Catecholaminergic Vasopressors Reduce Toll-Like Receptor Agonist-Induced Microvascular Endothelial Cell Permeability But Not Cytokine Production

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Jérémie Joffre, MD, PhD[1]
Elliot Lloyd, BA[1]
Erika Wong, MD[1,2]
Che Chung-Yeh, PhD[1]
Nina Nguyen, BA[1]
Fenguyn Xu, PhD[1]
Matthieu Legrand, MD, PhD[1]
Judith Hellman, MD[1]

OBJECTIVES: Catecholaminergic vasopressors are the cornerstone of circulatory shock management. Nevertheless, catecholamines have problematic side effects, arousing a growing interest in noncatecholaminergic agents such as vasopressin or angiotensin-II. However, their respective effects on sepsis-associated microvascular endothelial dysfunction such as permeability or inflammation remain elusive. We investigated the role of catecholamines and other vasopressors on Toll-like receptor agonists-induced microvascular endothelial permeability and inflammation.

SETTING: University research laboratory/cell research.

SUBJECTS: Human pulmonary microvascular endothelial cells from multiple donors.

INTERVENTION: Confluent monolayers of human pulmonary microvascular endothelial cells were treated with Toll-like receptor agonists (lipopolysaccharide, Poly[I:C], or tripalmitoyl-S-glyceryl cysteine) in the presence or absence of epinephrine, norepinephrine, vasopressin, and angiotensin-II. Permeability was inferred from transendothelial resistance, measured using electrical cell impedance sensing, where decreased transendothelial resistance is consistent with increased permeability. Cell-cell junction molecule expression was assessed via immunofluorescence microscopy and flow cytometry. We quantified cytokines in supernatants of Toll-like receptor agonist-treated human pulmonary microvascular endothelial cells.

MEASUREMENTS AND MAIN RESULTS: Epinephrine and norepinephrine both ameliorate lipopolysaccharide, polyinosinic:polycytidylic acid, or tripalmitoyl-S-glyceryl cysteine–induced reductions in transendothelial resistance, a surrogate for endothelial permeability. In contrast, the noncatecholaminergic agents, vasopressin, and angiotensin-II did not affect Toll-like receptor agonists-induced reductions in transendothelial resistance. β1- and β2-adrenergic receptor antagonists reduced the effects of the catecholamines on transendothelial resistance, whereas α-adrenergic receptor antagonists did not. We observed that epinephrine and norepinephrine induced actin cytoskeletal rearrangement and normalized the membrane expression of proteins involved with adherens-junctions (vascular endothelial-cadherin) and tight-junctions (zona occludens-1). Despite having a substantial effect on endothelial permeability, epinephrine and norepinephrine did not affect human pulmonary microvascular endothelial cell survival or production of interleukin-8, interleukin-6, or monocyte chemoattractant protein-1 (CCL-2) induced by Toll-like receptor agonists,

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suggesting that these functions are regulated separately from permeability.

CONCLUSIONS: Our findings demonstrate that treatment with epinephrine or norepinephrine strongly reduces endothelial permeability induced by agonists of multiple Toll-like receptors (Toll-like receptor-2, Toll-like receptor-3, Toll-like receptor-4) in vitro. Our studies suggest that both β1- and β2-adrenergic receptors mediate the stabilizing effects of epinephrine and norepinephrine on the endothelial barrier.

KEY WORDS: catecholamines; endothelial cells; inflammation; permeability; sepsis; vasopressors

Despite prompt care and improvements in sepsis management (1, 2), many patients develop a microcirculatory dysfunction and multiple organ failure (MOF). Thus, septic shock still carries unacceptably high mortality rates. Microvascular alterations play an essential role in developing organ failure in critically ill patients, especially in sepsis (3). Persistent microcirculatory alterations, such as mottling (4, 5), alterations in tissue saturation, or sublingual orthogonal polarization spectral, are associated with MOF and death, even though global hemodynamic and oxygenation variables are corrected (6–8). The first-line recommended vasopressor in septic shock is the catecholaminergic amine norepinephrine (9). In clinical trials, catecholaminergic and noncatecholaminergic agents can restore macrocirculatory variables, but thus far, no class of vasopressor has consistently exhibited superiority over the others in improving sepsis outcomes (10–15). The current expert recommendation to treat septic shock by adding either vasopressin or epinephrine to norepinephrine to raise mean arterial pressure to target is considered a "weak recommendation supported by a low quality of evidence" (16). Much remains to be understood about the physiologic and cellular effects of different vasopressors, including their impact on the endothelial functions and organ failure. Sepsis-induced microvascular endothelial dysfunction is a key feature of sepsis-related organ failure (17). Furthermore, endothelial permeability, which is regulated by adherens (cadherin-cadherin) and tight junction (claudin-occludin) proteins, leads to capillary leak that can worsen tissue hypoperfusion by increasing interstitial pressure. Notably, the effects of different classes of vasopressors on the function of microvascular endothelial cells are poorly defined. Here, we explored the microvascular endothelial cell effects of catecholaminergic (epinephrine and norepinephrine) and noncatecholaminergic vasopressors such as vasopressin and angiotensin-II. We tested the effects of catecholaminergic and noncatecholaminergic vasopressors in vitro on permeability, cytokine and chemokine production, and survival of primary human pulmonary microvascular endothelial cells (HMVEC) activated by microbial and endogenous inflammatory agonists.

METHODS

Cell Culture and Stimuli

Primary HMVEC from male and female cadavers were purchased (Lonza, Basel, Switzerland) and used at passage 3–6. HMVEC were plated and grown to 90–100% confluence before treatment (at 37°C, 5% Co_2), using the appropriate medium (endothelial cell growth medium [EGM]–2) (Lonza). Cells were incubated with lipopolysaccharide (LPS) (1 μg/mL; Sigma-Aldrich, St. Louis, MO), tripalmitoyl-S-glyceryl cysteine (Pam3Cys, 10 μg/mL; Abcam, Cambridge, United Kingdom), polyinosinic:polycytidylic acid (10 μg/mL; Abcam), recombinant human interleukin (IL)–1β (10 ng/mL; R&D Systems, Minneapolis, MN), recombinant human tumor necrosis factor (TNF)–α (10 ng/mL; R&D Systems), or cytomix (TNF-α, interferon-γ, and IL-1-β; R&D Systems). Simultaneously or after 8 hours, cells were stimulated with epinephrine (Sigma-Aldrich), norepinephrine (Hospira, Lake Forest, IL), vasopressin (Anaspect, Fremont, CA), or angiotensin-2 (Sigma-Aldrich) from 0.1 to 100 μM, encompassing clinically relevant concentration. The following reagents have been used to decipher the involvement of different adrenergic receptors (ARs): dobutamine, atenolol, propranolol (Cerilliant, Round Rock, TX), ICI-118,551 (Tocris, Bristol, United Kingdom), phentolamine methanesulfonate salt (PMS; Sigma-Aldrich), and phenylephrine (Hikma, Eatontown, NJ). UCSF's Institutional Review Board waived the need for approval to work with deidentified human cells purchased from a vendor.

Electric Cell-Substrate Impedance Sensing

HMVEC barrier integrity was inferred from transendothelial resistance (TER) measured using electrical

cell impedance sensing (ECIS) ZΘ TEER technology (Applied Biophysics Inc., Troy, NY). ECIS measures a cell monolayer's electrical impedance in real time and at multiple electrical frequencies (18, 19). A decrease in TER reflects an increase in permeability. A 96W20idf plate (Applied Biophysics Inc.) was stabilized with cysteine (10 mM) for 10 minutes. Cells were then seeded in 300 µL EGM-2 at a density of 60,000/cm^2. Cells were considered confluent upon reaching stable baseline resistances at 4,000 Hz, an optimal frequency specific for endothelial cells to be modeled using ECIS Software V.1.2.163.0. PC (Applied Biophysics Inc.) (20). Endothelial monolayers were then stimulated with inflammatory agonists and simultaneously or sequentially with the drugs described above, using four to eight replicates per condition. **Supplemental Figure 1** (Supplemental Digital Content 1, http://links.lww.com/CCM/G58; **legend**, Supplemental Digital Content 12, http://links.lww.com/CCM/G69) describes how we measured the area under the curve as an integrative marker of permeability and the maximal conductance peak.

Flow Cytometry

After detachment with the trypsin-free reagent Accutase (Innovative Cell Technologies INC., San Diego, CA), single cells in suspension were labeled with <FITC> -CD102 (Clone CBR-IC1/2) (Biolegend, San Diego, CA), <eFluor450> -CD31 (Clone WM59) (ebiosciences, San Diego, CA), and <PE> -CD144 (Clone 16B-1) (Invitrogen, Carlsbad, CA). Single-cell suspensions stained with fluorophore-conjugated antibodies were acquired the same day using an LSRII Fortessa (BD Biosciences, San Jose, CA) flow cytometer and analyzed with FlowJo software (Miltenyi, Bergisch Gladbach, Germany).

Immunofluorescence Microscopy

HMVEC were grown to confluence on LabTek II chamber slides (Nunc, thermofischer scientific, Waltham, MA) that had been precoated with collagen and then treated for 6 hours with LPS in the presence and absence of catecholaminergic agents, and antagonists and agonists of α- and β-AR. Cells were then fixed with 4% paraformaldehyde for 15 minutes and permeabilized with 0.5% Triton X-100 in phosphate-buffered saline (PBS). After blocking with 1% bovine serum albumin in PBS, cells were incubated with either anti–zona occludens (ZO)-1-AlexaFluor594 (Invitrogen), F-actin (rhodamine phalloidin; Invitrogen), or anti–vascular endothelial (VE)-cadherin (Santa Cruz Biotechnologies) followed by AlexaFluor488-tagged secondary antibody. Nuclei were counterstained with 4′6-diamidino-2-phenylindole. Slides were visualized with a Cytation 5 Biotek (Fischer scientific, Waltham, MA) and recorded at 20X objective on Gen5 Image+ Software (Biotek,Winooski, VT).

Immunoassays

Endothelial cell culture supernatants were collected at different timepoints and stored at –80°C until analysis. Cytokines were measured using Duoset ELISA Kit (R&D Systems) for IL-8, IL-6, and CCL-2, according to the appropriate dilution and following recommendations of the manufacturer.

Statistics

Results are reported as means (± SD), data were analyzed using one-way ordinary Kruskal-Wallis with multiple Dunn's post hoc test or a formal combined analysis of variance when multiple donors were analyzed on the same graph. *p* values of less than 0.05 were considered statistically significant. Statistics and graphical representations were performed using GraphPad Prism 8.0 (Graph Pad Software, San Diego, CA). Each experiment has been replicated at least twice on different donors from both genders.

RESULTS

Catecholaminergic Vasopressors, But Not Vasopressin or Angiotensin-2, Reduce Toll-Like Receptor Agonist-Induced Permeability on HMVEC

To assess for microvascular endothelial barrier dysfunction, we grew HMVEC to confluency on ECIS plates and stimulated them with LPS (Toll-like receptor [TLR]–4 agonist) in the presence or absence of increasing concentrations of each vasopressor. We observed that epinephrine and norepinephrine at concentrations from 0.1 to 100 µM significantly reduced LPS-induced permeability without an apparent concentration-dependent effect (**Fig. 1**, ***A*** and ***B***) (**Supplemental Fig. 2**, ***A*** and ***B***, Supplemental Digital Content 2, http://links.lww.com/CCM/G59; legend, Supplemental Digital Content 12,

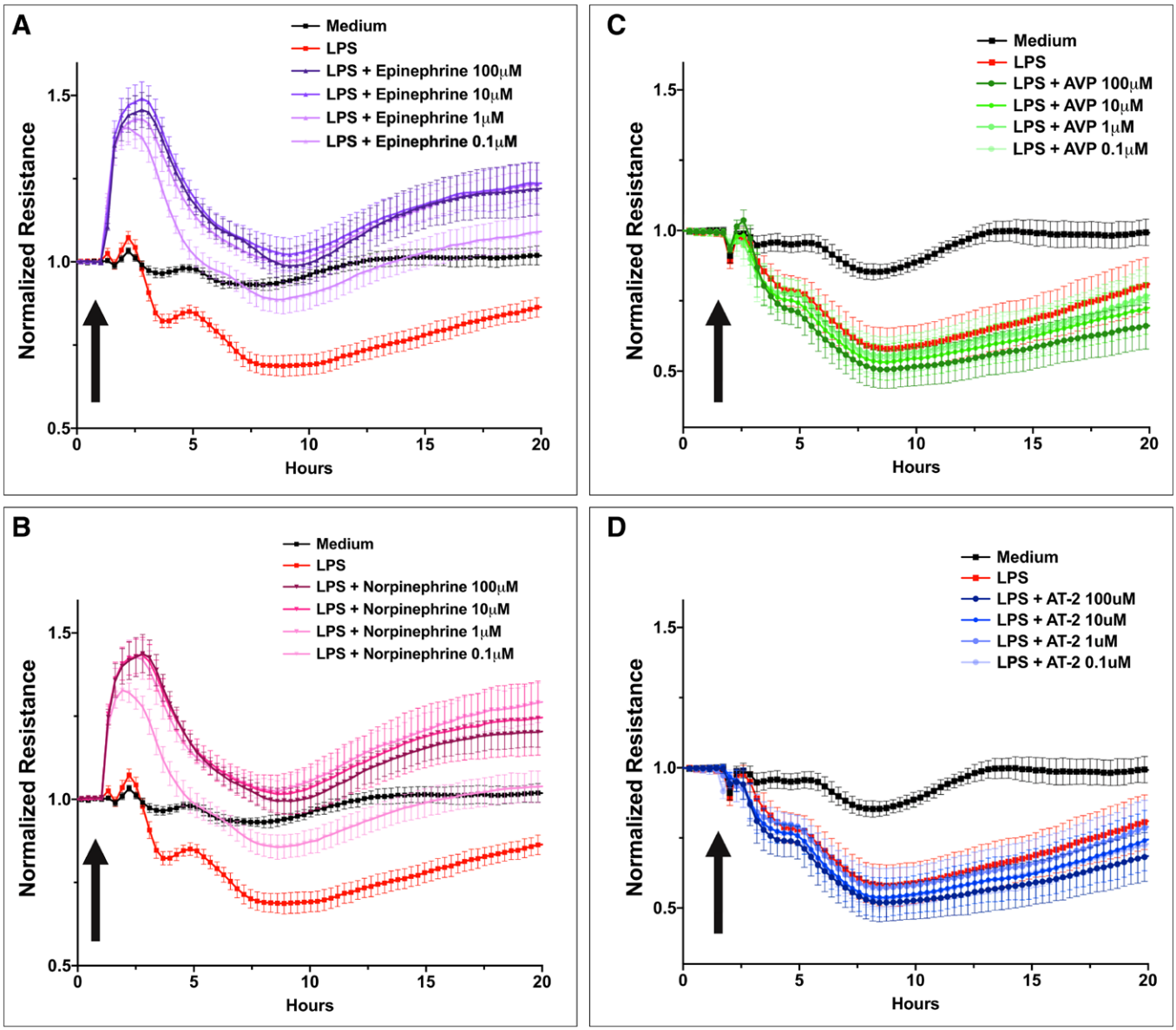

Figure 1. Effect of vasopressors on lipopolysaccharide (LPS)–induced permeability of human pulmonary microvascular endothelial cell (HMVEC) effects of increasing concentrations of epinephrine (**A**), norepinephrine (**B**), vasopressin (AVP) (**C**), an angiotensin (AT)–2 (**D**) on HMVEC LPS-induced permeability. HMVEC were simultaneously coincubated with indicated concentrations of vasopressors and LPS (1 μg/mL). *Arrows* indicate the time of addition of treatments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, any condition versus LPS.

http://links.lww.com/CCM/G69). Conversely, neither vasopressin nor angiotensin-2 affected LPS-induced permeability at any concentrations (**Fig. 1**, ***C*** and ***D***) (**Supplemental Fig. 2**, ***C*** and ***D***, Supplemental Digital Content 2, http://links.lww.com/CCM/G59; legend, Supplemental Digital Content 12, http://links.lww.com/CCM/G69). Despite substantial variability between the human donors regarding the intensity of response to both LPS and vasopressors, all donors studied showed the same pattern (**Supplemental Fig. 3**, Supplemental Digital Content 3, http://links.lww.com/CCM/G60; legend, Supplemental Digital Content 12, http://links.lww.com/CCM/G69). Overall, we estimate that 10 μM of catecholamines reduce LPS-induced permeability about 73.2% (± 24.9%; $p = 0.01$) for epinephrine and 70.8% (± 25.9%; $p = 0.02$) for norepinephrine. Similar results were observed in HMVEC treated with TLR-3 agonist (Poly[I:C]) (**Supplemental Fig. 4**, Supplemental Digital Content 4, http://links.lww.com/CCM/G61; legend, Supplemental Digital Content 12, http://links.lww.com/CCM/G69) and TLR-2 agonist (Pam3Cys) (**Supplemental Fig. 5**, Supplemental Digital Content 5, http://links.lww.com/CCM/G62; legend, Supplemental Digital Content 12,

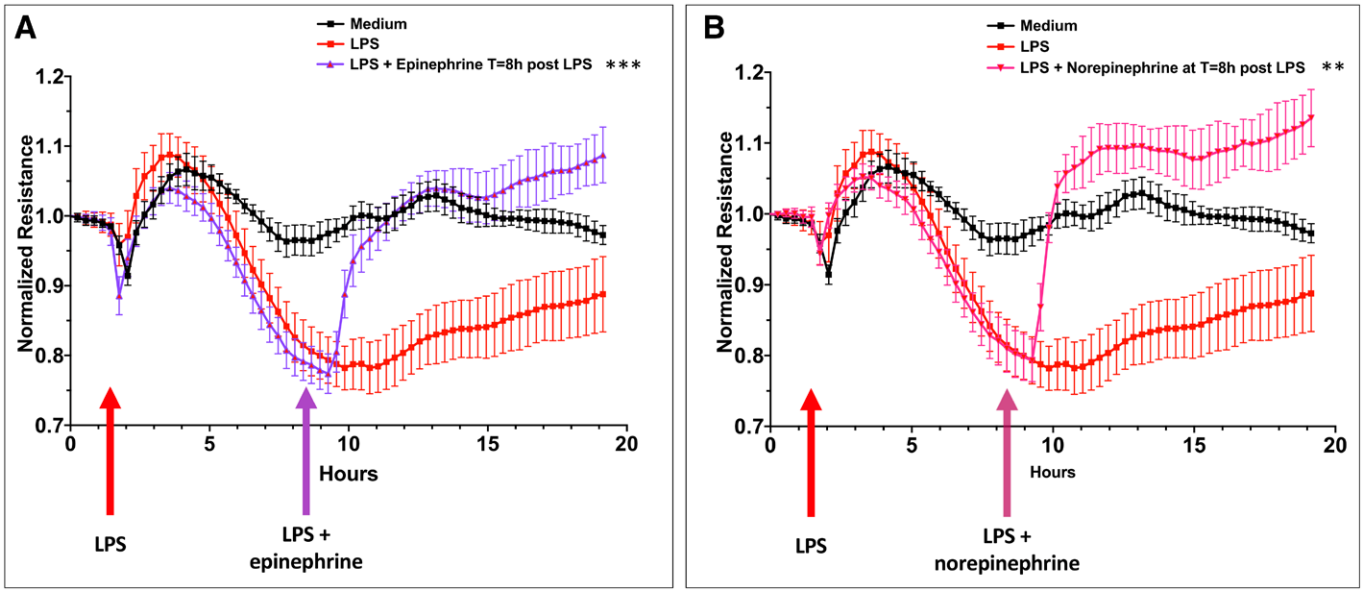

Figure 2. Posttreatment with catecholaminergic vasopressors reverses lipopolysaccharide (LPS)–induced permeability of human pulmonary microvascular endothelial cell. Epinephrine (10 μM) (**A**) and norepinephrine (10 μM) (**B**) were added to wells 8 hr after the addition of LPS (1 μg/mL). *Arrows* indicate the respective time of addition of treatments. $^{**}p < 0.01$, $^{***}p < 0.001$, area under the curve group LPS versus LPS + catecholamine at 8 hr.

http://links.lww.com/CCM/G69). Epinephrine and norepinephrine also significantly reduced permeability induced by non-TLR ligands endogenous mediators: IL-1β, TNF-α, and Cytomix (Supplemental Fig. 5, Supplemental Digital Content 5, http://links.lww.com/CCM/G62; legend, Supplemental Digital Content 12, http://links.lww.com/CCM/G69). Neither vasopressin nor angiotensin-2 had significant effect on permeability induced by these inflammatory agonists (Supplemental Fig. 5, Supplemental Digital Content 5, http://links.lww.com/CCM/G62; legend, Supplemental Digital Content 12, http://links.lww.com/CCM/G69). The effects of catecholamines on permeability induced by IL-1β, TNF-α, and cytomix are smaller compared with their effects on TLR agonists-induced permeability (LPS, Poly[I:C], and Pam3Cys). We speculate that these differences may be due to apoptosis of HMVEC treated with IL-1β, TNF-α, and cytomix, but not by the TLR agonists. Notably, the addition of catecholamines 8 hours post LPS challenge, when permeability was maximal (resistance nadir), restored the endothelial monolayer to its baseline resistance, in approximately 1 hour (**Fig. 2**). Combining a catecholaminergic and a noncatecholaminergic agent does not further modify the permeability effect than treatment with catecholamines alone, whether they were added simultaneously or sequentially (**Supplemental Fig. 6**, Supplemental Digital Content 6, http://links.lww.com/CCM/G63; legend, Supplemental Digital Content 12, http://links.lww.com/CCM/G69).

β-ARs, But Not α-Adrenergic, Are Responsible for the Reduced LPS-Induced Permeability of Catecholamines

Given their affinity for α-AR, we initially hypothesized that catecholamines reduce endothelial permeability induced by inflammatory agonists via α-AR signaling. However, the competitive blockade of α1- and α2-receptors using PMS (10 μM) did not reverse the effects of epinephrine or norepinephrine (**Fig. 3**, ***A*** and ***B***). PMS alone had no impact on endothelial barrier maintenance at baseline or in LPS-stimulated cells (**Supplemental Fig. 7*A***, Supplemental Digital Content 7, http://links.lww.com/CCM/G64; legend, Supplemental Digital Content 12, http://links.lww.com/CCM/G69). In addition, the pure α-AR agonist phenylephrine did not modify LPS-induced permeability (**Fig. 3*C***). Conversely, the simultaneous addition of the nonselective β-AR antagonist, propranolol (10 or 25 μM), to LPS + epinephrine/norepinephrine fully abrogated the effect of these catecholamines (**Fig. 4**, ***A1*** and ***A2***). We also observed that propranolol on its own transiently reduces TER and that propranolol prohibits full recovery of HMVEC from LPS-induced permeability (**Supplemental Fig. 7*B***, Supplemental Digital Content 7,

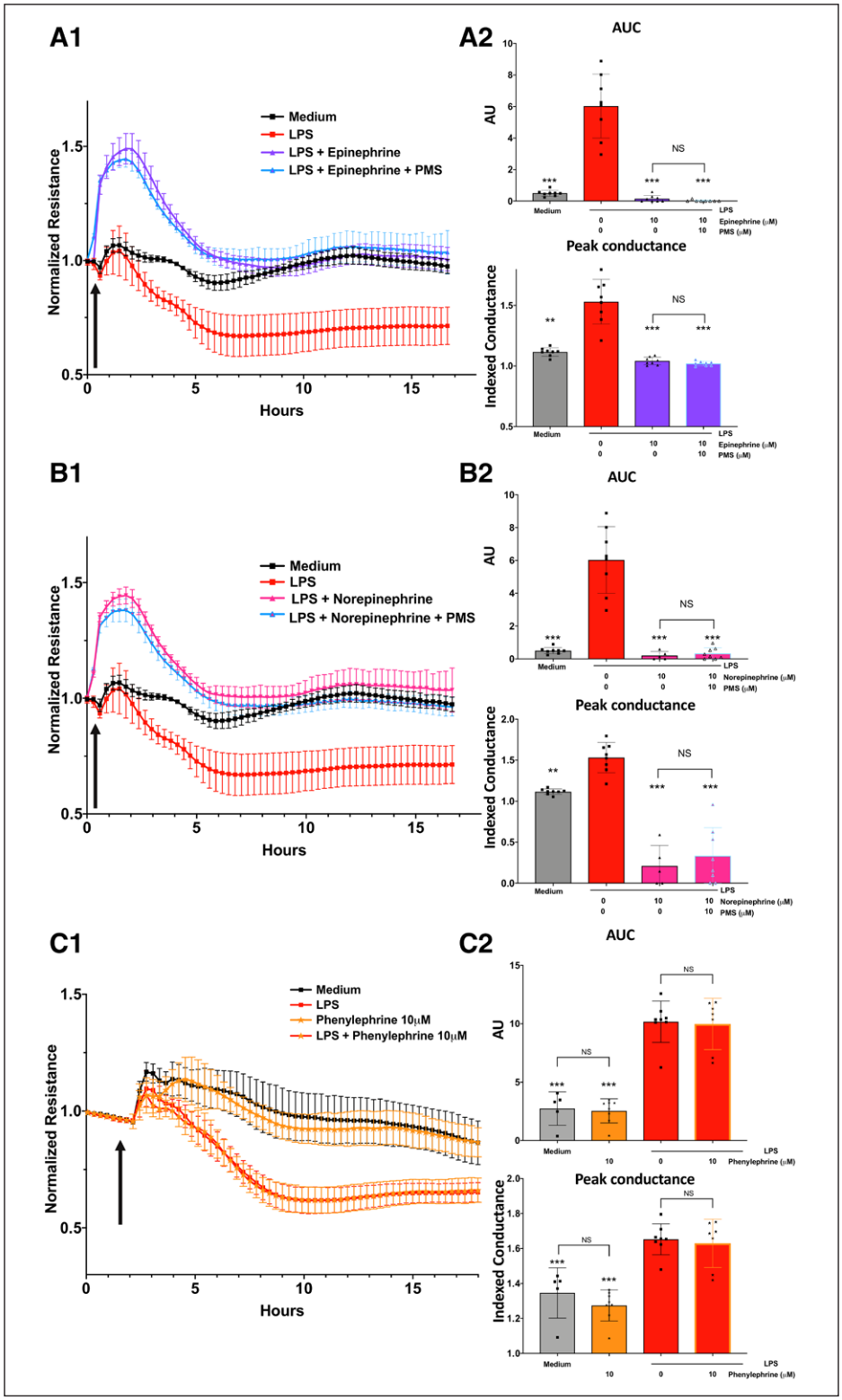

Figure 3. Catecholamines normalize human pulmonary microvascular endothelial cell (HMVEC) permeability independently of the α-adrenergic receptor. Synchronous HMVEC stimulation with phentolamine methanesulfonate salt (PMS) (10 μM) (an α1 and α2 receptors blocker) does not reverse the protective effect of epinephrine (10 μM) (**A1** and **A2**) nor norepinephrine (10 μM) (**B1** and **B2**). Pure α-agonist phenylephrine does not modify lipopolysaccharide (LPS)–induced permeability (1 μg/ml) (**C1** and **C2**). *Arrows* indicate the time of addition of treatments. $^{**}p < 0.01$, $^{***}p < 0.001$, any condition versus LPS. AUC = area under the curve.

http://links.lww.com/CCM/G64; legend, Supplemental Digital Content 12, http://links.lww.com/CCM/G69). We also observed that the selective β1-antagonist atenolol (10 or 25 μM) and selective β2-antagonist ICI-118,551 (10 or 25 μM) both abrogated norepinephrine's effect, whereas only ICI-118,551 counteracted the effect of epinephrine (**Fig. 4**, ***B1*** and ***B2***). Neither atenolol nor ICI-118,551 on their own affected TER (**Supplemental Fig. 7**, ***C*** and ***D***, Supplemental Digital Content 7, http://links.lww.com/CCM/G64; legend, Supplemental Digital Content 12, http://links.lww.com/CCM/G69). Finally, selective β2-agonist albuterol and β1-agonist dobutamine significantly reduced the LPS-induced permeability (**Supplemental Fig. 7**, ***E*** and ***F***, Supplemental Digital Content 7, http://links.lww.com/CCM/G64; legend, Supplemental Digital Content 12, http://links.lww.com/CCM/G69).

Catecholaminergic Vasopressors Restore Adherens- and Tight Junction Membrane Protein Organization Via Actin-Filament Rearrangement

Immunofluorescence of HMVEC, 6 hours post stimulation, revealed that LPS directly induces gaps in cell-cell junctions and reduces membrane VE-cadherin and ZO-1 expression. We observed that treatment with catecholaminergic agents (10 μM) reduces LPS-induced formation of cell-cell junction gaps and restores the expression of VE-cadherin and ZO-1. Catecholaminergic agents also

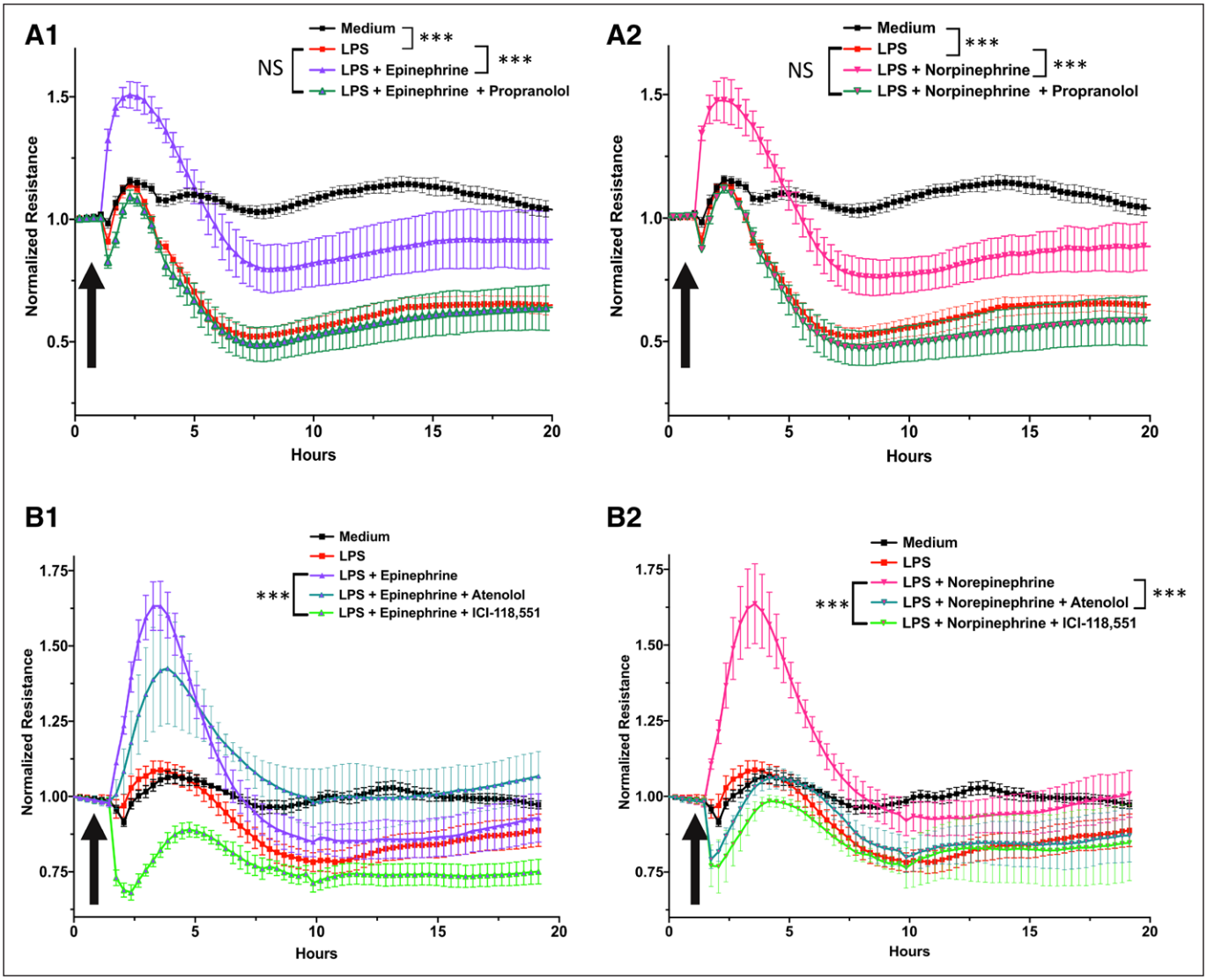

Figure 4. Catecholamines reduce lipopolysaccharide (LPS)–induced human pulmonary microvascular endothelial cell (HMVEC) permeability via β1- and β2-adrenergic receptors. Synchronous HMVEC stimulation with propranolol (25 μM) (a β1 and β2 receptors blocker) fully abrogates the antipermeability effects of epinephrine (10 μM) (**A1**) and norepinephrine (10 μM) (**A2**). β2-antagonist ICI-118,551 (25 μM) fully abrogates the reduced permeability induced by epinephrine (10 μM) and norepinephrine (10 μM), β1-antagonist atenolol (25 μM) reverses the effects of norepinephrine (but not epinephrine) in reducing LPS-induced permeability (1 μg/mL) (**B1** and **B2**). *Arrows* indicate the time of addition of treatments. $^{**}p < 0.01$, $^{***}p < 0.001$. NS = not significant.

induced substantial changes in the F-actin submembrane cytoskeleton. Indeed, treatment with epinephrine or norepinephrine induced actin-filament contraction with a reinforcement under the membrane and parallel rearrangement in "railroad tracks." Cotreatment with propranolol fully reversed the cytoskeletal changes induced by catecholamines and augmented HMVEC monolayer disruption. Representative images are shown in **Figure 5**. Catecholaminergic vasopressor-induced restoration of VE-Cadherin membranous expression was confirmed by flow cytometry (**Supplemental Fig. 8**, Supplemental Digital Content 8, http://links.lww.com/CCM/G65; legend, Supplemental Digital Content 12, http://links.lww.com/CCM/G69).

Treatment of HMVEC With Catecholaminergic Vasopressors Does Not Modify LPS or Pam3Cys-Induced Inflammatory Mediators Secretion or Cell Survival

The microvascular endothelium is a critical component of the immune response in sepsis and acute sterile inflammation. We, therefore, assessed the effects of vasopressors on HMVEC production of IL-6, IL-8, and CCL-2, which are involved in the recruitment and transendothelial

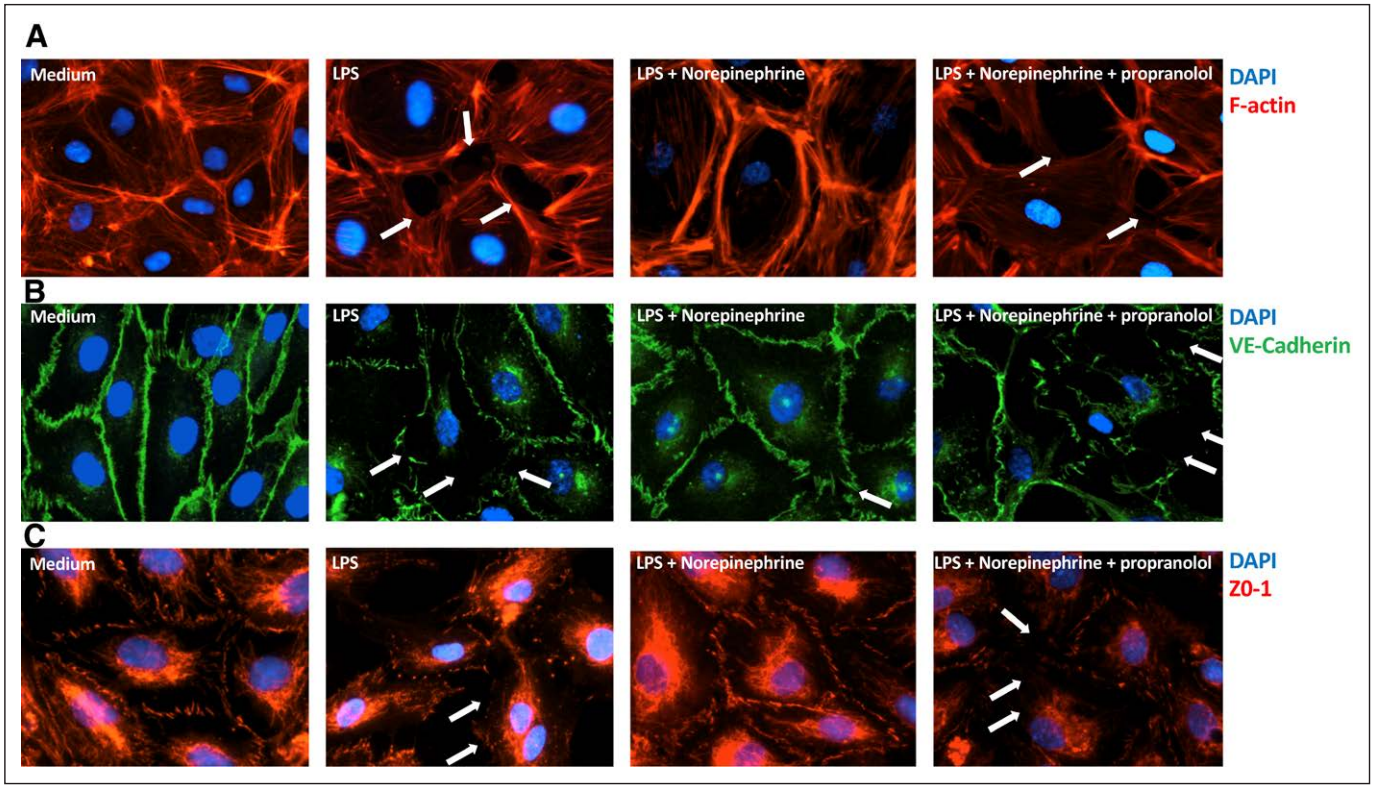

Figure 5. Catecholaminergic vasopressors restore adherens- and tight-junctions of lipopolysaccharide (LPS)–treated human pulmonary microvascular endothelial cell (HMVEC) via actin-filament rearrangement. Representative pictures of F-actin (**A**), VE-cadherin (**B**), and ZO-1 (5C) immunofluorescent staining on HMVEC, after 6 hr of stimulation with LPS (1 μg/mL). *Arrows* indicate gaps in cell-cell junctions. DAPI = 4′6-diamidino-2-phenylindole.

migration of monocytes and neutrophils. We observed no significant effects of epinephrine and norepinephrine on LPS-induced IL-6, IL-8, or CCL-2 at any time point. In contrast, angiotensin-2 significantly augmented LPS-induced IL-6 and CCL-2 at 24 hours (**Supplemental Fig. 9**, Supplemental Digital Content 9, http://links.lww.com/CCM/G66; legend, Supplemental Digital Content 12, http://links.lww.com/CCM/G69). Similar results were observed with Pam3Cys (**Supplemental Fig. 10**, Supplemental Digital Content 10, http://links.lww.com/CCM/G67; legend, Supplemental Digital Content 12, http://links.lww.com/CCM/G69). None of the vasopressors affected HMVEC survival as assessed by crystal violet assays (**Supplemental Fig. 11**, Supplemental Digital Content 11, http://links.lww.com/CCM/G68; legend, Supplemental Digital Content 12, http://links.lww.com/CCM/G69).

DISCUSSION

Permeability and endothelial dysfunction are hallmarks of sepsis and are being explored as targets for sepsis therapies. Our study indicates that catecholamines, which are widely used in sepsis and other forms of shock for their vasoconstrictive and inotropic effects, can directly reduce endothelial cell permeability induced by bacterial and viral TLR agonists. Catecholamines also limit permeability induced by proinflammatory cytokines. Our results raise the possibility that catecholamines may affect the course of shock not only through their effects on vascular tone and cardiac contractility but also by reducing microvascular endothelial permeability.

During sepsis, microbial products directly stimulate endothelial cells through pattern-recognition receptors (PRRs) (21, 22) and downstream inflammatory pathways mediated by nuclear factor kappa B and the mitogen-activated protein kinases (23). Engagement of endothelial cell PRRs promotes a proinflammatory phenotype with increased production of cytokines, chemokines and procoagulant factors, and up-regulated expression of proadhesive molecules and antifibrinolytic factors. Additionally, damaged glycocalyx and endothelial cell apoptosis lead to an increase in permeability to proteins and fluids, causing interstitial edema (17, 24). Our data indicate that

catecholaminergic vasopressors significantly decrease TLR-agonist–induced permeability, whereas noncatecholaminergic vasopressors do not. Even when LPS-induced endothelial permeability is at its peak, the addition of catecholamines restores TER within minutes of introduction. The effect of catecholamines on permeability is preserved in a vast range of concentrations (from 0.1 to 100 μM). Furthermore, no additive or synergistic effect was observed when combining different classes of vasopressors. Our data using selective pharmacologic antagonists implicate β1 and β2-AR in the endothelial stabilizing effects of catecholamines. We observed that antagonists for both β1 and β2 reversed the antipermeability effect of norepinephrine, whereas only β2-antagonist reversed the antipermeability effects of epinephrine. This dichotomy may be due to the 10- to 100-fold stronger affinity of epinephrine for the β2 receptor than the β1 receptor, leading to negligible effects of selective blockade of β1 on the stabilizing effects of epinephrine.

Catecholamines rapidly restore the endothelial barrier within minutes. This suggests that protein-protein interactions may be responsible for the restoration, rather than transcriptional regulation of genes involved in permeability. β-ARs have been reported to play a role in forming focal adhesions and dynamic remodeling of the actin cytoskeleton. Activation via the Gs subunit of β2-AR has been shown to enhance intracellular cyclic adenosine monophosphate (cAMP) generation transiently. Intracellular cAMP activates protein kinase A, which stabilizes the actin cytoskeleton and counteracts the cell retractile force (25–27). β-AR signaling is also mediated via the β-arrestin 2 and p115RhoGEF complexation, activating the membrane RhoA-kinase (28, 29). Inhibition of Rho-kinase has been demonstrated to increase LPS-induced endothelial permeability (30). Thus, β-AR blockade induces intracellular gaps via actin myosin-driven endothelial retraction, and conversely, we observed in our study that its activation reduces LPS-induced permeability through cytoskeletal rearrangement and contributes to the maintenance of endothelial barrier properties under baseline conditions (31).

Although this is an in vitro study of primary human endothelial cells, we speculate that our results may have relevance to the choice and timing of initiation of vasopressors in humans with shock. Endothelial barrier dysfunction leading to vascular leak and tissue edema is believed to promote organ failure in many conditions, including septic shock, acute respiratory distress syndrome (ARDS), ischemia-reperfusion, and noninfectious shock (e.g., pancreatitis, trauma) (32). Restoration of endothelial functions is considered a potential target of resuscitation strategies (33). Concerns with reducing permeability center around the concept that vascular permeability may serve a beneficial role in recruiting leukocytes to sites of infection and injury by promoting leukocyte-transendothelial migrations, which are necessary to fight infection and initiate repair of injured tissues. Several studies, however, have challenged the notion that vascular leak is required for leukocyte tissue migration (34, 35) and have documented that fluid leakage, leukocyte recruitment, and transendothelial migration are regulated separately (36–38).

Early fluid loading is a cornerstone of hemodynamic septic and nonseptic vasoplegic shock management. However, fluid resuscitation protocols are intensely debated, regarding the importance of achieving hemodynamic stability versus the risk of overloading. Observational studies suggest that insufficient fluid volume, in early phase of septic shock resuscitation, is deleterious (39–41). Conversely, numerous studies also suggest that high-volume resuscitation and a positive fluid balance might be harmful (42–44), supporting a more restrictive approach to fluid resuscitation (45–48). Reports in humans with septic shock suggest that early treatment with norepinephrine may be beneficial (49–51). Notably, in the CENSER study, patients receiving early norepinephrine had a lower occurrence of pulmonary edema (14.4 % vs 27.7 %; $p = 0{,}004$) despite receiving the equivalent volumes of fluids (51). Our results raise the possibility that early catecholamine administration may limit the capillary leak syndrome induced by sepsis and therefore limit interstitial edema.

Despite having substantial effects on TLR-agonist–induced permeability, we observed that the catecholaminergic vasopressors did not affect TLR-agonist–induced production of cytokines and chemokines by HMVEC. Our data suggest that TLR-dependent permeability and inflammation are independently regulated in microvascular endothelial cells (52). This suggests that it may be possible to develop endothelial based therapies that prevent or treat vascular leakage, without impairing the endothelium's

immunoinflammatory response. Although Staedtke et al (53) reported that epinephrine increases IL-6 secretion by peritoneal macrophages and T cells, but not endothelial cells, it has been recently published that norepinephrine infusion correlates with a "anti-inflammatory cytokine profile" in septic mice and healthy human volunteers following LPS challenge (54). Also, the effects of catecholamines on inflammation mediators might depend on the cell type and the inflammatory conditions. In our study, we found that high concentrations of noncatecholaminergic vasopressors (angiotensin-2 and to a lesser extent vasopressin) increased LPS- and Pam3Cys-induced production of IL-6 and CCL-2 at 24 hours. In vitro, the absence of vasopressors' clearance makes it difficult to fully mimic the potential in vivo effects. However, experimental studies reported that continuous infusion of angiotensin-2 in mice dose-dependently increases intra-aortic IL-6 production and recruitment of macrophages suggesting a vascular induction of chemokines (55, 56) and supporting our findings.

β-blockers have been proposed as potential therapeutic adjuvants for septic shock because they have been reported to decrease cardiac oxygen consumption, limit hyperglycemia, reduce the catabolic response to sepsis, and alleviate Type-1 T-helper inflammation (57–59).

Our study raises the possibility that β-blockers might exacerbate vascular leakage in sepsis. Our study has several limitations. First, we worked only with lung derived microvascular cells. We focused on lung-derived-EC because of their susceptibility to capillary leakage driving acute lung injury and ARDS in critical illness. However, given the heterogeneity of microvascular beds we can't extrapolate these findings to other organs (60, 61). Second, we explored only barrier and inflammatory functions, but the response of the microvascular endothelium to inflammation is multifaceted. We did not study the effects of vasopressors on other essential functions, such as of glycocalyx maintenance, coagulation, fibrinolysis, and leukocyte adhesion (17). Assessing endothelial barrier function in vivo is extremely challenging. Our in vitro system has the advantage that it allows real-time assessment of the effect of vasopressors on endothelial barrier function regardless of changes in vasomotor tone. But, it cannot pretend to fully reproduce the integrative effects of vasopressors in a critically ill patient's pulmonary microcirculation. Finally, although we observed a similar effect of LPS and catecholamines in every donor tested, we noticed substantial interindividual variability in the intensity of the permeability response to LPS and catecholamines.

CONCLUSIONS

Our findings demonstrate that treatment of cultured primary human lung microvascular endothelial cells with epinephrine or norepinephrine strongly reduces endothelial permeability induced by agonists of multiple TLRs. Furthermore, posttreatment with catecholamines many hours into treatment with LPS restores endothelial permeability to baseline. Both β1- and β2-AR, but not α-AR, mediate the stabilizing effects of epinephrine and norepinephrine on the endothelial barrier. Our results suggest that epinephrine and norepinephrine may act to restore vascular barrier integrity during sepsis and shock, in addition to their known effects on vasomotor tone and cardiac function.

1 *Department of Anesthesia and Perioperative Care, University of California, San Francisco, CA.*

2 *Division of Pediatric Critical Care, UCSF Benioff Children's Hospitals, San Francisco, CA.*

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For information regarding this article, E-mail: Jeremie.joffre@ucsf.edu

REFERENCES

1. Kaukonen KM, Bailey M, Suzuki S, et al: Mortality related to severe sepsis and septic shock among critically ill patients in Australia and New Zealand, 2000-2012. *JAMA* 2014; 311:1308–1316
2. Dellinger RP, Hussain S: From Barcelona to New York: 15 years of transition of sepsis performance improvement. *J Thorac Dis* 2017; 9:3453–3455
3. Sakr Y, Dubois MJ, De Backer D, et al: Persistent microcirculatory alterations are associated with organ failure and death in patients with septic shock. *Crit Care Med* 2004; 32:1825–1831
4. Ait-Oufella H, Lemoinne S, Boelle PY, et al: Mottling score predicts survival in septic shock. *Intensive Care Med* 2011; 37:801–807
5. Preda G, Bourcier S, Joffre J, et al: Mottling score is associated with 28-day mortality in critically ill patients with sepsis. *Minerva Anestesiol* 2017; 83:664–666
6. Ait-Oufella H, Joffre J, Boelle PY, et al: Knee area tissue oxygen saturation is predictive of 14-day mortality in septic shock. *Intensive Care Med* 2012; 38:976–983
7. Rovas A, Seidel LM, Vink H, et al: Association of sublingual microcirculation parameters and endothelial glycocalyx dimensions in resuscitated sepsis. *Crit Care* 2019; 23:260
8. Johansson PI, Stensballe J, Ostrowski SR: Shock induced endotheliopathy (SHINE) in acute critical illness - a unifying pathophysiologic mechanism. *Crit Care* 2017; 21:25
9. Rhodes A, Evans LE, Alhazzani W, et al: Surviving sepsis campaign: international guidelines for management of sepsis and septic shock: 2016. *Intensive Care Med* 2017; 43:304–377
10. Khanna A, Ostermann M, Bellomo R: Angiotensin II for the treatment of vasodilatory shock. *N Engl J Med* 2017; 377:2604
11. Sakr Y, Reinhart K, Vincent JL, et al: Does dopamine administration in shock influence outcome? Results of the sepsis occurrence in acutely ill patients (SOAP) Study. *Crit Care Med* 2006; 34:589–597
12. Myburgh JA, Higgins A, Jovanovska A, et al; CAT Study investigators: A comparison of epinephrine and norepinephrine in critically ill patients. *Intensive Care Med* 2008; 34:2226–2234
13. Russell JA, Walley KR, Singer J, et al; VASST Investigators: Vasopressin versus norepinephrine infusion in patients with septic shock. *N Engl J Med* 2008; 358:877–887
14. Marks JA, Pascual JL: Selepressin in septic shock: Sharpening the VASST effects of vasopressin?*. *Crit Care Med* 2014; 42:1747–1748
15. Laterre PF, Berry SM, Blemings A, et al: Effect of selepressin vs placebo on ventilator- and vasopressor-free days in patients with septic shock: The SEPSIS-ACT Randomized Clinical Trial. *JAMA* 2019; 322:1476–1485
16. Demiselle J, Fage N, Radermacher P, et al: Vasopressin and its analogues in shock states: A review. *Ann Intensive Care* 2020; 10:9
17. Joffre J, Hellman J, Ince C, et al: Endothelial responses in sepsis. *Am J Respir Crit Care Med* 2020; 202:361–370
18. Tiruppathi C, Malik AB, Del Vecchio PJ, et al: Electrical method for detection of endothelial cell shape change in real time: Assessment of endothelial barrier function. *Proc Natl Acad Sci USA* 1992; 89:7919–7923
19. Meng J, Roy S: Study of epithelium barrier functions by real-time TER measurement. *Bio Protoc* 2016; 6:e1815
20. Szulcek R, Bogaard HJ, van Nieuw Amerongen GP: Electric cell-substrate impedance sensing for the quantification of endothelial proliferation, barrier function, and motility. *J Vis Exp* 2014; 28:51300
21. Salvador B, Arranz A, Francisco S, et al: Modulation of endothelial function by toll like receptors. *Pharmacol Res* 2016; 108:46–56
22. Khakpour S, Wilhelmsen K, Hellman J: Vascular endothelial cell toll-like receptor pathways in sepsis. *Innate Immun* 2015; 21:827–846
23. Wang W, Deng M, Liu X, et al: TLR4 activation induces nontolerant inflammatory response in endothelial cells. *Inflammation* 2011; 34:509–518
24. Colbert JF, Schmidt EP: Endothelial and microcirculatory function and dysfunction in sepsis. *Clin Chest Med* 2016; 37:263–275
25. Rangarajan S, Enserink JM, Kuiperij HB, et al: Cyclic AMP induces integrin-mediated cell adhesion through Epac and Rap1 upon stimulation of the beta 2-adrenergic receptor. *J Cell Biol* 2003; 160:487–493
26. Qiao J, Huang F, Lum H: PKA inhibits RhoA activation: A protection mechanism against endothelial barrier dysfunction. *Am J Physiol Lung Cell Mol Physiol* 2003; 284:L972–L980
27. Moore TM, Chetham PM, Kelly JJ, et al: Signal transduction and regulation of lung endothelial cell permeability. Interaction between calcium and cAMP. *Am J Physiol* 1998; 275:L203–L222
28. Holinstat M, Mehta D, Kozasa T, et al: Protein kinase calpha-induced p115RhoGEF phosphorylation signals endothelial cytoskeletal rearrangement. *J Biol Chem* 2003; 278:28793–28798
29. Ma X, Zhao Y, Daaka Y, et al: Acute activation of β2-adrenergic receptor regulates focal adhesions through βArrestin2- and p115RhoGEF protein-mediated activation of RhoA. *J Biol Chem* 2012; 287:18925–18936
30. Xiaolu D, Jing P, Fang H, et al: Role of p115RhoGEF in lipopolysaccharide-induced mouse brain microvascular endothelial barrier dysfunction. *Brain Res* 2011; 1387:1–7
31. Spindler V, Waschke J: Beta-adrenergic stimulation contributes to maintenance of endothelial barrier functions under baseline conditions. *Microcirculation* 2011; 18:118–127
32. Lee WL, Slutsky AS: Sepsis and endothelial permeability. *N Engl J Med* 2010; 363:689–691
33. Hernández G, Ospina-Tascón GA, Damiani LP, et al; The ANDROMEDA SHOCK Investigators and the Latin America Intensive Care Network (LIVEN): Effect of a resuscitation strategy targeting peripheral perfusion status vs serum lactate levels on 28-day mortality among patients with septic shock:

The ANDROMEDA-SHOCK randomized clinical trial. *JAMA* 2019; 321:654–664

34. Martin TR, Pistorese BP, Chi EY, et al: Effects of leukotriene B4 in the human lung. Recruitment of neutrophils into the alveolar spaces without a change in protein permeability. *J Clin Invest* 1989; 84:1609–1619
35. Wessel F, Winderlich M, Holm M, et al: Leukocyte extravasation and vascular permeability are each controlled *in vivo* by different tyrosine residues of VE-cadherin. *Nat Immunol* 2014; 15:223–230
36. Sugiyama MG, Armstrong SM, Wang C, et al: The Tie2-agonist vasculotide rescues mice from influenza virus infection. *Sci Rep* 2015; 5:11030
37. Gutbier B, Jiang X, Dietert K, et al: Vasculotide reduces pulmonary hyperpermeability in experimental pneumococcal pneumonia. *Crit Care* 2017; 21:274
38. London NR, Zhu W, Bozza FA, et al: Targeting Robo4-dependent slit signaling to survive the cytokine storm in sepsis and influenza. *Sci Transl Med* 2010; 2:23ra19
39. Kuttab HI, Lykins JD, Hughes MD, et al: Evaluation and predictors of fluid resuscitation in patients with severe sepsis and septic shock. *Crit Care Med* 2019; 47:1582–1590
40. Leisman DE, Doerfler ME, Schneider SM, et al: Predictors, prevalence, and outcomes of early crystalloid responsiveness among initially hypotensive patients with sepsis and septic shock. *Crit Care Med* 2018; 46:189–198
41. Lane DJ, Wunsch H, Saskin R, et al: Association between early intravenous fluids provided by paramedics and subsequent in-hospital mortality among patients with sepsis. *JAMA Netw Open* 2018; 1:e185845
42. Sakr Y, Rubatto Birri PN, Kotfis K, et al; Intensive Care Over Nations Investigators: Higher fluid balance increases the risk of death from sepsis: Results from a large international audit. *Crit Care Med* 2017; 45:386–394
43. Tigabu BM, Davari M, Kebriaeezadeh A, et al: Fluid volume, fluid balance and patient outcome in severe sepsis and septic shock: A systematic review. *J Crit Care* 2018; 48:153–159
44. Marik PE, Byrne L, van Haren F: Fluid resuscitation in sepsis: The great 30 mL per kg hoax. *J Thorac Dis* 2020; 12:S37–S47
45. Byrne L, Van Haren F: Fluid resuscitation in human sepsis: Time to rewrite history? *Ann Intensive Care* 2017; 7:4
46. Maitland K, Kiguli S, Opoka RO, et al; FEAST Trial Group: Mortality after fluid bolus in African children with severe infection. *N Engl J Med* 2011; 364:2483–2495
47. Andrews B, Semler MW, Muchemwa L, et al: Effect of an early resuscitation protocol on in-hospital mortality among adults with sepsis and hypotension: A Randomized Clinical Trial. *JAMA* 2017; 318:1233–1240
48. Boyd JH, Forbes J, Nakada TA, et al: Fluid resuscitation in septic shock: A positive fluid balance and elevated central venous pressure are associated with increased mortality. *Crit Care Med* 2011; 39:259–265
49. Bai X, Yu W, Ji W, et al: Early versus delayed administration of norepinephrine in patients with septic shock. *Crit Care* 2014; 18:532
50. Ospina-Tascón GA, Hernandez G, Alvarez I, et al: Effects of very early start of norepinephrine in patients with septic shock: A propensity score-based analysis. *Crit Care* 2020; 24:52
51. Permpikul C, Tongyoo S, Viarasilpa T, et al: Early use of norepinephrine in septic shock resuscitation (CENSER). A randomized trial. *Am J Respir Crit Care Med* 2019; 199:1097–1105
52. Filewod NC, Lee WL: Inflammation without vascular leakage. Science fiction no longer? *Am J Respir Crit Care Med* 2019; 200:1472–1476
53. Staedtke V, Bai RY, Kim K, et al: Disruption of a self-amplifying catecholamine loop reduces cytokine release syndrome. *Nature* 2018; 564:273–277
54. Stolk RF, van der Pasch E, Naumann F, et al: Norepinephrine dysregulates the immune response and compromises host defense during sepsis. *Am J Respir Crit Care Med* 2020; 202:830–842
55. Gomolak JR, Didion SP: Angiotensin II-induced endothelial dysfunction is temporally linked with increases in interleukin-6 and vascular macrophage accumulation. *Front Physiol* 2014; 5:396
56. Han Y, Runge MS, Brasier AR: Angiotensin II induces interleukin-6 transcription in vascular smooth muscle cells through pleiotropic activation of nuclear factor-kappa B transcription factors. *Circ Res* 1999; 84:695–703
57. Oberbeck R, Kobbe P: Beta-adrenergic antagonists: Indications and potential immunomodulatory side effects in the critically ill. *Curr Med Chem* 2009; 16:1082–1090
58. Schmitz D, Wilsenack K, Lendemanns S, et al: Beta-Adrenergic blockade during systemic inflammation: Impact on cellular immune functions and survival in a murine model of sepsis. *Resuscitation* 2007; 72:286–294
59. Elenkov IJ, Wilder RL, Chrousos GP, et al: The sympathetic nerve–an integrative interface between two supersystems: The brain and the immune system. *Pharmacol Rev* 2000; 52:595–638
60. Aird WC: Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms. *Circ Res* 2007; 100:158–173
61. Klijn E, Den Uil CA, Bakker J, et al: The heterogeneity of the microcirculation in critical illness. *Clin Chest Med* 2008; 29:643,viii–654, viii