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Proteolytic Activity Attenuates the Response of Endothelial Cells to Fluid Shear Stress

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Abstract

Recent evidence indicates that several experimental pathophysiological conditions are associated with elevated protease activity in plasma, which impacts endothelial function. We hypothesize that extracellular structures bound to the endothelial cell (EC) membrane may be degraded by proteolytic activity and cause the cells to respond abnormally to physiological shear stress (12 dyn/cm²). To test this hypothesis, cultured bovine aortic endothelial cells (BAECs) were exposed to low levels of a serine protease, trypsin. Extracellular mechanosensor densities of the glycocalyx and vascular endothelial growth factor receptor 2 (VEGFR-2) were determined. Metabolic dysfunction was tested by examining insulin receptor and glucose uptake levels. Protease treatment impaired the cells' ability to align in the direction of fluid flow after 12 hours of shear stress; however, cells realigned after an additional 12 hours of shear stress with protease inhibition. Proteases caused reduction in the densities of glycocalyx, VEGFR-2, and insulin receptor in static and shear conditions, except for static VEGFR-2 cells. Under static conditions, protease-treated endothelial cells had reduced glucose uptake compared to untreated controls. Under shear, however, glucose uptake for protease-treated BAECs was greater than untreated controls. In conclusion, protease activity in plasma alters the exofacial membrane components of ECs and may interfere with mechanotransduction.

Keywords

Mechanotransduction; VEGFR-2; insulin resistance; lectin; glycocalyx; autodigestion

Introduction

Cardiovascular functions are compromised in many diseases with a progression towards cell dysfunctions and eventually organ failure. We recently proposed a new hypothesis, termed the *Autodigestion Hypothesis*, suggesting that proteolytic activity originating from several sources, including the intestine, may damage extracellular components of cells, thereby disturbing their functions.^{1,2} In physiological shock, the mucosal barrier of the intestine, which serves to compartmentalize digestive enzymes, becomes compromised.^{3,4} As a result, digestive proteases penetrate into the intestinal wall and the circulation, resulting in increased proteolytic activity in plasma.⁵ As a result, ECs may exhibit morphological changes, such as hyperpermeability, accelerated cell detachment, apoptosis^{6,7} and increased insulin resistance.^{8–10} In chronic diseases, e.g. experimental hypertension and diabetes^{11,12}, enhanced protease activity levels are also observed in association with proteolytic receptor cleavage and loss of cell functions.^{11,13,14} There has been no study on the effect of

degrading enzyme activity on EC structure and function, such as cell orientation in response to fluid shear stress or glucose transport.

To gain insight into the mechanisms and degree to which extracellular protease may compromise endothelial functions, we examined the response of ECs to fluid shear stress *in vitro* in the presence of a selected serine protease, trypsin. We determined EC alignment, the density of selected extracellular mechanosensors (the glycocalyx, vascular endothelial growth factor receptor 2, i.e. VEGFR-2, and insulin receptor), and glucose transport in ECs after exposure to physiological levels of fluid shear with the addition of proteolytic activity. The results indicate that ECs are damaged by low levels of proteases, resulting in impaired responses to physiological shear stress and metabolic dysfunction.

Materials and Methods

Cell Culture Preparation

Bovine aortic endothelial cells (BAECs), which respond robustly to fluid shear stress, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Omega, Tarzana, CA), 1 mM sodium pyruvate (Invitrogen, Carlsbad, CA), 1 mM L-glutamine (Invitrogen), and 1% penicillin-streptomycin (Invitrogen). Cell cultures were grown on 10-cm dishes at 37 °C in a humidified 95% air, 5% CO₂ incubator. Cell passage was completed by applying 0.75 ml of 0.05% trypsin in ethylene-diamine-tetraacetic acid (Invitrogen) to confluent layers, and cells were split in a ratio of 1:4.

Calibration of Protease Activity

N- α -benzoyl-D L-arginine-p-nitroanilide (BAPNA) substrate was added to known trypsin concentrations ranging logarithmically from 110 μ M to 11 nM buffered in 1% serum to prevent the collapse of the glycocalyx¹⁵, and absorbance was read after 90 minutes. The trypsin activity was measured at 2.1 μ M, which is the optimum concentration exceeding the buffering capacity (Fig. 1).¹⁶ Complete inhibition of trypsin activity was achieved when serum was increased to 10% by volume of the media.

Shear Stress Experiments

A recirculating flow system was constructed to apply fluid shear stress on a confluent monolayer of BAECs. Cells were seeded on fibronectin (Invitrogen) coated slides at 1 μ g/cm² on a 75 mm x 38 mm glass slide. Fibronectin was diluted in Dulbecco's Phosphate-Buffered Saline (DPBS) and allowed to settle on the glass slide for one hour prior to cell seeding.

A parallel plate flow system was used to apply shear stress to the cultured BAECs. The shear stress applied to the cells was 12 dyn/cm² for all experiments assuming steady flow between two parallel plates for a Newtonian viscous fluid.

Static and sheared ECs were divided into three groups. In the first group, ECs were sheared for 12 hours in 1% serum medium (SH cells). ECs in the second group were sheared in 1% serum media for 12 hours with 2.1 μ M trypsin (P/SH cells). The ECs in the final group were sheared for 24 hours; the first 12 hours of this period were in the presence of 2.1 μ M trypsin and the second 12 hours the trypsin was inhibited by addition of 10% serum by volume to the media (P-I/SH cells). Studies on static unsheared cells were completed with matching media conditions conducted concurrently (ST, P/ST, P-I/ST cells, respectively). At the end of each treatment, cells were fixed with 4% paraformaldehyde for 15 minutes and washed twice with DPBS before storing at 4o C. ECs exposed to protease for 12 hours were also checked for cell death using propidium iodine.

Analysis of Cell Alignment and Elongation

Images were digitally analyzed (ImageJ, <http://rsbweb.nih.gov/ij/>). Cell area (a) and perimeter (p) were measured (Fig. 2a) to determine a circularity index (S) for the cell:

$$S = p/2 \sqrt{a\pi}$$

The orientation of the cells was classified by measuring the alignment angle θ of the cell (Fig. 2b). The maximum linear distance between two points on the cell periphery was determined. The cell alignment angle was designated as the angle made with the direction of fluid flow and this line.

The cell images were digitizing to an 8-bit map (1 to 256 digital units), and the light background was subtracted. On each image, individual cells were randomly selected using the polygon tool, and the average pixel intensity for the cell labeling density was measured.

Glucose Uptake

Endothelial cells were seeded onto fibronectin-coated coverslips thickness 1 (Fisher, Pittsburg, PA) and subjected to shear stress or static conditions. They were washed three times with PBS and then incubated with glucose-free media (Invitrogen) containing 10 μ M G-NBD-deoxyglucose conjugated with fluorescein (465 nm excitation/540 nm emission) for 30 minutes. The cells were washed with PBS twice and stored in glucose-free media until imaged using a confocal microscope (single z-sections at 60x).

Immunohistochemistry and Quantification of VEGFR-2, Insulin Receptor, Lectin Binding Levels

All reactions were carried out at room temperature unless indicated otherwise. After treatment, the cells were fixed for 15 minutes with 4% paraformaldehyde in DPBS followed by two washes (5 min each) with PBS. The slides were rinsed with hydrogen peroxide (0.15%) in water for 10 minutes, washed with DPBS for 5 minutes, and blocked with 5% normal goat serum (Vector Laboratories, Inc., Burlingame, CA) for one hour. Extracellular VEGFR-2 (Genetex, Irvine, CA) antibody diluted 1:100 was applied to each slide and incubated at 4°C on a rotary shaker overnight. Controls with irrelevant chicken antibody control and without primary antibody were prepared concurrently. Following incubation, the slides were washed for five minutes three times with DPBS. The secondary antibody, chicken IgY Fc (Genetex), was diluted 1:200 in PBS and applied to the slides for one hour. The slides were washed three additional times in DPBS (5 minutes each). ImmPact Dab (Vector Laboratories) was used to develop the staining. The slides were dehydrated with an ethanol gradient and then cleaned in xylene solution before mounting with hard-set mounting media (Vector Laboratories) containing a nuclear label (4',6-diamidino-2-phenylindole, Dapi) to confirm uniform cell density.

Insulin receptor staining was performed as described above with the exception of blocking with 10% horse serum for 30 minutes and addition of a primary antibody against the extracellular domain of the insulin R α (N-20, dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA) that was applied overnight at 4 °C on a rotary shaker. The controls consisted of an irrelevant rabbit antibody and no primary antibody. Anti-rabbit secondary reagent Immpress Kit (Vector Labs) was applied for 30 minutes and visualized with a peroxidase substrate (ImmPact DAB, Vector Labs). Slides were mounted as described above.

FITC-lectin from *Bandeiraea simplicifolia* (Sigma-Aldrich, St. Louis, MO) was incubated at 20 µg/ml in PBS containing 0.1 mM CaCl₂, 0.1 mM MgCl₂, and 0.1 mM MnCl₂ for one hour and washed with PBS containing 0.1 mM CaCl₂, 0.1 mM MgCl₂, and 0.1 mM MnCl₂ twice before mounting.¹⁷

To quantify the immunolabeling intensity, light absorption A_c was calculated according to $A_c = -\ln(I/I_0)$ where I is the intensity of the cell and I_0 the intensity for a white background (digital value = 255). FITC-lectin labeling and novobiocin-brilliant green (NBG) -glucose uptake nuclear regions were excluded during the analysis and the images were inverted to calculate the light absorbance.

Statistical Analysis

Measurements are presented as mean $\pm$ standard deviation. Comparison between groups was carried out between the sheared groups without and with protease. Comparison between two groups (shear/static) was made by t-test with a Bonferroni correction. Mann Whitney tests for non-Gaussian distributions were applied for the difference between the alignment angle and circularity index to account for the non-normal distribution with significances determined at $p < 0.01$. The labeling intensities for the 12-hour control (SH or ST), 12-hour protease exposure (P/SH or P/ST), and the 12-hour protease exposure followed by 12-hour protease inhibition (PI/SH or P-I/ST) were compared between all the shear groups and all the static groups using ANOVA. $p < 0.0001$ was considered statistically significant by Bonferroni correction t-test and indicated by * on the graphs. All experiments were repeated at least three times on separate days.

Results

Protease Activity Disrupts Endothelial Cell Alignment to Shear Stress

The characteristic response of ECs to fluid shear stress was attenuated when the cells were sheared in the presence of trypsin activity. The average alignment angle for the P/SH cells was significantly elevated and statistically normally distributed similar to the ST cells, whereas SH and P-I/SH cell angles were shifted towards lower values, i.e., aligned with the direction of shear stress (Fig. 3a, Table 1). The circularity index of ECs was reduced in P/SH compared to SH and P-I/SH cells (Fig 3b, Table, 1). None of the treatment groups exhibited cell death as determined by the apoptosis marker propidium iodide (results not shown).¹⁸

VEGFR-2 and Glycocalyx Label Density

The VEGFR-2 density was upregulated by exposure to shear stress in the absence of protease (Fig. 4c). The density of the VEGFR-2 extracellular domain label was not significantly altered in ST and P/ST cells, but significantly increased (18%) for P-I/ST cells compared to P/ST cells (Fig. 4a&c). P-I/SH cells showed increased VEGFR-2 expression by 50% over P/SH cells to levels similar to SH cells. There was no significant correlation between VEGFR-2 labeling intensity and cell alignment angle or circularity index.

The lectin label absorbance of the glycocalyx decreased by approximately 25% for P/ST and P/SH cells compared to their respective control ST and SH cells (Fig. 4b&d). The glycocalyx label density returned to values similar to the controls after protease inhibition for PI/ST and P-I/SH cells. We found no significant correlation between glycocalyx label intensity values and alignment angle.

Glycocalyx and VEGFR-2 intensities were measured on individual cells. There was no correlation between the intensity of the lectin label for the glycocalyx with VEGFR-2 among individual cells for any of the conditions (results not shown).

Insulin Receptor Density and FITC-Glucose Uptake

P/ST and P/SH cells had lower levels of insulin receptor density with reductions of 13% and 31%, respectively, compared to cells without protease exposure. The P-I/ST cell label density returned and, therefore, was not significantly different from the ST cells. P-I/SH cells did not have a significant increase in receptor density compared to P/SH cells (Fig. 5a&c).

This protease-induced reduction of the insulin receptor density on the ECs, which would lead to reduced glucose transport into the cytoplasm, was seen only in P/ST cells (Fig. 5b). Glucose uptake increased by 95% P/SH cells compared to SH cells. After protease inhibition, glucose transport levels of P-I/SH cells matched that of control SH cells (Fig. 5d).

Discussion

The current results show that trypsin-derived proteolytic activity at low micromolar concentrations reduces the ability of ECs to align in the direction of fluid shear stress. This effect was reversed after blockage of the protease activity. The exposure to trypsin also reduced the glycocalyx and the extracellular domain density of the insulin receptor in both P/ST and P/SH cells. Extracellular VEGFR-2 labeling decreased only in P/SH but not P/ST cells, which suggests that VEGFR-2 density is not impacted by trypsin activity.

Endothelial cells tend to elongate when pro-angiogenic cues are present, e.g. shear stress and VEGF.¹⁹ Since cell alignment is time-dependent, we selected a time point (12 hours) at which alignment was not yet complete, as shown in Fig. 3. The average circularity index for P/SH cells was greater than that for ST cells, indicating that the trypsin-treated sheared cells could still respond to elongation cues.

The vascular endothelial growth factor receptor 2 (VEGFR-2) is important in vascular endothelial cell survival, permeability, migration, and proliferation.^{20–22} The ability of VEGFR-2 to transduce shear stress by phosphorylation of its receptor through functional integrin association during angiogenesis has been related to the shear patterns.^{23,24} While the trypsin levels selected for the current study is insufficient to detach cells, integrins could still be a target for digestion by trypsin. P/SH cells responded less to shear stress, and the lack of response may impact the ability for these cells to upregulate VEGFR-2 expression.²³ In unsheared cells, VEGFR-2 labeling density did not show a significant difference between ST and P/ST cells, but had a significant increase by 18% in P-I/ST cells when the media was supplemented with serum to inhibit the protease activity and stimulate cell growth (Fig. 4a&c.).

The glycocalyx, another shear stress transducer, forms a negatively charged carbohydrate layer that lines the endothelium and has been found to be important in the orientation of endothelial cells in response to shear.²⁵ The glycocalyx density as detected by lectin labeling was reduced in the presence of trypsin in P/SH and P/ST cells; this density was restored when trypsin was blocked. Inter-alpha trypsin inhibitor is covalently embedded in the glycocalyx and may also block the circulating trypsin when it enters the glycocalyx network.²⁶

The ability of trypsin to diffuse through the glycocalyx towards the cell membrane may impact the degree of protein cleavage on the cell surface. The glycocalyx pores are approximately 10 nm in diameter and trypsin, with a diameter of ~5 nm, is subject to restricted diffusion. Trypsin has to diffuse through the full thickness of the porous network of glycocalyx fibers before being able to interact with cell membrane components, such as transmembrane receptors.²⁷ In the process, trypsin may be cleaving core glycoproteins that

anchor the glycocalyx to cause a decrease in the overall density of the glycocalyx. The presence of the glycocalyx may limit the kinetics of trypsin transport in such a small domain to cause functional loss. Sheared ECs develop a thickened glycocalyx, which may further reduce the ability of trypsin to diffuse through it and affect surface receptors.^{28,29} Proteases that penetrate the glycocalyx to reach the cell surface may continue to diffuse through a 20–100 nm opening into the caveolae to interact further with additional receptors.³⁰ VEGFR-2 and insulin receptors reside in the caveolae.^{31,32}

The insulin receptor, which is a transmembrane tyrosine kinase composed of two alpha and two beta subunits³³, binds insulin to activate the glucose transporter-4 for uptake of glucose into the cell.³⁴ Interference with this pathway leads to hyperglycemia. Insulin resistance occurs in several chronic models of hyperglycemia and may be caused by proteolytic cleavage of the extracellular domain of the insulin receptor, as has been detected in experimental hypertension and shock models.^{11,12,16} Cleavage of the insulin receptor may reduce the ability of the cell to take up glucose during shock and account for the hyperglycemia of patients in intensive care.⁸

The current results support the hypothesis that trypsin reduces the density of the extracellular domain of the insulin receptor of static and sheared cells, possibly by proteolytic cleavage. The glucose uptake was reduced in P/ST cells in conjunction with a decreased density of the insulin extracellular domain. The process was reversible since blockade of protease activity restored the receptor density. Restoration of the receptor density during the 12-hour inhibition period may result from *de novo* synthesis of the receptor.

In contrast to P/ST compared to ST, glucose uptake in P/S cells was nearly doubled SH cells' uptake. The decrease in the insulin receptor density by trypsin in sheared ECs was less than that in ST cells, suggesting that fewer insulin receptors were cleaved by the protease in the sheared cells. Additionally, an increase in metabolic needs of ECs during the alignment with the fluid shear stress may explain why all ECs exposed to shear stress have increased glucose uptake compared to the corresponding static cells. There was no evidence for apoptosis of the ECs in response to protease. The occurrence of apoptosis would have been accompanied by uncontrolled entry of glucose into the cell cytoplasm.

The trypsin concentrations chosen for these experiments are greater than the current concentrations measured in the normal plasma of rats and humans which are on the order of ng/ml as compared to the concentration in the current experiments, 50 µg/ml (2.4 µM).³⁵ In physiological shock, the serine protease activity is considerably elevated and comparable to the concentration used in these current experiments.¹⁶ The trypsin concentrations used in the present study were less than those measured in the intestine, 300 µg/ml (9.6 µM), and hence ECs in a permeable intestine may be exposed to intestinal proteases at the order of magnitude used in the current experiments.³⁶ The purpose of selecting a higher concentration of trypsin was to demonstrate the potential damage that proteases can cause to ECs. In chronic conditions, trypsin exposure even at lower concentrations may gradually damage the EC over a longer period of time. Such chronic conditions remain to be investigated. Proteolytic activity at higher than 2.1 µM in the 1% serum media may also disturb the junctional proteins that cause leakage and weaken integrin attachments resulting in reduced cell adhesion.

The interaction of trypsin with a receptor involves the cleavage after arginine and lysine amino acids. More complicated protein structures such as receptors may not have these amino acids accessible for the active site of trypsin to bind to complete the peptide hydrolysis.³⁷ Protecting lysine and arginine amino acids in the protein structure may serve to

reduce degradation of receptors.³⁸ These high-probability cleavage sites may be protected by the secondary protein structure against enzymatic degradation by trypsin. Significant proportions of the extracellular domains of VEGFR-2 and insulin, 13% and 6% respectively, are composed of arginine and lysine residues, which could be targets of trypsin degradation.

In summary, our *in vitro* investigation demonstrates a mechanism for the potential loss of important endothelial responses. The inability for ECs to align to the direction of shear stress and reduction of metabolic function in response to protease activity are just two cases where proteases affect the cellular function. Since there are many possibilities for the endothelial cell's extracellular structures to be damaged by elevated serine protease activity in plasma, additional *in vivo* investigations on this potentially important topic are warranted.

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Figure 1.

Trypsin activity absorbance (A) fit by the best fit line where $A=0.12 \cdot x^{0.73}$ with R^2 value of 0.96. The arrow indicates the absorbance at a concentration of 2.1 μM trypsin which was used in the experimental model.

Figure 2.

(a) Example of the endothelial cell perimeter, p , and area, a , measurements. (b) Determination of the cell alignment angle with the orientation of the flow (arrow). Points 1 and 2 represent the maximum distance on the cell's perimeter connected by a line. The alignment angle, θ , is determined from the line between points 1 and 2 and the flow direction.

Figure 3.

Histograms and fitted (lognormal) probability density function for (a) the alignment angle, θ , (left column) and (b) circularity index, S , (right column). SH cells have lower alignment angles and increased circularity indices (first row) compared with P/SH cells (second row). Note the right shift in P/SH cells, indicating reduced alignment. * $p < 0.01$ for comparison of median values between P/SH and SH cells. $n = 100$ cells/group. The best fit for the log-normal functions to the histogram are similar for the SH and P-I/SH cells, indicating the expected cell elongation is induced during shear exposure. ST cells exhibited similar trends to the endothelial cells sheared in the presence of protease.

Figure 4.

(a) and (c) Images of VEGFR-2 extracellular labeling. VEGFR-2 staining for SH cells (flow direction indicated by the arrow) was reduced in P/SH cells only. Histograms show measurements of the labeling density as determined with light absorption. The length bars are 10 μ m. (b) and (d) Images of lectin labeled cells. Lectin label density was reduced in P/ST and P/SH cells. The length bar is equal to 5 μ m. * p <0.0001. The numbers in the bars indicate the number of cells measured.

Figure 5.

(a) and (c) Images of insulin receptor after labeling with an antibody against the extracellular receptor domain. Insulin receptor staining for SH was reduced in both P/SH and P/ST cells (flow direction specified by the arrow), but recovered in the P-I/ST group. Histograms show the labeling intensity levels for the images on the left. The scale equals 10 μ m. (b) and (d) Images of NBD-glucose labeled cells. Glucose uptake was reduced in the P/ST but not in P/SH cells. The length scale equals 5 μ m. * $p < 0.0001$. The number of cells measured are indicated in the bars.