Published in final edited form as:

Cell Mol Bioeng. 2009 September 1; 2(3): 320–331. doi:10.1007/s12195-009-0073-7.

Stretch and Shear Interactions Affect Intercellular Junction Protein Expression and Turnover in Endothelial Cells

Danielle E. Berardi^{1,2} and John M. Tarbell²

¹Department of Bioengineering, Pennsylvania State University, University Park, PA 16802

²Department of Biomedical Engineering, City College of New York, New York, NY 10031

Abstract

Complex hemodynamics play a role in the localization and development of atherosclerosis. Endothelial cells (ECs) lining blood vessel walls are directly influenced by various hemodynamic forces: simultaneous wall shear stress (WSS), normal stress, and circumferential stress/strain (CS) due to pulsatile flow, pressure, and diameter changes. ECs sense and transduce these forces into biomolecular responses that may affect intercellular junctions. In this study, a hemodynamic simulator was used to investigate the combined effects of WSS and CS on EC junctions with emphasis on the stress phase angle (SPA), the temporal phase difference between WSS and CS. Regions of the circulation with highly negative SPA, such as the coronary arteries and carotid bifurcation, are more susceptible to the development of atherosclerosis. At 5 h, expression of the tight junction protein zonula occludens-1 was significantly higher for the atheroprotective SPA = 0° compared to the atherogenic SPA = −180° while the apoptosis rate was significantly higher for SPA = −180° than SPA = 0°. This decrease in tight junction protein and increase in apoptosis and associated leaky junctions suggest a decreased junctional stability and a higher paracellular permeability for atherogenic macromolecules for the atherogenic SPA = −180° compared to SPA = 0°.

Key terms

Stress phase angle (SPA); Hemodynamics; Atherosclerosis; Permeability; Apoptosis; ZO-1

Atherosclerosis is an inflammatory disease that affects arteries through a cascade of events that involves lipid infiltration and accumulation in blood vessel walls. As these fatty lesions advance and calcify, arterial lumens may narrow or vulnerable plaques may rupture, resulting in heart attacks and strokes. According to the American Heart Association, cardiovascular disease, including coronary heart disease and stroke, is the leading cause of death in the United States.⁴⁵ Areas of the vasculature that are prone to atherosclerosis include the proximal coronary arteries, the carotid bifurcation, and other arterial bifurcations and curves,²⁰ where the complex interaction of hemodynamic forces plays a role in the localization and development of disease.

In arteries, the hemodynamic forces involved are wall shear stress (WSS), pressure (*P*), and circumferential stress/strain (CS), as shown in Figure 1A. WSS is the frictional fluid stress that acts tangential to the apical surface of ECs in response to blood flow, whose mean component

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Correspondence name and address: John M. Tarbell, City College of New York, Department of Biomedical Engineering, Steinman Hall T-401, 140th Street and Convent Avenue, New York, NY 10031, 212-650-6841, 212-650-6727, tarbell@ccny.cuny.edu.

The original publication is available at www.springerlink.com.

<http://www.springerlink.com/content/2n06374rq5142322/?p=566270903f914692bacb6c45512c77a9&pi=5>

ranges from 5–40 dyn/cm².³⁴ P is the normal stress that acts perpendicular to ECs and is balanced by circumferential stress (hoop stress) which induces circumferential strain, CS. CS is synchronous with diameter variation and is defined as $(D_{\max} - D_{\min}) / D_{\text{mean}}$, where D is the diameter. CS varies between 2–18% over a cardiac cycle for elastic arteries.¹⁹ WSS and CS are oscillatory functions of time due to the pulsatile nature of blood flow in the circulation, and as shown in Figure 1B, there may be a temporal phase angle between them that has been referred to as the stress phase angle, SPA.⁴³

SPA is associated with the localization of atherosclerosis in the circulation, with areas of highly negative SPA being more susceptible to the disease.^{28, 29, 43, 44, 50} For example, the outer wall of the abdominal aortic bifurcation ($\text{SPA} \cong -100^\circ$),^{28, 29} the outer wall of the carotid artery bifurcation sinus ($\text{SPA} \cong -180^\circ$),⁵⁰ and both the outer wall ($\text{SPA} \cong -220^\circ$) and inner wall ($\text{SPA} \cong -250^\circ$) of the curved coronary artery⁴⁴ are prone to disease. These highly negative SPA values are in contrast to those associated with regions spared of disease ($\text{SPA} \cong 0^\circ$): small straight arteries and veins⁴³ and the carotid sinus inner wall.⁴⁴ *In vivo* data³⁹ clearly shows that there is an out-of-phase relationship between P (that drives CS) and flow in the coronary arteries (that drives WSS) that is distinct from the relationship in the aorta. During systole, while the aortic blood pressure and flow are highest, the coronary blood flow is low and the pressure is high. Furthermore, unlike WSS, SPA, through its dependence on the impedance phase angle (IPA, phase angle between pressure and flow), responds to systemic changes: SPA is more negative in hypertension and more negative in arteries than in veins.^{28, 44}

In vitro studies using bovine aortic endothelial cells (BAECs) on elastic tubes showed that highly negative SPA conditions suppressed anti-atherogenic endothelial nitric oxide synthase (eNOS) gene expression and vasodilator nitric oxide (NO) metabolite production while the pro-atherogenic vasoconstrictor endothelin-1 (ET-1) gene expression and metabolite production increased between 4 and 12 h of exposure to mechanical forces.^{12–14, 43} These results were confirmed by an *in vivo* study in rabbit arteries,¹⁵ where this atherogenic gene profile (low eNOS, high ET-1) was exhibited in the coronary arteries and the opposite pattern was observed in the aorta. Additionally, this *in vivo* study showed that EC alignment and elongation were similar in both the atherogenic coronary arteries and the normal straight aorta while the intercostal ostia, where flow is disturbed, had random orientation and lower aspect ratio. This suggested that the WSS environments of the coronaries and aorta were similar and that the difference in SPA was critical in stimulating diverse gene expression profiles. Together, these studies showed that asynchronous SPA induces an atherogenic gene expression profile both *in vitro* for $\text{SPA} = -180^\circ$ and *in vivo* in coronary arteries where SPA is expected to be highly negative.⁴⁴

Adjacent ECs are connected through cell-cell adhesion with tight junctions (TJs) and adherens junctions (AJs) both playing a role in the paracellular permeability of the endothelium. TJ proteins, including occludin21 and zonula occludens-1 (ZO-1),⁴⁸ form a seal between adjacent ECs and limit the free transport of solutes, cells, and other molecules through the paracellular pathway.¹ Furthermore, the integrity of the AJs, and the presence of the characteristic protein, vascular endothelial cadherin (VE-cadherin), is required for TJ organization and maintenance²⁷ and may affect the TJ-controlled paracellular permeability barrier.⁵¹ Together these junctions maintain endothelial paracellular permeability properties under normal conditions, but in disease states, such as atherosclerosis, these junctions may be altered, allowing larger molecules, even low density lipoprotein (LDL), across the endothelium. Enhanced LDL permeability is normally associated with the presence of leaky junctions,^{4, 5, 33, 52} which are infrequent, transiently leaky interendothelial clefts associated with cells in a state of turnover due to the processes of mitosis or apoptosis,⁵⁵ or associated with tricellular corners,^{3, 54} where the protein tricellulin is localized.²⁴ A recent *in vitro* study showed that leaky junctions account for more than 90% of LDL transport under convective conditions.⁵

When subjected to WSS alone, ECs remodel by elongating in the direction of shear, which may require alteration of transmembrane proteins, such as occludin and VE-cadherin, at cell-cell contacts while leaving cytoplasmic proteins, such as ZO-1, unchanged. This remodeling also leads to increased permeability over short time periods (1–4 h).^{6, 18, 25, 41, 47} At longer times (24 h) there is a gain of occludin at cell borders associated with a decreased permeability.^{8, 10, 11} Related observations are that WSS decreases plasma membrane localization of VE-cadherin at 1–8 h of exposure but then increased plasma membrane localization of VE-cadherin is observed as the cells reorganize at 24–72 h.^{37, 40, 53} Physiologic levels of steady WSS (15–25 dyn/cm²) applied for 24–48 h aligned and elongated ECs in the flow direction,³⁰ decreased cell proliferation,^{26, 31} and reduced the rate of apoptosis.^{22, 26} Therefore, it is expected that leaky junctions, and increased endothelial permeability, would be more prevalent in regions of low WSS and separated flow than in regions of higher, unidirectional WSS. Thus, EC proliferation and apoptosis are altered by shear stress alone and may be changed in different ways under more complex hemodynamic conditions.

Studies of CS by itself for 24 h or longer have investigated EC shape and have noted cell alignment and elongation perpendicular to the stretch direction¹⁶ and proliferation at a faster rate⁴⁹ and apoptosis at a lower rate^{22, 35} than static controls. A recent study with BAECs showed that TJ protein expression (occludin and ZO-1) increased along with a reduction in occludin phosphorylation and an increase in ZO-1 phosphorylation after 24 h of 5% strain at 1 Hz.⁹ These conditions also resulted in increased localization of occludin and ZO-1 at the cell borders, which correlated to a decrease in dextran permeability after the cells were re-plated on porous supports. Therefore, CS may play a role in EC permeability and should be considered along with WSS to obtain a complete picture. However, to date, there are no known deformable, yet permeable, substrates available to directly study the effects of stretch on permeability or transport across ECs.

As an alternative to subjecting ECs to WSS or CS alone, we have applied these pulsatile forces simultaneously *in vitro*, either completely in-phase (SPA = 0°) or out-of-phase (SPA = -180°) with each other, to more accurately simulate the physiologic forces the endothelium is exposed to *in vivo*. We have examined the intercellular junction proteins and cell turnover rates as a means of indirectly assessing the effects of combined WSS and CS on the endothelial transport barrier to macromolecules. We find that SPA = -180° suppresses ZO-1 protein expression and increases apoptosis rate compared to SPA = 0°. The results suggest enhanced permeability to macromolecules under the atherogenic SPA = -180° condition.

Materials and Methods

Cell Culture and Hemodynamic Conditions

BAECs were isolated and grown from bovine thoracic aortas obtained from a local slaughterhouse as described previously.⁴⁶ BAECs used for experiments were more than 98% pure and were used from passage 6–9. In preparation for tube experiments, clear non-cytotoxic silicone elastic tubes (Sylgard 184 Silicone Elastomer Kit) were produced using manufactured molds, tested for their longitudinal stiffness, and thoroughly cleaned before use.^{12–14, 43} Fibronectin (30 µg/ml, Sigma) was applied to these tubes for 1 h in a humidified 37 °C 5% CO₂-95% air incubator, after which BAECs were seeded twice at a density between 4 and 6 × 10⁴ cells/cm². Tubes were fed with MEM-10% FBS, which was replaced with fresh media every other day, and were ready for the experiment on the fourth day post-seeding.

Once the ECs on the tubes grew to confluence, they were inserted into the experimental flow loop that has been thoroughly described elsewhere.^{12–14} While in a laminar flow hood, the tubes were aseptically connected to the flow loop polypropylene tubing before being transported and set up in the hemodynamic device. The pH of the experimental media was kept

constant at 7.2 using a controller connected to pH probes in the media reservoirs and an external CO₂ tank. The viscosity of the experimental media was increased to 3 cP using dextran (1%, 11×10^6 Da, Sigma), which was added to MEM without phenol red and supplemented with 1% PS and 1% BSA (MEM-dextran). The entire system was enclosed in a dark, temperature stable box (37 °C), except for the computer that recorded flow, pressure, and diameter readings from the sensors.

Since fluid flow (Q), WSS, and CS (diameter variation, D) were functions of time, they were decomposed into mean $\pm$ oscillatory (sinusoidal) components.¹⁴ Therefore, using this device, the tubes were subjected to a mean laminar flow component producing a WSS of 10 dyn/cm² with an oscillatory component of ± 10 dyn/cm² and a diameter variation, or CS, of $\pm 4\%$ with the mean value being 4%, corresponding to the increase in diameter when the tubes were pressurized to 70 ± 20 mmHg. The flow loop produced steady shear stress cases as well as pulsatile flow cases. All pulsatile flow cases had identical WSS, CS, and P waveforms with a SPA of either 0° or -180° . Separate controls were performed in an incubator: static controls at 0 mmHg and pressurized controls at 70 mmHg. Note that the controls and flow cases had drastically different volumes, 15 ml and 200 ml, respectively. To account for this volume difference, separate sham controls were run to examine the possible dilution effect on soluble mediators, which showed negligible influences.

Protein Extraction and Western Blotting Analysis

At the end of certain experiments, protein from the ECs was extracted and quantified using Western blots.¹⁸ First, the EC monolayers on the tubes were washed 2–3 times with PBS, followed by an ice-cold final wash with PBS that included 0.2 mM PMSF. After blotting the tube ends with a paper towel to remove the excess wash solution, ice-cold Stuart extraction buffer (0.2% SDS, 100 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 2 mM EDTA, 10 mM HEPES, 1 mM Na₃VO₄, 10 mM NaF, 1 mM benzamidine, 1 EDTA-free Mini Complete protease inhibitor cocktail tablet, and 1 mM PMSF) or RIPA extraction buffer (150 mM NaCl, 50 mM Tris, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1/10th protease inhibitor cocktail tablet, 1 mM PMSF, 1 mM Na₃VO₄) was applied to the cells. The tubes were massaged for 1 min to remove the cells from the tube walls, and the contents were collected into microcentrifuge tubes. Protein was further extracted for 10 min as the microcentrifuge tubes were rocked on ice blocks or sonicated for 1 min. These tubes were centrifuged at 13,000 rpm for 10 min at 4 °C to pellet the insoluble material. Aliquots of the supernatants were frozen separately at -80°C and were later used to determine the protein concentration of the supernatant using the BioRad DC protein assay. The frozen samples were diluted appropriately with Stuart solution on the day that the gel electrophoresis was run so that each sample lane was loaded with the same amount of protein and equal amounts of 2X sample buffer (2 ml glycerol, 0.2 g SDS, 1 ml 1M Tris pH 6.8, 0.018 g bromophenol blue, and 0.2 ml β -mercaptoethanol) or 5X sample buffer (312 mM Tris, 50% glycerol, 10% SDS, 0.05% bromophenol blue, and 25% β -mercaptoethanol). These samples were vortexed briefly to mix the contents, boiled for 5 min, and centrifuged at 13,000 rpm for 10 min before loading equal amounts into the gel.

Acrylamide-Bis gels were cast, with the resolving gel being 8% and the stacking gel being 4–10%. Again, the gels were loaded with equal amounts of protein in each sample. SDS-PAGE (SDS-polacrylamide gel electrophoresis) ran for 3.5 h at 90 mA in SDS running buffer using a Protean II system (BioRad) or 1.75 h at 100 V using a Mini-Protean 3 Cell (BioRad). After equilibration, proteins were transferred to Immun-Blot PVDF membranes (BioRad) overnight at 10V in CAPS transfer buffer with 10% methanol using a Trans-Blot Cell (BioRad) or 1.5 h at 100 V using a Mini Trans-Blot Cell (BioRad). Membranes were blocked with 5% milk in Tris buffered saline with Tween 20 (TBS-T) for 1 h, followed by 3 washing steps of 15, 5, and

5 min with TBS-T. After the membranes were divided, rabbit polyclonal primary antibodies – anti-occludin (1:1000; Zymed), anti-ZO-1 (1:1000; Zymed), anti-VE-cadherin (1:1000; Cayman), and anti- β -actin (1:5000; Cell Signaling) – were diluted in 1% milk in TBS-T and rocked 3–24 h with ice blocks. After another washing phase, donkey peroxidase-linked anti-rabbit secondary antibody (1:4000; GE Healthcare) was diluted in 1% milk in TBS-T and added and rocked for 1.5 h. Another washing step was performed before the membranes were subjected to enhanced chemilluminescence (ECL) detection reagents (GE Healthcare) for 1 min or ECL Advance detection reagents for 5 min (GE Healthcare). Shortly after, these membranes were subjected to film (Kodak BioMax Light film) in a darkroom; the developed film was scanned into a computer; and the protein content was quantified using Scion Image software (Scion Corp.) for densitometry measurements. A BioRad ChemiDoc XRS detection system with Quantity One version 4.5 software (BioRad) was also used to capture images. For each day's experiment, ratios were taken with respect to that day's static control.

Immunocytochemistry

At the end of some experiments, junction proteins were fluorescently stained with antibodies and visualized using fluorescence microscopy.¹⁸ First, the EC-laden tubes were washed twice with PBS and fixed in 1% paraformaldehyde for 10 min. They were then cut into sections, opened, and flattened before being permeabilized with 0.2% Triton X-100 in PBS (ZO-1) or 100% methanol (VE-cadherin) for 10 min and blocked with 10% BSA and 0.1% Triton X-100 in PBS (ZO-1) or 5% BSA and 0.1% Triton X-100 in PBS (VE-cadherin) for 1 h. After washing with 0.1% Triton X-100 in PBS, the tubes were incubated with polyclonal rabbit anti-ZO-1 (1:200) and anti-VE-cadherin (1:100) primary antibodies overnight at 4 °C. Following another set of washings with 0.1% Triton X-100 in PBS, the cells were subjected to Alexa Fluor® 488 donkey anti-rabbit (1:500 to 1:200; Invitrogen) secondary antibody for 1.5 h and washed again with 0.1% Triton X-100 in PBS. Tube pieces were then mounted with Aqua-Poly/Mount between glass slides and coverslips, with the coverslips touching the cells. These slides were imaged using a Nikon Eclipse TE2000-E inverted fluorescence microscope with a Photometrics Cascade 650 camera (Roper Scientific) and MetaVue 6.2r2 imaging software (Universal Imaging). At least three 20X and three 40X images were taken for each tube sample.

Using these images for ZO-1 and VE-cadherin at 5 and 12 h, certain image features were counted using Image J software (NIH Image). These features included breaks or discontinuities between cells, holes between cells, and discontinuities at tricellular corners. For each feature, entire 40x images were analyzed, counted, and averaged over all the images taken for each sample. Using the ZO-1 immunocytochemistry images to outline cell boundaries, EC elongation and alignment were also assessed using Image J software. The angle, or cell orientation angle, was measured between the cell major axis and the flow direction. This angle approached 0° as the cell completely aligned with the direction of flow. The aspect ratio was calculated as the major axis / minor axis, and increased as the cell became more elongated. The circularity, or cell shape index, was calculated as 4π (cell area) / (cell perimeter)². This value approaches one when the cell is a perfect circle and zero as the cell becomes completely elongated. For each reported value, five cells per 40x image were measured and averaged from the best of three images taken for each sample.

Cell Turnover Detection

At the end of some experiments, cell turnover was analyzed through immunostaining and fluorescence microscopy in order to determine mitosis and apoptosis rates. Tubes were washed and fixed with 1% paraformaldehyde for 10 min. Then they were cut, opened, flattened, permeabilized with 100% methanol for 10 min, and blocked with 1% BSA and 0.1% Triton X-100 in PBS (mitosis) or 5% BSA and 0.1% Triton X-100 in PBS (apoptosis) for 1 h. After washing with 0.1% Triton X-100 in PBS, tubes were incubated with monoclonal mouse anti-

MPM-2 (1:100; Upstate) (mitosis) or polyclonal rabbit anti-cleaved caspase-3 (1:500; Cell Signaling) (apoptosis) primary antibodies overnight at 4 °C. Following another set of washings with 0.1% Triton X-100 in PBS, the cells were subjected to Alexa Fluor 488 donkey anti-mouse (1:200; Invitrogen) or Alexa Fluor® 488 donkey anti-rabbit (1:500; Invitrogen) secondary antibodies for 1.5 h and washed again with 0.1% Triton X-100 in PBS. Tube pieces were then mounted with Aqua-Poly/Mount between glass slides and coverslips, with the coverslips touching the cells. These slides were imaged using a Nikon Eclipse TE2000-E inverted fluorescence microscope with a Photometrics Cascade 650 camera (Roper Scientific) and MetaVue 6.2r2 imaging software (Universal Imaging). At least three 20X and three 40X images were taken for each tube sample, and cells stained for mitosis and apoptosis were counted for the entire tube sample since they were brighter than the rest of the cell population. The percentages of mitotic and apoptotic cells were individually calculated from the number of fluorescent positively-stained cells relative to the total cell count for each tube sample. These percentages were averaged for each experimental case for at least three tube samples. No protein extraction or Western blot analysis was required for mitosis and apoptosis quantification.

Statistical Analysis

Relative protein expression levels were denoted as mean $\pm$ standard error of the mean. The p-values were generated using Minitab's General Linear Model, which performs univariate analysis of variance, with Tukey's multiple comparison tests. p-values < 0.05 indicated statistical significance, or that there were differences between the means being compared.

Results

Relative Protein Expression

As shown in Figure 2A, the relative protein expression of ZO-1 for SPA = 0° and SPA = -180° were significantly increased for both 5 and 12 h relative to 1 h that was below static control in both cases. These observations may be due to tight junction breakdown during remodeling at early times, followed by a reinforcement of the junctions against mechanical stress at later times. More specifically, 5-h ZO-1 relative protein expression was significantly higher for the atheroprotective SPA = 0° case when compared to the atherogenic SPA = -180° case, which was at the SC expression level. This ZO-1 finding at 5 h is consistent with the hypothesis that SPA = -180° is the more atherogenic state relative to the SPA = 0° case. This result suggests that EC junctions were less intact and less stable at SPA = -180°; and indirectly indicates that the monolayer would be more permeable for SPA = -180° than SPA = 0°.

Occludin and VE-cadherin did not show statistically significant changes between the two SPA cases at any time point. 1-h Western blots for occludin could not be completed due to problems with the available occludin antibody as it changed and lost reactivity in BAECs. As shown in Figure 2B, there was a greater increase in relative occludin protein expression at 5 h than at 12 h, which was significantly higher for the SPA = 0° case. For VE-cadherin relative protein expression, as shown in Figure 2C, there was an increase in protein at 12 h and when compared to 1 h this increase was statistically significant for SPA = -180° and SS.

Protein Localization and Cell Alignment

At both 5 and 12 h, ZO-1 and VE-cadherin protein were localized at the cell borders as expected (Figure 3 and Figure 4). Immunocytochemistry for occludin could not be completed due to problems with the available occludin antibody as it changed and lost reactivity in BAECs. The protein distribution and appearance were subtly different between flow cases relative to controls but not between SPA cases. The quantification of breaks, holes, and tricellular corners showed no statistically significant differences between the two SPA cases at either time point.

using both proteins as markers, as shown in Figure 5. However, at 5 h (Figure 5A), the ZO-1 staining showed that the average number of breaks was significantly higher than both the number of holes and tricellular corners. Meanwhile, for 5-h VE-cadherin staining (Figure 5B), the average number of holes between cells was significantly higher for both SPA cases compared to SC and PC, and the average number of discontinuities at tricellular corners was significantly increased for all three flow cases, including the two SPA cases, compared to SC. In addition, the average number of breaks was significantly higher than both holes and tricellular corners for SC.

Subjecting ECs to 5 and 12 h of sinusoidal flow conditions did not significantly alter the angle of cell alignment relative to the flow direction between the SPA = -180° and SPA = 0° cases (Figure 6A). The same 5- and 12-h exposure did not significantly alter the aspect ratio (Figure 6B) and the circularity factor (Figure 6C) between the SPA = -180° and SPA = 0° cases. These values were relatively consistent for all experimental conditions at 5 and 12 h, indicating few shape changes at these time point.

Apoptosis and Mitosis

In Figure 7A, we observe that 5-h apoptosis percentages were affected by the various mechanical conditions. In particular, the 5-h apoptosis values for the atherogenic SPA = -180° case were significantly higher than for the SPA = 0° case. In addition, the SPA = 0° apoptosis value was close to that of the SS case, and both values were significantly lower than SC and PC. Furthermore, the SPA = -180° apoptosis value was significantly higher than the SS apoptosis value, and the SS apoptosis percentage was significantly lower than either SC or PC apoptosis percentages. These apoptosis percentages at 5 h were an order of magnitude lower than those found *in vivo*,³² although control values for 12-h apoptosis (0.4%) agreed with *in vivo* literature. A possible explanation for this finding may be that during apoptosis, there was some shedding of apoptotic ECs either during the experiment or during the later immunostaining processing as apoptotic ECs would be less adherent to the tube surface. These difficulties with cell adhesion on tubes limited the completion of the 12-h apoptosis studies, indicating the general difficulty in extending the duration of these experiments in the current system. However, the statistically significant increase in apoptosis for SPA = -180° relative to SPA = 0° agrees with our hypothesis that the SPA = -180° case is pro-atherogenic compared to the SPA = 0° case because EC monolayers would be more permeable due to a higher incidence of leaky junctions associated with apoptotic cells.

As shown in Figure 7B, after 5 and 12 h there were changes in the mitosis percentages although mitosis for the atherogenic SPA = -180° case was statistically unchanged compared to the atheroprotective SPA = 0° case. While mitosis for SPA = 0° and SS were at the same level and were both significantly lower than PC at 5 h, at 12 h the SPA cases were similar to SC and PC. All of the mitosis percentages at 5 h were of the same order of magnitude as those found *in vivo*.^{7, 33} Furthermore, there were no statistically significant differences between the 5- and 12-h time points for each experimental case.

Discussion

EC tight junctions and adherens junctions play important roles in the regulation of paracellular permeability. Through Western blot analysis for occludin, ZO-1, and VE-cadherin, the relative protein expressions at 1, 5, and 12 h were compared for the atherogenic SPA = -180° case and the atheroprotective SPA = 0° case (Figure 2). Immunocytochemistry for ZO-1 and VE-cadherin allowed for the visualization of the local distribution of these proteins in ECs (Figure 3 and Figure 4). Occludin was not included in this immunocytochemistry analysis and 1-h Western blot analysis because the available antibody stained BAECs poorly after the antibody changed and lost reactivity in BAECs. While there were limited statistically significant

differences observed for the various protein expression and localization studies at different time points, ZO-1 protein expression at 5 h was significantly higher for the SPA = 0° case relative to the SPA = -180° case. These results support the hypothesis that SPA = -180° has a more atherogenic profile than SPA = 0° because the decreased expression of tight junction protein would be expected to yield a more permeable state. Unfortunately, it was not possible to run the experiments for longer than 12 h due to technical difficulties involving cell adhesion with the current system.

Small molecules pass through TJs, while water and larger molecules pass through the breaks in TJs that are estimated to be 20 nm in width.⁵ When subjected to physiologic steady WSS, previous studies showed that TJ protein, gene expression, and permeability varied in diverse trends depending on the cell type and time of exposure. Using BAECs, less than 5 h of physiologic steady WSS increased water,^{6, 41, 47} dextran,⁴² and albumin²⁵ permeability with varying effects on TJ protein expression – quickly increasing occludin phosphorylation, decreasing occludin content, and not changing ZO-1 content¹⁸ or transiently increasing occludin phosphorylation and not changing occludin content.⁴¹ It should be noted that these WSS studies were performed on flat permeable filter membranes and not impermeable silicone tubes that eliminated physiologic transmural flow through the monolayer as in the present study. Even without WSS but with chemical treatments, permeability to dextran increased when occludin was removed from TJs and ZO-1 was partially redistributed,³⁶ and water transport was reduced as occludin content increased and its phosphorylation decreased.¹ Thus, the statistically significant reduction of ZO-1 protein expression at 5 h for SPA = -180° compared to SPA = 0° (Figure 2A) and relatively unchanged occludin expression (Figure 3A) may be characteristic of increased paracellular permeability through the TJ. However, the loss of statistical significance at 12 h as well as the relative lack of statistically significant changes for the immunocytochemistry image features (Figure 5 and Figure 6), make it difficult to draw further conclusions.

Disruptions in adherens junctions may allow for the passage of larger molecules the size of LDL (22 nm), and VE-cadherin is the key protein in AJ maintenance.¹⁷ Physiologic steady WSS applied for up to 12 h reduced⁴⁰ or did not change^{37, 53} VE-cadherin protein expression, and its border localization^{37, 40} became punctate, discontinuous, and sparse. Our results for SPA = 0° and -180° also showed little change in VE-cadherin protein expression up to 12 h (Figure 2C), as well as more discontinuous border localization (Figure 4), and an increased number of holes between cells and at tricellular corners for both SPA cases (Figure 5B). The increased presence of holes in the VE-cadherin immunocytochemistry images, would suggest higher paracellular permeability.⁵⁴

EC junctions remodel in response to mechanical forces and this remodeling may play a role in the regulation of paracellular permeability. Through immunocytochemistry image analysis for ZO-1 and VE-cadherin, the angle of cell alignment, the aspect ratio, and the circularity or shape index were calculated at 5 and 12 h and compared for the atherogenic SPA = -180° case and the atheroprotective SPA = 0° case, but there were no statistically significant differences in any of these characteristics observed for these two SPA cases (Figure 6).

From parallel plate WSS studies by many others,^{2, 30} it is clear that 24 h of physiologic steady WSS significantly increases EC alignment and elongation while 5 h does not significantly induce these changes. It was also found that at least 18 h of 15 dyn/cm² WSS in the parallel plate device was needed to observe these morphological changes with the same cell type². Furthermore, ECs are known to orient perpendicular to the stretch direction¹⁶ and in the flow direction in a SPA $\cong$ 0° state run for 24 h.³⁸ Therefore, with our combined CS and WSS cases, possible reasons for the lack of change in alignment and elongation are that the experiments were not run long enough, the mean WSS or CS were not high enough, and the combination

of WSS and CS effects may not be additive when $SPA \neq 0^\circ$. However, the fact that there were no significant morphological changes between the two SPA cases even though there were differences in protein expression and gene expression^{12, 13} is consistent with the *in vivo* result that both the atherogenic coronary arteries and the normal straight aorta had similar cell alignment and aspect ratio in the flow direction even though there were significant differences in gene expression for these two regions.¹⁵

Another pathway that regulates paracellular permeability is through the presence of leaky junctions, which are influenced by the cell turnover processes of mitosis and apoptosis. Immunocytochemistry for ECs after 5 and 12 h of exposure to $SPA = -180^\circ$ and $SPA = 0^\circ$ allowed for the calculation of apoptosis and mitosis percentages (Figure 7). While there were limited statistically significant differences observed, the fact that apoptosis significantly increased for $SPA = -180^\circ$ relative to $SPA = 0^\circ$ supports the hypothesis that $SPA = -180^\circ$ is more atherogenic than $SPA = 0^\circ$.

Small molecules pass through tight junctions, and water and larger molecules, below the size of LDL, pass through the normal breaks in TJs.⁵ However, minor disruptions in adherens junctions may allow for the passage of larger molecules the size of LDL. A more likely pathway for LDL transport would be the leaky junctions,^{4, 5, 52, 55} which not only cause disruptions in AJs, as in the case of apoptosis,²³ but which provide for even larger pathways in the case of mitosis.^{5, 55} These characteristics help explain why the atherogenic $SPA = -180^\circ$ case would be characterized by a more permeable state because of the increase in cell turnover and the differences in EC junctional protein expression and localization.

Taken together, the decrease in ZO-1 relative junction protein expression and the increase in apoptosis at 5 h for the atherogenic, out-of-phase $SPA = -180^\circ$ case relative to the atheroprotective, in-phase $SPA = 0^\circ$ case, suggest a higher paracellular permeability as a result of decreased junctional stability. These results should be interpreted with caution until longer experiments can be completed since the current system has limitations due to reduced cell adhesion on tubes at long duration. Furthermore, these results may be clarified by increasing the time of SPA exposure and/or the magnitudes of the mean and oscillatory components of WSS and CS at these same SPAs.

Acknowledgments

This work was supported by National Institutes of Health National Heart, Lung, and Blood Institute Grants RO1-HL35549 and RO1-HL086543. Thanks to Michael Dancu for help with the dynamic simulator and the supply of tubes.

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A: hemodynamic forces

B: stress phase angle

Figure 1. Simultaneous WSS and CS on ECs is characterized by SPA

A: Arterial blood vessel walls are composed of different layers, with focus here on the intima, the innermost single layer of ECs that is directly exposed to blood flow. The endothelium is very sensitive to the hemodynamic forces to which it is subjected. Wall shear stress (WSS) is the frictional stress that acts tangential to the apical surface of the ECs in response to blood flow. Pressure (P) is the normal stress that acts perpendicular to the ECs and is balanced by circumferential stress (hoop stress) which induces circumferential strain (CS) that is synchronous with diameter variation.

B: The stress phase angle (SPA) characterizes the temporal phase difference between the oscillatory WSS and CS waveforms, with negative SPA indicating WSS leading CS.

A: ZO-1 **B: occludin** **C: VE-cadherin** **Figure 2. Relative protein expression for ZO-1, occludin, and VE-cadherin**

A: ZO-1 relative protein content for 1, 5, and 12 h via Western blotting. All data were normalized to SC (static control) and presented as mean $\pm$ standard error of the mean. + $p < 0.05$ compared to 1 h; * $p < 0.05$ between SPA cases (0° and -180°); ** $p < 0.05$ compared to SC; *** $p < 0.05$ compared to SS (steady shear stress); **** $p < 0.05$ compared to PC (pressurized control); n: 3–12.

B: Occludin relative protein content for 5 and 12 h via Western blotting. All data were normalized to SC and presented as mean $\pm$ standard error of the mean. ++ $p < 0.05$ compared to 5 h; n: 8–14.

C: VE-cadherin relative protein content for 1, 5, and 12 h via Western blotting. All data were normalized to SC and presented as mean $\pm$ standard error of the mean. + $p < 0.05$ compared to 1 h; ** $p < 0.05$ compared to SC; **** $p < 0.05$ compared to PC; n: 3–15.

A: 5-hour ZO-1 **B: 12-hour ZO-1** **Figure 3. Immunocytochemistry images for ZO-1**

Labeled arrows indicated breaks in immunostaining between cells (B), holes between cells (H), and small holes at tricellular corners (T).

A: Sample images for ZO-1 protein localization at 5 h via immunocytochemistry. For SPA = 0°, SPA = -180°, and SS (steady shear stress), the flow was in the direction from the bottom to the top of the page. Stretch was in the perpendicular direction for the two SPA cases. 40x images; SC is static control, PC is pressurized control.

B: Sample images for ZO-1 protein localization at 12 h via immunocytochemistry. For SS, the flow was in the direction from the bottom to the top of the page. The 12-h SPA cases were not shown due to experiment complications. 40x images.

A: 5-hour VE-cadherin **B: 12-hour VE-cadherin** **Figure 4. Immunocytochemistry images for VE-cadherin**

Labeled arrows indicated breaks in immunostaining between cells (B), holes between cells (H), and small holes at tricellular corners (T).

A: Sample images for VE-cadherin protein localization at 5 h via immunocytochemistry. For SPA = 0°, SPA = -180°, and SS (steady shear stress), the flow was in the direction from the bottom to the top of the page. Stretch was in the perpendicular direction for the two SPA cases. 40x images; SC is static control, PC is pressurized control.

B: Sample images for VE-cadherin protein localization at 12 h via immunocytochemistry. For SS, the flow was in the direction from the bottom to the top of the page. The 12-h SPA cases were not shown due to experiment complications. 40x images.

A: 5-hour ZO-1

B: 5-hour VE-cadherin

Figure 5. 5-h image analysis for ZO-1 and VE-cadherin

A: Average number (over all the images taken for each sample) of breaks in ZO-1 staining between cells, holes between cells, and small holes at tricellular corners grouped for each experimental condition. All data were presented as mean $\pm$ standard error of the mean. B represents $p < 0.05$ compared to average number of breaks in junctions; $n = 3$; SPA cases are 0° and -180°, SS is steady shear stress, SC is static control, PC is pressurized control.

B: Average number (over all the images taken for each sample) of breaks in VE-cadherin staining between cells, holes between cells, and small holes at tricellular corners grouped for each experimental condition. All data were presented as mean $\pm$ standard error of the mean.

** $p < 0.05$ compared to SC; **** $p < 0.05$ compared to PC; B represents $p < 0.05$ compared to average number of breaks in junctions; n: 3–4.

A: cell alignment (angle) **B: cell elongation (aspect ratio)** **C: cell elongation (circularity factor)** **Figure 6. 5- and 12-h cell characterization**

A: Angle of cell alignment relative to the direction of flow at 5 and 12 h showed no statistically significant differences for SPA = -180° compared to SPA = 0° via ZO-1 immunocytochemistry imaging analysis. Note that an angle of 0 indicates perfect alignment in the flow direction. All data were presented as mean $\pm$ standard error of the mean. **** $p < 0.05$ compared to PC (pressurized control); n: 4–9; SS is steady shear stress, SC is static control.

B: Aspect ratio at 5 and 12 h showed no statistically significant differences for SPA = -180° compared to SPA = 0° via ZO-1 immunocytochemistry imaging analysis. All data were presented as mean $\pm$ standard error of the mean. $p > 0.05$ for all cases; n: 4–9.

C: Circularity factor at 5 and 12 h showed no statistically significant differences for SPA = -180° compared to SPA = 0° via ZO-1 immunocytochemistry imaging analysis. Note that a circularity of 0 indicated an elongated polygon and 1 indicated a circle. All data were presented as mean $\pm$ standard error of the mean. $p > 0.05$ for all cases; n: 4–9.

A: apoptosis

B: mitosis

Figure 7. SPA effects on apoptosis and mitosis

A: 5-h exposure to experimental conditions significantly increased apoptosis for SPA = -180° compared to SPA = 0° via immunocytochemistry. All data were presented as mean $\pm$ standard error of the mean. * $p < 0.05$ between SPA cases; ** $p < 0.05$ compared to SC (static control); *** $p < 0.05$ compared to SS (steady shear stress); **** $p < 0.05$ compared to PC (pressurized control); n: 5–6.

B: 5- and 12-h exposure to experimental conditions did not significantly alter mitosis for each experimental case relative to time. All data were presented as mean $\pm$ standard error of the mean. **** $p < 0.05$ compared to PC; n: 4–12.