The Endothelial Glycocalyx Protects Against Myocardial Edema

Bernard M. van den Berg, Hans Vink, Jos A.E. Spaan

Myocardial tissue edema attributable to increased microvascular fluid loss contributes to cardiac dysfunction after myocardial ischemia, cardiopulmonary bypass, hypertension, and sepsis. Recent studies suggest that carbohydrate structures on the luminal surface of microvascular endothelium are essential to prevent tissue edema. We carefully preserved these structures for visualization with electron microscopy, revealing that the rat myocardial capillary endothelial surface layer is coated with a 0.2- to 0.5-μm-thick carbohydrate layer and that its degradation instantly results in notable myocardial tissue edema.

Materials and Methods

Perfusion and Tissue Preparation

In accordance with institutional guidelines, hearts of anesthetized male Wistar rats (body weight 250 to 350 g) were exposed, and after transecting the vena cava, the aorta was cannulated retrogradely at the aorta/brachiocephalic trunk bifurcation. Perfusion started with the removal of blood by oxygenated calcium-free cardioplegic solution containing 0.1% BSA (CCS-BSA) (pH 7.4, 37°C) at 8 mL/min for 3 minutes, 54 ± 1.9 mm Hg (mean ± SE). Next, hearts were perfused with a phosphate-buffered fixative (pH 7.4) containing 30 mmol/L MgCl₂ at 8 mL/min (62 ± 1.4 mm Hg) in which after 2 minutes Alcian blue 8GX (Sigma) was added (final concentration, 0.05%) and continued for the next 30 minutes at room temperature (79 ± 5.3 mm Hg). Tissue segments were postfixed with 1% osmium tetroxide (Electron Microscopy Sciences) and 1% lanthanum nitrate (Sigma) in water for 1 hour at room temperature followed by 1% aqueous Uranyl acetate for 1 hour and processed additionally for electron microscopy. Digital pictures of ultra-thin sections of capillaries were obtained with a built-in Megaview II CCD camera and processed with analySIS image-analytical software (both from Soft Imaging Systems GmbH).

Hyaluronidase Treatment of Rat Heart

Before fixation and staining, hearts were perfused with oxygenated CCS-BSA (37°C) containing 25 IU/mL of hyaluronidase (bovine testis, fraction IV-S, Sigma), benzamidene-HCL (1 mmol/L, Sigma), and 6-amino-n-caproic acid (5 mmol/L, Sigma) for 1 hour at 8 mL/min. Control hearts were perfused with the preceding solution, except hyaluronidase, as shown above.

Data Analysis

Pictures were analyzed using Image-Pro Plus software version 3.0 (Media Cybernetics). Mean glycocalyx thickness of 429 normal and 196 hyaluronidase-treated capillaries was determined to be the distance between luminal membrane and the optical background density plus 2×SD, representing ~95% of detectable stained structures. Results are given in box plots with 5th and 95th percentiles shown, and difference in glycocalyx thickness of normal and hyaluronidase-treated capillaries was assessed by means of Mann-Whitney U nonparametric test.

Endothelial cell thickness was determined by subtracting the inner from the outer capillary diameter, with diameters calculated from measured perimeters of endothelial cells without a nucleus shown. Endothelial cell thickness was expressed as mean ± SD.

Pericapillary space was determined by subtracting the outer capillary diameter from the inner diameter of surrounding myocardial tissue. Distributions of calculated pericapillary spaces given in 0.2-μm intervals are expressed as a median value, and difference between normal and hyaluronidase-treated capillaries was assessed by means of Mann-Whitney U nonparametric test.

A discussion of detailed methods of perfusion and tissue preparation and gold-labeled lectin binding can be found in the online data supplement, available at http://www.circresaha.org.

Results

Luminal endothelial surfaces of stained myocardial capillaries were coated with evenly distributed discrete hairy-like bushes (Figures 1A and 1C, left). Colocalization of lectins C. ensiformis and S. tuberosum to these extended stained structures confirmed its saccharine nature (see the online data supplement and online Figure 1). Enzymatic degradation with hyaluronidase before fixation and staining resulted in a condensed endothelial cell surface staining (Figures 1B and 1C, right), confirming the presence of surface-bound hyaluronan on normal myocardial capillaries. Normal myocardial
capillary glycocalyx, with a median overall surface coat 182 to 512 nm thick (95th percentile), was significantly higher \((P<0.001)\) than the glycocalyx found on hyaluronidase-treated capillaries of 77 to 201 nm thick (Figure 2).

Enzymatic degradation of the endothelial glycocalyx had no effect on endothelial cell thickness. With a mean endothelial cell thickness of 0.18±0.04 μm (n=270) and 0.16±0.03 μm (n=124) in normal and hyaluronidase-treated capillaries, respectively, these cells were well within the normal range. However, the interstitial space between capillaries and their surrounding tissue was significantly \((P<0.001)\) affected on treatment with hyaluronidase (Figures 3A and 3B). Although most of the normal vessels were surrounded by an interstitium with a median dimension of 0.28 μm (Figure 3A), distribution of the interstitial space in hyaluronidase-treated capillaries was clearly shifted to the right and resulted in a median value of 0.46 μm (Figure 3B).

**Discussion**

The observed dimension of the stained structures displayed a median thickness of 0.2 to 0.5 μm (95th percentile) that is consistent with previous, indirect estimates of 0.3 to 1.0 μm in mammalian skeletal muscle tissues. Hyaluronidase treatment altered the hairy-like structures into a condensed stained layer with a median thickness of 0.08 to 0.2 μm, resembling earlier electron-microscopic visualizations of the glycoca-
lyx. Moreover, hyaluronidase treatment significantly increased the pericapillary space, indicating a protective role for hyaluronan in preventing tissue edema.

Although these electron micrographs show for the first time hairy-like stained structures of a hyaluronate nature on the luminal endothelial cell surface, the presence of luminal bound hyaluronan is suggested previously by others using colloidal gold-labeled hyaluronan binding proteins. Furthermore, elongated structures of a hyaluronate nature have been demonstrated within filamentous plugs from fenestrated capillaries and as part of a pericellular matrix from smooth muscle cells.

![Figure 2](image-url)  
**Figure 2.** Distribution of glycocalyx thickness within normal capillaries (no treatment) and hyaluronidase-treated capillaries. Box plots indicate median values with 5th and 95th percentiles. $^P<0.001$ by means of Mann-Whitney U nonparametric test.

![Figure 3](image-url)  
**Figure 3.** Normalized frequency distribution of pericapillary spaces of normal (no treatment) and hyaluronidase-treated capillaries. Difference in pericapillary space between normal and hyaluronidase-treated capillaries was assessed by means of Mann-Whitney U nonparametric test.
The capillary endothelial cell thickness was not affected on hyaluronidase treatment of the endothelial glycocalyx and remained well within the normal range of between 0.17 and 0.23 μm. Intravital measurements revealed that local hyaluronidase treatment only increased glycocalyx permeability for dextran molecules of 70 and 145 kDa, but larger ones still remained excluded from the glycocalyx and did not move across the capillary wall. These functional data support the possibility that hyaluronidase can affect glycocalyx permeability in the absence of evident endothelial membrane damage and associated gross increases in capillary wall permeability.

With regard to the observed heterogeneity in stained structures, different local conditions within the coronary vascular bed itself may account for the observed variety in glycocalyx staining. Besides the topological structure of the coronary arterial tree as a major determinant of a heterogeneous blood flow distribution, other factors of influence include networks, 19 which result in greater red cell deformation, and reduced glycocalyx compression at high flow rates. 5,20 Heterogeneously distributed shear forces might cause a natural heterogeneity in glycocalyx thickness or result in differences in Alcian blue staining and in turn loss of carbohydrate structures.

Numerous factors are described that may modulate the integrity of the endothelial cell glycocalyx. Enzymatic degradation of carbohydrate brushes clearly increases the dimension of the pericapillary interstitial space, indicating that microvascular endothelial carbohydrate damage during ischemia reperfusion, 11 during hypoxia, 12 and after exposure to atherogenic plasma levels of oxidized low-density lipoprotein 21,22 most likely contributes to the associated tissue edema. Present results give additional evidence for the importance of a thick glycocalyx in the control of vascular integrity. 7,8

Conclusion
Electron micrographs demonstrated that surface-bound hyaluronan is a major determinant of the endothelial glycocalyx and that its degradation from the coronary endothelial surface results in myocardial tissue edema. Additional discussion paragraphs can be found in the online data supplement, available at http://circres.ahajournals.org.

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References
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Materials and Methods.

**Animal preparation.** Male Wistar rats (body weight 250-350 g) were anesthetized by intraperitoneal administration of 1.6 ml per kilogram Aescoket Plus (60 mg ketamine, 2 mg xylazine-base, 0.4 mg atropine-base; Aesulaap BV, Boxtel, The Netherlands). After trachea was cannulated for respiration (1.5 ml stroke volume at 95 times per minute), hearts were exposed by thoracotomy. All procedures were in accordance with institutional guidelines.

**Perfusion and tissue preparation.** After transecting the vena cava, the aorta was cannulated in retrograde at the aorta/brachiocephalic trunk bifurcation and lumen of left ventricle was drained. Perfusion of hearts at a constant flow of 8 ml/min was started with removal of blood by oxygenated calcium-free cardioplegic solution (CCS, in mmol/L: 5.55 glucose, 114 NaCl, 10 KCl, 1.18 KH₂PO₄, 1.17 MgSO₄·7H₂O, 25 NaHCO₃, 5.0 HEPES, 0.025 EDTA) containing 0.1% bovine serum albumin (CCS-BSA; pH 7.4, 37°C) for 3 minutes, 54 ± 1.9 mm Hg (mean ± SE). Next, fixation staining with 1% glutaraldehyde and 4% paraformaldehyde in a 84 mM sodium dihydrogen phosphate buffer (fixative, pH 7.4) containing 30 mmol/L MgCl₂ (62 ± 1.4 mm Hg), after 2 minutes Alcian blue 8GX¹ (Sigma Chemical Co., St. Louis, Missouri) was added (final concentration 0.05%) and continued for the next 30 minutes at room temperature (79 ± 5.3 mm Hg). Dissected tissue segments of approximately 1 x 2 mm, from the left free ventricle wall were fixated for another 60 minutes in fixative alone. Tissue segments were post-fixed with 1% aqueous osmium tetroxide (Electron Microscopy Sciences, Fort Washington, Pennsylvania) and 1% lanthanum nitrate hexahydrate² (Sigma) for 1 hour at room temperature, followed by en-bloc contrasting with 1% aqueous Uranyl acetate for 1 hour and dehydration steps in ethanol and propylene oxide and embedding in Epon 812 (Serva Feinbiochemica, Heidelberg, Germany). Ultra-thin (80 nm) sections of capillaries in cross-section on copper grids were examined in a Phillips EM-
420 electron microscope set at 100 kV. Digital pictures were obtained with a build-in
Megaview II CCD camera and processed with analySIS® image-analytical software (both
from Soft Imaging Systems GmbH, Münster, Germany).

**Gold-labeled lectin binding.** Colloidal gold-labeled lectins were from EY Laboratories Inc.,
San Mateo, California. Prior to fixation and staining rat hearts (n = 2) were perfused with 0.6
μg per ml of *Canavalia ensiformis* (ConA/10nm; carbohydrate specificity: α-D-mannose, α-
D-glucose, N-acetyl-α-D-glucosamine³) and *Giffonia simplicifolia* (GS-1/15nm;
carbohydrate specificity α-galactose, α-N-acetylglucosamine⁴), or *Solanum tuberosum*
(STA/10nm; N-acetyl-β-D-glucosamine oligomers⁵) and *Triticum vulgaris* (WGA/15nm;
carbohydrate specificity N-acetyl-β-D-glucosamine, N-acetylnearaminic acid⁶) conjugates in
CCS (37°C) containing 0.1% of acetylated BSA (BSA-c; Aurion, Wageningen, The
Netherlands) for 3 minutes at 8.0 ml/min.

**Hyaluronidase treatment of rat heart.** Prior to fixation and staining, rat hearts (n = 2) were
perfused with oxygenated CCS-BSA (37°C) containing 25 IU/ml of hyaluronidase (bovine
testis, fraction IV-S, Sigma), benzamidine-HCL (1mM, Sigma), and 6-amino-n-caproic acid
(5mM, Sigma) for 1 hour at 8 ml/min. Control hearts (n = 2) were perfused with the
preceding solution, except hyaluronidase, as shown above.

**Data analysis.** Digital pictures from capillaries were analyzed using Image-Pro Plus
software version 3.0 (Media Cybernetics, Silver Spring, Pennsylvania). Mean glycocalyx
thickness was determined to be the distance between luminal membrane and the optical
background density + 2 x SD, which represents about 95% of detectable stained structures.
Glycocalyx thickness of 429 normal- and 196 hyaluronidase treated capillaries were given in
box plots with 5- and 95 percentiles shown. Difference in glycocalyx thickness of normal- and hyaluronidase treated capillaries was assessed by means of Mann-Whitney U non-parametric test.

Endothelial cell thickness was determined by subtracting the inner from the outer capillary diameter, with diameters calculated from measured perimeters of endothelial cells without a nucleus shown. Endothelial cell thickness was expressed as mean ± SD.

The pericapillary space was determined by subtracting the outer capillary diameter from the inner diameter of surrounding myocardial tissue as a measure of tissue edema. Distribution of calculated pericapillary spaces are given in 0.2 μm intervals and expressed as a median value. Difference in pericapillary spaces of normal- and hyaluronidase treated capillaries was assessed by means of Mann-Whitney U non-parametric test.

Results

To confirm carbohydrate specific staining of Alcian blue 8GX, colloidal gold-labeled lectins were infused prior to the fixation-staining procedure. Co-localization of lectins *C. ensiformis* (ConA/10nm) and *S. tuberosum* (STA/10nm) to these extended stained structures confirmed its saccharine nature. Online Figure 1A and 1B, respectively. In contrast, lectins *Griffonia simplicifolia* (GS-1/15nm) and *Triticum vulgaris* (WGA/15nm) were found only in close proximity of the endothelial cell-surface.

Discussion.

Magnesium in the phosphate-buffered fixative with Alcian blue used, allows staining of all possible glycosaminoglycans, including hyaluronan. As a consequence, the thixotropic properties of the solution are increased which tend to aggregate the Alcian blue molecules.
However, these thixotropic properties are diminished by the flow-controlled perfusion system. Consequently, the solution and perfusion conditions are optimal for a direct availability of Alcian blue with anionic residues.

Using intravital microscopy selective permeability barrier properties of the glycocalyx were found to be notably decreased after hyaluronidase treatment\(^7^8\). The present approach to stabilize the anionic carbohydrates by Alcian blue revealed predominantly hyaluronan. Presumably the presence of hyaluronan masks other structures near the endothelial cell surface, such as, heparan- and chondroitin sulfate containing proteoglycans and glycoproteins. In addition, glycosaminoglycans intermingled with hyaluronan may have been lost upon fixation and staining with Alcian blue.

Preliminary observations by our group suggest a similarity of glycocalyx in capillaries and large vessels throughout the whole body.
Online Figure 1A. Localization of colloidal gold-labeled lectins *Canavalia ensiformis* (ConA/10nm, arrow) and *Griffonia simplicifolia* (GS-1/15nm, arrowhead), and B. *Solanum tuberosum* (STA/10nm, arrow) and *Triticum vulgaris* (WGA/15nm, arrowhead) within Alcian Blue 8GX stained structures (Bar = 0.1μm).
References.


