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Change in shear stress ($\Delta\tau$)/hydraulic conductivity (L_p) relationship after pronase treatment of individual capillaries *in situ*

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Abstract

A complex glycoprotein meshwork covers the inner wall of blood vessels and is implicated in mechanotransduction of fluid shear stress (τ). A relationship between $\Delta\tau$ and capillary L_p has been established. The purpose of this study was to evaluate L_p in response to $\Delta\tau$ after exposing the capillary lumen to a mild, non-specific protease selected to disrupt its inner matrix. We hypothesized that L_p would not correlate with $\Delta\tau$ after enzyme treatment. Frogs (*Rana pipiens*, $n=69$) were pithed and the mesentery was exteriorized. L_p was assessed at 30 cm H₂O using the modified Landis technique after an abrupt, square wave $\Delta\tau$ produced by a physiologically relevant increase in pressure. Perfusate solutions were 10 mg·ml⁻¹ BSA/frog Ringer's (Control) or 0.1 mg·ml⁻¹ pronase in BSA/Ringer's (1 min) then BSA/Ringer's alone (Test). Mean ($\pm$ SE) control L_p following $\Delta\tau$ was $2.2 \pm 0.2 \times 10^{-7}$ cm·s⁻¹·cm H₂O⁻¹ and individual values correlated positively with $\Delta\tau$ ($r=0.85$, $P<0.0001$, $n=41$). After pronase, mean Test L_p ($17.6 \pm 2.5 \times 10^{-7}$ cm·s⁻¹·cm H₂O⁻¹) was higher compared to control and $\Delta\tau/L_p$ plots revealed two subsets of capillaries. L_p correlated strongly with $\Delta\tau$ in capillaries with diameters ≤ 15 μ m ($r=0.91$, $P=0.0006$, $n=14$) and also in a second subset of capillaries with diameters >15 μ m ($r=0.96$, $P=0.0001$, $n=8$). Slopes were 3.9- and 8.7-fold higher, respectively, compared to control. These data suggest a protective role for luminal constituents of intact capillaries. Mechanisms involved in capillary responses to flow-induced, mechanical stimuli may be located in the cellular structures that form capillaries.

Keywords

mechanotransduction; blood flow; permeability; filtration; water; endothelial cells

Introduction

Endothelial cells line the entire cardiovascular system and comprise the major cell type of capillaries. A meshwork of glycoconjugates, the glycocalyx, and an array of receptor proteins coat the luminal surface of capillaries. These constituents of the capillary lumen play key roles in many vascular functions including coagulation, suppression of thrombosis, inhibition of smooth muscle cell proliferation (Marcum and Rosenberg, 1987), and inflammation (Ley; 1996; Springer, 1995).

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The capillary lumen and its glycoprotein and protein constituents are subjected continuously to changing physical forces, shear stress (τ) and pressure, as well as to a dynamic chemical environment. τ also impacts composition of the glycocalyx (Arisaka et al., 1995) and can change its thickness along the vascular tree (Haldenby et al., 1994). Studies performed on vessels from different levels of the cardiovascular tree indicate that perturbing the glycocalyx with ischemia/reperfusion, hypoxia, low-density lipoproteins, atherogenic shear stress profiles, hyperglycemia, or enzymes appear to induce endothelial vulnerability (Gouverneur et al., 2006). Endothelial vulnerability is associated with a variety of pathological conditions.

Microvessel permeability also has been associated with the luminal glycocalyx (Baldwin and Thurston, 2001; Curry and Michel, 1980; Michel and Curry, 1999). van den Berg et al. (2003) used hyaluronidase to target hyaluronan, a component of the glycocalyx, and demonstrated that enzyme digestion not only decreased thickness of the glycocalyx, but also increased the pericapillary space, indicative of myocardial tissue edema.

Only two studies have tested function of individual capillaries *in situ* following enzyme treatment. Adamson (1990) reported that in the presence of albumin pronase, a non-specific protease, disrupted the luminal meshwork and increased hydraulic conductivity (L_p) of frog mesenteric capillaries. This result is consistent with the tissue edema formation seen after enzyme treatment in the whole rat heart (van den Berg et al., 2003). In contrast, plasmin treatment did not change L_p in the same tissue preparation (Clough and Michel, 1991).

Regarding fluid filtration and τ , studies of endothelial cell monolayers suggest that τ modulates hydraulic conductivity (L_p) (Sill et al., 1995). *In situ* studies indicate that capillary L_p correlates with relative change in τ in mesenteric capillaries of frog (Williams, 1999) and rat (Kim et al., 2005). Consistent with the culture and capillary data are reports from whole organ preparations where permeability to surface area product increased with changes in flow (Rippe et al., 1978), red blood velocity in skeletal muscle capillaries correlated with permeability to Cr-EDTA (Shibata and Kamiya, 1992), and capillary filtration coefficient rose rapidly with exercise (Björnberg, 1990). In individual capillaries, the slope of the relationship between L_p and relative change in τ can be altered by the rate at which the mechanical stimulus is presented to each capillary (Williams, 2003).

With the emerging importance of the inner lining of blood vessels to health and disease, the purpose of this study was to further investigate the $\Delta\tau/L_p$ relationship in individual, living capillaries by perturbing luminal constituents enzymatically in the presence of albumin. We hypothesized that the $\Delta\tau/L_p$ relationship would be uncoupled after constituents of the capillary lumen were disrupted with brief, mild exposure to a non-specific protease. Contrary to our prediction, the $\Delta\tau/L_p$ relationship was maintained and strengthened after pronase treatment compared to control. Additionally, two capillary subsets were revealed. Slopes of the $\Delta\tau/L_p$ relationship were 3.9- and 8.7-fold higher (greater sensitivity to $\Delta\tau$) for pronase-treated capillaries compared to control. Portions of these results have been presented previously (Williams et al., 2004; Williams et al., 2005; Williams and Flood, 2006).

Materials and methods

Male North American leopard frogs (*Rana pipiens*, JM Hazen, Alburg, VT; CD Sullivan, Nashville, TN) were maintained in a controlled environment (15°C, 12 hr light:12 hr dark). All frogs were exposed to gentamicin (1.3 mg·ml⁻¹, bath water, 24 hrs) upon arrival at the animal care facility and were housed (6 to 10 frogs/container) with free access to fresh, antibiotic-free water and a dry area for at least 1 wk before the experiments. Frogs were fasted for 4 days prior to experiments. The Animal Care and Use Committee at the University of Missouri – Columbia approved all animal housing and handling procedures.

Intravital Video Microscopy

Animal Preparation—Each frog was pithed cerebrally and cotton was placed in the cranial cavity. A right lateral incision was made through the skin and abdominal wall. Blood samples were collected for measurement of protein concentration (Bio-Rad, Richmond, CA), hematocrit (micro-capillary reader, IEC, Needham Heights, MA), hemoglobin (Drabkins Reagent, Sigma, St. Louis, MO), red blood cell counts, and white blood cell counts (bright line hemacytometer, Hausser Scientific, Horsham, PA). A loop of intestine was exteriorized and coaxed gently around a polished quartz pillar to maintain blood perfusion to the tissue. The mesentery and intestine remained moist and cool (14 to 16°C) by air-equilibrated frog Ringer's solution flowing continuously across the tissue. Temperature was monitored with a thermocouple wire (type-T, Digi-Sense®, Cole Parmer, Vernon Hills, IL) anchored under the tissue.

Capillary Identification—Transilluminated sections of mesentery were inspected for capillaries using an inverted, compound microscope (UM 10x long working distance objective, 0.22 NA, Diavert, Leitz). Microvascular segments with approximately 500 to 1,000 m between branch points were defined as individual capillaries. Capillaries were devoid of vascular smooth muscle cells, contained flowing frog red blood cells (fRBC) and had no rolling or sticking WBC. At the branching ends of each capillary the direction of fRBC flow was noted and each capillary was categorized (Chambers and Zweifach, 1944). TC (true capillaries with divergent flow at one end and convergent flow at the other end) and VC (venular capillaries with convergent flow at both ends) were used in this study.

Video Imaging—The image of each capillary was displayed on a video monitor (PM-125, Ikegami Tsushinki Co., JN) with a miniature high resolution CCD camera (TM-7CN, PULNiX America Inc., Sunnyvale, CA) coupled to the microscope. The camera was equipped with an electronic shutter controller (SC-745) operating at a speed of 1/10,000 s, which allowed either fRBC or human red blood cells (hRBC) to be visualized clearly. Experiments were video recorded in real time (Panasonic AG-6300, Matsushita Electric Industries, JN) with a video timer (0.01 s, VTG-33, For-A, JN) superimposed onto the image of the capillary (final magnification, 500x). Clips of video recorded data were digitized (media converter DVMC-DA2, Sony) and archived on an external hard drive (LaCie, Ltd.). The scale used for all measurements was calibrated to a stage micrometer (0.01 mm, Meiji Techno, JN).

Glass Microtools—Micropipettes, occluders, and restraining rods were created with a pipette puller (Model PB-7, Narishige, Tokyo, JN) from acid-rinsed, borosilicate glass (1.5 mm, TW150-4, World Precision Instruments, WPI, Sarasota, FL). Each micropipette was ground to a single bevel (12 to 20 m tip OD) using a Narishige grinding wheel (Model EG-4, 0.3 m abrasive film, Thomas Scientific, Swedesboro, NJ). A sealed, acrylic chamber (WPI) held each micropipette in position and a water manometer was attached to the chamber to maintain pressure and flow. Three micromanipulators (Prior, Stoelting Co., Wood Dale, IL) were used to adjust the glass microtools in the X-Y-Z planes.

Solutions

Frog Ringer's was prepared fresh daily from 5x concentrated stock solution (pH 7.4 at 15°C) and contained (in mM) NaCl (111.0), KCl (2.4), MgSO₄ (1.0), CaCl₂ (1.1), glucose (5.0), NaHCO₃ (2.0), and N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid (HEPES)/Na-HEPES (5.0).

Dialyzed (6,000 to 8,000 MWCO, Spectra/Por membrane, Spectrum, Houston, TX) bovine serum albumin (BSA, 10 mg·ml⁻¹; A-4378, Sigma Chemical Co., St. Louis, MO) dissolved in frog Ringer's was used as the control solution. Fresh stock solution of pronase E from

Streptomyces griseus (1.0 mg·ml⁻¹; 4 U·mg⁻¹, P-5147, Sigma Chemical Co.) was made fresh daily and diluted before each experiment in 10 mg·ml⁻¹ BSA/frog Ringer's to a final concentration of 0.1 mg·ml⁻¹. hRBC were obtained from a finger prick, washed 3 times in Ringer's, and added to each pipette solution (2 to 3% vol/vol hematocrit). All solutions were maintained on ice until use and visible bubbles were removed when each micropipette was back-filled with solution.

For additional control experiments, pronase was deactivated by boiling for 20 min and tested in capillaries. Pronase also was mixed as usual with hRBC and BSA, kept on ice for 30 min, and centrifuged through a 10,000 MW cut-off filter. The filtrate was added to hRBC and BSA and tested in capillaries.

Mechanical Stimulus

Abrupt $\Delta\tau$ and τ Time Curves—Each capillary was cannulated at 8 cm H₂O (5.9 mm Hg, *in situ* TC pressure) and low flow was established for 2 min (Steady State 1). Next, manometer pressure was changed abruptly to 30 cm H₂O (22.1 mm Hg) to produce the mechanical stimulus, $\Delta\tau$. The higher flow was maintained for 2 min (Steady State 2) at which point the capillary was occluded to measure volume flux/surface area (J_v/S) also at 30 cm H₂O. It was assumed that filtration during occlusion reflected filtration at Steady State 2.

A time curve for τ was generated for each capillary from measures of the free-flowing hRBC. Plateaus for Steady State 1 and Steady State 2 were identified by visual inspection from these curves. The magnitude of the mechanical stimulus ($\Delta\tau$) was calculated from the plateau values as:

$$\Delta\tau = \tau^{\text{Steady State 2}} - \tau^{\text{Steady State 1}} \quad (1)$$

The intent of each experiment always was to introduce a square wave stimulus; however, deviations from intent do occur with work *in situ*. As such, the actual stimuli were categorized into square wave, overshoot, or undershoot patterns. As described previously (Williams, 2003), a square wave designation was assigned when τ at 15 s was within 2.0 dynes·cm⁻² of the mean value for Steady State 2. Capillaries were excluded from the $\Delta\tau/L_p$ analysis due to technical problems, which included overshoot or undershoot stimulus patterns and/or interruption of Steady State 2 by a clogged pipette.

Estimates of Capillary Fluid τ From Measures of RBC Velocity (v), Capillary Diameter, and Apparent Viscosity

v —The method used to measure v of RBC has been described in detail (Williams, 1999; Williams, 2003). Briefly, instantaneous RBC velocity (v^i) was measured directly on the video monitor in the frame-by-frame mode as the distance traveled by a single hRBC in time (dx/dt , m·s⁻¹).

$$v^i = dx / dt \quad (2)$$

A correction factor (CF; Michel et al., 1974) was calculated from hRBC radius (R , m) and each capillary radius (r , m, measured simultaneously with v^i) as:

$$CF = 2(1 - [(R)^2 / 2(r)^2]) \quad (3)$$

v was then calculated from v^i and CF assuming a centered RBC within the vessel.

$$v(\mu\text{m} \cdot \text{s}^{-1}) = v^i / CF \quad (4)$$

Capillary Diameter—Diameter was measured from video recordings at 3 sites spaced ≈ 100 μ m apart along the capillary and averaged.

Apparent Viscosity— η^{apparent} was estimated from protein concentration (frog plasma, before, and BSA/Ringer's, after cannulation; Chick and Lubrzynska, 1914) as,

$$\eta^{\text{apparent}}(\text{poise}) = (5.7 \times 10^{-5}[\text{protein, mg} \cdot \text{ml}^{-1}]^{37^\circ\text{C}}) + 0.0089, \quad (5)$$

and corrected to 15°C using the ratio between viscosity of water at 37 and 15°C (Weast, 1970). Use of protein concentration provided an estimate for the lower limit of capillary fluid viscosity.

τ —Shear rate was calculated from v and r as:

$$\text{Shear Rate (s}^{-1}\text{)} = 4v/r. \quad (6)$$

τ was then calculated as:

$$\tau(\text{dynes} \cdot \text{cm}^{-2}) = (\text{Shear Rate})(\eta^{\text{apparent}}). \quad (7)$$

Calculation of Volume Flux (J_v) per Surface Area (S) and Hydraulic Conductivity (L_p) From Measures of Capillary Diameter and J_v

J_v —The modified Landis technique (Landis, 1927; Michel et al., 1974) was selected to measure J_v to ensure that the downstream end of each capillary remained intact prior to and during the mechanical $\Delta\tau$ stimulus. After the $\Delta\tau$ protocol a glass occluder, which had been positioned over the downstream end of the capillary perpendicular to the longitudinal axis of the microvessel, was lowered gently onto the capillary to occlude it and trap the hRBC. Movement of the trapped hRBC acted as an index of fluid flux across the capillary wall. Visual inspection verified successful occlusion. Time course data were obtained from serial occlusions, all at 30 cm H_2O , moving the occluder progressively upstream along the capillary towards the micropipette, with one J_v measurement per occlusion and a minimum of 12 μ m between each occlusion site.

Capillary Diameter—Diameter was measured 3 times at 3 time points during occlusion of the capillary to verify that, within the limits of detection and resolution of the video system (2 μ m), S remained constant. Capillary volume and S were calculated from capillary radius (r , cm). A cylindrical capillary geometry was assumed.

J_v/S — J_v/S was calculated from hRBC velocity measured during the occlusion ($dx/dt^{\text{occlusion}}$, $\text{cm} \cdot \text{s}^{-1}$), capillary length measured between the occluder and each marker cell at the onset of the dx/dt measurement (x_o , cm), and the volume to surface area ratio ($r/2$, cm).

$$J_v/S(\text{cm} \cdot \text{s}^{-1}) = (dx/dt^{\text{occlusion}})(1/x_o)(r/2) \quad (8)$$

$dx/dt^{\text{occlusion}}$ and x_o were measured on 3 hRBC (spaced ≈ 50 μ m apart) at 3 time points (2.0, 2.3, and 2.6 s). Thus, 9 individual values of J_v/S were obtained from the first occlusion to decrease the standard deviation of the mean and increase precision.

L_p —Assuming a linear relationship between J_v/S and capillary pressure (P_c) as per the Starling equation, the slope obtained from regression analysis of J_v/S and P_c was used to estimate capillary filtration coefficient, L_p ($\text{cm} \cdot \text{s}^{-1} \cdot \text{cm H}_2\text{O}^{-1}$).

$$J_v/S = L_p[(P_c - P_i) - \sigma(\pi_c - \pi_i)] \quad (9)$$

where $(P_c - P_i)$ and $(\pi_c - \pi_i)$ are the differences in hydrostatic (P) and oncotic (π) pressures between the capillary lumen (c) and interstitium (i) of the mesentery and sigma (σ) is the reflection coefficient of the capillary barrier to protein. With an exteriorized mesentery, interstitial pressure is negligible and with fresh, protein-free frog Ringer's solution superfusing the tissue continuously, oncotic pressure differences are difficult to estimate. Calculating interstitial pressure from mesenteric protein concentration altered values for L_p by $0.1 \times 10^{-7} \text{ cm} \cdot \text{s}^{-1} \cdot \text{cm H}_2\text{O}^{-1}$, a difference that was within the error of the method. Adamson (1990) reported that effective oncotic pressure after pronase was not statistically different from control. In the present study, pronase combined with abrupt shear stress change in this study produced changes in L_p that declined rapidly in time (Figure 3). Under circumstances where a rapid change in flux occurs, Neal and Michel (1995) have used zero as the x-axis intercept ($\sigma(\pi_c - \pi_i)$). A procedure we have used here and in the past, which conservatively underestimates each value for L_p (Williams, 1999; Williams, 2003).

Each of the 9 measurements of J_v/S obtained during the first occlusion were calculated to L_p at 30 cm H_2O and averaged for a given capillary.

Experimental Protocols

Two protocols were used to evaluate pronase and the $\Delta\tau/L_p$ relationship (Figure 1). Control Group capillaries experienced the same mechanical manipulation as their paired Test Group. Variability due to differences in technical skill was minimized with the same individual (DAW) performing all cannulations and experimental protocols. To minimize measurement error and bias, a second individual (MHF) measured RBC velocity, capillary diameter, protein concentration, and J_v .

Protocol 1 (One Capillary Per Frog)—Frogs were divided into Control and Test Groups (see Table 1, Figure 1). Each capillary in the Control Group was cannulated (low flow, 2 min) with a pipette that contained BSA/frog Ringer's solution. The abrupt $\Delta\tau$ protocol described above was followed and J_v/S was measured (L_p^{Cap1}). The pipette was removed and the same capillary was recannulated with BSA/Ringer's (low flow, 1 min). The pipette was removed again, the capillary recannulated again (low flow, 1 min), and the abrupt $\Delta\tau$ protocol was repeated. J_v/S was measured a second time ($L_p^{\text{RecannCap1}}$).

The same initial steps were followed in the Test Group to obtain L_p^{Cap1} using BSA/Ringer's solution and the abrupt $\Delta\tau$ protocol. After L_p^{Cap1} was assessed the capillary was recannulated with a pipette containing pronase/BSA solution. The pronase solution flowed continuously (no occlusion) through the capillary (low flow, 1 min) and was removed promptly. Next, the capillary was recannulated with a pipette containing BSA/frog Ringer's solution (low flow, 1 min) and the abrupt $\Delta\tau$ protocol was repeated. J_v/S was measured a second time ($L_p^{\text{RecannCap1}}$).

Protocol 2 (Two Capillaries Per Frog)—Frogs were divided into Control and Test Groups (Table 1, Figure 1). For the Control Group, capillary 1 received BSA/frog Ringer's solution (low flow, 2 min) and the abrupt $\Delta\tau$ protocol. J_v/S was measured (L_p^{Cap1}). Next, capillary 2 was cannulated with BSA/Ringer's (low flow, 1 min). The pipette was removed and the capillary was recannulated with BSA/frog Ringer's (low flow, 1 min). The abrupt $\Delta\tau$ protocol was followed and J_v/S was measured (L_p^{Cap2}).

In the Test Group, capillary 1 received the same treatment as capillary 1 in the Control Group. Capillary 2, however, was cannulated initially with a pipette containing pronase (low flow, 1

min). After the pronase-containing pipette was removed, capillary 2 was recannulated with a BSA/frog Ringer's pipette and rinsed (low flow, 1 min). Capillary 2 received the abrupt $\Delta\tau$ protocol and J_v/S was measured (L_p Cap2). In general, only 1 capillary was visible on the video monitor (0.23 mm^2) and the second capillary was located in a separate section of the mesentery. For additional "vehicle" control experiments, Protocol 2 was followed and either deactivated pronase or filtered pronase were substituted for pronase.

Statistical Analyses

Data were tested for normality (JMP, SAS Institute, Inc.) and outliers identified from an outlier box plot. L_p values are reported from the first occlusion since the time course of L_p following $\Delta\tau$ is transient in non-pronase treated capillaries (Williams, 1999). Data are reported as mean $\pm$ SE. Paired t -tests were used to test for differences between means. Correlation coefficient, r , and linear regression analysis were used to evaluate the relationship between $\Delta\tau$ and L_p . A P -value of 0.05 was set before initiating the experiments to indicate significance.

Results

Frogs ($n = 69$; $33.2 \pm 0.8 \text{ g wt}$; $19.2 \pm 0.3 \text{ cm length}$; $62.7 \pm 1.2 \text{ bpm}$ pithed heart rate) were determined to be healthy by inspection. Table 2 contains summary data of measurements performed on blood and lymph fluid. Skin and abdominal cavity fluid could not be measured in all animals. One protocol was completed on 66 frogs and both protocols were accomplished on 3 frogs resulting in a total of 70 individual capillaries.

$\Delta\tau/L_p$ Relationship For Control (BSA/Ringer's) Capillaries

Capillary diameter averaged $14.2 \pm 0.5 \text{ m}$ (range 8.0 to 21.5 m). Estimates of τ *in situ* (before cannulation) ranged from 1.0 to $24.6 \text{ dynes}\cdot\text{cm}^{-2}$ with an average of $7.6 \pm 0.7 \text{ dynes}\cdot\text{cm}^{-2}$ ($v = 762.7 \pm 60.8 \text{ m}\cdot\text{s}^{-1}$; shear rate = $456.1 \pm 38.9 \text{ s}^{-1}$).

Capillary L_p (obtained from the first occlusion) averaged $2.50 \pm 0.17 \text{ cm}\cdot\text{s}^{-1}\cdot\text{cm H}_2\text{O}^{-1}$ ($n = 60$; range = 0.4 to 5.8×10^{-7} ; $n = 9$ outliers). L_p was $2.37 \pm 0.21 \times 10^{-7}$ for TC and $2.57 \pm 0.24 \times 10^{-7} \text{ cm}\cdot\text{s}^{-1}\cdot\text{cm H}_2\text{O}^{-1}$ for VC ($P = 0.61$) and decreased with time as published previously (Williams, 1999; data not shown). Figure 2, Panel A presents individual values for L_p plotted as a function of the square wave $\Delta\tau$ presented to each capillary. $\Delta\tau$ correlated ($r = 0.85$, $P < 0.0001$) with capillary L_p . Figure 2, Panel B shows the same L_p values and τ estimated after cannulation, before each capillary was stimulated. No relationship existed.

Time Course for Capillary L_p Following Pronase and $\Delta\tau$

L_p decreased with time, after pronase followed by $\Delta\tau$, in 6 capillaries (Figure 3) at an average rate of $0.15 \pm 0.03 \text{ } L_p \text{ units/s}$. The step change in τ occurred between 120 and 125 s before the first assessment of L_p . Oscillations in L_p (changes not accounted for by measurement error) were apparent for the 3 capillaries that displayed the greatest response. Due to the transient L_p time course, which is similar for control capillaries (Williams, 1999), values from the first assessment of L_p were selected to represent the acute response to pronase and abrupt $\Delta\tau$ (Figures 4, 5, and 6).

Average Capillary L_p Following Pronase Treatment Then $\Delta\tau$

Protocol 1 (One Capillary Per Frog)—Pooled *in situ* capillary diameter for Control and Test Groups averaged $14.8 \pm 0.9 \text{ m}$ ($n = 17$ capillaries). For the Control Group, average diameter before the initial cannulation ($14.2 \pm 1.5 \text{ m}$) did not differ statistically ($P = 0.68$) from that measured after recannulation and $\Delta\tau$ ($15.1 \pm 1.4 \text{ m}$). Similarly, for the Test Group, average

diameter before the initial cannulation (15.1 ± 1.1 m) was not different statistically ($P = 0.36$) from that measured after recannulation, pronase, and $\Delta\tau$ (17.0 ± 1.6 m).

Magnitude of $\Delta\tau$ was similar statistically between Cap1 and RecannCap1 in the Control Group (92.0 ± 10.9 versus 81.0 ± 14.6 dynes $\cdot$ cm $^{-2}$, respectively, $P = 0.56$) and, likewise, for the Test Group (47.6 ± 11.2 versus 54.0 ± 11.1 dynes $\cdot$ cm $^{-2}$, respectively, $P = 0.69$).

Figure 4 shows the average values of capillary L_p for Control and Test Groups. $L_p^{\text{RecannCap1}}$ for Control (BSA/Ringer's only) was 3.1-fold higher ($P = 0.05$) compared to L_p^{Cap1} . For the Test Group, $L_p^{\text{RecannCap1}}$, which was assessed after pronase treatment, also was higher (4.7-fold, $P = 0.004$) than L_p^{Cap1} (BSA/Ringer's).

Protocol 2 (Two Capillaries Per Frog)—Mean pooled baseline diameter for all capillaries studied using Experimental Protocol 2 was 14.1 ± 0.5 m ($n = 52$ capillaries). The diameters measured before and after $\Delta\tau$ were similar ($P = 0.52$) for capillaries in the Control Group (14.4 ± 1.0 and 15.6 ± 1.6 m, respectively, $n = 16$) and similar ($P = 0.94$) for those in the Test Group (13.6 ± 0.9 and 13.7 ± 0.6 m, respectively, $n = 36$).

Magnitude of $\Delta\tau$ was similar statistically between Cap 1 and Cap 2 for the Control Group (56.9 ± 12.2 versus 85.0 ± 18.0 dynes $\cdot$ cm $^{-2}$, $P = 0.22$) and also for Test Group (48.5 ± 6.1 versus 53.9 ± 6.0 dynes $\cdot$ cm $^{-2}$, $P = 0.53$).

Figure 5 presents average L_p for Control and Test Groups. L_p^{Cap1} did not differ ($P = 0.90$) from L_p^{Cap2} in the Control Group (BSA/Ringer's only). In contrast, for the Test Group, mild pronase treatment of the capillary lumen followed by an acute, abrupt $\Delta\tau$ resulted in 5.7-fold higher ($P < 0.0001$) L_p for Capillary 2 (pronase, L_p^{Cap2}) compared to Capillary 1 (BSA/Ringer's, L_p^{Cap1}).

Average L_p did not change following pronase that was deactivated by boiling ($2.0 \pm 0.3 \times 10^{-7}$ cm $\cdot$ s $^{-1}$ $\cdot$ cm H $_2$ O-1, $n = 4$, Capillary 2) versus control ($1.6 \pm 0.4 \times 10^{-7}$ cm $\cdot$ s $^{-1}$ $\cdot$ cm H $_2$ O-1, Capillary 1, $P = 0.37$). Average L_p following exposure to filtered pronase then $\Delta\tau$ was $3.3 \pm 1.1 \times 10^{-7}$ cm $\cdot$ s $^{-1}$ $\cdot$ cm H $_2$ O-1 ($n = 9$, Capillary 2) and was not different statistically compared to control, Capillary 1 ($2.2 \pm 0.4 \times 10^{-7}$ cm $\cdot$ s $^{-1}$ $\cdot$ cm H $_2$ O-1, $P = 0.37$).

In general, pronase treatment made the capillaries easier to work with, that is, they were less “sticky” at the occlusion site and tended not to clot. Additionally, the mesenteric tissue appeared white for control capillaries and for pronase-treated capillaries before $\Delta\tau$. After $\Delta\tau$ in pronase-treated capillaries, the tissues became spotty in appearance likely due to increased filtration rate.

$\Delta\tau/L_p$ Relationship Following Pronase Treatment

Figure 6 depicts L_p plotted as a function of square wave $\Delta\tau$ for control and pronase-treated capillaries. For control capillaries, $\Delta\tau$ correlated with L_p ($r = 0.85$, $P < 0.0001$). Diameter did not differentiate any subsets among the control capillaries.

For the pronase-treated capillaries shown in Figure 6, two subsets of capillaries were revealed and distinguished according to diameter that was above and below the maximum width of a fRBC (width, 15 m, length, 25 m). Correlations between $\Delta\tau$ and L_p existed for both capillary subsets (≤ 15 m diameter, $r = 0.91$, $P = 0.0002$ and > 15 m diameter, $r = 0.96$, $P = 0.0001$). Both subsets were more sensitive (greater slope compared to control) to $\Delta\tau$ when exposed to pronase. Slopes for the $\Delta\tau/L_p$ response curve were 0.065 for control (BSA/Ringer's), 0.254 for pronase-treated capillaries with diameters ≤ 15 m (3.9-fold increase above control), and 0.566 for pronase-treated capillaries with diameters > 15 m (8.7-fold increase above control).

For pronase-treated capillaries with diameters ≤ 15 m, the $\Delta\tau/L_p$ relationship plateaued (slope = 0.003) above 70 dynes-cm⁻² for $\Delta\tau$. Capillaries with diameters > 15 m were more sensitive (greater slope) to $\Delta\tau$ after pronase treatment compared to capillaries with diameters ≤ 15 m.

Figure 7 presents L_p data for capillaries tested with either deactivated/boiled pronase solution (Panel A) or filtered pronase solution (Panel B) followed by a square wave, abrupt $\Delta\tau$. Data obtained using deactivated pronase were indistinguishable from control data (Panel A). For capillaries tested with filtered pronase, the $\Delta\tau/L_p$ graph (Panel B) indicated that 4 out of 9 data points fell outside the data for their paired controls and outside the larger, comparison data set from Figure 2A, although the means were not statistically different (see Protocol 2 above).

Discussion

The present study contains two key points related to the physiology of individual TC and VC located in frog mesentery. First, control values for L_p obtained from the first occlusion of each capillary correlated positively with $\Delta\tau$. Second, the $\Delta\tau/L_p$ relationship was shifted left of control for pronase-treated capillaries indicating greater sensitivity to $\Delta\tau$ and providing one possible explanation for higher average L_p after pronase. Collectively, the data suggest that disrupting luminal constituents of intact capillaries could contribute to tissue edema formation possibly due, in part, to an accentuated response to $\Delta\tau$. The data imply a protective role for luminal constituents, which include, but are not limited to, the glycocalyx.

Control Values of L_p in Response To An Acute, Abrupt, Square Wave $\Delta\tau$

By definition, the increase in pressure above *in situ* that is used in the modified Landis technique to induce filtration also causes a $\Delta\tau$ along the capillary wall. Here, we calculated $\Delta\tau$ instead of $\Delta\tau\%$ (Williams, 1999; Williams, 2003) to minimize the impact of *in situ* τ estimates on the calculation. Each capillary was cannulated with the pressure manometer set low and then changed to 30 cm H₂O. As such, the magnitude of $\Delta\tau$ varied primarily with network resistance downstream (capillary length, diameter, and network geometry) and with slight differences in pipette resistance upstream. Widening the pressure gradient to increase flow produced the mechanical stimulus ($\Delta\tau$) and was selected for its physiological and pathophysiological relevance. Flow change due to pressure change occurs when microvascular arterioles are dilated leaving the downstream capillary bed unprotected. One *in vivo* clinical example would be release of external pressure applied to the skin after spinal cord injury.

L_p for control capillaries was obtained exclusively from the first occlusion of each capillary due to the transient, initial decline of L_p in time after $\Delta\tau$ (27). Minimizing variability from mixed stimulus patterns (28) revealed a significant relationship between $\Delta\tau$ and L_p for healthy, control capillaries where 52% of the variability in L_p was accounted for by the magnitude of the acute, abrupt, square wave $\Delta\tau$. The large data set in Figure 2A also provided context for evaluating the smaller data sets obtained from the deactivated and filtered pronase control experiments (Figures 7A and 7B).

Pronase Digestion Within the Capillary Lumen and the $\Delta\tau/L_p$ Relationship

We hypothesized originally that the non-specific protease, pronase, in the presence of albumin, would disorganize constituents of the capillary wall (consistent with electron micrographs (Adamson, 1990) and weaken or eliminate the $\Delta\tau/L_p$ relationship. In fact, pronase revealed two subsets of capillaries, each displaying strengthened relationships between $\Delta\tau$ and L_p where 83% and 93% of the variability in L_p was accounted for by the magnitude of the acute, abrupt, square wave $\Delta\tau$ stimulus in ≤ 15 m diameter and > 15 m diameter capillaries, respectively. Therefore, we reject our hypothesis and conclude from the data that individual capillaries displayed greater sensitivity to mechanical stimulation following mild pronase digestion.

We used an established protocol for pronase in individual frog mesenteric capillaries and our finding that L_p was higher after pronase (Figure 5) is consistent with previous results using this protocol (Adamson, 1990). With the same concentration and duration of pronase exposure, Adamson also reported disorganized cationized ferritin labeling of the glycocalyx with no report of changes in capillary ultrastructure, again, consistent with the functional data here. We did not observe any leak sites in the capillaries, which can be detected visually by the hRBC marker cells tracking to the wall upon occlusion. Our results extend the previous report by demonstrating a relationship between $\Delta\tau$ and L_p in pronase-treated capillaries. The data suggest an accentuated response to mechanical stimulation as one explanation for the higher L_p following pronase digestion.

Two additional control experiments were performed. The pronase protein itself, devoid of its enzymatic activity by boiling, did not produce a response different from control capillaries (Figure 7A). A second “vehicle” control experiment was performed to discount the possibility that vasoactive products had been cleaved from the perfusate solution albumin (Dull et al., 2003) and/or hRBC by pronase and were responsible for the response reported in Figure 6. Although about 40% of the capillaries appeared to respond to the pronase filtrate, the response was inconsistent with data falling within, above, and below the paired control data and the comparison data set (Figure 7B). As such, the magnitude of the L_p response to filtered pronase could not account for that of pronase. Future work is necessary to identify the cleavage product/s responsible for some of the observed responses.

The two capillary subsets that were distinguished by diameters above and below the maximum width of a fRBC may reflect differences in chronic shear stress and different adaptations to chronic flow among individual capillaries. This chronic state may be detectable only after the first, protective luminal layer has been disrupted. The chronic configuration of components embedded in the membrane and/or subcellular compartments of the capillary wall may, in turn, dictate each capillary response to $\Delta\tau$. Further study is required.

For our first investigation of mechanical stimulation after enzymatic disruption of the capillary, we targeted the entire capillary lumen and essentially treated it as a single entity. In actuality, the glycocalyx is composed of many individual molecules with varying functional roles (Henry and Duling, 1999). Previous work that utilized enzymes to target individual components of the glycocalyx increased pericapillary space (interstitial fluid) in cardiac tissue most likely indicating increased filtration (van den Berg et al., 2003). While results of our present study are consistent with the work in cardiac tissue, we cannot speculate from the present data about which luminal components are responsible for the increase in L_p due to the non-specific nature of pronase, *albeit* brief and mild. More specific studies are necessary.

Although correlations should be interpreted with caution, the results reported here do suggest that each capillary somehow sensed and responded, at least in part, to the physiologically relevant mechanical stimulation. The lack of relationship between L_p and pre-stimulus τ (Figure 2B), plus the strengthened relationship between $\Delta\tau$ and L_p (Figure 6) with pronase, as well as the variety of controls built into the experimental design collectively support the conclusion that $\Delta\tau$ accounted for 80 to 90% of the variability in the assessed endpoint, L_p , after pronase exposure. In contrast, since $\Delta\tau$ only accounted for 52% of the variance for the control capillaries (Figure 2A) these data suggest that constituents of the lumen impacted by pronase may contribute to a large portion of the variation in values of L_p assessed *in situ*.

Shear Stress Mechanotransduction and Clinical Significance

Evidence presented in this study indicates that constituents of the capillary lumen may be involved in blunting the mechanical transfer of τ stimuli in intact frog mesenteric capillaries.

Although predicting the precise location of the transducing structure or mechanism remains outside the limits of our data, we can suggest at least two possibilities.

The first line of reasoning locates the mechanotransducer in the lipid bilayer of the endothelial cell membrane. As such, luminal constituents may act as a physical buffer *in vivo* blunting the response of capillaries to fluctuations in fluid flow. Secomb et al. (2001) have suggested that when a meshwork is intact, τ is transmitted to the surface layer of endothelial cells, thus, minimizing direct stress on the cell membrane. Without the protective meshwork, $\Delta\tau$ would be transmitted to the membrane directly and responses accentuated, a prediction supported by data in the present study. A change in membrane fluidity, which is known to occur during exposure of cultured endothelial cells to fluid τ (Haidekker et al., 2000), may be involved in the response of L_p to $\Delta\tau$ when the lumen is disrupted and unable to perform its protective function. Further research is required.

Second, the glycocalyx may trigger signaling molecules such as nitric oxide. According to data from Mochizuki et al. (Mochizuki et al., 2003; isolated canine femoral arteries) and Florian et al. (Florian et al., 2003; cultured endothelial cells) disrupting components of the glycocalyx enzymatically prevented nitric oxide release. It would follow that if nitric oxide is released similarly in frog mesenteric capillaries, then the increased L_p after pronase implies an antagonistic role for nitric oxide with regard to barrier function. Release of nitric oxide consequent to an abrupt $\Delta\tau$ would inhibit an increase in L_p and minimize edema formation. Unpublished data from our laboratory indicated an inverse relationship between plasma nitrite/nitrate (NO_x) levels and L_p assessed on τ stimulated capillaries (Williams et al., 2003). As yet, direct measures of NO_x along with L_p assessment have not been performed.

Clinically, adverse consequences could result during drug trials if an interaction occurred between a particular drug and the mechanical, hemodynamic-induced force on the capillary wall. Responses due to chemical intervention could be underestimated or considered simplistically by not accounting for the impact of mechanical force. The interaction of changes in fluid τ with drugs and/or biologically active agents could accentuate tissue edema formation and should be taken into account when capillary responses to chemicals are tested. It is important to note that the two subsets of pronase-treated capillaries were revealed here only after examining the $\Delta\tau/L_p$ relationship. This additional level of complexity was not evident by mathematical averages alone. The $\Delta\tau/L_p$ relationship provides an important physiologically and pathophysiologically relevant tool for evaluating individual capillaries.

Methodological Considerations

Of importance methodologically was the negative result obtained with Experimental Protocol 1, which was only evident when a control group of animals was included in the experimental design. Values for L_p assessed after recannulation with BSA/Ringer's and after recannulation with pronase were essentially the same for both Control and Test Groups (Figure 4). Had the capillary been used as its own control (Cap 1, Test, Experiment 1) without a separate control group, the conclusion of an enzyme/shear stress effect would have been drawn. In contrast, Protocol 2 provided a very clear, definitive result (Figure 5) since capillaries in the Control Group plus the control capillaries in the Test Group were lower than the pronase capillaries in the Test Group. Analysis of the $\Delta\tau/L_p$ relationship (Figure 6) further indicated that pronase increased L_p above control likely from increased sensitivity to $\Delta\tau$.

Traditionally, it has been argued by others studying individual capillaries that tests of chemicals, including enzymes, should be performed on the same capillary for internal control. A separate control group of animals is seldom required. Experimental Protocol 1 was designed to adhere to this line of reasoning. With Control and Test Groups displaying similar results from Experiment 1 (although Cap 1 was significantly different from Recann/Cap 1 in the Test

Group), we next reasoned that the traditional approach had been accepted for testing the effects of chemical stimuli on capillary L_p , but may not be valid for evaluating mechanical stimuli (Williams, 2003). We tested our hypothesis a second way using two naïve capillaries in the same animal where neither had experienced a shear stress challenge prior to the experimental intervention (Protocol 2). A difference between control and pronase was evident. Future work will require this experimental approach for studying mechanical stimuli and capillary L_p .

As illustrated in Figure 1, a change in shear stress plus occlusion occurred in each capillary before recannulation in the Control (Cap 1), Experiment 1 protocol compared to low flow cannulation and then recannulation for Control (Cap 2), Experiment 2. These mechanical stimuli in addition to recannulation could have contributed to the higher second L_p in the Control Group of Experiment 1 (Figure 4) since recannulation alone did not appear to influence control L_p for the second capillary in Experiment 2. Additionally, the change in shear stress plus occlusion that was performed prior to pronase in the Test Group of Experiment 1 (Figure 4) appeared to blunt the response to pronase compared to that in Experiment 2 (Figure 5) consistent with the glycocalyx working through a signal transduction mechanism. Alternatively, the first increase in shear plus occlusion that occurred in Experiment 1 could have altered the glycocalyx so that less of the matrix was exposed to the subsequent pronase infusion.

In conclusion, pronase treatment of individual capillaries of frog mesentery resulted in increased sensitivity to acute, abrupt square wave $\Delta\tau$ as demonstrated by higher capillary L_p and greater slope of the $\Delta\tau/L_p$ relationship. The data implicate either primary or secondary involvement of luminal constituents of the capillary in transducing the mechanical force that is generated by blood flow changes *in vivo*.

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Experiment 1 Experiment 2 Key:

- ↓ = low flow cannulation (BSA/Ringer's)
- ↓ = low flow recannulation (BSA/Ringer's)
- = low flow cannulation (Pronase - Test)
- = low flow recannulation (Pronase - Test)
- ↑ = abrupt change in shear stress
- ◆ = L_p assessment (occlusion)

Cap, capillary

Figure 1.

Diagram of protocols for Experiment 1 and Experiment 2. Key defines symbols used in the diagram. Experiment 1, Test (Cap 1) also illustrates a 5 min time course.

Figure 2.

Detailed analysis of basal hydraulic conductivity (L_p) assessed on individual mesenteric capillaries. Panel A – L_p values as a function of the magnitude of an abrupt, square wave change in shear stress ($\Delta\tau$) (regression equation for solid line: $L_p = 0.049(\pm 0.008)(\Delta\tau) + 0.09(\pm 0.35)$, $R^2 = 0.52$, $P < 0.0001$, $n = 41$; intercept not different from zero, $P = 0.80$). Panel B – L_p values and shear stress (τ) after cannulation, before the square wave $\Delta\tau$ stimulus (dashed line: no relationship, $R^2 = 0.01$, $P = 0.48$).

Figure 3.

Time course of hydraulic conductivity (L_p) after mild pronase digestion followed by an abrupt change in shear stress ($\Delta\tau$) for 6 individual capillaries. Each symbol indicates data for one capillary. Arrows indicate pronase (pr) exposure time (60 s), which was followed by Ringer's at low flow for at least 1 min before $\Delta\tau$. Average time between $\Delta\tau$ and the first L_p assessment was 124.3 ± 1.7 s. Experimental Protocol 1 was used for 1 capillary (filled squares) and the other 5 were tested using Protocol 2.

Figure 4. Average hydraulic conductivity (L_p) assessed using Experimental Protocol 1. Data are mean $\pm$ SE, (n). Cap - capillary, recann - recannulation. *, $P < 0.05$.

Figure 5. Average hydraulic conductivity (L_p) assessed using Experimental Protocol 2. Data are mean $\pm$ SE, (n). Cap - capillary. **, $P < 0.0001$.

Figure 6.

Hydraulic conductivity (L_p) plotted as a function of the magnitude of an abrupt, square wave change in shear stress ($\Delta\tau$) for control (BSA/Ringer's) and pronase-treated capillaries.

Regression equations: BSA/Ringer's capillaries (solid line), $L_p = 0.065(\pm 0.010)(\Delta\tau) - 0.40$ (± 0.43), $R^2 = 0.72$, $P < 0.0001$, $n = 20$; Pronase-treated capillaries with diameters ≤ 15 m (dashed line), $L_p = 0.254(\pm 0.041)(\Delta\tau) - 4.46(\pm 1.80)$, $R^2 = 0.83$, $P = 0.0002$, $n = 14$; Pronase-treated capillaries with diameter > 15 m (dashed-dot line), $L_p = 0.566(\pm 0.063)(\Delta\tau) - 2.98$ (± 2.82), $R^2 = 0.93$, $P = 0.0001$, $n = 8$. Data for this smaller subset of control capillaries were included in the larger control data set pictured in Figure 2.

Figure 7.

Hydraulic conductivity (L_p) plotted as a function of the magnitude of an abrupt, square wave change in shear stress ($\Delta\tau$). Panel A – deactivated/boiled pronase-treated capillaries and matched control capillaries (BSA/Ringer's), Cap - capillary. Panel B – filtered pronase-treated capillaries and matched control capillaries (BSA/Ringer's). Data for all controls from Figure 1A (solid circles and line) are provided for context in both panels.

Table 1Experimental groups and pipette solutions used prior to abrupt $\Delta\tau$ stimulus.

Protocol #	Group	Capillary	Pipette Solution for L_p1	Pipette Solution for L_p2
1	Control	1	BSA/Ringer's	BSA/Ringer's
	Test	1	BSA/Ringer's	Pronase then BSA/Ringer's
2	Control	1	BSA/Ringer's	
		2	BSA/Ringer's	
	Test	1	BSA/Ringer's	
		2	Pronase then BSA/Ringer's	

L_p – hydraulic conductivity; BSA – bovine serum albumin

Table 2Systemic values for blood and lymph fluid sampled from *Rana pipiens*.

Plasma protein concentration (mg·ml ⁻¹)	22.5 ± 0.7 (69)
Abdominal cavity fluid protein concentration (mg·ml ⁻¹)	12.8 ± 0.6 (58)
Skin lymph fluid protein concentration (mg·ml ⁻¹)	14.2 ± 0.7 (34)
WBC (cells·mm ⁻³)	5,189.1 ± 205.1 (64)
RBC (cells·mm ⁻³)	331,866.7 ± 12,385.9 (60)
Hematocrit (%)	26.1 ± 0.7 (69)
Hemoglobin concentration (g·dl ⁻¹)	9.3 ± 0.5 (58)

Data are mean ± SE (n). WBC – white blood cells, RBC – red blood cells