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Fibrinogen Fragment X Mediates Endothelial Barrier Disruption via Suppression of VE-cadherin

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Abstract

Background: Major traumatic injury is associated with early hemorrhage-related and late-stage deaths due to multiple organ failure (MOF). While improvements to hemostatic resuscitation have significantly reduced hemorrhage-related deaths, the incidence of MOF among trauma patients remains high. Dysregulation of vascular endothelial cell (EC) barrier function is a central mechanism in the development of MOF; however, the mechanistic triggers remain unknown. Accelerated fibrinolysis occurs in a majority of trauma patients, resulting in high circulating levels of fibrin(ogen) degradation products, such as fragment X. To date, the relationship between fragment X and EC dysregulation and barrier disruption is unknown. The goal of this project was to determine the effects of fragment X on EC barrier integrity and expression of paracellular junctional proteins that regulate barrier function.

Materials and Methods: Human lung microvascular endothelial cells (HLMVECs) were treated with increasing concentrations of fragment X (1, 10, and 100 μg/mL), and barrier function

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was monitored using the xCELLigence live-cell monitoring system. Quantitative PCR (qPCR) was performed to measure changes in EC expression of 84 genes. Immunofluorescent (IF) cytostaining was performed to validate qPCR findings.

Results: Fragment X treatment significantly increased endothelial permeability over time ($p<0.05$). There was also a significant reduction in VE-cadherin mRNA expression in fragment X-treated HLMVECs compared to control ($p=0.01$), which was confirmed by IF staining.

Conclusions: Fragment X may induce EC hyperpermeability by reducing VE-cadherin expression. This suggests that a targeted approach to disrupting EC-fragment X interactions could mitigate EC barrier disruption, organ edema, and MOF associated with major trauma.

Keywords

endotheliopathy; trauma; fibrinogen degradation products; hyperfibrinolysis; hyperfibrinogenolysis; endothelial permeability

INTRODUCTION

Trauma has been the leading cause of death in the United States for individuals aged 1 through 44 years over the past 3 decades.[1] Dysregulation of the coagulation system has been identified as a mechanistic driver of both early hemorrhage-related deaths and late-stage deaths due to multiple organ failure (MOF).[2,3] While improvements to hemostatic resuscitation have significantly reduced hemorrhage-related deaths, the incidence of MOF among patients who survive initial hemorrhage remains high.[4] Identification of mechanisms responsible for MOF after traumatic injury could lead to novel medical interventions to improve long-term outcomes in trauma patients.

The vascular endothelium is a key determinant of normal organ function through its tightly controlled regulation of coagulation, inflammation, and barrier integrity.[5] In particular, dysregulation of vascular barrier function is a unifying mechanism in the pathogenesis of trauma-related MOF.[3] The vascular barrier is comprised, in part, of endothelial cells (ECs) connected by junctional proteins that restrict the movement of fluid and proteins into tissues.[6] Vascular endothelial (VE)-cadherin is an EC-specific adherens protein essential in maintaining cell-cell adhesions.[6,7] It is expressed in all tissue types throughout the body, making it among the most important regulators of barrier integrity.[8] As such, changes in the cellular environment that result in reductions in VE-cadherin expression or shedding of soluble VE-cadherin result in vascular barrier disruption that increases fluid leakage into the extravascular space and subsequent tissue edema that contributes to the development of MOF.[9–11] Although marked reductions in VE-cadherin expression and vascular barrier integrity have been reported following trauma and hemorrhage,[12] the mechanistic triggers remain unknown.

Hyperfibrinolysis, defined as accelerated clot breakdown, is a major contributor to coagulopathic bleeding after trauma, particularly among those patients in hemorrhagic shock. A recent multicenter retrospective analysis found that 75% of trauma patients had evidence of moderate fibrinolysis, and 15% showed evidence of severe hyperfibrinolysis.[13,14] During fibrinolysis and fibrinogenolysis, profibrinolytic enzymes

convert circulating plasminogen to plasmin, which then cleaves fibrinogen into fragment X and fibrin into D-dimers. Further cleavage of fragment X generates fragments D and E. These fragments, referred to as fibrin(ogen) fragments/degradation products (FDPs), are present at high levels in the plasma after traumatic injury[15] and can circulate via the bloodstream to distant vascular beds.[9] Prior work has shown that incorporation of fragment X into a developing fibrin network results in clots with reduced tensile strength that are more susceptible to lysis, contributing to an increased risk of bleeding.[16] To date, it remains unknown whether fragment X can modulate EC function.

The goals of our study were to examine the effects of fragment X on EC barrier integrity and EC expression of genes that regulate permeability and other cellular functions. We hypothesized that fragment X would induce disruption of EC barrier function, thereby providing a novel link between trauma, vascular barrier disruption, and MOF.

METHODS

Cell culture

Human lung microvascular endothelial cells (HLMVECs) (Cell Applications, Inc., San Diego, CA) derived from a single male donor were cultured using microvascular endothelial cell basal media (Cell Applications, Inc.) supplemented with microvascular endothelial cell growth supplement (Cell Applications, Inc.) per vendor's instructions.

Endothelial permeability

HLMVECs were seeded at a concentration of 14,000 cells per well into a 16-well gold electrode-equipped E-plate (ACEA BIO, San Diego, CA) that was pretreated with attachment factor solution (Cell Applications, Inc.). Cells grew for 48 hours to become confluent, as signified by a plateau of electrical impedance, and then switched to low serum media for 2 hours. Cells were subsequently treated with vehicle (media alone) or fragment X (Bio-Rad Laboratories Inc., Hercules, CA) at 1 μg/mL, 10 μg/mL, or 100 μg/mL in media, and EC barrier function was monitored for 24 hours. Treatment doses of fragment X were selected based on concentrations of total FDPs reported in admitted trauma patients and are similar to past reports of EC treatment with FDPs.[15,17] Electrical impedance was measured and recorded every 10 minutes by cell-microelectronic sensing (xCELLigence RTCA, ACEA BIO). The cell permeability index was used to assess EC barrier function and was defined as the change in electrical impedance from baseline to 24 hours post-treatment. Data were normalized to vehicle-treated well.

Microarray

HLMVECs were seeded in 6 well plates at a density of 300,000 cells/well and grown to 80% confluence. HLMVECs were treated with vehicle (media alone) or fragment X (100 μg/mL) for 4 hours at 37°C. Total RNA was then extracted and quantified using an RNeasy Mini Kit (QIAGEN, Germantown, MD) and Thermo Fisher NanoDrop Spectrophotometer (Waltham, MA), respectively. DNA was eliminated using the RNase-Free DNase Set (QIAGEN), and the RT^2 First Strand Kit (QIAGEN) was used to facilitate the reverse transcription reaction. Quantitative real-time PCR (qPCR) using the RT^2 SYBR Green qPCR

Mastermix (QIAGEN) was performed per vendor's protocol using the RT^2 Profiler PCR Array for Endothelial Cell Biology (PAHS-015ZC-12, QIAGEN) to assess changes in EC expression of 84 EC-specific gene targets in response to fragment X compared to control; housekeeping genes, such as beta actin and glyceraldehyde-3-phosphate dehydrogenase, was also assessed. A gene list is provided in Table 1. Amplification was performed using the Applied Biosystems StepOnePlus real-time PCR system with the following parameters: 10 minutes at 95°C, 15 seconds at 95°C, and 1 minute at 60°C for 40 cycles.

Immunocytostaining and microscopy

HLMVECs were seeded into a 24-well culture plate pretreated with attachment factor solution at a concentration of 75,000 cells per well and allowed to grow for 48 hours at 37°C. Cells were washed once with Hanks' Balanced Salt Solution (Cell Applications, Inc.) and then treated with vehicle (media alone) and fragment X at 1 μg/mL, 10 μg/mL, or 100 μg/mL (200 μL final volume, in duplicate). Cells were incubated for 4 hours at 37°C. Media was then aspirated, and cells were washed once with phosphate-buffered saline (PBS) (Lonza, Walkersville, MD) before being fixed with 4% paraformaldehyde (PFA) (Acros Organics, Branchburg, New Jersey)-supplemented PBS for 30 minutes at room temperature. The PFA-supplemented PBS was then aspirated, and the cells were washed in PBS for 5 minutes (3 times), then 2% goat serum (Thermo Fisher Scientific) diluted in PBS for 60 minutes. The cells were incubated overnight at 4°C with a 1:800 dilution of primary antibody against VE-cadherin (Life Technologies Corp., Carlsbad, CA) in 2% goat serum. The following day, primary antibody solution was aspirated, and the cells were washed in 0.1% bovine serum albumin (BSA)-supplemented PBS for 5 minutes (3 times), then a 1:500 dilution of secondary antibody goat anti-mouse IgG (Molecular Probes, Inc., Eugene, OR) to 2% goat serum for 60 minutes, and finally 0.1% BSA-supplemented PBS for 5 minutes (3 times).

After the final wash was aspirated, the cells were mounted with Vectashield Antifade Mounting Media with 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc. Burlingame, CA) onto a glass slide. The mounted cells were stored covered overnight at 4°C to allow for best staining. The cells were visualized and photographed using fluorescence microscopy (A1/A1R Microscope System, Nikon, Dexter, MI) and NIS-Elements software. VE-cadherin staining was quantified using NIS-Elements BR Analysis 4.50.00 64-bit software to determine fluorescence intensity using binary area normalized to number of cells per image.

Statistical analysis

Continuous data were assessed for distribution normality using the Shapiro-Wilk test and Quantile-Quantile plots. All continuous data were normally distributed. Statistical differences in normalized cell index between vehicle- and fragment X-treated cells were assessed at each time point with one-way analysis of variance (ANOVA) using Tukey's adjustment for multiple comparisons. Differences in gene expression between vehicle- and fragment X-treated cells were determined using two-sided Student's *t* tests. Effects of fragment X treatment on VE-cadherin staining were determined with one-way ANOVA using Tukey's adjustment for multiple comparisons.

RESULTS

Effects of fragment X on EC barrier function

To investigate the effects of fragment X on EC barrier function, we utilized human primary lung microvascular ECs. Lung ECs were selected as lungs are rich in ECs and among the first organs to be affected following trauma and hemorrhagic shock.[18] Fragment X treatment of lung ECs resulted in a reduction in endothelial barrier function over time, with significant changes between vehicle and fragment X groups (1 and 100 μg/mL) observed at both 12 and 24 hours (Figure 1A). The most pronounced changes were observed 24 hours after treatment, with fragment X having a significant effect at 1 and 100 μg/mL as compared to vehicle (Figure IB).

Effects of fragment X on expression of EC-specific gene targets

In order to examine the effects of fragment X on EC gene transcription, HLMVECs were treated with 100 μg/mL of fragment X, the dose at which we observed the most significant increases in endothelial permeability, and gene expression measured by qPCR using the RT^2 Profiler PCR Array for Endothelial Cell Biology. Genes for which we observed a significant or ≥ 2-fold change in expression from vehicle are reported (Figure 2). Fragment X induced a 2.24-fold increase in EC expression of vascular endothelial growth factor A (VEGFA; $p<0.01$) and 1.31-fold increase in occludin ($p<0.01$). In addition, fragment X treatment resulted in a 0.69-fold decrease in von Willebrand factor (vWF; $p<0.01$) expression, 0.19-fold decrease in prostacyclin synthase ($p<0.01$), 0.51-fold decrease in vascular cell adhesion molecular 1 (VCAM1; $p=0.49$), 0.68-fold decrease in endoglin ($p=0.01$), and 0.71-fold decrease in VE-cadherin ($p=0.01$). Notable changes that did not reach statistical significance included a 2.47-fold increase in tissue factor expression, 3.8-fold increase in E-selectin, 2.25-fold increase in interleukin (IL)-11, and 2.56- and 4.08-fold increases in endothelin 1 and 2, respectively. Collectively, fragment X induced a shift toward a gene signature that was general barrier disruptive, procoagulant, and anti-angiogenic.

Effects of fragment X on VE-cadherin expression

As discussed in the Introduction, VE-cadherin is a crucial protein for maintaining EC barrier integrity and ubiquitously expressed throughout the body. Our qPCR analyses identified significant reductions in VE-cadherin expression in fragment X-treated ECs (Figure 3A and described above). To validate and further investigate this, we performed immunofluorescent (IF) cytostaining and subsequent visualization by fluorescent microscopy. In the vehicle-treated cells, VE-cadherin exhibited clear, substantial paracellular staining, whereas cells treated with increasing doses of fragment X had decreased levels of VE-cadherin (Figure 3B). Representative images are provided in Figure 3C. While a slight yet significant increase in occludin gene expression was observed upon fragment X treatment in our qPCR analyses, no differences in occludin expression by IF were noted (data not shown).

DISCUSSION

Vascular endothelial barrier disruption is a central mechanism in the development of MOF after severe trauma and hemorrhage. Fibrinolysis and fibrinogenolysis are hallmark

features of traumatic coagulopathy that result in significant increases in circulating levels of FDPs.[15,19] Fragment X is a product of fibrinogenolysis that contributes to overall clot instability, yet its role in modulating EC function is unclear.[16] Here, we found that fragment X treatment results in disruption of endothelial barrier integrity, which is associated with a decrease in EC expression of VE-cadherin. In addition to impacting VE-cadherin, fragment X also induced significant changes in several other genes that regulate EC-mediated coagulation, inflammation, angiogenesis, and vasoconstriction.

Fragment X is a large fibrinogen degradation product that is missing a portion of the COOH-terminal of the fibrinogen α chain. It has been found to circulate at high levels in patients undergoing tissue plasminogen activator (tPA) therapy and remains in systemic circulation for 24 hours.[20] Importantly, fragment X retains its polymerization potential and can, therefore, be incorporated into developing fibrin clots.[16] Past work has demonstrated that this results in formation of unstable clots that are more susceptible to tPA-mediated degradation.[16,21] Our data demonstrate that fragment X can also interact with ECs, modulating gene transcription and increasing endothelial permeability. Combined, these data indicate that fragment X could be a mechanistic driver of both coagulopathy and endotheliopathy, linking early hemorrhage and hyperfibrinolysis with late-stage MOF. Interestingly, the data herein were generated using microvascular ECs derived from lungs, an organ that is particularly rich in tPA, the enzyme that drives plasmin generation and subsequent production of FDPs.[3] Lung edema is also a hallmark feature of MOF. Our findings that lung ECs are sensitive to FDP-mediated changes in barrier function indicate that localized fibrinolysis in tPA-rich organs could be a mechanistic underpinning driving susceptibility to trauma-induced organ injury.

While this is the first study to report that fragment X can induce endothelial permeability, these data are in agreement with past findings that demonstrate a role for other FDPs in vascular dysfunction. Fragment E has been shown to directly bind EC VE-cadherin, thus destabilizing the endothelial barrier and promoting leukocyte recruitment and transmigration.[9,22,23] It has also been shown to bind peritoneal macrophages and induce secretion of pro-inflammatory cytokines IL-1β and IL-6.[24] In addition, Ge et al have shown that fibrinogen fragment D can promote endothelial permeability through augmenting EC production of urokinase plasminogen activator, which increases cell-mediated plasmin generation and extracellular matrix degradation.[25,26] Furthermore, fibrinogen itself has been shown to modulate EC function via its interaction with both VE-cadherin and ICAM-1 to induce endothelial permeability and bridge leukocyte adhesion to the endothelium, respectively.[27,28] In contrast to these findings, other reports have demonstrated that fully intact fibrinogen can confer endothelial protection through the endothelial PAK1/Cofilin signaling pathway,[29,30] and its fragment Bβ15-42 has vascular protective qualities, preserving EC barrier function in animal models of dengue and septic shock and reducing neutrophil infiltration into the end organs during hemorrhagic shock.[9,31,32] Thus, the degree of fibrin(ogen) proteolysis and the resultant fragment structure uniquely determines the function of the fragment and its impact on the endothelium.

The findings herein highlight a potential opportunity for future development of therapeutics to modulate fragment X release or its interaction with ECs. Importantly, empiric or goal-

directed administration of the antifibrinolytic tranexamic acid (TXA) is a currently accepted strategy for attenuating plasmin generation and subsequent fibrin(ogen)olysis and release of fragment X after major trauma. Recent data indicate that, *in vitro*, TXA can mitigate EC glycocalyx degradation and preserve barrier integrity[33,34]; however, in prospective studies, there is no observed difference in EC activation markers between trauma patients who do and do not receive TXA.[11] Our data demonstrating fragment-X-induced endothelial barrier disruption is a possible explanation.

TXA is a lysine analog that occupies lysine binding sites on tPA, plasminogen, and plasmin, preventing its direct binding to fibrin(ogen) via kringle domain interactions instead of active site inhibition.[35–37] As such, lysine analogs are unable to prevent fragment X formation by plasmin because the same kringle domain interactions are not required for fragment X formation.[38] Fragment X is also generated rapidly, within 30 minutes of tPA administration,[16,20] which could indicate that fragment X is already present in sufficient amounts to induce EC damage prior to administration of antifibrinolytic therapy. Thus, novel strategies to disrupt the interaction between ECs and fragment X could provide an alternative approach. Currently, the EC receptor for fragment X is unknown, and thus, future work from our team will focus on identifying this receptor in addition to fragment X domains that interact with ECs. In addition, we seek to determine the precise signaling events induced by fragment X that result in reduced VE-cadherin expression and the impact of blocking these interactions *in vitro* and *in vivo.*

This study has several limitations. First, fragment X doses for our study were selected based on total FDP levels in plasma after trauma and *in vitro* studies using other FDPs. Our knowledge of plasma fragment X levels in trauma patients is limited by availability of high-throughput mass spectrometry screening approaches capable of quantifying fragment X in small sample volumes. Second, our studies utilized lung-derived microvascular ECs, and data could differ using cells from different vascular beds. In addition, our findings are restricted to *in vitro*, static culture systems that will require future translation into animal models. Finally, this study utilized primary ECs harvested from a single male donor. A male donor was selected given that between 75-80% of highest-level trauma activations are men.[39–41] However, past work has demonstrated that females are protected from hemorrhage-related deaths and less likely to develop complications during hospitalization. While recent findings have identified platelet hyper-reactivity in female injured patients compared to males,[42] little is known about the sex-specific responses of ECs in this setting. Diebel et al demonstrated that treatment with estrogen, but not testosterone, reduced hypoxic injury to ECs.[43] These data could indicate that expression of sex-specific hormones could prime or modulate how ECs respond to major trauma and hemorrhage. Our future experiments will address how biological factors like sex and age dictate endothelial gene expression and functional changes in response to fragment X.

CONCLUSIONS

Fragment X induces EC barrier disruption, which is associated with loss of VE-cadherin expression. Future identification of a targeted approach to prevent the interaction between

fragment X and ECs could mitigate vascular barrier disruption and subsequent organ edema and MOF following severe trauma.

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AVAILABILITY OF DATA –

Data will be made available upon reasonable request.

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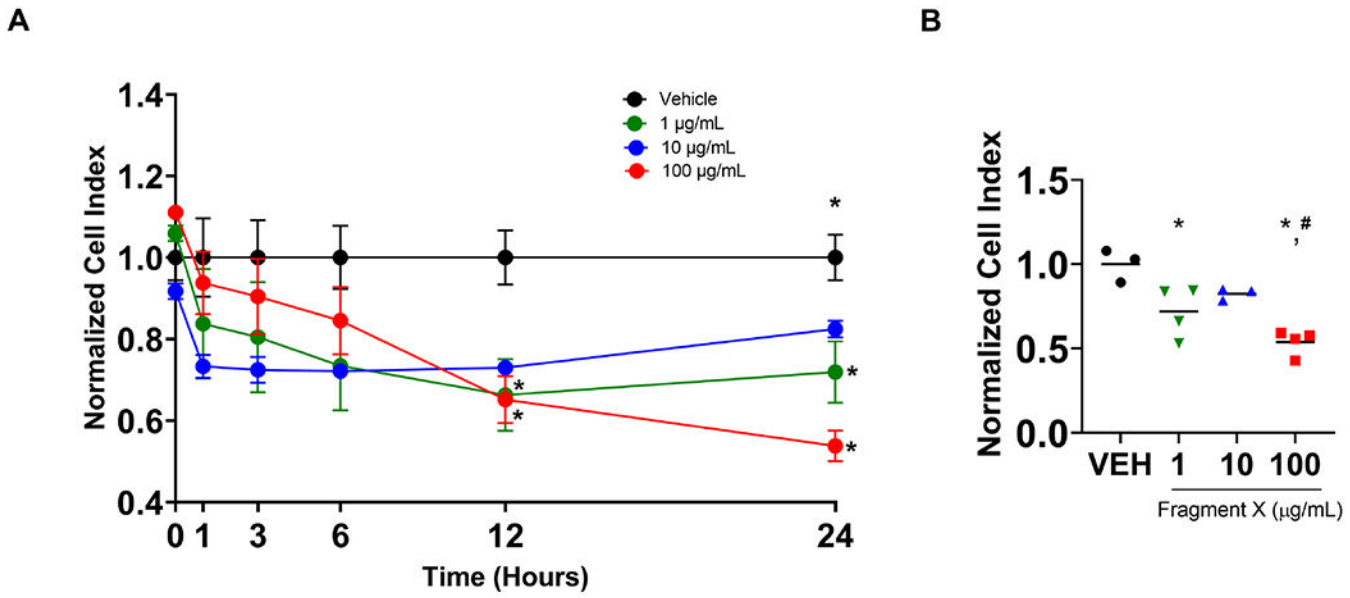

Figure 1. Impact of Fragment X on Endothelial Barrier Function.

Lung microvascular endothelial cells were treated with vehicle (black) or 1 (green), 10 (blue), or 100 (red) µg/mL of fragment X, and barrier function was monitored using XCelligence for 24 hours. Data are expressed as mean and standard deviation (A). Data were normalized to vehicle and cell index (i.e., cell connectivity) at 24 hours. Data are represented as a dot plot with bars denoting the mean values (B). N=3-4 independent XCelligence experiments performed in technical triplicates. * denotes $p<0.05$ compared to vehicle. # denotes $p<0.05$ compared to 10 µg/mL. VEH=vehicle

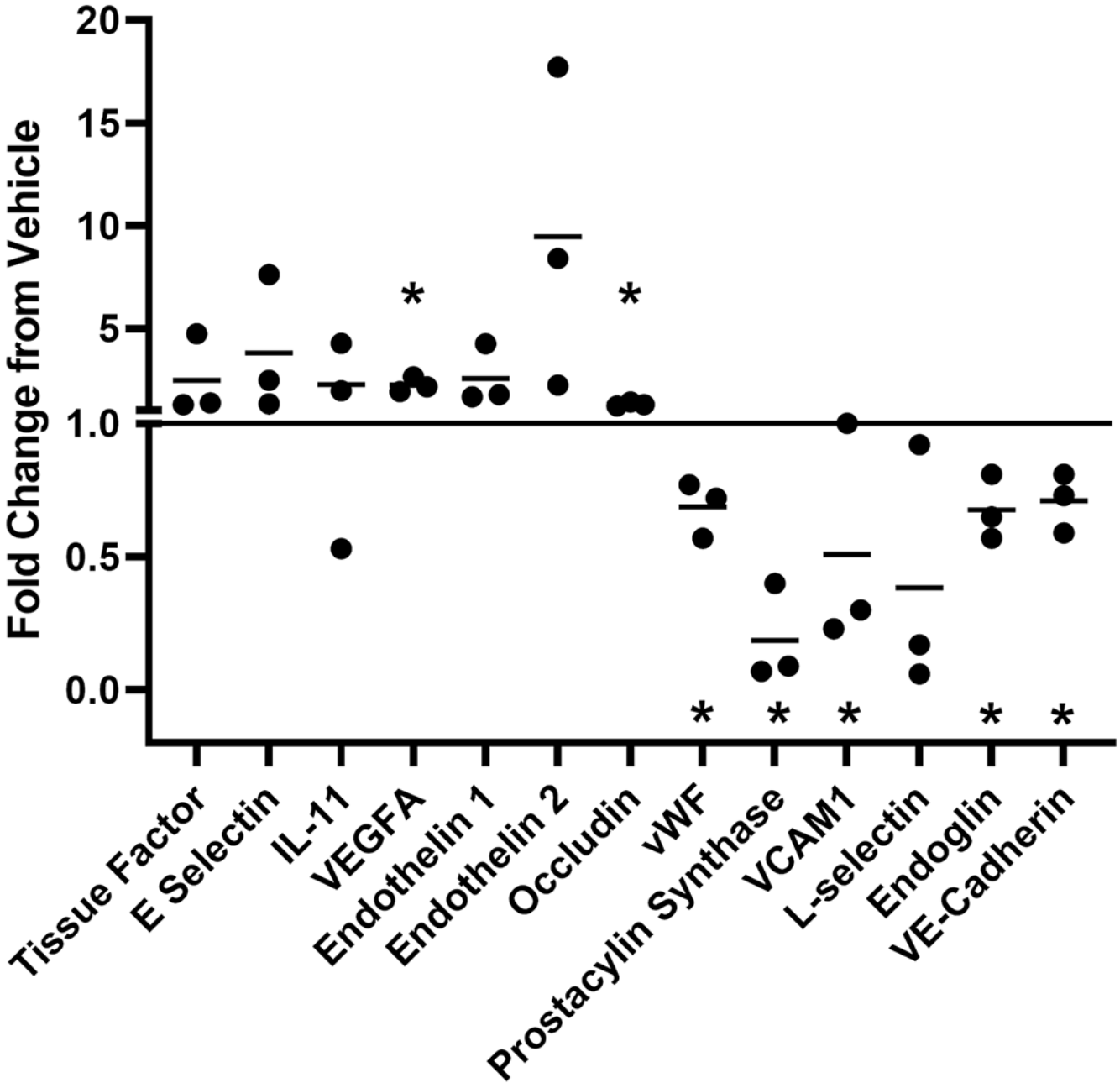

Figure 2. Effect of Fragment X on Endothelial Gene Transcription.

RNA was collected from lung microvascular endothelial cells 4 hours after treatment with vehicle or 100 μg/mL of fragment X. Gene expression was assessed using the RT^2 Profiler PCR Array for Endothelial Cell Biology. Data are expressed as fold change from vehicle. Data are expressed as dot plots with bars denoting mean values. Cell treatments, RNA collection, and microarray analyses were performed as N=3 independent biological replicates. Microarray samples were run in technical duplicate and biological triplicate. * denotes $p<0.05$ compared to vehicle. IL-11=interleukin 11; VEGFA=vascular endothelial growth factor A; vWF=von Willebrand factor; VE-Cadherin=vascular endothelial cadherin

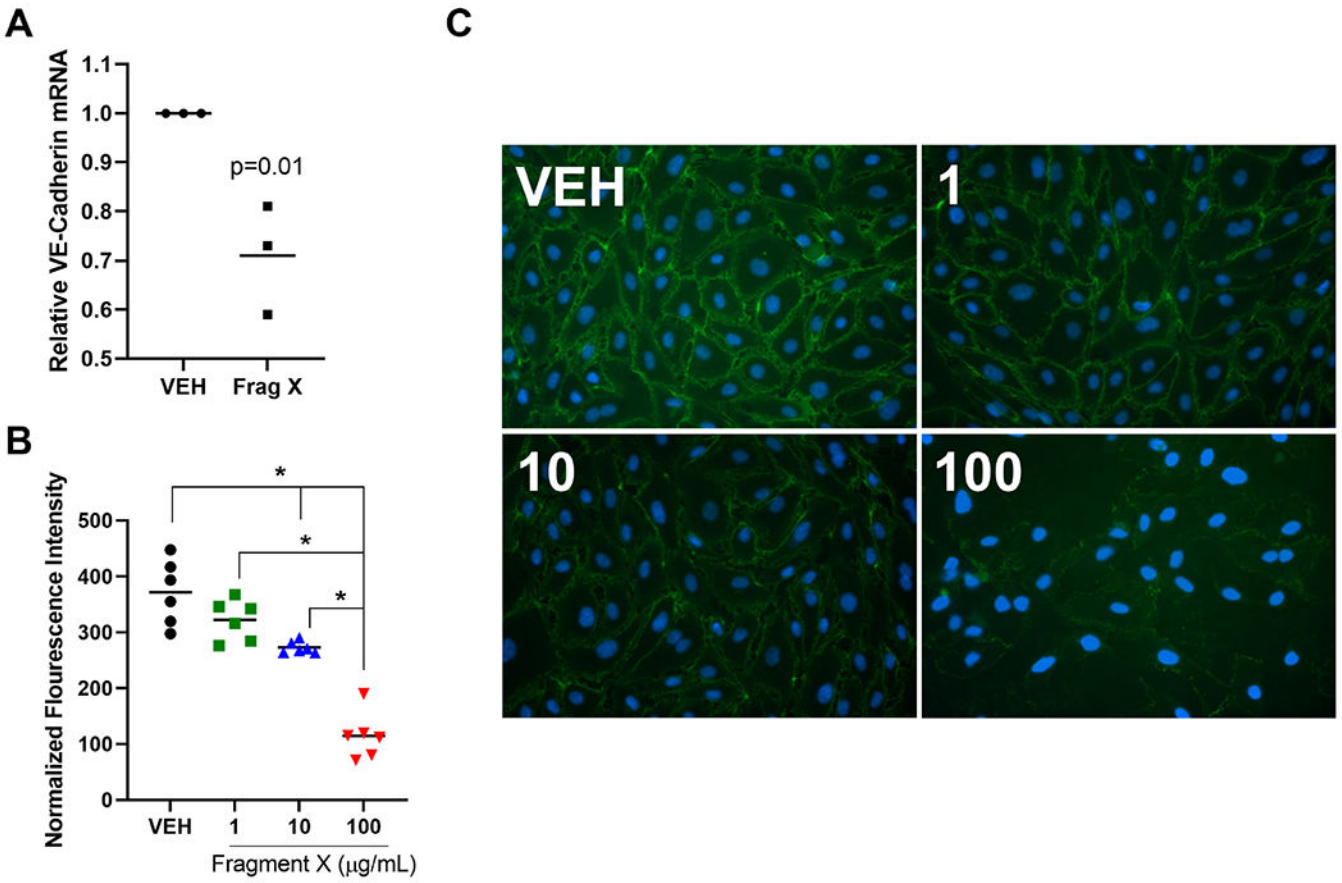

Figure 3. Effect of Fragment X on Endothelial VE-Cadherin Expression.

Vascular endothelial (VE)-cadherin mRNA levels assessed by RT^2 Profiler PCR Array for Endothelial Cell Biology relative to vehicle are illustrated. Data are expressed as dot plots with bars denoting mean values. Experiments were performed in biological triplicate (A). Lung microvascular endothelial cells were treated with vehicle or 1, 10, or 100 μg/mL of fragment X followed by immunostaining with anti-VE-cadherin antibodies and fluorescence intensity quantified by NIS-Elements and normalized to the cell number. Data are expressed as dot plots with bars denoting mean values (B). Representative images are provided (C). Cellular treatments, staining, and fluorescence quantification were performed as N=6 independent biological replicates. * denotes $p<0.05$ between groups. VEH=vehicle

Table 1.

RT^2 Profiler PCR Array for Endothelial Cell Biology Gene Targets

Gene	Description	Category
ACE	Angiotensin I converting enzyme 1	Vascular tone
AGT	Angiotensinogen	Vascular tone
AGTR1	Angiotensin II receptor, type 1	Vascular tone
ANGPT1	Angiopoietin 1	Vascular tone
CCL2	Chemokine (C-C motif) ligand 2	Chemokine
CCL5	Chemokine (C-C motif) ligand 5	Chemokine
CDH5	VE-cadherin	Vascular tone
CX3CL1	Chemokine (C-X3-C motif) ligand 1	Chemokine
EDN1	Endothelin 1	Vascular tone
EDN2	Endothelin 2	Vascular tone
ENG	Endoglin	Vascular tone
F2R	Protease-activated receptor 1	Coagulation
F3	Tissue factor	Coagulation
ICAM1	Intercellular adhesion molecule 1	Cytokine
IL11	Interleukin 11	Cytokine
IL1B	Interleukin 1, beta	Cytokine
IL3	Interleukin 3 (colony-stimulating factor, multiple)	Cytokine
IL6	Interleukin 6 (interferon, beta 2)	Cytokine
IL7	Interleukin 7	Cytokine
NOS3	Nitric oxide synthase 3 (endothelial cell)	Vascular tone
OCLN	Occludin	Vascular tone
PDGFRA	Platelet-derived growth factor receptor, α polypeptide	Chemokine
PECAM1	Platelet/endothelial cell adhesion molecule	Chemokine
PLAT	Tissue plasminogen activator	Coagulation
PLAU	Urokinase plasminogen activator	Coagulation
PLG	Plasminogen	Coagulation
PROCR	Endothelial protein C receptor	Coagulation
PTGIS	Prostaglandin I2 (prostacyclin) synthase	Chemokine
SELE	Selectin E	Chemokine
SELL	Selectin L	Chemokine
SELPLG	Selectin P ligand	Chemokine
SERPINE1	Plasminogen activator inhibitor type 1	Coagulation
TFPI	Tissue factor pathway inhibitor	Coagulation
THBD	Thrombomodulin	Coagulation
TNF	Tumor necrosis factor	Cytokine
VCAM1	Vascular cell adhesion molecule 1	Chemokine

Gene	Description	Category
VEGFA	Vascular endothelial growth factor A	Vascular tone
VWF	Von Willebrand factor	Coagulation