Lung endothelial heparan sulfates mediate cationic peptide-induced barrier dysfunction: a new role for the glycocalyx

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Submitted 22 January 2003; accepted in final form 11 May 2003

Lung endothelial heparan sulfates mediate cationic peptide-induced barrier dysfunction: a new role for the glycocalyx. Am J Physiol Lung Cell Mol Physiol 285: L986–L995, 2003. First published May 16, 2003; 10.1152/ajplung.00022.2003.—The endothelial glycocalyx is believed to play a major role in microvascular permeability. We tested the hypothesis that specific components of the glycocalyx, via cytoskeletal-mediated signaling, actively participate in barrier regulation. With the use of polymers of arginine and lysine as a model of neutrophil-derived inflammatory cationic proteins, we determined size- and dose-dependent responses of cultured bovine lung microvascular endothelial cell permeability as assessed by transendothelial electrical resistance (TER). Monomers of L-arginine and L-lysine did not alter barrier function, suggesting a cross-linking requirement of cell surface “receptors”. To test the hypothesis that glycosaminoglycans (GAGs) are candidate receptors for this response, we used highly selective enzymes to remove specific GAGs before polyarginine (PA) treatment and examined the effect on TER. Heparinase III attenuated PA-induced barrier dysfunction by 50%, whereas heparinase I had no effect. To link changes in barrier function with structural alterations, we examined actin organization and syndecan localization after PA. PA induced actin stress fiber formation and clustering of syndecan-1 and syndecan-4, which were significantly attenuated by heparinase III. PA-induced cytoskeletal rearrangement and barrier function did not involve myosin light chain kinase (MLCK) or p38 MAPK, as ML-7, a specific MLCK inhibitor, or SB-20358, a p38 MAPK inhibitor, did not alter PA-induced barrier dysfunction. In summary, lung endothelial cell heparan sulfate proteoglycans are key participants in inflammatory cationic peptide-induced signaling that links cytoskeletal reorganization with subsequent barrier dysfunction.

endothelium; inflammation; permeability

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monocytes and T cells (34). Importantly, HBP/CAP37 has been reported to account for all of the vascular permeability-enhancing activity of activated neutrophils (12). Whether receptors for HBP/CAP37 and other inflammatory cationic peptides are present on the luminal surface of vascular endothelium has not been established. However, the strong cationic nature of HBP in conjunction with intrinsic heparin-binding characteristics suggests that a search for the HBP receptors could potentially include cell surface glycosaminoglycans (GAGs), including heparin/heparan sulfate-like GAGs (HSGAGs), implicating a component of the endothelial glyocalyx, a thick surface layer of proteoglycans whose anionic GAG side chains form an entangled meshwork on the cell surface (36). The adsorption of serum proteins onto the GAG chains results in the formation of a gel-like layer that creates a direct physical barrier to both water and protein transport into the intercellular junction (6, 15). In this study, we hypothesized that specific components of the glyocalyx may play an active role in barrier regulation through GAG-mediated interactions with soluble cationic ligands (like HBP), thereby activating signaling pathways associated with the cytoplasmic domain of the proteoglycan core protein. We now report size- and polymer-specific effects of diverse cationic peptides on endothelial cell cytoskeletal rearrangement and transmonolayer permeability. Our results indicate that cell surface HSGAGs are actively involved in barrier regulation and directly mediate, in part, cationic peptide-induced signaling that leads to increases in endothelial permeability and cytoskeletal reorganization. These data further suggest that the syndecan family of heparan sulfate proteoglycans participate in polycation-induced signaling and highlights a novel role for the glyocalyx.

METHODS

Cell culture and reagents. Bovine lung microvascular endothelial cells (BLMVEC) were obtained from American Type Culture Collection (Rockville, MD) and Vec Technologies (Rensselaer, NY) and cultured in DMEM and MCDB-131, respectively, and supplemented with 10% fetal calf serum. Cells were used from passages 6–10. All reagents were from Sigma Chemical (St. Louis, MO) unless otherwise stated. Heparinase I (EC 4.2.2.7) and heparinase III (EC 2.2.2.8) were purchased from Sigma Chemical and Seikagaku America (Falmouth, MA). Polyclonal antibodies to syndecan-1 and anti-syndecan-4 antibodies followed by anti-IgG-fluorescein isothiocyanate (FITC) were purchased from Sigma Chemical (St. Louis, MO). Monoclonal antibody (BB4) to syndecan-1 was localized by incubating monolayers with anti-syndecan-1 antibodies followed by anti-IgG-FITC-labeled secondary antibody. Cell monolayers were then examined by fluorescence microscopy (Nikon Eclipse TE-200 inverted microscope).

Measurement of transendothelial electrical resistance. Endothelial cells were grown to confluence in polycarbonate wells containing evaporated gold microelectrodes (surface area 10⁻⁶ cm²) in series with a larger gold counter electrode (surface area 1 cm²) connected to a phase-sensitive locked-in amplifier, as we and others have previously described (10, 13). Measurements of transendothelial electrical resistance (TER) were performed using an electrical cell-substrate impedance sensor (Applied BioPhysics, Troy, NY). Briefly, current was applied across the electrodes by a 4,000-Hz alternating current voltage source with an amplitude of 1 V in series with a 1-MΩ resistor to approximate a constant current source (+1 mA). The in-phase and off-phase voltages between the electrodes were monitored in real time with the lock-in amplifier and subsequently converted to scalar measurements of transendothelial impedance, of which resistance was the primary focus. TER was monitored for at least 30 min to establish a baseline resistance that, for BLMVEC, was typically 6–10 × 10⁴ Ω. Each monolayer served as its own control, and percent change in TER for groups was pooled and expressed as means ± SE.

GAG analysis. To quantify the amount of GAG chain removed from the cell surface by enzymatic treatment, we measured the release of ³⁵S-labeled heparan and chondroitin sulfate and remaining cell-associated radioactivity. Briefly, endothelial cells were cultured in 12-well plates and labeled for 60 h with 30 μCi/ml ³⁵S sulfate in sulfate-free medium and were then washed three times with PBS. The cells were then incubated for an additional 24 h in complete medium; before enzyme assay, cells were washed three times with sulfate-free medium. Enzyme solutions were placed onto confluent monolayers of BLMVEC, and aliquots of medium were collected over a 3-h period and counted. Cells were then lysed overnight with 0.1 N NaOH, and total cell-bound radioactivity was counted. All enzyme assays were done in triplicate. The amount of radioactivity released by each enzyme was expressed as the percentage of total cell counts.

Heparan sulfate disaccharide analysis was performed as previously described (20). Briefly, endothelial cells were digested with a mixture of heparinase I, II, and III with fragments separated by capillary electrophoresis. Disaccharides were identified by comparison against comigration of known standards.

Immunofluorescence microscopy. Confluent monolayers of human lung microvascular endothelial cells were cultured on glass coverslips and treated with polyarginine (PA; 90 kDa, 50 μg/ml) for 30 min and 1 h. Human lung microvascular endothelial cells were chosen for these parallel studies because commercially available anti-syndecan antibodies do not cross-react with bovine syndecans. Cells were fixed with 3.7% paraformaldehyde, permeabilized with 0.25% Triton X-100, and incubated with Texas red-phalloidin (Molecular Probes, Portland, OR) to visualize filamentous actin. Syndecan was localized by incubating monolayers with anti-syndecan-1 and anti-syndecan-4 antibodies followed by anti-IgG-FITC-labeled secondary antibody. Cell monolayers were then examined by fluorescence microscopy (Nikon Eclipse TE-200 inverted microscope).

Statistics. For all TER measurements, each monolayer served as its own control, and percentage change from baseline was determined. Data from each group were pooled and analyzed by one-way ANOVA, and groups were compared with a Student-Neuman-Keuls test using Sigma Stat (SPSS, Chicago, IL). Data are presented as percent change in TER ± SE.

RESULTS

Polycationic peptides increase endothelial permeability. We examined the role of the polycationic peptides PA and polylysine (PL) on endothelial permeability as measured by TER. Both PA (90 kDa) and PL (84 kDa) produced rapid decreases in TER as demonstrated in Fig. 1, A and B, with maximal permeability occurring at a concentration of 50 μg/ml. By comparison, dextran 70, a neutral polymer of similar size (70 kDa), did not
affect TER values at concentrations of 10, 50, and 100 μg/ml (data not shown).

We next tested the effect of polycation size on endothelial barrier function and demonstrated the multivalent requirement of cationic peptides for this response. Monomers of L-arginine failed to alter TER (Fig. 2A), whereas PA at each size tested (11.8, 38, and 90 kDa) produced maximal reductions in TER (Fig. 2A). Consistent with these results, L-lysine monomers did not significantly alter TER (Fig. 2B), whereas larger polymers of lysine (32 and 84 kDa) both reduced TER maximally (Fig. 2B). The observation that monomers of L-arginine and L-lysine failed to increase endothelial permeability supports the notion that signal activation may require cross-linking of cell surface domains. Although the polymers of arginine and lysine used in these studies were both polydisperse, the response to the smallest PA (11.8 kDa) differed dramatically from the response to the smallest PL (7.3 kDa). PA (11.8 kDa) induced maximal barrier dysfunction, whereas the 7.3-kDa PL polymer did not affect lung TER (Fig. 2B), suggesting specificity in the response evoked by interaction between the cationic peptide structure and the unknown binding site(s) on the endothelial surface.

HSGAGs mediate PA-induced barrier dysfunction. We hypothesized that cationic peptides mediate cellular effects via interaction with anionic GAGs present on the endothelial surface composed primarily of HSGAGs and chondroitin sulfate-like GAGs. To examine the dependence of cationic peptide-induced decrease in TER on endothelial GAGs, we selectively removed either HSGAGs or chondroitin sulfate GAGs via enzymatic cleavage using heparinase III and chondroitinase ABC, respectively. Removal of either heparan sulfates or chondroitin sulfates had no effect on baseline TER; however, the removal of cell surface heparan sulfate with heparinase III significantly attenuated PA-induced permeability (~50%; Fig. 3), whereas chondroitin sulfate removal did not alter PA-induced barrier dysfunction (Fig. 3). We next quantified the amount of heparan sulfate removed by heparinase III pretreatment to link enzyme activity with the alterations in PA-induced barrier dysfunction. Heparinase III (15 mU/ml) pretreatment removed ~67% of the releasable heparan sulfate pool (Table 1). Given this close linkage between the amount of heparan sulfate removed and the magnitude of attenuation of PA-induced barrier dysfunction, these data suggest that heparan sulfate proteoglycans are key participants in cationic peptide-induced endothelial barrier dysfunction.

To exclude persistent HSGAGs not removed with heparinase III as a possible explanation for the remaining 50% of the PA-induced barrier dysfunction...
that was refractory to heparinase III, we pretreated monolayers with heparinase I alone and in combination with heparinase III, before PA stimulation. Heparinase III cleaves primarily between unsulfated or monosulfated saccharides within the GAG chain, whereas heparinase I generally cleaves at more highly sulfated saccharides (28). Heparinase I alone had no effect on PA-induced barrier dysfunction, and the mix of heparinase I and III provided no additional attenuation of the PA response compared with heparinase III alone. Enzyme activity assays demonstrated that heparinase I released 40% of labeled heparan sulfate (Table 1). The combination of heparinases I and III had no effect on PA-induced TER beyond that of heparinase III alone (Fig. 3), and enzyme activity assays demonstrated no additional release when the enzymes were used in combination. We next examined the role of chondroitin sulfate-like GAGs by predigesting the cell surface with chondroitinase ABC lyase (2.5 mU/ml) before PA treatment. This dose of chondroitinase is severalfold higher than previously used (16) to remove endothelial cell surface chondroitin sulfate. Pretreatment of BLMVEC with chondroitinase had no effect on PA-induced permeability (Fig. 3), and enzyme activity assays demonstrated that chondroitin sulfate-like GAGs only accounted for 15% of cell surface-labeled GAG (Table 1).

To correlate these enzyme results with HSGAG structure, we characterized the disaccharide composition of the HSGAGs from lung microvascular endothelial cells by capillary electrophoresis (20). These studies demonstrated (Table 2) that 41% of the disaccharides were monosulfated and 59% were unsulfated disaccharides. The predominance of monosulfated and unsulfated disaccharides directly correlates with the observed activity of heparinase III (28) and the observed attenuation of the PA-induced permeability response by heparinase III pretreatment.

HSGAG sulfation level does not affect PA-induced permeability. To investigate the structural requirements of HSGAGs in mediating PA-induced signaling, we pretreated monolayers for 24 h with sodium chlorate in sulfate-free RPMI 1640, which reduces endothelial HSGAGs by 65% (Table 2) and chondroitin sulfation by 90% (16). Chlorate treatment failed to alter either baseline TER or PA-induced barrier dysfunction (Fig. 3), suggesting that simple charge interaction alone is not solely responsible for mediating PA binding and associated signal activation but requires an additional specific structural characteristic of the repeating disaccharide units.

Anti-syndecan and anti-heparan sulfate antibody cross-linking and endothelial barrier function. The size-dependent effect of polycations on barrier dysfunc-
Table 1. Quantification of enzyme activity

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Concentration, mU/mL</th>
<th>%35SO4 Released</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparinase III</td>
<td>15</td>
<td>67</td>
</tr>
<tr>
<td>Heparinase I</td>
<td>15</td>
<td>40</td>
</tr>
<tr>
<td>Heparinase I + III</td>
<td>15 + 15</td>
<td>62</td>
</tr>
<tr>
<td>Chondroitinase</td>
<td>2.5</td>
<td>16</td>
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35SO4-releasable activity from bovine lung microvascular endothelial cells by glycosaminoglycan-degrading enzymes (see METHODS).

Table 2. Heparan sulfate disaccharide composition

<table>
<thead>
<tr>
<th>Disaccharide</th>
<th>BLMVEC in MCDB-131</th>
<th>BLMVEC in SF-RPMI 1640</th>
<th>BLMVEC in SF-RPMI 1640 + Chlorate, 50 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔU2SHNS,6S</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ΔU2SHNS</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ΔUH30,6S</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ΔUHNS,6S</td>
<td>0.83</td>
<td>0.63</td>
<td>0.25</td>
</tr>
<tr>
<td>ΔU3HNS,6S,6S</td>
<td>12.91</td>
<td>14.46</td>
<td>5.56</td>
</tr>
<tr>
<td>ΔU3HNS,6S</td>
<td>27.77</td>
<td>26.23</td>
<td>11.27</td>
</tr>
<tr>
<td>ΔUH4Nc</td>
<td>57.87</td>
<td>58.31</td>
<td>82.73</td>
</tr>
</tbody>
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Heparan sulfate from bovine lung microvascular endothelial cells (BLMVEC) was digested completely, and disaccharides were identified by capillary electrophoresis. Disaccharide composition was measured in cells cultured in standard culture medium (MCDB-131), in sulfate-free RPMI 1640 (SF-RPMI 1640), and sulfate-free RPMI 1640 plus 50 mM sodium chlorate for 24 h. U, uronic acid, H, glucosamine, 2S, 2-O-sulfate, 6S, 6-O-sulfate, NAc, N-acetyl, NS, N-sulfate.
syndecan-1 and syndecan-4 localization and actin organization in response to PA challenge. In control endothelial cells, syndecan-1 distribution was limited to the cell periphery (Fig. 4A), and cells demonstrated the typical peripheral actin band with a few centrally located stress fibers (Fig. 4B). Treatment of endothelial monolayers with PA (90 kDa, 50 μg/ml) caused a significant change in syndecan-1 distribution, from a continuous peripheral pattern to a punctuate pattern consistent with clustering (Fig. 4C), and altered the polymerized actin cytoskeleton from a peripheral distribution to predominately cytoplasmic stress fibers (Fig. 4D). These changes in syndecan-1 and actin were associated with gap formation between adjacent endothelial cells (Fig. 4D, arrows), consistent with the associated increase in monolayer permeability reflected by TER.

Under basal conditions, syndecan-4 staining localized to the cell periphery, as did actin, specifically at areas of cell-cell contact; there was also significant perinuclear staining (Fig. 5, A and C). After PA treatment, syndecan-4 was notably absent from cell-cell contacts and demonstrated reduced staining in the perinuclear region but demonstrated enhanced localization in a linear pattern throughout the cytoplasm (Fig. 5B), suggestive of colocalization with actin (Fig. 5D) and/or microfilaments. Thus both syndecan-1 and syndecan-4 demonstrated significant changes in localization after PA stimulation, and these changes occurred in association with dramatic actin stress fiber formation.

Heparinase III pretreatment abolishes PA-induced actin cytoskeletal reorganization and syndecan local-

Fig. 4. A: immunofluorescence images of syndecan-1 showing peripheral staining pattern in control cells. B: control cell actin staining showing cortical actin and some stress fiber. C: PA (90 kDa, 50 μg/ml, 30-min incubation) induced syndecan-1 clustering around periphery and caused loss of cortical actin with concurrent actin stress fiber formation (D) that is associated with intercellular gap formation (arrows). E: Hep III pretreatment (15 mU/ml for 1.5 h) attenuated PA effects on syndecan-1 localization and prevented loss of cortical actin and also prevented intracellular gap formation (F). Heparinase treatment alone had no effect on syndecan-1 or localization (not shown).
To confirm that HSGAGs were directly involved in mediating syndecan clustering and actin reorganization, cell surface heparan sulfates were removed with heparinase III before treatment with PA. Immunofluorescent studies revealed that removal of cell surface heparan sulfates completely abolished PA-induced actin reorganization, syndecan-1 clustering, and interendothelial gap formation (Fig. 4, E and F), whereas heparinase III treatment alone had no effect on actin or syndecan-1 localization (data not shown). Heparinase III pretreatment also attenuated PA-induced syndecan-4 clustering and actin reorganization (Fig. 5, E and F).

**Signaling pathways involved in cationic peptide-mediated barrier dysfunction.** Increases in endothelial cell stress fibers implicates an activation of the endothelial cell contractile apparatus. We examined the role of two major signaling pathways known to contribute to endothelial cell stress fiber formation, myosin light chain kinase (MLCK), and p38 MAPK (8, 11) on cationic peptide-mediated endothelial permeability. To examine the role of MLCK on myosin light chain phosphorylation, we first assessed the levels of myosin light chain in response to numerous agonists (41) and found that PA did not increase the level of phosphorylation of myosin light chain (data not shown). Consistent with these results, 1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine HCl (ML-7), a specific MLCK inhibitor, was used at a concentration (20 μM) that has been shown to completely inhibit MLCK phosphorylation in response to numerous agonists (41) and failed to alter PA-induced barrier dysfunction as assessed by
TER (Fig. 3), suggesting that PA acts through MLCK-independent pathways to modulate cytoskeletal organization and barrier function. We have also previously reported a p38 MAPK pathway (11, 32) leading to marked stress fiber not associated with myosin light chain phosphorylation. To assess this signaling pathway, we tested SB-20358, a p38 MAPK inhibitor, which failed to affect the PA response on TER (Fig. 3). The concentration of SB-20358 used (20 μM) has been shown to significantly inhibit p38 MAPK (29). These results suggest a novel mechanism of stress fiber formation that does not require an increase in either MLCK or p38 MAPK activity.

**DISCUSSION**

In this study, we demonstrate that endothelial HSGAGs mediate cationic peptide-induced signaling that leads to endothelial cytoskeletal rearrangement and barrier dysfunction. This cationic peptide-mediated loss in barrier function was associated with syndecan-1 and syndecan-4 clustering and actin stress fiber formation; removal of cell surface HSGAGs attenuated these responses. Together, these observations suggest that syndecan heparan sulfates serve as an endothelial cell surface binding domain for cationic peptides and initiate stimulus/coupling responses that signal via the actin cytoskeleton to increase endothelial permeability. However, it is likely that there are additional participants involved in this complex signaling pathway that lead to changes in endothelial barrier function. These data provide the first account in identifying an endothelial-surface binding domain for inflammatory cationic peptides and provide mechanistic insight for the recent observations of Gautum et al. (12), who demonstrated that the neutrophil-derived cationic peptide, HBP, is responsible for the increase in vascular permeability produced by endothelial cell interaction with activated neutrophils.

Like HBP/CAP37, PA and PL are highly cationic molecules that have been utilized as models of neutrophil- and eosinophil-derived cationic peptides (30, 39). PL promotes edema formation when injected intradermally (30) and increases lung epithelial permeability when instilled into the intra-alveolar space (39). The mechanism(s) responsible for these effects was unknown, and the direct effects of cationic peptides on endothelial and epithelial cells could not be separated from secondary paracrine mediators, such as mast cell degranulation or macrophage activation. Polycation-induced permeability was postulated to occur through charge-mediated binding to anionic sites on the cell surface, presumably sulfated proteoglycans (30). The cellular effects of polycations are size dependent (30), suggesting that cationic peptides cross-link cell surface domains, and the greater the number of cross-linked “receptors” the greater the intensity of signal amplification. Our data support this concept, since monomers of L-arginine and L-lysine failed to alter endothelial barrier properties, whereas larger polymers induced maximal signal activation and greater changes in permeability.

Our results with the small polycation polymers require specific comment as the 11.8-kDa polymer of arginine induced maximal barrier dysfunction, whereas the smallest lysine polymer (7.3 kDa) did not alter endothelial permeability. Because the cationic peptides used in this study were all polydispersed, we expected similar results for these two peptides. The difference between the size response of PA (11.8 kDa) and PL (7.3 kDa) suggests there is structural specificity of the putative binding domain for these basic amino acid residues. The lack of effect of sodium chloride on PA-induced barrier dysfunction further suggests that the interaction of PA with its receptor involves more complex stereoselectivity as opposed to simple charge interaction.

The specificity for arginine-mediated binding to heparan sulfate has been reported by Liu and colleagues (24), who demonstrated that the binding of tissue factor pathway inhibitor-2/matrix-associated serine protease inhibitor to heparan sulfate and dermatan sulfate occurred selectively through arginine-mediated interaction. Likewise, Michel and colleagues (27) demonstrated that serum albumin reduces capillary permeability via arginine-mediated interaction with the endothelial cell surface, whereas modification of lysine residues had no effect on the ability of albumin to modulate permeability; similar results were obtained when modified albumins were tested on the permeability of cultured endothelial cells (35). Last, Vepa et al. (40) demonstrated that PA was more potent in activating endothelial cell phospholipase D compared with PL. Thus our data are consistent with a number of reports demonstrating that whereas arginine and lysine residues carry the same electrical charge, other structural characteristics allow endothelial proteins and HSGAGs to discriminate between these residues.

A specific focus of this work was to characterize the endothelial receptor for inflammatory cationic peptides with endothelial HSGAGs as likely candidates. Endothelial cells express three HSGAGs, including perlecan, syndecan, and glypican. Of the three, only the syndecans are known to influence cytoskeletal organization, cell-cell adhesion, and motility (42). Given that these processes are associated with changes in vascular permeability (7), syndecans were the most likely candidate molecule in our search for a cationic peptide receptor. To establish that syndecans participate in cationic peptide-induced signaling, we examined syndecan localization after stimulation with PA and found PA induced 1) actin stress fiber formation, 2) syndecan-1 to cluster around the cell periphery, and 3) syndecan-4 to distribute in a centralized linear pattern, suggestive of localization with actin or microfilaments and/or microtubules. Because antibody-mediated cross-linking of syndecan has been associated with syndecan clustering and subsequent stress fiber formation (38), we hypothesize that cationic peptides cross-link heparan sulfates to promote similar changes in
syndecan localization and actin organization. Syndecan-1 clustering precedes and likely mediates actin reorganization in response to PA as removal of heparan sulfates by heparinase III completely abolished both syndecan clustering and actin stress fiber formation while reducing the barrier disruption of PA by 50%. This suggests that other cell surface domains may be involved and/or other signaling pathways are activated that affect permeability independent of actin reorganization. Removal of chondroitin sulfate-like GAGs with chondroitinase did not effect PA-mediated permeability changes.

We offer three possible explanations regarding the complex relationship between the enzyme activity data (Table 1), the compositional analysis (Table 2), and the effects of enzyme pretreatment (Fig. 3) on PA-mediated barrier disruption. Briefly, heparinase III released 67% of labeled heparan sulfates and reduced PA-induced barrier dysfunction by 50%. Thus there is a close correlation between the amount of heparan sulfate released and the attenuation of the effects of PA on TER. Heparinase I, however, released 40% of labeled heparan sulfates but had no effect on attenuating PA-induced barrier dysfunction. The simplest explanation for this observation is that the heparan sulfates released by heparinase I were not carried on syndecans but, perhaps, carried by glypicans or perlecan and, therefore, not involved in barrier regulation. An alternative explanation is that the additional 27% of the heparan sulfates released by heparinase III (compared with heparinase I) surpassed a crucial cell surface GAG content that is required to participate in cross-linking or activation of intracellular signals that are associated with barrier regulation. Last, heparan sulfates can have similar global composition but possess markedly different focal sequences, referred to as “fine structure”. These differences in heparan sulfate focal sequence have been shown to differentially alter endothelial responses to fibroblast growth factor-mediated signaling (2). Thus the heparan sulfates removed by heparinase I may have had focal sequences that did not participate in barrier regulation.

HSGAG-mediated signaling has been well documented as numerous growth factors, including the fibroblast growth factor family and vascular endothelial growth factor-165 (4, 14) as well as matrix elements (5, 18), require HSGAGs as coligands for maximal receptor activation. Langford and colleagues (21) also demonstrated that both the number and position of heparan sulfates on the syndecan core protein can influence heparan-mediated signaling. Last, the fine structure of the heparan sulfates also influences heparan-mediated signaling and cell behavior (37). Thus through undetermined mechanisms, the heparan sulfates can modulate syndecan-induced signal activation and subsequent cell function.

Our data suggest that PA-induced cross-linking of syndecan heparan sulfates activate signaling pathways associated with syndecan clustering and actin cytoskeletal reorganization. The mechanism(s) responsible for clustering remains largely unknown. Polycations, like PA and PL, activate endothelial cell phospholipase D apparently through a protein kinase C (PKC)-dependent mechanism (40) such that activation of PKC may be an initial event. The signaling pathways leading to actin reorganization via syndecan-1 likely include Src family of kinases, cortactin, tubulin, and microfilament-regulated processes, whereas syndecan-4-mediated actin organization involves PKC-and phosphatidylinositol 4,5-bisphosphate-dependent processes (42). It is well known that endothelial cell actin reorganization and permeability is strongly influenced by endothelial-specific MLCK activity (9); in general, alterations in endothelial permeability occur via activation of MLCK-dependent or p38 MAPK pathways (7, 28). We examined the activity of MLCK in response to PA treatment and found no increase in myosin light chain phosphorylation, and ML-7, a specific inhibitor of MLCK, had no effect on PA-induced barrier dysfunction. We also tested the p38 MAPK inhibitor, SB-203580, and found that it had no affect on PA- or PL-mediated permeability. These observations suggest that heparan sulfate-directed signaling may act through novel mechanism(s) to induce contractile activity and cytoskeletal rearrangement. Currently, we are exploring this exciting possibility.

In summary, we have identified HSGAGs as an endothelial receptor for cationic peptides and have found that signals activated via HSGAG cross-linking result in cytoskeletal reorganization and subsequent barrier dysfunction. Syndecan-1 and syndecan-4 heparan sulfates appear to be mediators for these events, although we cannot exclude a role for other accessory participants. These data identify another important step in understanding mechanism(s) involved in MN-induced inflammation and associated changes in vascular permeability and highlight a proinflammatory role for specific components of the glycocalyx.

The authors thank Drs. Anna Birukova, Alexander Verin, and Louis Romer for assistance with the immunofluorescence images and Christopher Hubert for assistance with manuscript preparation. Current address for R. O. Dull: Univ. of Utah, Dept. of Anesthesiology, Salt Lake City, UT 84132.

DISCLOSURES

This work was supported through National Heart, Lung, and Blood Institute Grant KO8 HL-68063 and a Johns Hopkins Clinician Scientist Award (to R. O. Dull).

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