Microvascular and capillary perfusion following glycocalyx degradation

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Submitted 12 October 2006; accepted in final form 2 March 2007

Microvascular and capillary perfusion following glycocalyx degradation. J Appl Physiol 102: 2251–2259, 2007. First published March 8, 2007; doi:10.1152/japplphysiol.01155.2006.—Systemic parameters and microvascular and capillary hemodynamics were studied in the hamster window chamber model before and after hyaluronan degradation by intravenous injection of Streptomyces hyaluronidase (100 units, 40–50 U/ml plasma). Glycocalyx permeation was estimated using fluorescent markers of different molecular size (40, 70, and 2,000 kDa), and electrical charge. Systemic parameters (blood pressure, heart rate, blood gases) and microhemodynamics (vascular tone, velocity, and blood flow) remained statistically unchanged after injection of hyaluronidase, compared with inactivated hyaluronidase. Conversely, capillary hemodynamics were drastically affected. Functional capillary density, the capillaries perfused with red blood cells (RBCs), decreased by 35%, capillary Hct of the remaining functional capillaries increased from 16 to 27%, and penetration of 70-kDa fluorescent marker increased. Furthermore, plasma-only perfused capillaries statistically increased 30 min after hyaluronidase. The decrease in functional capillary density accounted for an increased RBC flux in the remainder of the capillaries, since the same number of RBCs had to traverse a reduced number of capillaries. Flux balances showed a reduction from baseline of 11% for the RBC flux and 20% for the plasma flux after treatment. These discrepancies are within the margin of error of the techniques used and could be explained by accounting for RBC over-velocity compared with plasma. These findings suggest that the decrease in the glycocalyx leads to capillary perfusion impairments.

functional capillary density; plasma volume; hyaluronan; capillary flow; red blood cell flux; tissue perfusion

ENDOTHELIAL CELL GLYCOCALYX is a network of polysaccharides that projects from the cellular surface of the endothelium into the vessel lumen. It was first identified by special electron microscopic staining techniques nearly 40 years ago (20). It has been shown to consist of proteoglycans and glycoproteins attached to the endothelial cell membrane and to bind a large number of plasma proteins essential for vascular function (4, 27, 33). It is reported that it may extend as much as 0.4–0.5 μm into the microvascular lumen, which would cause this material to occupy between 10 and 20% of vascular volume (10, 20, 32).

The glycocalyx extent has been documented primarily by visual evidence in microscopic studies and analysis of microhemodynamic changes induced by techniques that presumably change the extent of the glycocalyx. Desjardins and Duling (5) induced a significant increase in local capillary red blood cell (RBC) flux in the cremaster muscle by the intra-arteriolar injection of heparinase, while RBC velocity, capillary, and arteriolar diameter were unaltered. Smith et al. (28) induced a substantial change in the thickness of the endothelial surface layer by local degradation via illumination of the exposed tissue with light from a mercury vapor arc when the animal was infused with FITC 70-kDa dextran. They used intravital microscopic image velocimetry to determine fluid velocity profiles in the cell-free layer near the vessel wall and found the presence of a surface layer that impedes flow near the vessel wall of venules (28). Long et al. (19), using microscopic techniques based on the trajectory analyses of microscopic particles in blood, were able to estimate the cell-free layer thickness in microvessels (>20 μm diameter).

Glycocalyx studies generally focus on events in single microvessels or global changes in whole body glycocalyx volume. Few studies report microhemodynamic effects of systemic glycocalyx degradation (9, 10, 31, 33). Henry and Duling (10) administered hyaluronidase systemically, finding that this treatment increased the penetration of FITC-dextrans (70 and 145 kDa) to regions presumed to be occupied by the endothelial glycocalyx, but not RBCs or high-molecular-weight FITC-dextrans (580 and 2000 kDa). Interventions where the glycocalyx was affected systemically were carried out by Constantinescou et al. (4), who administered oxidized low-density lipoproteins and reduced the effective thickness of the glycocalyx as a consequence of oxygen-derived free radicals, causing significant increases in capillary tube hematocrit (Hct) and RBC flux. Electron microscopy studies of rat myocardial capillary endothelial surfaces show that the degradation of the glycocalyx instantly results in notable myocardial tissue edema (31). Short-term hyperglycemia rapidly decreases the ability of the glycocalyx to exclude 70-kDa dextran and decreases the lineal density of capillaries or number of capillaries with RBC flow per unit volume of tissue [functional capillary density (FCD)] (35). FCD is an important indicator of animal survival and microvascular metabolic maintenance in studies of resuscitation following hemorrhage (2, 15), being a more reliable indicator of survival than blood flow or tissue oxygen tension (2, 14).

In the present study, we addressed the question of whether hyaluronan degradation by Streptomyces hyaluronidase increases permeation of plasma components (and added tracer markers), changing capillary perfusion, organ flow distribution, and microhemodynamics. We hypothesize that the structural organization of the microvascular endothelial glycocalyx, determined in part by hyaluronan, influences capillary hemodynamics and FCD. To test this hypothesis, we used the
Hamster chamber window model to determine: 1) glycocalyx permeability measured by fluorescent markers with different molecular masses and electrical charge (24, 32); and 2) capillary perfusion and microhemodynamics.

**METHODS**

**Animal preparation.** Animal handling and care followed the “NIH Guide for the Care and Use of Laboratory Animals.” The experimental protocol was approved by the local animal care committee. Investigations were performed in male Golden Syrian Hamsters fitted with a dorsal window chamber (6). This model has been extensively used for investigations of the intact microvasculature of adipose, subcutaneous tissue, and skeletal muscle in conscious animals (6). A complete description of the preparation is given in Ref. 3. Pentobarbital sodium (50 mg/kg ip) anesthesia is used for window implantation and for carotid artery and jugular vein catheterization. Four to five days after the initial surgery, the microvasculature was examined, and only animals passing an established systemic and microcircular inclusion criteria, which includes having tissue void of low perfusion, inflammation, and edema, were entered into the study (3). All experiments were performed in awake hamsters without anesthesia and with baseline hemodynamics similar to that of previous studies with this model.

**Systemic parameters, blood chemistry, and biophysical properties.** Mean arterial pressure (MAP) and heart rate (HR) were recorded continuously (MP 150, Biopac System, Santa Barbara, CA). Arterial blood was collected in heparinized glass capillaries (0.05 ml) and immediately analyzed for arterial PO2, arterial PCO2, base excess, and pH (Blood Chemistry Analyser 248, Bayer, Norwood, MA). The comparatively low arterial PO2 and high arterial PCO2 of these animals are a consequence of their adaptation to a fossorial environment. Hct was measured from centrifuged arterial blood samples taken in heparinized capillary tubes (25 μl, ~50% of the heparinized glass capillary tube is filled). Hb content was determined spectrophotometrically from a single drop of blood (H-B hemoglobin, Henoque, Stockholm, Sweden).

**Plasma viscosity.** Plasma samples were obtained by centrifugation of the heparinized blood samples (2 ml) and separation of the supernatant plasma. The viscosity of plasma was determined with a cone and plate viscometer at a shear rate of 160 s−1 at 37°C (Dv-II+ Viscometer, Brookfield Engineering Laboratories, Middleboro, MA).

**Macromolecule penetration.** Fluorescent-marker suspension (5 mg/ml 40-kDa Texas Red dextran neutral, 5 mg/ml 70-kDa fluorescein dextran anionic, 5 mg/ml 70-kDa rodamine B dextran neutral, 5 mg/ml 2-MDa tetramerilrhodamine dextran anionic, and 5 mg/ml Alexa Fluor 680 albumin obtained from Molecular Probes, Eugene, OR) in 1% albumin-MOPS solution (0.25% bovine serum albumin, morpholinepropanesulfonic acid, buffer at 2 mM, normotonic, and pH 7.3; Angus Buffers & Biochemicals, Niagara Falls, NY) was used to guarantee pH stability of the fluorochromes. Fluorescent-marker suspension was given in bolus doses (100 μl) via jugular catheter. Blood samples (50 μl, heparinized glass capillary) were collected 2 min before dye fluorescent-marker infusion (background fluorescence) and 2 and 5 min after. Blood was sampled (40 μl, ~80% of the heparinized glass capillary tube is filled), and Hct was determined after capillary centrifugation. Capillary plasma (30–40 μl) was foil wrapped and stored at −20°C until analysis. Dye concentration measurements were performed after slow defrosting of the plasma samples at room temperature, transferred to a 10-μl quartz microcuvette (NSG Precision Cell, Farmingdale, NY), and the fluorescent signals were determined by an automated fluorescent system spectrofluorometer FluoMax-2 (Horiba) (25). Dye concentration was determined by linear regression of measured concentration at 2 and 5 min. Dye fluorescence calibration was performed by determination of fluorescent signals for each fluorescent marker. Fluorescent signatures were deconvolved by multiple nonlinear regression analysis, using the fluorescence of individual components to obtain plasma concentration of each fluorescent marker (maximum residual of multiple linear regression analysis was set as 5%). Each fluorochrome emission signature was assumed as Gaussian distribution, with mean value equal to wavelength with maximal fluorescence obtained during calibration (70-kDa fluorescein dextran = 492 nm; 70-kDa rodamine B dextran = 557 nm; 2-MDa tetramerilrhodamine dextran = 569 nm; 40-kDa Texas Red dextran = 596 nm; Alexa Fluor 680 albumin = 682 nm).

Previous studies report that 40-kDa dextran equilibrates quickly (penetrates) within the glycocalyx, whereas 70-kDa dextran does not penetrate the intact glycocalyx (32). Dextran (70 kDa) was used to obtain an estimate of total permeable volume of plasma and degraded glycocalyx (32). This approach does not calculate the glycocalyx volume; it uses the glycocalyx-permeable volume to macromolecules as an estimator for the degraded glycocalyx volume (24, 32). Concentration of 2-MDa dextran was used to establish plasma volume at each time point. The concentrations of dextrans allow us to calculate the changes in glycocalyx-permeable volume consequence degradations by hyaluronidase.

**Microhemodynamic parameters.** Arterial and venular blood flow velocity were measured using the photodiode cross-correlation technique (12) (Fiber Optic Photo Diode and Velocity Tracker model 102B; Vista Electronics, San Diego, CA). The centerline velocity (V) was corrected according to vessel size to obtain the mean blood velocity (18). The video image shearing technique was used to measure vessel diameter (D) online (11). Blood flow was calculated from the measured parameters as Q = πVX(D/2)2.

**Measurements of microvascular oxygen tension.** High-resolution noninvasive microvascular PO2 measurements were made using phosphorescence quenching microscopy (PQM) (13, 29). PQM is based on the oxygen-dependent quenching of phosphorescence emitted by albumin-bound metallocorporphin complex after pulsed light excitation. PQM is independent of the dye concentration within the tissue and is well suited for detecting hypoxia because its decay time is inversely proportional to the PO2 level, causing the method to be more precise at low PO2 values. This technique is used to measure both intravascular and extravascular PO2, since the albumin-dye complex continuously extravasates from the circulation into the interstitial tissue (13, 29). Tissue PO2 was measured in tissue regions in between functional capillaries. PQM allows for precise localization of the PO2 measurements without subjecting the tissue to injury. PO2 measurements were performed only at 90 min after resuscitation, at the last observation time point. These measurements provide a detailed understanding of microvascular oxygen distribution and indicate whether oxygen is delivered to the intestinal areas.

**FCD.** FCD is defined as the number of capillary segments with transit of at least a single RBC in a 60-s period per microscopic observation field. Detailed mappings were made of the chamber vasculature so that the same vessels studied at baseline could be followed throughout the experiment. This parameter is evaluated in 12 successive microscopic fields (totaling a region of ~1.6 mm2, 420 × 320 μm each) by systematically displacing the microscopic field of view by a field width in successive steps in the lateral direction. The first field is chosen by a distinctive anatomic landmark (i.e., large microvascular bifurcation) to easily and quickly reestablish the same observation fields at each observation time point. Each field has between 5 and 12 capillary segments with RBC flow. FCD (cm−1), i.e., total length of RBC-perfused capillaries divided by the microscopic field area of view, is evaluated by measuring and adding the length of capillaries with RBC transit in the field of view.

**Plasma perfused capillaries.** Fluorescence microscopy using epillumination for excitation and detection of fluorescein dextran was used to study plasma-only perfused capillaries. No additional fluorescent dextran was administered to previously injected animals to determine the penetration of fluorescent markers. Capillaries without flow were determined by comparing the detailed maps of the chamber...
vasculature drawn and recorded at baseline and the information of RBC and plasma-perfused capillaries.

**Capillary Hct.** Offline, frame-by-frame analyses of videotaped images were used to count the number of RBCs in capillary segments (40 \( \mu \text{m} \) long) over an observation period of 20 s. Final values are based on the mean of three measurements from the same capillary segment during the same time point (5). Capillary tube Hct was calculated from the number of cells in a specific length, the RBC mean corpuscular volume (58 \( \mu \text{m}^3 \) for the hamster) (26), and the capillary diameter.

**Capillary RBC velocity.** RBC velocity was estimated by determining the time required for a RBC to move a set distance (40 \( \mu \text{m} \)) over an observation period of 20 s. In most instances, RBC velocities were measured in the same capillary segment used to determine the capillary tube Hct.

**Plasma velocity by labeled platelets.** Blood was obtained and heparinized from a donor hamster, and platelet-rich interface was obtained upon blood centrifugation at 200 \( g \) for 20 min and collected and transferred to a fresh tube. Platelets were stained with 50 \( \mu \text{g} \) 5-(and-6)-carboxyfluorescein diacetate (Molecular Probes, Eugene, OR), labeled platelets were washed three times to remove excess dye and heparin, and lastly, resuspended in PBS to a final concentration of 5 \( \times 10^8 \) cells/ml. The 5-(and-6)-carboxyfluorescein diacetate-stained platelets (100 \( \mu \text{L} \), 5 \( \times 10^8 \) platelets per time point) were intravenously injected. Platelets were allowed a period of 5 min to recirculate until analysis of the labeled platelet velocity in the plasma-only perfused capillary was started. Off-line, frame-by-frame analysis of dye videotaped images included the quantitative assessment of platelet velocity during an observation period of 20 s.

**Enzyme dose response.** Streptomyces hyaluronidase (Sigma Chemical, St. Louis, MO) was given in bolus doses via the jugular catheter. At 10, 20, 30, 60, and 90 min after enzyme infusion, FCD and fluorescent marker penetration were measured and recorded. Enzyme dosage was tested with doses of 50, 100, and 150 units in saline, equivalent to at least 20, 40, and 60 U/ml plasma, respectively (based on a 2.5-ml plasma volume in a 60- to 70-g hamster).

**Enzyme treatment and controls.** Streptomyces hyaluronidase was given in bolus doses (0.1 ml) via jugular catheter. At 30, 60, and 90 min after enzyme infusion, fluorescent marker penetration, capillary perfusion, and microhemodynamics were measured. Control experiments were performed by replacing hyaluronidase with heat-inactivated hyaluronidase (95°C for 10 min).

**Experimental groups.** Animals were randomly divided before infusion into the following experimental groups: 1) hyaluronidase, and 2) control.

**Experimental setup and procedure.** The unanesthetized animals were placed in a restraining tube with a longitudinal slit from which the window chamber protruded. The animals were given 30 min to adjust to the tube environment before baseline systemic parameters (MAP, HR, blood gases, and Hct) were measured. The conscious animal in the tube was then fixed to the microscopic stage of a stereomicroscope equipped with a transillumination intravital microscope (BX51WI, Olympus, New Hyde Park, NY). The tissue image was projected onto a charge-coupled device camera (COHU 4815, San Diego, CA) connected to a videocassette recorder (AG-7355; JVC, Tokyo, Japan) and viewed on a monitor. Microvascular diameter, velocity, FCD, and oxygen tension measurements were carried out using a 40× (LUMPF-L-WIR, numerical aperture 0.8, Olympus) water immersion objective, Capillary Hct, capillary RBC velocity, and plasma velocity measurements were carried out using a 60× (LUMPF-L-WIR, numerical aperture 0.9, Olympus) water immersion objective. Detection of RBC passage was enhanced by increasing contrast between RBCs and tissue using a BG12 (420 nm) bandpass filter. The camera used had a frame size of 720 \( \times 480 \) at a rate of ~30 frames/s, which produced images of microcirculation, depending on the magnification used: 40× (175 \( \times 140 \mu \text{m} \)) and 60× (117 \( \times 94 \mu \text{m} \)).

Fields of observation and vessels were chosen for study at locations in the tissue where the vessels were in sharp focus. Detailed mappings were made of the chamber vasculature to record the vessel location and ensure that the same microvessels were studied throughout the experiment.

**Data analysis.** Results are presented as means (SD), unless otherwise denoted. Figures 1–3 are presented as box-whisker plots separating the data into quartiles, with the top of the box defining the 75th percentile, the line within the box giving the median, and the bottom of the box showing the 25th percentile. The upper whisker defines the 95th percentile; the lower whisker, the 5th percentile. Data within each group were analyzed using one-way ANOVA and, when appropriate, post hoc analyses performed with the Bonferroni’s multiple-comparison tests. Mann-Whitney test was used for comparisons between systemic parameters among groups. All data are presented as absolute values and ratios relative to baseline values. A ratio of 1.0 signifies no change from baseline, while lower and higher ratios are indicative of changes proportionally higher and lower than baseline. The same vessels and functional capillary fields were followed, allowing direct comparisons (repeated measurements) to their baseline levels, producing more robust statistics for small sample populations. Kurtosis test was used to characterize the relative peakedness or flatness of microvascular measurements compared with the normal distribution. Positive kurtosis indicates a relatively peaked distribution; negative kurtosis indicates a relatively flat distribution. Skewness test (skewness statistic) was used to characterize the degree of asymmetry of microvascular measurements around its mean. Positive skewness indicates a distribution with an asymmetric tail extending toward more positive values; negative skewness indicates a distribution with an asymmetric tail extending toward more negative values. All statistics were calculated using GraphPad Prism 4.01 (GraphPad Software, San Diego, CA). Fluorescent multiple nonlinear regression was calculated using OriginPro 7.5 software (Originlab, Northampton, MA). Changes were considered statistically significant if \( P < 0.05 \).

**RESULTS**

A dose of 100 units of hyaluronidase produced the greatest decreased FCD, increased capillary Hct, and penetration of fluorescent markers (70-kDa dextran anionic and neutral; albumin) into the glyocalyx volume. Dose of 50 units of hyaluronidase produced minor changes in FCD and capillary Hct, and 150 units decreased FCD and increased capillary Hct and penetration of fluorescent markers no further than 100 units. Therefore, using higher dosages was not justified. Similar findings were reported by Henry and Duling (10), who showed that 40–50 U/ml of hyaluronidase in plasma in the hamster cremaster microcirculation reduced 70- and 140-kDa dextran exclusion zones. This finding and our prior observations suggest that hyaluronan and other glycoconjugates assemble a matrix structure on the endothelial surface.

Animals were assigned randomly to the experimental groups: hyaluronidase \( [n = 18; 64.8 \text{ g (SD 3.9)}] \) and control \( [n = 18; 65.1 \text{ g (SD 4.4)}] \). Microcirculation was studied in all animals. Nine animals in each group were used for glyocalyx volume, three animals in each group were used for microvascular \( \text{PO}_2 \) and plasma viscosity determinations, and the remaining six animals of each group were used for only microcirculation studies. Each animal within the group was statistically similar (\( P > 0.30 \)) in systemic and microcirculation parameters at each time point compared with all of the other animals within its group.

**Macrohemodynamics and systemic parameters.** Throughout the entire course of the experiment, MAP, HR, arterial Hct, Hb,
and blood gases remained stable and did not significantly change from baseline. Blood-gas parameters (PO2 and PCO2) were different between groups at 45 min after injection. There were no statistically significant changes in systemic parameters between experimental groups at baseline and 90 min. Microhemodynamic and blood-gas parameters are presented in Table 1.

**Microhemodynamics.** Changes in vascular tone, velocity, and blood flow in the hamster window model were not statistically significant between experimental groups or among time points within the group. Hyaluronidase infusion produced increased spread of velocity distribution for arterioles and venules. Microvascular measurements confirmed findings of flow distribution using microspheres for the window model, showing no significant changes in microvascular flow (Fig. 1). Kurtosis and skewness for microhemodynamics were drastically affected 30 min after hyaluronidase infusion [diameter (-0.60/-0.22), velocity (-0.08/0.94), and flow (-0.04/0.78)] compared with inactivated hyaluronidase at an identical time [diameter (0.37/0.50), velocity (-0.02/-0.60), and flow (-0.45/0.64)]. Analysis of this result indicates a nonconsistent response in diameters with a tendency to increase blood velocity and consequently blood flow post-hyaluronidase infusion.

**Capillary perfusion.** Changes in RBC-perfused capillaries, plasma-only perfused capillaries, and capillaries without plasma or RBC flow are summarized in Fig. 2. Infusion of hyaluronidase significantly decreased FCD (capillary perfused with RBC) after 30, 60, and 90 min (P < 0.05). FCD was more severely decreased 60 min after infusion of hyaluronidase compared with 30 and 90 min after infusion. Hyaluronidase FCD was decreased at 30 and 60 min after infusion compared with control (P < 0.05). Plasma-only perfused capillaries increased at 30, 60, and 90 min for hyaluronidase, and at 60 and 90 for control (P < 0.05). Plasma-only perfused capillaries were statistically increased at 60 min after hyaluronidase infusion compared with other time points studied. Plasma-only perfused capillaries were increased after 60 and 90 min after inactivated hyaluronidase infusion (control). Hyaluronidase treatment increased plasma-only perfused capillaries at 30 and 60 min after infusion compared with control (P < 0.05). Nonflowing capillaries increased at 60 and 90 min after infusion of hyaluronidase and at 90 for control animals (P < 0.05). Nonflowing capillaries increased at 60 and 90 min after infusion of hyaluronidase; infusion of plasma-inactivated hyaluronidase increased the nonflowing capillaries 90 min after infusion. Hyaluronidase treatment increased nonflowing capillaries at 60 and 90 min after infusion compared with control (P < 0.05). Before enzyme infusion, capillary RBC velocity was 127 μm/s (SD 29) (n = 22). Sixty minutes after enzyme infusion, capillary RBC velocity for the control group was 118 μm/s (SD 32) (n = 18) and 82 μm/s (SD 25) (n = 14) for the hyaluronidase group (P < 0.05 compared with before infusion and control). Plasma velocity in capillaries perfused with only plasma 60 min after enzyme infusion was 159 μm/s (SD 36) (n = 12) for hyaluronidase (P < 0.05) compared with RBC capillary velocity for hyaluronidase, control, and before infusion.

**Capillary Hct.** Infusion of hyaluronidase increased capillary Hct at 30 and 60 min (P < 0.05) (Fig. 3). Inactivated hyaluronidase did not change capillary Hct. Changes in capillary Hct were significant between hyaluronidase and control at 30 and 60 min after infusion (P < 0.05).

**Plasma viscosity.** Plasma viscosity was measured from blood samples obtained from three treated animals at 45 min after treatment, the time of the maximal hyaluronidase effect on glyocalyx volume. Plasma viscosity at baseline is 1.20 cP (SD 0.7) (from previous studies), and, at 45 min after treatment, the time of maximal effect on FCD, it was 1.35 cP (SD 0.6), a difference that was statistically significant.

**Microvascular PO2.** There was no statistical difference between groups in the blood PO2 measured in arterioles and venules, 45 min after infusion. Tissue PO2 at baseline was 21 Torr (SD 2), and, at 45 min after hyaluronidase administration, it was 20 Torr (SD 2), which was not statistically different.

**Effect on macromolecule penetration.** At baseline, the permeable volume to 40-kDa dextran was calculated using plasma concentration. This volume was significantly larger than the permeable volumes for 70-kDa dextran (anionic and neutral), albumin, and 2-MDa dextran. Dextran (40 kDa) was permeable within the endothelium glyocalyx. The second dose of fluorescent marker was infused 45 min after infusion of active or inactivate hyaluronidase. After infusion, 40-kDa dextran-permeable volume remained the largest permeable volume compared with the other markers (anionic and neutral 70-kDa dextran, albumin, and 2-MDa dextran). The hyaluronidase group (45 min after infusion) presented an increased permeable volume to 70-kDa dextran (anionic and neutral) and albumin compared with baseline and compared with control (P < 0.05), showing an increased penetration of molecules of ~70 kDa (macromolecules) into the glyocalyx. Molecules of different natures (dextran and albumin) and independent of the electrical charge (anionic and neutral 70-kDa dextran) infiltrated within.

### Table 1. Laboratory parameters

<table>
<thead>
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<th>Parameter</th>
<th>Baseline</th>
<th>45 min</th>
<th>90 min</th>
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<tr>
<td>Hct, %</td>
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<td>48 (2)</td>
<td>48 (3)</td>
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<tr>
<td>Hb, g/dl</td>
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<td>Heart rate, beats/min</td>
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<td>430 (38)</td>
<td>447 (45)</td>
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<tr>
<td>PaCO2, Torr</td>
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<td>50.2 (5.2)</td>
<td>47.4 (5.5)*</td>
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<tr>
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<td>7.36 (0.02)</td>
<td>7.34 (0.02)</td>
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<td>BE, mmol/l</td>
<td>3.0 (1.7)</td>
<td>3.9 (2.0)</td>
<td>1.0 (2.2)</td>
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</table>

Values are means (SD). Hct, systemic hematocrit; Hb, hemoglobin content of blood; MAP, mean arterial blood pressure; PaO2, arterial partial O2 pressure; PaCO2, arterial partial pressure of CO2; BE, base excess. *P < 0.05, hyaluronidase vs. control in the same time period.

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the endothelial glycocalyx volume after hyaluronidase infusion. Plasma-permeable volume for large molecules measured with 2-MDa dextran did not change. A third dose of fluorescent marker was infused 90 min after infusion of active or inactive hyaluronidase. At 90 min, 70-kDa dextran and albumin-permeable volumes for control and hyaluronidase were similar to the volumes measured at baseline, indicating that the effects induced by the hyaluronidase infusion were transitory. Figure 4A shows dye concentration, and Fig. 4B shows permeable volume of the different fluorescent macromolecules for control and hyaluronidase at baseline, 45 and 90 min after infusion. Estimated glycocalyx-impermeable volumes (40-kDa dextran, 70-kDa dextran, or albumin) are presented in Fig. 4C. Plasma volumes were calculated from concentration of 2-MDa dextran and were 1.79 ml (SD 0.52) for control and 1.76 ml (SD 0.37) for hyaluronidase at baseline.

DISCUSSION

The principal finding of this study is that glycocalyx hyaluronan degradation by Streptomyces hyaluronidase caused a significant change in the distribution of RBCs in the capillary network. The decrease in FCD was accompanied by an increase in capillary Hct of the remaining functional capillaries and an increase in plasma-only perfused capillaries. These two effects approximately balanced each other, such that the decrease in FCD (capillaries with RBC flow) accounted for the increase in RBC flux (RBC velocity \times capillary Hct) in the remaining capillaries, since the same number of RBCs had to traverse a smaller number of capillaries, while the fluid displaced by the increased number of RBCs in the functional capillaries was shunted into the capillaries with plasma flow only. Since neither arteriolar nor venular flow was significantly changed, capillary blood flow as a whole did not change, a finding also reported by Desjardins and Duling (5). Similar changes of capillary Hct were reported before and after partial glycocalyx degradation (4, 5). Decrease in FCD was also reported after induced glycocalyx injury by acute hyperglycemia (35), hyaluronidase infusion (10, 33), and increased permeability by macromolecules. There were no significant changes due to the ionic nature of the dextran marker used.

The significant decrease in FCD was not accompanied by a change of tissue PO2, a result that is in accordance with the concept that these tissues are predominantly supplied with oxygen by the arterioles (30).

Glycocalyx integrity was measured by the changes in permeation differences of fluorescent markers. Permeation increased 45 min after hyaluronidase infusion; a 20% increase in macromolecule permeability augmented the effective plasma volume for molecules up to 65 kDa (20% of the plasma volume, Fig. 4). Macrovascular hemodynamics (MAP, HR, cardiac output, and vascular resistance) and microhemodynamics remained unchanged after hyaluronidase compared with inactivated hyaluronidase. Glycocalyx permeability is determined by hyaluronan, while its volume is regulated by proteoglycans (24, 32). The total plasma volume measured by 70-kDa dextran in these experiments is estimated to be \(\sim 1.95 \text{ ml} \) (or a total blood volume of 3.75 ml). The glycocalyx volume averages \(~0.75 \text{ ml} \) or \(~20\% \) of vascular volume, which is smaller than the value of 35% estimated by Nieuwdorp et al. (23) in humans. In our experiments, the glycocalyx-impermeable vol-

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Fig. 1. Relative changes from baseline in arteriolar and venular hemodynamics. Dotted line represents baseline level. Diameters [\(\mu\text{m} \text{, means (SD)} \)] in A (arteriolar) and B (venular) for baseline in each group: hyaluronidase [arterioles (A): 54.8 (SD 6.4), \(n = 62\); venules (V): 61.7 (SD 7.6), \(n = 68\); control [A: 55.4 (SD 7.2), \(n = 54\); V: 59.8 (SD 6.7), \(n = 56\)]. Red blood cell (RBC) velocities [mm/s, means (SD)] in C (arteriolar) and D (venular) for baseline in each animal group: hyaluronidase [A: 4.6 (SD 1.0); V: 2.5 (SD 0.6)]; control [A: 4.5 (SD 1.5); V: 2.6 (SD 0.5)]. Calculated flows [nl/s, means (SD)] in E (arteriolar) and F (venular) for baseline in each animal group: hyaluronidase [A: 12.8 (SD 2.9); V: 6.7 (SD 2.5)]; control [A: 6.7 (SD 1.6); V: 3.2 (SD 1.1)]. \(n\) = Number of vessels studied.
volume was reduced to 0.40 ml at the time of the maximal effect, indicating that as much as 0.35 ml of hyaluronan (or ~50% of the glycocalyx volume) are cleaved by hyaluronidase, increasing effective plasma-permeable volume to low-molecular-weight molecules. The increase in blood viscosity was found to be the same as that attained by adding hyaluronic acid to plasma to achieve a concentration of 1% by weight. Thus the concentration of hyaluronan on the surface of the endothelium appears to be on the order of 5% by weight.

Remarkably, there was no significant difference in the estimated glycocalyx-permeable volume measured using anionic and neutral 70-kDa dextran. On the other hand, as found in previous studies, albumin provided a lower estimate of the glycocalyx-permeable volume as a consequence of its slow diffusion into the endothelial surface layer (32).

The finding of the decrease in FCD coupled with the increase in Hct in the remaining capillaries suggests that reduction of the glycocalyx, resulting in increased capillary Hct, is not sufficient per se to account for the disparity between tube and discharge Hct found in capillaries. FCD is defined as the density of capillaries with the passage of RBCs (Fig. 5). Capillaries with the passage of only plasma may be functional. However, plasma flow is difficult to discern, and there is substantial evidence that the definition based on RBC flow is functionally adequate to describe the relationship between tissue survival and FCD (14). In most tissues at rest, 100% of the capillaries are perfused with blood, and increases are seldom seen and do not exceed >105%. Determinants of FCD include capillary pressure, endothelial swelling, and leukocyte and RBC adhesion. Hemodynamic effects at bifurcations cause changes in Hct in the subsequent branching and could change FCD, if RBCs were completely prevented from entering a slowly flowing branch. The extent of the glycocalyx should also play a role in determining capillary Hct and change of FCD. It could be argued that degradation of the extent of the glycocalyx would facilitate the entrance of RBCs into a branching segment. However, the present study and that of Zuurbier et al. (35) show the opposite effect.

To determine whether the increase in plasma-perfused capillaries is due to displacement of fluid from RBC-perfused capillaries with greater Hct values, we performed a RBC and plasma flux balance. The nominal value of RBC flux was calculated using data on FCD and Hct. The result was set at 100% for the 30-min baseline. The corresponding value for treated tissue was 89%, a difference well within error due to the approximations made. The same calculation made to determine the flow of plasma shows that, at 30 min, total plasma flow in the capillaries was 80% of baseline. In this calculation, it is assumed that the capillary cross-sectional area is increased in accordance with the glycocalyx reduction. A factor not taken...
into consideration is the higher velocity of RBCs relative to plasma, which has been estimated to be as much as 29% (1), and hence would significantly reduce the contribution of plasma flow in the capillaries with RBC flow. Furthermore, measurements were not perfectly synchronized, the maximal effect on hemodynamics being at 30 min, while the maximal effect on the glycocalyx was determined at 45 min, with most of the effects being over at 90 min.

Notably, neither systemic Hct nor arteriolar flows changed due to treatment. Since changes in glycocalyx integrity minimally affect larger vessel volumetric flow, it would seem that there was the same arteriolar flux of plasma and RBCs at baseline and at the time of the maximal effect of hyaluronidase (30 min), indicating that the observed changes in FCD must result in a redistribution of RBCs and plasma flux in the capillary network, as evidenced by the microhemodynamic results of this study.

Fig. 4. Plasma fluorescent marker concentration and estimated macromolecule-permeable volume. A: macromolecule concentrations for inactivated hyaluronidase infusion (control) and after hyaluronidase infusion (hyaluronidase). Shadow areas at base of bars at 45 and 90 min indicate background dye concentrations before second and third dose of fluorescent markers. B: distribution volume of macromolecules after hyaluronidase infusion. 40-kDa dextran (Dex_{40k}), a glycocalyx-permeable molecule diffused free through the proteoglycans network on the endothelial surface layer. 70-kDa dextran and albumin (Alb) do not penetrate the glycocalyx and are retained in the vasculature, whereas the 40-kDa dextran penetrates the glycocalyx within minutes (24, 32). From the concentrations and the known amount of infused dextrans and albumin, the distribution volumes for 40-kDa dextran, 70-kDa dextran [neutral (Dex_{70kN}) and anionic (Dex_{70kA})], and albumin were calculated for each experiment. C: estimated glycocalyx-impermeable volume after hyaluronidase infusion. Changes in concentration of 70-kDa dextran (neutral and anionic) and albumin compared with 40-kDa dextran (glycocalyx permeable) were used to calculate glycocalyx-impermeable volume [volume 40-kDa dextran / H10021 70-kDa volume (neutral or anionic) or volume albumin]. Dex_{2M}, 2-MDa dextran. †P < 0.05 relative to baseline; *P < 0.05 among groups.

Fig. 5. Micrographs of changes in capillary perfusion after hematocrit. A: functional capillaries perfused with RBCs. B: capillaries perfused with only plasma and without perfusion. Capillaries perfused with only plasma had fluorescent platelets moving with the plasma. Capillaries without perfusion had a low fluorescence level and do not have any cell flow. C: representation of all capillaries in the microphotographs. Lines illustrate the capillary network: black lines show capillaries with RBC flow, dark gray line with plasma flow, and light gray line without flow.
The approximate balance between capillaries with flowing RBCs and those empty of RBCs showing the flow of fluorescent marker capillaries indicates that some of the capillaries become nonfunctional without being occluded. The cause behind the decrease of RBC perfusion of a substantial number of capillaries due to the introduction of hyaluronidase is not visually apparent. The thickness of the tissue hinders the optical resolution, rendering ineffective the gains attainable with proper tissue illumination via the use of a substage condenser positioned and matched to produce maximal resolution by the objective. As a whole, the change in blood vessel dimensions (arterioles, venules) due to the glyocalyx degradation should be small. At the capillary level, which is the largest area of the cardiovascular system, an increase in plasma-permeable volume corresponds to an increase in capillary vessel diameter, a change of ~0.2–0.3 μm for a capillary diameter of 5 μm, which is typical for this tissue [measured capillary diameter was 5.1 μm (SD 0.5) for hyaluronidase and 4.9 μm (SD 0.4) for control].

Flow velocity in the non-RBC flowing capillaries was statistically higher than in functional capillaries, suggesting that increasing the population of RBC empty-flowing capillaries may be due to hydrodynamic effects rather than anatomical changes. It is possible that the capillary flow distribution characterized by nearly 100% FCD is the result of a tissue microvascular adaptation process involving hydrodynamic effects at bifurcations, and the remodeling of capillary diameters so that each bifurcation presents the distribution of hydraulic resistance that ensures the entrance of RBCs to each daughter branch, resulting in all capillaries being perfused by RBCs. Changing the vessel diameter introduces subtle differences in the distribution of capillary hydraulic resistances and entry conditions that may cause a departure from the optimal steady-state distribution of RBCs, resulting in the decrease of FCD. As an example of this effect, Kim et al. (17) found that reduction of flow alone decreased FCD due to increased plasma skimming (i.e., phase separation), rather than red cell stasis in capillaries (17).

There are no reports in the literature on the effects of hyaluronidase on FCD. However, Zuurbier et al. (35) found the decrease of glyocalyx-impermeable volume (due to short-term hyperglycemia) lowers FCD. These authors propose this may be due in part to increased leukocyte adhesion (16). It has also been proposed that the decrease of proteoglycans and glycoproteins in the glyocalyx reduces the endothelium-derived nitric oxide release via mechanotransduction, causing vasoconstriction (7, 22). However, our experiments did not find evidence of pressure changes, indicating that the change in FCD was not related to significant changes in peripheral vascular resistance, as seen in other studies (8). These considerations suggest that changes in FCD may be directly related to effects at the capillary level. Changes in the glyocalyx lead to significant changes in transendothelial permeability. However, it could also involve the endothelial cell membrane permeability. A change in endothelial cell permeability can lead to endothelial swelling, which has been shown to be a factor in determining FCD (21). The possibility of a heterogeneous response in the capillary network cannot be excluded.

Plasma (and therefore blood viscosity) increased after administration of hyaluronidase due to increased levels of circulating hyaluronan, leading to the increase of plasma protein content. In principle, this may be a negative effect contributing to the decrease of FCD. However, studies by Tsai et al. (29) have shown that increasing plasma viscosity can be of significant benefit, causing shear stress-related vasodilation and maintenance of FCD. Therefore, the negative effects arising from acute glyocalyx degradation may be, in part, mitigated by the increase in blood and plasma viscosity.

In conclusion, changes in the permeability of macromolecules (infiltration) in the glyocalyx, induced by the introduction of hyaluronidase, increased the permeable plasma volume in which molecules of up to 65 kDa can be in solution. This effect is usually interpreted as a change in glyocalyx integrity and/or a decrease in glyocalyx volume. This effect is accompanied by a significant redistribution of RBC flow in the capillary network, leading to a significant increase in the presence of RBC empty, flowing capillaries. There is a corresponding decrease in FCD, showing that alterations in the glyocalyx cause capillary perfusion to become less than optimal. This result suggests a mechanism by which glyocalyx impairment may lead to disease conditions.

ACKNOWLEDGMENTS

The authors thank Froilan P. Barra and Cynthia Walser for the surgical preparation of the animals.

GRANTS

This work has been supported by Grants R01-HL76182 to A. G. Tsai, and R24-64395, R01-62354, and R01-62318 to M. Intaglietta.

DISCLAIMER

M. Intaglietta is a member of the Board of the La Jolla Bioengineering Institute.

REFERENCES