UltraRapid Communication

Heparan Sulfate Proteoglycan Is a Mechanosensor on Endothelial Cells

Jeffry A. Florian, Jason R. Kosky, Kristy Ainslie, Zhengyu Pang, Randal O. Dull, John M. Tarbell

Abstract—The objective of this study was to test whether a glycosaminoglycan component of the surface glycocalyx layer is a fluid shear stress sensor on endothelial cells (ECs). Because enhanced nitric oxide (NO) production in response to fluid shear stress is a characteristic and physiologically important response of ECs, we evaluated NO_x (NO_2^- and NO_3^-) production in response to fluid shear stress after enzymatic removal of heparan sulfate, the dominant glycosaminoglycan of the EC glycocalyx, from cultured ECs. The significant NO_x production induced by steady shear stress (20 dyne/cm^2) was inhibited completely by pretreatment with 15 mU/mL heparinase III (E.C.4.2.2.8) for 2 hours. Oscillatory shear stress (10 ± 15 dyne/cm^2) induced an even greater NO_x production than steady shear stress that was completely inhibited by pretreatment with heparinase III. Addition of bradykinin (BK) induced significant NO_x production that was not inhibited by heparinase pretreatment, demonstrating that the cells were still able to produce abundant NO after heparinase treatment. Fluorescent imaging with a heparan sulfate antibody revealed that heparinase III treatments removed a substantial fraction of the heparan sulfate bound to the surfaces of ECs. In summary, these experiments demonstrate that a heparan sulfate component of the EC glycocalyx participates in mechanosensing that mediates NO production in response to shear stress. The full text of this article is available online at http://www.circresaha.org. **(*Circ Res*. 2003;93:e136-e142.)**

Key Words: shear stress ■ endothelial cells ■ heparan sulfate ■ nitric oxide ■ glycocalyx

The inner surfaces of blood vessels are lined with a monolayer of endothelial cells (ECs) that is continually exposed to the mechanical shearing forces (stresses) of blood flow. Variations in shear stress magnitude as well as temporal and spatial distribution have been shown to induce alterations in endothelial permeability and hydraulic conductivity,[1–3] cytoskeletal structure,[4–7] surface adhesion molecule expression,[8] and gene expression.[9,10] In addition, the exposure of endothelial cells to shear (both steady and oscillatory) has been shown to alter the production of vasoregulating agents of which nitric oxide (NO) is perhaps the most notable.[11–13]

NO is a vasodilator produced by the conversion of L-arginine to L-citrulline that is catalyzed by endothelial nitric oxide synthase (eNOS). NO modulates vascular tone by eliciting relaxation of smooth muscle cells while inhibiting smooth muscle cell growth.[14] NO production responds to changes in shear stress in a biphasic manner in human umbilical vein endothelial cells (HUVECs) and bovine aortic endothelial cells (BAECs).[11,12] There is an initial rapid NO production phase that is G protein and Ca^{2+}-dependent and is influenced by rate of change of shear and not the shear level per se. The subsequent phase is characterized by a lower rate of NO production rate that is G protein and Ca^{2+}-independent but is shear level dependent.[12,15] Both phases of the NO response are mediated by mechanotransduction, or the transmission of the fluid shear force to the cellular surface.[16] The hypothesis of this study is that a specific component of the plasma membrane surface layer, termed the glycocalyx, is in fact the mechanosensor for the NO response.

The surface of endothelial cells is decorated with a wide variety of membrane-bound macromolecules, including glycoproteins, proteoglycans, and their associated glycosaminoglycan (GAG) side chains that constitute the glycocalyx. Heparan sulfates are the most common endothelial cell-surface GAG, comprising some 50% to 90% of the GAG pool,[17] and they have been the most extensively studied GAG in regard to their ability to function as signal transduction molecules.[18] Heparan sulfate proteoglycans (HSPGs) can be divided into three different groups based on their protein backbones (syndecans, glypicans, and perlecans). The least common HSPG, perlecan, consists of only one variant with a total of five GAG linkages and is located within the EC basement membrane. Syndecans form the largest group of HSPGs on the endothelial surface and are distinguished by highly variable extracellular domains[19] and by their status as the only HSPG that penetrates the cytoplasm, allowing for

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interaction with the cytoskeleton.[20] Glypicans form the second most common HSPG group, and possess structural similarity, typically differing only in the number of GAG attachment sites. These molecules are bound to the surface through a COOH-terminal GPI (glycosyl phosphatidyl inositol) anchor.[21]

There are several possible mechanisms by which the glycocalyx might serve as a mechanotransducer. First, the glycocalyx acts as a transport boundary between blood flow and the endothelial surface, and as such, it is privy to differing concentrations of agonists.[22–24] Alterations in flow could affect the distribution of agonists in this layer, indirectly transmitting flow conditions to the cell. Alternatively, the membrane-attached proteoglycans may be physically displaced when exposed to shear. This proteoglycan displacement could be transmitted to the cellular surface, provoking an intracellular response.[16] To efficiently transmit shear stress from flowing blood by this mechanism, the membrane-bound proteins should possess an extended extracellular domain for shear sensing along with an intracellular, cytoplasmic sequence for direct transmittance.[25] Such an arrangement is typical of syndecans on the cellular surface,[19] which have an extensive GAG component to sense shear and a cytoplasmic domain that is linked with known signaling elements such as G-protein receptors, including those that form a cytoplasmic bond with eNOS,[16,25] and cytoskeletal elements like actin that can transduce physical forces throughout the cell[26–28] to affect cell function. However, it has not yet been conclusively demonstrated that there is a dominant apical distribution of syndecans on endothelial cells.

In order to assess the role of the glycocalyx in shear-induced NO production, we employed an engineered shearing device to expose monolayers of BAECs to defined shear stress (steady and oscillatory). Heparinase III was used to selectively deplete the glycocalyx of the heparan sulfate GAG component. The NO production rate in response to shearing conditions was determined for heparinase treated and untreated monolayers, and it was observed that heparinase treatment completely inhibited shear-induced NO production.

Materials and Methods

Chemicals

The following chemicals were obtained from Sigma Chemical Co: bovine serum albumin (BSA, Fraction V, 30% solution), minimal essential media (MEM), fetal bovine serum (FBS), L-glutamine, penicillin-streptomycin, fibronectin, trypsin-EDTA, Dulbecco's phosphate-buffered saline (DPBS) without Ca^{2+} and Mg^{2+}, Hank's balanced salt solution (HBSS), heparinase III from *Flavobacterium heparanum*, sodium nitrate, sodium nitrite, β-nicotinamide adenine dinucleotide phosphate (β-NADPH), *N*-(1-naphthyl)ethylenediamine (NED), flavin adenine dinucleotide (FAD), sulfanilamide, nitrate reductase, 2,3-diaminonaphthalene (DAN), *Bandeiraea simplicifolia* biotinylated lectin, bradykinin (BK), and histamine.

Polycarbonate Transwell filters (Costar, 0.4-μm pore size, 24.5-mm diameter) were obtained from Fisher. Heparan sulfate primary antibody (HepSS-1 mouse anti-mouse IgM) was obtained from US Biological. Alexa Fluor 488-labeled secondary antibodies (goat anti-mouse IgM) and NeutriAvidin Alexa-Fluor 350 avidin conjugate were obtained from Molecular Probes.

Cell Culture

Bovine aortic endothelial cells (BAECs) were harvested from bovine thoracic aortas as described by Sill et al.[30] Briefly, after removal, the aorta was transported in ice-cold HBSS containing 1% penicillin-streptomyocin. For cell harvesting, the aorta was rinsed twice with HBSS while the fat was separated from the aorta. Subsequently the aorta was split longitudinally between the intercostal arteries and formed into a trough. A solution of 60 mL of complete media (MEM w/ 10% FBS, 1% L-glutamine, and 1% penicillin-streptomyocin) was added to 1 mL of Liberase Blendzyme 2 (Roche Diagnostics Corporation), and subsequently, 10 mL of this solution was added to the luminal surface. This solution was allowed to incubate at room temperature for 40 minutes, then the solution was collected, centrifuged, and the pellet was resuspended and plated on a T-25 flask. Five more washings involving 10 mL of the Blendzyme cocktail were repeated, with incubations of 5 minutes at room temperature. Each time, the solution was pipetted off the aorta, centrifuged, and the resulting pellet was resuspended and plated on a T-25 flask.

Cells on flasks were grown in MEM supplemented with 10% FBS, penicillin (50 U/mL), streptomyocin (50 μg/mL), and L-glutamine (2 mmol/L) at 37°C in 5% CO_2. To examine the effect of shear stress on cellular response, BAECs (passage 5 to 9) were seeded onto fibronectin-coated slides (30 μg/slide) at a cell density of 0.25×10^6 cells/cm^2. Before cell seeding, however, the slides were glued to the bottom of a 24.5 mm diameter Transwell filter holder (after removal of the filter) with a silicone elastomer (Sylgard). The cylindrical filter holder could then be used to support the cells when defined shear stress was applied using the rotating disk apparatus described below. These slides mounted on the filter holder were used in experiments 3 to 4 days after cell seeding.

Shear Apparatus

A detailed description of the shear apparatus used in these experiments can be found in Sill et al.[2] Briefly, a cylindrical disk (radius=12 mm) was supported above the monolayer at a distance of approximately 251 μm. This disk was rotated by a motor drive to produce a defined shear stress that varies linearly from zero at the center to a maximum value at the edge (mean value is two-thirds the maximum value). All subsequent values of shear stress will be reported as the maximum value. To obtain oscillatory shear stress, we varied the rotational speed of the shear disk by adding a sinusoidal waveform to the steady rotational motion. Steady shear experiments were conducted at 20 dyne/cm^2. Oscillatory shear was produced by using a steady shear component of 10 dyne/cm^2 and an oscillatory component of 15 dyne/cm^2 (equivalent to a shear stress of 10 ± 15 dyne/cm^2 at 1 Hz) as described in Hillsley and Tarbell.[29]

Just before a shear experiment, the monolayer was rinsed twice with the experimental media consisting of phenol red free MEM (PRF-MEM), supplemented with penicillin, streptomyocin, L-glutamine, and 1% BSA, and after washing, 2 mL of experimental media were added to the top of the monolayer that had a typical cell density of 0.25×10^6 cells/cm^2. The slide on the cylindrical filter holder was positioned beneath the shear apparatus and raised into position using an adjustable stage. After shear was initiated, 300 μL samples were taken at 0, 5, 15, 30, 60, 120, and 180 minutes after application of shear and replaced with an equal volume of fresh media. Due to this dilution of media, the measured values of concentration were adjusted by the dilution factor (1.7/2.0) and reported as the undiluted (cumulative) values.

Nitrite/Nitrate Determination

In this procedure, samples were first reduced with nitrate reductase, converting free NO_3^- to NO_2^-. Four microliters of the enzymatic mixture, containing 50 μmol/L β-NADPH and 5 mmol/L FAD, was mixed with 5 μL of KH_2PO_4 buffer (pH 7.5) and 50 μL of sample and incubated at 37°C for 3 hours. A fluorometric assay with accuracy down to 10 nmol/L was then used.[30] This quantification is based on the reaction of nitrite with 2,3-diaminonaphthalene (DAN), which produces the fluorescent compound, 1-(H)-naphthotriazole. After the reduction step, 10 μL of fresh DAN solution (0.05 mg/mL

in 0.62 mol/L HCl) was added to each well and refrigerated at 20°C for 10 minutes. Next, 10 μL of 2.8 N NaOH was added to terminate the reaction. Fluorescent readings were obtained using a Packard FluoroCount fluorometer with excitation at 360 nm and emission at 425 nm (corresponding to the fluorescent spectra peaks of 1-(H)-naphthotriazole) and Plate Reader Version 3.0 software. Nitrite standards using $NaNO_3$ in PRF-MEM with 1% BSA were run over the range of 12 nmol/L to 40 μmol/L. To convert measured sample concentrations in molar units (moles/10^3 mL) to the normalized units used in presenting the results (moles/10^6 cells), we multiplied by the volume of media on top of the cell monolayer (2 mL) to determine the number of moles present and next divided by the area of the monolayer (4.52 cm^2) and finally by cell density (0.25×10^6 cells/cm^2). Using these conversion factors, the concentration at the upper end of the standard curve (40 μmol/L) becomes 70.8 nmol/10^6 cells.

Heparinase Treatment

Heparinase III (heparinase; E.C.4.2.2.8) is an enzyme selective for heparan sulfate within the glycocalyx. For heparinase treatment, 2 mL of a solution containing 15 mU/mL of heparinase in experimental media were placed on the monolayer and incubated for 2 hours before experimentation. After exposure to heparinase, the monolayer was washed twice in experimental media and then exposed to shear stress as described above. The solution of heparinase was reconstituted from aliquots prepared within 2 months of the experiment, 15 minutes before application.

Protease Activity

Heparinase was assayed for protease activity using a RediPlate 96-EnzCheck Protease Assay kit from Molecular Probes. The protease assay kit is capable of detecting a broad variety of proteases including metallo-, serine, acid and sulfhydryl proteases. The assay was performed in accordance with manufacturer's instructions. The protease activity of the 15-mU/mL heparinase III was compared against known concentrations of chymotrypsin and pronase at 1, 3, and 24 hours. All samples of chymotrypsin and pronase were run in triplicate.

Bradykinin and Histamine Treatments

Bradykinin (BK) and histamine were utilized to test the viability of the endothelial cells' NO production machinery after enzymatic treatments. For experiments involving BK, the 2-mL starting volume contained BK at a concentration of 5 nmol/L. Similarly, the media used to replace samples in these experiments also contained BK at a concentration of 5 nmol/L. Related experiments utilized a 2-mL starting volume containing 100 μmol/L histamine with replacement media at the same concentration.

Fluorescent Labeling of Heparan Sulfate and Chondroitin Sulfate

In order to assess the effectiveness of heparinase in removing heparan sulfate from the glycocalyx and leaving chondroitin sulfate unaffected, fluorescent antibody imaging was used. BAECs were grown to confluence on slides (usually 3 to 5 days). For heparan sulfate imaging, slides were incubated with 2 mL of a 15-mU/mL heparinase solution for 2 hours. Control slides were left untreated. The monolayers were then washed with DPBS and a solution of 2 μL HepSS-1[31] was diluted to 1 mL using DPBS and applied to the monolayers. After a 15-minute incubation period, 2 μL Alexa Fluor 488-labeled secondary antibody was diluted to 1 mL with DPBS and applied to the monolayers for another 15-minute period. For chondroitin sulfate imaging, slides were incubated with 2 mL of a 15-mU/mL heparinase solution for 2 hours. Control slides were left untreated. The monolayers were then washed with DPBS and a solution of 0.005 mg/mL of biotinylated lectin in DPBS was applied. This lectin binds specifically to the *N*-acetyl-D-galactosamine residue present on chondroitin sulfate. After a 15-minute incubation period, a solution of 0.005 mg/mL fluorescent-labeled avidin conjugate in DPBS was applied to the monolayers for another 15-minute period. Then the monolayers were washed twice with DPBS and imaged using wide field fluorescence at 40× on an Olympus BX60 digital microscope with an exposure of 750 ms. Overall fluorescence intensity was calculated for a series of image fields, using Image Pro Plus. Comparison of the fluorescence intensity of the untreated and treated image fields provided an estimate of the degree of heparan sulfate and chondroitin sulfate removal associated with the heparinase treatment.

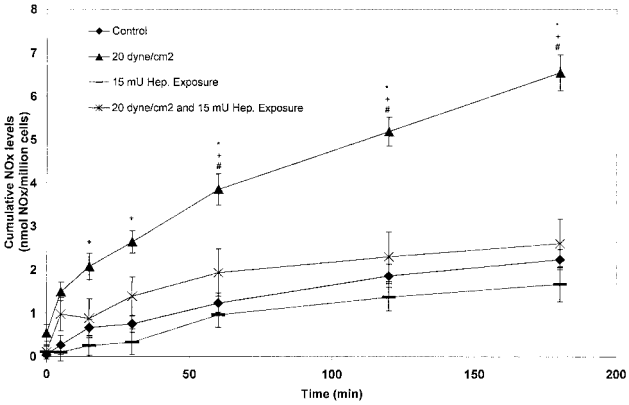

Figure 1. Effect of steady shear stress on NO_x production after heparinase treatment. NO_x production induced by 20 dyne/cm^2 steady shear stress (n=7) was significantly greater than control (*$P<0.05$, n=14), control with heparinase exposure (+$P<0.05$, n=13), and steady shear with heparinase treatment (#$P<0.05$, n=7). Treatment with heparinase substantially inhibited overall NO_x production. Data are represented as mean±SE.

Data Presentation and Statistical Analysis

Significant differences between group means were evaluated by repeated measures analysis where cumulative NO_x concentration was analyzed by a two-way (time and concentration) ANOVA using Minitab. NO_x concentrations were reported as mean±SE. A level of $P<0.05$ was considered significant for statistical analysis.

Results

Steady Shear Experiments

EC monolayers were exposed to 20 dyne/cm^2 steady shear stress with and without heparinase pretreatment, and stationary controls with and without heparinase pretreatment were run concurrently (Figure 1). Henceforth the conditions will be abbreviated as follows: stationary control (C), control with heparinase pretreatment (CH), steady shear of 20 dyne/cm^2 (SS), and steady shear of 20 dyne/cm^2 with heparinase pretreatment (SSH).

SS NO_x production was biphasic with an elevated production rate in the first 5 minutes that tapered off to a sustained rate by 60 minutes. SS NO_x concentration was elevated significantly relative to C NO_x concentration ($P<0.05$ after 30 minutes) and was 3-fold higher at 180 minutes.

The SSH response was dramatically inhibited, reaching a final concentration at 180 minutes that was not significantly different from C ($P>0.98$) or CH ($P>0.35$). The SSH response was significantly lower than the SS response after 60 minutes ($P<0.05$). C and CH concentrations were not significantly different from each other ($P>0.95$ at all times).

Oscillatory Shear Experiments

EC monolayers were exposed to oscillatory shear stress with a mean component of 10 dyne/cm^2 and a sinusoidal compo-

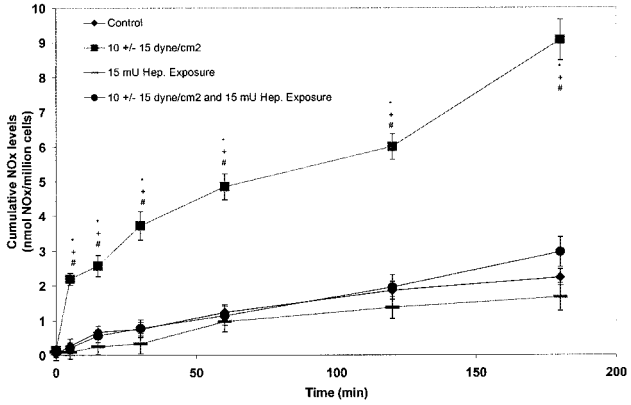

Figure 2. Effect of oscillatory shear stress on NO_x production after heparinase treatment. NO_x production induced by 10±15 dyne/cm^2 oscillatory shear stress (n=7) was significantly elevated above control (*P<0.01, n=14), control with heparinase exposure (+P<0.01, n=13), and oscillatory shear with heparinase exposure (#P<0.01, n=7). Treatment with heparinase greatly inhibited NO_x production. Data are presented as mean±SE.

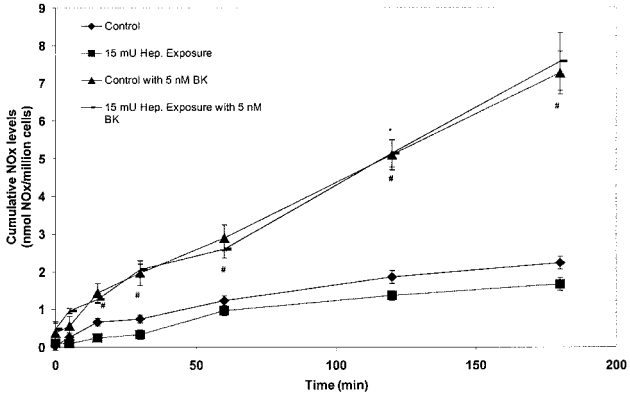

Figure 3. Effect of bradykinin on NO_x production after heparinase treatment. After application of 5 nmol/L BK, controls (n=5) displayed a significant increase in NO_x production in comparison to controls not exposed to BK (*P<0.05, n=14). After heparinase treatment, cells exposed to 5 nmol/L BK (n=5) displayed a significant increase in comparison to heparinase treated cells without BK (#P<0.05, n=7). Controls exposed to BK and heparinase treated cells exposed to BK had the same NO_x production (P>0.99). Data are presented as mean±SE.

nent of 15 dyne/cm^2 (10±15 dyne/cm^2) so that shear stress reversal (shear in the direction opposite the mean shear) would occur over a portion of the cycle (Figure 2). The abbreviations OS and OSH will be used to denote experiments with oscillatory shear and oscillatory shear with heparinase pretreatment, respectively.

OS experiments were characterized by rapid NO_x production in the first 5 minutes with a lower but sustained rate at later times. The final NO_x concentration at 180 minutes (9.07±0.60) was higher than in the SS experiments (6.54±0.42) and 4.1-fold higher than in C experiments. OS NO_x concentration was significantly higher than C NO_x concentration at all times (P<0.01).

Treatment with heparinase (OSH) completely eliminated the NO_x response to shear stress (Figure 2). The OSH response was significantly lower than the OS response at all times (P<0.01) and was reduced by 94% at 180 minutes.

Bradykinin and Histamine Experiments

EC monolayers were exposed to 5 nmol/L BK, and significant NO_x production was induced over a 3-hour period (Figure 3). The NO_x produced after 3 hours of exposure to BK was similar to the NO_x produced after 3 hours of exposure to steady or oscillatory shear stress (cf, Figures 1 and 2). When ECs were pretreated with heparinase, the NO_x produced in response to BK exposure was nearly identical to that of untreated cells (P>0.99 for all times). The BK response was significantly greater than the control (no BK) for both heparinase pretreated (P<0.05) and untreated (P<0.05) monolayers.

NO_x production in response to histamine (100 μmol/L) without heparinase reached a concentration of 4.50±0.36 (n=3) nmol NO_x/million cells after 3 hours of exposure, whereas the 3-hour response to the same dose of histamine after heparinase treatment was 3.95±0.71 (n=3) nmol NO_x/million cells. These responses were not significantly different from each other (P>0.23), but they were significantly different from the control response (no histamine or heparinase) of 1.45±0.34 (n=3) nmol NO_x/million cells (P<0.002).

Verification of Heparan Sulfate Removal by Heparinase III

Previously, Haldenby et al[32] reported that heparinase treatments were capable of removing a substantial fraction of heparan sulfate proteoglycans. To verify the removal associated with our heparinase pretreatment, both treated and untreated EC monolayers were labeled with an antibody specific to heparan sulfate and visualized with a fluorescently labeled secondary antibody before and after pretreatment. Representative images demonstrating this process are displayed in Figure 4. Clearly there was a substantial reduction of heparan sulfate after heparinase treatment that was quantified as a 45.9±13.8% (n=3) reduction in fluorescence intensity relative to the untreated controls. To verify that the heparinase pretreatment did not remove chondroitin sulfate proteoglycans, both treated and untreated EC monolayers were labeled with a biotinylated lectin that binds specifically to the *N*-acetyl-D-galactosamine residue present on chondroitin sulfate and visualized with a fluorescent labeled avidin conjugate. Representative images demonstrating this process are displayed in Figure 5. There was a negligible reduction of chondroitin sulfate after heparinase treatment that was quantified as a 1.0±1.4% (n=4) reduction in fluorescence intensity.

Protease Activity of Heparinase III

There was no detectable protease activity in the heparinase (15mU/mL) at any time interval tested. Specifically, after 3 hours of incubation in the protease assay, chymotrypsin protease activity varied from 214 to 420 fluorescence units as the concentration increased from 0.16 to 1.25 μmol/L. For the same concentration range, pronase activity varied between 135 and 520 fluorescence units. These data demonstrate the lower and upper limits of sensitivity of the assay (135 to 520 fluorescence units). Five replications of heparinase (15 mU/mL) incubation (3 hours) in the protease assay produced activity readings between

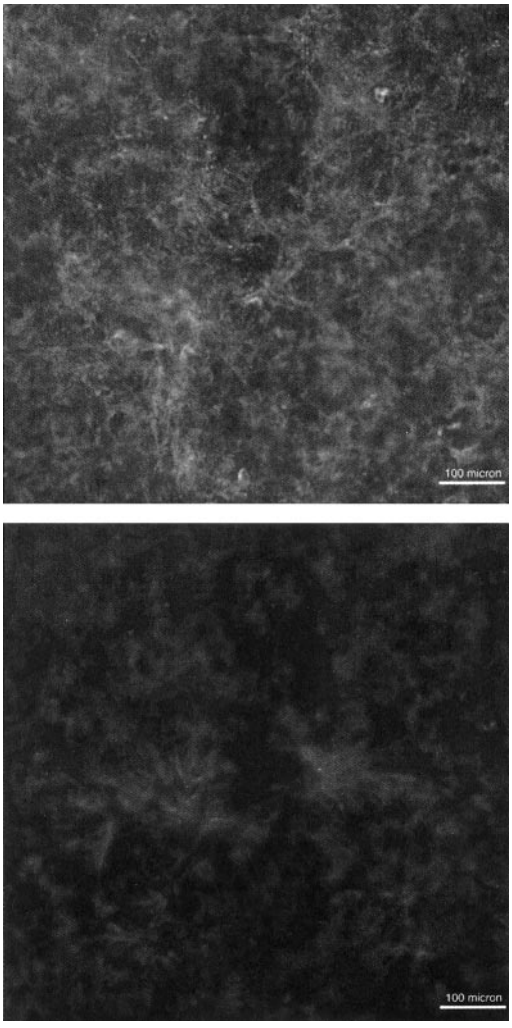

Figure 4. Images of fluorescent antibody to heparan sulfate without (top) and with (bottom) treatment with 15 mU/mL of heparinase for 2 hours.

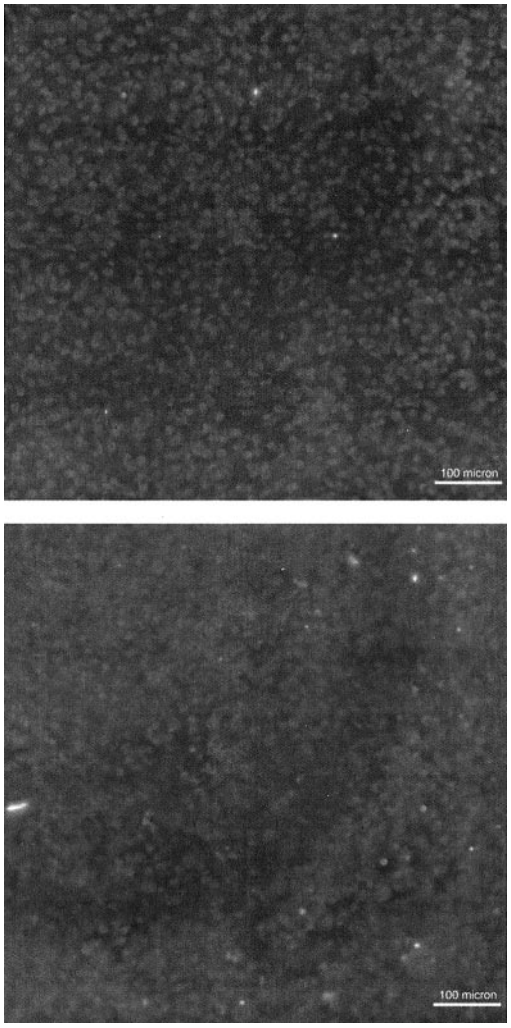

Figure 5. Images of fluorescent avidin conjugate bound to chondroitin sulfate without (top) and with (bottom) treatment with 15 mU/mL of heparinase for 2 hours.

104 and 121 fluorescence units, demonstrating that no detectable protease activity was present.

Discussion

A number of studies using cultured ECs have reported shear-induced NO_x production. Kuchan and Frangos[12] using human umbilical vein endothelial cells (HUVECs) exposed to steady shear observed a biphasic response characterized by an initial burst of NO_x production within minutes followed by a more gradual NO_x release over hours. It was also demonstrated that the initial response was G protein and Ca^{2+}-dependent, but independent of shear magnitude in the range of 6 to 25 dyne/cm^2.[12,15]

In contrast, the secondary response was G protein and Ca^{2+}-independent and shear level dependent. Frangos et al[33] also demonstrated that the initial elevated response was a result of the high rate of change of shear stress associated with the step change from zero to elevated (steady) shear stress. The initial response could be eliminated by ramping up the shear to the same steady level in a gradual fashion.[33] Chang et al[11] exposed BAECs to step changes in shear stress and observed a biphasic response of NO_x production similar to that reported for HUVECs.[12] In the present study, NO_x production in response to a step change to steady shear stress displayed the characteristic biphasic response (Figure 1) that has been described in earlier studies. NO_x production under oscillatory shear conditions was also characterized by distinct biphasic behavior with an elevated rate in the first 5 minutes followed by a more gradual production out to 3 hours (Figure 2). The mechanisms associated with the two phases of the oscillatory response have not been investigated, but it seems likely that the initial burst of production is associated with the sudden start up of the shear stress process.

Previous experiments have demonstrated that heparinase, an enzyme specific for heparan sulfates in the glycocalyx, has the ability to alter the thickness[34,35] and barrier function of the glycocalyx.[36] Dull et al[37] used the same concentration of heparinase as in the present study, and observed 67% removal of labeled sulfate ($^{35}SO_4$) in bovine lung microvascular endothelial cells. Haldenby et al[32] demonstrated that heparinase treatments were able to reduce fluorescence intensity by 27% to 51% using fluorescence-labeled wheat germ agglutinin in vitro. Results from the present experiments (Figure 4) indicated a reduction of

45.9±13.8% in the fluorescence intensity associated with a heparan sulfate antibody, which is consistent with previous studies. The specificity of the heparinase enzyme was further verified by demonstrating a negligible degradation of chondroitin sulfate (Figure 5) and undetectable protease activity. The effects of other glycoprotein-specific enzymes on the structure and function of the glycocalyx have also been examined. Desjardins and Duling[34] reported that neuraminidase, papain, pronase E, and clostripain had no effect on capillary tube hematocrit, a marker of glycocalyx thickness. Henry and Duling[38] observed that hyaluronidase increased access of 70- and 145-kDa dextrans to the endothelial surface layer but not larger dextrans or red blood cells.

Having demonstrated that our EC monolayers produced NO_x in response to shear stress in a manner consistent with previous reports in the literature and that we were able to substantially reduce the heparan sulfate GAG component of the glycocalyx through heparinase pretreatment, we were able to address the hypothesis that this GAG component is a shear stress sensor. After treatment with heparinase, NO_x production under steady shear stress was substantially inhibited (Figure 1). Oscillatory shear stress experiments with heparinase-treated EC monolayers were characterized by complete inhibition of NO_x production at all times (Figure 2). The viability of the NO production apparatus of our ECs after treatment with heparinase was also investigated by exposure to bradykinin and histamine in the absence of shear stress. We observed that BK (Figure 3) and histamine induced significant NO_x production that was not altered by pretreatment with heparinase. This indicates that heparinase treatment did not have any inhibitory effects on NO_x production unless flow (shear) was present. These experiments support our hypothesis that heparan sulfate is the shear sensor for NO production by EC.

An earlier study used the enzyme neuraminidase (0.2 U/mL) to remove sialic acid residues from saline-perfused rabbit mesenteric arteries and observed that flow-dependent vasodilation was abolished by a 30-minute pretreatment with the enzyme.[39] Because flow-dependent vasodilation is mediated by NO release in many arteries, and neuraminidase degrades the surface glycocalyx, this study is consistent with our observations. However, the interpretation of this whole artery study is less clear. The enzyme may have also affected the smooth muscle cells in the artery and thus the effect cannot be uniquely associated with the endothelium. In addition, the protease activity of neuraminidase was not assessed in that study as it was for heparinase in the present work.

There are also important differences in the structural alterations generated by various enzymes and their subsequent impact on flow-mediated signals that may be transduced into a biological response. As mentioned previously, neuraminidase digestion of the capillary surface was not associated with changes in capillary tube hematocrit and thus not likely to have caused marked changes in glycocalyx thickness. Heparinase digestion, however, was clearly associated with changes in glycocalyx thickness and glycocalyx barrier function. We believe these two observations are complimentary. Constituents of the glycocalyx form an entangled matrix covering the endothelial surface, and it is highly likely that mechanical properties of this surface layer are imparted not only by the scaffolding components, such as heparan sulfates, but also by associated macromolecules including sialic acid-containing glycoproteins, proteoglycans, and oligosaccharides that are degraded by neuraminidase. We have demonstrated that heparan sulfates are involved in flow-mediated mechanotransduction, but we do not exclude the possibility that other components of the glycocalyx participate in this process.

It is interesting to note that by removing only 45% of cell surface heparan sulfate, we could completely abolish shear-induced NO production. The ability of 45% heparan sulfate removal to totally abolish NO production suggests that a threshold of transmitted stress must be exceeded to stimulate NO production. Many signaling pathways require a "threshold level " of stimulation that must be attained in order to provide activation. We believe that the mechanotransduction system is no different. Partial removal of the mechanosensor may reduce the transmitted signal below the threshold for response. This is of course speculative because we do not yet know the details of the signal transduction mechanism downstream of the mechanosensor.

The underlying model associated with our hypothesis that heparan sulfate is a shear sensor is the following: as blood flow interacts with proteoglycans extending from the endothelial surface, a shearing force is applied to the outstretched heparan sulfate GAG component, displacing the core proteins from their unsheared orientation. This displacement is transmitted into the cell provoking a response.

Of particular interest in regard to NO production are G protein–linked receptors, which interact with G protein complexes. G proteins, in turn, are believed to be involved in eNOS activation. In HUVECs, the first phase of the NO response to a step change in shear stress is both G protein and Ca^{2+}-dependent, whereas the later, sustained phase does not depend on G proteins or Ca^{2+}.[15] We have observed in BAECs that the sustained phase is also inhibited by removal of the heparan sulfate shear sensor, suggesting that linkages in addition to G proteins are affected. A possible explanation lies in the myristoylation site at the N-terminus of eNOS, which may anchor itself to the plasma membrane, or possibly even to proteoglycans, which penetrate the plasma membrane.[40] The orientation of this linkage would likely be independent of both Ca^{2+} and G proteins. Further studies are required to evaluate this hypothesis.

A unifying hypothesis of mechanotransduction would likely require the involvement of cytoskeletal elements in series with the cell surface shear sensor. The arrangement of heparan sulfate as a shear sensor with subsequent signaling through the cytoskeleton is attractive for several reasons. First, heparan sulfates are abundant on the endothelial surface and, at least one class of HSPG, ie, syndecans, is known to associate with cytoskeletal elements including actin, either directly or through associated actin-binding proteins. Secondly, the NO pathway in endothelial cells has been shown to be regulated by the state of local actin filaments,[41] thus providing a mechanistic framework to link cell-surface heparan sulfates to the nitric oxide pathway. Support for this notion can be derived from studies on FGF-mediated signaling, where FGF-induced vasodilatation is mediated by NOS activation and requires heparan sulfates as a coligand; over expression of syndecan-4 enhances FGF-mediated NOS activity, as does the addition of exogenous heparan sulfate,[42] demonstrat-

ing the importance of heparan sulfate as a cofactor in NOS activation. Thus, physical association, whether direct or indirect, of heparan sulfates with actin and NOS provide a strong conceptual framework to evaluate mechanotransduction mechanisms. Studies are underway to evaluate the structural mechanisms underlying heparan sulfate-mediated mechanotransduction. To this end, we have mutants of syndecan-1 that have the heparan sulfate attachment sites deleted and a mutant with the cytoplasmic domain truncated. These mutant syndecans should allow us to further define the role of heparan sulfates, proteoglycan core proteins, and cytoskeletal interactions in endothelial mechanotransduction.

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