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Protective Mechanism of the Selective Vasopressin V_{1A} Receptor Agonist Selepressin against Endothelial Barrier Dysfunction

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ABSTRACT

Sepsis and septic shock are among the most common causes of death in the intensive care unit; advanced therapeutic approaches are thus urgently needed. Vascular hyperpermeability represents a major manifestation of severe sepsis and is responsible for the ensuing organ dysfunction and failure. Vasopressin V_{1A} receptor ($V_{1A}R$) agonists have shown promise in the treatment of sepsis, increasing blood pressure, and reducing vascular hyperpermeability. The effects of the selective $V_{1A}R$-selective agonist selepressin have been investigated in an in vitro model of thrombin-, vascular endothelial growth factor–, angiopoietin 2–, and lipopolysaccharide (LPS)-induced pulmonary microvascular endothelial hyperpermeability. Results suggest that selepressin counteracts the effects of all four endothelial barrier disruptors in a concentration-dependent manner, as reflected in real-time measurements of vascular permeability by means of transendothelial electrical resistance. Further, selepressin protected the barrier integrity against the LPS-mediated corruption of the endothelial monolayer integrity, as captured by VE-cadherin and actin staining. The protective effects of selepressin were abolished by silencing of the vasopressin $V_{1A}R$, as well as by atosiban, an antagonist of the human $V_{1A}R$. p53 appears to be involved in mediating these palliative effects, since selepressin strongly induced its expression levels, suppressed the inflammatory RhoA/myosin light chain2 pathway, and triggered the barrier-protective effects of the GTPase Rac1. We conclude that $V_{1A}R$-selective agonists, such as selepressin, may prove useful in the improvement of endothelial barrier function in the management of severe sepsis.

SIGNIFICANCE STATEMENT

A cardinal sign of sepsis, a serious disease with significant mortality and no specific treatment, is pulmonary endothelial barrier dysfunction that leads to pulmonary edema. Here, we present evidence that in cultured human lung microvascular endothelial cells, the synthetic, selective vasopressin V_{1A} receptor agonist selepressin protects against endothelial barrier dysfunction caused by four different edemogenic agents, suggesting a potential role of selepressin in the clinical management of sepsis.

Introduction

Sepsis is an excessive systemic inflammatory response to infectious and noninfectious causes that may progress to multiple organ failure and death. Although sepsis is an important medical issue causing 270,000 deaths in the United States each year (Rhee et al., 2017), there have been few recent advances in the development of novel sepsis therapeutics. The need for new therapies is urgent.

Endothelial dysfunction is an important aspect of sepsis and leads to anomalies of vascular barrier function (Kumar et al., 2009). The endothelium is a cellular system lining the interior wall of blood vessels, thus establishing a barrier between circulating blood and organ parenchyma (Barabutis et al., 2016). This crucial vascular barrier is also responsible for the transport of essential nutrients and gases and is indispensable to proper organ function.

The consequences of severe endothelial dysfunction in sepsis include dysregulated hemostasis and vascular reactivity, tissue edema, and organ failure, leading to mortality (Hawiger et al., 2015; Walborn et al., 2019). Strategies improving endothelial barrier function and reducing vascular hyperpermeability could play an important role in improving sepsis outcomes.

Vasopressin receptor agonists, including arginine vasopressin (AVP) and the vasopressin prodrug terlipressin, improved outcomes in patients with sepsis (Russell et al., 2017). The efficacy of vasopressin agonists is likely to result in part from vasoconstriction elicited by the $V_{1A}R$, preventing sepsis-associated hypotension, although other mechanisms also appear to play a role. Vasopressin and terlipressin activate multiple members of the vasopressin receptor family, which elicit varying responses, including the V_2 receptor (V_2R),

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ABBREVIATIONS: Ang, angiopoietin; AVP, arginine vasopressin; ECIS, electric cell-substrate impedance sensing; HLMVEC, human lung microvascular endothelial cell; TEER, transendothelial electrical resistance; $V_{1A}R$, vasopressin V_{1A} receptor; VEGF, vascular endothelial growth factor; V_2R, vasopressin V_2 receptor.

which stimulates antidiuretic responses and the release of clotting factors that may be detrimental in sepsis, suggesting that a V_{1A}R-selective agonist such as selepressin may be superior to vasopressin. In ovine sepsis studies, selepressin dramatically reduced vascular hyperpermeability in addition to maintaining blood pressure, resulting in reduced pulmonary edema, lower inflammatory cytokine levels, and improved organ function compared with vasopressin (He et al., 2016). In a phase IIa clinical trial, selepressin reduced catecholamine use in patients with sepsis while maintaining pressure and reducing net fluid balance (Russell et al., 2017). The positive impact of selepressin on vascular hyperpermeability in sepsis and superiority to vasopressin support the potential therapeutic importance of selective V_{1A}R agonism in this setting and suggest that action in the vascular endothelium may play a role in the impact of selepressin on vascular hyperpermeability.

To examine the mechanism by which selepressin might improve vascular barrier function and reduce vascular hyperpermeability, its impact on endothelial barrier function was investigated in an in vitro model of vascular hyperpermeability. We previously reported the characterization of human lung microvascular endothelial cells as a model of vascular permeability, responding to stimuli that cause vascular hyperpermeability with increased permeability in vitro (Catravas et al., 2010). We have recently published that wild-type p53 regulates the opposing actions of ras homolog family member A (RhoA) and ras-related C3 botulinum toxin substrate 1 (Rac1) and protects the endothelium against inflammatory insults due to bacterial infections (Barabutis et al., 2015). This action of p53 involves inhibiting the actin-severing activity of cofilin (Barabutis et al., 2018a), as well as preventing the barrier-disruptive effect of myosin light chain 2 (MLC2) phosphorylation (Barabutis et al., 2019).

The pathophysiology of sepsis appears to involve edemogenic agents acting by a variety of mechanisms to disrupt endothelial barrier function, including thrombin, VEGF, angiopoietin (Ang) 2, and LPS. Thrombin induces endothelial hyperpermeability via the catalytic activity of the enzyme and activation of the thrombin receptor (Rabiet et al., 1996), and a similar effect is exerted upon the activation of the VEGF receptor, affecting the actomyosin cytoskeleton (Siejka et al., 2012). Ang2 levels are elevated in sepsis (Fisher et al., 2016), and Ang2 binding to the TEK tyrosine kinase (Tie2) receptor triggers endothelial permeability as well (David et al., 2013). The bacterial toxin LPS is a potent inflammatory stimulus that binds to the toll-like receptor 4 receptor to trigger vascular hyperpermeability. Agents to address endothelial hyperpermeability in sepsis should ideally demonstrate the ability to prevent the action of a broad variety of edemogenic agents such as these.

In the present study, we examine the mechanism of selepressin action in this vascular endothelial model by exposing primary human lung microvascular endothelial cells to thrombin, VEGF, Ang2, and LPS. Real-time measurements of endothelial permeability by means of transendothelial electrical resistance showed that pretreatment of human lung microvascular endothelial cells with selepressin prevented endothelial barrier dysfunction inflicted by all four edemogenic agents. These protective actions appear to involve induction of p53 expression and suppression of ras homolog family member A (RhoA) and MLC2 activation, as well as activation of the barrier-protective action of ras-related C3 botulinum toxin substrate 1 (Rac1) and enhancement of the expression of VE-cadherin. Collectively, this study supports the hypothesis that selepressin may be efficient in reducing the vascular hyperpermeability associated with sepsis and that it may prove a useful component in the management of patients with sepsis.

Materials and Methods

Reagents. p53 (9282s), and p-myosin light-chain 2 antibodies were obtained from Cell Signaling (Danvers, MA). B-actin antibody (P8999) and CelyticM lysis reagent (C2978) were purchased from Sigma-Aldrich (St Louis, MO). Secondary mouse and rabbit antibodies were purchased from Licor (Lincoln, NE). Pierce bicinchoninic acid protein assay kit and nitrocellulose membranes were obtained from Fisher Scientific (Pittsburgh, PA). VE-cadherin and F-actin antibodies were from Abcam, 4',6-Diamidino-2-Phenylindole (DAPI) and Alexa Fluor secondary antibodies were from Thermo Fisher Scientific, and Texas Red-X phalloidin was from Life Technologies. Tween 20, bovine serum albumin, PBS, tris-buffered saline, and ProLong Gold antifade reagent were purchased from Thermo Fisher Scientific. Paraformaldehyde was obtained from Electron Microscopy Sciences, and Triton X-100 was from Aqua Solutions.

VEGF, LPS, thrombin, and Ang2 were obtained from Sigma-Aldrich. Selepressin was provided by Ferring Pharmaceuticals, and its structure and synthesis have been previously published (Wisniewski et al., 2011).

Rac1 Activity Assay. Rac1 activation was detected by the Rac1 pull-down activation assay (#BK035; Cytoskeleton, Denver, CO). Briefly, 500 μg of cell lysates was incubated with GST-Rhotekin-RBD fusion protein and was coupled to glutathione resin. After precipitation, the complexes were washed with the lysis buffer, eluted on SDS-PAGE sample buffer, immunoblotted, and probed with Rac1 antibody. Aliquots were taken from supernatants prior to precipitation and were used to quantify total Rac1.

RhoA Activity Assay. RhoA activation was detected by the RhoA pull-down activation assay (#BK036; Cytoskeleton). Five hundred micrograms of cell lysates was incubated with GST-Rhotekin-RBD fusion protein and was coupled to glutathione resin. The complexes were then washed with radioimmunoprecipitation assay buffer, eluted on SDS-PAGE sample buffer, immunoblotted, and probed with RhoA antibody. Aliquots were taken from supernatants prior to precipitation and were used to quantify total RhoA.

Transfections. siRNA against arginine vasopressin receptor 1 (sc-29767) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An irrelevant small interfering RNA (siRNA) from the same source that does not lead to any specific degradation of any known cellular mRNA was used as a control (sc-37007). For transfection, the siRNA was diluted in Opti-MEM I Reduced Serum Medium, and Lipofectamine 2000 was diluted in an equal volume of Opti-MEM I and incubated for 30 minutes at room temperature. The oligomer-Lipofectamine complexes were added to cells that had reached 70% confluence and were cultured in media free of antibiotics. The medium was replaced 8 hours after the transfection (Barabutis et al., 2013).

Cell Culture. In-house harvested and identified human lung microvascular endothelial cells (HLMVECs) were isolated and maintained in M199 media supplemented with 20% FBS and antibiotics/antimycotics, as described previously (Catravas et al., 2010). HLMVECs used in these studies were from multiple adult male donors (age <65 years). They were harvested from fresh, discarded lung tissue obtained from lobectomy or pneumonectomy procedures. Mouse endothelial cells were grown in Lonza EGM-2 medium (CC-3202).

Protein Isolation and Western Blot Analysis. Proteins were isolated from cells or tissues using CelLyticM Lysis Reagent or radioimmunoprecipitation assay buffer. Protein-matched samples

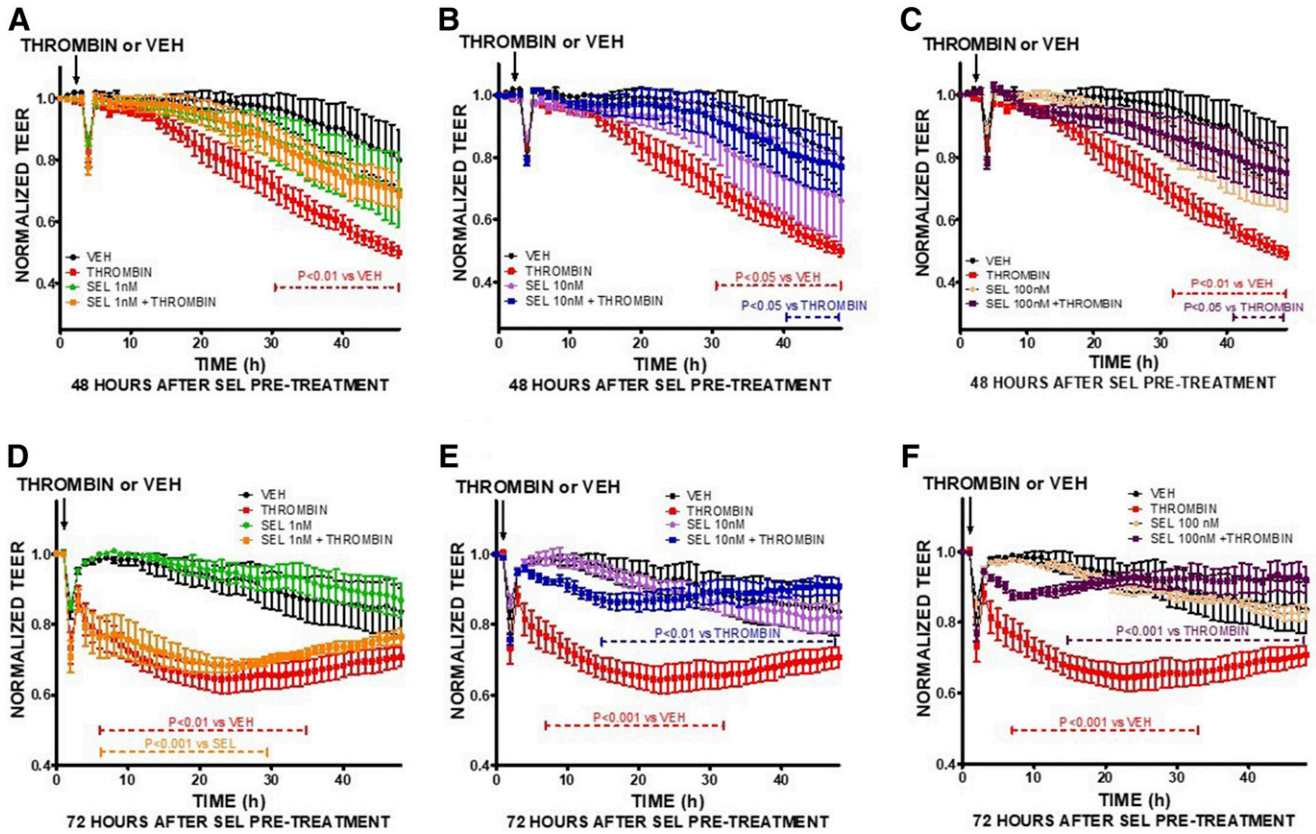

Fig. 1. Protective effect of selepressin (SEL) against thrombin-induced endothelial barrier dysfunction. HLMVECs were pretreated for 48 hours with either vehicle (VEH; 0.01% DMSO) or 1 nM (A), 10 nM (B), 100 nM (C) selepressin and were exposed to either vehicle (PBS) or thrombin (1 U/ml). A gradual increase in endothelial permeability (reduced TEER) was observed in the thrombin-treated cells, which was reduced in selepressin-treated cells. Similarly, HLMVECs were pretreated for 72 hours with either vehicle (0.01% DMSO) or 1 nM (D), 10 nM (E), 100 nM (F) selepressin and were exposed to either vehicle (PBS) or thrombin (1 U/ml). A gradual increase in endothelial permeability (reduced TEER) was again observed in thrombin-treated cells, which was significantly prevented in 10 and 100 nM selepressin-treated cells. In all the cases, $N = 4$ per group. Means ± S.E. Two-way ANOVA for repeated measures followed by Bonferroni post-hoc test. Text color reflects the group that is being compared. Arrow indicates the time of addition of either thrombin or vehicle.

(40 μg per lane) were separated by electrophoresis through 12% SDS-PAGE Tris·HCl gels. Wet transfer was used to transfer the proteins onto nitrocellulose membranes. The membranes were incubated for 1 hour at room temperature in 5% nonfat dry milk in Tris-buffered saline–0.1% (v/v) Tween 20. The blots were then incubated at 4°C overnight with the appropriate primary antibody (1:1000; for β-actin, the concentration was 1:5000). The signal for the immunoreactive proteins was developed by using the appropriate secondary antibody (1:5000) and visualized in a LICOR Odyssey CLx imaging system (Barabutis et al., 2018a).

Measurement of Endothelial Barrier Function. The barrier function of confluent endothelial cell monolayers was estimated by electric cell-substrate impedance sensing utilizing an ECIS model 1600R in confluent monolayers per the instructions of the manufacturer (Applied Biophysics, Troy, NY). All experiments were conducted with cells that had reached a steady-state resistance of at least 800 Ω (Barabutis et al., 2018a). To avoid interruption of data recording, selepressin was not washed out prior to agonist administration.

Confocal Microscopy. Confocal microscopy was performed using an Olympus FluoView FV10i confocal microscope with a 488-nm excitation filter for VE-cadherin (Alexa Fluor 488 Ab), 595-nm for F-actin (Texas Red Ab), and 359-nm filter for 4',6-Diamidino-2-Phenylindole (DAPI) (nuclei). Each coverslip was divided into 49 squares, and 10 squares were chosen at random and analyzed under 60× magnification, making 10 images diagonally of each square. For the quantitative calculation of stained proteins, mean density of VE-cadherin (green color) was measured on the periphery of each cell, and F-actin (red color) of throughout each cell by Fiji ImageJ software.

Densitometry/Statistical Analysis. ImageJ software (National Institutes of Health) was used to perform densitometry of immunoblots. All data are expressed as mean values ± S.E.M., and n represents the number of experimental repeats. Two-way ANOVA for repeated measures with Bonferroni post hoc test was used in Figs. 1–3 and 5. One-way ANOVA for independent samples with Bonferroni post hoc test was used in all other figures. A value of $P < 0.05$ was considered significant. GraphPad Prism 4 (version 4.03; Graph Pad Software, Inc., CA) was used for data analysis.

Results

The expression of $V_{1A}R$, $V_{1B}R$, V_2R, and oxytocin receptor mRNA and protein in HLMVECs was first confirmed by real-time quantitative polymerase chain reaction (qPCR) and Western blotting, respectively (data not shown). We then investigated the possible barrier-protective effects of selepressin using ECIS. In pilot experiments, we did not observe protective actions with selepressin pretreatment periods of less than 48 hours. Thus, all subsequent studies were performed after a 48- or 72-hour pretreatment of selepressin prior to the addition of other agonists.

Selepressin Pretreatment Ameliorates Thrombin-Induced Endothelial Barrier Dysfunction in a Concentration--Dependent Manner. HLMVECs were seeded on gold electrode arrays and were exposed for 48 (Fig. 1, A–C) or 72 hours (Fig. 1, D–F) to either vehicle (0.01% DMSO) or 1 (Fig. 1, A and

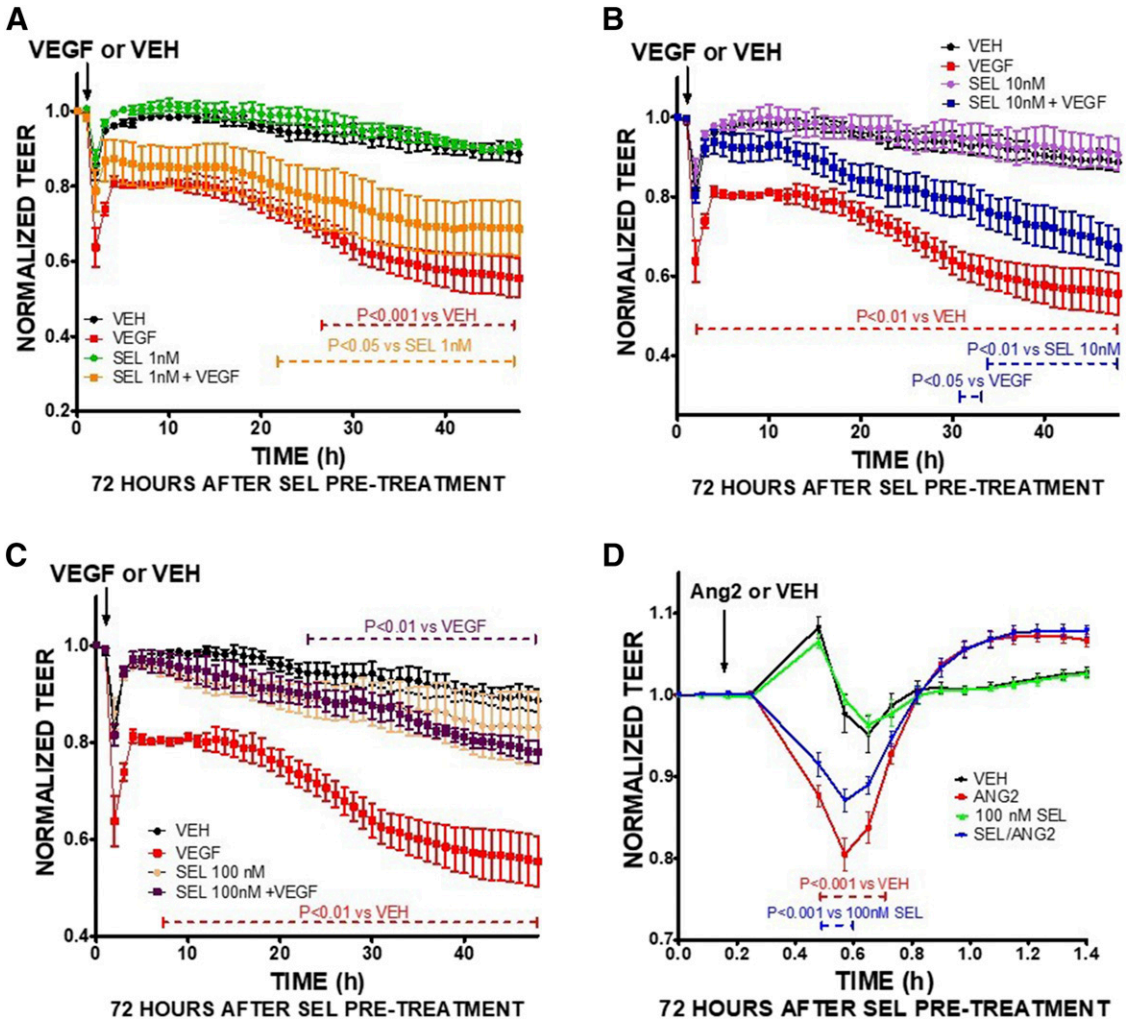

Fig. 2. Protective effect of selepressin against VEGF- and angiopoietin 2–induced endothelial barrier dysfunction. HLMVECs were pretreated for 72 hours with either vehicle (VEH; 0.01% DMSO) or 1 nM (Fig. 2A), 10 nM (B), or 100 nM (C) selepressin (SEL) and were exposed to either vehicle (PBS) or VEGF (100 ng/ml). A gradual increase in endothelial permeability (reduced TEER) was observed in the VEGF-treated cells, which was concentration dependently reduced in selepressin-treated cells. Similarly, HLMVECs were pretreated for 72 hours with either vehicle (0.01% DMSO) or 100 nM selepressin (D) and were exposed to either vehicle (PBS) or Ang2 (400 ng/ml). Selepressin significantly suppressed Ang2-induced hyperpermeability. $N = 4$ per group. Means ± S.E. Two-way ANOVA for repeated measures followed by Bonferroni post hoc test. Text color reflects the group that is being compared. Arrow indicates the time of addition of VEGF, Ang2, or vehicle.

D), 10 (Fig. 1, B and E), or 100 nM (Fig. 1, C and F) of selepressin prior to PBS (vehicle) or thrombin (1 U/ml) treatment. Transendothelial electrical resistance (TEER) was monitored continuously for the indicated times. In all cases, the cells pretreated with selepressin were less susceptible to thrombin than those exposed to PBS (Fig. 1). The effect of selepressin was concentration-dependent; virtually complete protection was obtained with exposure to either 10 or 100 nM of selepressin (e.g., Fig. 1, E and F vs. Fig. 1D).

Selepressin Pretreatment Ameliorates VEGF-Induced Barrier Dysfunction in a Concentration-Dependent Manner. HLMVECs were seeded on gold electrode arrays and were exposed for 72 hours to either vehicle (0.01% DMSO) or 1 (Fig. 2A), 10 (Fig. 2B), or 100 nM (Fig. 2C) of selepressin prior to PBS (vehicle) or VEGF (100 ng/ml) treatment. Cells pretreated with selepressin were variably resistant to VEGF-induced hyperpermeability (Fig. 2). Selepressin exerted a clear concentration-dependent protective effect; cells that were pretreated with 100 nM of selepressin were virtually unaffected by VEGF (Fig. 1C).

Selepressin Pretreatment Ameliorates the Angiopoietin 2–Induced Barrier Dysfunction in a Concentration-Dependent Manner. After HLMVECs formed confluent monolayers, they were pretreated for 72 hours with 100 nM of selepressin and exposed to 400 ng/ml of Ang2. Ang2 produced a very rapid, significant decrease in TEER, reflective of impaired barrier function. Selepressin partially, but significantly, reduced the Ang2 effect (Fig. 2D).

Selepressin Pretreatment Ameliorates the LPS-Induced Vascular Barrier Dysfunction in a Concentration-Dependent Manner. HLMVECs were seeded on gold electrode arrays and were exposed for 72 hours to either vehicle (0.01% DMSO) or 1 (Fig. 3A), 10 (Fig. 3B), or 100 nM (Fig. 3C) of selepressin prior to PBS (vehicle) or LPS (1 EU/ml) treatment. As published previously, LPS profoundly reduced TEER values. Selepressin effectively prevented the LPS-triggered barrier disruption in a concentration-dependent manner (Fig. 3).

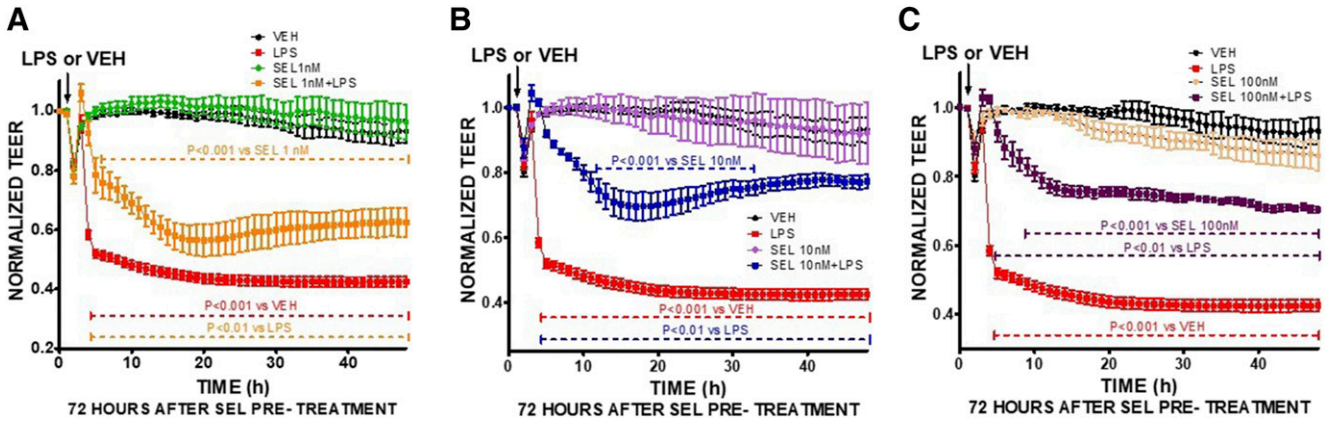

Fig. 3. Protective effect of selepressin against LPS-induced endothelial barrier dysfunction. HLMVECs were pretreated for 72 hours with either vehicle (VEH; 0.01% DMSO) or 1 nM (A), 10 nM (B), and 100 nM (C) selepressin (SEL) and were exposed to either vehicle (PBS) or LPS (1 EU/ml). A gradual increase in endothelial permeability (reduced TEER) was observed in LPS-treated cells, which was significantly suppressed in all selepressin-treated cells. $N = 4$ per group. Means ± S.E. Two-way ANOVA for repeated measures followed by Bonferroni post hoc test. Text color reflects the group that is being compared. Arrow indicates the time of addition of either LPS or vehicle.

To better visualize the barrier-protective effects of selepressin, we calculated the efficiency of protection at different concentrations and pretreatment times and against the three barrier disruptors studied. Efficiency was calculated at the time of maximal response (nadir TEER value) to the edemogenic agent as the ratio of the difference in TEER values between each selepressin plus edemogenic agent sample from the average of all four edemogenic agent alone samples to the average of all four vehicle values; this ratio was then multiplied by 100 to produce percent efficiency of protection. As shown in Fig. 4, selepressin expressed different protective profiles against the three disruptors and after different times of pretreatment. Selepressin appeared most effective against thrombin-induced barrier dysfunction and after 72 hours of pretreatment.

The Protective Effects of Selepressin against the LPS-Induced Barrier Dysfunction Are Mediated by the Vasopressin Receptor Activation. HLMVEC were pretreated for 72 hours with vehicle (0.01% DMSO) or a cocktail of selepressin (100 nM) and the vasopressin receptor antagonist atosiban (1 μM) before exposure to vehicle (PBS) or LPS (1 EU/ml). Figure 5A demonstrates that atosiban suppressed the protective effect of selepressin against LPS. To further investigate, confluent HLMVECs were transfected with either irrelevant (non-targeting control-NTC) siRNA or siRNA that specifically targets the $V_{1A}R$ for 72 hours. Transfected cells were then exposed to either selepressin or vehicle for an additional 72 hours prior to LPS (1 EU/ml) or vehicle (PBS) treatment. As shown in Fig. 5B, silencing of the $V_{1A}R$ abolished the protective effect of selepressin in LPS-triggered endothelial barrier dysfunction.

Selepressin Prevents the LPS-Induced Loss of VE-Cadherin and Cortical Actin. Endothelial cells were seeded onto glass slides and left to grow till confluence. They were then exposed for 72 hours to 0.01% DMSO (control) or four different doses (1, 10, 100, and 1000 nM) of selepressin before LPS (1 EU/ml) or vehicle (PBS) treatment. The cells were then fixed and incubated with VE-cadherin and actin antibodies, and cortical VE-cadherin and actin staining was observed by confocal microscopy (Fig. 6A) and quantified by ImageJ (Fig. 6, B and C). LPS profoundly reduced both cortical VE-cadherin staining and the ratio of cortical to total actin staining. Selepressin exerted a concentration-dependent protective effect against these effects of LPS.

Silencing of the Vasopressin Receptor 1A Does Not Abolish the Protective Effects of Selepressin against the LPS-Induced Loss of VE-Cadherin Staining. Confluent HLMVECs were transfected for 48 hours with $V_{1A}R$ siRNA or irrelevant siRNA. After the transfections, the cells were treated with either vehicle (0.01% DMSO) or 100 nM selepressin for 72 hours before LPS or vehicle exposure. Figure 7A demonstrates that, surprisingly, silencing of the $V_{1A}R$ did not prevent selepressin from protecting against LPS-induced loss of VE-cadherin cortical staining. To confirm the

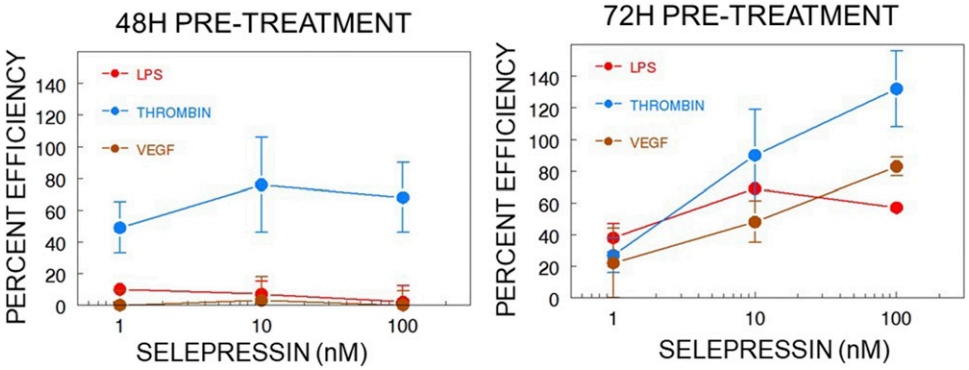

Fig. 4. Efficiency of selepressin blockade of increased permeability with edemogenic agents. Based on data in Figs. 1–3, the percent efficiency of blockade of increased permeability was calculated for each concentration of selepressin and thrombin, VEGF, or LPS, either with 48 or 72 hours pretreatment with selepressin.

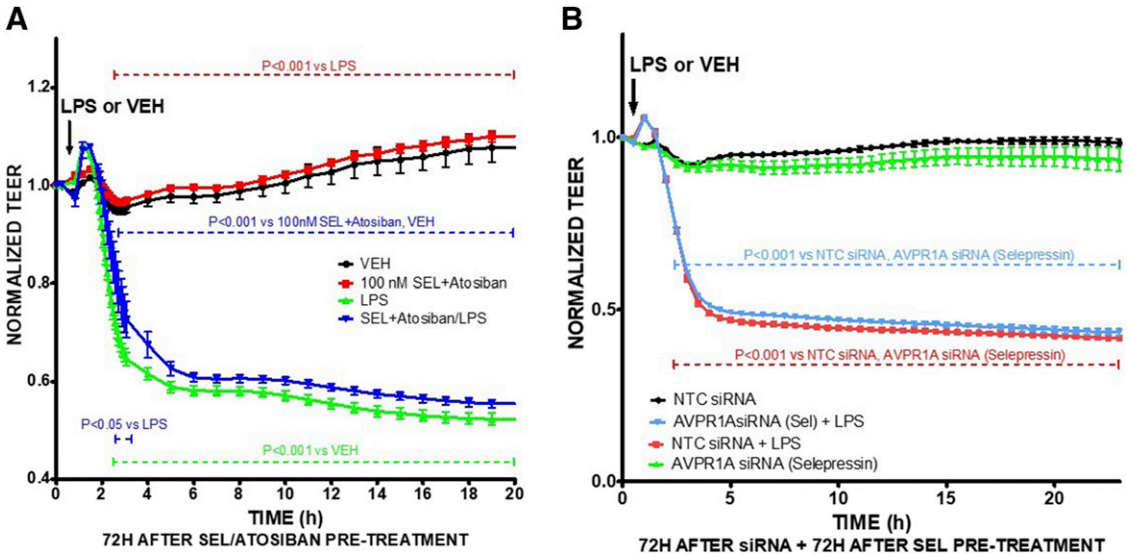

Fig. 5. Vasopressin receptor inhibition abolishes the protective effects of selepressin toward LPS-induced endothelial hyperpermeability. (A) HLMVECs were pretreated with selepressin (SEL; 100 nM) and the $V_{1A}R$ antagonist atosiban (1 μM) or 0.01% DMSO [vehicle (VEH)] prior to vehicle (PBS) or LPS (1 EU/ml) exposure. Atosiban abolished the protective effects of selepressin against LPS, seen in Fig. 3. (B) HLMVECs were transfected with an irrelevant siRNA (siCTR) or siRNA against the $V_{1A}R$ ($siV_{1A}R$) and then pretreated for 72 hours with either selepressin or vehicle (0.01% DMSO) prior to vehicle (PBS) or LPS (1 EU/ml) exposure. Treatment with $siV_{1A}R$ abolished the protective effects of selepressin against LPS, seen in Fig. 3. $N = 4$ per group. Means ± S.E. Two-way ANOVA for repeated measures followed by Bonferroni post hoc test. Text color reflects the group that is being compared. Arrow indicates the time of addition of either LPS or vehicle. NTC, non-targeting control.

effects of selepressin on barrier integrity in these experiments, cellular gap formation was measured and expressed as percent area covered by endothelial cells. As shown in Fig. 7, A and B, right panel, in the presence of an irrelevant siRNA, LPS significantly reduced the amount of area on the slide covered by endothelial cells, and this was not affected by selepressin treatment in the presence of $V_{1A}R$ siRNA.

Selepressin Induces the Expression of the Barrier-Protective p53. HLMVECs were treated with vehicle (0.01% DMSO) or different concentrations of selepressin (1, 10, 100, and 10000 nM) for 24 or 48 hours. The earlier 24-hour pretreatment time point was tested in case the p53 effect occurred earlier than the observed barrier-protective effect. Selepressin significantly induced p53 expression levels, but only after treatment of 48 hours at 100 nM (Fig. 8A).

Selepressin Induced the Activation of Rac1 and Prevented the LPS-Induced Barrier Disruptive Effects of RhoA/MLC2 Activation. HLMVECs were treated for 48 hours with selepressin (100 nM) or vehicle (0.01% DMSO) before exposure to vehicle (PBS) or LPS (1 EU/ml). In agreement with results from the ECIS studies, selepressin induced the activation of Rac1, which is known to exert prominent barrier-protective effects (Fig. 8B). Furthermore, selepressin suppressed the LPS-induced RhoA activation

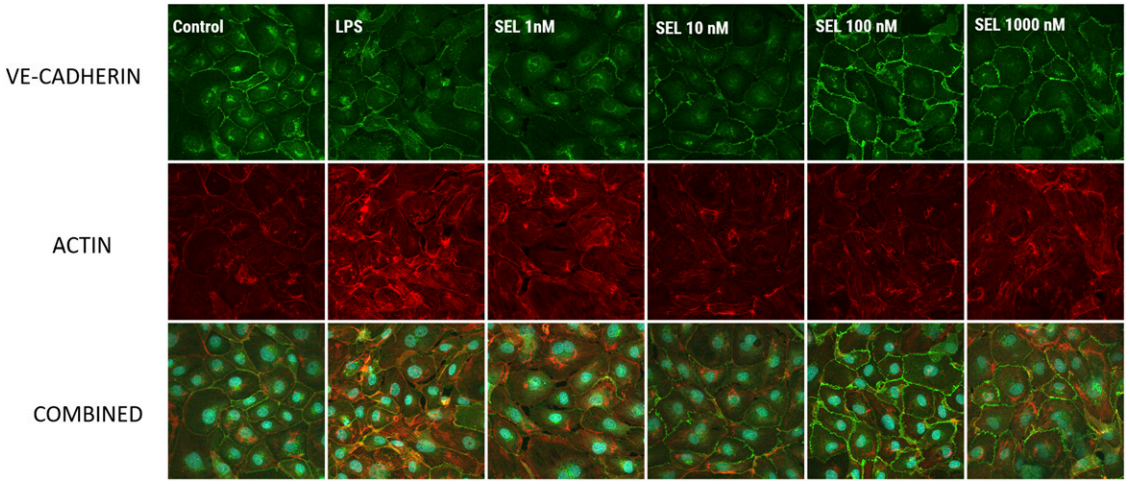

Fig. 6. Selepressin (SEL) protects HLMVECs against LPS-induced VE-cadherin and actin reorganization. HLMVECs grown on glass coverslips were treated with PBS (vehicle) or LPS (1 EU/ml) after 72 hours of pretreatment with either 0.01% DMSO (vehicle) or selepressin (1, 10, 100, 1000 nM). The cells were then fixed and double-stained for VE-cadherin and actin. Quantification of cortical VE-cadherin staining and of the cortical-to-total-actin distribution is shown in panels (B) and (C), respectively. #$P < 0.05$ vs. LPS, *$P < 0.05$ vs. vehicle. Means ± S.E.M. Three slides per group, 10 observations per slide. One-way ANOVA for independent samples followed by Bonferroni post hoc test.

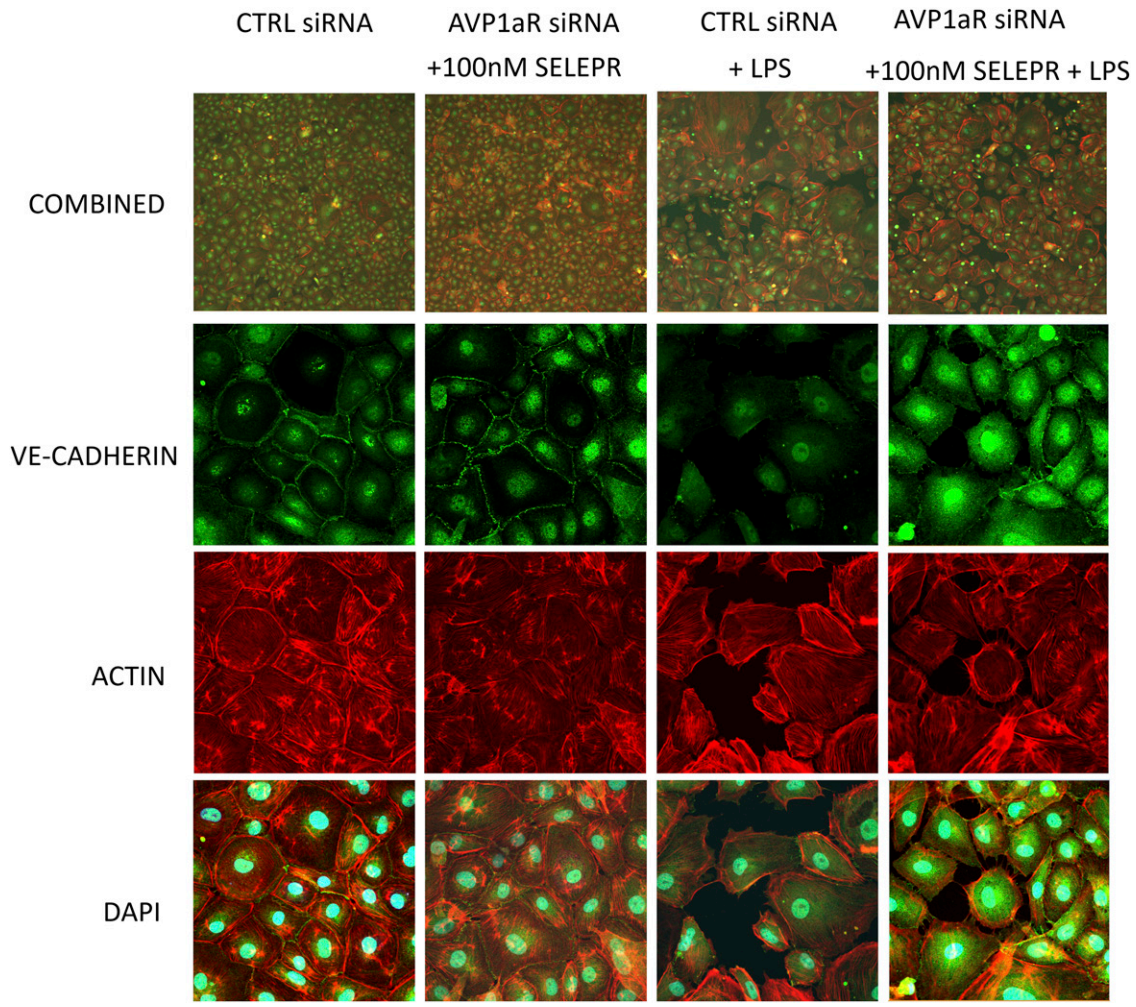

Fig. 7. Silencing of vasopressin receptor 1a does not prevent the protective effects of selepressin against LPS–VE-cadherin reorganization but prevents selepressin (SEL) antagonism of LPS-induced gap formation. HLMVECs grown on glass coverslips were treated with irrelevant, control siRNA, or siRNA against the $V_{1A}R$ for 72 hours. After the transfection, the cells were exposed to either vehicle (0.01% DMSO) or selepressin (100 nM) prior to vehicle (PBS) or LPS (1 EU/ml) exposure. Cells were then fixed and double-stained for VE-cadherin and actin. Quantification of cortical VE-cadherin staining and endothelial gaps (expressed as percent coverage of slide surface by cells) is shown in (B). #$P < 0.05$ vs. negative control (Neg. Cont.) siRNA + LPS, *$P < 0.05$ vs. AVPR1A siRNA + SEL. Means ± S.E.M. Three slides per group, 10 observations per slide. One-way ANOVA for independent samples followed by Bonferroni post hoc test. AVPR1A, arginine vasopressin receptor 1A.

(Fig. 8C) and reduced the LPS-triggered MLC2 phosphorylation (Fig. 8D).

Discussion

Sepsis and its more severe form, septic shock, are among the most common causes of death among hospitalized patients in the intensive care unit (Vincent et al., 2006). Because of the severe outcomes of septic shock and the need for new treatments, the endothelial barrier–protective properties of a novel selective $V_{1A}R$ agonist, selepressin, was tested against several known edemogenic agents. We chose selepressin over other $V_{1A}R$ agonists (e.g., vasopressin or terlipressin) because of its selectivity for the $V_{1A}R$ (Laporte et al., 2011) and tested the compound in vitro at concentrations relevant for its effects in vivo. In contrast to vasopressin and terlipressin, selepressin does not activate the V_2R that mediates antidiuretic effects (Laporte et al., 2011) and release of von Willebrand factor (Rehberg et al., 2012) and induces NO-mediated vasodilation (Kaufmann et al., 2003), effects that could accelerate the progression of septic shock by exacerbating oliguria, procoagulation, and vasodilation (Russell et al., 2017).

It has been suggested that selepressin could be used as a titrated first-line vasopressor in the treatment of severe sepsis and that it may be advantageous over the mixed $V_{1A}R$/V_2R agonist AVP, since selepressin may also reduce sepsis-induced vascular hyperpermeability. In a sheep model of sepsis induced by *Pseudomonas aeruginosa* pneumonia, characterized by pronounced vascular hyperpermeability, selepressin's antivascular hyperpermeability effect manifested itself by dramatically reduced cumulative fluid intake and cumulative fluid balance (Maybauer et al., 2014). This was associated with a significant blunting of the fall in total plasma protein concentration and oncotic pressure. AVP was not as effective as selepressin at blocking vascular hyperpermeability in that model. The difference in efficacy at blocking vascular hyperpermeability between selepressin and AVP appeared to be related to the pronounced agonist activity of AVP at the V_2R (Wisniewski et al., 2009; Laporte et al., 2011). Indeed, addition of the selective V_2R agonist

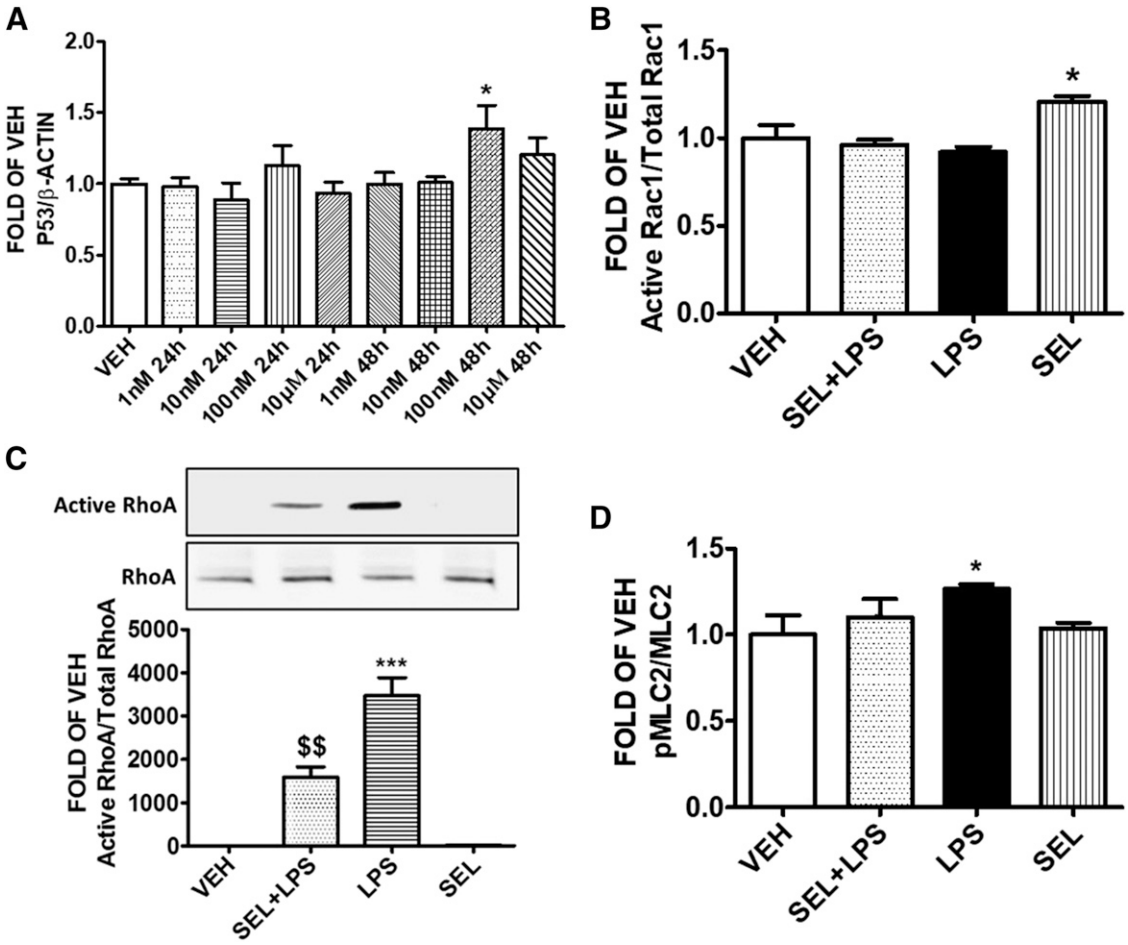

Fig. 8. Signaling pathways involved in the protective effects of selepressin (SEL) against LPS-induced endothelial barrier dysfunction. (A) Quantification of Western blot analysis of p53 expression after treatment of HLMVECs with either vehicle (VEH; 0.01% DMSO) or selepressin (1, 10, 100, and 10,000 nM) for 24 or 48 hours. p53 band signal intensity was analyzed by densitometry. Protein levels were normalized to β-actin. $^{*}P < 0.05$ vs. vehicle. Means ± S.E.M. (B) Quantification of Western blot analysis of total and active Rac1 after treatment of HLMVECs with selepressin (100 nM) or vehicle (0.01% DMSO) prior to exposure to LPS (1 EU/ml for 1 hour) or vehicle (PBS). Signal intensity was analyzed by densitometry. Protein levels were normalized to total Rac1. $^{*}P < 0.05$ vs. vehicle. Means ± S.E.M. (C) Western blot analysis of active RhoA and total RhoA levels after treatment of HLMVECs with selepressin (100 nm) or vehicle (0.01% DMSO) for 48 hours and post-treated with LPS (1 EU/ml) or vehicle (PBS) for 1 hour. The blots shown are representative of three independent experiments. Protein levels were normalized to RhoA. $^{***}P < 0.001$ vs. vehicle; $^{\$\$}P < 0.01$ vs. LPS. (D) Quantification of Western blot analysis of phospho-MLC2 after treatment of HLMVECs with selepressin (100 nM) or vehicle (0.01% DMSO) for 48 hours prior to exposure to LPS (1 EU/ml for 1 hour) or vehicle (PBS). Protein levels were normalized to total MLC2. $^{*}P < 0.05$ vs. vehicle. Means ± S.E.M. of three independent experiments. One-way ANOVA for independent samples followed by Bonferroni post hoc test.

1-desamino-8-d-arginine vasopressin (dDAVP) to the selepressin treatment decreased the amplitude of the antivascular hyperpermeability effect down to a level observed with AVP treatment (Maybauer et al., 2014). In a recent clinical trial, selepressin treatment did not differ from placebo in the number of ventilator-free days, vasopressor-free days, or 90-day mortality of patients with sepsis; however, it increased 24-hour urine output, decreased 24-hour net fluid balance, and decreased cardiovascular dysfunction for the first 48 hours. Selepressin also increased mean arterial pressure and lowered norepinephrine requirement for the first 6 hours of treatment (Laterre et al., 2019). These findings agree with the animal data, as well as with our findings that selepressin strengthens endothelial barrier function.

Vascular hyperpermeability due to increased endothelial permeability results in organ dysfunction with a subsequent increased mortality in sepsis and septic shock (Aslan et al., 2017). Thus, the in vitro barrier dysfunction in HLMVECs is a reasonable model for the barrier-protective actions of selepressin in human lungs during sepsis, and perhaps in other vascular beds (Murphy et al., 2009). Of particular relevance is the fact that, in a sheep model of septic shock induced by fecal peritonitis, selepressin significantly reduced pulmonary edema, whereas either AVP or norepinephrine was ineffective in that respect (He et al., 2016). In the present study, a variety of hyperpermeability inducers were used to compromise the human endothelium and trigger endothelial hyperpermeability, including thrombin, VEGF, angiopoietin 2, and LPS.

Endothelial dysfunction induced by thrombin plays an important role in sepsis. Bacterial toxins and cytokines lead to expression of tissue factor on the surface of damaged endothelium and circulating mononuclear cells (Levi and van der Poll, 2017). Tissue factor expression results in activation of the coagulation cascade, which leads to increased thrombin formation. On the other hand, the regulatory physiologic anticoagulation system is impaired. Activation of the endogenous fibrinolysis may be insufficient to counteract

the ongoing coagulation. The fibrin clot formed as a result of the thrombin burst is not effectively removed, the consequence being intravascular thrombus deposition (Levi et al., 2002; Taylor and Kinasewitz, 2002). Besides its hemostatic activities, thrombin is also involved in perpetuating the inflammatory reaction and induces cytokine expression in monocytes and endothelial cells via the protease-activated receptors (Kataoka et al., 2003; Wang and Reiser, 2003). The inflammatory reaction and microvascular thrombus formation contribute to organ dysfunction and sepsis (Petros et al., 2012). Here, the edemogenic effects of thrombin are effectively suppressed by selepressin, further supporting our original hypothesis. Cells subjected to a 48-hour pretreatment with selepressin were more susceptible to thrombin than those pretreated with selepressin for 72 hours. In future studies, it will be useful to investigate whether these differences correlate with changes in Rac1/RhoA signaling.

VEGF signaling has also been closely linked to endothelial dysfunction in sepsis. Increased VEGF expression has been associated with several instances of severe sepsis and septic shock (Karlsson et al., 2008; Whitney et al., 2018). Furthermore, circulating levels of endothelium-derived soluble vascular endothelial cell growth factor receptor-1 and urokinase-type plasminogen activator have been associated with organ dysfunction and mortality in patients with sepsis (Shapiro and Aird, 2011). Others have reported that VEGF might contribute to the development of acute lung injury in sepsis (Wada et al., 2013). Thus, in an LPS-induced endotoxemic rat model, it was reported that plasma levels of VEGF and its permeability-mediating receptor vascular endothelial cell growth factor receptor-2 were upregulated over time (Jesmin et al., 2012). Another study suggested that since the VEGF-A axis is involved in the control of microvascular permeability and in the pathogenesis of conditions associated with endothelial barrier disruption such as sepsis, it could be used as marker for sepsis (Alves et al., 2011). Interestingly, it was suggested that the anti-VEGF bevacizumab could improve survival in vivo in experimental sepsis (Jeong et al., 2013). In the present study, VEGF was employed as one of four edemogenic agents. Selepressin was able to concentration dependently counteract the effects of VEGF on pulmonary endothelial monolayers, thus supporting the hypothesis that it may reduce vascular hyperpermeability in sepsis.

Ang2 also regulates endothelial function, inducing endothelial dysfunction and increasing vascular permeability in sepsis. In healthy tissue, angiopoietin 1 agonizes the Tie2 receptor, maintaining vascular function, but in inflammatory conditions, Ang2 is elevated, blocking Tie2 activation and increasing endothelial permeability (Fisher et al., 2016; Akwii et al., 2019). Ang2 is elevated in sepsis and contributes to the pathophysiology of sepsis in animal models (David et al., 2012), leading to targeting of Tie2 signaling in sepsis by a variety of therapeutic approaches. The reduction in Ang2-induced vascular hyperpermeability by selepressin further supports its potential for intervening in sepsis.

The bacterial endotoxin LPS is elevated in the bloodstream in systemic infections, released from Gram negative bacteria, and is a common inflammatory stimulus in experimental studies examining the impact of inflammation on the vascular endothelium. The impact of LPS on vascular hyperpermeability has been extensively studied (Liu et al., 2015), and LPS stimulates host inflammatory signaling molecules, such as thrombin, VEGF, and Ang2 in sepsis (Jeong et al., 2013; Ziegler et al., 2013; Brauckmann et al., 2019). The demonstration of reduced LPS-induced endothelial hyperpermeability with selepressin indicates once again the broad potential for its positive action in sepsis and allows further examination of the mechanisms involved.

We also began exploring the intracellular signaling pathways mediating the barrier-protective effects of selepressin. Originally recognized for its role in cell cycle regulation and associated with cellular responses to stress from DNA damage, p53 is increasingly recognized to play a role in responses to a wide variety of stress. p53 is involved in the regulation of vasopressin secretion from the hypothalamo-hypophyseal system (Hernandez et al., 2015), and we have shown that p53 promotes endothelial barrier enhancement and protection from several edemogenic agents, including LPS (Barabutis et al., 2015, 2018a). Here, selepressin upregulated p53 expression in a time-dependent manner, suggesting a potentially important role of p53 in selepressin's barrier-protective actions. Moreover, p53 regulates cytoskeletal distribution and reorganization (Barabutis et al., 2018b), including inhibition of RhoA and mediation of Rac1 signaling (Barabutis et al., 2018a). It was recently shown that p53-null mice express less lung phosphocofilin, a downstream Rac1 target, compared with wild-type mice (Uddin et al., 2020). Indeed, p53 induction by either heat shock protein 90 inhibition (Barabutis et al., 2019) or growth hormone releasing hormone antagonists (Uddin et al., 2019) suppressed the LPS-induced MLC2 and cofilin activation (Barabutis, 2020). In agreement with our hypothesis and the known actions of p53, selepressin effectively antagonized the LPS-induced RhoA activation, prevented consequent MLC2 activation (phosphorylation), and prevented LPS-induced Rac1 downregulation. The blockade of some but not all aspects of selepressin signaling by $V_{1A}R$ siRNA suggests multiple signaling mechanisms may be involved. The improvement of endothelial barrier function by selepressin in the presence of this broad variety of edemogenic factors acting by varying mechanisms supports the central role of this mechanism as a point of intervention in endothelial dysfunction.

More research would be useful to further elucidate the mechanism by which $V_{1A}R$ mediates barrier protection, investigating for example the mechanism by which $V_{1A}R$ agonism alters p53 signaling. The 48- to 72-hour time frame required for cells to be exposed to $V_{1A}R$ agonism prior to the edemogenic factors suggests that intracellular calcium flux mediated by the $V_{1A}R$ is not sufficient for the protective effect to be observed; a slower downstream signaling process, such as alteration in gene expression, perhaps related to the action of p53 as a transcriptional regulator, may play a role. Understanding this process may also aid in the translation of these findings to the clinical setting, whether or not longer exposure to selepressin results in greater efficacy in vivo through increased barrier protection. These findings may also play a role in other therapeutic settings in which $V_{1A}R$ agonism or endothelial barrier dysfunction play important roles, such as ascites and cirrhotic liver disease.

In conclusion, an endothelial barrier–protective effect of selepressin has been demonstrated for the first time, providing a rationale for the observed in vivo inhibition of sepsis-induced vascular hyperpermeability and adding to the emerging body of evidence that substantiates the beneficial use of selepressin in patients with sepsis and septic shock.

Authorship Contributions

Participated in research design: Catravas, Croston, Reinheimer.
Conducted experiments: Barabutis, Marinova, Solopov.
Contributed new reagents or analytic tools: Reinheimer.
Performed data analysis: Barabutis, Marinova, Solopov, Uddin.
Wrote or contributed to the writing of the manuscript: Barabutis, Marinova, Solopov, Croston, Reinheimer, Catravas.

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