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Ethanol's Effects on Transient Receptor Potential Channel Expression in Brain Microvascular Endothelial Cells

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Abstract

Ethanol (EtOH), the main ingredient in alcoholic beverages, is well known for its behavioral, physiological, and immunosuppressive effects. There is evidence that EtOH acts through protein targets to exert its physiological effects; however, the mechanisms underlying EtOH's effects on inflammatory processes, particularly at the blood-brain barrier (BBB), are still poorly understood. Transient receptor potential (TRP) channels, the vanguards of human sensory systems, are novel molecular receptors significantly affected by EtOH, and are heavily expressed in brain microvascular endothelial cells (BMVECs), one of the cellular constituents of the BBB. EtOH's actions on endothelial TRP channels could affect intracellular Ca^{2+} and Mg^{2+} dynamics, which mediate leukocyte adhesion to endothelial cells and endothelial permeability at the BBB, thus altering immune and inflammatory responses. We examined the basal expression profiles of all 29 known mammalian TRP channels in mouse BMVECs and determined both EtOH concentration- and time-dependent effects on TRP expression using a PCR array. We also generated an in vitro BBB model to examine the involvement of a chosen TRP channel, TRP melastatin 7 (TRPM7), in EtOH-mediated alteration of BBB permeability. With the exception of the *akyrin* subfamily, members of five TRP subfamilies were expressed in mouse BMVECs, and their expression levels were modulated by EtOH in a concentration-dependent manner. In the in vitro BBB model, TRPM7 antagonists further enhanced EtOH-mediated alteration of BBB permeability. Because of the diversity of TRP channels in BMVECs that regulate cellular processes, EtOH can affect $\text{Ca}^{2+}/\text{Mg}^{2+}$ signaling, immune responses, lysosomal functions as well as BBB integrity.

Keywords

Ethanol; Transient receptor potential channels; Brain microvascular endothelial cells; Blood-brain barrier

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Compliance with Ethical Standards

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest The authors declare that they have no conflict of interest.

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Background

Ethanol (EtOH), the main ingredient in alcoholic beverages, is an immune system modulator known for its behavioral and psychological effects (Romeo et al. 2010). However, the underlying mechanisms by which EtOH exerts its various effects are still not well understood. Although recent evidence indicates that EtOH acts at the molecular level (Harris et al. 2008; Howard et al. 2011a, b), there is still a fundamental gap in our knowledge concerning EtOH's potential cellular targets and its subsequent physiological effects.

Alcoholic beverages differ in their EtOH concentration, or alcohol-by-volume (ABV) content, and EtOH's effects appear to be concentration-dependent. We previously reported that binge consumption of alcoholic solutions with a high EtOH concentration causes more pronounced immune responses than those with a low EtOH concentration, even when the amount of EtOH intake is the same (Liu et al. 2011; Sarkar et al. 2012). Compared to other substances of abuse, such as morphine and methamphetamine that have dissociation constants (K_d) for their respective targets in the nanomolar (nM) to micromolar (μ M) ranges (Wolozin and Pasternak 1981; Nguyen et al. 2005), EtOH appears to have particularly low affinity for its molecular targets. EtOH exerts robust effects only at relatively high concentrations; intoxication occurs with a blood level of ~17 mM, whereas ~190 mM is required for anesthesia (Alifimoff et al. 1989).

Ethanol exerts its myriad effects through a variety of protein targets (Haorah et al. 2005, 2007, 2008; Harris et al. 2008; Howard et al. 2011a, b), particularly ion channels (Howard et al. 2011a, b). One such target is a group of cell membrane ion channels called transient receptor potential (TRP) channels. TRP channels constitute a large and functionally diverse superfamily of cation cell membrane channel proteins that are expressed in many cell types, and are generally described as the vanguard of our sensory systems because they respond to a variety of intra- and intercellular stimuli (Clapham 2003; Voets et al. 2005). Seven subfamilies of TRP channels have been identified based on their specific agonists: ankyrin (TRPA), canonical (TRPC), melastatin (TRPM), mucolipin (TRPML), nonechanopotential or NOMPC (TRPN), polycystin (TRPP), and vanilloid (TRPV); mammals do not have representatives in the TRPN subfamily (Montell 2005; Ramsey et al. 2006). The sequence identity of these subfamilies is as low as 20% (Montell 2005; Ramsey et al. 2006). Several TRP channels, including TRPV1–4, TRPM8, and TRPA1, are involved in temperature sensing (Patapoutian et al. 2003; Bandell et al. 2007) and reception of noxious and immunologic stimuli (Clapham 2003; Voets et al. 2005; Cortright and Szallasi 2009).

EtOH has been reported to significantly affect various TRP channels, particularly the thermo-sensing and nociceptive TRPA1, TRPV1, and TRPM8 channels. EtOH activates TRPA1 (Matta et al. 2008) and TRPV1 channels and selectively excites sensory neurons (Cornett et al. 2008), whereas EtOH inhibits menthol-induced responses in TRPM8 channels (Benedikt et al. 2007). EtOH can activate TRPV1 channels, eliciting a burning sensation in an EtOH concentration-dependent manner (Trevisani et al. 2002). EtOH can also potentiate the response of TRPV1 to capsaicin, a TRPV1 agonist, as well as protons and heat, lowering the heat activation threshold from 42 °C to 34 °C (Trevisani et al. 2002, Cornett et al. 2008).

Animal behavioral assessment also suggests a role for TRPV1 in both EtOH-induced intoxication (Blednov and Harris 2009) and in avoidance of EtOH (Ellingson et al. 2009).

TRP channels mediate certain immune responses, such as cytokine production and leukocyte-endothelial adhesion (LEA), the initial step in the inflammatory process (Brown et al. 2008; Gaskin et al. 2011; Berrouit et al. 2012). TRPs are found in various cell types, including brain microvascular endothelial cells (BMVECs) (Nilius et al. 2003; De Bock et al. 2013) that, together with astrocytes and pericytes, form the blood-brain barrier (BBB), which is essential in protecting the central nervous system (CNS) from pathogen invasion. BMVECs are the primary cell type responsible for eliciting immune responses at the BBB, and we have focused our study on endothelial TRP expression in response to EtOH.

Activation of TRP channels by various extracellular stimuli, including EtOH, induces an influx of calcium ions (Ca^{2+}), which can subsequently increase LEA. Conversely, EtOH is an immunosuppressant that can inhibit LEA by suppressing TRP channel expression. We have reported that the effects of binge EtOH exposure on immune responses are concentration-dependent (Liu et al. 2011; Sarkar et al. 2012). However, the mechanisms that underlie EtOH's effects on inflammatory processes are still poorly understood.

In this study, we investigated the EtOH concentration-dependent effects on TRP channels in mediating neuroimmune responses in mouse BMVECs. We first measured the level of all 29 known mammalian TRP channels in mouse BMVECs and then examined the effects of EtOH on the expression of these mammalian TRP channels using real-time PCR array. Our results showed that TRP channels in five out of six mammalian subfamilies were expressed in BMVECs, and the expression levels of many of these channels were affected by EtOH in a concentration-dependent manner. EtOH modulation of various TRP channels at the protein level was also demonstrated. Expression of TRP melastatin 7 (TRPM7) in mouse BMVECs was most abundant among all TRP channels. Its expression was significantly down-regulated by high-concentration EtOH at both mRNA and protein levels. Suppressed function of the TRPM7 channel in mouse BMVECs, by treatment with various antagonists, was shown to lead to elevation of the permeability of BMVEC barrier, suggesting involvement of TRPM7 in EtOH-mediated compromised BBB integrity.

Methods

Cell Culture and Treatment

Mouse BMVECs were purchased from Cell Biologics (Chicago, IL), seeded at a density of 5×10^4 cells/well (passage 6) in 6-well plates containing 4 mL/ well of fresh media, and grown until 70% confluency. Fresh media containing either control vehicle (phosphate buffered saline, PBS) or various concentrations of EtOH (4.35, 8.7, 17.4, or 26.1 mM) was added, and the cells were incubated for 24 h at 37 °C. In a parallel experiment, the cells were treated with 17.4 mM EtOH, and incubated at 37 °C for 0.5 min (control), 2, 24, and 36 h.

After incubation, the cultures were examined with a phase contrast microscope to ensure that the monolayers had remained intact. The supernatant from each well was then discarded, and each well was washed twice with PBS. Endothelial cells were detached with PRG-2

trypsin/EDTA. Trypsin was then inactivated by addition of cell culture media containing 10% fetal bovine serum (FBS). Cell suspensions were then centrifuged, supernatants discarded, and cells re-suspended in culture media. Cell viability was determined by Trypan blue dye exclusion assay using a hemacytometer under light microscopy.

Real-Time PCR Array and Data Analysis

Based on existing literature, we designed a real-time PCR array to include all 29 mammalian TRP genes, and the array was custom-made by SABiosciences/Qiagen (Frederick, MD). Real-time PCR was performed using an ABI Prism 7900HT Fast Detection System (Applied Biosystems, Foster, CA). The threshold and baseline values were set manually, according to the manufacturer's instructions, and the threshold cycle value (Ct) data were analyzed on the manufacturer's website (<http://www.sabiosciences.com/pcr/arrayanalysis.php>). RNA expression of each gene was normalized using two from a set of five housekeeping genes (*β-actin*, *β-2 microglobulin*, *glyceraldehyde-3-phosphate dehydrogenase*, *β-glucuronidase*, and *heat shock protein 90α*) as controls. The relative expression of each gene, compared with the expression of the controls, was calculated using the 2^{-Ct} method. A > 2-fold difference, compared to the control, was interpreted as differential regulation, with positive values indicating up-regulation and negative values indicating down-regulation.

Quantitative Multiplex Analysis of TRP Channels

BMVECs were treated with 0, 17.4, or 52.2 mM EtOH for 24 h. The membrane proteins were extracted using a Mem-PER Plus Membrane Protein Extraction Kit (Thermo Scientific, Rockford, IL). TRP channel protein expression in the BMVECs was quantified using Meso Scale Discovery multi-spot assay (MSD, Rockville, MD) (Freebern et al. 2005). Five, 10, and 20 µg of total membrane proteins were coated on a MSD high-bind plate; thermo solubilization buffer from the extraction kit was used as a blank. The plate was blocked with 150 µl of 5% bovine serum albumin [BSA] (Gold Biotechnology, St. Louis, MO) in 1× PBS, and washed with 200 µl PBS-T (PBS + 0.1% Tween-20). Twenty-five microliters of rabbit anti-mouse TRP antibodies (Alomone Labs, Jerusalem, Israel) was then added to each well at a concentration of 1 µg/mL in PBST-1% BSA, followed by 25 µl of SULFO-tagged anti-rabbit IgG antibody (1 µg/mL in PBST-1% BSA). The plate was sealed and shaken for 2 h at room temperature, then washed three times with 200 µl PBS-T. MSD read buffer T (150 µl) was added to each well, and the plate was read immediately on a SI2400 Sector Imager (Meso Scale Discovery, Rockville, Maryland). The signals from the membrane protein extracts were normalized to those of the blanks, and the relative expression of TRP channel proteins in the EtOH-treated endothelial cells was compared to that of the same proteins in the PBS-control cells.

Construction of an In Vitro BMVEC Barrier and Permeability Assay

An in vitro BMVEC barrier was constructed using Corning Transwell inserts [insert area = 0.33 cm²; pore size = 0.4 µm] (Corning Life Sciences, Tewksbury, MA). A published protocol (Takata et al. 2013; Molino et al. 2014) was used with modifications. Briefly, the isolated and cultured mouse BMVECs (purchased from Cell Biologics, Chicago, IL) (Passage 5 or 7; 5×10^4 total cells per insert in 300 µl of cell culture medium supplied by the vendor) were seeded on the inside of the insert, and 1200 µl of cell culture medium was

added to the bottom side of the transwell. The transwell plates were then incubated at 37 °C with 5% CO₂. The BMVEC barrier formed within 3 d, as confirmed by daily Transendothelial Electrical Resistance (TEER) measurements. Designated concentrations of ethanol were added to both the inserts and bottom of the wells, as well as cell culture incubator trays. To measure the permeability of the BMVEC barrier, 50 µg/mL of Evans Blue Dye (EBD) (Sigma-Aldrich, St. Louis, MO) was added to the upper insert only, and the models were incubated in a humidified cell culture incubator at 37 °C with 5% CO₂ for 24 h. One hundred microliter samples from both the upper insert and the lower chamber were transferred to a clear 96-well plate. Absorbance of EBD was measured at 630 nm using a SpectraMax 190 Microplate Reader (Molecular Devices, Sunnyvale, CA). The permeability was calculated as the amount of EBD passing across the endothelial layer in 24 h (Freese et al. 2014). Permeability coefficient was measured and calculated as described by Deli et al. (Deli et al. 2005). Briefly, the Clearance (cm³) of tracer diffusing from the luminal (insert) to abluminal chamber was calculated by the following formula: Clearance (cm³) = [Concentration]_{Abluminal} × [Volume]_{Abluminal} / [Concentration]_{Luminal}. [Concentration]_{Luminal} is the initial tracer concentration within the insert loaded with tracer; [Concentration]_{Abluminal} is the tracer concentration in the abluminal chamber following treatment. During the experiment, the tracer volume diffused from luminal (insert) to abluminal increased linearly with time. Permeability-surface area product is abbreviated as PS. PS_{time-plotted} was calculated by Clearance (cm³) divided by time (s). PS_{endothelial} was calculated by 1/PS_{endothelial} = 1/PS_{time-plotted} - 1/PS_{insert}. PS_{endothelial} divided by the surface area generated the permeability coefficient (cm/s). For Trpm 7 inhibition, three different Trpm 7 Channel Blockers, NS 8593, CyPPA and SKA-31 (Alomone Labs, Jerusalem, Israel) were added to the inserts at 50 µM, together with a tracer Sodium Fluorescein (NaF) at 10 µg/mL, and incubated in a humidified cell culture incubator at 37 °C with 5% CO₂ for 24 h. One hundred microliter samples from both the upper insert and the lower chamber were transferred to a clear 96-well plate. Fluorescence of NaF was measured using a Gemini EM microplate reader (Molecular Devices, Sunnyvale, CA) with an excitation wavelength of 480 nm and an emission wavelength of 535 nm. The permeability was calculated as the amount of NaF passing across the endothelial layer in 24 h [34].

Statistical analysis: All data were expressed as the mean ± standard deviation (S.D.). Groups were compared using a Student's *t*-test or one-way analysis of variance (ANOVA), followed by a Dunn's post hoc test. Unless otherwise indicated, comparisons between groups were made using a Student's *t* test; *p* < 0.05 was considered significant.

Results

Expression of TRP Channels in Mouse BMVECs

Five of the 29 TRP genes, *Trpm4*, *Trpm7*, *Trpm11*, *Trpm12* and *Trpp2*, were highly expressed in mouse BMVECs, whereas *Trpc1*, *Trpc2*, *Trpc5*, *Trpc7*, *Trpm2*, *Trpm5*, *Trpv2*, *Trpv4* and *Trpp5* were moderately expressed; other TRP genes were not detected in mouse BMVECs (Fig. 1). The expression levels (2^{-Ct} values) of TRP genes relative to those of control housekeeping genes are listed in Supplemental Table 1 of Online Resource 1.

EtOH Concentration- and Time-Dependent TRP Gene Expression

EtOH concentration-dependent TRP gene expression in BMVECs was determined at 24 h after treatment with 0 (PBS control), 4.35, 8.7, 17.4, or 26.1 mM EtOH. Time-dependent TRP gene expression was determined at 0.5 min (control), 2, 24, and 36 h after treatment with 17.4 mM EtOH. TRP mRNA expression in the six subfamilies was as follows (See Supplemental Table 2 of Online Resource 1).

Ankyrin (TRPA) Subfamily—The *Trpa1* gene, the single member of this subfamily, was not expressed in mouse BMVECs, with or without EtOH treatment.

Canonical (TRPC) Subfamily—Among the TRPC subfamily genes, *Trpc1* expression showed a > 2 fold increase after treatment with 26.1 mM EtOH for 24 h compared to control (Fig. 2a). There was a significant increase in *Trpc1* expression at 2 h following addition of 17.4 mM EtOH, followed by a decline in expression at 24 h, with only a slight increase observed after 36 h (Fig. 2b). *Trpc2* expression was increased at 24 h after treatment with 4.35 mM and 17.4 mM EtOH compared to PBS control (Fig. 2a); whereas there was no difference in expression when compared to 17.4 mM EtOH treatment for 0.5 min (Fig. 2b). *Trpc5* expression decreased following treatment with 4.35 mM EtOH for 24 h compared to PBS control. *Trpc5* expression decreased at 24 h after treatment with 17.4 mM EtOH, then increased at 36 h when compared to 17.4 mM EtOH treatment for 0.5 min. There was no difference in *Trpc7* expression at 24 h following treatment with 4.35–26.1 mM EtOH compared to PBS control (Fig. 2a). Following treatment with 17.4 mM EtOH, *Trpc7* expression was decreased at 24 h and 36 h compared to the 0.5 min control (Fig. 2b). Other TRPC subfamily members were not expressed, with or without EtOH treatment.

Melastatin (TRPM) Subfamily—In the TRPM subfamily, expression of *Trpm4* was significantly affected by EtOH. Compared to the PBS control, there was a 5.8-fold and a 4.3-fold decrease in *Trpm4* expression following treatment with 8.7 mM and 17.4 mM EtOH at 24 h, respectively (Fig. 3a). *Trpm2* was also decreased by 17.4 mM EtOH at 24 h (Fig. 3a). When compared to a 0.5 min treatment, *Trpm1*, *Trpm2*, and *Trpm5* were decreased at 2 h – 36 h by 17.4 mM EtOH. *Trpm7* was increased at 2 h after treatment with 17.4 mM EtOH, and then the expression level decreased over time (Fig. 3b). Other TRPM subfamily genes were not significantly affected by EtOH.

Vanilloid (TRPV) Subfamily—Among the six members in the TRPV subfamily, only *Trpv2* and *Trpv4* were expressed in BMVECs. Compared to PBS control, *Trpv2* was not affected by treatment with 4.35–26.1 mM EtOH for 24 h. However, *Trpv2* was initially increased at 2 h after treatment with 17.4 mM EtOH, and then leveled out at 24 h and 36 h when compared to a 0.5 min treatment. *Trpv4* expression decreased 2.1-fold and 2.7-fold following treatment with 8.7 mM and 17.4 mM EtOH for 24 h, respectively, compared to PBS control (Fig. 4). Following treatment with 17.4 mM EtOH, expression of *Trpv4* increased for 24 h – 36 h compared to the 0.5 min control (Fig. 4).

Mucolipin (TRPML) Subfamily—All TRPML subfamily members (*Trpml1–3*) were expressed in mouse BMVECs, and their expression was affected by EtOH. *Trpml1*

expression increased at 2 h after 17.4 mM EtOH treatment compared to the 0.5 min control, and then declined over time (Fig. 5b). *Trpm2* expression decreased after treatment with 8.7 mM EtOH at 24 h compared to PBS control (Fig. 5a); whereas *Trpm2* expression increased 5.4-fold at 2 h after 17.4 mM EtOH treatment, then decreased 3.4-fold and 4.7-fold at 24 h and 36 h, respectively, when compared to the 0.5 min control (Fig. 5b). *Trpm3* expression gradually decreased over 36 h following treatment with 17.4 mM EtOH compared to the 0.5 min control (Fig. 5b).

Polycystin (TRPP) Subfamily—*Trpp2* and *Trpp5* were expressed in mouse BMVECs. *Trpp2* expression decreased 3.3-fold at 24 h after treatment with 17.4 mM EtOH compared to PBS control (Fig. 6a). *Trpp5* expression decreased after 26.1 mM EtOH treatment compared to PBS control (Fig. 6a); however, its expression gradually increased over 36 h after 17.4 mM EtOH treatment compared to the 0.5 min control (Fig. 6b).

EtOH Effects on TRP Channel Protein Expression

The modulation of TRP channel protein expression by EtOH was confirmed by multiplex analysis using MSD multi-spot assays. After the BMVECs were treated with 17.4 mM EtOH for 24 h, TRPC2 protein expression was reduced by approximately 6%, TRPM2 protein was significantly down-regulated by more than 50%, TRPM7 protein decreased by more than 20%, whereas TRPV4 protein was up-regulated by 15% (Fig. 7a). At 24 h after treatment with 52.2 mM EtOH, both TRPM5 and TRPV2 increased by more than 60%, whereas TRPV4 protein was up-regulated by 19% (Fig. 7b). The significant down-regulation of TRPM2, moderate down-regulation of TRPM7, and up-regulation of TRPV2 and TRPV4 were observed at both mRNA and protein levels. Other TRP channels were either not detected by MSD assays, or the changes in expression levels were not statistically significant.

EtOH Effects on In Vitro Endothelial Barrier Permeability and BBB Integrity

Based on EtOH's down-regulation of the relatively high expression of TRPM7 in mouse BMVECs at both mRNA and protein levels, we chose to use TRPM7 to examine the involvement of this TRP channel in maintaining BBB integrity. An in vitro model of a BMVEC barrier was constructed and treated with 0 (control) 17.4 mM, 52.2 mM or 104.4 mM EtOH for 24 h. The passage of a small tracer, Evans Blue Dye (EBD), was used to assess the permeability of the BMVEC barrier. After treatment with 0 mM, 17.4 mM, 52.2 mM and 104.4 mM Ethanol for 24 h, the resistance to permeability caused by BBB was decreased significantly ($p < 0.05$). (Fig. 8a and Supplemental Table 5a of Online Resource 1).

Involvement of TRPM7 in the BBB Permeability and Integrity In Vitro

Three different TRPM 7 Channel Blockers, NS 8593, CyPPA and SKA-31, were used to inhibit TRPM 7 in the in-vitro BBB model. The permeability of the barrier formed by mouse BMVECs increased significantly ($p < 0.05$), compared to control wells without TRPM 7 blockers. In the presence of 50 μ M of each of the three blockers, NS8593, CyPPA, and SKA-31, the permeability of the BMVEC barrier significantly increased in 24 h (Fig. 8b and Supplemental Table 5b of Online Resource 1).

Discussion

As molecular sensors responsible for detection of environmental conditions, such as temperature, mechanical stress, pheromones, taste, and pain, TRP channels are generally expressed in sensory neurons (Clapham 2003). TRP channels are also expressed in many different types of non-neuronal tissues, such as smooth muscle (Guibert et al. 2011), liver (Rychkov and Barritt 2011), kidney (Mene et al. 2013), and immune organs (Rychkov and Barritt 2011), and their functions are diverse. For example, TRPM4 is a Ca^{2+} -activated Na^+ channel; TRPML1 is a nonselective channel inhibited by acidic pH; TRPC1, TRPV4, and TRPP2 are Ca^{2+} permeable channels; and TRPM7 contains a C-terminal Ser/Thr α -kinase domain and is permeable to both Ca^{2+} and Mg^{2+} (Ramsey et al. 2006).

EtOH strongly suppresses innate immunity. Among the three basic components of the BBB, BMVECs are the primary cells that initiate immune responses. EtOH acts on temperature and nociceptive TRPV1, TRPM8, and TRPA1 channels. However, the expression of these TRP channels was not detected in BMVECs in our real-time PCR array experiments, indicating that EtOH's effects on BMVECs may not be mediated by these three particular channels. We did find that several other TRP channels within the TRPC, TRPM, TRPV, TRPML, and TRPP subfamilies are expressed in BMVECs, and that their expression is markedly affected by EtOH in a concentration-dependent manner at both mRNA and protein expression levels. Generally speaking, protein detection techniques are lower in sensitivity than PCR array technique in measuring mRNA. In addition, the availability and suitability of antibodies limits the successful quantification of proteins. For TRP channel proteins, we were able to detect and quantify protein expression using the MSD assay, and those data were consistent with mRNA expression as determined by PCR array.

Among members of the canonical subfamily, we found that *Trpc1*, *Trpc2*, *Trpc5*, and *Trpc7* genes were expressed in BMVECs, and that *Trpc1* and *Trpc2* expression was increased by EtOH. The canonical TRP channels are mainly responsible for Ca^{2+} entry into the endothelium and are activated by phospholipidase C (PLC) β and γ pathways, lipids (diacylglycerol and arachidonic acid), and intracellular calcium (Ca^{2+}) depletion (Nilius et al. 2003), and may form heteromers (Nilius et al. 2007).

The melastatin subfamily represents a diverse group of structurally and functionally distinct ion channels, from Ca^{2+} impermeable TRPM4 and TRPM5, to highly $\text{Ca}^{2+}/\text{Mg}^{2+}$ permeable TRPM6 and TRPM7 (Kraft and Harteneck 2005). The Ca^{2+} permeable, non-selective *Trpm2* gene, which was slightly decreased by EtOH, is highly expressed in immunocytes and the channel is involved in inflammation processes (Sano et al. 2001; Heiner et al. 2003; Kraft et al. 2004). TRPM2 protein is also required for lipopolysaccharide (LPS)-induced production of pro-inflammatory cytokines, IL-6, IL-8, IL10, and TNF- α (Wehrhahn et al. 2010). TRPM2 appears to be involved in endothelial barrier regulation and permeability (Hecquet et al. 2008, 2010). Thus, by decreasing *Trpm2* gene expression, EtOH may suppress inflammatory responses in BMVECs.

We found that *Trpm4* gene, one of the most highly expressed TRP channels in BMVECs, was significantly decreased by EtOH. TRPM4 is a Ca^{2+} -activated monovalent (Na^+ and K^+)

ion channel whose activation is responsible for depolarization of the cell membrane, thus impacting cell physiology, including immune responses (Guinamard et al. 2010, 2011). The membrane depolarization facilitated by TRPM4 channel controls Ca^{2+} overload and oscillation. Reduction in *Trpm4* expression causes a large intracellular Ca^{2+} influx and enhances IL-2 production (Launay et al. 2004). Recently, *Trpm4* gene was also found to be essential for oxidative stress-enhanced endothelial cell migration (Sarmiento et al. 2014). Therefore, the substantial decrease in *Trpm4* expression by EtOH in BMVECs may impact pro-inflammatory responses.

Trpm7, a gene highly expressed in mBMVECs and modulated by EtOH, produces a unique channel that contains a C-terminal Ser/Thr α -kinase domain (Perraud et al. 2011) that is highly permeable to both Ca^{2+} and Mg^{2+} and is critical in maintaining Mg^{2+} homeostasis in the cells (Paravicini et al. 2012). The TRPM7 protein modulates endothelial behavior, and changes in expression may impair endothelial functions (Baldoli et al. 2013). The TRPM7 channel plays an essential role in immune responses, mainly through mediating the intracellular Mg^{2+} level (Brandao et al. 2013). Down-regulation of TRPM7 could lead to a decrease in intracellular Mg^{2+} , causing cell cycle arrest and reduced cell proliferation (Sahni et al. 2010). Disruption of endothelial Mg^{2+} homeostasis resulting from reduction of TRPM7 protein could also be one of the mechanisms by which EtOH causes immunosuppression.

We demonstrated in our in vitro BMVEC barrier model that down-regulation of TRPM7 expression in BMVECs by EtOH had profound effects on endothelial permeability similar to the effects caused by TRPM7 channel blockers, suggesting that blocking TRPM7 channels could disrupt tight junctions between endothelial cells, allowing more molecules to pass through due to decreased expression of occludin, claudin-5, and ZO-1. Thus, TRPM7 is a possible mediator that links EtOH's detrimental effects to BBB integrity.

Among the six members of the vanilloid subfamily, only *Trpv2* and *Trpv4* were expressed in mouse BMVECs. TRPV1–4 channels are sequentially homologous and functionally similar as heat sensors (Kauer and Gibson 2009). The Ca^{2+} -permeable TRPV2 and TRPV4 channels are believed to mediate Ca^{2+} influx at the BBB, which may affect BBB integrity (Brown et al. 2008). Since *Trpv1* expression was not detected in BMVECs, it is likely that *Trpv2* and/or *Trpv4* may mediate EtOH's effects in BMVECs. Down-regulation of *Trpv2* and *Trpv4* expression by EtOH may, therefore, inhibit inflammatory responses and suppress innate immune responses at the BBB.

All three members of the mucolipin subfamily were expressed in mouse BMVECs, with *Trpm1* being particularly abundant. Expression of all three was decreased by EtOH over time. *Trpm1* is a non-selective intracellular channel located on the lysosomal membrane. *Trpm1* is permeable to Ca^{2+} , Fe^{2+} , and Zn^{2+} , and is important for endocytosis and endosomal/lysosomal function (Grimm et al. 2012; Venkatachalam et al. 2013). TRPML proteins appear to function in membrane trafficking ((Dong et al. 2010), and we found that these genes are highly expressed in BMVECs. The exact physiological role of TRPML channels remains elusive, but the reduced expression of these channels by EtOH could cause

a malfunction in endo-lysosomal transport, intracellular vesicular fusion processes, and lysosomal metal ion transport (Kiselyov et al. 2011).

Polycystin subfamily channels are associated with polycystic kidney disease (Ko and Park 2013; Mene et al. 2013). TRPP subfamily members were abundantly expressed in mouse BMVECs, and *Trpp2* was significantly affected by EtOH. *Trpp2*, together with *Trpc1*, is responsible for the transient influx of Ca^{2+} in BMVECs, nitrous oxide synthesis, and actin cytoskeletal rearrangement, leading to damage to the BBB upon stretch-induced injury (Berrout et al. 2012). A decrease in *Trpp2* expression induced by EtOH, therefore, would inhibit inflammatory and innate immune responses in BMVECs.

Conclusion

In summary, EtOH may have much more profound actions on BMVECs than currently known. Our data indicate that EtOH can impair the BBB integrity via its actions on the expression of TRPM7. Figure 9 depicts promising TRP channels involved in alcohol mediated brain dysfunction. We found members of TRP canonical, melastatin, vanilloid, mucolipin and polycystin to be expressed in mouse BMVECs. Ample literatures have reported TRPA1, TRPV1 (Trevisani et al. 2002, Cornett et al. 2008, Matta et al. 2008) and TRPM8 (Benedikt et al. 2007) channels have a role in alcohol mediated brain dysfunction by animal behavior assessment. In this study, we have shown that EtOH mediated downregulation of TRPM7 expression leads to BBB dysfunction as a result of the loss of endothelial cell integrity. TRPM7 controls Ca^{2+} and Mg^{2+} homeostasis, with its activity being regulated by intracellular Mg^{2+} (Runnels et al. 2002; Kozak et al. 2005; Gwanyanya et al. 2006; Langeslag et al. 2007; Macianskiene et al. 2008; Xie et al. 2011; Oh et al. 2012). In addition, our antagonist/agonist approach has confirmed that changes in expression of TRPM7 impairs the integrity of the BBB. Taken together with the literatures, our study has suggested that EtOH's actions might be possibly in line with involvement of intracellular Mg^{2+} in regulation of TRPM7 expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Expression levels of 29 mammalian TRP genes in mouse BMVECs. Genes with high expression levels: *Trpc1*, *Trpm4*, *Trpm7*, *Trpm11*, and *Trpp2*. Genes with moderate expression levels: *Trpc2*, *Trpc5*, *Trpc7*, *Trpm1*, *Trpm2*, *Trpm3*, *Trpm5*, *Trpv2*, *Trpv4*, *Trpm12*, *Trpm13*, and *Trpp5*. Genes with no detected expression: *Trpa1*, *Trpc3*, *Trpc4*, *Trpc6*, *Trpm6*, *Trpm8*, *Trpv1*, *Trpv3*, *Trpv5*, *Trpv6*, *Trpp1*, and *Trpp3*. ($n = 3$ technical triplicates)

Fig. 2.

a Concentration-dependent expression of TRPC subfamily genes in BMVECs following a 24 h treatment with 0 (PBS control), 4.35, 8.7, 17.4, or 26.1 mM EtOH. **b** Time course of TRPC subfamily gene expression in BMVECs treated with 17.4 mM ETOH for 0.5 min (control), 2, 24, or 36 h. * $p < 0.05$, ** $p < 0.01$

Fig. 3.

a Concentration-dependent expression of TRPM subfamily genes in BMVECs following a 24 h treatment with 0 (PBS control), 4.35, 8.7, 17.4, or 26.1 mM EtOH. **b** Time course of TRPM subfamily gene expression in BMVECs treated with 17.4 mM ETOH for 0.5 min (control), 2, 24, or 36 h. *p < 0.05, **p < 0.01

Fig. 4.

a Concentration-dependent expression of TRPV subfamily genes in BMVECs following a 24 h treatment with 0 (PBS control), 4.35, 8.7, 17.4, or 26.1 mM EtOH. **b** Time course of TRPV subfamily gene expression in BMVECs treated with 17.4 mM ETOH for 0.5 min (control), 2, 24, or 36 h. * $p < 0.05$, ** $p < 0.01$

Fig. 5.

a Concentration-dependent expression of TRPML subfamily genes in BMVECs following a 24 h treatment with 0 (PBS control), 4.35, 8.7, 17.4, or 26.1 mM EtOH. **b** Time course of TRPML subfamily gene expression in BMVECs treated with 17.4 mM ETOH for 0.5 min (control), 2, 24, or 36 h. *p < 0.05, **p < 0.01

Fig. 6.

a Concentration-dependent expression of TRPP subfamily genes in BMVECs following a 24 h treatment with 0 (PBS control), 4.35, 8.7, 17.4, or 26.1 mM EtOH. **b** Time course of TRPP subfamily gene expression in BMVECs treated with 17.4 mM ETOH for 0.5 min (control), 2, 24, or 36 h. * $p < 0.05$, ** $p < 0.01$

(a) 17.4 mM, 24 h

(b) 52.2 mM, 24 h

Fig. 7. Fold change in various TRP channel protein expression in BMVECs following a 24 h treatment with 17.4 mM (a) or 52.2 mM (b) EtOH. * $p < 0.05$, ** $p < 0.01$

Fig.8.

Endothelial cell barrier permeability following treatment with **a)** different concentrations of EtOH or **b)** the TRPM7 antagonists, NS8593, CyPPA, or SKA-31. * $p < 0.05$; ** $p < 0.01$. **a** Mouse BMVECs at Passage 7 were seeded into Corning transwell inserts seated in 24-well plates to construct an in-vitro Blood-Brain Barrier (BBB) model. The cells were allowed to grow in the inserts for three days to form a barrier. The same number of wells without cells were used as background. The wells were then treated with 0 mM, 17.4 mM, 52.2 mM and 104.4 mM EtOH for 24 h, and to determine permeability, 50 ng/ μ l Evans Blue Dye (EBD) was added into the inserts at the same time and the model was incubated at 37 $^{\circ}$ C for 24 h. The permeability coefficient was then calculated. *P*-value was calculated using student's *t* test. **b** Mouse BMVECs at Passage 5 were seeded into Corning transwell inserts seated in 24-well plates to construct an in-vitro Blood-Brain Barrier (BBB) model. The cells were allowed to grow in the inserts for three days to form a barrier (BBB). Wells were then treated with three different Trpm 7 Channel Blockers, NS 8593, CyPPA and SKA-31 at 50 μ M for 24 h, and the permeability assay was then performed to determine the effect of TRPM7 blockers on the BBB. *P*-values were calculated using student's *t* test

Fig. 9.

Promising TRP channels involved in alcohol mediated brain dysfunction. TRP channel genes that were found to be expressed in mouse BMVECS as shown in Fig. 1 (black). TRPA1, TRPM8, and TRPV1 channels have been implicated to have a role in alcohol mediated brain dysfunction by animal behavioral assessments (red). EtOH mediated TRPM7 expression downregulation leads to BBB dysfunction by loss of endothelial cell integrity (blue)