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Protective mechanism of the selective vasopressin V_{1A} receptor agonist selepressin against endothelial barrier dysfunction

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

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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-, VEGF-, angiopoietin 2-, and 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/MLC2 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.

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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 $V_{1A}R$ 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.

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Introduction

Sepsis is an excessive systemic inflammatory response to infectious and noninfectious causes that may progress to multiple organ failure and death. While sepsis is an important medical issue causing 270,000 deaths in the US 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, leading to anomalies of vascular barrier function (Kumar et al., 2009). The endothelium is a cellular system lining the interior wall of blood vessels and 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 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 sepsis patients(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, though other mechanisms also appear to play a role. Vasopressin and terlipressin activate multiple members of the

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vasopressin receptor family which elicit varying responses, including the V_2 receptor (V_2R), which stimulates anti-diuretic responses and the release of clotting factors that may be detrimental in sepsis, suggesting 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 cytokines levels, and improved organ function compared to vasopressin(He et al., 2016). In a Phase IIa clinical trial, selepressin reduced catecholamine use in sepsis patients, 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 RhoA and 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 MLC2 phosphorylation(Barabutis et al., 2019).

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The pathophysiology of sepsis appears to involve edemogenic agents acting by a variety of mechanisms to disrupt endothelial barrier function, including thrombin, VEGF, angiopoietin 2 (Ang2), 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 Tie2 receptor triggers endothelial permeability as well(David et al., 2013). The bacterial toxin LPS is a potent inflammatory stimulus binding to the TLR4 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 RhoA and MLC2 activation, as well as activation of the barrier protective action of 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 septic patients.

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Materials and Methods

Reagents: p53 (9282s), and p-myosin light-chain 2 antibodies were obtained from Cell Signaling (Danvers, MA, USA). B-actin antibody (P8999) and CelyticM lysis reagent (C2978) were purchased from Sigma-Aldrich (St Louis, MO, USA). Secondary mouse and rabbit antibodies were purchased from Licor (Lincoln, NE, USA). Pierce BCA protein assay and nitrocellulose membranes were obtained from Fisher Scientific (Pittsburgh, PA, USA). VE Cadherin and F actin antibodies were from Abcam; DAPI and Alexa Fluor 2ry antibodies from Thermo Fisher Scientific, Texas Red-X phalloidin from Life Technologies. Tween 20, bovine serum albumin, PBS, TBS and ProLong Gold antifade reagent were purchased from Thermo Fisher Scientific. Paraformaldehyde was obtained from Electron Microscopy Sciences and Triton X-100 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, USA). Briefly, 500µg 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, Denver, CO, USA). 500µg of cell lysates was incubated

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with GST-Rhotekin-RBD fusion protein and was coupled to glutathione resin. The complexes were then washed with RIPA 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 AVPR1 (sc-29767) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An irrelevant siRNA from the same source that does not lead to any specific degradation of any known cellular mRNA was used as 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 min 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 h after the transfection (Barabutis et al., 2013).

Cell Culture: In-house harvested and identified human lung microvascular endothelial cells (HLMVEC) were isolated and maintained in M199 media supplemented with 20% FBS and antibiotics/anti-mycotics, as described previously (Catravas et al., 2010). HLMVEC 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 RIPA buffer. Protein-matched samples (40 μg per lane) were separated by electrophoresis through 12% SDS-PAGE Tris·HCl gels. Wet

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transfer was used to transfer the proteins onto nitrocellulose membranes. The membranes were incubated for 1 h at room temperature in 5% nonfat dry milk in Tris-buffered saline-0.1% (vol/vol) 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, USA). All experiments were conducted with cells which 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 488 nm excitation filter for VE Cadherin (Alexa Fluor 488 Ab), 595m for F-Actin (Texas Red Ab) and 359 nm filter for DAPI (nuclei). Each coverslip was divided into 49 squares and 10 squares were chosen at random and analyzed under 60x 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.

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Densitometry/statistical analysis: ImageJ software (National Institute of Health) was used to perform densitometry of immunoblots. All data are expressed as mean values ± S.E.M. (standard error of mean), and *n* represents the number of experimental repeats. Two-way ANOVA for repeated measures with Bonferroni post-hoc test was used in Figures 1, 2, 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, USA) was used for data analysis.

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Results

The expression of $V_{1A}R$, $V_{1B}R$, V_2R, and oxytocin receptor mRNA and protein in HLMVEC was first confirmed by real-time 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 following a 48- or 72-hour pretreatment of selepressin, prior to the addition of other agonists.

Selepressin pre-treatment ameliorates thrombin-induced endothelial barrier dysfunction in a concentration dependent manner. HLMVEC were seeded on gold electrode arrays and were exposed for 48 (Figures 1A, 1B, 1C) or 72 hours (Figures 1D, 1E,1F) to either vehicle (0.01% DMSO) or 1nM (Figure 1A, 1D), 10nM (Figure 1B, 1E) or 100nM (Figures 1C, 1F) 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 100nM of selepressin (e.g., Figures 1E, 1F vs. 1D).

Selepressin pre-treatment ameliorates VEGF-induced barrier dysfunction in a concentration dependent manner. HLMVEC were seeded on gold electrode arrays and were exposed for 72 hours to either vehicle (0.01% DMSO) or 1 nM (Figure 2A), 10 nM

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(Figure 2B), or 100 nM (Figure 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 (Figure 1C).

Selepressin pre-treatment ameliorates the angiopoietin-2-induced barrier dysfunction in a concentration dependent manner. After HLMVEC 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 (Figure 2D).

Selepressin pre-treatment ameliorates the LPS-induced vascular barrier dysfunction in a concentration dependent manner. HLMVEC were seeded on gold electrode arrays and were exposed for 72 hours to either vehicle (0.01%DMSO) or 1 nM (Figure 3A), 10 nM (Figure 3B), or 100 nM (Figure 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 (Figure 3).

To better visualize the barrier protective effects of selepressin, we calculated the efficiency of protection at different concentrations, 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

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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 % efficiency of protection. As shown in Figure 4, selepressin expressed different protective profiles against the three disruptors and following different times of pretreatment. Selepressin appeared most effective against thrombin-induced barrier dysfunction and following 72 hours of pre-treatment.

The protective effects of selepressin against the LPS-induced barrier dysfunction are mediated by the vasopressin receptor activation. HLMVEC were pre-treated 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 HLMVEC were transfected with either irrelevant (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 Figure 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 4 different doses (1, 10, 100, and 1000 nM) of selepressin, before LPS (1 EU/ml) or vehicle (PBS) treatment. The cells were

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then fixed, incubated with VE-cadherin and actin antibodies, and cortical VE-cadherin and actin staining was observed by confocal microscopy (Figure 6A) and quantified by ImageJ (Figures 6B and 6C). LPS profoundly reduced both cortical VE-Cadherins 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 HLMVEC were transfected for 48 hours with $V_{1A}R$ siRNA or irrelevant siRNA. After the transfections, the cells were treated to 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 effects of selepressin on barrier integrity in these experiments, cellular gap formation was measured and expressed it as percent area covered by endothelial cells. As shown in Figure 7A and 7B, 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. HLMVEC were treated with vehicle (0.01% DMSO), or different concentrations of selepressin (1 nM, 10 nM, 100 nM, and 1000 nM) for 24 or 48 hours. The earlier 24h 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 for 48

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hours at 100 nM (Figure 8A).

Selepressin induced the activation of Rac1 and prevented the LPS-induced barrier disruptive effects of RhoA/MLC2 activation. HLMVEC 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 (Figure 8B). Furthermore, selepressin suppressed the LPS-induced RhoA activation (Figure 8C) and reduced the LPS triggered MLC2 phosphorylation (figure 8D).

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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). Due to the severe outcomes of septic shock and the need of 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), testing 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), 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, pro-coagulation, 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 anti-vascular-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

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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 dDAVP to the selepressin treatment decreased the amplitude of the anti-vascular-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 septic patients, however it increased 24-hr urine output, decreased 24-hr 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 six 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 HLMVEC 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, while either AVP or norepinephrine were 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 hyper-permeability, 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 (TF) on the surface of damaged

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endothelium and circulating mononuclear cells(Levi and van der Poll, 2017). TF 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 (PAR)(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 48h-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 (sVEGFR-1, sFlt-1) and urokinase-type plasminogen activator (uPA) have been associated with organ dysfunction and mortality in septic patients(Shapiro and Aird, 2011). Others have reported that VEGF might contribute to the development of acute lung injury

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(ALI) 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 VEGFR-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; Barabutis et al., 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 phospho-cofilin, a downstream Rac 1 target, compared to 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

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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 hours timeframe 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 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 add to the emerging body of evidence that substantiates the beneficial use of selepressin in patients with sepsis and septic shock.

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AUTHORSHIP CONTRIBUTIONS:

Participated in research design: Catravas, Croston and Reinheimer

Conducted experiments: Barabutis, Marinova and Solopov.

Contributed new reagents: Reinheimer.

Performed data analysis: Barabutis, Uddin, Marinova and Solopov.

Wrote or contributed to the writing of the manuscript: Barabutis, Marinova, Croston, Reinheimer, Catravas and Solopov.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1: Protective effect of selepressin against thrombin-induced endothelial barrier dysfunction. Human lung microvascular endothelial cells (HLMVEC) were pretreated for 48 h with either vehicle (0.01% DMSO) or 1 nM **(Figure 1A)**, 10 nM **(Figure 1B)**, 100 nm **(Figure 1C)** selepressin and were exposed to either vehicle (PBS) or thrombin (1 unit/ml). A gradual increase in endothelial permeability (reduced TEER) was observed in the thrombin- treated cells, which was reduced in selepressin-treated cells. Similarly, HLMVEC were pretreated for 72 h with either vehicle (0.01% DMSO) or 1 nM (**Figure 1D)**, 10 nM **(Figure 1E)**, 100 nm (**Figure 1F)** selepressin and were exposed to either vehicle (PBS) or thrombin (1 unit/ml). A gradual increase in endothelial permeability (reduced TEER) was again observed in thrombin-treated cells, which was significantly prevented in 10 nM and 100 nM selepressin-treated cells. In all the cases, N=4 per group. Means ± SE. 2-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.

Figure 2: Protective effect of selepressin against VEGF- and angiopoietin 2-induced endothelial barrier dysfunction. HLMVEC were pretreated for 72 h with either vehicle (0.01% DMSO) or 1 nM (**Figure 2A)**, 10 nM **(Figure 2B)**, 100 nm **(Figure 2C)** selepressin 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, HLMVEC were pretreated for 72 h with either vehicle (0.01% DMSO) or 100 nM selepressin

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(Figure 2D) and were exposed to either vehicle (PBS) or angiopoietin 2 (Ang2; 400 ng/ml). Selepressin significantly suppressed Ang2-induced hyperpermeability. 2-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.

Figure 3: Protective effect of selepressin against LPS-induced endothelial barrier dysfunction. HLMVEC were pretreated for 72 h with either vehicle (0.01% DMSO) or 1 nM **(Figure 3A)**, 10 nM **(Figure 3B)**, and 100 nm **(Figure 3C)** selepressin 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 ± SE. N = 4 per group. Means ± SE. 2-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.

Figure 4: Efficiency of selepressin blockade of increased permeability with edemogenic agents. Based on data in figures 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 hours or 72 hours pretreatment with selepressin.

Figure 5: Vasopressin receptor inhibition abolishes the protective effects of selepressin towards LPS-induced endothelial hyper-permeability. A) HLMVEC were pretreated with selepressin (100 nM) and the V_{1A}R antagonist, atosiban (1 μM), or 0.01% DMSO (vehicle) prior to vehicle (PBS) or LPS (1EU/ml) exposure. Atosiban abolished the protective effects of selepressin against LPS, seen in Figure 3. **B)** HLMVEC were

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transfected with an irrelevant siRNA (siCTR) or and 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 Figure 3. N = 4 per group. Means ± SE. 2-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.

Figure 6: Selepressin protects HLMVEC against LPS-induced VE-cadherin and actin reorganization. HLMVEC grown on glass coverslips were treated with PBS (vehicle) or LPS (1EU/ml) after 72h 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 VEH. Means ± SEM. Three slides per group, ten observations per slide. One-way ANOVA for independent samples followed by Bonferroni post-hoc test.

Figure 7: Silencing of vasopressin receptor 1a does not prevent the protective effects of selepressin against LPS-VE cadherin reorganization but prevents selepressin antagonism of LPS-induced gap formation. HLMVEC 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

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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) are shown in Panel B. #P<0.05 vs Neg. Cont. siRNA + LPS, *P<0.05 vs AVPR1A siRNA + SEL. Means ± SEM. Three slides per group, ten observations per slide. One-way ANOVA for independent samples followed by Bonferroni post-hoc test.

Figure 8: Signaling pathways involved in the protective effects of selepressin against LPS-induced endothelial barrier dysfunction. (A) Quantification of Western blot analysis of p53 expression after treatment of HLMVEC with either vehicle (0.01% DMSO), or selepressin (1, 10, 100 and 10000 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 ± SEM. **(B)** Quantification of Western blot analysis of total and active Rac1 after treatment of HLMVEC with selepressin (100 nM) or vehicle (0.01% DMSO) prior to exposure to LPS (1 EU/ml for 1h) or vehicle (PBS). Signal intensity was analyzed by densitometry. Protein levels were normalized to total Rac1. *P<0.05 vs vehicle. Means ± SEM. **(C)** Western blot analysis of active RhoA and total RhoA levels after treatment of HLMVEC with Selepressin (100nm) 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 HLMVEC with selepressin (100 nM) or vehicle (0.01% DMSO) for 48 h prior to exposure to LPS (1 EU/ml for 1h) or vehicle (PBS). Protein levels

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were normalized to total MLC2. *P<0.05 vs vehicle. Means ± SEM of three independent experiments. One-way ANOVA for independent samples followed by Bonferroni post-hoc test.

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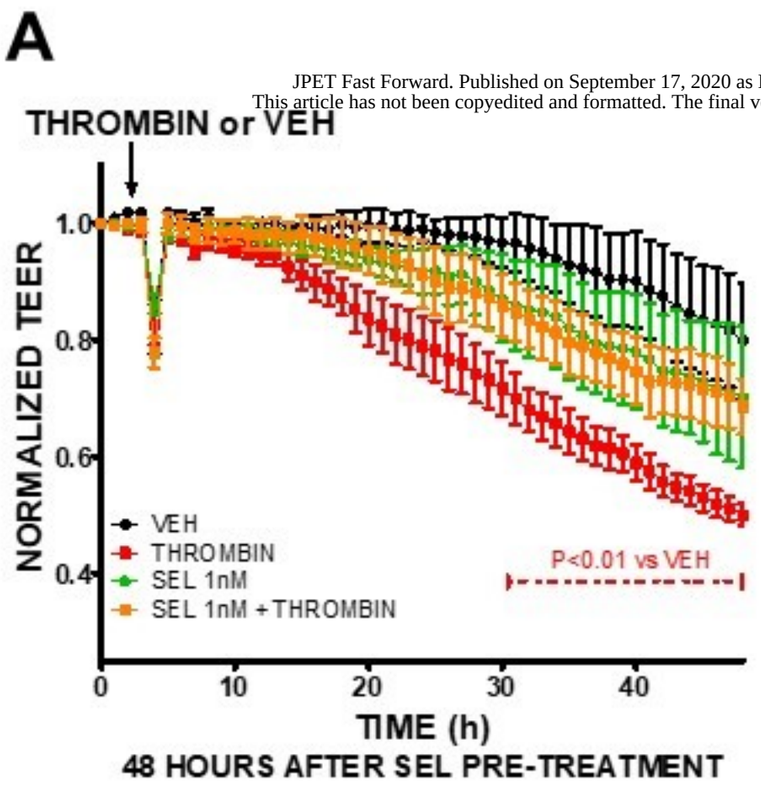
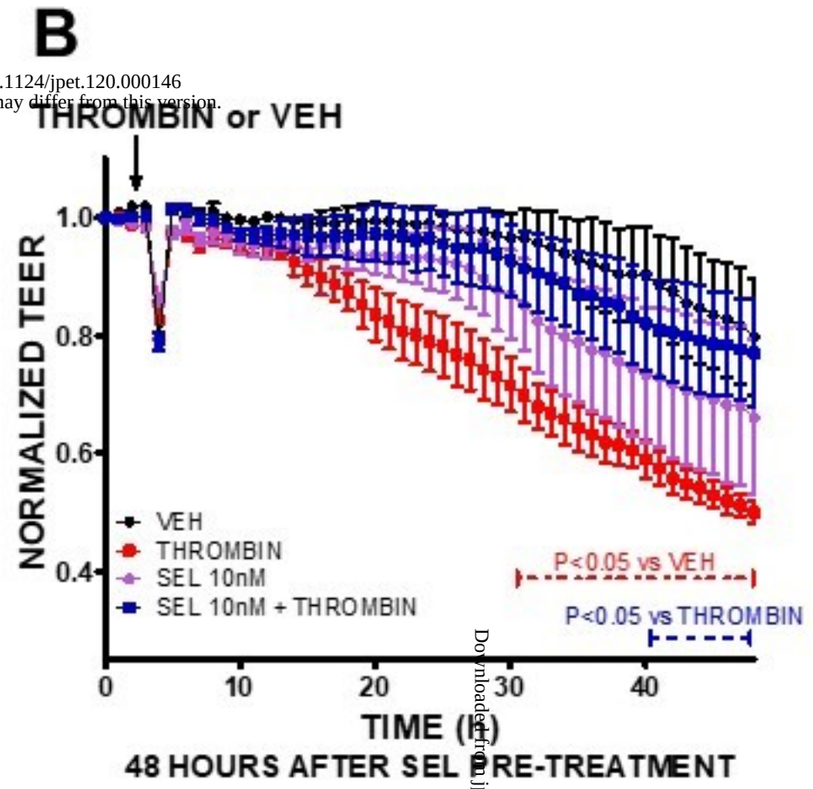
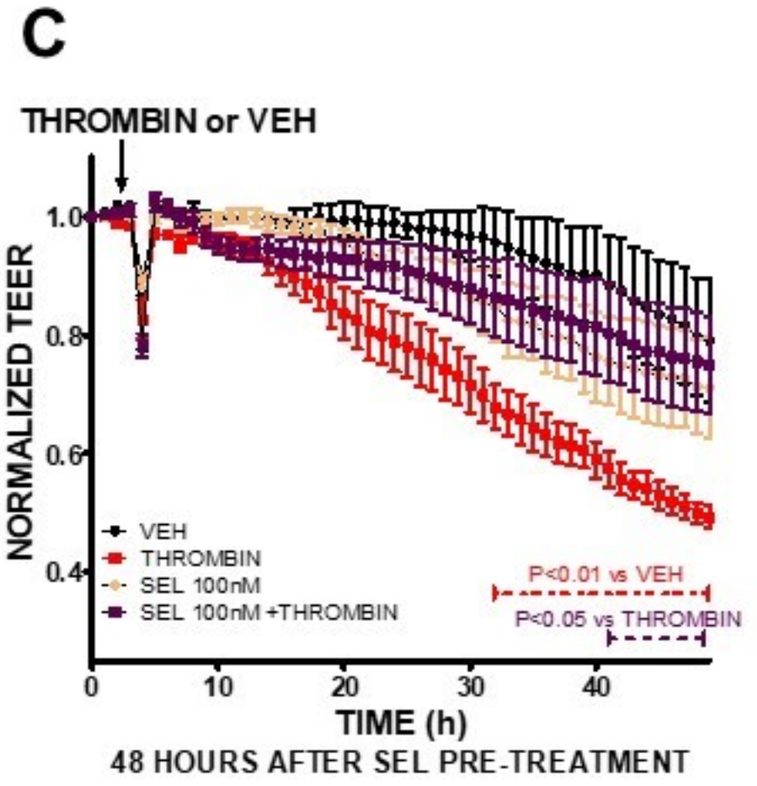
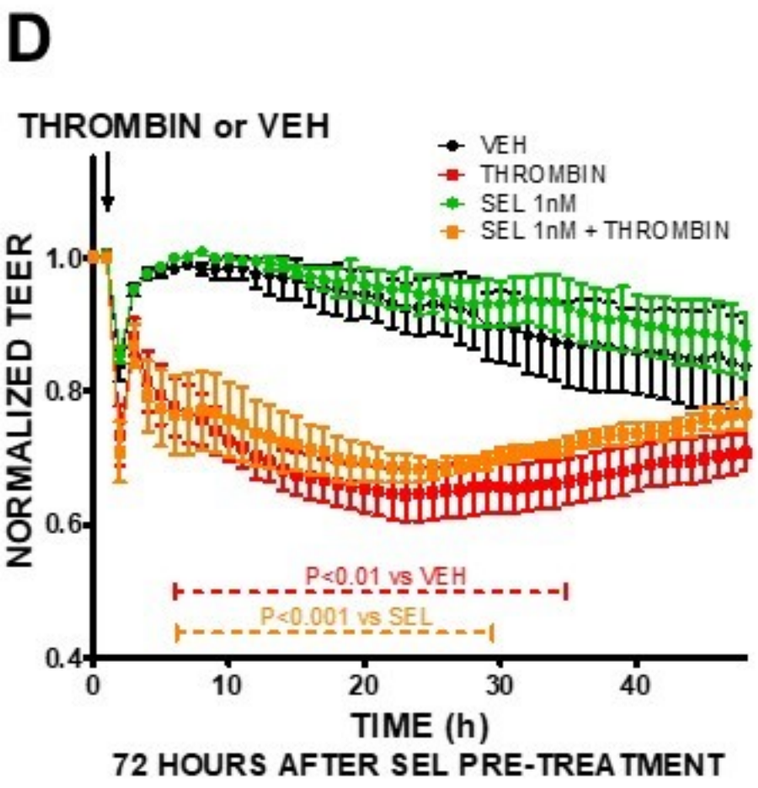
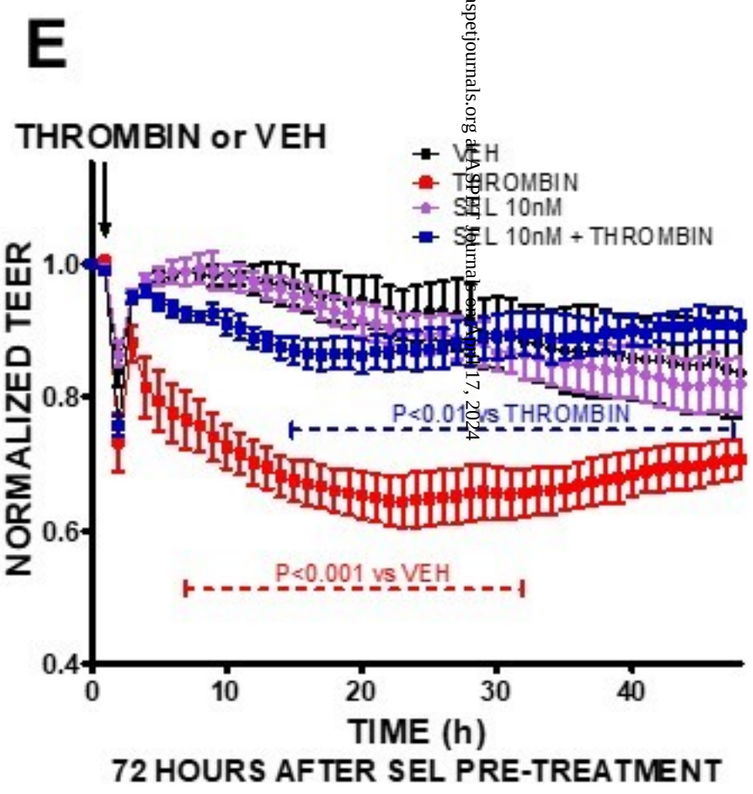










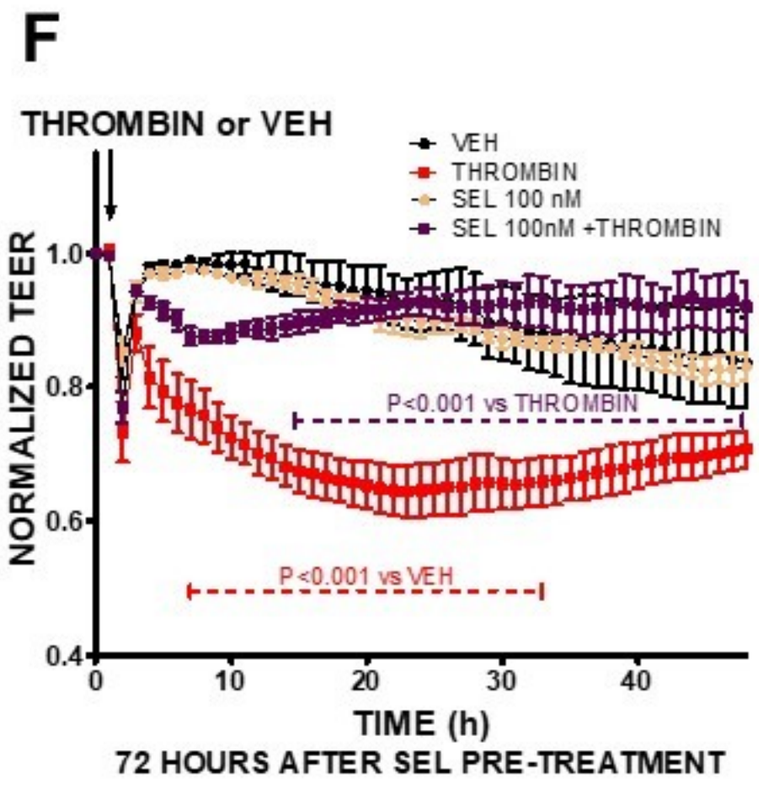
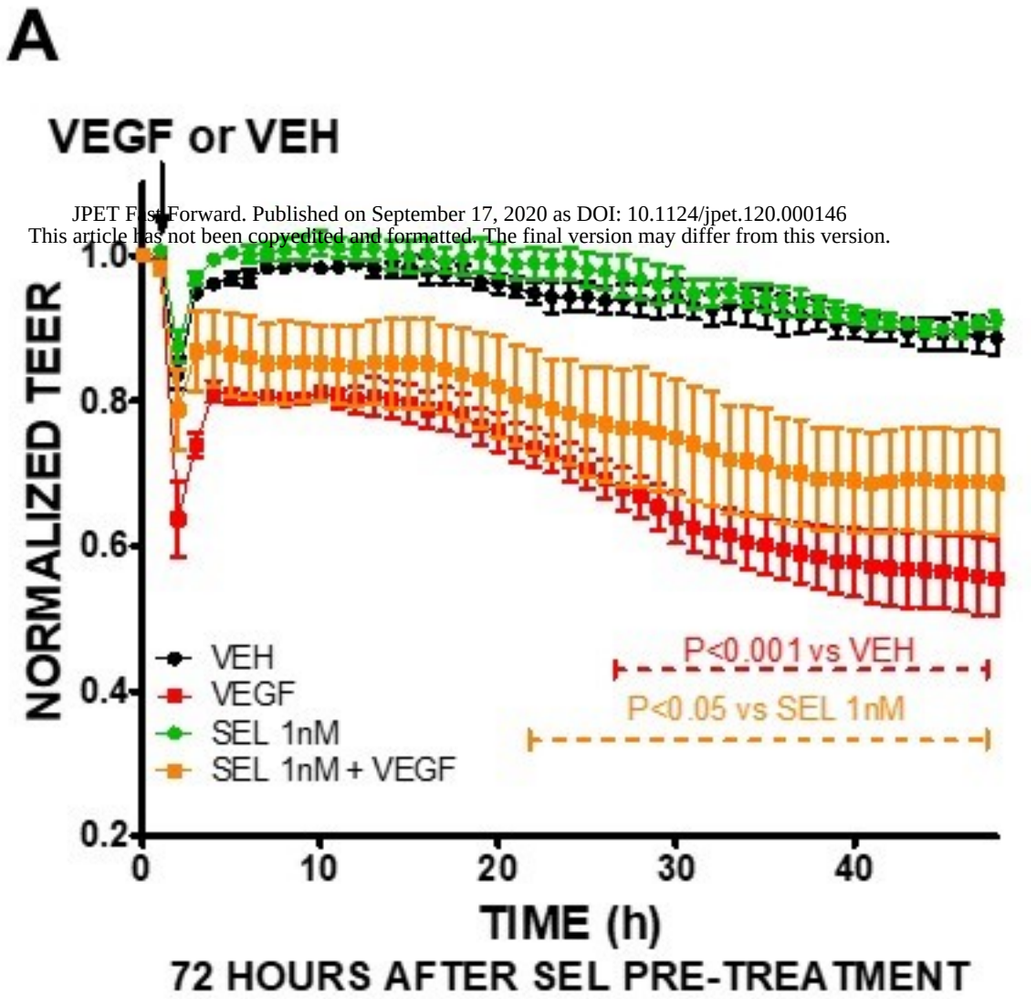
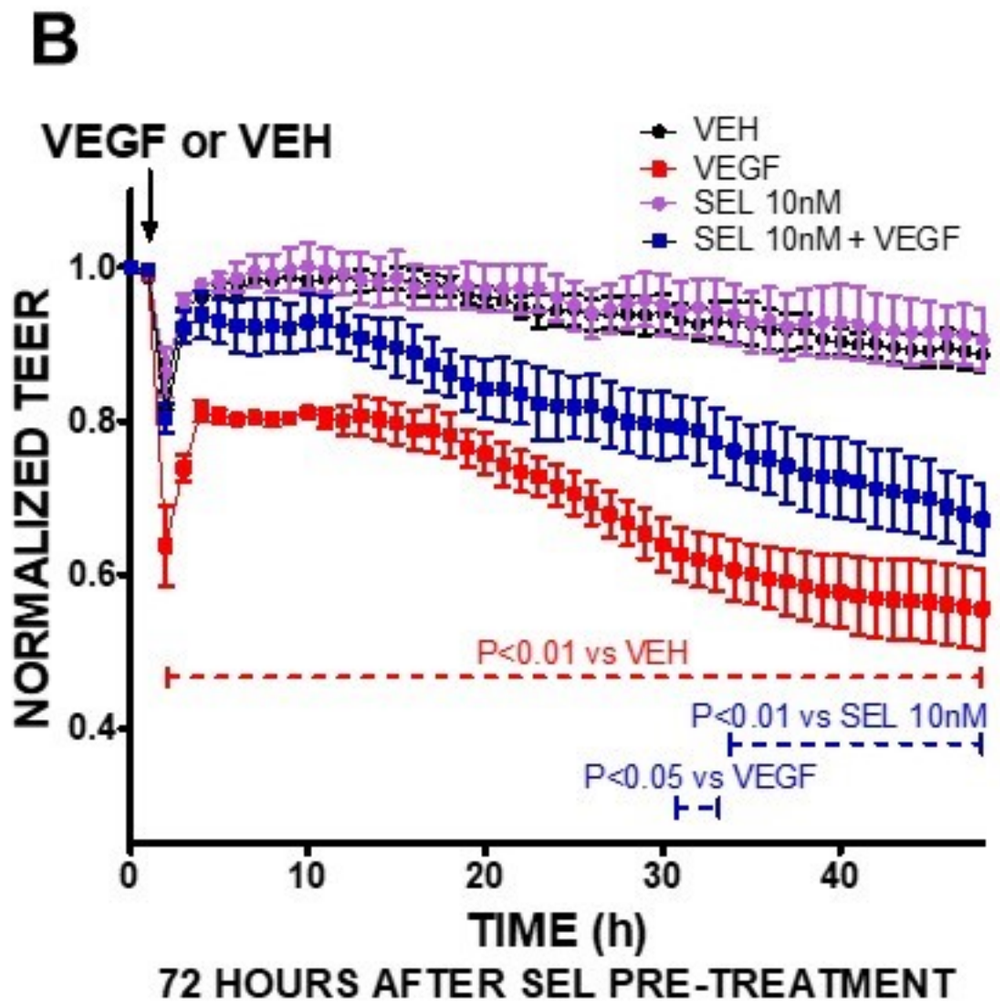
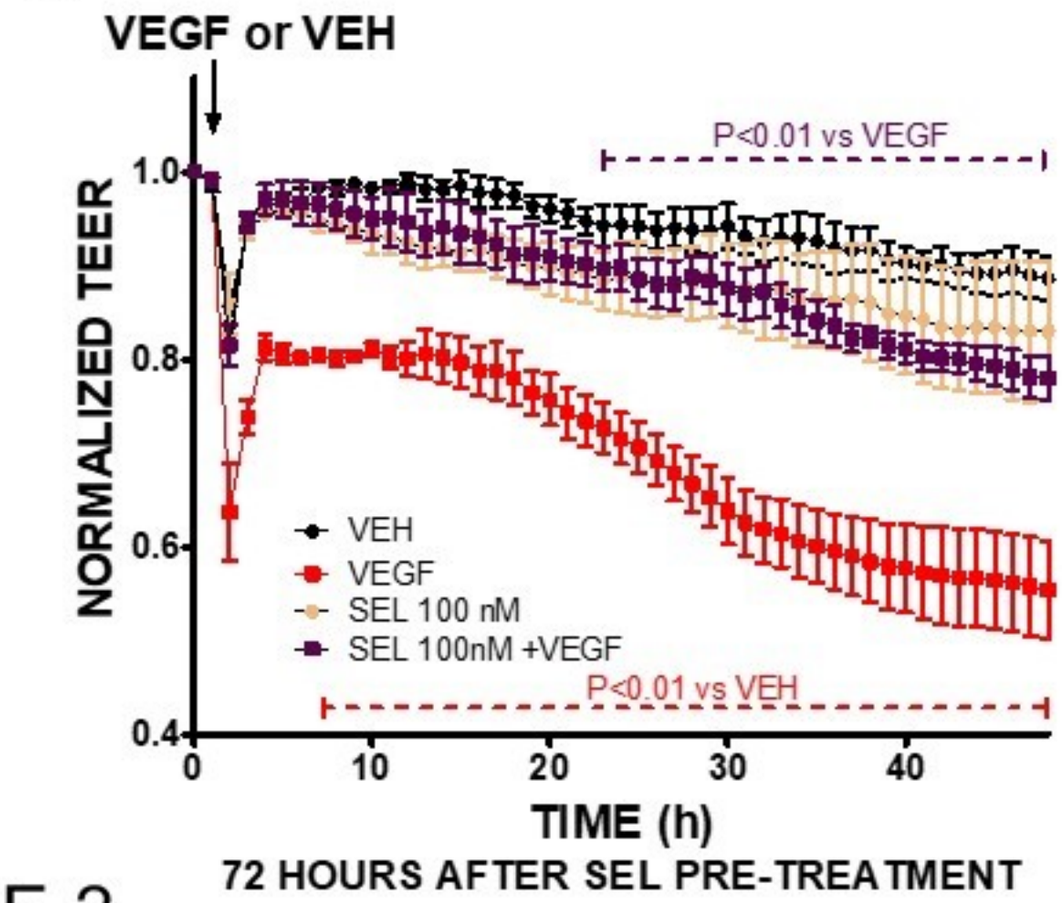


FIGURE 1







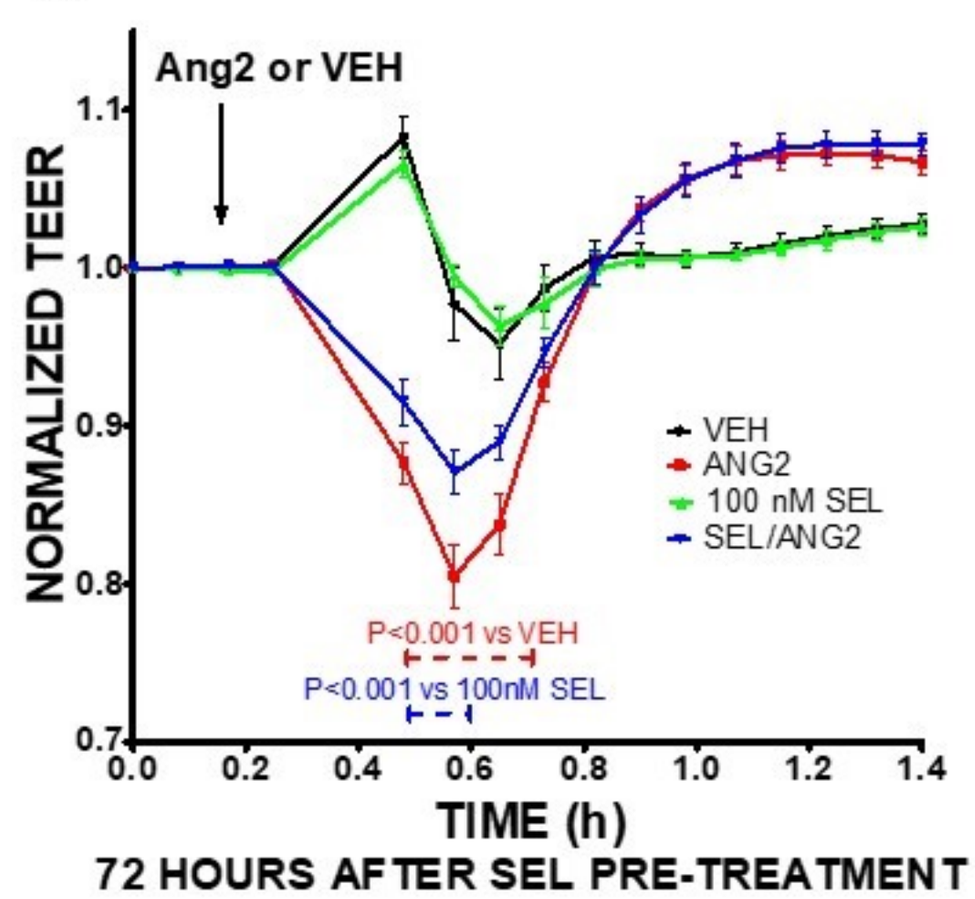


FIGURE 2

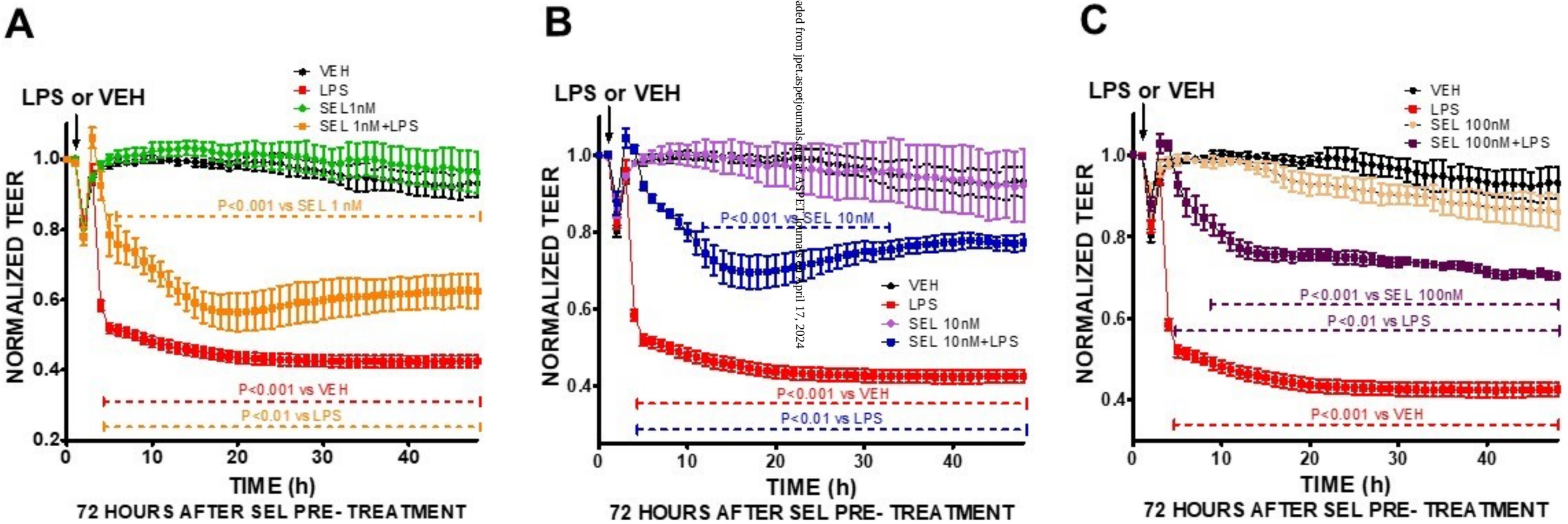
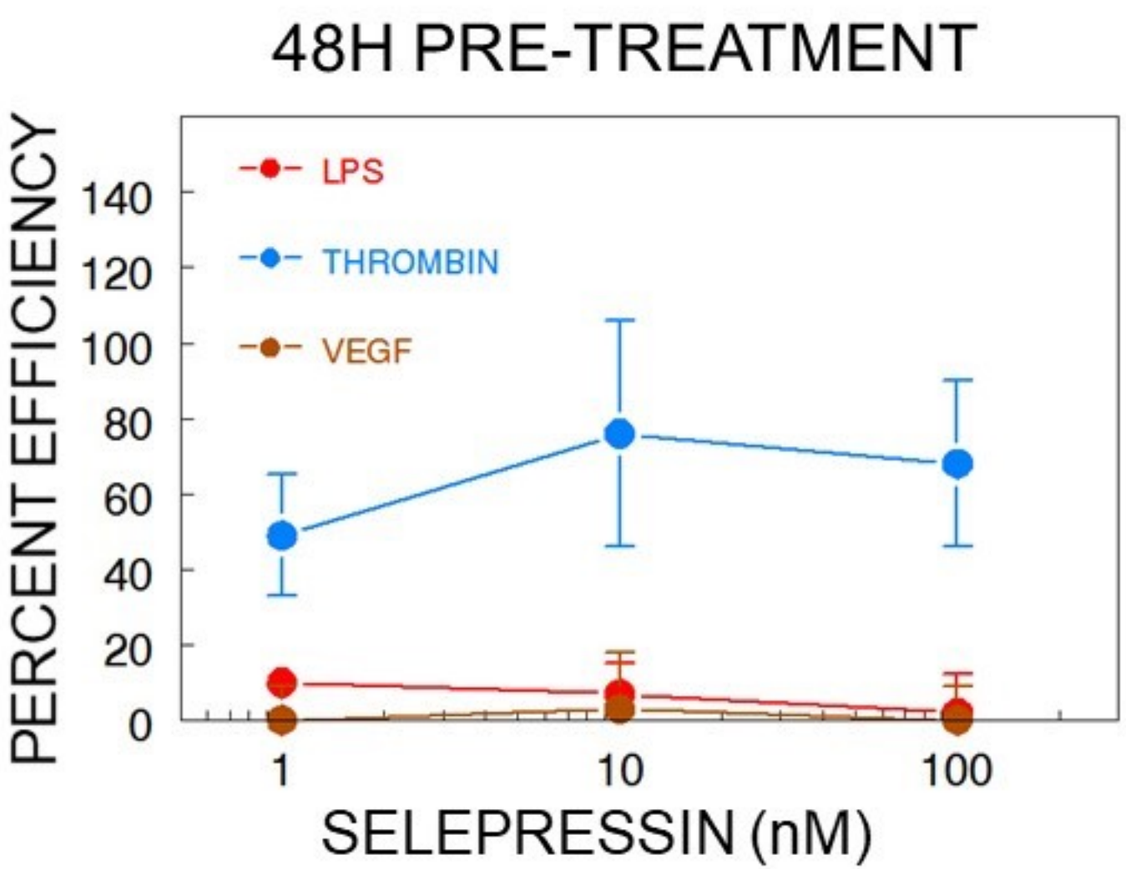


FIGURE 3



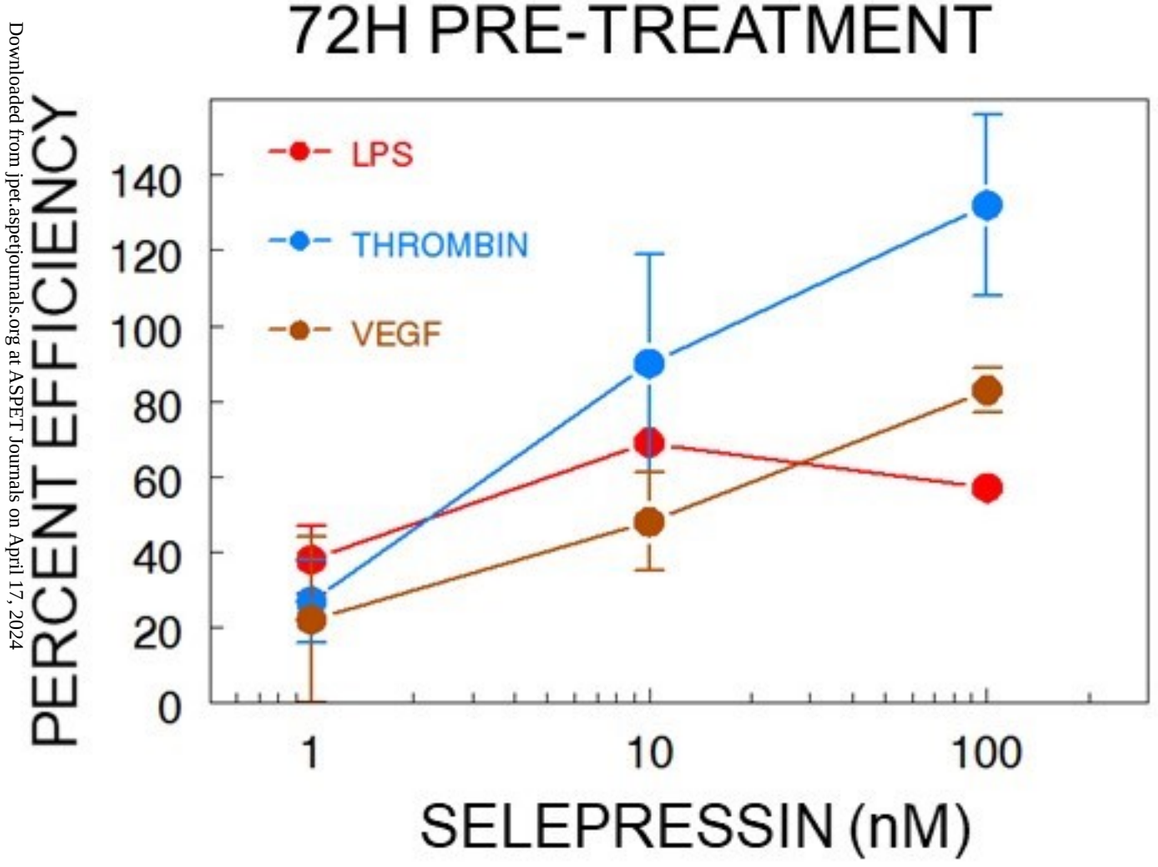
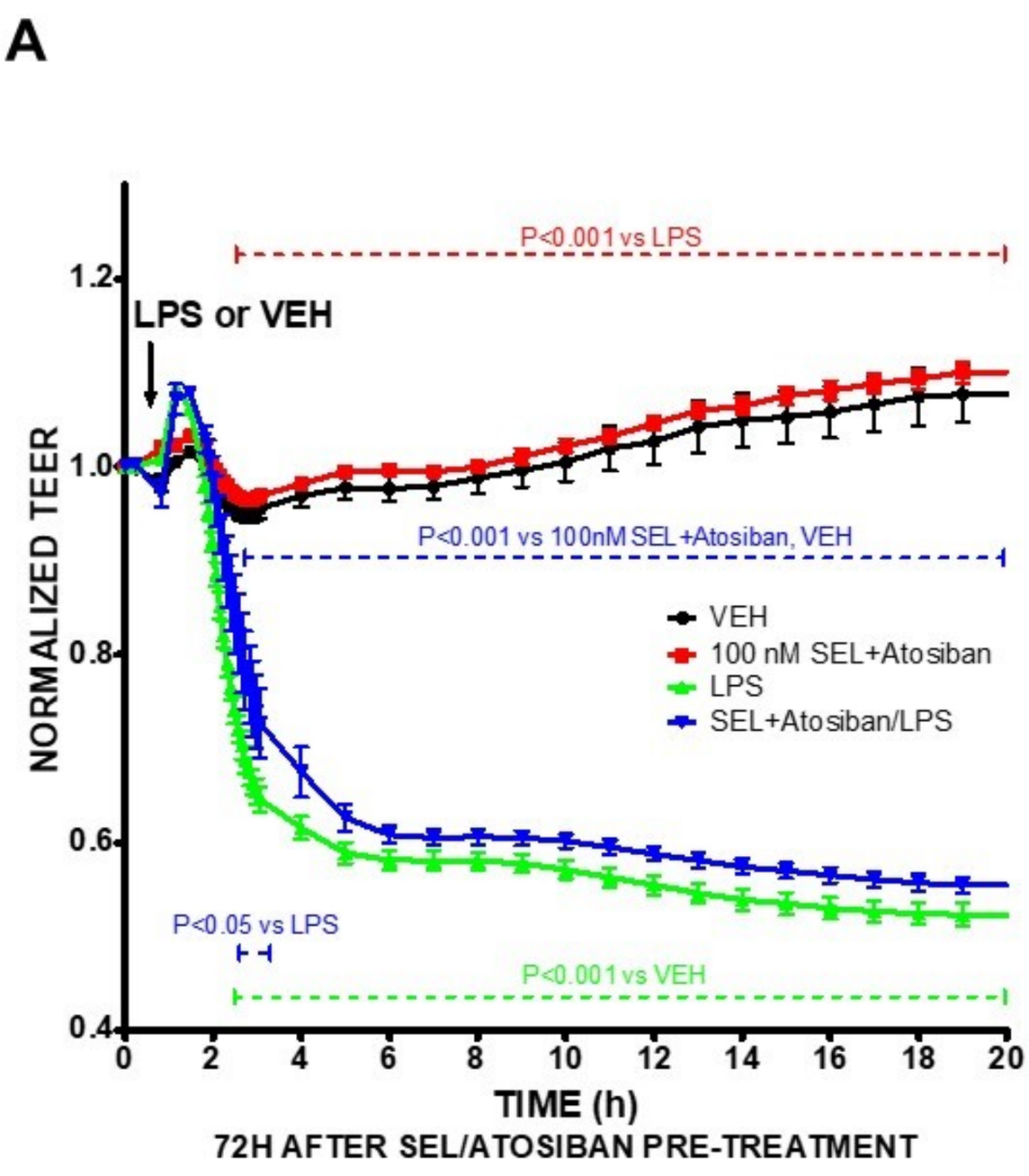


FIGURE 4



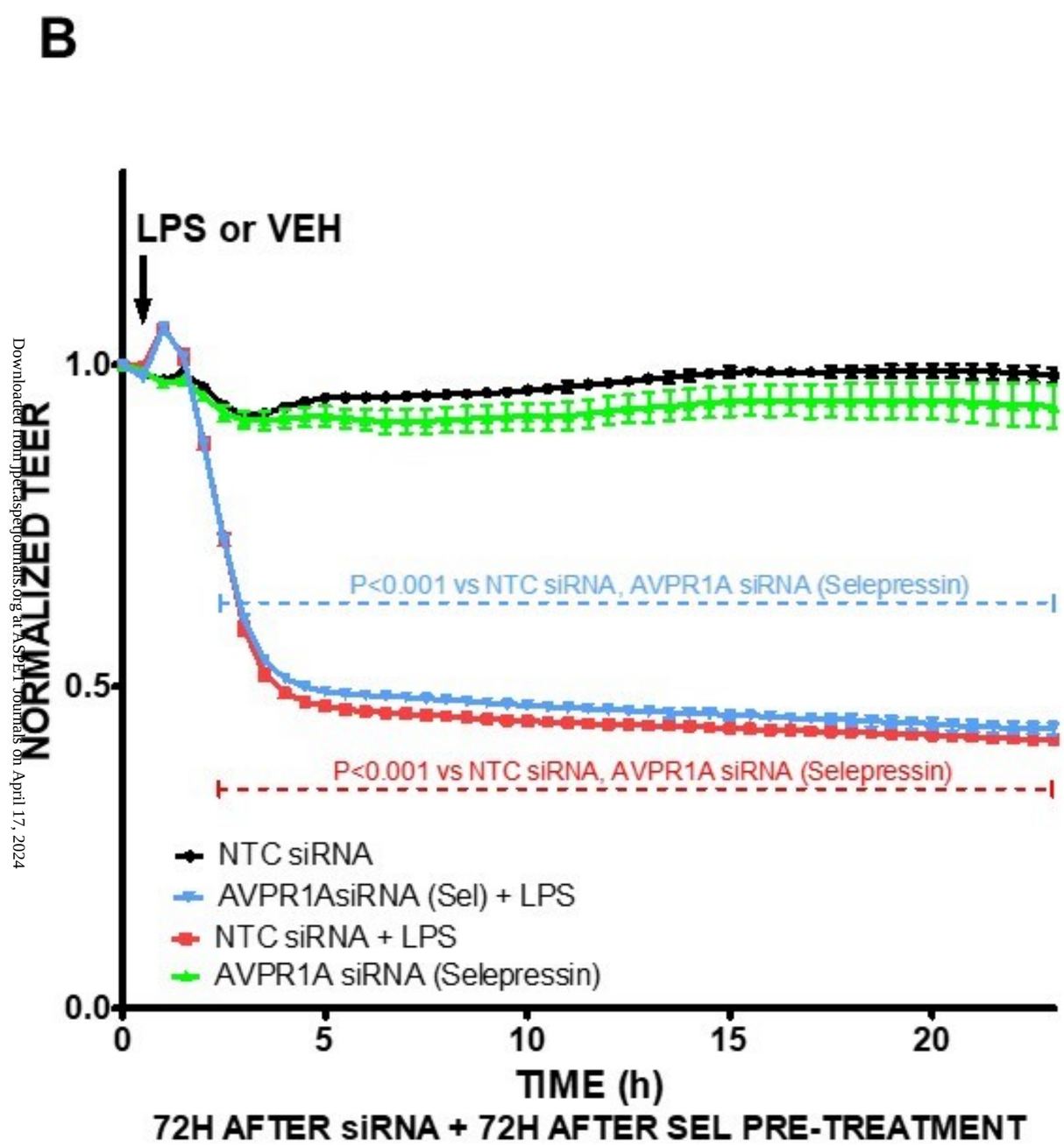
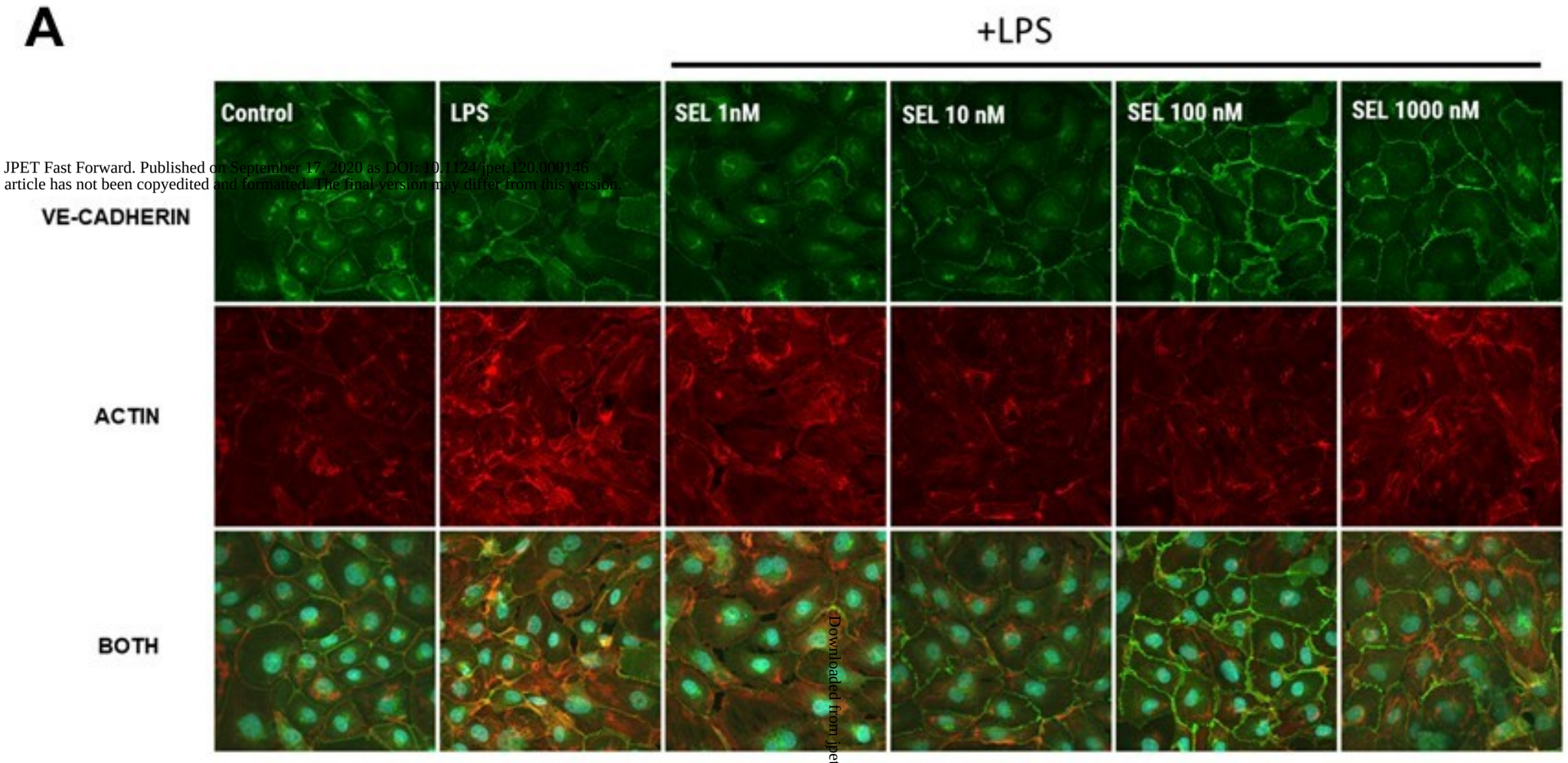
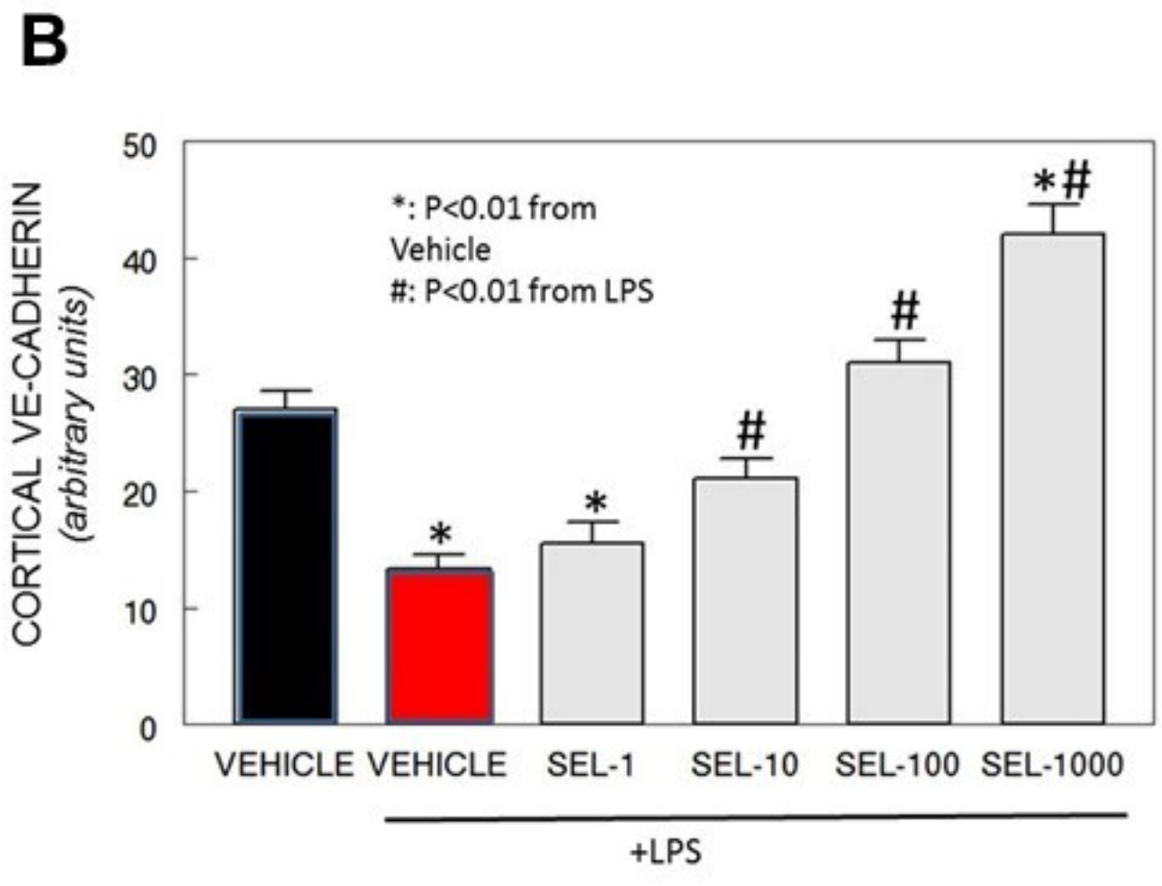


FIGURE 5





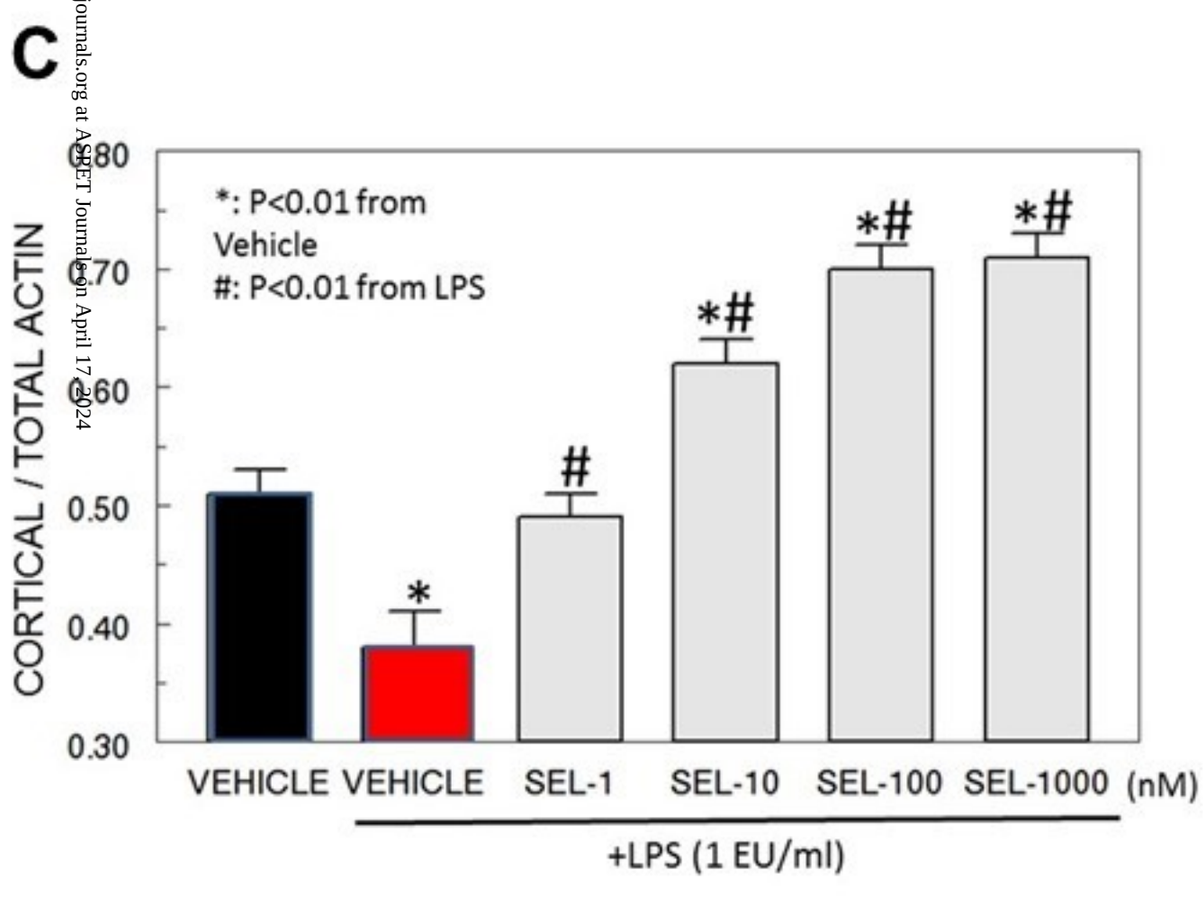


FIGURE 6

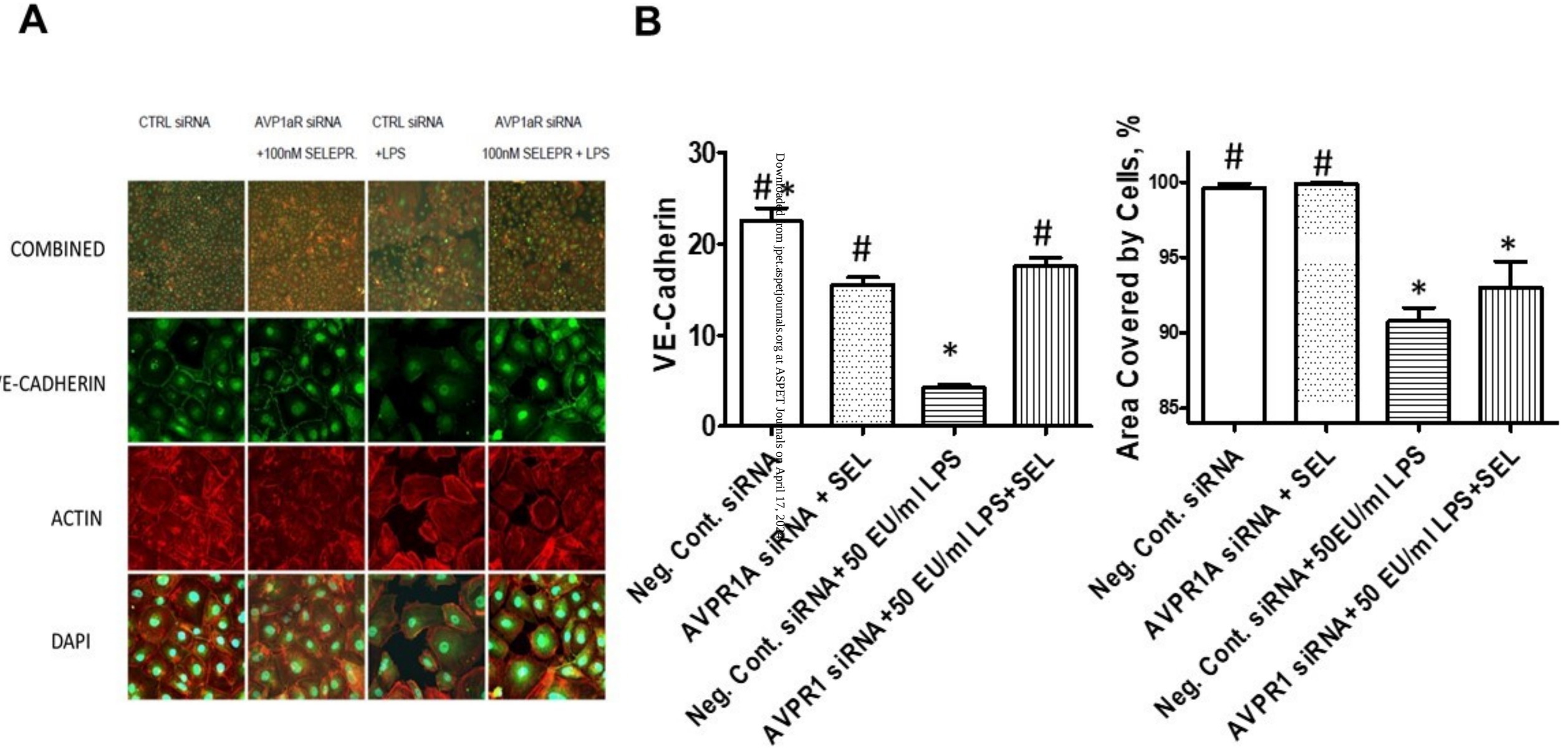


FIGURE 7

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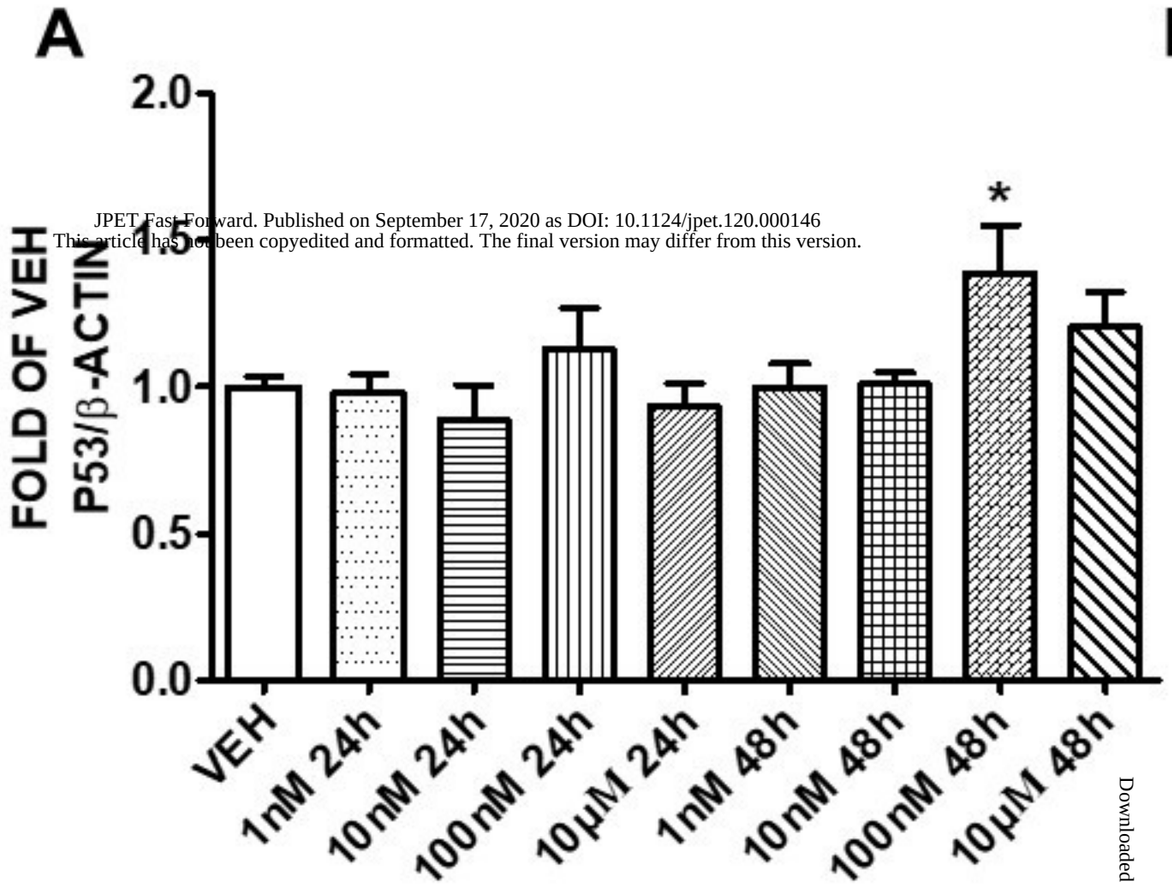

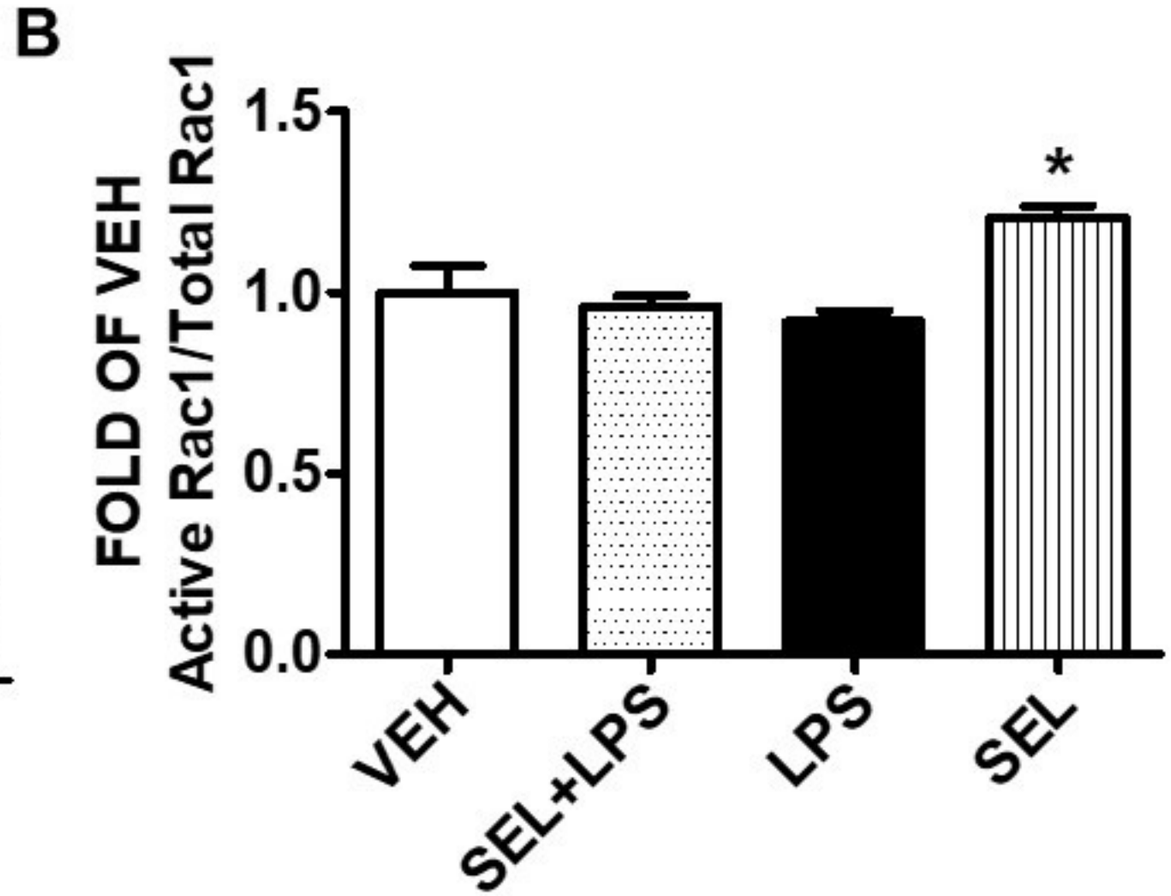

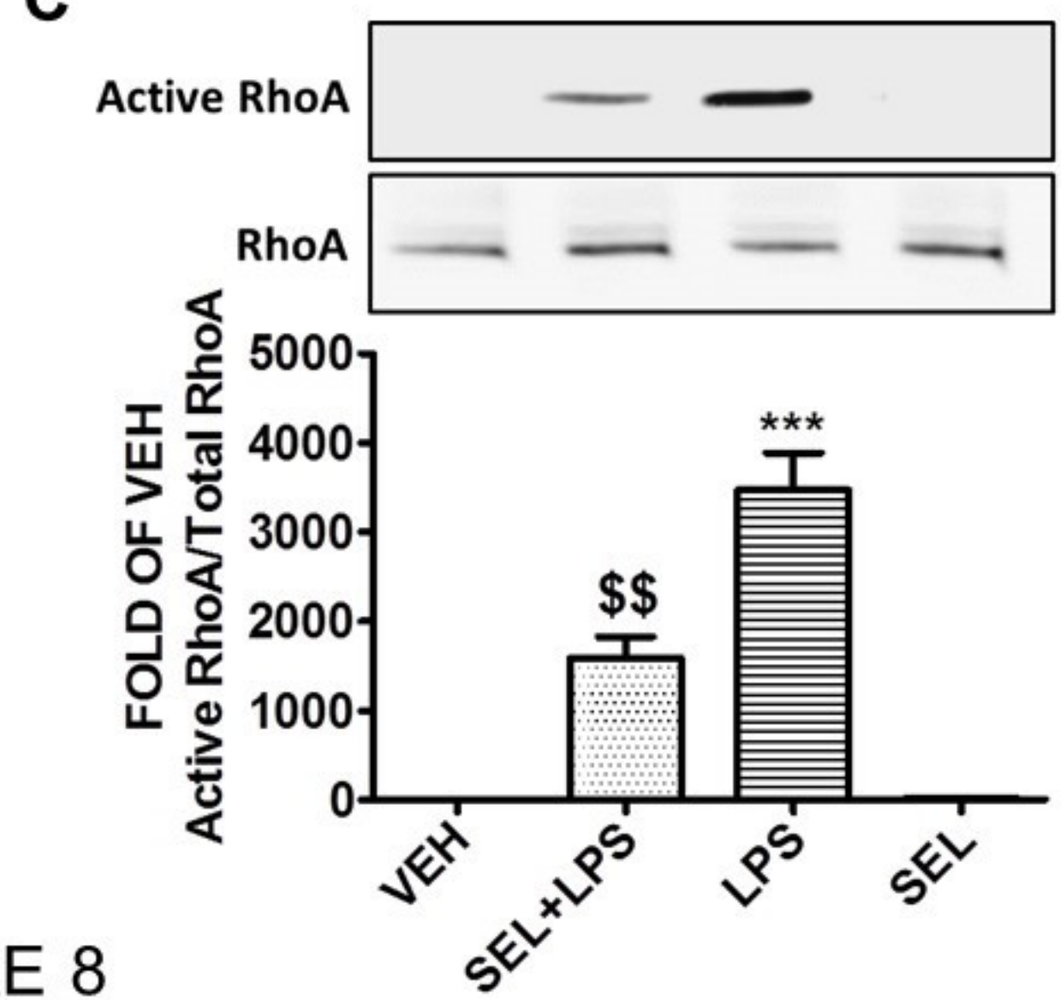

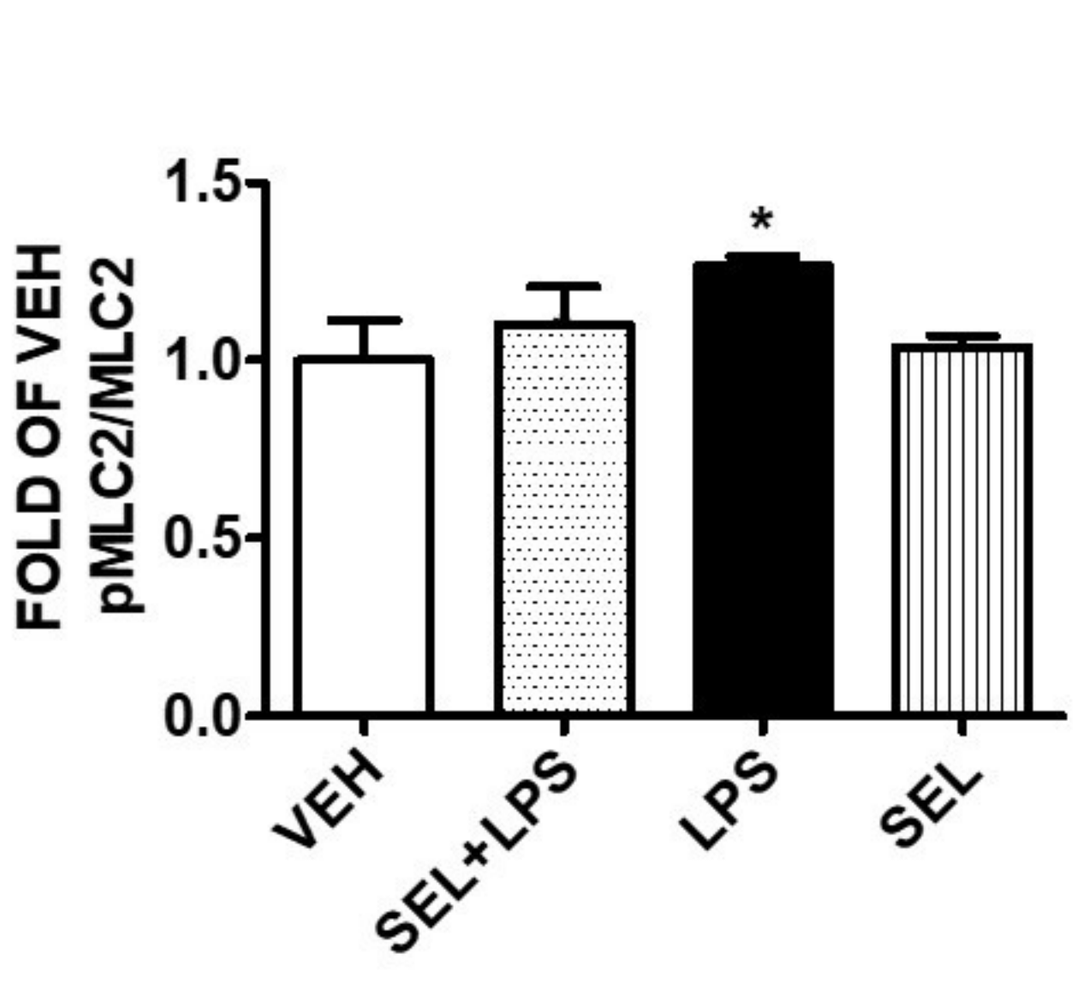

FIGURE 8