formation: a potential mechanism for valproic acid-induced neural tube defects. *Mol. Pharmacol.* 2006; 69:1304–1310. [PubMed: 16377765]
- Detich N, Bovenzi V, Szyf M. Valproate induces replication-independent active DNA demethylation. *J. Biol. Chem.* 2003; 278:27586–27592. [PubMed: 12748177]
- Dong E, Chen Y, Gavin DP, Grayson DR, Guidotti A. Valproate induces DNA demethylation in nuclear extracts from adult mouse brain. *Epigenetics.* 2010; 5:730–735. [PubMed: 20716949]
- Dong E, Nelson M, Grayson DR, Costa E, Guidotti A. Clozapine and sulpiride but not haloperidol or olanzapine activate brain DNA demethylation. *Proc. Natl. Acad. Sci. U.S.A.* 2008; 105:13614–13619. [PubMed: 18757738]
- Elanchezhian R, Palsamy P, Madson CJ, Lynch DW, Shinohara T. Age-related cataracts: homocysteine coupled endoplasmic reticulum stress and suppression of Nrf2-dependent antioxidant protection. *Chem. Biol. Interact.* 2012a; 200:1–10. [PubMed: 22964297]
- Elanchezhian R, Palsamy P, Madson CJ, Mulhern ML, Lynch DW, Troia AM, Usukura J, Shinohara T. Low glucose under hypoxic conditions induces unfolded protein response and produces reactive oxygen species in lens epithelial cells. *Cell Death Dis.* 2012b; 3:e301. [PubMed: 22513875]
- Farooq M, Kaswala RH, Kleiman NJ, Kasinathan C, Frederikse PH. GluA2 AMPA glutamate receptor subunit exhibits codon 607 Q/R RNA editing in the lens. *Biochem. Biophys. Res. Commun.* 2012; 418:273–277. [PubMed: 22266371]
- Glover SJ, Quinn AG, Barter P, Hart J, Moore SJ, Dean JC, Turnpenny PD. Ophthalmic findings in fetal anticonvulsant syndrome(s). *Ophthalmology.* 2002; 109:942–947. [PubMed: 11986102]
- Gurvich N, Tsygankova OM, Meinkoth JL, Klein PS. Histone deacetylase is a target of valproic acid-mediated cellular differentiation. *Cancer Res.* 2004; 64:1079–1086. [PubMed: 14871841]
- Hanhart J, Vinker S, Nemet A, Levartovsky S, Kaiserman I. Prevalence of Epilepsy among Cataract Patients. *Curr. Eye Res.* 2010; 35:487–491. [PubMed: 20465442]
- Harding HP, Novoa I, Zhang Y, Zeng H, Wek R, Schapira M, Ron D. Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol. Cell.* 2000; 6:1099–1108. [PubMed: 11106749]
- Hetz C, Chevet E, Harding HP. Targeting the unfolded protein response in disease. *Nat. Rev. Drug Discov.* 2013; 12:703–719. [PubMed: 23989796]
- Ikesugi K, Yamamoto R, Mulhern ML, Shinohara T. Role of the unfolded protein response (UPR) in cataract formation. *Exp. Eye Res.* 2006; 83:508–516. [PubMed: 16643900]
- Isaac NE, Walker AM, Jick H, Gorman M. Exposure to phenothiazine drugs and risk of cataract. *Arch. Ophthalmol.* 1991; 109:256–260. [PubMed: 1671548]
- Kato S, Ohno K, Ihara N. [Breeding of cataract rat strain (ICRF/Kmu/Yg)]. *Jikken Dobutsu.* 1990; 39:295–298. [PubMed: 2361529]
- Kawai Y, Arinze IJ. Valproic acid-induced gene expression through production of reactive oxygen species. *Cancer Res.* 2006; 66:6563–6569. [PubMed: 16818628]
- Kerr M, Gil-Nagel A, Glynn M, Mula M, Thompson R, Zuberi SM. Treatment of behavioral problems in intellectually disabled adult patients with epilepsy. *Epilepsia.* 2013; 54(Suppl 1):34–40. [PubMed: 23458464]
- Kim SH, Choi YM, Chung S, Uhm DY, Park MK. Two different Ca²⁺-dependent inhibitory mechanisms of spontaneous firing by glutamate in dopamine neurons. *J. Neurochem.* 2004; 91:983–995. [PubMed: 15525352]
- Kinoshita A, Kitaoka T, Oba K, Amemiya T. Bilateral drug-induced cataract in a patient receiving anticonvulsant therapy. *Jpn. J. Ophthalmol.* 2004; 48:81–82. [PubMed: 14767660]
- Kiviluoto S, Vervliet T, Ivanova H, Decuypere JP, De Smedt H, Missiaen L, Bultynck G, Parys JB. Regulation of inositol 1,4,5-trisphosphate receptors during endoplasmic reticulum stress. *Biochim. Biophys. Acta.* 2013; 1833:1612–1624. [PubMed: 23380704]

- Kuwabara T, Fukushima T, Makino K, Kondo H. Epileptic seizure, cataract, and tongue atrophy during the 8 years after electrical brain injury. *Intern. Med.* 2009; 48:1179–1182. [PubMed: 19571455]
- Lou MF. Redox regulation in the lens. *Prog. Retin. Eye Res.* 2003; 22:657–682. [PubMed: 12892645]
- Marchion DC, Bicaku E, Daud AI, Sullivan DM, Munster PN. Valproic acid alters chromatin structure by regulation of chromatin modulation proteins. *Cancer Res.* 2005; 65:3815–3822. [PubMed: 15867379]
- Mathers W, Kattan H, Earll J, Lemp M. Development of presenile cataracts in association with high serum levels of phenytoin. *Ann. Ophthalmol.* 1987; 19:291–292. [PubMed: 3662311]
- Milutinovic S, D'Alessio AC, Detich N, Szyf M. Valproate induces widespread epigenetic reprogramming which involves demethylation of specific genes. *Carcinogenesis.* 2007; 28:560–571. [PubMed: 17012225]
- Morishima N, Nakanishi K, Takenouchi H, Shibata T, Yasuhiko Y. An endoplasmic reticulum stress-specific caspase cascade in apoptosis. Cytochrome c-independent activation of caspase-9 by caspase-12. *J. Biol. Chem.* 2002; 277:34287–34294. [PubMed: 12097332]
- Na L, Wartenberg M, Nau H, Hescheler J, Sauer H. Anticonvulsant valproic acid inhibits cardiomyocyte differentiation of embryonic stem cells by increasing intracellular levels of reactive oxygen species. *Birth Defects Res. Part A Clin. Mol. Teratol.* 2003; 67:174–180. [PubMed: 12797459]
- Narasimhan M, Patel D, Vedpathak D, Rathinam M, Henderson G, Mahimainathan L. Identification of novel microRNAs in post-transcriptional control of Nrf2 expression and redox homeostasis in neuronal, SH-SY5Y cells. *PLoS One.* 2012; 7:e51111. [PubMed: 23236440]
- Pagani M, Fabbri M, Benedetti C, Fassio A, Pilati S, Bulleid NJ, Cabibbo A, Sitia R. Endoplasmic reticulum oxidoreductin 1-beta (ERO1-Lbeta), a human gene induced in the course of the unfolded protein response. *J. Biol. Chem.* 2000; 275:23685–23692. [PubMed: 10818100]
- Palsamy P, Ayaki M, Elanchezhian R, Shinohara T. Promoter demethylation of *Keap1* gene in human diabetic cataractous lenses. *Biochem. Biophys. Res. Commun.* 2012; 423:542–548. [PubMed: 22683333]
- Papa FR. Endoplasmic reticulum stress, pancreatic beta-cell degeneration, and diabetes. *Cold Spring Harb. Perspect. Med.* 2012; 2:a007666. [PubMed: 22951443]
- Richardson BC. Role of DNA methylation in the regulation of cell function: autoimmunity, aging and cancer. *J. Nutr.* 2002; 132:2401S–2405S. [PubMed: 12163700]
- Rogawski MA. Revisiting AMPA receptors as an antiepileptic drug target. *Epilepsy Curr.* 2011; 11:56–63. [PubMed: 21686307]
- Rohde C, Zhang Y, Reinhardt R, Jeltsch A. BISMA--fast and accurate bisulfite sequencing data analysis of individual clones from unique and repetitive sequences. *BMC Bioinformatics.* 2010; 11:230. [PubMed: 20459626]
- Rushmore TH, Morton MR, Pickett CB. The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *J. Biol. Chem.* 1991; 266:11632–11639. [PubMed: 1646813]
- Sands SA, Guerra V, Morilak DA. Changes in tyrosine hydroxylase mRNA expression in the rat locus coeruleus following acute or chronic treatment with valproic acid. *Neuropsychopharmacology.* 2000; 22:27–35. [PubMed: 10633488]
- Sawada J, Yamashita T, Aizawa H, Aburakawa Y, Hasebe N, Kwak S. Effects of antidepressants on GluR2 Q/R site-RNA editing in modified HeLa cell line. *Neurosci. Res.* 2009; 64:251–258. [PubMed: 19447293]
- Scorrano L, Oakes SA, Opferman JT, Cheng EH, Sorcinelli MD, Pozzan T, Korsmeyer SJ. BAX and BAK regulation of endoplasmic reticulum Ca²⁺: a control point for apoptosis. *Science.* 2003; 300:135–139. [PubMed: 12624178]
- Shah NM, Rushworth SA, Murray MY, Bowles KM, MacEwan DJ. Understanding the role of NRF2-regulated miRNAs in human malignancies. *Oncotarget.* 2013; 4:1130–1142. [PubMed: 24029073]
- Shao CH, Wehrens XH, Wyatt TA, Parbhu S, Rozanski GJ, Patel KP, Bidasee KR. Exercise training during diabetes attenuates cardiac ryanodine receptor dysregulation. *J. Appl. Physiol.* 2009; 106:1280–1292. [PubMed: 19131475]

- Shih M, David LL, Lampi KJ, Ma H, Fukiage C, Azuma M, Shearer TR. Proteolysis by m-calpain enhances in vitro light scattering by crystallins from human and bovine lenses. *Curr. Eye Res.* 2001; 22:458–469. [PubMed: 11584346]
- Singh B, Ronghe AM, Chatterjee A, Bhat NK, Bhat HK. MicroRNA-93 regulates NRF2 expression and is associated with breast carcinogenesis. *Carcinogenesis.* 2013; 34:1165–1172. [PubMed: 23492819]
- Singh G, Bell GS, Driever PH, Sander JW. Cancer risk in people with epilepsy using valproate-sodium. *Acta Neurol. Scand.* 2012; 125:234–240. [PubMed: 22077648]
- Singh G, Driever PH, Sander JW. Cancer risk in people with epilepsy: the role of antiepileptic drugs. *Brain.* 2005; 128:7–17. [PubMed: 15574465]
- Stephen LJ, Brodie MJ. Pharmacotherapy of epilepsy: newly approved and developmental agents. *CNS Drugs.* 2011; 25:89–107. [PubMed: 21254787]
- Tu BP, Weissman JS. The FAD- and O(2)-dependent reaction cycle of Ero1-mediated oxidative protein folding in the endoplasmic reticulum. *Mol. Cell.* 2002; 10:983–994. [PubMed: 12453408]
- Tu BP, Weissman JS. Oxidative protein folding in eukaryotes: mechanisms and consequences. *J. Cell Biol.* 2004; 164:341–346. [PubMed: 14757749]
- Tunnicliff G. Actions of sodium valproate on the central nervous system. *J. Physiol. Pharmacol.* 1999; 50:347–365. [PubMed: 10574466]
- Vire E, Brenner C, Deplus R, Blanchon L, Fraga M, Didelot C, Morey L, Van Eynde A, Bernard D, Vanderwinden JM, Bollen M, Esteller M, Di Croce L, de Launoit Y, Fuks F. The Polycomb group protein EZH2 directly controls DNA methylation. *Nature.* 2006; 439:871–874. [PubMed: 16357870]
- Wlodarczyk BJ, Palacios AM, George TM, Finnell RH. Antiepileptic drugs and pregnancy outcomes. *Am. J. Med. Genet. A.* 2012; 158A:2071–2090. [PubMed: 22711424]
- Yang M, Yao Y, Eades G, Zhang Y, Zhou Q. MiR-28 regulates Nrf2 expression through a Keap1-independent mechanism. *Breast Cancer Res. Treat.* 2011; 129:983–991. [PubMed: 21638050]

Highlights

- Valproic acid (VPA) activates ER stress mediated UPR in human lens epithelial cells
- VPA induces Ca^{2+} release from ER to cytoplasm in human lens epithelial cells
- VPA suppresses Nrf2 dependent antioxidant protection in human lens epithelial cells
- VPA demethylates *Keap1* promoter by modifying DNA demethylation enzyme expressions

Fig. 1.

Activation of ER stress mediated UPR-specific proteins in HLECs treated with 20 mM VPA. Immunoblot of p-PERK, PERK, p-eIF2α, eIF2α, IRE1α, ATF6, ATF4, BiP, and CHOP in HLECs treated with 20 mM VPA for 0, 2, 4, 8 and 12 h. GAPDH was probed as a loading control. The data are presented as mean $\pm$ SD from three independent experiments. One-way ANOVA followed by *post hoc* test least significant difference was used to determine the statistical significance: * p <0.05.

Fig. 2. ER- Ca^{2+} mobilization, ROS overproduction, and cell death in HLECs treated with 20 mM VPA. A and B. Time-lapse confocal images of HLECs showing the mobilization of Ca^{2+} from ER, when challenged with 20 mM VPA for about 5 min. Three independent experiments were performed and all of which were exhibited similar pattern of Ca^{2+} release from ER when challenged with 20 mM VPA. In the graph, “a” denotes the 1st dose of VPA and “b” denotes the 2nd dose of VPA. C. Immunoblot of SERCA in HLECs treated with 20 mM VPA for 3 days, and 1 $\mu\text{g}/\mu\text{L}$ tunicamycin for 1 day. GAPDH was probed as a loading control. D. Photomicrographs of $\text{H}_2\text{-DCFH-AD}$ staining for ROS production in HLECs treated with 20 mM VPA for 12 h. E. Photomicrographs of Ethidium homodimer-III staining for cell death in HLECs treated with 20 mM VPA for 12 h. The data are presented as mean $\pm$ SD from three independent experiments. A student's *t* test was used to determine the statistical significance: * $p < 0.05$.

Fig. 3.

Levels of ER localized oxidative enzymes in HLECs treated with 20 mM VPA. Immunoblot of Ero1-L α , Ero1-L β , and PDI in HLECs treated with 20 mM of VPA for 12 h. GAPDH was probed as a loading control. The data are presented as mean $\pm$ SD from three independent experiments. A student's *t* test was used to determine the statistical significance: **p*<0.05.

Fig. 4.

Suppression of Nrf2 dependent antioxidant protection in HLECs treated with 20 mM VPA. Immunoblot of Nrf2, Keap1, GR and Catalase in HLECs treated with 20 mM VPA for 3 days and 1 $\mu\text{g}/\mu\text{L}$ tunicamycin for 1 day. GAPDH was probed as a loading control. The data are presented as mean $\pm$ SD from three independent experiments. A student's *t* test was used to determine the statistical significance: $*p < 0.05$.

Fig. 5.

Loss of *Keap1* promoter DNA methylation in HLECs treated with VPA and tunicamycin. A. The methylation pattern in the *Keap1* promoter of control HLECs show highly methylated CpG dinucleotides in the region between -433 and -96 of *Keap1* promoter. B. The methylation pattern in the *Keap1* promoter of HLECs treated with 20 mM VPA for 3 days show highly demethylated CpG dinucleotides in the region between -433 and -96 of *Keap1* promoter. C. The methylation pattern in the *Keap1* promoter of HLECs treated with 1 μ g/ μ L tunicamycin for 1 day show moderately demethylated CpG dinucleotides in the region between -433 and -96 of *Keap1* promoter. Red colored box denotes methylcytosine, and blue colored box denotes unmethylated cytosine, respectively. The color gradient bar, which represents the red region, has more methylated CpGs and blue region has more unmethylated CpGs. D. Total percentage of methylated CpGs present in the region between -433 and -96 of *Keap1* promoter of HLECs treated with 20 mM VPA for 3 days and 1 μ g/ μ L tunicamycin for 1 day. E. RT-qPCR analyses of HLECs treated with 20 mM VPA for 1 day and 1 μ g/ μ L tunicamycin for 1 day showing the increased expressions of *Keap1* and *Nrf2* mRNAs. Fold change in the gene expressions were normalized with the internal control, *Gapdh*. The data are presented as mean $\pm$ SD from three independent experiments. A student's *t* test was used to determine the statistical significance: **p*<0.05.

Fig. 6.

Altered expressions of passive and active DNA demethylation pathway enzymes in HLECs treated with VPA. A. RT-qPCR of passive DNA demethylation pathway enzymes, such as *Dnmt1*, *Dnmt3a*, and *Dnmt3b*, and active DNA demethylation pathway enzyme, *Tet1* in HLECs treated with 20 mM VPA for 3 days and 1 μ g/ μ L tunicamycin for 1 day. Fold change in the gene expressions were normalized with the internal control, *Gapdh*. B. Immunoblot of passive DNA demethylation pathway enzymes, *Dnmt1*, *Dnmt3a*, and *Dnmt3b*, and active DNA demethylation pathway enzyme, *TET1* in HLECs treated with 20 mM VPA for 3 days and 1 μ g/ μ L tunicamycin for 1 day. GAPDH was probed as a loading control. The data are presented as mean $\pm$ SD from three independent experiments. A student's *t* test was used to determine the statistical significance: **p*<0.05.

Table 1

List of primer sequences for bisulphite genomic DNA sequencing

Accession No.	Gene description	Gene symbol	Forward	Reverse	Product size
NM_203500.1	Kelch-like ECH-associated protein 1	<i>Kcap1</i>	ttttagttatttaggagggtgaggt	aaacccccctctcactat	337

The primer sequences for bisulfite genomic DNA sequencing were designed using the OligoPerfect™ Designer software with the instructions of Invitrogen for optimal primer design and were synthesized commercially.

Table 2

List of primer sequences for RT-qPCR

Accession No.	Gene description	Gene symbol	Forward	Reverse	Product size
NM_203500.1	Kelch-like ECH-associated protein 1	<i>Keap1</i>	gggtccctacagccaag	tgggggtccacagaagataaagc	66
NM_006164.3	Nuclear factor (erythroid derived 2)-like 2	<i>Nrf2</i>	acacgggtccacagctcacc	tgcctccaaagatgtaataca	96
NM_001130823.1	DNA (cytosine-5-)-methyltransferase 1	<i>Dnmt1</i>	cccaagaactaggattagagc	gggttgccctgggtgcttttc	71
NM_175629.1	DNA (cytosine-5-)-methyltransferase 3a	<i>Dnmt3a</i>	cctgaagcctcaagagcagct	tgggtctctcttctgtcttgc	94
NM_006892.3	DNA (cytosine-5-)-methyltransferase 3b	<i>Dnmt3b</i>	ggaaattagaatacaggaaatacga	aatttgcttgaggcgcttg	105
NM_030625.2	Tet methylcytosine dioxygenase 1	<i>Tet1</i>	tcgtgtgttgccctctgga	gcctttaaactttgggcttc	77
J02642.1	Glyceraldehyde-3-phosphate dehydrogenase	<i>Gapdh</i>	tggatcgtggaggactca	ggggccatcgacagcttc	69

The primer sequences for RT-qPCR were designed using the ProbeFinder software (version: 2.49) with the instructions of Roche for optimal primer design, and were synthesized commercially.