Transendothelial flow inhibits neutrophil transmigration through a nitric oxide-dependent mechanism: potential role for cleft shear stress

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Burns AR, Zheng Z, Soubra SH, Chen J, Rumbaut RE. Transendothelial flow inhibits neutrophil transmigration through a nitric oxide-dependent mechanism: potential role for cleft shear stress. Am J Physiol Heart Circ Physiol 293: H2904–H2910, 2007.—Endothelial cells in vivo are well known to respond to parallel shear stress induced by luminal blood flow. In addition, fluid filtration across endothelium (transendothelial flow) may trigger nitric oxide (NO) production, presumably via shear stress within intercellular clefts. Since NO regulates neutrophil-endothelial interactions, we determined whether transendothelial flow regulates neutrophil transmigration. Interleukin-1β-treated human umbilical vein endothelial cell (HUVEC) monolayers cultured on a polycarbonate filter were placed in a custom chamber with or without a modest hydrostatic pressure gradient (ΔP, 10 cmH2O) to induce transendothelial flow. In other experiments, cells were studied in a parallel plate flow chamber at various transendothelial flows (ΔP = 0, 5, and 10 cmH2O) and luminal flows (shear stress of 0, 1, and 2 dyn/cm²). In the absence of luminal flow, transendothelial flow reduced transmigration of freshly isolated human neutrophils from 57% to 14% (P < 0.05) and induced an increase in NO detected with a fluorescent assay (DAF-2DA). The NO synthase inhibitor l-NAME prevented the effects of transendothelial flow on neutrophil transmigration, while a NO donor (DETA/NO, 1 mM) inhibited neutrophil transmigration. Finally, in the presence of luminal flow (1 and 2 dyn/cm²), transendothelial flow also inhibited transmigration. On the basis of HUVEC morphometry and measured transendothelial volume flow, we estimated cleft shear stress to range from 49 to 198 dyn/cm². These shear stress estimates, while substantial, are of similar magnitude to those reported by others with similar analyses. These data are consistent with the hypothesis that endothelial cleft shear stress inhibits neutrophil transmigration via a NO-dependent mechanism.

endothelium; neutrophils; hydraulic conductivity; diapedesis; permeability

A hallmark feature of an acute inflammatory response is the extravasation of leukocytes, primarily neutrophils, at sites of tissue injury or infection. Through a series of coordinated adhesive events, neutrophils first tether, then roll, and finally arrest on the inflamed endothelial surface. Under favorable conditions, firmly adherent neutrophils undergo transendothelial migration (diapedesis) in response to locally derived chemotactic factors (e.g., interleukin-8 and platelet-activating factor) (18, 28), and neutrophils can utilize paracellular (i.e., between endothelial cells) and transcellular (i.e., direct penetration of a single endothelial cell) migration pathways (5).

The human umbilical vein endothelial cell (HUVEC) monolayer is a commonly used in vitro model for studying leukocyte trafficking, and it has proven to be remarkably predictive for leukocyte adhesion and migration events occurring in vivo (6). Indeed, many of the molecular mechanisms regulating neutrophil adhesion and migration have been elucidated using this model. When freshly isolated neutrophils are allowed to interact with a cytokine-activated (e.g., interleukin-1β) HUVEC monolayer, rapid and efficient neutrophil migration ensues. Several studies with this model have exposed the endothelium to parallel surface shear stress, to mimic the in vivo luminal shear stress induced by blood flow (1, 16, 20). However, the potential effect of another relevant physiological force, transendothelial flow, on neutrophil adhesion and transmigration has, to our knowledge, not been described previously.

Transendothelial (transvascular) flow is an important regulator of endothelial function. Increased transendothelial flow occurs in a variety of pathophysiological conditions. Sustained step increases in transendothelial pressure are associated with increased endothelial hydraulic conductivity (Lp) in vitro (9, 30) and in vivo (12). The associated increase in Lp is thought to involve a nitric oxide-dependent mechanism (9, 30). Nitric oxide (NO), synthesized in vascular endothelial cells by endothelial NO synthase (eNOS), reportedly inhibits neutrophil binding to endothelial cells and suppresses transendothelial migration. Indeed, mice deficient in eNOS show enhanced baseline leukocyte rolling and adhesion in mesenteric postcapillary venules (17). Similarly, NOS inhibitors increase leukocyte adhesion in cat (13) and rat mesenteric venules (25). The purpose of the present study was to determine whether neutrophil transendothelial migration is regulated by transendothelial flow and whether this regulation is mediated by endothelial-derived NO.

Materials and Methods

Endothelial cell culture. HUVECs were routinely harvested from 5–10 umbilical veins by collagenase perfusion as previously described (11). Pooled cells were grown to confluence in M199 (Gibco) containing 10% fetal bovine serum (FBS, Hyclone), 10% bovine calf serum (BCS, Hyclone), 50 µg/ml penicillin, 50 µg/ml streptomycin, 1% fungizone, 0.01% heparin, 10 mM HEPES (Gibco), and 50 µg/ml endothelial cell growth supplement (BD Biosciences). First-passage HUVECs were seeded at confluence (2.7 × 10⁶ cells/cm²) onto tissue culture polycarbonate filters (Snapwell, 0.4-µm pore size, 12-mm diameter; Corning, Corning, NY) precoated with 0.2% gelatin (Difco) and used at 6 days postseeding. In some experiments, first-passage HUVECs were grown on 12-mm glass coverslips precoated with glutaraldehyde-cross-linked gelatin (2).

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Neutrophil isolation. Human peripheral blood neutrophils were isolated from citrate-anticoagulated venous blood that was sedimented over 6% dextran (250,000 mol wt) and centrifuged over a gradient of Ficoll-Hypaque as previously described (3). The blood was obtained from healthy volunteers, under a protocol approved by the Institutional Review Board of Baylor College of Medicine. The isolation method routinely yields neutrophil purities >95% with, as determined by Trypan blue exclusion, >97% viability. Isolated neutrophils were washed and resuspended in Dulbecco’s phosphate-buffered saline (pH 7.4; Gibco BRL, Grant Island, NY) and kept at room temperature for up to 2 h before being used in the adhesion/transmigration assay.

Neutrophil adhesion and transmigration assays. Interleukin-1β (IL-1β)-treated (10 U/ml, 1.5 h) HUVEC monolayers growing on polycarbonate membrane filters (Snapwell inserts) were rinsed in MEM containing 0.5% human serum albumin (HSA) and placed in a custom chamber (Fig. 1A) filled with MEM-0.5% HSA without transendothelial pressure (∆P, 0 cmH2O). In some experiments, the chamber was modified to allow quantification of transendothelial volume flow for measurement of Lp, based on the method described by Sill et al. (27). For this purpose, a glass tube (2-mm inner diameter, 50-cm long; ACE Glass) was interposed between the subluminal chamber and the collecting reservoir. A small air bubble was inserted into the glass tubing, and fluid flow across the endothelium induced movement of the bubble. The displacement of the air bubble was tracked and recorded by a computer-controlled spectrophotometer (Pergamon) and converted to volume flux (Jv) according to

\[ J_v = \frac{(\Delta d/\Delta t)F/A}{\Delta t} \]  \hspace{1cm} (1)

where Jv is volume flux, \((\Delta d/\Delta t)\) is the bubble displacement per unit time, F is the cross-sectional area of the glass tubing, and A is the area of the monolayer. The device output represents 5-min average Jv data; details on the device and on this method to measure Lp are provided in the study by Sill et al. (27). Assuming an absence of oncotic pressure gradient across the monolayer (identical medium was present on the luminal and abluminal chambers), Lp was calculated based on the hydrostatic pressure gradient (∆P, 10 cmH2O) as

\[ L_p = \frac{J_v}{\Delta P} \]  \hspace{1cm} (2)

After the 1-h equilibration period, the monolayers were subjected to an additional 1.5 h in MEM-0.5% HSA with or without a transendothelial pressure gradient (10 cmH2O); the pressure gradient was applied by lowering the collecting reservoir’s fluid level to a position 10 cm beneath the fluid level covering the monolayer (Fig. 1B). Isolated neutrophils (1 × 10⁶/ml) were added to the fluid above the monolayer and allowed to settle and interact for 15 min (neutrophil-to-endothelial cell ratio, 2:1), after which the monolayer was fixed (0.05% glutaraldehyde) and silver stained (2). Silver staining not only revealed the location of endothelial borders, but it also labels adherent neutrophils that have yet to migrate across the monolayer; transmigrated neutrophils are not stained by silver. Under epifluorescence illumination, glutaraldehyde-fixed neutrophils appeared autofluorescent, and neutrophils above the monolayer (adherent) and neutrophils below the monolayer (transmigrated) were enumerated by light microscopy. The sum of adherent and transmigrated neutrophils represented the total number of interacting neutrophils. Neutrophils that settled on the monolayer but failed to adhere (noninteracting) were not enumerated, since they were washed away during the fixation/rinsing process. By a switch to bright-field optics, the position of neutrophils below the monolayer could be confirmed by the lack of silver staining. Percent transmigration was calculated as follows: 100 (transmigrated)/(adherent + transmigrated).

In some cases, a NOS inhibitor, L-NAME, or its inactive enantiomer, d-NAME (final concentration, 100 μM each; Calbiochem, La Jolla, CA), was added to the MEM-0.5% HSA immediately after the HUVEC monolayers were placed in the chamber. In these experiments, the MEM-0.5% HSA medium above the monolayer was also replaced with fresh medium containing L-NAME or d-NAME just before the addition of the neutrophils.

To examine the effect of exogenous NO on neutrophil transmigration as well as adhesion, HUVEC monolayers growing on glass coverslips were treated with and without IL-1β (10 U/ml, 2.5 h) and then exposed to 1 mM diethylenetriamine/NO (DETA/NO) for 1.5 h before a rinsing. Because cells were grown on glass coverslips, instead of Snapwell inserts, it is possible to assess both neutrophil adhesion and transmigration using static adhesion chambers as described previously (2). Briefly, neutrophils (10⁶/ml) were injected into each chamber and allowed to settle and interact for 500 s. At this time, using phase contrast optics, the total number of contacting neutrophils (phase bright appearance) in three 40× fields was determined. The chamber was then inverted for an additional 500 s (to allow nonadherent neutrophils to detach) and then inverted once more so that the number of adherent (phase bright appearance) and the number of transmigrated neutrophils (phase dark appearance) could be enumerated; neutrophils change from phase bright to phase dark as they migrate across the endothelium. Percent adhesion was calculated as follows: 100 (adherent + transmigrated)/(total contact). Percent transmigration was calculated as follows: 100 (transmigrated)/(adherent + transmigrated).

Semiquantitative analysis of NO production by HUVEC monolayers. To assess the effect of transmural pressure (10 cmH2O) on endothelial NO release, the NO-sensitive dye 4,5-diaminofluorescein diacetate (DAF-2DA; Calbiochem) was added (2.5 μM in MEM-0.5% HSA containing 0.05% DMSO) 1 h after the IL-1β-treated HUVEC monolayer was placed in the chamber. Transendothelial pressure (10 cmH2O) was then applied for 1.5 h. Each experiment was paired with a second monolayer studied concurrently, from the same batch of endothelial cells, that was maintained for 1.5 h in the absence of transendothelial pressure. At the end of the experiment, the chamber was disassembled, and the polycarbonate filter supporting the monolayer was removed, placed on a glass slide, and mounted with a coverslip. DAF-2DA fluorescence levels were recorded on a Delta-Vision Spectris microscope system (Applied Precision, Issaquah, WA), and background (baseline) fluorescence levels (monolayers treated with MEM-0.5% HSA plus 0.05% DMSO alone) were digitally subtracted using SoftWorx image analysis software.
Electron microscopy. HUVEC monolayers growing on polycarbonate filters were fixed (2 h) in buffered (0.1 M sodium cacodylate, pH 7.2) 2.5% glutaraldehyde followed by treatment with 1% tannic acid (5 min, to enhance cell membrane appearance) and postfixation (1 h) in 1% osmium tetroxide. Monolayers were then dehydrated in ethanol, embedded in epoxy resin (Araldite), and sectioned (80-nm thick) transversely on an RMC MT7000 ultramicrotome. Sections were stained with uranyl acetate and lead citrate and viewed on a JEOL 200CX transmission electron microscope operating at 80 keV. Photomicrographs (negatives) were scanned into Adobe Photoshop, and endothelial cleft dimensions (length and width) were measured. To obtain accurate magnification values of specimen negatives, photomicrographs of a carbon diffraction grating were used for calibration.

Statistics. Data are expressed as means ± SE. Statistical analyses involved one-way analysis of variance followed by an appropriate posttest (Dunnell’s or Student Newman-Keuls) for multiple comparisons, or paired Student t-tests for two-group comparisons. Statistical significance was set at P < 0.05.

RESULTS

Morphometric data for IL-1β-treated HUVEC monolayers grown on polycarbonate filters are shown in Table 1. Cell size (e.g., perimeter = 115.0 ± 1.7 μm) and shape (e.g., no. of tricellular corners = 5.1 ± 0.1) are similar to our previously reported measurements of resting HUVEC monolayers grown on glass coverslips (cell perimeter = 142 ± 0.4 μm, and no. of tricellular corners = 5.3 ± 0.1) (2). The median Lp across HUVEC monolayers after IL-1β treatment (4 h) was 6.5 × 10⁻⁷ cm² s⁻¹ cmH₂O⁻¹ (n = 8), similar to our previously reported Lp values (5.3–6.8 × 10⁻⁷ cm² s⁻¹ cmH₂O⁻¹) for resting HUVEC monolayers (4). Hence, HUVEC monolayers grown on polycarbonate filters resemble cells grown on glass coverslips, and IL-1β-treatment (4 h) does not appear to alter baseline Lp.

Consistent with previous studies (2, 3), neutrophil migration across IL-1β-activated HUVEC grown on polycarbonate filters was very efficient. In the absence of transendothelial flow (no transendothelial pressure gradient), ~50–60% of the contacting neutrophils migrated across the endothelial monolayer (Fig. 2). However, in the presence of transendothelial flow (transendothelial pressure gradient of 10 cmH₂O), we observed an eightfold reduction in neutrophil transmigration. The pressure gradient had no effect on the total number of interacting (adherent + transmigrated) neutrophils (Fig. 3).

Table 1. Morphometric endothelial parameters for shear stress estimates

<table>
<thead>
<tr>
<th>Cell Parameter</th>
<th>IL-1β-Treated (4 h) HUVEC</th>
</tr>
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<tbody>
<tr>
<td>No. of tricellular corners (n = 55)</td>
<td>5.1 ± 0.1</td>
</tr>
<tr>
<td>Area, μm² (n = 91)</td>
<td>680.7 ± 16.4</td>
</tr>
<tr>
<td>Perimeter, μm (n = 91)</td>
<td>115.0 ± 1.7</td>
</tr>
<tr>
<td>Nonjunctional cleft width, nm (n = 114)</td>
<td>12.7 ± 0.4*</td>
</tr>
<tr>
<td>Total cleft depth, nm (n = 12)</td>
<td>1.200 ± 282.7</td>
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</tbody>
</table>

Data are means ± SE. HUVEC, human umbilical vein endothelial cells. *Cleft width not associated with endothelial tight junctions, adherens junctions, or gap junctions. For light microscopy measures (tricellular corners, area, and perimeter), n reflects the no. of cells analyzed from 6 monolayers. For electron microscopy cleft measures (width and depth), 12 clefts were analyzed from 2 monolayers. For cleft width, n reflects no. of measurements; for cleft depth, n reflects no. of clefts.

On the basis of a report by Tarbell et al. (30) showing that sustained step increases in transendothelial pressure are associated with a NO-dependent increase in endothelial Lp, and numerous reports showing that NO inhibits neutrophil binding to endothelial cells and suppresses transendothelial migration (13, 17, 25), we wished to determine whether NO plays a role in the diminished neutrophil transmigration that occurs in the presence of transendothelial pressure. Figure 2 shows that in

Fig. 2. Influence of transendothelial flow on neutrophil transendothelial migration and effects of a nitric oxide synthase (NOS) inhibitor. In the absence or presence of a transendothelial pressure gradient (10 cmH₂O, n = 12 per group), freshly isolated human neutrophils were added to IL-1β-activated HUVEC monolayers. In some cases, the endothelium was pretreated with L-NAME (an inhibitor of NOS) or D-NAME (an inactive enantiomer of L-NAME), and transendothelial migration was assessed (n = 6 per group). Data are shown as means ± SE. *P ≤ 0.05 for comparisons noted on the figure.

Fig. 3. Effect of transendothelial flow and a NOS inhibitor on neutrophil (polymorphonuclear neutrophil; PMN) adhesion to endothelium. Freshly isolated neutrophils were added to IL-1β-treated HUVEC monolayers in the absence or presence of a transendothelial pressure gradient (10 cmH₂O, n = 12 per group). In some cases, the endothelium was pretreated with L-NAME (an inhibitor of NOS) or D-NAME (an inactive enantiomer of L-NAME). The total no. of adherent + transmigrated neutrophils/mm² was determined (n = 6 per group). NS, not significant statistically.
the presence of L-NAME (100 μM), a NOS inhibitor, transendothelial pressure (10 cmH2O) had no inhibitory effect on neutrophil transmigration. The specificity of NOS inhibition was confirmed using D-NAME, an inactive enantiomer of L-NAME, which failed to prevent the reduction in neutrophil transendothelial migration that occurred in the presence of a transendothelial pressure gradient. The combination of the transendothelial pressure gradient plus L-NAME or D-NAME had no effect on the total number of neutrophils (adherent + transmigrated) interacting with the monolayer (Fig. 3). Thus the transendothelial pressure-induced decrease in neutrophil transmigration (Fig. 2) required NOS and was not a result of diminished neutrophil adhesion.

In other in vitro systems, increased transendothelial pressure can lead to reduced endothelial Lp. The tightening of the endothelial barrier under pressure has been termed the “sealing effect” (27, 29), and it could potentially contribute to reduced neutrophil transmigration. To exclude this possibility, we monitored the change in Lp following the application of a transendothelial pressure gradient (10 cmH2O; see Fig. 4). Over the course of 90 min, the sealing effect in these IL-1β-treated HUVEC monolayers was negligible and thus unlikely to be involved in the observed transendothelial pressure-induced reduction in neutrophil transmigration (Fig. 2).

Since it has also been reported that NO increases baseline human neutrophil adhesion to resting HUVEC monolayers (23), while inhibiting spontaneous neutrophil transendothelial migration (22), we attempted to determine whether NO alone, in the absence of transendothelial pressure, affects neutrophil adhesion and transmigration on IL-1β-activated HUVEC monolayers. The experimental condition employed involved culturing the HUVEC monolayers on glass coverslips (rather than polycarbonate membrane filters) and assessing neutrophil transmigration in a specially constructed static adhesion chamber. Although this chamber cannot be used to study the effects of transendothelial pressure, it is ideally suited to study the effects of exogenous NO on neutrophil interactions with endothelial cells, because neutrophil adhesion, in addition to transmigration, can be evaluated and expressed as a percentage of the number of neutrophils contacting the monolayer. Figure 5 shows that in the absence of transendothelial pressure, ~80% of the neutrophils adhered to the IL-1β-activated HUVEC monolayers, and ~50% of the adherent neutrophils migrated across the monolayer. Pretreatment of the IL-1β-activated HUVEC monolayers, but not neutrophils, with DETA/NO (a NO donor) significantly impaired neutrophil transmigration but had no effect on neutrophil adhesion. It is worth noting that the DETA/NO effect appears to be specific to the endothelium, since neutrophils treated with DETA/NO did not exhibit impaired transmigration. Additional experiments on resting (i.e., not treated with IL-1β) HUVEC monolayers confirmed that neutrophil transmigration did not occur, and adhesion was minimal (4.5 ± 1.3%). Moreover, neutrophil adhesion was unaltered (2.4 ± 0.4%) by pretreatment of the endothelium with DETA/NO. Thus DETA/NO treatment of IL-1β-activated HUVEC monolayers inhibits neutrophil transmigration, but not adhesion, through an endothelial-dependent mechanism.

To confirm that transendothelial flow upregulates endothelial NO production, IL-1β-activated HUVEC monolayers growing on polycarbonate membrane filters were subjected to a transendothelial pressure gradient (10 cmH2O) for 1.5 h. Following a staining with DAF-2DA, the resulting fluorescence emissions were recorded and used as an indication of NO expression levels. Figure 6 demonstrates that transendothelial flow significantly (P < 0.05) increased DAF-2DA fluorescence (indicative of NO production) 1.8-fold compared with IL-1β-activated monolayers not exposed to transendothelial flow.

Finally, we wished to determine whether transendothelial flow can inhibit neutrophil transmigration under conditions where the endothelium is also subjected to luminal (i.e., parallel) flow, as is the case in vivo. Figure 7 shows that transendothelial pressure (5 or 10 cmH2O) effectively inhibited neutrophil transendothelial migration on IL-1β-treated HUVEC monolayers subjected to venous levels of luminal shear stress (1 or 2 dyn/cm²). The magnitude of the inhibitory response was similar to that seen in the absence of luminal shear stress.

DISCUSSION

The results in this paper support the conclusion that neutrophil transendothelial migration is inhibited by transendothelial flow, and this inhibition is mediated by endothelial-derived NO. The data supporting these conclusions are as follows.

1) Transendothelial flow significantly reduced neutrophil trans-
transmigration was markedly inhibited by DETA/NO pretreatment. The physiological significance of this observation becomes apparent when one begins to examine the mechanism behind the inhibitory action of transendothelial pressure on IL-1β-stimulated neutrophil transmigration. Specifically, in the presence of a modest pressure gradient (10 cmH2O), DAF-2DA fluorescence (an indicator of NO production) increased (~2-fold), whereas neutrophil transmigration decreased (~4-fold). Importantly, pretreatment with the NOS inhibitor L-NAME prevented this pressure-induced reduction in neutrophil transmigration. Collectively, the observations demonstrate that NO is a key inhibitor of neutrophil transmigration, and transendothelial pressure is a physiological trigger that increases endothelial NO levels.

In vivo, endothelial cells are exposed to both surface flow and transendothelial pressure. Although there are numerous examples of in vitro studies that model leukocyte interactions with endothelial cells under conditions of hydrodynamic flow (e.g., parallel plate studies), very few have considered the effect of transendothelial pressure. Most of the previous in vitro studies that deal with pressure and its effect on the endothelium involve cells grown on nonporous substrates, so fluid filtration (transendothelial flow) is not present. This is a very different situation than our study and those of Tarbell and colleagues (7, 27, 30) and Dull et al. (9), where fluid filtration is driven by modest transmural pressure (e.g., 10 cmH2O). In our model, this pressure gradient resulted in a transendothelial flow per unit surface area (Jv/s) of 6.5 \times 10^{-6} \text{ cm/s}, corresponding to a median Lp of 6.5 \times 10^{-7} \text{ cm/s cmH2O}^{-1}. This value is comparable to the values of 5.3–6.8 \times 10^{-7} \text{ cm/s cmH2O}^{-1} that we reported previously for Lp of resting HUVEC (4) and to the values of 4 \times 10^{-7} \text{ cm/s cmH2O}^{-1} reported by Tarbell et al. (30) for bovine aortic endothelial cells. Furthermore, these Lp values are relevant, as they are in the same order of magnitude as those found in rat mesenteric venules in vivo (26).

In the present study, the data reveal that a modest transendothelial pressure gradient had a pronounced inhibitory effect on neutrophil transmigration. Because the endothelial cells in this study were cultured on a porous polycarbonate filter, the 10-cmH2O pressure gradient resulted in a measurable transmigrational flow of 6.5 \times 10^{-6} \text{ cm/s}, which is driven by modest transmural pressure (e.g., 10 cmH2O). In vivo, endothelial cells are exposed to both surface flow and transendothelial pressure. Although there are numerous examples of in vitro studies that model leukocyte interactions with endothelial cells under conditions of hydrodynamic flow (e.g., parallel plate studies), very few have considered the effect of transendothelial pressure. Most of the previous in vitro studies that deal with pressure and its effect on the endothelium involve cells grown on nonporous substrates, so fluid filtration (transendothelial flow) is not present. This is a very different situation than our study and those of Tarbell and colleagues (7, 27, 30) and Dull et al. (9), where fluid filtration is driven by modest transmural pressure (e.g., 10 cmH2O). In our model, this pressure gradient resulted in a transendothelial flow per unit surface area (Jv/s) of 6.5 \times 10^{-6} \text{ cm/s}, corresponding to a median Lp of 6.5 \times 10^{-7} \text{ cm/s cmH2O}^{-1}. This value is comparable to the values of 5.3–6.8 \times 10^{-7} \text{ cm/s cmH2O}^{-1} that we reported previously for Lp of resting HUVEC (4) and to the values of 4 \times 10^{-7} \text{ cm/s cmH2O}^{-1} reported by Tarbell et al. (30) for bovine aortic endothelial cells. Furthermore, these Lp values are relevant, as they are in the same order of magnitude as those found in rat mesenteric venules in vivo (26).

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endothelial flow. Tarbell et al. (30) proposed that transendothelial flow of a similar magnitude resulted in significant endothelial cleft shear stress, with magnitudes similar to estimates of shear stress imposed by blood flow across the luminal endothelial surface (19). Since luminal shear stress stimulates NO release from cultured cells (7, 14, 24), it was reasonable to hypothesize that shear stress within the endothelial cleft also triggers NO production (30).

Taking the concept of endothelial cleft shear stress one step further, we propose that cleft shear stress-induced NO release accounts for inhibition of neutrophil transmigration by transendothelial flow in our model. On the basis of the ultrastructural data outlined in Table 1 and our measures of volume flux, we estimated endothelial cleft shear stress using two mathematical models of endothelial cleft structure, assuming that transendothelial flow is exclusively paracellular. Full details of this and other assumptions and limitations of these models have been published (8, 21, 30). Following is a brief description of shear stress estimates in our system, using a similar approach as that reported by Tarbell et al. (30).

Parallel slits. This model assumes that laminar flow of a Newtonian fluid occurs through long rectangular slits between parallel walls lining the intercellular clefts. In this model, shear stress ($\tau$) is calculated as

$$\tau = \frac{3\mu}{W}J_{v}(A/A_{cleft})$$

(3)

where $\mu$ is viscosity (we used 0.0069 dyn·s·cm$^{-2}$ for water at 37°C), $W$ is endothelial cleft half-width, $J_{v}$ is volume flux (measured at 6.5 × 10$^{-6}$ cm/s), $A$ is endothelial area, and $A_{cleft}$ is endothelial cleft area. The cleft area for rectangular slits is calculated as the product of cleft length (or cell perimeter) and cleft width ($2W$), as reviewed by Michel and Curry (21). However, to calculate cleft area as a function of endothelial area in a monolayer ($A_{cleft}$), we used cell perimeter times cleft half-width ($W$), since two adjacent endothelial cells share the same cleft area. This cleft area analysis does not account for the center of the corners where three endothelial cells meet (i.e., tricellular corners). Walker et al. (31) reported the width of that region to be 27.4 nm, using freeze fracture on pulmonary capillary endothelial cells. With that value for tricellular corner width and the assumption that each cell has five such corners (2), the estimated area of the corners is negligible, at <0.5% of the calculated total cleft area. Thus the use of $A_{cleft}$, as described above, and the substitution of the other parameters into Eq. 3 result in an estimated shear stress of 198 dyn/cm$^2$ with this model.

Another method to estimate shear stress with the parallel slit model depends on cleft morphometry and net pressure gradient and is independent of measured volume flux. In this calculation, shear stress is calculated as

$$\tau = \frac{\Delta P}{L}W$$

(4)

where $\Delta P$ is the hydrostatic pressure gradient across the cleft, $L$ is the cleft depth, and $W$ is cleft half-width as defined earlier. Assuming 10 cmH$_2$O as the pressure gradient across the cleft, and substituting $L$ and $W$ from Table 1, we estimated shear stress with Eq. 4 to be 49 dyn/cm$^2$.

Fiber matrix slit model. This model assumes that the slit is occupied by a fiber matrix array, consisting of glycosaminoglycan fibers, presumably associated with the endothelial cell surface glyocalyx and possibly the interendothelial cleft; detailed descriptions of this model are provided elsewhere (8, 21). To estimate cleft shear stress with this model, we applied the parameters used by Tarbell et al. (30), including fiber radius ($a_i$) of 0.6 nm, fiber spacing ($\Delta_i$) of 7 nm, and calculated specific hydraulic conductance ($K_p$) as

$$K_p = 0.0572a_i^2(\Delta/a_i)^{2.777}$$

(5)

Shear stress is calculated as

$$\tau = J_v(\mu/K_p)(A/A_{cleft})$$

(6)

Substitution of parameters, as done earlier, yielded an estimated shear stress of 157 dyn/cm$^2$ with the fiber matrix model.

The models described above, while simplified, offer relevant estimates of the potential magnitude of interendothelial cell shear stress induced by a modest pressure gradient. More elaborate models, combining aspects of the glyocalyx and cleft structure, are reviewed in the work by Michel and Curry (21). Of interest, recent work by Dull et al. (9) on bovine lung microvascular endothelial cells demonstrates that heparan sulfates mediate hydrostatic pressure gradient-induced increase in L$_p$ via a NO-dependent mechanism, suggesting a role for the endothelial glyocalyx as mechanotransducer of those responses. Conceivably, the endothelial glyocalyx may mediate the transendothelial flow-induced inhibition of neutrophil transmigration outlined in this study, although this remains to be demonstrated. A greater understanding of endothelial glyocalyx organization relative to interendothelial clefts under normal and inflammatory conditions is expected to provide additional insight.

The range of estimates of cleft shear stress in this study (49–198 dyn/cm$^2$) is comparable to values calculated with Eqs. 3–6, using the parameters reported by Tarbell et al. (30) on bovine aortic endothelial cells (40–98 dyn/cm$^2$) exposed to the same transendothelial pressure gradient of 10 cm H$_2$O. This analysis supports the notion that a modest transendothelial pressure gradient results in significant shear stress through the endothelial cleft. Cleft shear stress induced by transendothelial volume flow has received much less attention in the literature than shear stress on the endothelial surface induced by blood flow. Of interest, most published studies with cultured endothelium exposed to “venous” flow use a surface shear stress of $\sim$2 dyn/cm$^2$ (15, 16), an order of magnitude less than our lower estimates of cleft shear stress. In vivo, endothelial cells are typically exposed to both blood flow (surface shear stress) and transendothelial volume flow (cleft shear stress). Our data and those of Tarbell et al. (30) demonstrate that endothelial cells are responsive to transendothelial flow. Furthermore, we now show that the responses to transendothelial flow are present in the setting of luminal flow. Thus it is important to consider cleft shear stress as a relevant physical stimulus, in addition to surface shear.

Our analysis of the effects of transendothelial pressure has focused on transendothelial volume flow and the resultant cleft shear stress. Another potential effect of pressure is endothelial deformation induced by the hydrostatic pressure column on the endothelial cell surface. However, it is unlikely that this mechanism accounts for our results, since the endothelial chamber design (see Fig. 1) results in only 10% of the pressure gradient (i.e., 1 cm H$_2$O) applied to the endothelial cell surface. Simi-


