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Proteolytic Receptor Cleavage and Attenuated Endothelial Cell Response to Fluid Shear Stress in a Model for Autodigestion in Shock

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by

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The thesis of Angelina E. Altshuler is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

I dedicate this thesis to my family for their encouragement and support.

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LIST OF ABBREVIATIONS

BAEC	Bovine aortic endothelial cell
DPBS	Dulbecco's phosphate buffered saline
EC	Endothelial cell
MMP	Matrix metalloproteinase
PECAM-1	Platelet endothelial cell adhesion molecule 1
TIMP	Tissue inhibitor of metalloproteinase
VEGFR-2	Vascular Endothelial Growth Factor Receptor 2

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ABSTRACT OF THE THESIS

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In physiological shock, a leading cause of death, cell and tissue function are compromised. Once proteases enter into the circulation during shock, there may be proteolytic damage to the extracellular components of cells including cleavage of surface receptors and the glycocalyx of the endothelial cells (ECs). The endothelial cell is sensitive to physiological shear stress (typically 10-15 dyn/cm^2) and aligns in the direction of the fluid flow.

Sheared endothelial cells exhibited a higher tolerance to protease exposure than static cells. Extracellular damage to the endothelial cell by active proteases causes the ECs to not align in the direction of the fluid shear stress; however when the proteases were inhibited, ECs exposed to shear stress could realign with the direction of the flow. Receptor intensity of the mechanosensors VEGFR-2 and junctional PECAM-1 was reduced on both sheared and unsheared ECs. The insulin receptor surface density was also reduced for cells exposed to proteases.

In conclusion, proteases present in the circulation during shock may damage extracellular molecular membrane components on the cell and interfere with one of the most basic mechanotransduction mechanisms in endothelial cells, i.e. aligning in the presence of physiological shear stress.

I. INTRODUCTION

A. INTRODUCTION TO SHOCK AND THE AUTODIGESTION HYPOTHESIS

In shock the normal physiological state is severely perturbed and it may lead to death. There are multiple forms of shock and all are associated with high mortality (de Jonghe et al. 2009, Bilello et al. 2003, Gangemi et al. 2009). For example, nearly 215,000 deaths a year are caused by septic shock in intensive care units (Parrillo 2008). Hemorrhagic shock is the leading cause of death of humans under the age of 44 years (Jochberger et al. 2006). Shock is accompanied by a succession of organ dysfunctions referred to as *multiple organ failure*, which is the cause of the mortality in shock (Barie et al. 2009). The rapid progression of multiple organ failure and the generation of uncontrolled and widespread cell activation associated with the decline in cell and organ functionality has long been attributed to the intestine (Schmid-Schönbein 2007, Fukuda et al. 2004, Schmid-Schönbein, Kistler & Hugli 2001; Hou et al. 2009).

A number of hypotheses have been advanced to explain the rapid progression of cell and organ failure in shock. Shock has been hypothesized to be caused by endotoxins or cytokines (Fukuda et al. 2004). Leukocytes were hypothesized to cause organ damage and blocking leukocyte adhesion molecules would prevent the continued progression of inflammation and microvascular occlusion (Mitteregger et al. 1999, Barroso-Aranda et al. 1988). Oxygen free radical scavengers and nitric oxide modulators were hypothesized to contribute to the cell and organ failure (Waxman 1996, Rubanyi 1998). However, none of these hypotheses served to fully explain the cascade of events in shock that leads to multiple organ failure (Schmid-Schönbein 2009).

Recently, a new hypothesis has been advanced referred termed the *Autodigestion Hypothesis* suggesting that proteolytic activity damages extracellular components of cells and tissues thereby compromising homeostasis (Schmid-Schönbein 2009). This hypothesis is based on the idea that the primary source of the enzymatic activity necessary for nonselective degradation is in the intestine where proteins, lipids, and carbohydrates are degraded into absorbable components as part of normal digestion. While the intestine absorbs and transports digested nutrients into the blood stream, the powerful digestive enzymes synthesized by the pancreas are contained in the lumen of the intestine with minimal entry into the circulation. This containment of the pancreatic digestive enzymes is provided by the mucosal barrier. The mucosal barrier is composed epithelial cells and a mucin layer generated by goblet cells. The mucus layer protects the intestinal enterocytes and villi from damage by the digestive enzymes and bacteria contained in the intestine (Sheth et al. 2010). In shock, the mucosal barrier of the intestine is damaged allowing the digestive proteases to pass into the intestinal wall (Penn, Hugli & Schmid-Schönbein 2007, Rosario et al. 2004). Diseases such as pancreatitis where there is a secretion of digestive enzymes into the blood stream have similar consequences as shock (Wilson, Manji & Neoptolemos 1998). Since the mucosal barrier is essential for containing the proteases, under circumstances where this barrier function is compromised, the cells in the circulation including the endothelial cells become potentially exposed to proteolytic degradation (Sun et al. 2001).

Several recent shock studies have established a correlation between the ability of an animal to recover from shock and blockage of the digestive enzymes (Mitsuoka, Kistler & Schmid-Schönbein 2000, Ohnishi et al. 1985, Doucet et al. 2004, Fitzal et al.

2004). If the enzymes in the intestine are blocked during shock, cell and tissue injury is decreased. There are fewer activated leukocytes, fewer rolling leukocytes in venules, reduced apoptosis, in addition to a higher incidence of survival (Fitzal et al. 2002). Interestingly, several earlier studies provoked shock by inducing an elevated proteolytic activity (Balldin, Ohlsson 1981). Observing how the intestinal contents can directly activate a response in tissues and cells even though they are not in direct contact with the wall raises the question of how damaging the digestive enzymes can be to the lining of the circulation.

The Autodigestion Hypothesis may not be limited to shock, but other diseases may also be accompanied by elevated enzymatic activity in the circulation. However, shock may be one of the most intense forms of autodigestion.

B. ENDOTHELIAL DYSFUNCTION AND MECHANOTRANSDUCTION

Arteries consist of three main layers. The intima is lined by endothelial cells at the interface with the blood in the lumen. The media is composed of smooth muscle cells, which synthesize extracellular matrix and maintain vascular tone. The adventia is the outermost layer of the artery and contains connective tissues including macrophages, fat cells, and the vasa vasorum. Endothelial cells are located at the interface between the blood flow and the tissues and are exposed to fluid shear stresses. Endothelial cells also experience circumferential normal stresses (associated with stretch of the vessel wall) as well as circumferential stretch and compression during vasodilatation and vasoconstriction by the smooth muscle cells. There is fluid pressure (normal stress)

acting on the endothelium. Lastly, the blood flowing over the wall of the endothelium exerts a shear stress over the endothelium (Sato, Ohashi 2005).

Since the endothelium interfaces with the blood flow and protease activity in blood is elevated in a state of shock, the focus of this thesis will be on the response of these cells after protease exposure. In hemorrhagic shock, endothelial cells exhibit hyperpermeability and an increased rate of apoptosis (Childs et al. 2007, Maier, Bulger 1996, Matsuda et al. 2010, Masini et al. 2006). Since endothelial cells form a monolayer in the interior of the blood vessel, cell detachment from the vessel leads to morphological changes and ultimately apoptosis. Activating endothelial apoptosis correlates with the level of permeability of the endothelium (Grunenfelder et al. 2001, Senthil et al. 2006). The function of endothelial cells is compromised and the number of endothelial progenitor cells is also decreased in septic shock (Luo et al. 2009). However, no hypothesis exists that is universally accepted which can provide a mechanism which causes the dysfunction of the endothelial cells during shock.

Endothelial cells in regions of laminar flow respond to shear stress by orientating in the direction of the flow. In static states, endothelial cells tend to form cobblestone patterns. Therefore, in vascular regions with disturbed flow direction and magnitude, the endothelial cells do not remodel. In the arterial tree, branching points are susceptible due to a disturbed fluid shear stress (Chien 2008, Chiu, Usami & Chien 2009, Davies 2009). Shear stress modulates endothelial cell phenotype and affects protein expression promoting cells to reside in the anti-inflammatory state. Laminar compared to disturbed flow applied to endothelial cells affects: vasoactive response, EC turnover, macromolecular permeability, ability to uptake LDL, synthesis of DNA, phenotype,

expression of adhesion molecule, inflammatory and chemokine genes, antioxidant genes, white blood cell adhesion and platelet aggregation (Fisher et al. 2001). Cell turnover is faster resulting in leakier endothelial lining where in more extreme circumstances permeability of the endothelium is increased 50-100% in some regions (Weinbaum et al. 1985). In vascular areas of disturbed flow, some of the hemodynamic non-uniformities are caused by flow separation, transient flow reversals, and on average a lower shear stress (Davies 2008).

When gene expression in endothelial cells derived from disturbed flow and uniform flow were compared in swine aortas the disturbed flow genes exhibited an overall gene upregulation. Specific genes encoding proteins that are involved in adhesion, apoptotic, cytoskeleton, extracellular matrix, immune response, inflammation, proliferation, signal transduction, and transcription genes were upregulated.

At branch points in arteries, there tends to be back flow and regions with lower shear stress. The branch points have characteristics that make them more susceptible to inflammation, notability a chronic state of atherosclerosis. Inflammatory genes are up-regulated, monocyte adhesion increases, stress fibers are misaligned, permeability is increased, and the cells express an overall pro-atherogenic phenotype compared to endothelial cells under laminar flow (Chien 2008, Gonzales, Wick 1996, Chien 2007). Though the regions of disturbed flow correspond with non-aligned endothelial cells, the local hemodynamics dictate a range of gene, morphological, and protection in response to the flow patterns (Davies 2008, Chien 2007). The distinct phenotypes of the endothelial cells may also be important in protecting humans in more acute conditions such as shock.

As mentioned, when exposed to fluid shear stress, endothelial cells respond by aligning with the direction of the fluid flow. The particular gene expressions and morphology have been well studied (Chiu, Usami & Chien 2009, Laughlin, Newcomer & Bender 2008). There are several classes of extracellular molecules that are hypothesized to take part in sensing fluid shear stress.

i. GLYCOCALYX

The glycocalyx, which is composed of a polysaccharide matrix that covers the surface of the endothelial cells, is a selective protein filter and has been shown to aid in the mechanotransduction of endothelial cell. If the layer is intentionally cleaved, the mechanical response of the endothelial cells decreases (Chappell et al. 2008, Florian et al. 2003, Florian et al. 2003). The glycocalyx is anchored by extracellular proteins called lectins which may also play a role in regulating the uniformity of the glycocalyx (Collard et al. 2001).

In many cases of shock, there is evidence that the deterioration of the glycocalyx is partially attributed to endothelial dysfunction detected (Chappell, Westphal & Jacob 2009). In septic shock, the glycocalyx layer was decreased in shock animals compared to controls (Gotloib et al. 1992). The mechanotransduction capabilities of endothelial cells could be compromised when exposed to uninhibited proteolytic activity.

ii. INTEGRINS

Integrins, which mediate endothelial cell attachment, participate in mechanotransduction mechanisms that signal the endothelial cell to align with the

direction of the shear stress (Shyy, Chien 2002). The transmembrane domain of the integrin has the ability of transducing intracellular signals that switch the cell to the anti-inflammatory state (Radel, Rizzo 2005). Integrins also attach to extracellular membrane components such as fibronectin, laminin, and collagen (Giancotti, Ruoslahti 1999). Shear stress activates integrins to bind to the substrate and increase in density. This has been shown by a variety of studies that block the integrin signaling pathways by either antibodies or peptides (Shyy, Chien 2002). Since integrins form the interface between the cell and the attachment surface, the molecular attachments made are critical to the cell's homeostasis. As observed, cells that are sheared have more attachments to the substrate than unsheared cells (Urbich et al. 2000).

In cell cultures, the serine protease trypsin is a widely used enzyme to detach cells prior to reseeding (instead of scraping the cells). The concentrations and duration of trypsin exposure are minimized to reduce damage to the extracellular components of the cells because extreme exposure is linked with apoptosis (Marthinuss, Andrade-Gordon & Seiberg 1995). The strength of attachments was tested by exposing freshly seeded cells with damaged attachments to high shear stress levels, observing cell spreading area, and integrin density (Brown et al. 2007). ECs exposed to higher trypsin concentrations had a decreased integrin density (Brown et al. 2007). In cell culture, it is typical to allow recently passaged cells to reestablish their integrin attachments with the substrate for approximately one hour before imposing any mechanical stresses to the cells. Additionally, the cell may also need to synthesize and repair damaged surface receptors that may have been degraded during the trypsinization process. Although concentrations of trypsin used in cell culture are much higher than those in the plasma, it does not

eliminate the possibility for acute damage of extracellular receptors by plasma with increased proteolytic activity. Endothelial dysfunction and permeability attributed to shock may contribute to symptoms like increased permeability, loss of functional extracellular components, and inflammation.

iii. VASCULAR ENDOTHELIAL GROWTH RECEPTOR 2 (VEGFR-2)

The receptor VEGFR-2 is a type III kinase receptor important in vascular endothelial cell survival, permeability, migration, and proliferation (Holmes et al. 2007, Yamamoto et al. 2005). The extracellular region contains seven immunoglobulin-like domains connected to a short transmembrane domain to the intracellular region containing the tyrosine kinase domain (Shibuya 2002). When VEGFR-2 is inhibited, cell survival decreases (Shimotake et al. 2010).

VEGFR-2 is able to transduce shear stress, and the endothelial cell's response to flow occurs within the first 15 seconds by phosphorylation of the receptor (Tzima et al. 2005a). The gene expression for VEGFR-2 receptor is significantly increased during exposure to 24 hour shear stress (Chen et al. 2001). Shear stress activates the VEGFR-2 receptor and induces VE-cadherin-β-catenin-VEGFR-2 complex (Shay-Salit et al. 2002). VEGFR-2 and integrins have a functional association during angiogenesis related to the shear patterns (Somanath, Malinin & Byzova 2009). Integrins are necessary for the mechanical stimulation of VEGFR-2 but not the chemical stimulation (Wang et al. 2007b).

In addition, VEGFR-2 controls some inflammatory genes necessary for reducing inflammation like the transcription factor NFκB, which controls the transcription of DNA

in response to stress (Liu et al. 2008). Blocking VEGFR-2 by an inhibitor disrupts the shear-induced translocation of NFκB (Wang et al. 2009). However, this mechanism can also be inhibited by reduction of the extracellular binding site of the receptor on the endothelial cell. There have been no studies on the effect that shock conditions may have on this receptor.

iv. PLATELET ENDOTHELIAL CELL ADHESION MOLECULE (PECAM-1)

PECAM-1 contributes to a variety of important events including leukocyte migration through the walls of the endothelium and mechanotransduction (Woodfin, Voisin & Nourshargh 2007). PECAM-1 has a molecular weight of 130 kDa. It consists of 6 extracellular folds and typically binds with itself, a process that is of importance in leukocyte, platelet, and endothelial interactions. Cells with PECAM-1 gene deletion are unable to orient their actin fibers in the direction of the fluid flow (Tzima et al. 2005c).

PECAM-1 plays an important role in facilitating the migration of monocytes and leukocytes through the endothelium (Muller 2003). While the typical location of PECAM-1 is between the cell junctions, PECAM-1-mediated leukocyte migration occurs when intracellular stores of the PECAM-1 move to the cell surface (Dangerfield et al. 2002). This phenomenon has not been documented in endothelial cells.

PECAM-1 has been monitored in ischemia-reperfusion injuries and when blocked produce anti-inflammatory characteristics. Blocking the binding site of PECAM-1 with an antibody reduces the severity of the ischemia-reperfusion injury (Sun et al. 2001). Once the PECAM-1 molecule is activated and in abundance, inflammation may be present (Turegun et al. 1999).

In shock, the inflammatory cascade is clearly activated and associated with increased expression of molecules. For example after 90 minutes of shock, the PECAM-1 labeling levels in the tissues increased (van Meurs et al. 2008). The tissues in these experiments were fixed with acetone, which permeabilizes the membrane, so the antibody would detect intracellular and extracellular PECAM-1.

Studies in mouse knockout models indicate that PECAM-1 is critical to vascular remodeling (Chen, Tzima 2009). The vascular remodeling capability of cells without PECAM-1 was reduced. Inward remodeling of PECAM-1 was associated with surface adhesion molecule expression, leukocyte adhesion, and NFκB expression. All of these are important factors in upregulation of the inflammatory cascade. The same has not been tested in shock animals, but monocyte adhesion is reduced in areas of laminar flow where there is a constant shear stress applied to the endothelial cells (Tedgui, Mallat 2001). The role of PECAM-1 in shock as an inflammatory mediator and mechanotransducer suggests that it may be damaged by proteolytic activity and is therefore a target for the current investigation.

C. SERINE PROTEASES AND MATRIX METALLOPROTEINASES

The pancreas is a major source of serine proteases (chymotrypsin, trypsin, and elastase) produced in the acinar cells in the proenzyme form. The proenzymes are secreted into the small intestine through pancreatic ducts and are activated by the change to a basic pH in the small intestine. Once activated, serine proteases are extremely powerful enzymes cleaving the carboxyl side after one individual amino acid (i.e. trypsin cleaves after lysine and arginine amino acid residues). The high concentration and

activity of these enzymes in the intestine is necessary to digest food to absorbable monomeric components (monosaccharides, amino acids, etc.). The digestive proteases are compartmentalized in the lumen of the small intestine by a protective mucosal barrier, which prevents them from entering the wall of the intestine under normal physiological conditions. When the intestinal wall is damaged, inflammation and disease risks arise (Turner 2009). If the process of the high turnover of mucin and epithelial cells in disrupted, the endothelial cells are susceptible to injury (Wang et al. 2007a).

The serine proteases can be inhibited by a variety of small molecules. In the plasma, anti-α-trypsin blocks trypsin. Anti-α-trypsin may be degraded by plasmin (Lambin, Audran & Steinbuch 1977). In vitro, diatomic cations such as calcium and magnesium also have the capability to inhibit the activity of the serine proteases. Serine proteases also have the capability to degrade each other if the necessary cleavage site is exposed. Trypsin has been hypothesized as the culprit in many diseases due to the potential degradation power of this enzyme (Sha, Ma & Jha 2009).

Synthetic protease inhibitors such as ANGD and gabexate mesilate have been effective in blocking serine protease and reducing inflammation during shock (Mitsuoka, Schmid-Schönbein 2000, Harada, Okajima & Kushimoto 1999).

Matrix metalloproteinases (MMPs) are another important family of degrading proteases. They have a zinc catalytic center and are endopeptidases. Similar to serine proteases, MMPs are synthesized in the pro-enzyme form and can be activated. MMPs have been found to be activated by serine proteases (Lindstad et al. 2005, Kistler, Hugli & Schmid-Schönbein 2000). MMPs primarily function to remodel tissues and extracellular components. They are synthesized on many different types of cells

including leukocytes and endothelial cells. MMP-1, MMP-2 and MMP-9 are synthesized by endothelial cells (Arenas et al. 2004, Genersch et al. 2000, Hanemaaijer et al. 1998).

MMPs also have a specific class of inhibitors called tissue inhibitors of metalloproteinases (TIMPs). The balance between MMPs and TIMPs is critical to regulating their activity (Verma, Hansch 2007). Many synthetic drugs such as statins and doxycycline are capable of inhibiting MMPs (Hanemaaijer et al. 1998). Blocking the MMPs (e.g. with doxycycline) has tremendous impact on other factors associated with inflammation, such as hypertension (DeLano, Schmid-Schönbein 2008).

D. PLASMA PROTEASE ACTIVITY

There is evidence for elevated proteolytic activity in the plasma of shock animals. If the pancreatic enzymes are blocked prior to inducing shock in the rat model, the activity in the plasma is reduced (DeLano). Amylase and lipase activity levels were found higher in shock patients (Malinoski et al. 2009). The plasma of shock animals has been shown to increase endothelial permeability, although the mechanism is not established (Magnotti et al. 1998). Digestive enzymes may be involved in generating inflammatory mediators in the circulation (Mitsuoka, Schmid-Schönbein 2000). The presence of proteolytic activity has been documented in the plasma of rats. Other supporting evidence that pancreatic activity instigates the inflammatory response is the observation of increased MMP-9 activity on endothelial cells of shock animals (Rosario et al. 2004). MMPs are activated by serine proteases. Since serine proteases activate other proteases on both the endothelial surface and on other cells in the circulation, the

cascade results in an amplification of inflammation in the cardiovascular system (Kistler, Hugli & Schmid-Schönbein 2000).

Aside from shock, evidence from several studies indicates there is serine protease activity in the plasma of healthy mammals. Enzymatic activity of rat plasma measured over a course of 2 years for trypsin and elastase were recorded to change with the aging process (Paczek, Michalska & Bartlomiejczyk 2009). In healthy humans over a wide array of ages, the protease activity of elastase and plasmin increased where the production of α-1-antitrypsin increased with age (Paczek, Michalska & Bartlomiejczyk 2008). Measuring low levels of protease activity in whole blood is currently limited by the sensitivity of the detection of substrates to values of the order of nanograms (Lefkowitz et al. 2010). Enzyme activities below these values are currently largely undetected.

In hypertension, plasma protease activity was found also to be elevated (DeLano, Schmid-Schönbein 2008, Tran, Delano & Schmid-Schönbein 2010). Pancreatitis, a severe disease in which proteases are released from an inflamed pancreas, also has high protease activity in the plasma. Complexes of trypsin and inhibitor have been documented in pancreatic cases to be 182±53 ng/ml in surviving animals and 542±356 ng/ml in dying animals (Largman, Reidelberger & Tsukamoto 1986).

The dramatic effect of plasma with proteolytic activity on surface receptor and endothelial function raises many unexplored questions. Are extracellular components being cleaved and does this compromise the mechanical response of the endothelial cells in contact with plasma containing protease activity?

E. SOLUBLE RECEPTORS: EVIDENCE OF RECEPTOR CLEAVAGE

Protein fragments have been detected in the circulation of mammals with inflammatory diseases. Increased vascular permeability and macrophage-induced regions correlated with elevated P-selectin levels (Kisucka et al. 2009). Soluble insulin receptors measured in human plasma samples were associated with Type II diabetes (Soluble Insulin Receptor Study Group 2007). Plasma of obese patients contained higher levels of soluble VEGFR-2 receptor and correlated to diabetes occurrences (Wada et al. 2009). While soluble receptors are documented, there is no accepted mechanism for how the fragments appear in the plasma.

In physiological shock, soluble ICAM-1 (inter-cellular adhesion molecule 1) and ELAM-1 (endothelial leukocyte adhesion F molecule 1) have also been detected (Hein et al. 2005). In septic shock, circulating endothelial cells have been documented (Mutunga et al. 2001). Furthermore, there are many physiological processes that could potentially be explained by a receptor cleavage mechanism.

F. INSULIN RECEPTOR AND HYPERGLYCEMIA IN SHOCK

The insulin receptor is a transmembrane tyrosine kinase composed of two alpha and two beta subunits (Blanquart, Achi & Issad 2008). The insulin receptor binds the hormone insulin, which activates the glucose transporter-4 to uptake glucose into the cell (Dugani et al. 2008). Once glucose enters the cell, it can be either stored or broken down. When there is an interference with this pathway, glucose remains in the blood causing hyperglycemina or high plasma glucose.

While the most frequent discussion about insulin resistance is in the context of cardiovascular diseases and metabolic syndrome, a less known case of hyperglycemia, a condition with high glucose concentrations in the plasma, has been documented in shock patients and the critically ill (Fahy, Sheehy & Coursin 2009, Xu et al. 1996, Zhai, Messina 2009). The incidence of diabetes in hospitalized patients is between 12.3-25% (Clement et al. 2004).

In hypertension, proteolytic activity has been documented in the plasma of the spontaneous hypertensive rat (SHR). The density of the extracellular insulin receptor was found to be reduced in the SHR compared to control animals (DeLano, Schmid-Schönbein 2008). There is a possibility that endothelial cell functions are compromised when exposed to proteases. The elevated proteolytic activity in shock plasma derived from the intestine may be compromising the function of the insulin receptor.

G. SUMMARY

Although the mechanotransduction of endothelial cells is relatively well understood, the endothelial response to shear stress in the presence of proteolytic activity has not been explored. In shock, the powerful proteolytic enzymes that are an integral part of the digestive system are no longer compartmentalized in the intestine and have been observed to enter the general circulation. The presence of proteases with an increased activity is associated with damage of extracellular surface components that are critical for cell function and survival. The insulin receptor, VEGFR-2, and PECAM-1 are candidate receptors out of a multitude of extracellular receptors that could potentially be

enzymatically damaged on the endothelial cells. It is uncertain if fluid shear stress exposure protects the endothelium from proteolytic activity in the circulation.

H. HYPOTHESIS

1. Endothelial cells exposed to proteases are subject to membrane damage resulting in conformational changes and loss of the extracellular domain of membrane receptors.
2. Endothelial cells sheared in the presence of proteases will not align with the fluid shear stress.
3. Sheared endothelial cells exhibit a reduced degree of proteolytic damage of surface receptors compared to unsheared endothelial cells.

I. OBJECTIVE

Investigate to what degree proteolysis by digestive enzymes causes injury to the endothelium leading to a shift in cell morphology, surface receptor density, and response to fluid shear stress.

J. SPECIFIC AIMS

1. Investigate the effects of proteolytic cleavage on morphology (area & perimeter) of static vs. pre-sheared ECs.
2. Investigate response of ECs to fluid shear stress with and without concurrent protease exposure.

3. Investigate cleavage of the extracellular domain of the following receptors in the presence of trypsin:
    a. PECAM-1 (CD-31)
    b. VEGFR-2 (Flk-1)
    c. Insulin receptor (CD-220)

II. Materials and Methods

A. INTRODUCTION

In order to facilitate investigation of endothelial cell responses in the presence of elevated proteolytic activity to similar levels found in shock, cultured bovine aortic endothelial cells (BAECs) were grown and used in these experiments. The cell's ability to align in response to shear stress was investigated with and without receptor cleavage upon exposure to proteases. Detection of extracellular receptor density was carried out for receptors involved in the shear response (PECAM-1 and VEGFR-2) and in metabolic physiological function (insulin receptor).

B. CELL CULTURE PREPARATION

Bovine aortic endothelial cells (BAECs) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Omega), 1 mM sodium pyruvate (Invitrogen), 1 mM L-glutamine (Invitrogen), and 1% penicillin-streptamycin (Invitrogen). Cell cultures were grown on 10 cm dishes at 37 ºC in a humidified 95% air, 5% CO_2 incubator. Cell passage was completed by applying 0.75 ml of 0.05% trypsin in EDTA (Invitrogen) to confluent layers and cells were split at a ratio approximately of 1:4.

C. FLUID SHEAR STRESS APPLICATION

A recirculating flow system was constructed in order to impose fluid shear stress on a confluent monolayer of BAECs. Cells were seeded on fibronectin (Invitrogen) coated slides at 1 μg/cm^2 on a 75 mm by 38 mm glass slide. The fibronectin was diluted in PBS and allowed to set on the glass slide for 1 hour prior to cell seeding (Iuliano, Saavedra & Truskey 1993).

A silicone gasket with a thickness of 0.250 mm was sandwiched between the endothelial cell coated slide and an acrylic rectangular flow chamber. The device was secured with four large clips. The purpose of the gasket was to provide a confining space for which culture medium could flow through the inlet over the cells to the outlet of the flow chamber. The flow was generated by a hydrostatic pressure difference between one upper and one lower reservoir. The flow system was contained in a temperature controlled box at 37 ºC. The circulating medium was ventilated with humidified 5% CO_2 and 95% air.

The shear stress applied to the cells was 12 dyn/cm^2 for all experiments calculated from the following equation derived from steady Poiseuille flow between two parallel plates:

$$\tau = \frac{6\mu Q}{wh^2}$$

where Q is the flow rate, w is the width of the chamber, h is the height of the chamber, μ is the viscosity, and τ is the shear stress.

D. PROTEASE ASSAYS

i. BAPNA ASSAY

To test the range of inhibition of 1% reduced serum media, N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA) stock solution of 20 mM was made in dimethyl sulfoxide. The samples were prepared by mixing 10 µl of 2% fetal bovine serum with a dilution of trypsin ranging from 110 µM to 11 nM. The controls were 1% serum without trypsin, 1.1 M trypsin in DPBS and DPBS. Samples were loaded into a 96 well plate. Data was acquired from readings taken every 15 minutes at room temperature at 305 nm wavelength. Samples were run in multiples of four.

ii. CASEIN ASSAY

Simulation of proteolytic activity was carried out by first determining the minimum amount of protease addition to see activity in the casein substrate red fluorescence assay (Invitrogen). Concentrations tested ranged from 50 µg/L to 7.36 ng/L by a four dilution in DPBS to view the range of activity. The samples were loaded into a black walled clear bottom plate and the fluorescence was measured at 589 excitation and 617 emission for 1.5 hours reading every 2 minutes.

E. TRYPSINIZATION RESPONSE

Cells were either grown to a confluent layer or cells were sheared for 48 hours in growing medium. Cells were rinsed with DPBS 3 times. After washing, 21 µM (0.05%), 2.1 µM (0.005%), or 0.21 µM (0.0005%) of trypsin in DPBS was applied to the static cells for a duration ranging from 6-10 minutes and placed under a 10x objective for

contrast microscopy imaging kept inside a temperature control box at 37º Celsius. The sheared cells were exposed to 0.21 μM trypsin for 20 minutes. Perimeter and area were quantified over a preselected time interval.

i. F-ACTIN ANALYSIS

AlexaFluor 488-conjugated phalloidin (Molecular PROBES; Eugene, OR) was used to label F-actin. Prior to labeling, cells were washed three times with DPBS and incubated with 0.21 μM trypsin in DPBS for 15 minutes. After 15 minutes, the reaction was stopped by washing the cells with 10 ml of 10% serum media followed by a DPBS wash. ECs were fixed in 4% paraformaldehyde for 15 minutes, washed twice with PBS, and permeabilized with 0.1% Triton X-100 for 5 minutes followed by two more washes with PBS. The stock solution of 6.6 μM was diluted to 166.5 nM in PBS. 400 μL of the working solution was applied to each slide for 20 minutes. Following incubation, slides were rinsed 3 times with PBS and mounted with DAPI hard set mounting media (Vector Laboratories).

F. PROTEASE EXPOSURE TIME COURSE DURING SHEAR STRESS APPLICATION

i. PREALIGNMENT PRIOR TO TRYPSIN EXPOSURE

Endothelial cells were prepared on glass slides and sheared at 12 dyn/cm^2 for 16 hours in 1% serum media. Control cells were exposed to shear stress without exposure to proteases and the experimental group was exposed to proteases continuously at low activity throughout the 16 hour shear period (**Figure 1, design 1**).

ii. **EXPOSURE TO PROTEASES SHEAR RESPONSE OF ENDOTHELIAL CELLS**

ECs were seeded onto glass slides and washed once with DPBS before shearing at 12 dyn/cm^2 for 24 hours in the flow system. The shear fluid was reduced serum to a final concentration of 1% and all other components remained constant. During the final 90 minutes of the flow exposure, trypsin stock solution was added to the flow loop media to a final concentration of 2.1 μM trypsin (**Figure 1, design 2**).

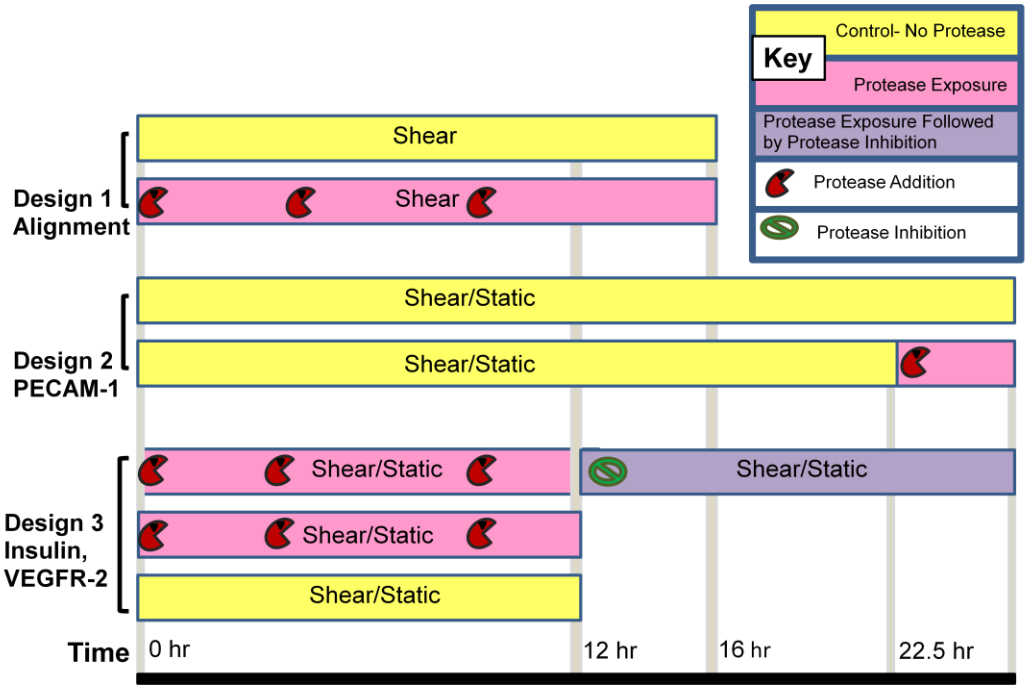

Figure 1: Time course for three protocols of endothelial cell groups exposed to shear stress and proteases. Each horizontal bar represents a different cell culture.

iii. PROTEASE EXPOSURE REALIGNMENT STUDIES

ECs were seeded onto glass slides and groups were prepared in duplicate. Four slides were sheared in parallel at 12 dyn/cm^2 for 12 hours with the addition of a final

trypsin concentration of 2.1 μM of the total 1% media volume. After twelve hours, two slides were fixed with 4% paraformaldehyde for 15 minutes and washed twice with DPBS before storing at 4º Celsius. The chambers were replaced with two new slides and the shearing media was adjusted to 10% serum. After 12 hours, these slides were also fixed by the same method. Static controls were exposed in duplicate to the same protease levels, quenched by the same amount of serum (**Figure 1, design 3**).

G. CELL MORPHOLOGY AND IMMUNOHISTOCHEMISTRY

i. HEMATOXYLIN AND EOSIN LABELING

After exposure, cells were fixed immediately with methanol at -20º Celsius on ice for 10 minutes followed by three washes with DPBS. The slides were stained for 10 seconds with hemotoxylin, rinsed in water and then stained for 10 seconds with eosin followed by a water rinse. The cells were then dehydrated in increasing ethanol concentrations 70%, 90%, 95%, and 100% and washed with xylene. After drying, slides were mounted with Vector Hard Set Mounting Media and sealed with nail polish.

ii. IMMUNOHISTOCHEMISTY FOR PECAM-1

Labeling for platelet endothelial cell adhesion molecule 1 (PECAM-1, CD-31) by immunohistochemistry was completed for sheared (24 hour) and static (0 hour) cells with and without trypsin exposure. Afterwards the cells were fixed in -20º C methanol for 15 minutes on ice. Cells were washed with DPBS and incubated with 0.5% hydrogen peroxide solution for 10 minutes. After two 5 minute washes with DPBS, cells were blocked with 10% horse serum diluted in PBS for 30 minutes. Cells were incubated with

the PECAM-1 primary antibody stock concentration of 1 mg/ml (Abbiotec) for 90 minutes at a dilution of 1:200 in 10% horse serum in a humidified chamber at room temperature. Control slides were incubated at a comparable concentration with an irrelevant rabbit antibody (Santa Cruz Biotechnologies) at an equivalent concentration. Following primary antibody incubation, slides were washed with PBS twice for 5 minutes. Antirabbit secondary reagent (Immpress Kit from Vector Labs) was applied for 30 minutes. Slides were washed again twice for 5 minutes. Immpact Daab (Vector Labs) was applied. Once slides reached the appropriate color, they were rinsed with tap water. Slides were dehydrated with an ethanol gradient and then cleaned in xylene solution. Slides were mounted using hard set mounting media (Vector Labs) containing Dapi.

iii. IMMUNOHISTOCHEMISTRY FOR VEGFR-2

After treatment, the cells were fixed for 15 minutes with 4% paraformaldehyde in DPBS followed by 2 washes each 5 minutes with PBS. Slides were incubated with 0.5% hydrogen peroxide in water for 10 minutes on a rotary shaker. The slides were washed with DPBS for 5 minutes. After the wash, 5% normal goat serum (Vector Labs) was applied to the slides for 1 hour. After blocking, extracellular VEGFR-2 (Genetex) antibody diluted 1:100 was applied to each slide and incubated at 4º Celsius on a rotary shaker overnight. Labeling control of irrelevant chicken antibody and a no primary control were carried out concurrently. Following incubation, slides were washed for five minutes three times with DPBS. The secondary antibody chicken IgY Fc (Genetex) was diluted 1:100 in PBS and applied to the slides for 1 hour. Slides were washed in DPBS for 5 minutes each 3 more times. Immpact Dab (Vector Labs) was applied. Once slides

reached the appropriate color, they were rinsed with tap water. Slides were dehydrated with an ethanol gradient and then cleaned in xylene solution. Slides were mounted using hard set mounting media (Vector Labs) containing Dapi.

iv. IMMUNOHISTOCHEMISTRY FOR INSULIN RECEPTOR

After treatment, slides were fixed for 15 minutes with 4% paraformaldehyde in DPBS followed by two five minutes washes with DPBS. Slides were incubated with 0.5% hydrogen peroxide in water for 10 minutes on a rotary shaker. The slides were washed with DPBS for 5 minutes. After the wash, 10% horse serum was applied to the slides for 30 minutes. Addition of the primary antibody insulin Rα extracellular (N-20; Santa Cruz Biotechnology) in a dilution of 1:100 was applied. The controls were an irrelevant rabbit antibody and a no primary control. After 1 hour incubation, the slides were washed three times with DPBS for 5 minutes each wash. Antirabbit secondary reagent (Immpress Kit from Vector Labs) was applied for 30 minutes. Slides were washed again twice with DPBS for 5 minutes. Immpact Dab (Vector Labs) was applied. Once slides reached the appropriate color, they were rinsed with tap water. Slides were dehydrated with an ethanol gradient and then cleaned in xylene solution. Slides were mounted using hard set mounting media (Vector Labs) containing Dapi.

H. QUANTIFICATION OF LABEL DENSITY

All image processing was completed using Image J (http://rsbweb.nih.gov/ij/).

i. ANALYSIS OF CELL ALIGNMENT AND ELONGATION

Cell area, A, and perimeter, P, was measured digitally (**Figure 2A**). The two parameters were also used to determine a *circularity* index of the cell:

$$S = \frac{1}{4\pi}\frac{P^2}{A}$$

where P is the cell perimeter, A is the cell area, and S is the circularity index. The orientation of the cells was classified by measuring the alignment angle θ of the cell as shown in **Figure 2B**. The spatial linear regression between the maximum distance between two points of the cell was estimated. The angle which this made with the fluid flow as designated the alignment angle.

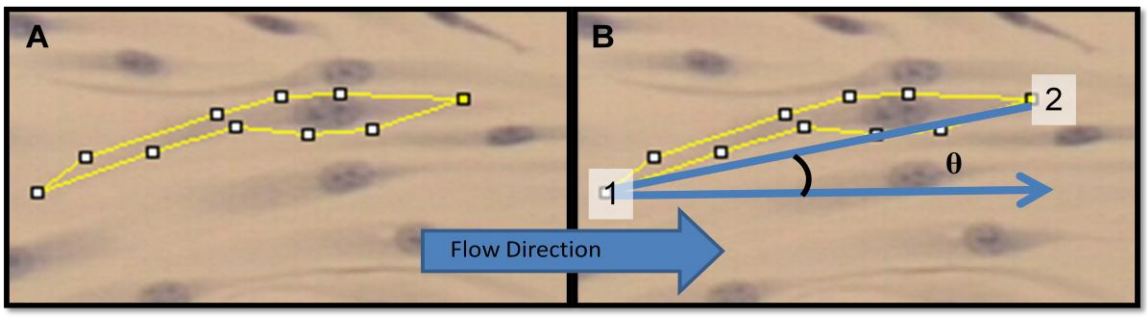

Figure 2: A. Example of the measurement of the perimeter, P, and cell area, A. B. Sample measurement of the cell alignment angle with the orientation of the flow. Points 1 and 2 represent the maximum distance on the cell's perimeter. The upper blue line represents the segment connecting 1 and 2. The blue arrow denotes the direction of the flow and shows how much the cell deviates from the fluid shear stress.

ii. PECAM-1 LABELING ANAYLSIS

After labeling, a minimum of 8 frames of each slide was digitized at 20x magnification (**Figure 3A**). The light background was first subtracted from the image

(**Figure 3B-C**). The image was then inverted to show a white background so that the maximum value of 255 pixel intensity corresponds to white (**Figure 3D**). Multiple images were taken from the same slide of PECAM-1 staining. From each frame, the PECAM-1 staining was quantified from the same region of the frame. The junctional PECAM-1 labeling was selected by tracing around the perimeter of the staining in the magnified view (**Figure 3F-H**). On selected positively stained area, the images pixel intensity was recorded and combined for all frames.

The pixel intensity values of multiple image frames were combined for the each cell group as basis to display the pixel intensity distribution. The threshold intensity was selected based on the prerequisite that all regions contribute to below the threshold and that it was at least two standard deviations below the mean pixel intensity from the overall histogram for the region. The percent of pixels below this value were calculated and compared.

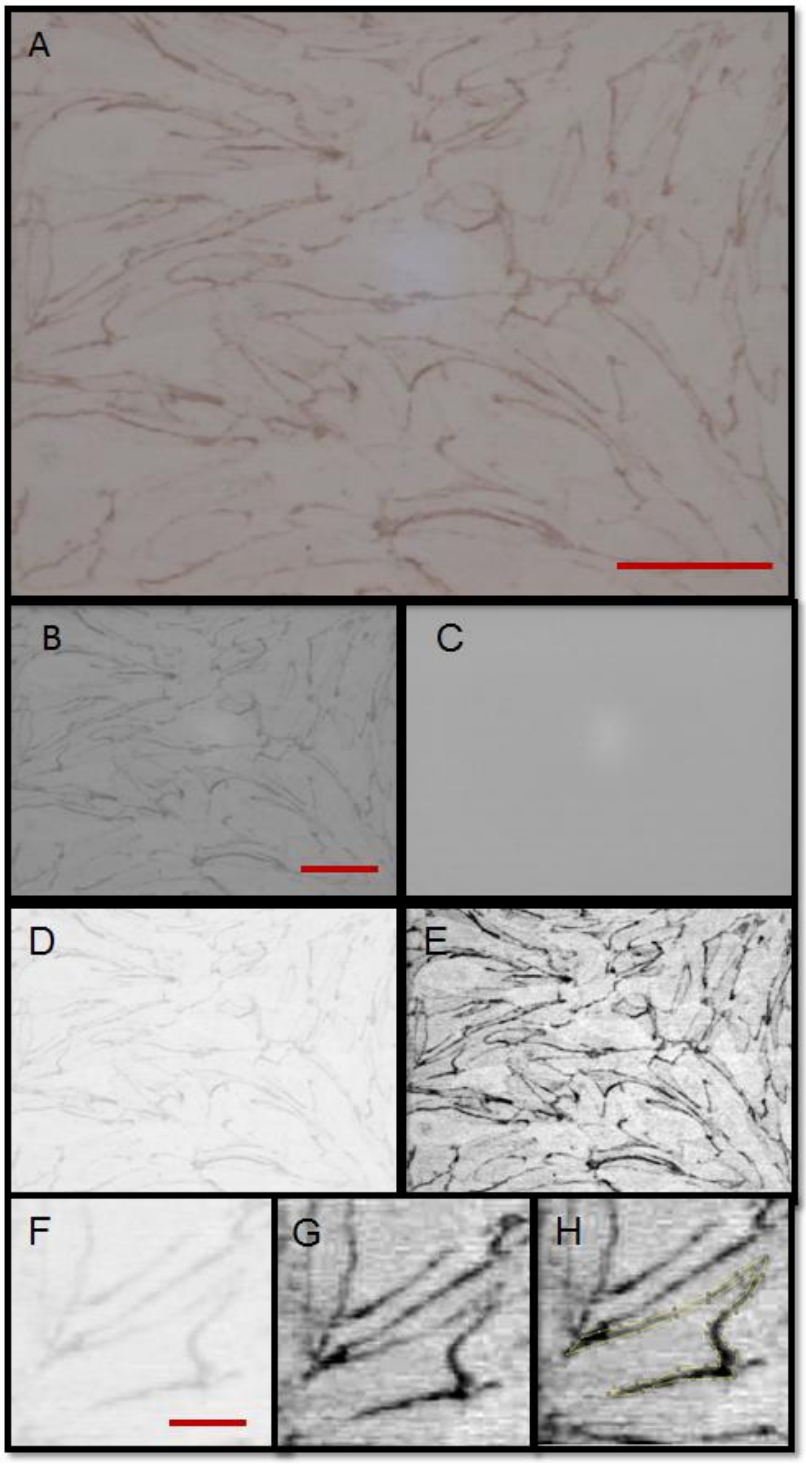

Figure 3: Measurement of the PECAM-1 label intensity. A. Original image. B. Image converted to 8 bit. C. Background image. D. Inverted result of background subtraction. E. Enhanced image. F. Magnified image. G. Enhanced image. H. Selection of PECAM-1 junctional staining. Scale corresponds to 100 µm.

iii. IMAGE ANALYSIS FOR INSULIN RECEPTOR AND VEGFR-2 DENSITY

The images were processed after digitizing to 8 bit map (1 to 256 digital units) and subtracting the light background (**Figure 4A**). On each image, individual cells were randomly selected using the polygon tool (ImageJ), and the average pixel intensity was measured for at least 100 cells in each group (**Figure 4B**).

The light absorption of a selected cell, A_c, was calculated according to $A_c=-\ln(I/I_o)$ where I is the intensity of the tissue and I_o the intensity for a white background (255).

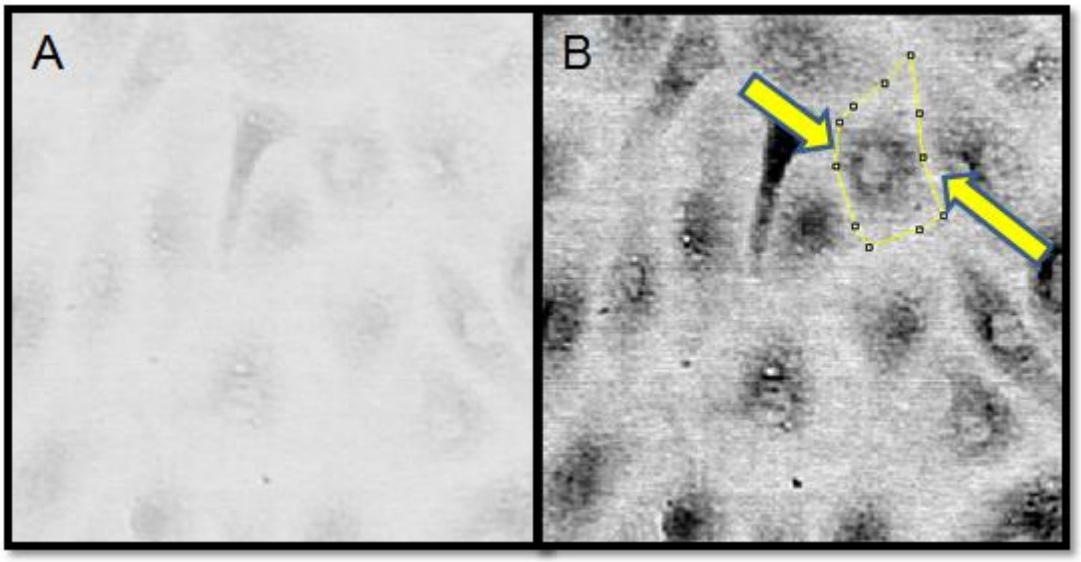

Figure 4: A. Digital image of endothelial cell labeled with antibody against after background subtraction. B. Same image with enhanced contrast and circled cell. Arrows indicate the cell perimeter selected.

I. STATISTICAL ANALYSIS

All measurements are presented as mean±standard deviation. Linear regression was used to best fit the calibration line for the trypsin activity for casein.

For Protocol 1 (**Figure 1**), comparison between groups was carried out between the sheared control group and the sheared with protease group. Mann Whitney tests for non–Gaussian distributions were applied for the difference between the alignment angle and circularity index. A p-value<0.01 was considered statistically significant.

Protocol 2 (**Figure 1**) compares ECs sheared (with and without protease exposure) and static cells (with and without protease exposure). A student t-test was performed between the groups. A p-value <0.05 was considered statistically significant.

Protocol 3 (**Figure 1**) compares the non-Gaussian distributions of alignment angle and circularity index the same way as for Protocol 1. The labeling intensities for the 12 hour control (shear/static), 12 hour protease exposure (shear/static), and the 12 hour protease exposure followed by 12 hour inhibition (shear/static) were compared between all the shear groups and all the static groups using ANOVA. Comparison between two groups for (shear/static) was made by a t-test with a Bonferroni correction. A p-value <0.05 was considered statistically significant.

III. Results

A. ANALYSIS OF PROTEASE ACTIVITY FOR SHOCK MODEL

In order to determine typical protease activity in the circulation, the serine protease trypsin was added to 1% reduced serum media to determine the optimal concentration of trypsin in the current experiments. A fluorescent casein substrate was used to determine the range of trypsin activity in PBS and described by an exponential relationship (**Figure 5**).

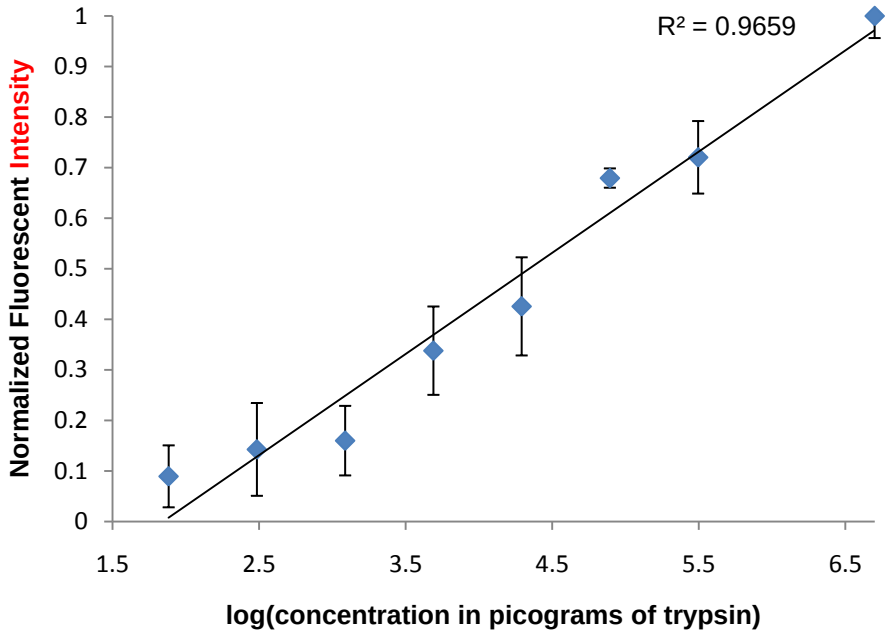

Figure 5: Linear correlation between the log concentration of trypsin and fluorescence intensity of casein substrate after 60 minute incubation at 37º C. The range of trypsin concentration is between 5 µg/mL (2.1 µM) and 80 pg/mL (33.6 pM).

Figure 6 shows the BAPNA substrate activity over a range of trypsin concentrations between 11 μM to 11 nM**.** A trypsin concentration of 2.1 μM was at the threshold of measurable activity.

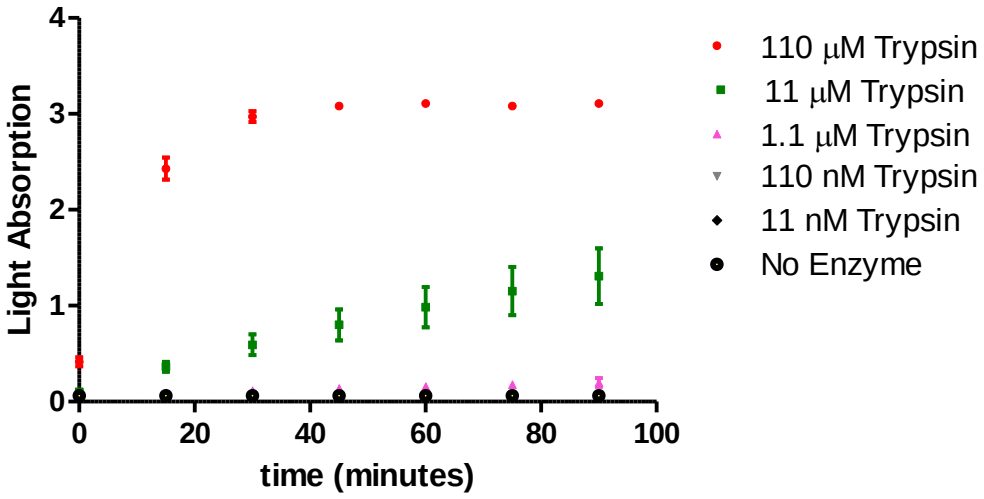

Figure 6: The kinetic assay for BAPNA for a range of trypsin concentrations between 110 μM to 11 nM in 1% serum media. The activity decreases rapidly 1.1 μM below the threshold of the substrate.

B. RESPONSE OF ENDOTHELIAL CELLS TO TRYPSIN

Direct effects of uninhibited trypsin on endothelial cells under both static and sheared conditions were explored first. The time course for individual static endothelial cells upon exposure to uninhibited trypsin at 21 μM, 2.1 μM, or 0.21 μM is shown in **Figures 7-9** for the normalized area, normalized perimeter, and circularity index. Area and perimeter decreased more rapidly with increased concentration and the circulatory

index converged to 1. ECs initially adhered to the glass surface exhibit a period of slow retraction followed by a steep decrease in cell area as a function of trypsin concentration. ECs exposed to the greatest concentration of trypsin (21 μM) had the greatest change in area, perimeter, and reached a circularity index of 1 the fastest **Figure 10-11**. The circularity index approached 1 (a circle) for concentrations of 21 μM and 2.1 μM but not for 0.21 μM (**Figure 12**).

Sheared single cell time course analysis indicated the need to extend the observation period to see a trend (**Figure 13**). Comparing pre-sheared cells to static cell's response to 0.21 μM trypsin exposure revealed a faster decrease in both the area and perimeter for the static cells compared to the sheared cells (**Figure 14**).

F-actin labeling of cells exposed to 0.21 μM trypsin shows more decrease in junctions between the cells. Undisturbed focal adhesion sites are brighter and are still attached even though the majority of the cell is retracting as the trypsin cleaves critical points of attachment as indicated by the arrows (**Figure 15**).

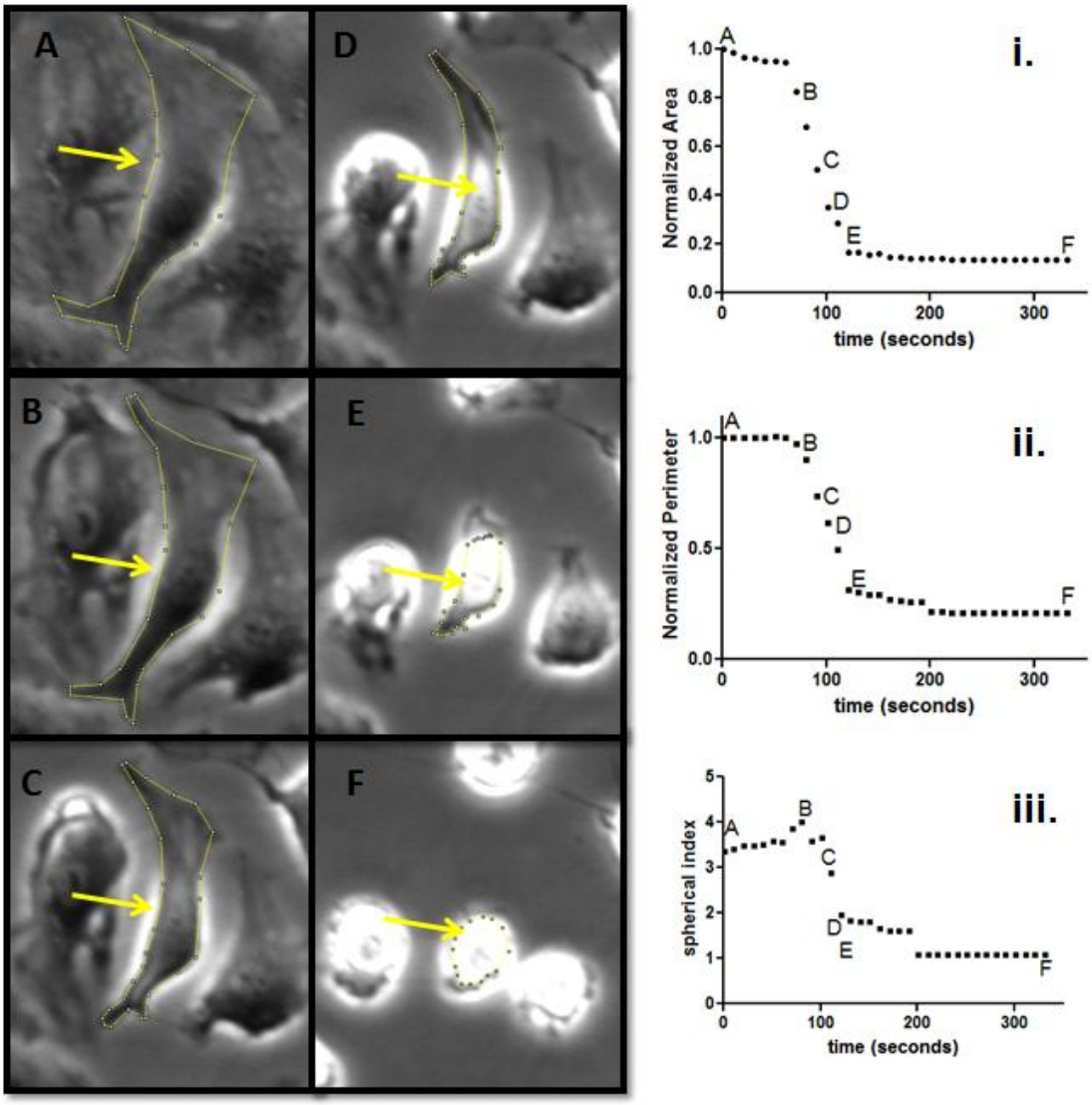

Figure 7: 21 μM trypsin exposure to static endothelial cells. Micrographs A-F on the left correspond to the time courses on the right where the yellow arrow indicates the same cell through the trypsin exposure. Area and perimeter retraction occurred within the first 110 seconds (i, ii). The cell's circularity index initially increased followed by a decrease converging to 1 (iii).

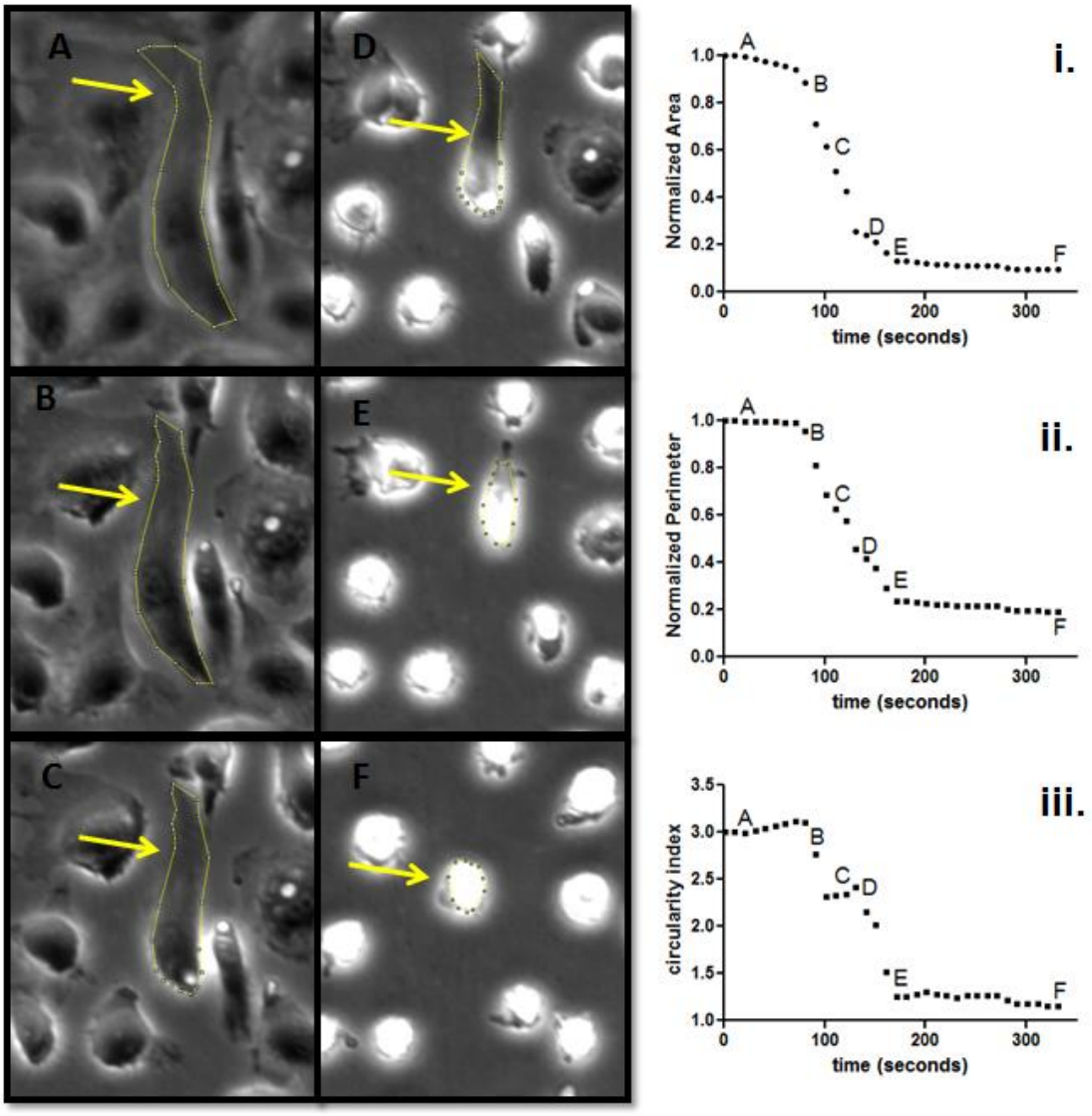

Figure 8: 2.1 μM trypsin exposure to static endothelial cells. Micrographs A-F on the left correspond to the time courses on the right where the yellow arrow indicates the same cell through the trypsin exposure. Area and perimeter retraction occurred within the first 180 seconds (i, ii). The cell's circularity index initially increased followed by a decrease converging to 1 (iii).

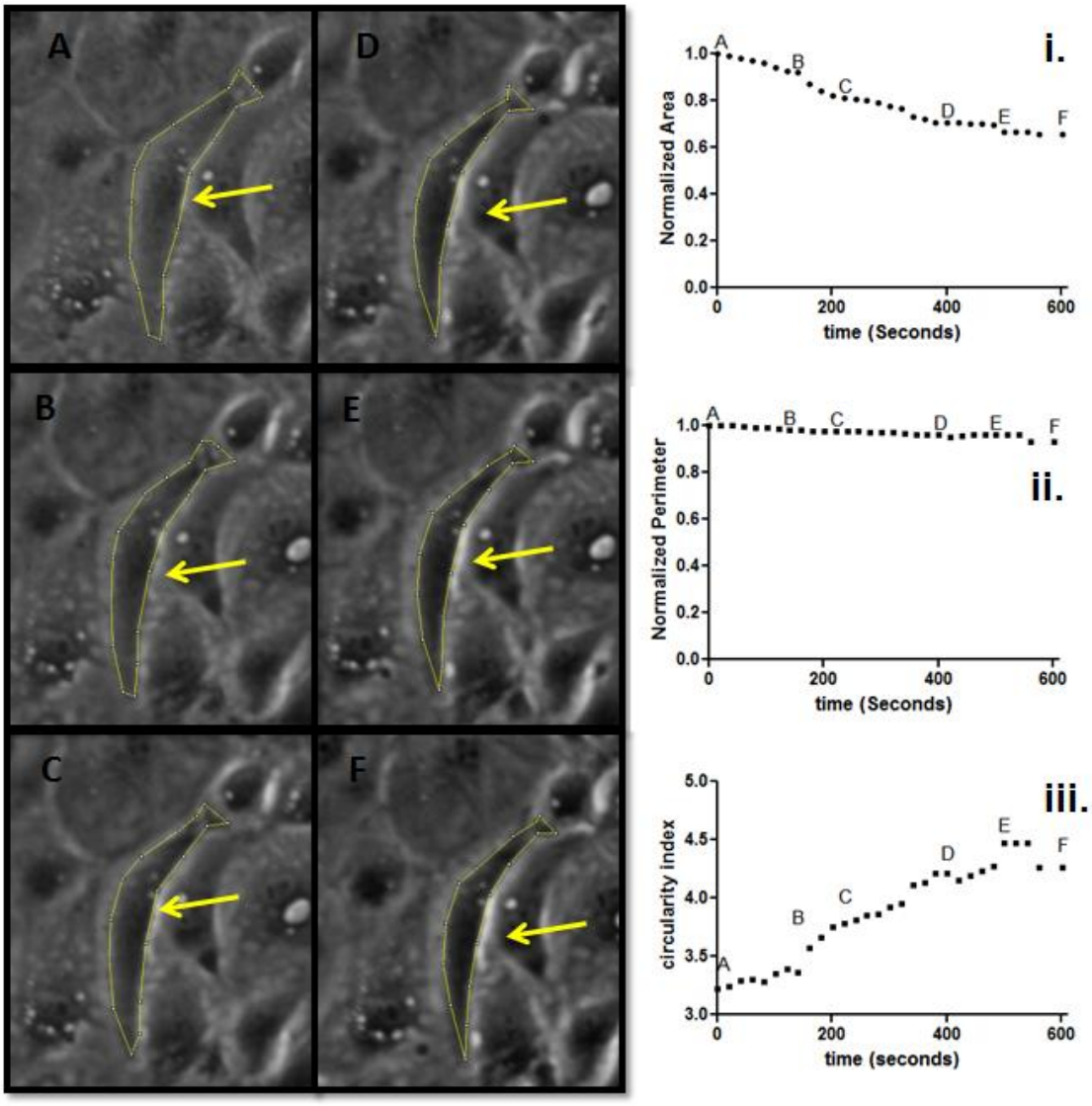

Figure 9: 0.21 μM trypsin exposure to static endothelial cells. Micrographs A-F on the left correspond to the time courses on the right where the yellow arrow indicates the same cell through the trypsin exposure. Area and perimeter retraction occurred within the first 110 seconds (i, ii). The cell's circularity index initially increased followed by a decrease converging to 1 (iii).

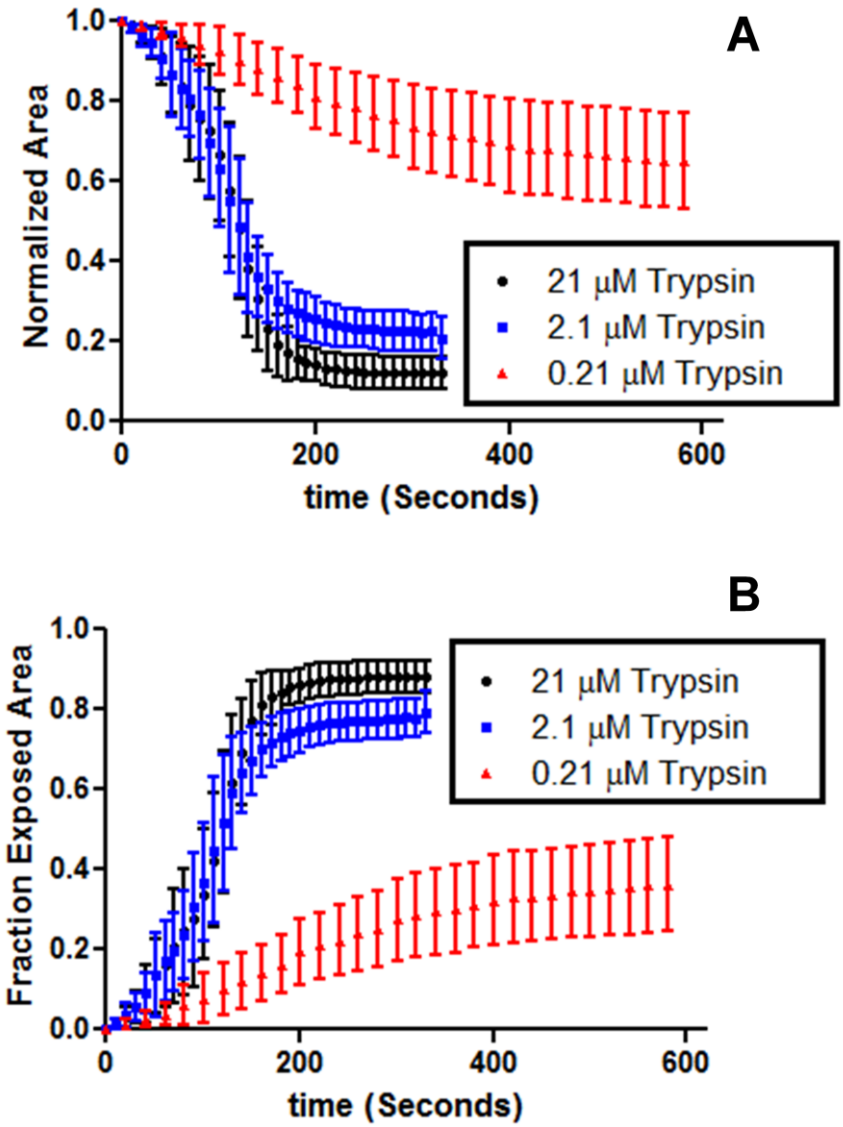

Figure 10: A. Cell area normalized relative to the cell area at the initial time 0 for endothelial cells under static conditions exposed to three trypsin concentrations. B. Fraction of exposed surface during trypsin exposure. (n=12 cells for each concentration).

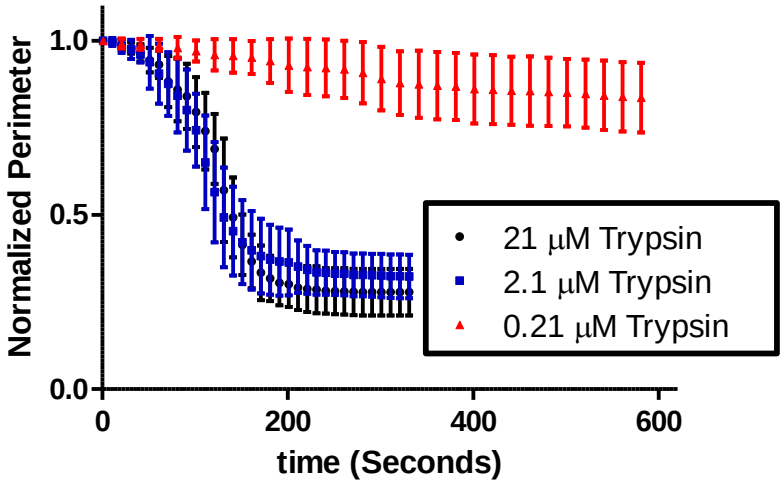

Figure 11: Normalized perimeter to initial measurement changes for static cells exposed to three trypsin concentrations. (n=12 cells/concentration).

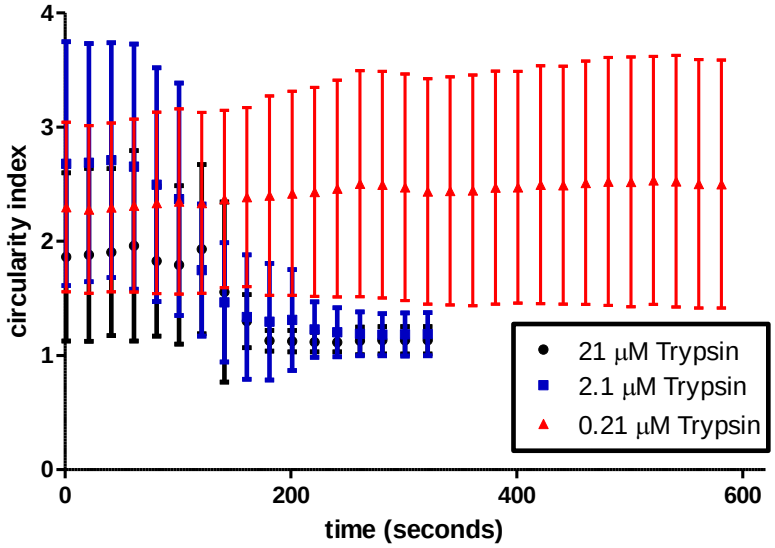

Figure 12: Circularity index for three concentrations of trypsin. 21 M and 2.1 M concentrations converge to approximately 1 indicating a circular shape of the cell. (n=12 cells/concentration).

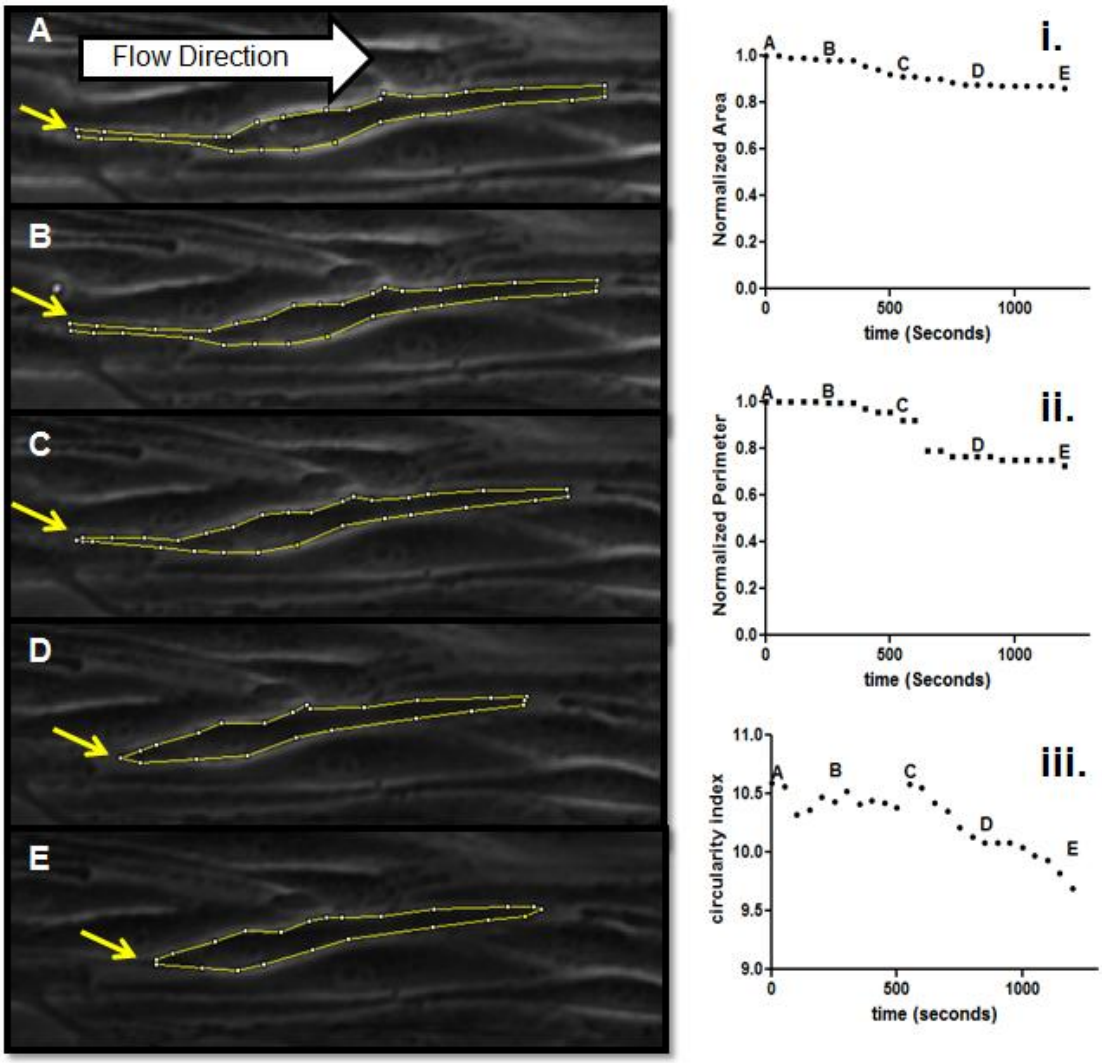

Figure 13: 0.21 μM trypsin exposure to presheared endothelial cells. Micrographs A-F on the left correspond to the time courses on the right where the yellow arrow indicates the same cell through the trypsin exposure. Area and perimeter retraction occurred within the first 180 seconds (i, ii). The cell's circularity index initially increased followed by a decrease converging to 1 (iii).

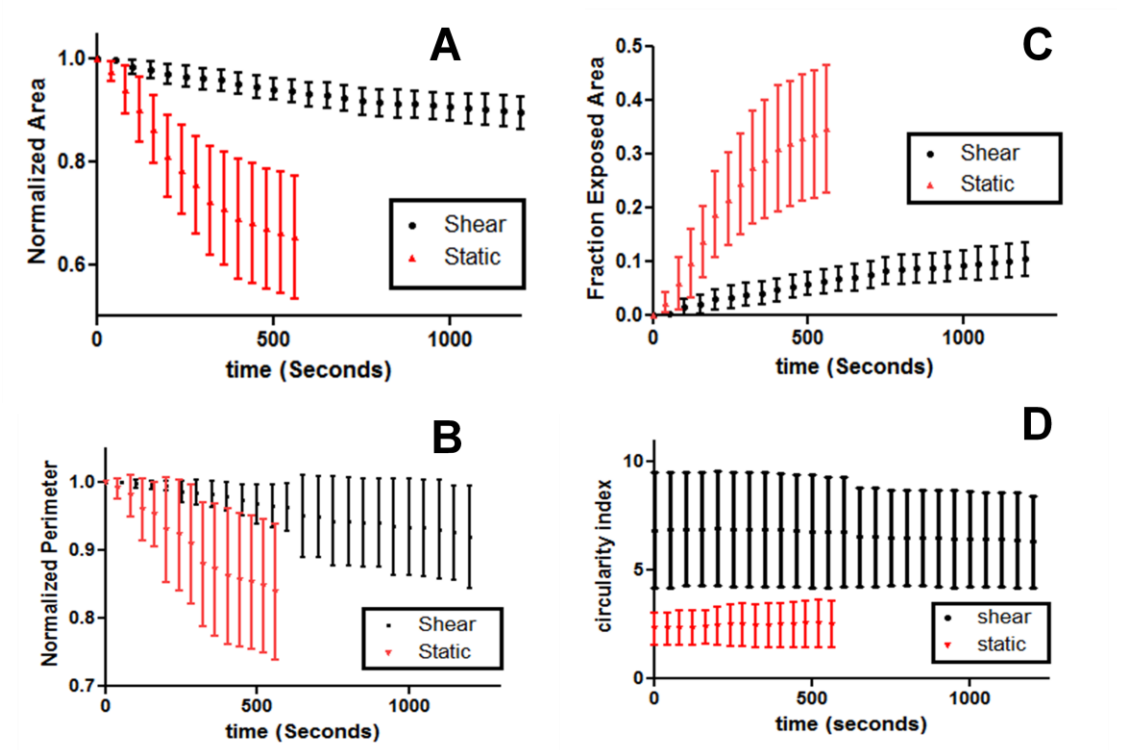

Figure 14: A. Timecourse of the normalized cell area under shear and static conditions for 0.21 μM trypsin exposed cells. A&B . Area and perimeter decrease faster for static cells than sheared cells. C. Fraction of exposed area was not as large for sheared endothelial cells. D. Sheared endothelial cells had an increased circularity index corresponding to the elongation of the cells. (n=12 cells/group).

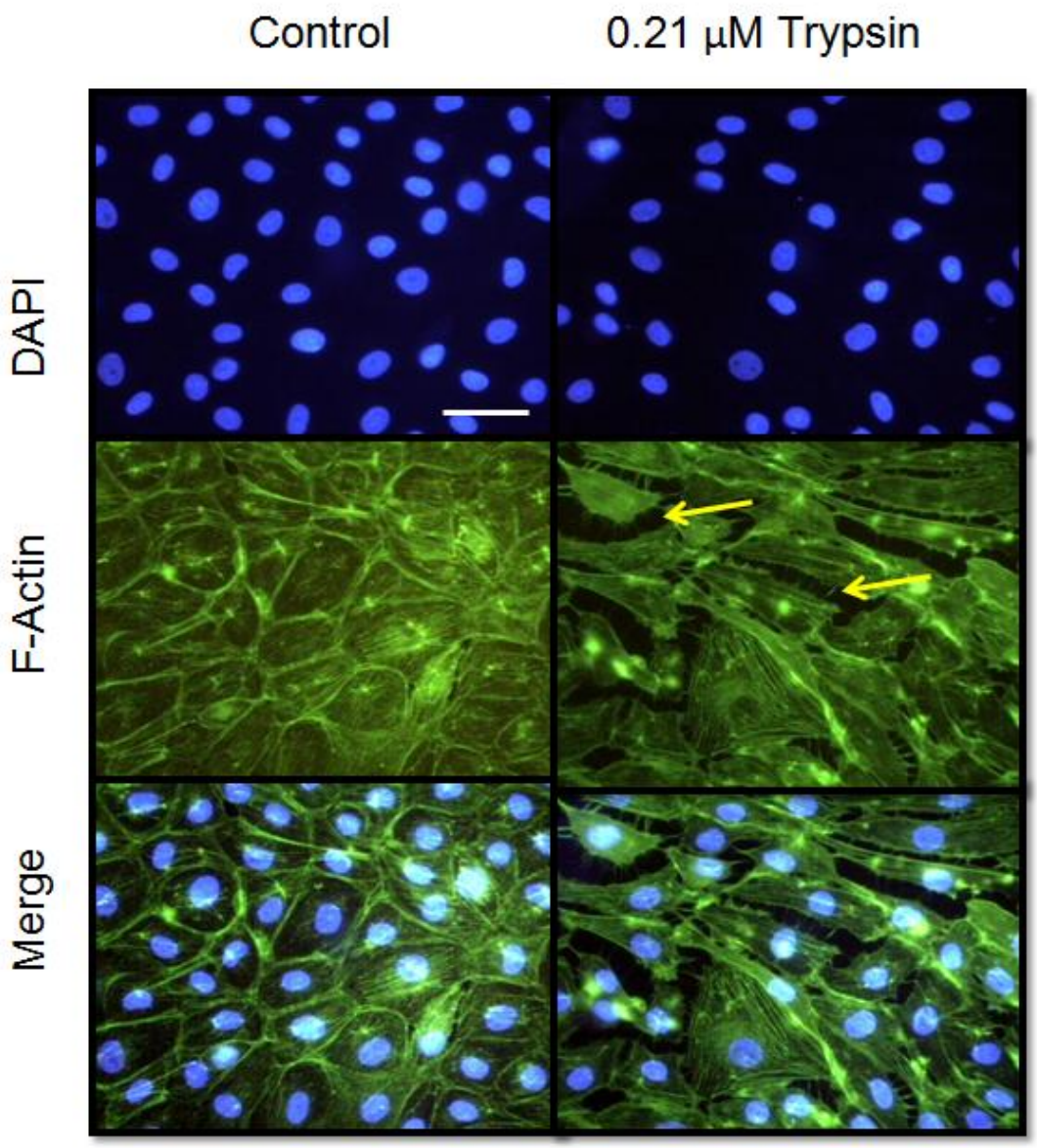

Figure 15: Micrographs of endothelial cells with F-actin labeling after exposure with 0.21 µM trypsin for 15 minutes. The yellow arrow points to the F-actin connected to focal adhesions before complete cell detachment. Length scale equals 50 µm for all images.

C. SHEAR RESPONSE IN THE PRESENCE OF PROTEASE

The characteristic responses of endothelial cells to fluid shear stress were diminished with the addition of protease concentrations of 2.1 μM after 16 hours of 12 dyn/cm^2 shear stress application. There is a reduced cell alignment assessed by the alignment angle and circularity index from micrographs such as that shown in **Figure 16**. The non-Gaussian distribution of the alignment angle favored the lower angles for cells not exposed to proteases (**Figure 17-18**). The circularity index was reduced for protease exposed cells (**Figure 19-20**).

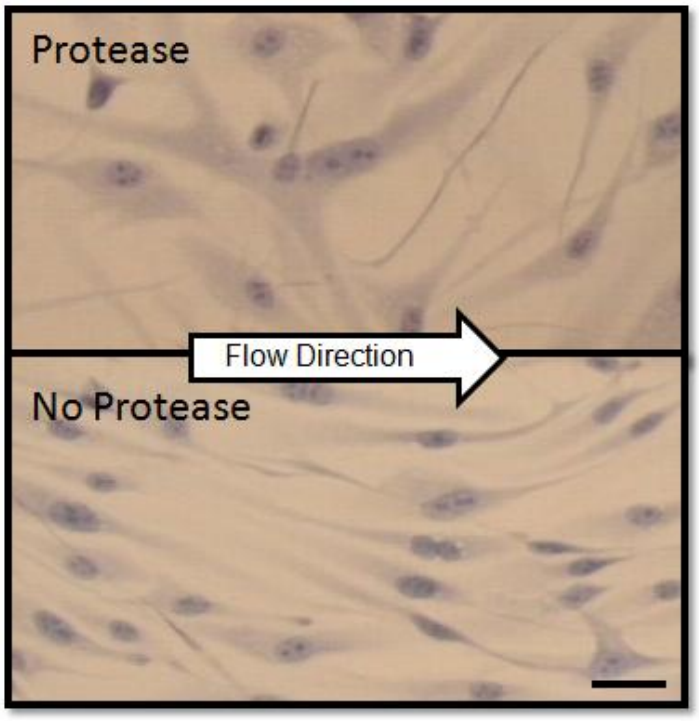

Figure 16: Endothelial cells exposed to the direction of the fluid shear stress of 12 dyn/cm^2. Protease expose cells show reduced alignment with the fluid flow. Scale bar corresponds to 20 μm.

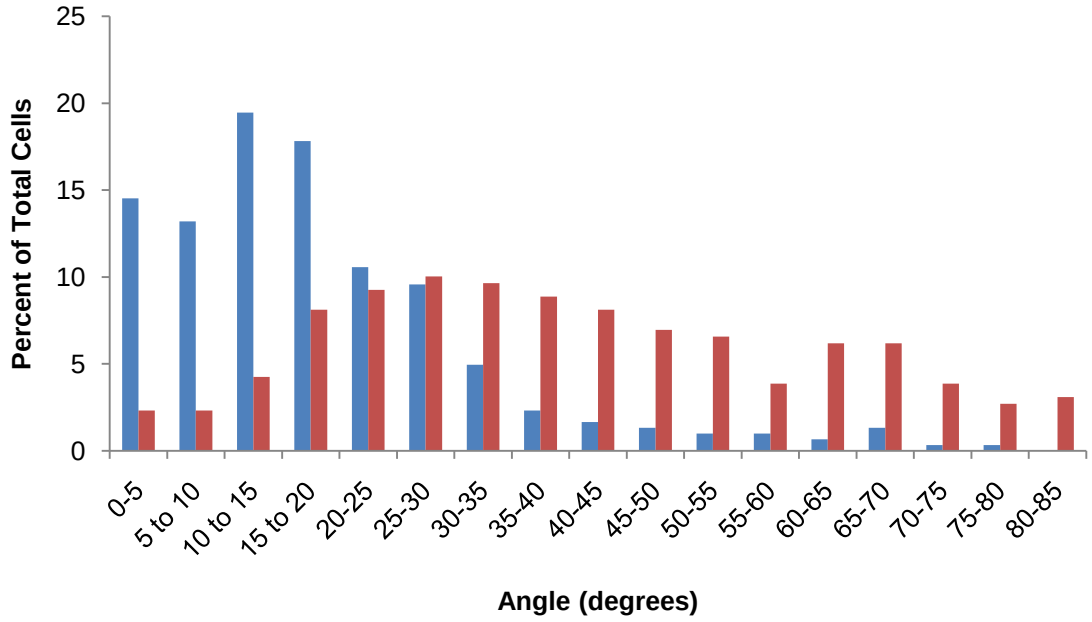

Figure17: Histogram of the alignment angle of the cells with the direction of the flow after application of the fluid shear (12 dyn/cm^2) for 16 hours.

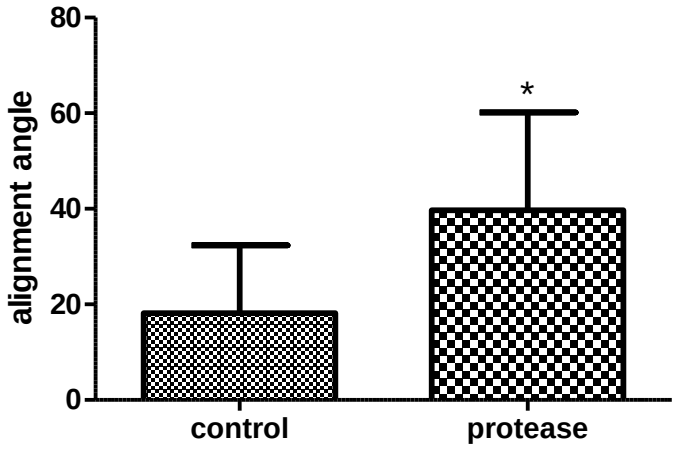

Figure 18: Alignment angle of cells sheared with protease (protease, n=303 cells) and without protease (control, n=259). *p<0.0001 by a Mann Whitney test.

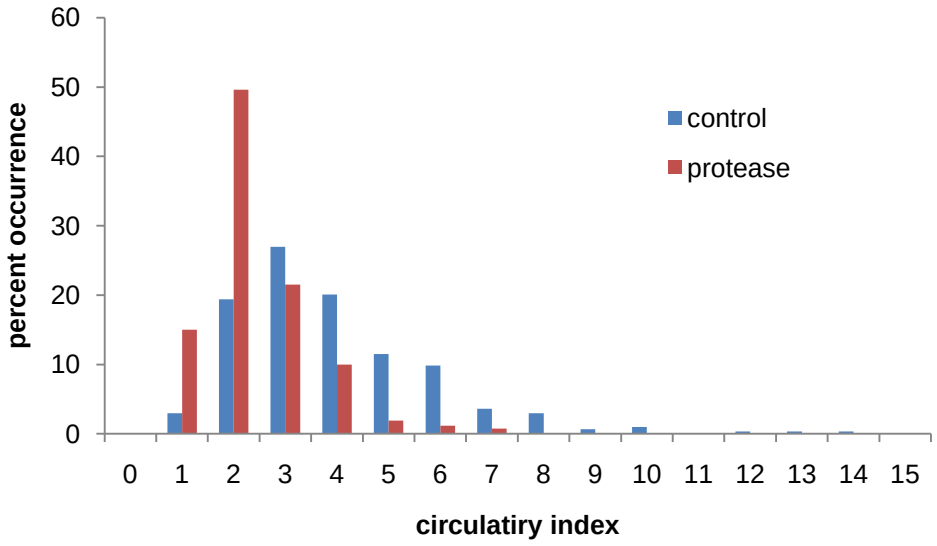

Figure 19: Histogram of the circularity index after application of the 12 dyn/cm^2 of laminar fluid shear stress for 16 hours.

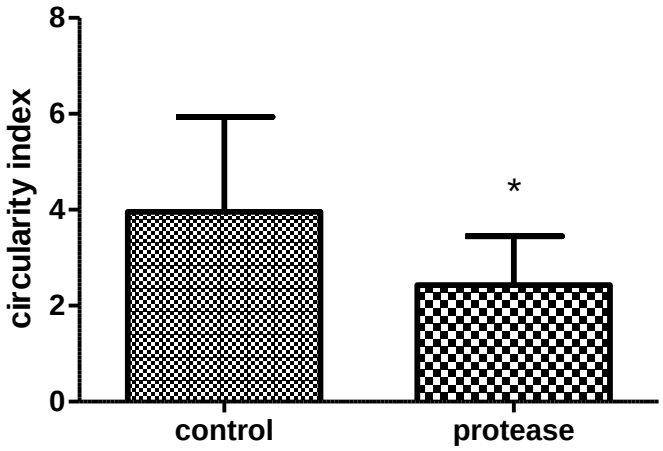

Figure 20: Circularity index of cells sheared in the presence of (protease, n=303) and cells sheared without protease (control, n=259). *p<0.0001 by a Mann Whitney test.

D. MECHANOSENSOR DAMAGE DURING PROTEOLYTIC EXPOSURE: PECAM-1

Comparing the total static label intensity distribution for PECAM-1 between static and trypsin exposed cells (**Figure 21-22**), there was a significant difference between the two groups when the threshold of labeling intensity was selected at 200 pixels (**Figure 23**). This threshold serves to limit the analysis to only the junctional regions of the endothelial cells where the majority of PECAM-1 is localized (see Methods). The reduced junctional labeling can be seen on original images (**Figure 24**). There was a 68% reduction in PECAM-1 label intensity below threshold at the junctions in the protease exposed cells.

Cells sheared with and without protease (**Figures 25-26**) had a PECAM-1 label intensity at the junctions (i.e. below 200 pixels) which is on average 85% less in the presence of protease (**Figure 27-28**).

The PECAM-1 label intensities for ECs not exposed to protease and exposed to protease were not significantly different, even though the endothelial cells that were sheared had increased label intensity (**Figure 29-30**).

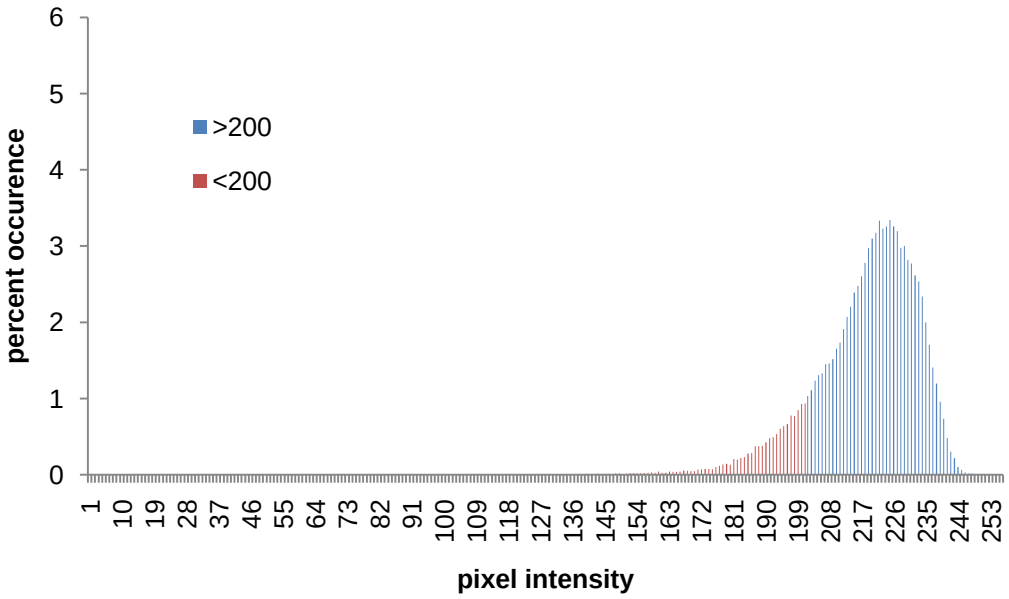

Figure 21: The intensity PECAM-1 labeling of cells exposed to static conditions in the absense of protease exposure. Below the 200 signal intensity threshold of the total area selected are in red.

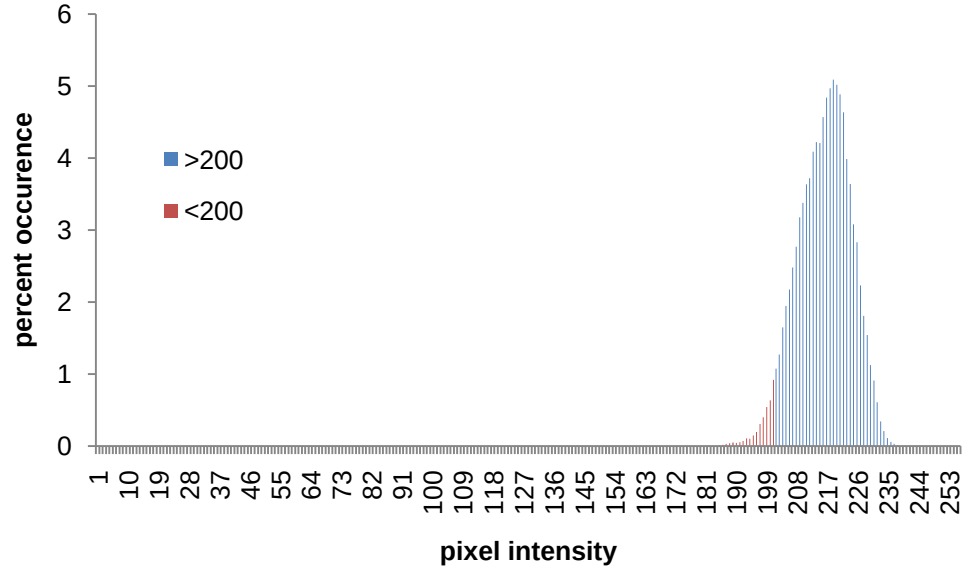

Figure 22: The intensity PECAM-1 labeling of cells exposed to static conditions in the presense of protease exposure. Below the 200 signal intensity (red) is reduced in the presense of protease.

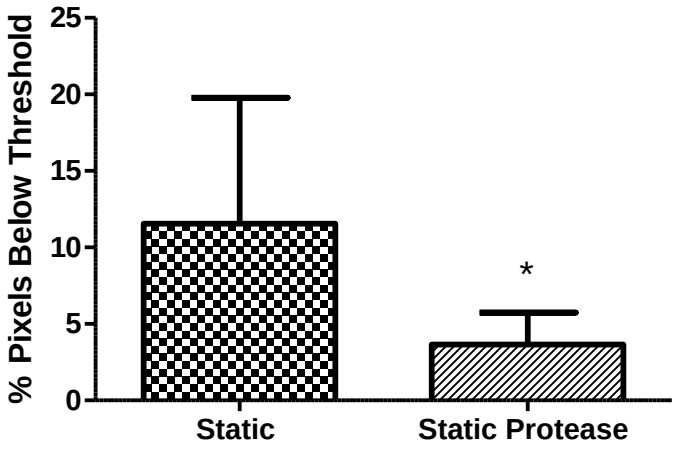

Figure 23: Signal intensity at the EC junctions (below 200 pixel) after exposure to static conditions without (n=6 frames) and with protease (n=7), *p<0.04 by a t-test.

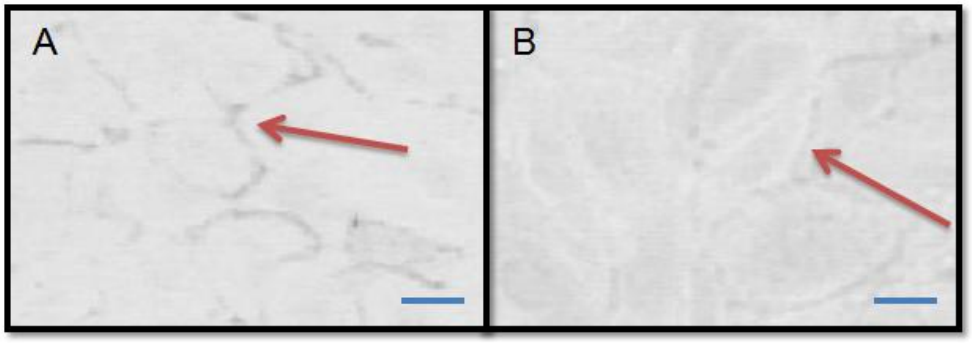

Figure 24: Comparison of the PECAM-1 label intensity of static cells without (A) and with (B) 1.5 hours to protease. The arrows point to the region of PECAM-1 label at the cell junctions which is reduced in the cells exposed to protease. Length scale equals 20 μm.

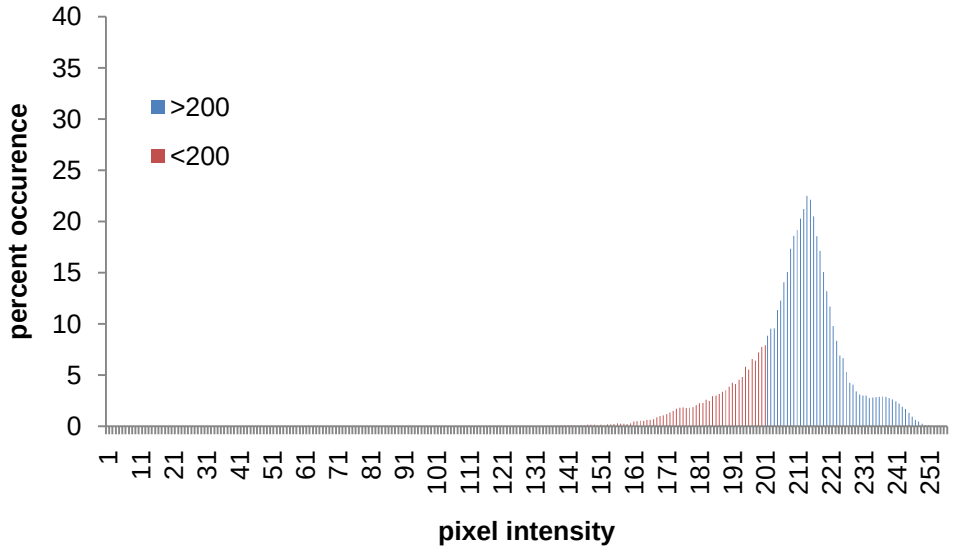

Figure 25: The intensity PECAM-1 labeling of cells exposed to shear conditions in the absense of protease exposure. Below the 200 signal intensity threshold of the total area selected are in red.

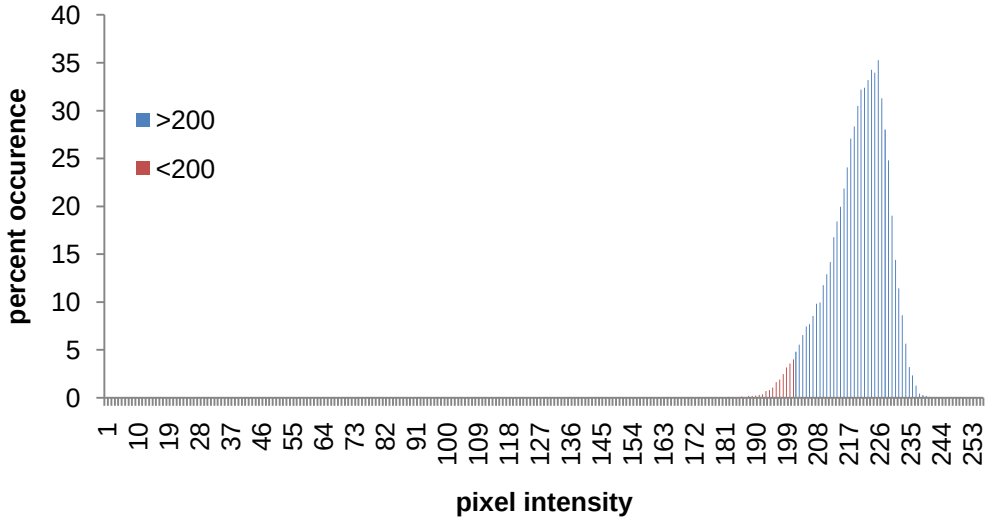

Figure 26: The intensity PECAM-1 labeling of cells exposed to shear conditions in the presense of protease exposure. Below the 200 signal intensity (red) is reduced in the presense of protease.

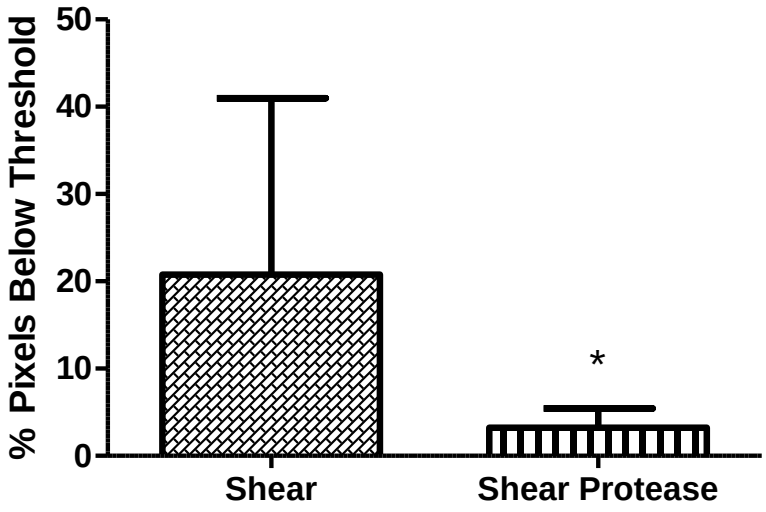

Figure 27: PECAM-1 label intensities at cell junctions labeling for sheared cells below the threshold. Cells sheared without (left, n=10 frames) and with protease (right, n=8 frames) were significantly different, *p<0.03.

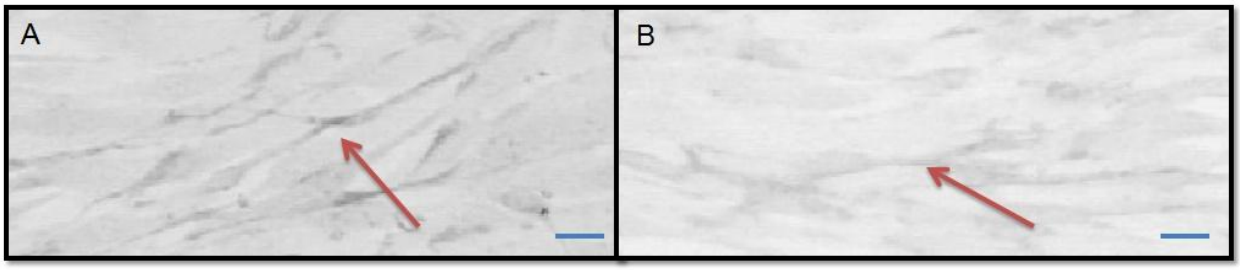

Figure 28: Comparison of the PECAM-1 label intensity of sheared cells without (A) and with (B) 1.5 hours to protease. Notice the ECs are elongated due to preshearing but the labeling intensities are decreased in the protease treated ECs. Length of scale bar equals 20 µm.

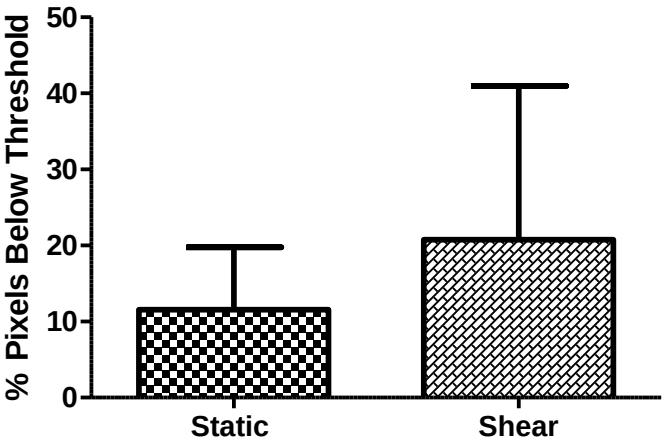

Figure 29: PECAM-1 label density on static and sheared ECs. The mean values were not significantly different between the two groups

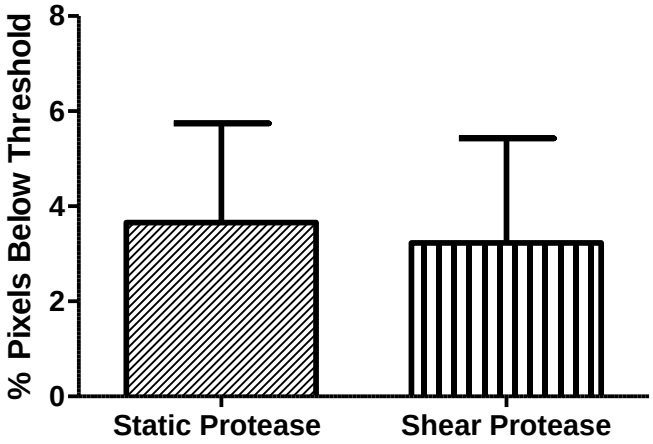

Figure 30: Comparison of the labeling intensities of junctional PECAM-1 on static and sheared cells after exposure to protease labeling. The mean values were not significantly different.

E. MECHANOSENSOR DAMAGE DURING PROTEOLYTIC EXPOSURE: VEGFR-2

Protocol 3 was used to investigate the VEGFR-2 expression and alignment (**Figure 1**). The alignment of cells was reduced with larger average alignment angle for cells exposed to proteases (**Figure 31-32**). The circularity index is also shifted towards lower values for protease exposed cells (**Figure 33-34**).

VEGFR-2 intensity was reduced after proteolytic exposure. Endothelial cells exposed to sheared and static conditions (**Figures 35 & 37**) had significantly different absorbance/cell when comparing the protease exposed groups to the groups not exposed to proteases (**Figure 36 & 38**). There was on average a 30% reduction in absorbance in the sheared cells with protease compared to the control sheared cells. After protease exposure cells were inhibited for 12 hours, and the absorbance for VEGFR-2 extracellular labeling doubled. Comparing static cells in the presence of protease to the cells in the presence of protease and followed by 12 hours of protease inhibition, the VEGFR-2 extracellular density increased 20%.

When comparing control static cells with the control sheared cells, there was a significant increase in labeling in the 12 hour sheared cells (**Figure 39**). However, the sheared cells exposed to proteases had a decreased labeling intensity compared to the controls (**Figure 40**)**.** These labeling experiments were repeated twice on separate days with similar results.

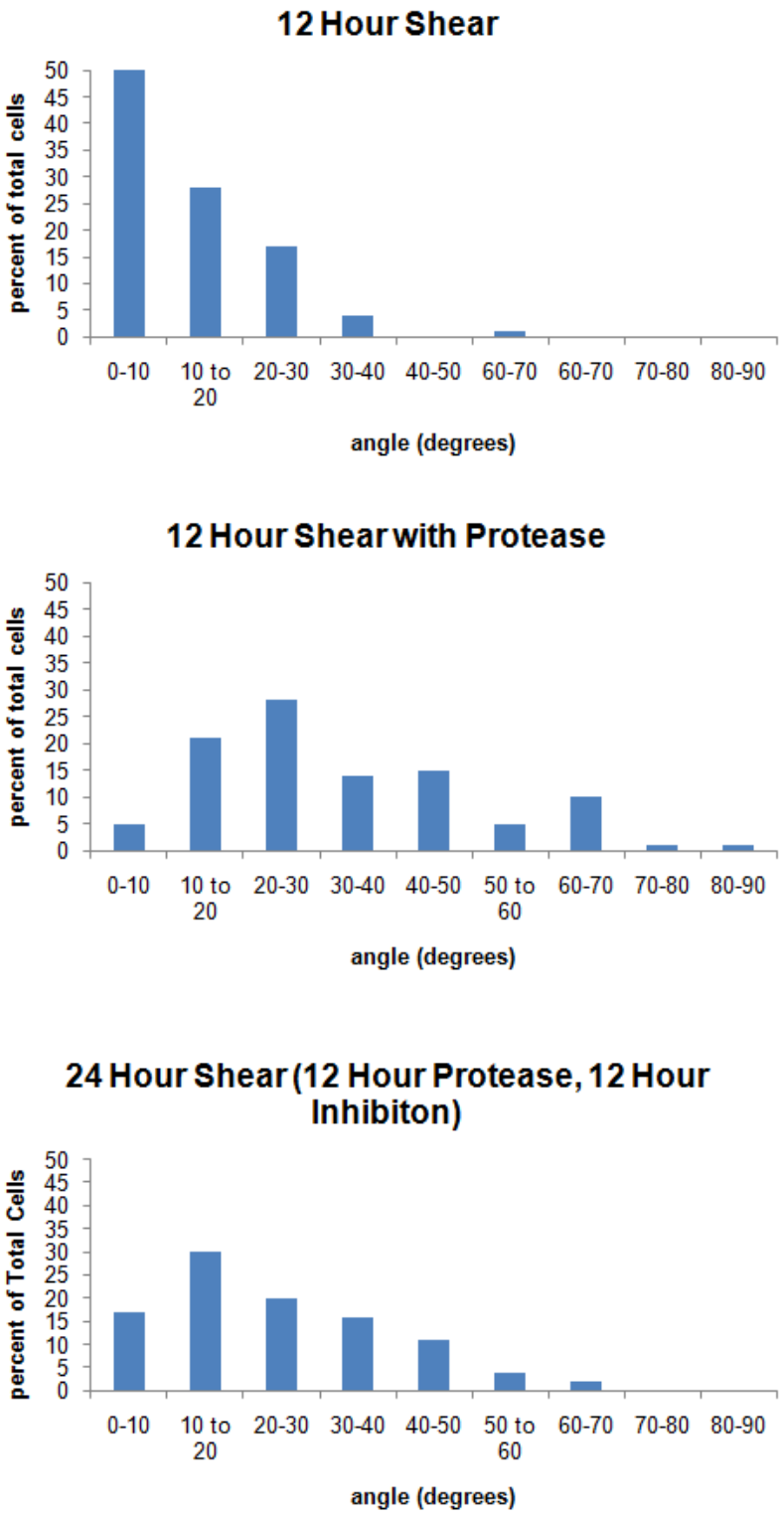

Figure 31: Comparison of the alignment angle distributions for three sheared groups.

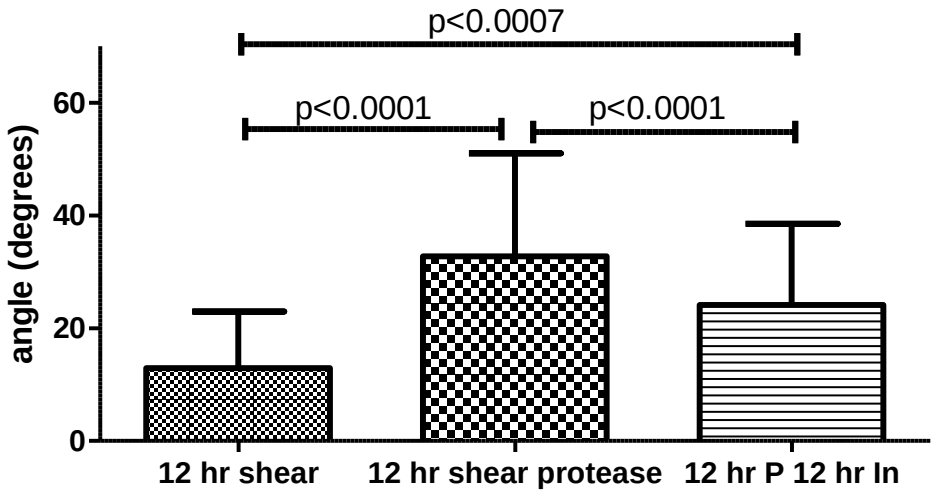

Figure 32: Comparison of the alignment angle in sheared cells (12 hr shear, n=100), sheared cells exposed for 12 hours to proteases (12 hr static protease, n=100), and cells sheared for 12 hours with protease exposure followed by 12 hours with protease inhibition (12 hr P 12 hr In, n=100) was significantly different by a Kruskal-Wallis test, $p<0.0001$.

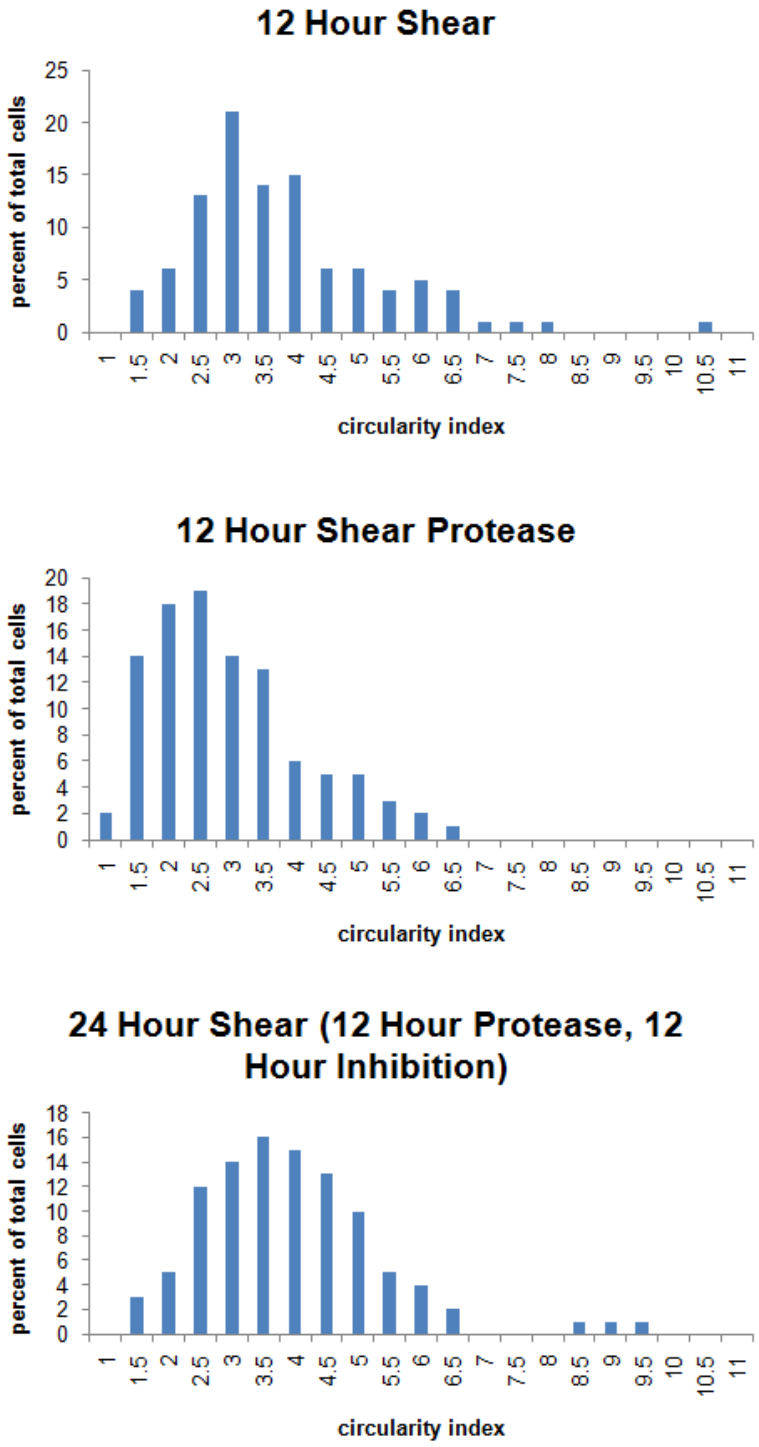

Figure 33: Comparison of the circularity index distributions for three sheared groups.

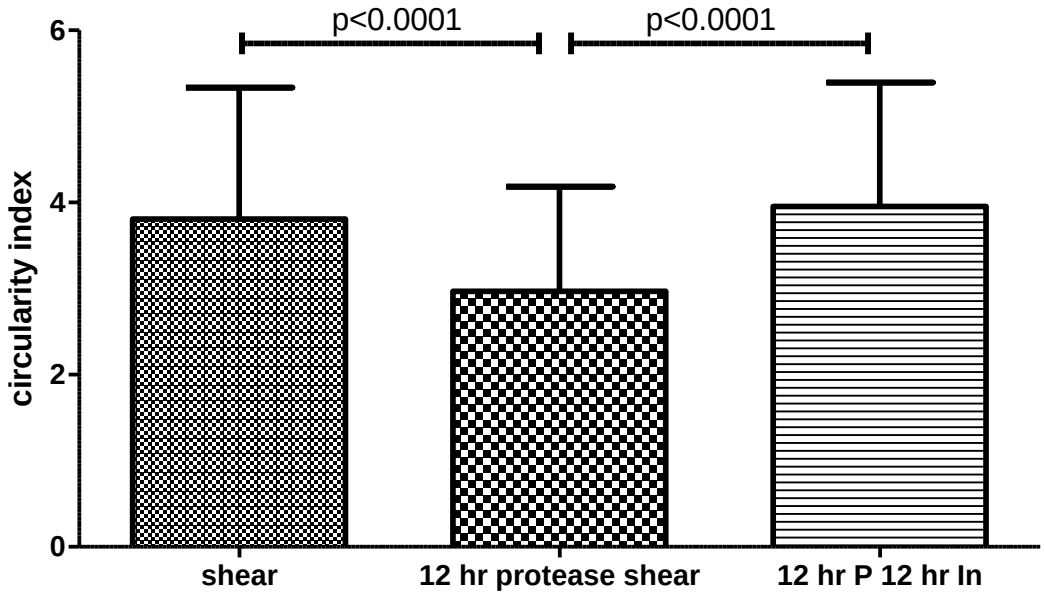

Figure 34: Circularity index distributions for sheared cells (shear, n=100), sheared cells exposed for 12 hours to proteases (12 hr protease shear, n=100), and cells sheared for 12 hours with protease exposure followed by 12 hours with protease inhibition (12 hr P 12 hr In, n=100) were different between the three groups by a Kruskal Wallis test; $p<0.0001$.

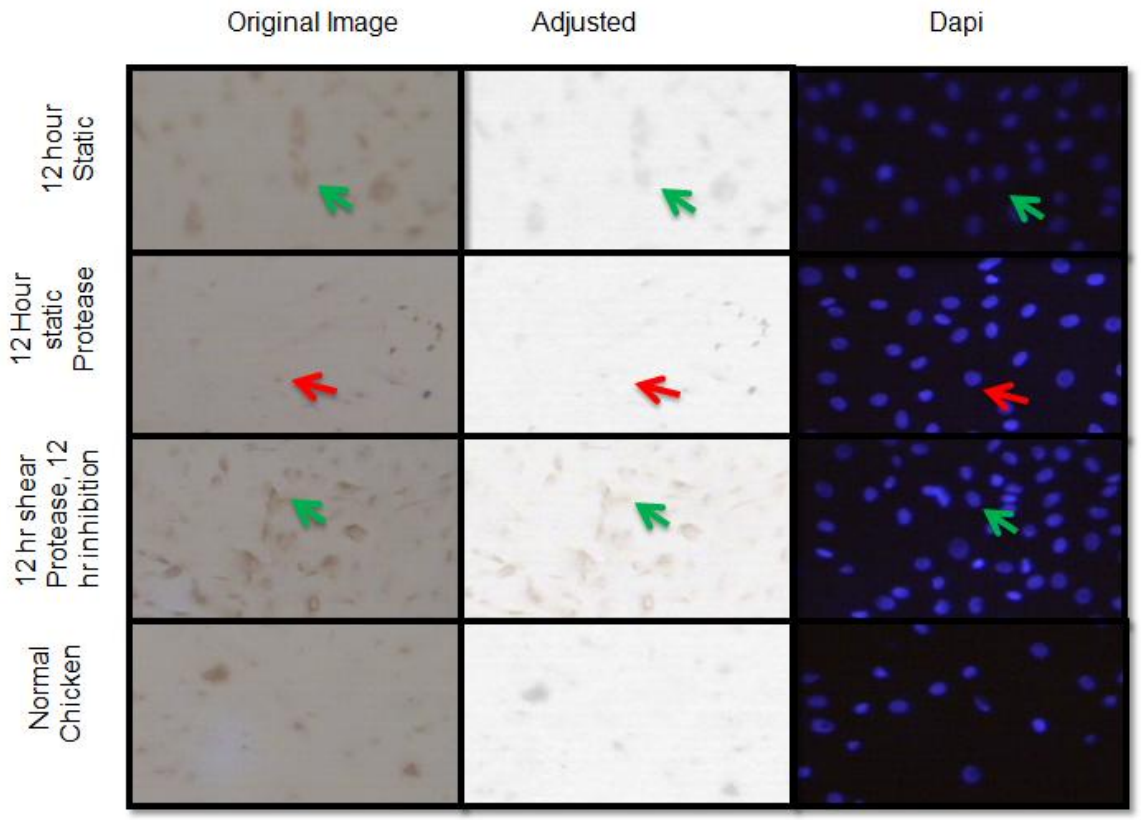

Figure 35: Representations of the static endothelial cells with VEGFR-2 labeling. Adjusted micrographs have background subtracted and are inverted. Dapi serves to confirm the presence of the cells on the slide. Green arrows indicate positive labeling. Red arrows indicate the negative cell labeling.

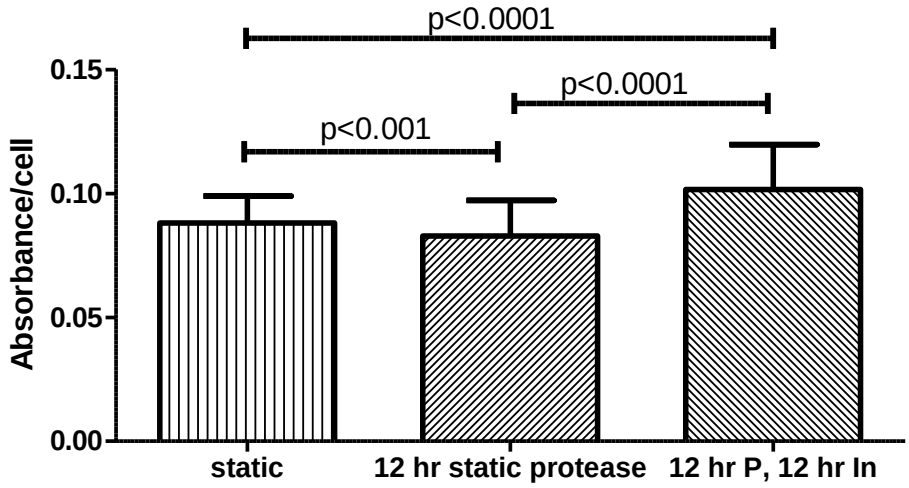

Figure 36: VEGFR-2 labeling intensity in static cells (n=147), cells exposed for 12 hours to proteases (12 hr static protease, 165) and to 12 hours protease exposure followed by 12 hour with protease inhibition (12 hr P 12 hr In, n=107). ANOVA analysis between the groups was statistically significant, $p<0.0001$.

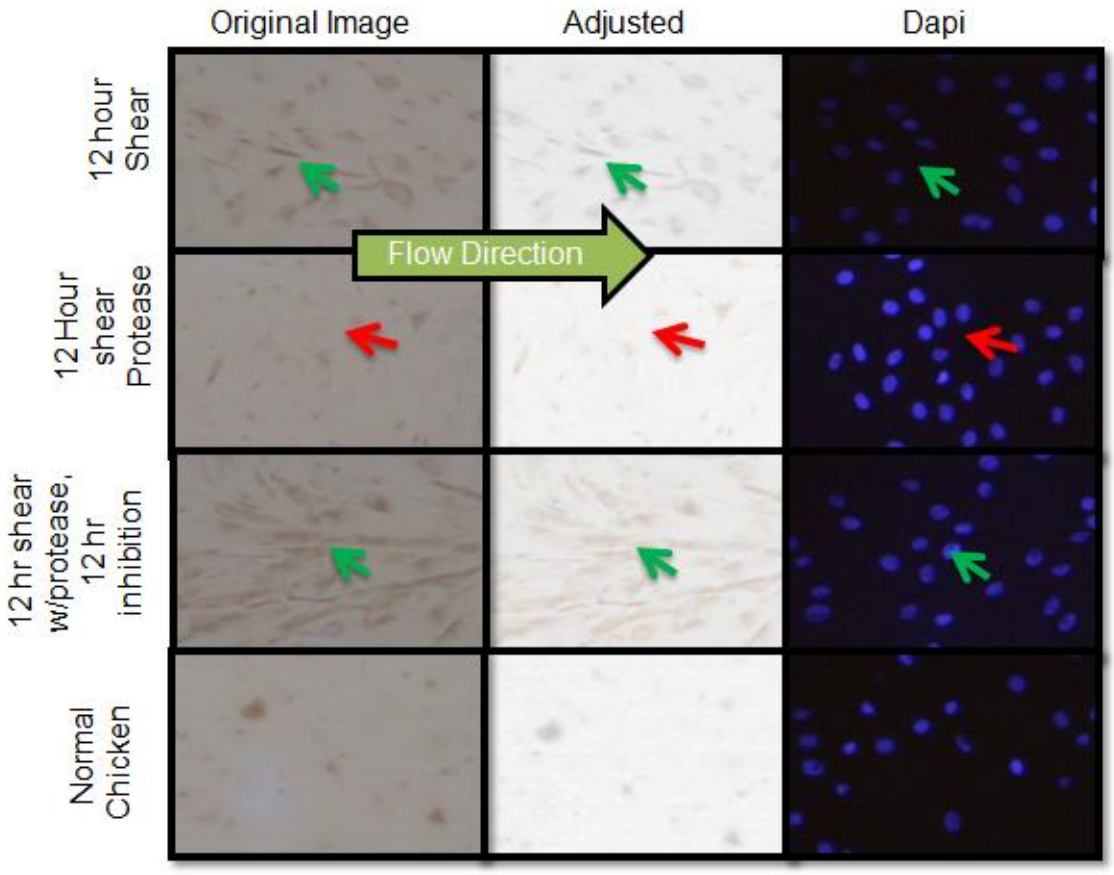

Figure 37: Representations of the sheared endothelial cells with VEGFR-2 labeling. Adjusted micrographs have background subtracted and are inverted. Dapi serves to confirm the presence of the cells on the culture. Green arrows indicate positive labeling. Red arrows indicate the negative cell labeling.

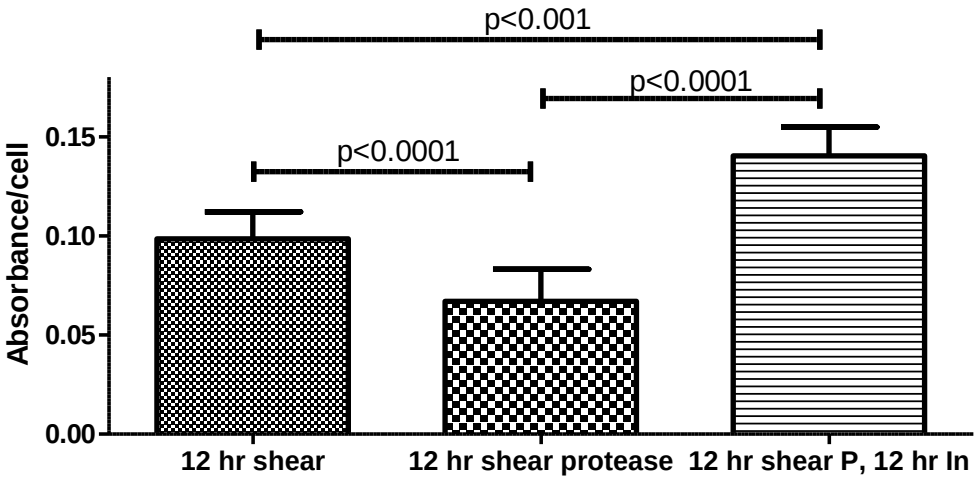

Figure 38: VEGFR-2 labeling intensities in cells exposed for 12 hours to shear conditions (12 hr shear, n=100), 12 hours to shear conditions in the presence of proteases (12 hr shear protease, 100), and to 12 hours shear conditions with protease followed by 12 hours with protease inhibition (12 hr shear P 12 hr In, 100). ANOVA analysis between the groups was statistically significant, $p<0.0001$.

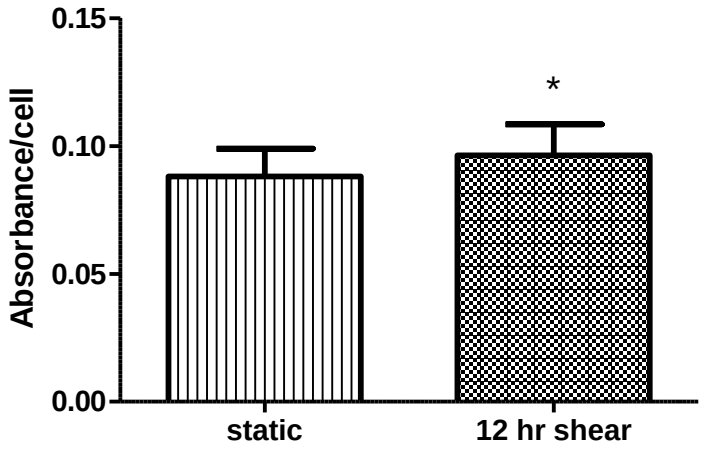

Figure 39: Comparing static cells to 12 hour sheared cells for VEGFR-2 extracellular receptor expression was statistically significant, *p<0.0001.

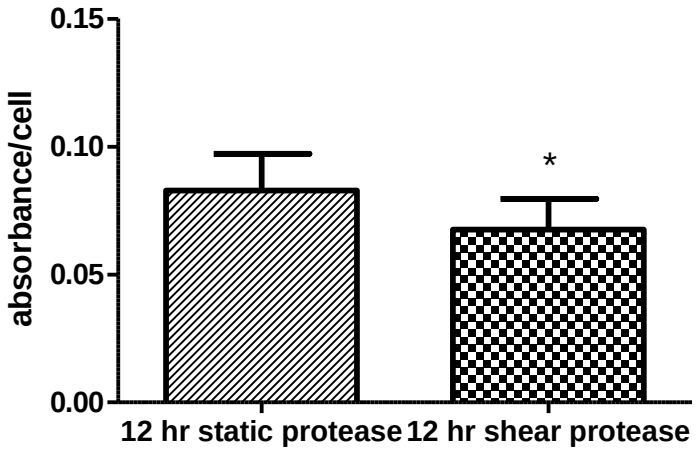

Figure 40: Comparing static cells to 12 hour sheared cell both in the presence of protease for VEGFR-2 receptor labeling was statistically significant, *p<0.0001.

F. INSULIN RECEPTOR DAMAGED BY PROTEOLYTIC CLEAVAGE

To investigate the potential for receptor damage during a proteolytic exposure, the properties of alignment were quantified to confirm the cells did not align in the direction of the flow (**Protocol 3, Figure 1**).

Insulin extracellular receptor labeling was higher in ECs not exposed to proteases (**Figure 41-42**). Static cells exposed for 12 hour to trypsin had a 30% reduction in density of the extracellular domain of the insulin receptor. After protease inhibition for 12 hours, the cells were able to recover their receptor density by 25%.

The sheared cells also followed a similar labeling pattern (**Figure 43-44**). Cells sheared in the presence of protease had on average a 12% reduction in signal intensity. After the cells treated with protease were inhibited for 12 hours, the signal increased slightly.

When comparing the shear with the static control without protease there was less signal intensity in the sheared cells (**Figure 45**). For the protease exposed cells, there was no significant difference in the labeling intensity (**Figure 46**).

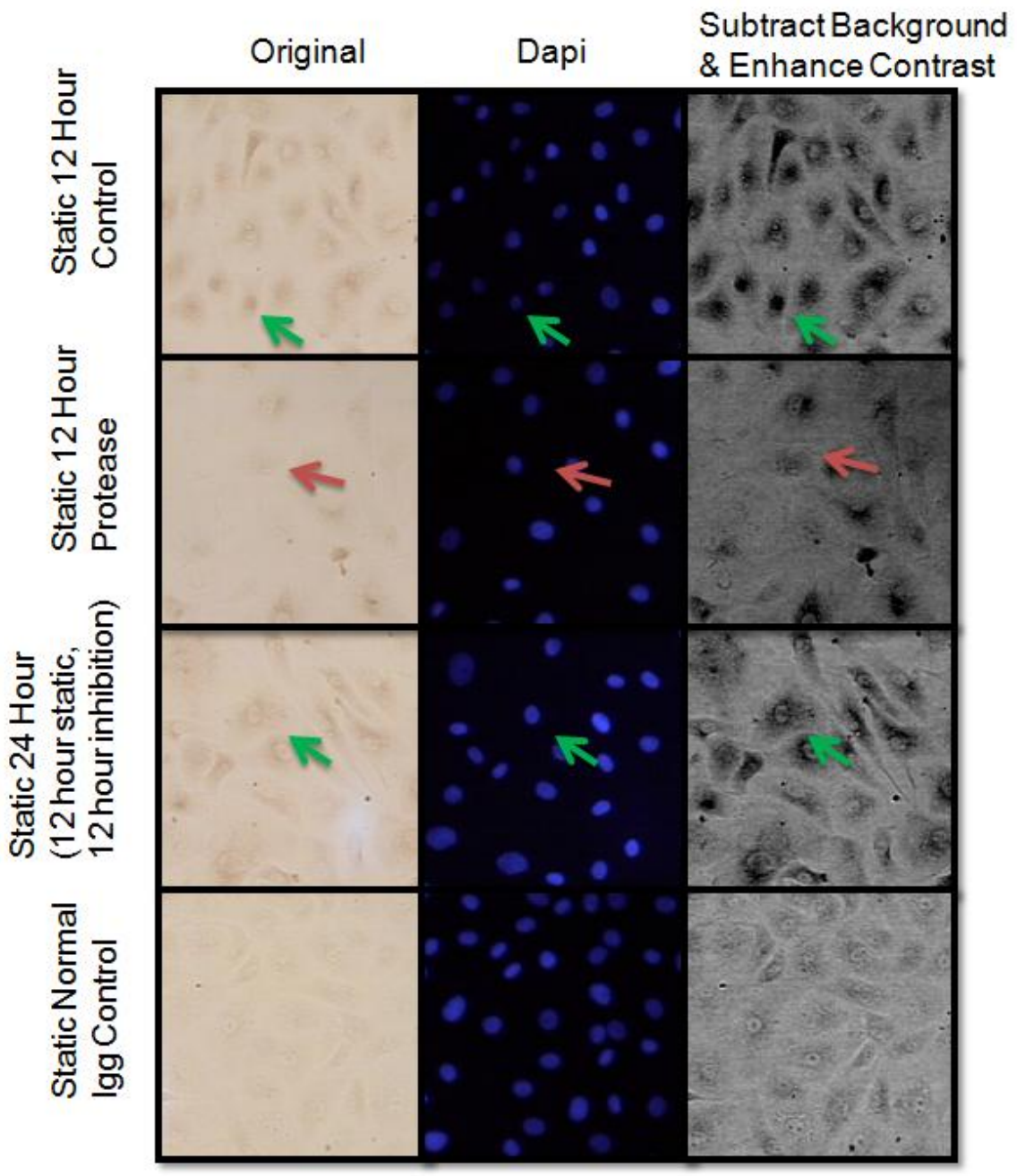

Figure 41: Comparing the static groups for extracellular insulin receptor labeling. Red arrows indicate negative labeling and green arrows indicate positive insulin labeling.

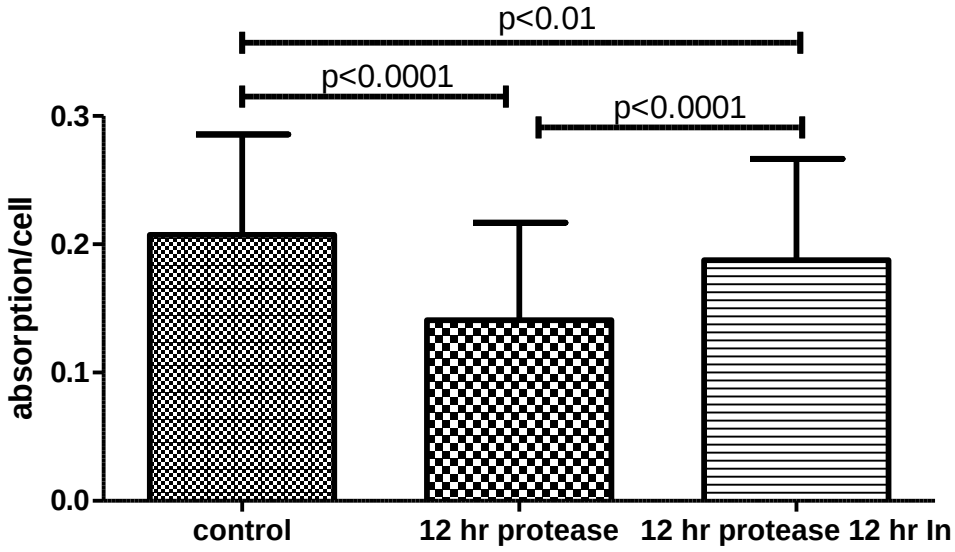

Figure 42: Comparing the three groups of insulin receptor density for static cells (control, n=158), static protease exposed for 12 hours (12 hr protease, n=283) and static cells exposed to protease for 12 hours followed by 12 hours of inhibition (12 hr protease 12 hr In, n=195). ANOVA analysis between the groups was statistically significant, $p<0.0001$.

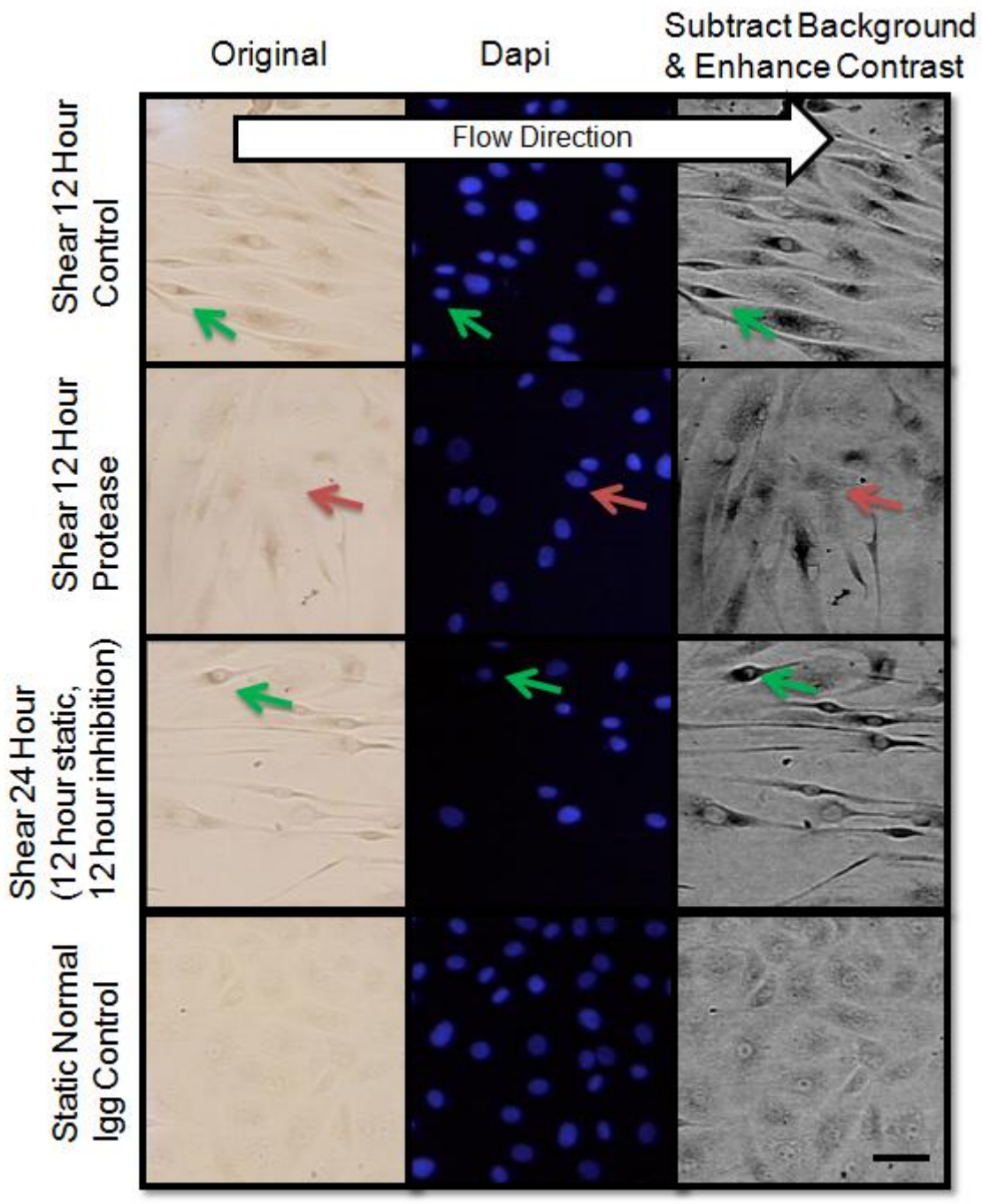

Figure 43: Comparing cells sheared with and without protease exposure for extracellular insulin labeling. Red arrows indicate negative labeling where green arrows indicate positive labeling. Also, the note the cells were able to orientate with the fluid flow direction of the shear stress. The scale bar corresponds to 50 μm for all panels.

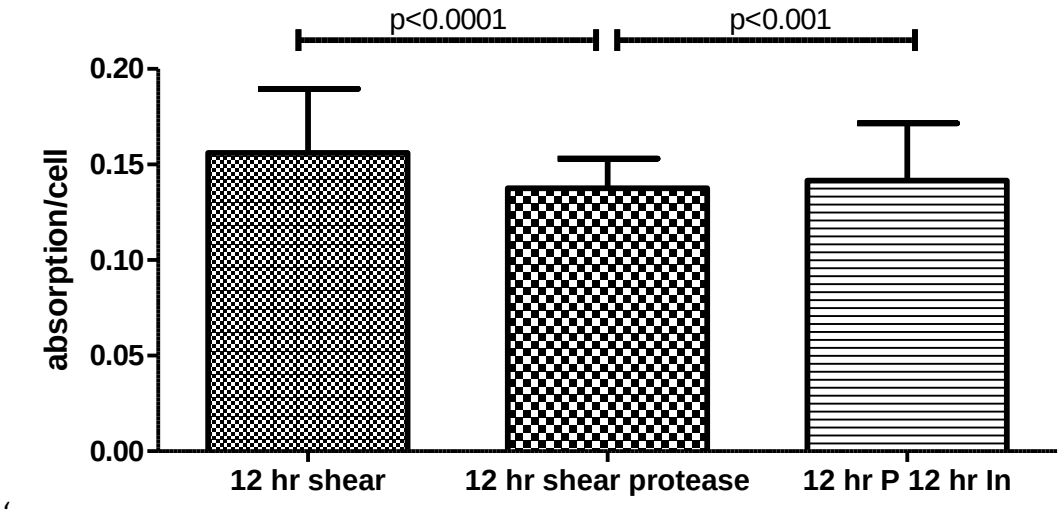

Figure 44: Comparing the sheared insulin absorption levels for cells sheared for 12 hours (12 hr shear, n=196), cells sheared in the presence of protease for 12 hours (12 hr shear protease, n=128), and cells sheared in the presence of protease for 12 hours followed by 12 hours inhibition (12 hr P 12 hr In, n=253). The difference between groups was significant by ANOVA analysis, $p<0.0001$.

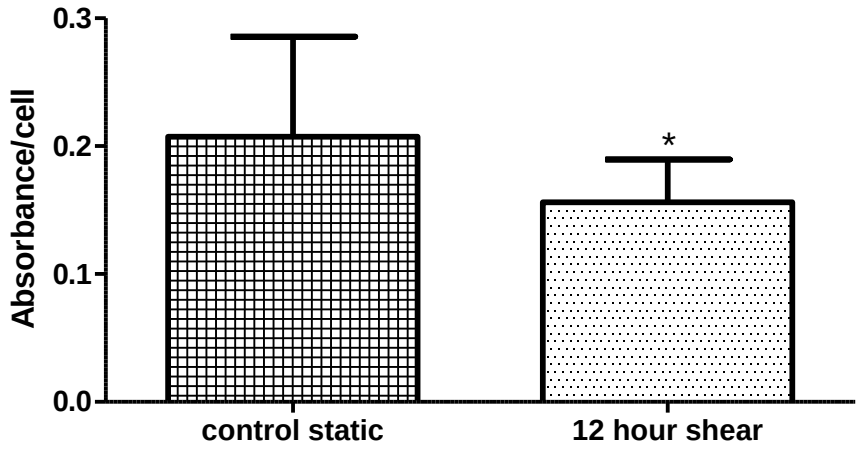

Figure 45: The insulin receptor labeling was greater for control cells than sheared cells, *p<0.0001.

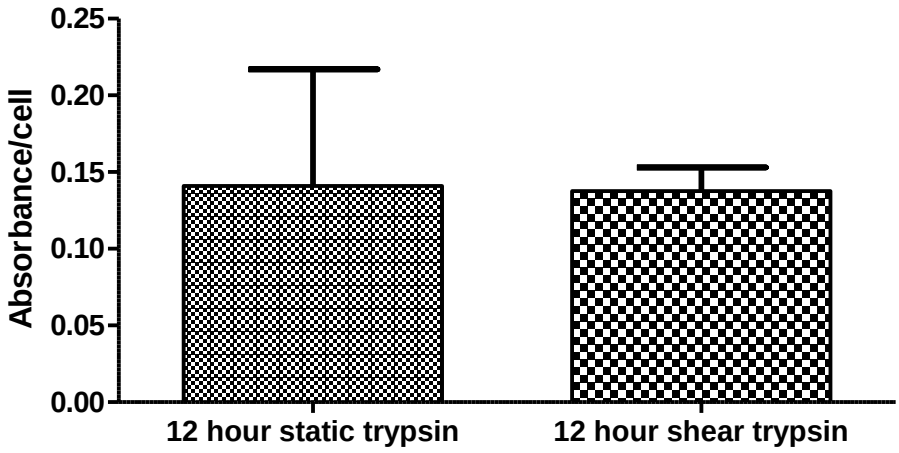

Figure 46: The labeling between the protease exposed shear and static cells yielded no significant difference between the groups.

IV. DISCUSSION

A. SUMMARY

Sheared ECs are more resistant to morphological changes (area, perimeter, and circularity index) than static cells when exposed to protease activity. Endothelial cells exposed to fluid shear stress concurrently with serine protease exposure do not respond to the direction of the shear stress. The extracellular components of endothelial cells can be damaged by protease activity disrupting the normal surface receptor density (PECAM-1, VEGFR-2, and insulin receptor). Proteolytic damage of surface receptors may be a partial cause of the reduced ECs response to fluid shear stress in the presence of proteolytic activity.

B. ACUTE PROTEASE EXPOSURE STUDIES

The protease concentrations chosen for these experiments were 10-100 times greater than any concentration measured in the plasma of rats and humans which are on the order of ng/ml whereas the trypsin concentrations that were applied in these experiments were 50 μg/ml (2.4 μM) (Paczek, Michalska & Bartlomiejczyk 2009). In the shock animals, the serine protease activity present was comparable to the concentration used in the experiments (DeLano, ongoing experiments). However, the experimental trypsin concentrations were less than those measured in the intestine, 300 μg/ml (9.6 μM), exposure of the endothelium to intestinal proteases may reach this order of magnitude (Waldo et al. 2003). The purpose of selecting a high concentration of trypsin was to demonstrate the potential damage of the protease to the ECs. In chronic

conditions, the trypsin exposure may continuously damage endothelial cell over a longer period of time at lower concentrations.

Higher concentrations of trypsin promoted an increase in cell area not covered by ECs and a decrease in circulatory index which could contribute to the increase in circulating endothelial cells in shock and the elevated permeability of the endothelium (Mutunga et al. 2001). Proteolytic activity may disturb the junctional proteins and weaken integrins attachments. Even a small weakening of the endothelium could result in further tissue damage. Sheared cells morphology did not change as rapidly to proteolytic attack possibility because sheared cells have an increased adherence to the surface (Urbich et al. 2000).

C. ENDOTHELIAL CELL RESPONSE TO SHEAR STRESS IN THE PRESENCE OF PROTEASE

Endothelial cells exposed to trypsin proteolytic activity while simultaneously being exposed to shear stress of 12 dyn/cm^2 did not align to the same degree in the direction of the flow compared to the controls cells. Alignment angle and circularity index distributions had large standard deviations because the cells were allowed a minimal time needed to reach alignment without all cells fully responding yet. If the duration of shear stress application were shorter, not enough ECs would show indications of alignment to differentiate between protease exposed cells. If the time were increased, most of the cells would be aligned and no difference would be detected. The average angle of alignment was close to 45 degrees, which represents a random orientation between 0-90 degrees.

Not only was the shear response reduced with the addition of protease activity to the shearing media, the PECAM-1 receptor and VEGFR-2 receptor density were decreased in two different experimental designs combining the concurrent application of fluid shear stress and protease exposure.

The reduction in extracellular labeling caused by the proteolytic activity could increase soluble PECAM-1, which has been associated with inflammation (Figarella-Branger et al. 2006, Serebruany et al. 1999). Others have documented an increased overall cell labeling of PECAM-1 in tissues of shock animals (Weis, Bohnert 2008). Since the protein quantification was done in the cell junctions, this increase could be attributed to new protein synthesis of the molecule repairing the molecule after damage. Even after cells were aligned before protease exposure, the extracellular PECAM-1 labeling decreased compared to presheared cells, which indicated that the shear was alone not enough to prevent proteolytic damage of junctional proteins.

The reduced alignment and VEGFR-2 labeling intensity in the sheared case correlate with the previous observations that VEGFR-2 is a mechanosensor (Chen et al. 2001, Wang et al. 2002). When the protease damaged cells were shifted into a medium with inhibition of the proteases and continuation of shear, the orientated in the direction of the flow and the VEGFR-2 receptor density regained, suggesting that the lack of protease promoted the reorientation and increase of receptor density on the ECs. The protease could contribute to the extracellular damage of the mechanosensors and others have documented the importance of these receptors for mechanotransduction (Shyy, Chien 2002, Tzima et al. 2005b).

Exposure to protease compromised the extracellular insulin receptor density. Cleavage of the insulin receptor is an alternative explanation for the inability for the cell to uptake glucose during shock and account for the hyperglycemia measured in intensive care patients (Fahy, Sheehy & Coursin 2009). Reduction in receptor density would increase the concentration of glucose in the blood, but since the receptor could be regenerated by *de novo* synthesis, the process is reversible and normal glucose uptake could be restored.

D. EXPERIMETNAL LIMITIATONS

The evidence of receptor density reduction for VEGFR-2, PECAM-1, and the insulin receptor by binding antibodies targeted against the extracellular domain suggests the extracellular domain was cleaved. To further investigate this issue, Western Blots were performed using the same extracellular domain targeted antibodies as immunhistochemistry. While I could not detect the cleaved fragments in a Western Blot, a silver stain gel showed many additional protein cleavage products in the supernatant. Since trypsin is a nonspecific degrading enzyme, fragments that are cleaved are still susceptible for continued degradation once released from the cell membrane, therefore increasing the challenge of detecting cleaved fragments. If more specific enzymes were found to degrade extracellular proteins, detecting the fragment would be feasible.

There are some shortcomings with immunohistochemistry, which could induce error into the results by the nonlinearity of the colormetric DAB staining. There also were no significant labeling differences between cells sheared with protease and static cells. This may be caused by the high trypsin concentration which may degrade the

accessible molecules where a slightly lower protease concentration could be more selective. Also, the labeling for VEGFR-2 and insulin was carried out after fixing the cells in paraformaldehyde. Since no permeabilization was used, the intracellular antigen exposure was minimized.

E. CONCLUSIONS

There are many potential opportunities for endothelial cell damage by serine proteases during shock causing endothelial dysfunction, damage to receptors, and reduced response to shear stress. In the model of physiological shock, autodigestion by pancreatic enzymes in the circulation can severely impact the homeostasis of the endothelial cells.

V. FUTURE STUDIES

The model developed for protease exposure to cultured endothelial cells exemplifies how tolerant the cells are to relatively high levels of proteolytic activity, much exceeding any physiological concentration that would appear in the plasma. However, the endothelial dysfunction that was observed *in vitro* in the shock model for autodigestion opens many possibilities for the potential hazards of protease activity on extracellular components. Verifying the receptor cleavage in an *in vivo* shock model would confirm the loss of receptor density.

The autodigestion hypothesis may serve as a potential explanation for the low levels of sVEGFR-detected in the plasma. Not only would the cell's function be compromised but the receptor fragment may influence downstream signaling. For example soluble VEGFR-2 fragments inhibit angiogenesis in tumors (Kou et al. 2004). Soluble VEGFR-2 has been detected in the plasma of both mice and humans; however whether the mechanism for how the receptor fragment enters the circulation via mRNA posttranslational splicing or proteolytic cleavage is still debated (Ebos et al. 2004).

Other extracellular membrane components may be susceptible to proteolytic attack and have their function compromised. Chronic conditions such as hypertension and obesity where there is a consistent low protease activity can theoretically damage receptors (DeLano, Schmid-Schönbein 2008, Tran, Delano & Schmid-Schönbein 2010, Chen et al. 2008). There is already evidence revealing the cleavage of several receptors in chronic disease states where the protease concentrations are low, but consistent. For instance, the spontaneously hypertensive rat has elevated proteolytic activity in the

plasma. As a result, cleavage of several receptors has been documented including that of the insulin receptor and VEGFR-2 (Tran, Delano & Schmid-Schönbein 2010).

If the mechanism for the receptors to enter the circulation is by circulating proteases, it would be useful to know what part of the protein is cleaved by the protease. Identifying the fragment would be a useful in identifying the site in the protein at risk to cleavage. Soluble fragments measured in the circulation *in vivo* could be used for potential patient diagnostics. Determining the potential damage of many key receptors on the endothelial cell by both MMPs and serine proteases would identify the potential receptors at risk to proteolytic cleavage.

After receptors are damaged, mRNA transcription is upregulated should be investigated. For several receptors where cleavage has been shown (VEGFR-2 and insulin), reverse transcription of the protein transcript could be completed after acute exposure to proteases. The experiment could be carried out on shear and static endothelial cells to see if sheared endothelial cells have a protective mechanism to proteolytic damage.

On the contrary, the mRNA can be silenced for select receptors with siRNA to see if the cell's mortality depends on key receptors ie. VEGFR-2 (Makino et al. 2007). The receptor density on the cell can be quantified by immunohistochemistry to see if receptors have the ability to repair after damage by proteases. Cell apoptosis levels can also be measured. Enzymatic degradation by serine proteases and MMPs can be observed for any of these experiments.

Cell lines could be genetically altered to include a fluorescent tag on the extracellular domain of the receptor. If the receptor is cleaved, the intensity would

decrease. This would be a potential method to measure in real-time the kinetics of the receptor cleavage and the dissociation of the cleavage fragment from the rest of the receptor.

There are many experiments that can be completed both in the context of physiological shock and chronic diseases to validate that receptor cleavage is the mechanism for loss of receptor density and endothelial dysfunction.

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