Differential effects of histamine and thrombin on endothelial barrier function through actin-myosin tension

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Moy, Alan B., Ken Blackwell, and Anant Kamath. Differential effects of histamine and thrombin on endothelial barrier function through actin-myosin tension. Am J Physiol Heart Circ Physiol 282: H21–H29, 2002.—We compared temporal changes in isometric tension in cultured human umbilical vein endothelial cells inoculated on a polymerized collagen membrane with changes in cell-cell and cell-matrix adhesion derived by a mathematical model of transendothelial cell resistance. Thrombin and histamine disrupt barrier function by targeting a greater loss in cell-cell adhesion, which preceded losses in overall transendothelial resistance. There were minor losses in cell-matrix adhesion, which was temporally slower than the decline in the overall transendothelial resistance. In contrast, thrombin and histamine restored barrier function by initiating a restoration of cell-matrix adhesion, which occurred before an increase in overall transendothelial resistance. Thrombin mediated a second and slower decline in cell-cell adhesion, which was not observed in histamine-treated cells. This decline in cell-cell adhesion temporally correlated with expressed maximal levels of tension development, suggesting that actin-myosin contraction directly strains cell-cell adhesion sites. Pretreatment of cells with ML-7 mediated more rapid recovery of cell-cell adhesion and had no effect on cell-matrix adhesion. Taken together, expression of actin-myosin contraction affects the restoration of barrier function by straining cell-cell adhesion sites.

Electrical resistance; analytic modeling

Edemagenic agents alter endothelial barrier function by remodeling centripetal-based cytoskeletal forces, which increase paracellular permeability. Additionally, these same edemagenic agents also activate the expression of centrifugal-based cytoskeletal forces, which promote the restoration of barrier function and limit the extent of inflammatory edema (12). A dynamic balance between centripetal and centrifugal forces at any moment in time defines the state of barrier function. The precise cytoskeletal elements and the mechanisms that regulate centripetal- and centrifugal-based forces are not well understood.

Majno et al. (7, 8) originally hypothesized that inflammatory mediators increased paracellular permeability by contraction of the actin-myosin cytoskeleton. Earlier reports (16, 22, 23) documented cell retraction by inducing actin-myosin contraction in permeabilized cultured endothelial cells exposed to low micromolar levels of calcium and ATP and myosin light chain kinase (MLCK). These reports demonstrated that, under these in vitro conditions, contraction of actin-myosin generates sufficient force to pull adjacent cells apart from each other. However, edemagenic stimuli typically increase cell calcium to nanomolar levels (3, 15), an issue that has challenged the notion that edemagenic agents disrupt barrier function through contraction of actin-myosin cross-bridges.

To precisely evaluate the contribution of actin-myosin contraction on barrier function in response to physiological stimuli, myosin ATPase activity needs to be quantitated in real time and correlated with an equally quantitative and dynamic measurement of cell adhesion. Measured levels of myosin light chain phosphorylation have been used to assess myosin ATPase activity. However, myosin ATPase activity is governed by the cooperative interaction between myosin heads, which increases exponentially once one myosin head is phosphorylated (13). Thus the relationship between MLC phosphorylation and myosin ATPase activity is nonlinear and represents an indirect and inaccurate measurement of myosin ATPase activity. Others have measured endothelial force by measuring the amount of wrinkling exerted by endothelial cells on a deformable silicone substratum (2, 9). Freely movable cell-collagen lattices in solution have also been used to measure endothelial contraction by measuring the reduction in the size of the lattice over periods of hours to days (5, 18). However, these assays suffer from significant limitation by being qualitative or incapable of dynamically and quantitatively measuring constitutive changes in myosin ATPase activity under steady-state conditions or in response to physiological or pharmacological stimuli.

To precisely understand how contractile forces disrupt barrier function, dynamic and quantitative measurements of cell adhesion are required. Traditional techniques of measuring voltage clamp, as previously reported in cultured epithelial cells (17, 19), lack the resolution to measure the small transendothelial resistances...
tance in cultured endothelial monolayers. Permeability assays lack the spatial and temporal resolution to measure endothelial-cell adhesion dynamically and quantitatively because of its dependence on diffusion or transport of macromolecules. To resolve these problems, we (10, 12) previously reported techniques that dynamically quantitate contractile forces by measuring isometric tension on collagen hydrogels and techniques that dynamically quantitate cell adhesion by measuring transendothelial resistance in cultured endothelial cells grown on a microelectrode.

We (12) previously reported that histamine and thrombin disrupt barrier function independent of contraction of the actin-myosin cytoskeleton. Thrombin is a particularly novel edemagenic molecule because it disrupts barrier function in association with contraction of the actin-myosin cytoskeleton (12). In contrast, histamine disrupts barrier function independent of active contraction of the actin cytoskeleton (12). On the basis of their differences on contractility, these mediators provide a useful strategy to understand the contribution of actin-myosin contraction on barrier function in the setting of edemagenic stimuli. We (10, 12) previously reported that thrombin mediates a more sustained decline in transendothelial resistance than histamine. Tension development is not necessary for the initial loss in cell adhesion. Tension development was temporally out of phase with overall changes in transendothelial resistance in thrombin-treated endothelial cells. At time points of maximal force expression, barrier function was well on its way toward being restored to its basal state, and at time points at which barrier function was restored to baseline conditions, centripetal force was still greatly expressed. Yet, inhibition of thrombin-mediated tension development accelerated the restoration of barrier function. Taken together, inflammatory edema formation is clearly more complex than simple contraction of the actin-myosin cytoskeleton, and such contraction potentially affects the restoration, not the initial disruption, in barrier function. The next important question is how actin-myosin contraction affects the restoration of barrier function even though force generation does not temporally correlate with changes in transendothelial resistance.

Transendothelial resistance across a cell-covered electrode is a complex measurement dependent, in a large part, on cell-cell and cell-matrix adhesion (11). Thrombin-mediated force generation could affect the restoration of barrier function by straining cell-cell or cell-matrix adhesion. We recently validated an analytical approach that simultaneously compares the change in cell-cell and cell-matrix adhesion as it temporally changes with overall transendothelial resistance. This technique allows a more precise understanding of how changes in cell-cell and cell-matrix adhesion contribute to overall changes in barrier function (11). With the use of this approach, we were able to demonstrate that histamine transiently disrupts barrier function by first disrupting cell-cell adhesion but restores barrier function, in part, through direct effects on cell-matrix adhesion. We now use this analytical approach to evaluate how actin-myosin contraction delays the restoration of barrier function in response to thrombin. In this study, we demonstrate that actin-myosin contraction delays the restoration of barrier function by straining cell-cell adhesion sites.

**MATERIALS AND METHODS**

**Cell cultures.** Cultured human umbilical vein endothelial cells (HUVECs) were prepared by collagenase treatment of freshly obtained human umbilical veins as described (3). Harvested primary cultures designated for cell adhesion assays were plated on 60-mm tissue culture plates, which were coated with fibronectin (Collaborative Research; Bedford, MA). All cells were cultured in medium 199 and supplemented with 20% heat-inactivated fetal calf serum, basal medium Eagle vitamins and amino acids, glucose (5 mM), glutamine (2 mM), penicillin (100 μg/ml), and streptomycin (100 μg/ml). Cells were identified as endothelial cells by their characteristic uniform morphology and uptake of acetylated low-density lipoprotein and by indirect immunofluorescent staining for factor VIII. Cytochalasin D and histamine were obtained from Sigma (St. Louis, Mo). Human α-thrombin was obtained from Enzyme Research Laboratories (South Bend, IN). Vitrogen collagen (type 1, bovine dermal collagen) was obtained from Celsrix Pharmaceuticals (Santa Clara, CA). ML-7 was obtained from Calbiochem (La Jolla, CA).

**Cell adhesion assays.** Quantitative measurements in cell adhesion were measured in real time by quantitating transendothelial resistance using a previously reported technique in a 1-day-old postconfluent monolayer of cultured endothelial cells grown on the surface of microelectrodes coated with 100 μg/ml fibronectin (10–12). In this system, cells were inoculated on a small gold electrode (5 × 10^−4 cm^2) using culture medium as the electrolyte, and barrier function was measured dynamically by determining the electrical impedance of the cell-covered electrode. The total impedance of the monolayer is composed of the impedance between the ventral surface of the cell and the electrode, the impedance between the cells, and the impedance of the cell membranes, which is dominated by the membrane capacitance (C_m) (4). A 1-V, 4,000-Hz alternating current signal was supplied through a 1-MΩ resistor to approximate a constant current source. Voltage and phase data were measured with a SR580 lock-in amplifier (Stanford Instruments) and then later stored and processed with a personal computer. The in-phase voltage (proportional to the resistance) and the out-of-phase voltage (proportional to the capacitive reactance) were measured. We expressed cell adhesion as a function of resistance, which was expressed as a fractional change to the initial value at the moment test agents were given. Both histamine and thrombin mediate a transient decline in the fractional resistance. As a convention, we refer in the text to the disruption of barrier function as the negative slope of the overall transendothelial resistance, whereas restoration of barrier function refers to the positive slope of the overall transendothelial resistance.

**Mathematical model to resolve the experimental resistance into changes in cell-cell and cell-matrix adhesion.** We used a previously derived mathematical model to calculate specific cell-cell and cell-substrate adhesion (4, 11). The model is based on the notion that current flows radially from the ventral surface of the cell and the electrode, escapes between cells, and goes directly through the cell membrane by capacitive coupling. In this model, the total impedance across a cell-covered electrode is composed of the impedance
between the ventral surface of the cell and the electrode (cell-matrix resistance ($\alpha$)), the impedance between cells (cell-cell resistance ($R_b$)), the transcellular impedance ($Z_m$), and the impedance of a naked electrode ($Z_n$). For these calculations, the cells are regarded as circular disks and $Z_m$ is related to $C_m$, $\alpha$ can also be defined as follows: $R_c \sqrt{\rho h}$, where $R_c$ is the cell radius, $\rho$ is the resistivity of the medium, and $h$ is the average separation distance between the cell and the underlying matrix (4).

Because $Z_n$ and $Z_m$ are measured and $Z_n$ is the impedance of the two cell membranes in series, $\alpha$, $R_b$, and $C_m$ are the only adjustable parameters in the model. We first identified the values of $\alpha$, $R_b$, and $C_m$ before the addition of test agents. $\alpha$ and $R_b$ cannot be explicitly solved because they are dependent on modified Bessel functions and thus have to be derived by curve fitting. First, $Z_c$ and $Z_w$ were measured as a function of current frequency in untreated cells. By iteratively choosing values for $C_m$, $R_b$, and $\alpha$, we can arrive at the best fit to the experimental impedance. Because resistance is also dependent on the same values for $C_m$, $R_b$, and $\alpha$ as they are for impedance or reactance, $C_m$, $R_b$, and $\alpha$ were measured by finding the best fit of the calculated resistance to the experimental resistance.

We expressed barrier function from the experimental resistance rather than impedance for two reasons. First, physiological stimuli mediate greater effects on resistance than on impedance. Second, by measuring resistance, the membrane resistance of $Z_m$ is trivial because the model considers the membrane resistance and capacitance as a parallel circuit. The resistance was measured at 13 separate frequencies between 22 and 90,000 Hz and expressed as the ratio of normalized resistance of a cell-covered to cell-free electrode. Real-time changes in cell-cell adhesion ($R_b$) and cell-matrix adhesion ($\alpha$) in response to thrombin or other interventions were determined at a single frequency of 4,000 Hz.

**Measurement of isometric tension.** Isometric tension of cultured endothelial cells was measured as described by Bodmer et al. (1). Isometric tension was simultaneously monitored in two separate isometric vectors because endothelial cells express tension multiaxially (1). Force was averaged for both vectors and expressed as the fractional change of the maximal force generation in the absence of ML-7 or expressed as the fractional change of the constitutive force in the presence of ML-7.

**RESULTS**

**Relationship between thrombin-mediated tension development and transendothelial resistance.** To elucidate the complex interaction between force generation and barrier function in cultured endothelial cells, we first compared the temporal relationship between centripetal tension and overall transendothelial resistance. To measure centripetal tension, we measured isometric tension in postconfluent cultured endothelial cells inoculated on a polymerized type-1 collagen membrane. To dynamically measure endothelial barrier function, we monitored transendothelial resistance in 1-day-old postconfluent cultured HUVECs grown on a microelectrode. As shown in Fig. 1, 7 U/ml ($6 \times 10^{-8}$ M) thrombin immediately decreased transendothelial resistance before any significant increase in isometric tension. Thrombin maximally decreased barrier function within 60–90 s, whereas tension development was maximally expressed within 5–10 min. Furthermore, at time points at which thrombin maximally expressed tension, the restoration of barrier function was already initiated. Additionally, at time points at which barrier function had recovered to initial basal levels, force was still expressed at 50% of its maximal level. Thus temporal changes in force generation were out of phase with changes in overall barrier function, demonstrating that actin-myosin contraction was not sufficient to account for the disruption in transendothelial resistance or the overall change in barrier function at any moment in time.

**Effect of thrombin and histamine on endothelial cell-cell and cell-matrix adhesion.** Because force did not temporally correlate with overall transendothelial resistance, we examined whether force generation better correlated with local changes in cell adhesion. To elucidate the regions at which thrombin altered cell adhesion, we broke down transendothelial resistance into separate measurements of cell-cell and cell-matrix adhesion. We accomplished this approach by modeling transendothelial resistance as a circuit composed of a resistor and capacitor in series using a method we previously reported (11). This model is a function of $R_b$, which is dependent on cell-cell adhesion, and $\alpha$, which is dependent on cell-matrix adhesion. The utility of the model is its ability to predict the sites at which biological molecules alter barrier function.

With the use of an optimization approach that we previously described (11), thrombin mediated characteristic differences on $\alpha$ and $R_b$ during the disruption and restoration phase of barrier function (Fig. 2). During the initial disruption phase of barrier function, thrombin mediated rapid declines in both $R_b$ and $\alpha$, but with a greater fractional decline in $R_b$ (Fig. 2A). When
the same data are more closely examined over the first 60 s, the decline in $R_b$ preceded the decline in overall resistance (Fig. 2B). In contrast, thrombin mediated a decline in $\alpha$ that was slower and quantitatively less than the decline in the overall resistance. Taken together, the model predicts that thrombin initiates the disruption in barrier function by first and specifically disrupting cell-cell adhesion sites. However, thrombin decreased $\alpha$ either through a reactant loss of cell-cell adhesion, or the loss in cell-matrix adhesion is the target of signal transduction pathways, which proceed slower than the loss of cell-cell adhesion. Alternatively, thrombin decreased cell-matrix adhesion through both mechanisms.

In contrast, the restoration in overall barrier function appeared dependent on effects on cell-matrix adhesion (Fig. 2A). Increases in $\alpha$ preceded increases in overall transendothelial resistance. In contrast, after the initial rapid decline in $R_b$, thrombin mediated a second decline in $R_b$, at time points at which overall resistance was recovering. Interestingly, the rate of the second decline in $R_b$ was much slower than that of the initial decline in $R_b$. After a period of 10–15 min, thrombin mediated a restoration in $R_b$ that took several minutes to return to initial basal levels. These data suggest that mechanical effects directed at cell-matrix adhesion sites, not those directed at cell-cell adhesion sites, initiated the recovery phase of barrier function in response to thrombin.

There are some important similarities between the effects of histamine and thrombin on $\alpha$ and $R_b$ worth emphasizing. As reported previously (11) and again reported here in separate experiments (Fig. 3), histamine disrupted barrier function by disrupting $R_b$. Also, histamine initiated recoveries in $\alpha$ before recoveries in resistance, whereas the recovery in $R_b$ was temporally slower than the recovery of overall resistance. Taken together, both histamine and thrombin disrupt barrier function by rapidly disrupting cell-cell adhesion but initiate the recovery in barrier function through effects on cell-matrix interaction.

However, there were important differences in cell mechanics between histamine and thrombin. First, thrombin mediated a much greater fractional decline in $R_b$ than that observed in histamine-treated cells (Fig. 4A). Second, thrombin mediated a second and slower decline in $R_b$, which was not observed in histamine-treated cells. Third, thrombin mediated a greater decline in $\alpha$ in thrombin-treated cells than that observed in histamine-treated cells (Fig. 4B). Fourth, the restoration in $\alpha$ occurred more promptly in histamine-treated cells compared with thrombin-stimulated cells. Taken together, these data suggest that there may be common signal transduction mechanisms between histamine and thrombin that govern the disruption and restoration of barrier function. Yet, there may be different mechanisms between histamine and thrombin that may explain their quantitative and temporal differences on barrier function.

Correlation between tension development and cell-cell and cell-matrix adhesion. To elucidate how tension development affects the restoration of thrombin-mediated barrier dysfunction, we compared the temporal relationship between tension development and changes

![Fig. 2. A: effect of 7 U/ml thrombin on the fractional change in the overall resistance, cell-cell resistance ($R_b$), and cell-matrix resistance ($\alpha$) in 1-day-old postconfluent cultured HUVEC. Tracings represent the means ± SE of ≥5 separate experiments. B: data displayed in A for the first 60 s. See text for explanation.](http://ajpheart.physiology.org/)

![Fig. 3. Fractional change in overall transendothelial resistance, $\alpha$, and $R_b$ in 1-day-old postconfluent HUVEC exposed to 10 μM histamine. Tracings represent the average values at time points of 5 separate experiments. See text for explanation.](http://ajpheart.physiology.org/)
thrombin-treated cells exposed to ML-7 compared with cells not exposed to ML-7. Additionally, the second and slow decline in $R_b$ that was observed in cells stimulated with thrombin alone was absent in thrombin-stimulated cells that were pretreated with ML-7. Taken together, these data suggest that expression of actin-myosin tension development under thrombin-stimulated condition affects the recovery phase of barrier function by straining cell-cell adhesion sites.

On the basis of the predictions of the model, thrombin mediates several distinct centripetal and centrifugal forces that are either contractile dependent or contractile independent and are expressed at different time points during the disruption and the restoration phase of barrier dysfunction (Fig. 7). Thrombin disrupts barrier function through expression of contractile-independent centripetal forces, which are directed to a greater degree at cell-cell adhesion sites and to a

\[ \text{Fig. 5. A: temporal relationship between isometric tension and fractional change (of the initial resistance upon the addition of thrombin) in } \alpha \text{ and } R_b \text{ in cultured HUVEC stimulated with 7 U/ml thrombin. Tension is expressed as the fractional change of the maximal force. A 100\% change of the maximal force is equivalent to } \sim 100\% \text{ of the constitutive force. } B: \text{ temporal relationship between isometric tension and fractional change in } \alpha \text{ and } R_b \text{ in thrombin-treated HUVEC treated with 100 } \mu M \text{ ML-7. Tension is expressed as the fractional change of the constitutive force. The means } \pm \text{ SE are plotted for } \sim 5 \text{ separate experiments. See text for explanation.} \]
lesser degree at cell-matrix adhesion sites, consistent with the paradigm in histamine-stimulated cells. In contrast, the recovery phase of thrombin-mediated barrier dysfunction is dependent on several competing centripetal and centrifugal forces that are both contractile dependent and independent. Thrombin mediates a centripetal force directed at cell-cell adhesion sites that is contractile dependent. This centripetal force contributes to the more prolonged decline in transendothelial resistance. Thrombin then activates centrifugal forces, first at cell-matrix adhesion sites, and later at cell-cell adhesion sites, which are contractile independent and overcome all centripetal forces to restore barrier function to its initial basal state.

DISCUSSION

The precise contribution of actin-myosin contraction on barrier function under edemagenic stimuli is not well understood. Exogenous administration of MLCK and calmodulin induces endothelial-cell retraction in permeabilized endothelial cells (22). Exposure of permeabilized endothelial cells to low micromolar calcium levels induces cell retraction through a myosin-dependent pathway (16). These reports have been the basis of extrapolating the hypothesis that edemagenic stimuli induce cell retraction through activation of signal transduction pathways that increase cell calcium and activate MLCK-dependent centripetal tension development. However, there are two major criticisms of these models in extrapolating the role of actin-myosin contraction under physiological conditions. First, the plasma membrane is stripped away by detergent treatment, which removes the ability to measure signal transduction in intact cells in response to receptor-ligand interactions. Second, the dose of calcium and ATP used in permeabilized models is typically higher than that observed in intact cells in response to physiological or pharmacological stimuli.

In contrast, the contribution of actin-myosin contraction in governing barrier function in intact cells in response to edemagenic stimuli is comparably much different. We (12) previously reported that edemagenic agents such as histamine and thrombin disrupt barrier function independent of expressed actin-myosin contraction. Second, barrier function is fully restored to initial basal conditions at time points at which significant levels of force are still expressed in thrombin-treated cells. Clearly, actin-myosin contraction is not sufficient to account for all changes in endothelial barrier function. However, thrombin-mediated actin-myosin contraction contributes to a more sustained decline in barrier function compared with histamine-treated cells. Thus actin-myosin contraction expressed under physiological conditions affects the restoration phase of barrier function, not the disruption phase of barrier dysfunction. In these series of experiments, we demonstrated that expression of actin-myosin contraction modulates the restoration phase of thrombin-mediated barrier by disrupting cell-cell adhesion.
To elucidate the temporal and spatial effects by which actin-myosin contraction regulates barrier function, we temporally compared the effect of force generation on cell-cell and cell-matrix adhesion in thrombin-stimulated HUVEC. With the use of a previously described analytical model (11), we reported that histamine decreased barrier function by first disrupting cell-cell adhesion but restored barrier function by first engaging cell-matrix interaction. Histamine induced a reactive loss in cell-matrix adhesion through the mechanical coupling between cell-cell and cell-matrix sites by an intervening filamentous cytoskeleton. In the present study, thrombin disrupts barrier function in a manner similar to histamine except that it additionally disrupts cell-cell adhesion by expressed actin-myosin contractile forces.

The analytical approach is based on the mathematical modeling of a cell-covered electrode as a circuit composed of a resistor and capacitor in series that breaks down transendothelial resistance into separate resistance measurements that are dependent on cell-cell adhesion ($R_b$) and cell-matrix adhesion ($\alpha$). This approach provides several advantages over other methodologies. First, simultaneous and separate measurements of cell-cell and cell-matrix adhesion can be compared within the context of overall transendothelial resistance or barrier function. This is particularly necessary to evaluate the response to physiological or pharmacological stimuli that have a very rapid onset of action. Second, the approach can resolve spatial, temporal, and quantitative effects on cell-cell and cell-matrix adhesion.

Thrombin mediated distinct spatial and temporal effects in cell adhesion during the disruption and the restoration phase of barrier dysfunction. During the disruption phase, thrombin mediated rapid declines in cell-cell and cell-matrix adhesion, with much greater quantitative effects on cell-cell adhesion than on cell-matrix adhesion. Additionally, thrombin mediated different temporal effects in cell-cell and cell-matrix adhesion during the disruption of barrier function. Decreases in cell-cell adhesion preceded the loss of overall resistance. In contrast, loss of cell-matrix adhesion was temporally slower than the loss in overall transendothelial resistance. Taken together, these data suggest that thrombin disrupts barrier function by first and predominately targeting cell-cell adhesion sites. The loss of cell-matrix adhesion may be explained by three potential mechanisms. First, the decline in cell-matrix adhesion is a reactive effect in response to a primary decline in cell-cell adhesion. This is conceivable if cell-cell adhesion is mechanically coupled to cell-matrix adhesion sites by an intervening filamentous cytoskeleton. Alternatively, thrombin could specifically target the loss of cell-matrix adhesion but at a rate slower than that of cell-cell adhesion. Finally, the loss of cell-matrix adhesion could reflect the combination of both mechanisms.

Although the initial loss in $\alpha$ and $R_b$ in thrombin-stimulated cells showed temporal similarities with responses in histamine-treated cells, there were quantitative differences in these changes. Thrombin caused greater decreases in both $R_b$ and $\alpha$ in cultured monolayers than did histamine. The mechanisms for these differences between histamine and thrombin cannot be resolved by these studies. Others (20) have reported that histamine transiently decreased transcellular resistance in ECV304 cells transfected with cadherin-5 or E-cadherin. Additionally, we (11) previously reported similar quantitative decreases in overall resistance and $R_b$ between cells exposed to histamine and cells treated with an antibody that disrupts homotypic cadherin binding. Although the above reports support the notion that histamine disrupts barrier function by targeting the cadherin complex, there are some important differences between these two interventions. Histamine transiently creates intracellular gaps on the order of 80–100 nm between adjacent cells, which are below the resolution of light microscopy and scanning electron microscopy (11). Intercellular gaps induced by anti-cadherin antibodies produce irreversible and large gaps that are of several orders of magnitude greater than those seen with histamine (11). Taken together, these data suggest that histamine must activate other elements, other than cadherin, to mediate reversible reapposition at sites of cell-cell contact.

The mechanism of how thrombin disrupts barrier function through effects on cell-cell adhesion sites is not clear. Others (6) have reported altered distribution of cadherin staining in thrombin-stimulated endothelial cells. Ratcliffe et al. (14) reported reduced serine and threonine dephosphorylation of catenin (14). Ukopec et al. (18a) reported Src homology 2 phosphatase (SHP2) tyrosine phosphorylation and dissociation from vascular endothelial-cadherin complexes. The loss of SHP2 from the cadherin complexes correlated with increased tyrosine phosphorylation of $\beta$-catenin, $\gamma$-catenin, and p120-catenin (18a). The functional relevance of these morphological and biochemical changes on the cadherin-catenin system remains unclear. Also, the reason why thrombin mediates greater declines in $R_b$ and in the overall measured transendothelial resistance than histamine cannot be resolved from our present work.

Our model suggests several unique centrifugal and centripetal forces were expressed during the restoration phase in thrombin-treated cells in a temporal-dependent fashion. Some of these centripetal and centrifugal effects on cell adhesion overlap with responses observed in histamine-treated cells. In particular, the restoration in overall transendothelial resistance was preceded by increases in $\alpha$, which is consistent with the notion that direct effects at cell-matrix sites facilitate the restoration of barrier function. This notion is even more supported by the responses in thrombin-treated cells. Only increases $\alpha$ paralleled and preceded increases in the overall transendothelial resistance at time points at which $R_b$ was decreasing. Although the model predicts that direct effects on cell-matrix adhesion initiate the restoration phase of barrier function in thrombin and histamine-treated cells, the rate of re-
covery of cell-matrix adhesion was slower with thrombin than with histamine. Nonetheless, taken together, these data suggest that histamine and thrombin share common signaling mechanisms by which they transiently disrupt barrier function through a sequential loss of cell-cell adhesion and restore barrier function through effects on cell-matrix adhesion, followed by reapposition of cell-cell adhesion.

The most striking difference between histamine and thrombin during the restoration phase was the observed second and slower, or more prolonged, decline in $R_b$. Compared with changes in isometric tension, this second and maximal decline in $R_b$ temporally correlated with maximal increases in force generation in thrombin-stimulated cells. Consistent with the notion that this second decline in cell-cell adhesion was coupled to expressed actin-myosin tension, this phase was abolished by ML-7. Additionally, ML-7 accelerated the recovery phase of $R_b$. ML-7 did not alter $\alpha$ in any quantitative or temporal manner. This data suggests that expression of actin-myosin contraction under thrombin-stimulated conditions delayed the restoration of barrier function by specifically straining cell-cell adhesion sites while it had little effect in altering cell-matrix adhesion.

The impact of force generation on cell-cell adhesion is particularly remarkable because it suggests that the mechanical behavior of the cytoskeleton needs to be considered as a three-dimensional integrated mechanical system in which cell-cell adhesion sites are mechanically coupled to cell-matrix adhesion sites by an intervening filamentous cytoskeleton. This implies that deformations at local sites have to be interpreted within the context of three-dimensional deformations rather than on local deformations of membrane-cytoskeletal systems. This conclusion is based on several pieces of data. Our tension system was designed to detect the transmission of endothelial force to the underlying matrix. To disrupt cell-cell adhesion, these forces have to be transmitted along actin cables to strain cell-cell adhesion sites. In further support of an integrated mechanical system, we (11) reported that histamine and antibodies to cadherin induce a reactant decline in cell-matrix adhesion in postconfluent cells. When cell-cell adhesion sites are mechanically uncoupled from cell-matrix adhesion sites under subconfluent cultured conditions, histamine and antibodies to cadherin do not mediate loss in cell-matrix adhesion (11).

However, our data also suggest the possibility that mechanical forces directed at cell-matrix and at cell-cell adhesion sites may be compartmentalized and act independent of expressed actin-myosin contraction force to restore barrier function. The precise identity and the mechanisms by which cytoskeletal-based forces restore barrier function remains to be elucidated. However, several potential candidate proteins such as microtubules, intermediate filaments, actin, and several adhesion receptors like cadherin and integrins may be critical in regulating these processes.

It is reasonable to suspect that the material properties of the endothelial cell, particularly near the cell periphery, have to be different before and after exposure to thrombin. At time points at which barrier function is restored to initial basal levels, there are significant levels of expressed actin-myosin contraction. The restoration of barrier function is not simply attributed to a reduction in tension development. We (10) previously reported that cAMP-stimulation restored thrombin-mediated barrier function more promptly despite the fact that cAMP did not inhibit thrombin-mediated tension development. ML-7 reduced the temporal effects of thrombin on cell-cell adhesion. Centrifugal forces must abrogate the impact of the contractile force of actin-myosin from straining cell-cell adhesion sites. Taken together, these data suggest that mechanical forces have to be recruited to counterbalance the actin-myosin contractile forces as cell-cell reapposition occurs.

In summary, expression of actin-myosin contraction in thrombin-treated endothelial cells affects the restoration of barrier function. Although thrombin shares some temporal changes in cell-cell and cell-matrix adhesion with histamine, thrombin also mediates quantitative and temporal differences in cell-cell and cell-matrix adhesion compared with cells exposed to histamine. In particular, thrombin induced a more prolonged decline in cell-cell adhesion, which temporally correlated with tension development. Pharmacological inhibition of tension development had predominate effects on cell-cell adhesion and had no effect on cell-matrix adhesion. Taken together, these data demonstrate that expression of actin-myosin contraction affects the restoration of barrier function by straining cell-cell adhesion sites. Mechanical deformations of cytoskeletal-membrane interactions need to be considered as a model in which cell-cell and cell-matrix adhesion sites are mechanically coupled by an intervening filamentous cytoskeletal network.

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