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Sphingosine-1-phosphate protects against brain microvascular endothelial junctional protein disorganization and barrier dysfunction caused by alcohol

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Abstract

Objective: Sphingosine-1-phosphate (S1P) has known endothelial barrier protective properties, but whether this extends to the blood-brain barrier (BBB) is unclear. We hypothesized that alcohol-induced disruption of brain microvascular endothelial barrier function and junctional protein organization can be ameliorated by S1P treatment.

Methods: Cultured primary human brain microvascular endothelial cell (HBMEC) monolayers were used to characterize endothelial-specific mechanisms of BBB regulation. Transendothelial electrical resistance (TER) and apparent permeability coefficients for albumin, dextran-4 kDa, and sodium fluorescein were used as indices of barrier function. Junctional localization of Claudin-5, VE-cadherin and β -catenin were determined by immunofluorescence confocal microscopy. S1P was applied following treatment with alcohol.

Results: Alcohol significantly impaired HBMEC TER. Application of S1P after alcohol treatment resulted in a hastened recovery to the baseline HBMEC TER. Alcohol-treated HBMEC had a significantly higher mean permeability than control that was reversed by S1P. Alcohol caused formation of gaps between cells. Treatment with S1P (after alcohol) increased junctional localization of VE-Cadherin, Claudin-5 and β -catenin.

Conclusions: Alcohol impairs the barrier function and junctional organization of HBMEC monolayers. S1P enhanced barrier function and restored junctions in the presence of alcohol, and thus may be useful for restoring BBB function during alcohol intoxication.

Keywords

Sphingosine-1-phosphate; ethanol; blood-brain barrier; endothelial barrier function

INTRODUCTION

The blood brain barrier (BBB) performs an important role in maintaining homeostasis in the central nervous system. This selectively permeable layer is composed of endothelial cells that protect the brain from blood-borne cytotoxins and regulate the entrance of molecules,

while also ensuring the supply of nutrients for proper neural function. Brain microvascular endothelial cells are reported to have tighter intercellular junctions than peripheral endothelial cells [1].

Passage across the BBB may occur through two general routes. One of these routes is transcellular transport through the endothelial cells, either via channels or through vesicular-mediated mechanisms. The second general route of passage across the BBB is paracellular, or between endothelial cells, which is limited by the tightness of the junctional clefts [2]. The transcellular route has an important role in maintaining the basal permeability of the endothelium. However, under pathophysiological conditions, most of the flux of plasma fluid and proteins occur through paracellular cell-cell junctions [3]. These cell-cell connections are characterized by the presence of endothelial junctional proteins that are similar to those found in the classical tight junctions and adherens junctions in the epithelium. These transmembrane proteins seal the interendothelial space and maintain tight cell-cell contact, forming a continuous endothelium. Claudin-5 and Occludin are the major tight junction proteins, which are linked to the actin cytoskeleton by zonula occludens proteins (ZO-1 and ZO-2) [4]. Claudin-5 is expressed primarily in epithelial cells, contributing to epithelial barrier function [5]. However, in the BBB, claudin-5 plays a particularly important role in brain capillary endothelial cells, where this TJ protein is highly expressed [6]. Adherens junction proteins consist of glycoproteins of the cadherin superfamily. The endothelium expresses a specific cadherin (VE-cadherin) that is strictly located at the intercellular junctions of all types of endothelium [7]. VE-cadherin forms complexes with catenins such as β -catenin and α -catenin, which anchor it to the actin cytoskeleton. This association with catenins is necessary for junctional organization and for the adhesive function of these molecules [8].

The functional integrity and low permeability of the BBB depends in part on high levels of expression of junctional proteins, and their high degree of localization at the junctional clefts [9]. Disruption of these proteins by disease or certain drugs may result in increased BBB permeability, exposing the brain to cytotoxins and inflammatory mediators, and consequently impairing neural function [10]. Hence, understanding how various factors can affect BBB junctional proteins is critical for improving methods for prevention of neurological diseases and for the development of novel treatments.

The role of tight junction proteins in the blood-brain barrier has been extensively studied, given their high expression in brain microvessels. However, there is increasing evidence that adherens junction proteins also have important roles in maintaining the integrity of the BBB under pathophysiological conditions. For example, *in vitro* models of brain injury show reduced expression of both tight junctions and adherens junction proteins VE-cadherin and β -catenin in brain endothelial cells, which is accompanied by monolayer hyperpermeability [11,12]. Besides that, ischemic stroke in adult mice is shown to activate the VE-cadherin promoter and increase VE-cadherin expression within ischemic regions, suggesting that endothelial VE-cadherin is involved in the reconstitution of the BBB following brain injury [13]. The maturation of tight junction proteins in the brain has also been shown to be dependent on adherens junctions. In particular, the localization of β -catenin at the cellular membrane allows the maturation of claudin-5. When β -catenin is translocated to the nucleus

it mediates the transcriptional repression of claudin-5 in brain microvascular endothelial cells [10], illustrating how adherens junctions are necessary for the establishment of TJs in the BBB [14].

Alcohol intoxication not only impairs brain function, but also results in neuroinflammation and neurodegeneration in humans and animals [15]. The mechanisms involved in alcohol-induced pathologies include cellular changes, inflammasome activation, apoptosis, and reactive oxygen species (ROS) production [16]. Haorah et. al. (2005) showed that alcohol causes disruption of brain endothelial cell monolayers, accompanied by decreased expression of ZO-1, claudin-5 and occludin [17]. Despite these advances in understanding, no strategies to improve BBB function under such conditions have been advanced.

Our laboratory recently investigated the potential of the bioactive lipid sphingosine-1-phosphate (S1P) to reduce microvascular permeability caused by combined alcohol intoxication and traumatic injury [18,19]. S1P is an abundant sphingolipid present in plasma at nanomolar to micromolar concentrations [20], and has been shown to stabilize barrier function in human pulmonary artery endothelial cells and human umbilical vein endothelial cells (HUVEC) [21]. S1P also helps preserve the glycocalyx of intact microvessels to help maintain normal permeability [22]. In our recent study utilizing a conscious rodent model of combined alcohol intoxication and hemorrhagic shock, we found that S1P significantly reduced microvascular permeability that typically occurs during the resuscitation phase [18,19].

In the current study, we investigated the potential of S1P to enhance the barrier function of brain microvascular endothelial cell monolayer in the presence of alcohol. We assessed to what extent alcohol impairs barrier function and we hypothesized that S1P could ameliorate barrier dysfunction and junctional protein disruption caused by alcohol treatment. In addition, we also previously observed that a cAMP analog with endothelial barrier enhancing properties, 8-CPT-O-Me-cAMP (8-CPT), can restore barrier function in the presence of alcohol in HUVEC monolayers [23]. Therefore, for some experiments we included 8-CPT as a positive control of improved barrier function after alcohol treatment, and determined whether its barrier protective role also extends to brain endothelial cell monolayers.

MATERIALS AND METHODS

Endothelial Cell Culture

Human Brain Microvascular Endothelial Cells (HBMEC) were used as an *in vitro* BBB model. Cells were obtained from ScienCell Research Laboratories (Carlsbad, CA) and grown in 10 cm petri dishes in endothelial cell medium (ECM; ScienCell) supplemented with fetal bovine serum, endothelial growth supplement and penicillin/streptomycin (ScienCell), on 1.5% porcine gelatin matrix (Sigma-Aldrich, St. Louis, MO), in an incubator at 37 °C and 5% CO₂. HBMECs were used at passages 1 to 3.

Pharmacological Agents

The ethyl alcohol was obtained from Pharmco by Greenfield Global (Shelbyville, KY), the sphingosine-1-phosphate (S1P) was from Tocris Biotechne (Minneapolis, MN), and the 8-CPT-2'-O-me-cAMP-2'-O-Me-cAMP (8-CPT) was purchased from Merck-Millipore (Billerica, MA). Fluorescein isothiocyanate (FITC)-conjugated albumin, FITC-dextran and sodium fluorescein were purchased from Sigma-Aldrich (St. Louis, MO).

Alcohol Treatment and Assessment of Endothelial Barrier Function

We assessed endothelial barrier function using two distinct methods. The first was to determine transendothelial electrical resistance (TER) of confluent HBMEC monolayers using the Electric Cell-Substrate Impedance Sensing (ECIS) system (Applied Biophysics, Troy, NY) as previously described [24,25]. The advantages of this method are its sensitivity to detect small changes in barrier function due to nanometer-scale changes in the junctions between cells, and the ability to make moment-to-moment measurements, typically every 1–10 seconds apart [26]. Cells were subcultured onto gelatin-coated 8W1E ECIS electrode arrays and grown until 3 days post-confluence, allowing for junctional maturation. On the day of the experiment, the medium was changed to ECM and the arrays were attached to the ECIS station in a 37 °C, 5% CO₂ incubator and allowed to reach a steady TER of about 15,000 Ω at 4000Hz. The ECIS system allows the cells to be treated while simultaneously recording TER.

The second method we used to evaluate barrier function was to determine the apparent solute permeability (P_{solute}) of HBMEC monolayers to molecules of different sizes, as previously described [25,27,28]. Briefly, HBMEC were seeded on the upper chamber of gelatin-coated Transwell membranes (0.4 μm; Corning, Corning, MA), and allowed to grow until 3 days post-confluence so that they formed mature junctions. On the day of the experiment, the medium was changed to ECM. After 1 hour of baseline, a stock concentration of the molecular tracers sodium fluorescein (0.4 kDa) or sodium fluorescein isothiocyanate (FITC)-conjugated to albumin (66.4 kDa) or dextran (4 kDa) was added to the upper chamber. HBMECs were immediately treated with vehicle + vehicle (0.1% BSA in PBS), alcohol + vehicle, or alcohol + S1P. The S1P was added 5 minutes after alcohol treatment. After 60 minutes of total treatment, samples of media were collected from both upper (luminal) and lower (abluminal) chambers for fluorometric analysis. For the sodium fluorescein tracer, the samples were collected after 10 minutes of alcohol treatment, to avoid equilibration of this tracer in both chambers, which happens within less than an hour. The concentration of the tracer in each chamber was determined using a standard curve, and the permeability coefficient was calculated using the equation $P_{\text{solute}} = [(C_A/t) \times (1/A) \times (V/C_L)]$, where C_A is the abluminal concentration, t is the time (in seconds), A is the area of the membrane (in cm²), V is the volume of the abluminal chamber, and C_L is the luminal concentration.

Experimental Protocols

To assess whether alcohol caused a concentration-related response, the cells were treated with 50, 75 or 100 mM ethyl alcohol. These concentrations are equivalent to two to three times the legal limit for blood alcohol concentration in the United States. The alcohol was

diluted in 0.1% BSA dissolved in PBS (vehicle) prior to addition to the cell media. Controls were treated with isovolumic vehicle. To assess the effects of the barrier enhancers, cells were treated with either S1P (1 μ M), 8-CPT (100 μ M), or isovolumic vehicle (0.1% BSA in PBS). To test whether S1P could reverse any effects of alcohol, cells were treated with alcohol, followed by 1 μ M S1P or isovolumic vehicle, which were administered 5 minutes after alcohol treatment. 100 μ M 8-CPT was used as a positive control for barrier enhancement. It is worth noting that for S1P, the barrier-protective properties occur at physiological concentrations (0.1–1.0 μ M). At higher concentrations (10 μ M), S1P significantly can impair barrier function by disrupting ZO-1 structure and the distribution of F-actin in endothelial cells [29]. Therefore, the concentrations of S1P and 8-CPT used in this study were based on the effective concentrations for enhancing barrier function as previously reported [23,30].

Immunofluorescence Labeling and Laser Confocal Microscopy

To assess the impact of alcohol on intercellular junctions and the potential of S1P to restore junctional integrity, cultured brain endothelial cells were immunolabeled for VE-cadherin (C-19, Santa Cruz Biotechnology, Santa Cruz, CA), Claudin-5 (4C3C2, Invitrogen) and β -catenin (L54E2, Cell signaling) following treatment with vehicle + vehicle (0.1% BSA in PBS), alcohol + vehicle, or alcohol + S1P. S1P was added 5 minutes of treatment with alcohol. The cells were fixed for immunofluorescence labeling 15 minutes after alcohol was added, to match the time-point of maximum drop in TER caused by alcohol and maximum barrier function enhancement elicited by S1P, as observed in the ECIS assay. Briefly, HBMEC were seeded onto gelatin-coated glass coverslips (Fisher Scientific, Hampton, NH) and incubated in endothelial growth medium until confluent. The medium was changed to EBM and allowed to incubate for 1 hour before the treatment. After the various treatments, cells were fixed in 4% paraformaldehyde in PBS for 10 minutes, permeabilized in 0.1% Triton-X-100 in PBS for 10 minutes and blocked for nonspecific binding with 10% serum in PBS for 30 minutes. Cells were incubated with primary antibodies overnight at 4 °C. They were then washed 3X for 10 minutes with 0.1% Tween-20 in PBS, followed by incubation for 1 hour with Alexa 488- and Alexa-555-conjugated secondary antibodies (Life Technologies, Carlsbad, CA). The samples were washed 3X again and the coverslips were mounted onto microscope slides with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA). Images were collected with an Olympus FV1200 spectral inverted laser scanning confocal microscope (Olympus, Center Valley, PA), and the images were analyzed with NIH ImageJ software [31].

Propidium Iodide Viability Assay

In order to assess cell viability following alcohol treatment, we used propidium iodide (PI) staining, according to the manufacturer's protocol (Life Technologies, Carlsbad, CA). Briefly, HBMECs were seeded onto gelatin-coated 35-mm glass bottom dishes (MatTek Corporation, Ashland, MA) and allowed to mature for three days post-confluence. On the day of the experiment the medium was changed for basal endothelial medium and allowed to equilibrate for 1 hour. Cells were treated with vehicle + vehicle (0.1% BSA in PBS), alcohol + vehicle or alcohol + S1P. S1P was added 5 minutes after addition of alcohol and the total time of treatment was 6 hours. After the various treatments, cells were rinsed and

equilibrated with a PI solution for 5 minutes, according to the manufacturer's instructions. After 5 minutes, cell viability was assessed using an ASI Imaging Rapid Automated Modular Microscope system (ASI Imaging, Eugene, OR) with a 10X objective and Micromanager/ImageJ software for image collection. For each culture dish, five fields of view were selected at random and the number of PI-positive cells were counted and divided by the number of total cells.

Data Analysis

For time-course data, the tracings show mean TER over time for each experimental group. Other summarized data are presented as the mean $\pm$ SE. Values of N are for the number of cell assays, representing independent experiments. Differences between two groups were assessed using unpaired t-tests. When three or more groups were compared, analysis of variance (ANOVA), and when appropriate, post-hoc tests to compare treatment groups to the control (Dunnett's test) or to compare all groups (Tukey's test) were performed. For the permeability coefficient analysis, a two-way ANOVA was performed with the treatments (control, alcohol, and alcohol+S1P) and solutes (fluorescein, dextran-4kDa, and albumin) as factors, and Holm-Sidak multiple comparisons test used for post-hoc analysis. Significance was accepted at $p < 0.05$.

RESULTS

The direct impact of different alcohol concentrations (50, 75, and 100 mM) on barrier function of human brain microvascular endothelial cell monolayers was assessed using ECIS. Consistent a previous study on the effects of alcohol on BBB function [17], we found that alcohol causes a concentration-related decrease in HBMEC barrier function (Fig. 1). Alcohol applied at 75 mM and 100 mM alcohol significantly decreased TER compared to vehicle (0.1% BSA in PBS) control (Fig. 1b). The maximum drop in TER was within the first 15 minutes of treatment, and was followed by a sustained decrease in TER compared to baseline that lasted for over 2 hours (Fig. 1a). Cell viability was assessed using PI staining to determine if the drop in TER was due to the activation of cell death pathways. The results showed no significant difference in the percentage of PI-positive cells between alcohol-treated and control groups (data not shown), suggesting that the cells remain viable during alcohol-induced barrier dysfunction.

We next tested the well-characterized endothelial barrier enhancer S1P, to determine if its barrier-enhancing properties extend to HBMEC monolayers. S1P increased TER to 38% above baseline on average, while the positive control 8-CPT caused an average 28% increase (Fig. 2). Vehicle controls caused no change in TER. To examine whether S1P could rapidly restore barrier dysfunction caused by alcohol treatment, we first treated HBMEC monolayers with 75 mM alcohol to elicit a drop in TER, and added S1P 5 minutes later. S1P restored TER well above baseline levels (Fig. 3a). This response was quite rapid, and significantly reduced the amount of the time needed for TER to recover to at least baseline levels after the addition of alcohol (Fig. 3b). Treatment with 8-CPT also ameliorated alcohol-induced barrier dysfunction and decreased the time of recovery, compared to alcohol alone (Fig. 3c, d).

Because the TER changes we observed with alcohol were relatively small, and TER does not necessarily represent permeability to plasma proteins, we also determined how alcohol impacts permeability coefficients of HBMEC monolayers to molecules of three different sizes (Fig 4). Three groups were compared (control, alcohol, and alcohol + S1P), and two-way ANOVA revealed a significant difference due to these treatment groups, independent of which tracer was used ($p < 0.001$). Post-hoc analysis of the main treatment groups revealed that alcohol treatment significantly increased permeability compared to control, and that addition of S1P in the presence of alcohol significantly decreased permeability compared to alcohol alone (Fig. 4). It is worth noting that when a similar post-hoc analysis was performed with subgroups for each individual tracer, no significant differences were found, likely due to the smaller sample sizes in each subgroup.

To assess whether the alcohol-induced decrease in barrier integrity may be due to the disruption of junctional protein organization, we investigated the localization of Claudin-5, VE-cadherin, and β -catenin (Fig. 5). We also tested the ability of S1P treatment (5 min. after addition of alcohol) to rescue any changes caused by alcohol. In control HBMEC monolayers, claudin-5, VE-cadherin and β -catenin form a continuous border between cells, forming junctions that appear very tight. We observed that treatment with 75 mM alcohol for 15 minutes caused an apparent disruption on the normal junctional integrity of all three proteins. In many cells treated with alcohol, instead of the typical continuous junctional protein belt between cells, there was an apparent shift of the proteins to the cytoplasm. We also found that treatment with alcohol caused formation of gaps between HBMECs. In contrast, when S1P was added to alcohol-treated cells, these effects appeared to be reversed. To quantify our observations, we evaluated content of these proteins at the paracellular junctions by measuring the intensities of the immunofluorescence labeling with regions of interests drawn at cell borders. Alcohol treatment did not cause a significant change in junctional content of Claudin-5, VE-cadherin, and β -catenin (Fig. 5). However, addition of S1P after alcohol significantly elevated the intensities of all three when compared to the cells treated with alcohol alone (Fig. 5). These data suggest that the ability of alcohol to reduce barrier function and cause junctional gaps does not necessarily involve a significant loss of VE-cadherin, claudin-5, and β -catenin at intercellular junctions. However, the enhanced barrier function elicited by S1P appears to involve increasing or stabilizing the amounts of VE-cadherin, Claudin-5, and β -catenin within intercellular junctions.

DISCUSSION

Exogenous S1P has been shown to enhance endothelial barrier function at physiological concentrations, while prolonged or high-dose supplementation with S1P can reverse these beneficial effects [29,32]. Previous studies have typically shown a rapid and brief response caused by low-dose S1P treatment in HUVEC, significantly increasing the TER within 2 min, followed by a peak at 5 min, and a decline by 10 min [29,33]. Our current results extend the barrier-protective effects of S1P to HBMEC, and show some similarities with these previous results. For example, TER also starts to decrease 10 min after S1P treatment, although our current data show that S1P-induced enhancement of barrier function is sustained above baseline levels for at least 60 minutes in HBMEC monolayers (Fig. 2a).

The key finding in this study is that S1P can improve the integrity of HBMEC monolayers that have been exposed to alcohol-induced stress. We first demonstrated that alcohol disrupts barrier function in this model of the BBB, as evidenced by the drop in TER on HBMEC monolayers (Fig. 1). We also showed that S1P treatment, at a physiological concentration, reversed the effects of alcohol by significantly decreasing the time needed for recovery to baseline TER in alcohol-treated brain endothelial cell monolayers (Fig. 3). We found that these findings extend to solute permeability (Fig. 4). Furthermore, we observed that alcohol causes a displacement of junctional proteins from the cell borders to the cytoplasm, and causes gap formation between endothelial cells. S1P significantly reverses the effects of alcohol by recruiting these proteins back to the cell borders, significantly increasing their junctional content and reinforcing a tight endothelial monolayer (Fig. 5). A possible explanation for the increase in interendothelial junctional proteins above baseline levels after S1P treatment is that S1P recruits all the available Claudin-5, β -catenin, and VE-cadherin to the cell periphery, including protein molecules that were not yet localized to the cell border before alcohol treatment. This experimental evidence supports the overall concept that exogenous S1P has potential to protect barrier function of brain microvessels during alcohol intoxication.

Our laboratory has previously shown that alcohol increases microvascular leakage in the intestinal mesentery *in vivo*, and also impairs the barrier function of cultured endothelial cell monolayers, including HUVEC and human cardiac microvascular endothelial cells (HMVEC-c). Initially we found that the alcohol-induced decreases in TER could be mitigated with the cAMP analog, 8-CTP [23]. More recently, we demonstrated similar findings with S1P, which we chose for study because it is a typical endogenous bioactive lipid [18]. In the current study, we extend our observations to HBMEC. Our findings of alcohol-induced endothelial barrier dysfunction have some similarities with the findings reported by Haorah et al utilizing bovine brain endothelial cells, in which they observed a decrease in TER at 1 and 2 h post-alcohol treatment [17]. Our current data show that alcohol actually reduces barrier function in a much shorter time frame, causing a drop in TER within the first few minutes after alcohol treatment. This drop is accompanied by the formation of intercellular gaps, and even though our quantitative analysis did not reveal a significant change in the localization of junctional proteins in the first 10 minutes (Fig. 7), Haorah and colleagues reported that this effect is exacerbated with increasing time of alcohol exposure [17].

We have also expanded the knowledge of how S1P can improve barrier function of brain microvascular endothelial barriers by investigating quantitative analysis of confocal images of junctional proteins. An interesting observation was that junctional localization of important proteins such as claudin-5 and VE-cadherin is not significantly altered by alcohol treatment in our model. Nevertheless, intercellular gaps begin to form and likely account for the increases in permeability. With S1P treatment after alcohol, the junctional proteins VE-cadherin, claudin-5, and β -catenin had significantly higher localization in the junctions compared to alcohol alone, and gaps were no longer observed. This finding likely explains the enhanced TER, usually over baseline levels, observed after S1P treatment. It is also worth noting that in our previous study, we observed that 8-CPT also ameliorated VE-cadherin localization at junctions after alcohol treatment. In that study, in addition to

utilizing immunofluorescence labeling of native VE-cadherin, our laboratory was able to confirm the results with live cell imaging experiments utilizing HUVEC expressing VE-cadherin-GFP [23].

An important limitation of our study is that our model of the BBB utilized only HBMEC and not additional supporting cells that would be found in the brain. While this model enabled us to examine the direct impact of alcohol and S1P on brain endothelial cells, we would expect a tighter BBB *in vivo*. The observation that alcohol rapidly reduces TER of HBMEC and increases the permeability might not be relevant in the healthy brain, but is likely to be relevant in a “double-hit” scenario, such as when an injury occurs during alcohol intoxication, or if a chronic neuroinflammatory disease is already present, amplifying the overall detrimental challenge faced by the brain endothelial cells. In such situations, the BBB might become more permeable to larger molecules that typically do not enter the realm of the central nervous system in the presence of alcohol alone. We have observed this type of effect in the mesentery, in which alcohol intoxication exacerbates hemorrhagic shock and resuscitation-induced hypotension and mesenteric microvascular leakage *in vivo* [18]. Importantly, treatment with S1P can reverse these detrimental effects [18]. Based on these previous observations and the current findings, we conclude that there may be potential benefit of the use of S1P or activation of its receptors in conditions that involve alcohol-induced disruption of the BBB. We speculate that S1P-induced barrier enhancement may also be useful for “two hit” scenarios, which represent a topic for future investigation.

PERSPECTIVE

S1P likely acts through S1P receptor 1 (S1PR1) to exert its barrier enhancing functions [29]. Our study sheds light on the potential of S1P to reverse barrier dysfunction caused by insults like alcohol, and on the possible development of S1P-mediated therapies for impairments that involve BBB breakdown. The clinical relevance of the pathways activated by S1P receptor 1 can be highlighted by the currently approved use of its agonist FTY720, a S1P analog used to treat multiple sclerosis under the name Fingolimod [34].

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Fig. 1. Alcohol-induced endothelial barrier dysfunction in cultured human brain endothelial cell (HBMEC) monolayers. *A*. Traces from an individual experiment showing the time course of changes in transendothelial electrical resistance (TER) in HBMEC monolayers following application of vehicle (0.1% BSA in PBS) or 50, 75, or 100 mM alcohol. *B*. The mean maximum alcohol-induced changes in TER in HBMEC, observed 5 minutes post-alcohol treatment were compared. * $p < 0.05$, alcohol ($N = 18$ for each concentration) vs. vehicle control (0.1% BSA in PBS; $N = 12$).

Fig. 2.

S1P elevates barrier function of brain endothelial cell monolayers. **A.** Traces from an individual experiment showing the time course of changes in TER of HBMEC treated with 1 μ M S1P. **B.** The bar graph represents the mean maximum % increase in TER in HBMEC treated with 1 μ M S1P, observed 10 minutes after addition of S1P. * $p < 0.05$, S1P (N = 14) vs vehicle control (0.1% BSA in PBS; N = 9). **C.** Time course of changes in TER of HBMEC treated with 100 μ M of the Epac Activator 8-CPT, used as a positive control. **D.** The bar graph represents the maximum % increase in TER in HBMEC monolayers treated with 100

μM 8-CPT, observed 15 minutes post-treatment. $*p < 0.05$, 8-CPT-2'-O-me-cAMP (N = 13) vs vehicle control (0.1% BSA in PBS; N = 9).

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Fig. 3.

S1P restores TER in alcohol-treated human brain endothelial cell monolayers. **A.** Traces from an individual experiment show the time-course change in TER of HBMEC monolayers treated with alcohol followed by S1P (1 μ M) or vehicle (0.1% BSA in PBS). Alcohol (75 mM) was added at the 0 min time point, and caused a drop in TER. S1P, added 5 minutes later, enhanced barrier function and restored TER in alcohol-treated cells above baseline levels. **B.** Cells treated with S1P took significantly less time to recover back to baseline levels, compared to vehicle controls. * $p < 0.05$, alcohol + S1P (N = 14) vs alcohol + vehicle

controls (N = 14) (bar graph). *C.* 8-CPT restores TER in alcohol-treated HBMEC monolayers. Traces from an individual experiment show the time course change in TER of HBEMC treated with 75 mM alcohol followed by 100 μ M 8-CPT or vehicle (0.1% BSA in PBS). *D.* Cells treated with alcohol followed by vehicle took above 150 minutes to recover TER to baseline levels. Cells treated with alcohol followed by 8-CPT took significantly less time. * $p < 0.05$, alcohol + 8-CPT (N = 7) vs alcohol + vehicle controls (N = 9) (bar graph).

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Fig. 4.

Impact of alcohol treatment on HBMEC monolayer permeability. Monolayers were treated with vehicle + vehicle (0.1% BSA in PBS), 75 mM alcohol + vehicle, or 75 mM alcohol + 1 μ M S1P. Transwell permeability assays were performed to calculate the apparent permeability coefficients for FITC-albumin (66.4 kDa), FITC-dextran (4 kDa) and sodium (Na)-fluorescein (0.4 kDa). The permeability coefficients (P_{solute}) represent an average over 1 hour for FITC-albumin and FITC-dextran, while only a 10-min time period was used for sodium fluorescein due to its relatively high permeability. Two-way ANOVA analysis revealed a significant difference among the three treatment groups ($p < 0.001$). *Indicates $p < 0.05$ between the groups indicated in post-hoc analysis (Holm-Sidak test multiple comparisons test, all solutes included). A similar post-hoc analysis with subgroups for each individual tracer was also performed and revealed no significant differences. $N=3$ independent experiments (with 4 replicates within each experiment) were performed for the sodium fluorescein and FITC-dextran-4 kDa groups, and $N=4$ experiments were performed for the FITC-albumin group.

Fig. 5.

Alcohol causes intercellular gap formation, while S1P enhances paracellular protein organization at intercellular junctions in the presence of alcohol treatment **A**. Representative confocal immunofluorescence microscopy z-projections of claudin-5 (green) and VE-Cadherin (red) in confluent cultured HBMEC treated with vehicle + vehicle (0.1% BSA in PBS), alcohol + vehicle, and alcohol + S1P. Arrows indicate gap formation associated with alcohol treatment. Cells treated with 75 mM alcohol followed by 1 μ M S1P do not present gap formation. While the quantification (bar graphs) of VE-cadherin and claudin-5 at the

paracellular cleft do not show a significant change upon alcohol treatment, S1P treatment after alcohol significantly increases their localization at junctions compared to alcohol alone. Cell nuclei are labeled in blue. Scale bar = 45 μ m. * $p < 0.05$ alcohol + S1P (N = 7) vs. alcohol + vehicle (N = 8). *B.* Representative immunofluorescence microscopy images of β -catenin of confluent HBMEC monolayers treated with vehicle + vehicle, alcohol + vehicle, and alcohol + S1P. Arrows indicate gap formation associated with alcohol treatment. Treatment with S1P after alcohol was able to restore junctional integrity. Cell nuclei are labeled in blue. Scale bar = 45 μ m. Quantification of β -catenin at the paracellular junctions of HBMECs monolayers treated with vehicle + alcohol do not show a significant change compared to vehicle-only treated cells. However, S1P treatment significantly increases β -catenin content at the junctions (bar graph). Images were analyzed using ImageJ software, ** $p < 0.01$ alcohol + S1P (N = 7) vs. alcohol + vehicle (N = 8).