Mechanotransducuction in Endothelial and Inflammatory Cells

Steady and Unsteady Fluid Shear Control of Atherosclerosis

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I. Introduction

Atherosclerosis remains a leading cause of morbidity and mortality in the Western world (115, 126). It is a chronic systemic disease attributed to many well-identified risk factors (i.e. diabetes mellitus, hyperlipidemia, hypercholesteremia, hypertension, and cigarette smoking). Yet the formation of atherosclerotic lesions do not occur in a random fashion. The coronary arteries, the major branches of the aortic arch, and the abdominal aorta are particularly susceptible sites. Given the focal nature of plaque formation within these regions, it has long been suggested that certain characteristics of fluid shear stress unique to these regions may potentiate the early stages of atherogenesis independent of other risk factors. Detailed analyses of fluid mechanics in atherosclerosis-susceptible regions of the vasculature reveal a strong correlation between endothelial cell dysfunction and areas of low mean shear stress and oscillatory flow with flow recirculation. Conversely, steady shear stress stimulates cellular responses that are essential for endothelial cell function and are atheroprotective.

This chapter will provide a basic overview of fluid mechanics and the hemodynamic forces experienced by the vascular endothelium. We will review the fundamental differences between steady and unsteady fluid shear, and the controversial pro-atherosclerotic effect purported between temporal and spatial gradients of shear stress will also be specifically addressed. Finally, several of the major pro-atherosclerotic effects of unsteady flow on the endothelium, and the atheroprotective role of steady flow will be reviewed.

II. Hemodynamic forces and the endothelium

2.1 Localization of plaque development

Hemodynamic forces interacting with an active vascular endothelium have long been implicated in the nonrandom localization of atherosclerotic lesions. DeBakey *and al.* (29) have described four predominant regions of arterial plaque distribution: 1) the coronary arterial bed; 2) the major branches of the aortic arch; 3) the visceral arterial branches of the abdominal aorta; 4) the terminal abdominal aorta and its major branches. (Figure 1) The left coronary artery bifurcation into the left anterior descending and circumflex branches has a particular predilection for plaque formation (44). Lesions are distributed mainly along the outer walls of the bifurcation whereas the walls of the flow-divider and the inner walls further downstream are less affected (116). Detailed analyses of fluid mechanics in these atherosclerosis-susceptible regions of the vasculature have identified unique patterns of disturbed flow. These sites are characterized by regions of flow separation, recirculation, reattachment, and perhaps most importantly, significant temporal and spatial gradients of shear stress (52, 72).

The relationship of plaque localization to wall shear stress has received the greatest attention. Figure 2 is a diagrammatic representation of the flow fields around the carotid bifurcation

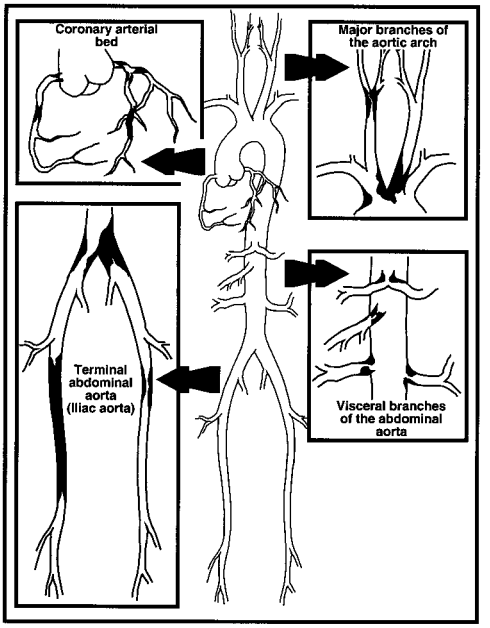

Figure 1: Predominant sites for the localization of atherosclerotic lesions. Reproduced with permission from DeBakey *et al.* 1985.

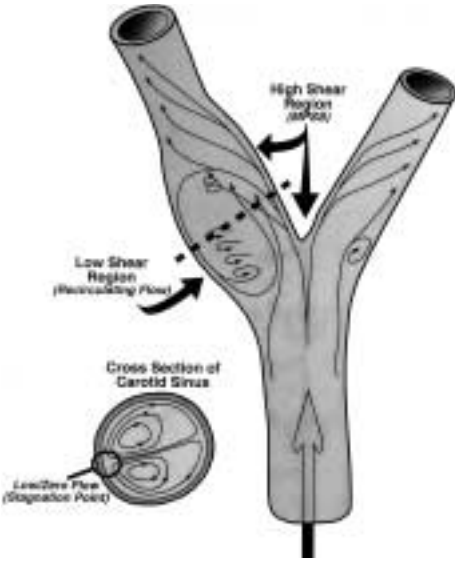

Figure 2: Diagrammatic representation of flow features at the carotid bifurcation. Reproduced with permission from Ku *et al.* 1985

(Figure 2.). In a strong correlation with plaque localization, a region of flow separation, recirculation, and reattachment is generated along the outer lateral wall of the carotid bifurcation. This site is also the region of lowest mean wall shear stress, and a site where significant temporal and spatial gradients are also generated (72, 129). In the region preceding the carotid bifurcation, and the region along the inner wall of the flow-divider side, flow patterns remain linear and laminar. Flow at these sites is primarily unidirectional, which means wall shear stress is high. The occurrence of plaque formation within these regions is correspondingly very low.

2.2 Physical hemodynamic force exerted on vessel wall

Mechanical forces are important modulators of endothelial cells. The endothelium responds rapidly and sensitively to the mechanical conditions created by blood flow (42, 109). As blood flows through a vessel, it exerts a physical force on the vessel wall. This force generates stress which can be resolved into two principal vectors. 1) The stress parallel to the vessel wall is defined as shear stress. This represents the frictional force that blood flow exerts on the endothelial surface of the vessel wall. 2) The stress perpendicular to the vessel wall is defined as tensile stress. This represents the dilating force of blood pressure on the vessel wall (Figure 3).

Whereas the entire blood vessel is exposed to the tensile stress of blood pressure, endothelial cells on the inner surface of the vessel wall are exposed to the largest frictional force of shear stress. There is also transmural flow within the interstitial space between the endothelium and the smooth muscle cells of the arterial wall. The magnitude of wall shear stress produced by transmural flow on smooth muscle cells has been estimated to be on the order of 1 dyne/cm^2, which is the range known to affect endothelial cells *in vitro* (123). Because of frictional resistance at the blood-endothelial interface, flow velocity is greater in midstream than at the lumen

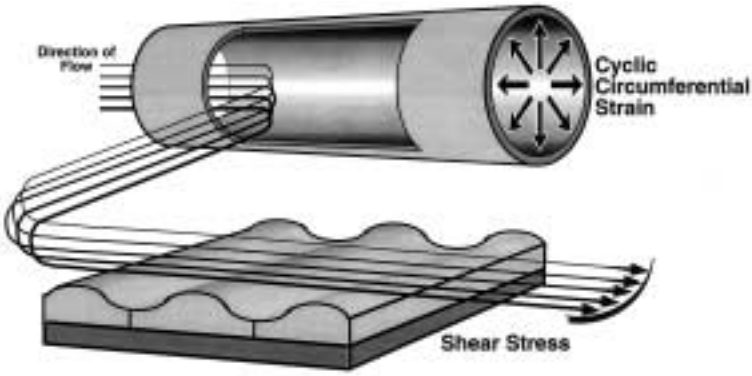

Figure 3: Diagrammatic representation of the two principal force vectors acting on the blood vessel wall. Wall shear stress: the tangential drag force of blood passing along the lumenal surface of the vessel (left). Pressure: the outward distribution of circumferential strain produced by pulsatile blood flow (right).

surface, and thus a gradient of velocities exists from the center of the vessel extending outward to the vessel wall. The magnitude of wall shear stress depends on how fast the fluid velocity increases when moving from the vessel wall towards the center of the vessel. The velocity gradient near the wall is the wall shear rate. Wall shear stress is measured close to the vessel wall, and for a cylindrical tube is calculated as:

$$\text{wall shear stress} = \frac{4\mu Q}{\pi r^3}$$

where μ is the fluid viscosity (poise), Q is the volume flow (cm^3/s), and r is the distance perpendicular to and away from the wall (cm). Wall shear stress is expressed as dynes/cm^2, and can be summarized as the change in blood velocity unit per change in the radial distance unit of the vessel wall.

Arterial blood flow *in vivo* is pulsatile. When pulsatile fluid displacement follows predictable paths, it is said to be laminar. Given the pulsatile nature of the cardiac cycle, the absolute shear stress varies throughout the cardiac cycle. In regions where stable flow is unidirectional, the time-averaged fluctuations in shear stress are positive. Mean positive shear stress greater than 6 dyne/cm^2 in magnitude (MPSS) predominates throughout much of the major arterial vasculature. Therefore, *in vivo* MPSS flow patterns are comprised of distinct superpositioned steady and temporal components. Frangos *and al.* have demonstrated that endothelial cells can discriminate between the superposition components of flow, and respond differently via distinct mechano-chemical transduction pathways (43).

The *in vivo* definition of MPSS should be distinguished from the steady shear stress flow patterns that are often used in *in vitro* experimental preparations. *In vitro* steady shear stress flow patterns are typically generated using a continues flow loop or a syringe pump programmed to deliver a specific and constant flow rate across a cultured endothelial monolayer in a geometrically uniform flow chamber. *In vitro* steady shear stress flow patterns produced in this manner generate a steady positive shear stress, and once flow is fully established, it is a temporally and spatially uniform shear stress. However *in vitro* preparations may unintentionally generate a significant temporal component with the sudden onset of flow. Furthermore, the interpretation of many *in vitro* studies may be complicated by undefined flow profiles, or lack of adherence to a uniform nomenclature to describe experimental flow profiles. Apparent conflicting findings reported between studies may often be reconciled when the details of the various flow regimes are closely scrutinized.

Departures from unidirectional flow occur mainly around branch points and distal to stenoses. In such locations, predictable secondary flow patterns of separation, reattachment and

recirculation tend to form (recirculating flow). At the point of flow reattachment, shear stress is zero (stagnation point). During the down-stroke of the systole phase in the cardiac cycle, a reversal of flow occurs, which alters the size and spatial migration of the secondary flow patterns (Figure 4) (52). Within regions of recirculating flow, significant temporal and spatial gradients of shear stress are also generated. Spatial shear stress gradients are defined as the difference of shear stress between two neighbouring cells, at the same point in time. Temporal shear stress gradients are defined as the increase or decrease of shear stress at the same location on a cell over a very short period of time (< 0.3sec). Spatial gradients are generated primarily within recirculation zones, and at bifurcation flow dividers. Given the pulsatile nature of blood flow *in vivo*, temporal gradients are generated throughout the vasculature to some degree, but they are significantly magnified within recirculation zones. Within recirculation zones, both maximal spatial and temporal gradients overlap each other (Figure 5) (60, 125).

The complex flow profiles within recirculation zones should not be confused with turbulent flow. Turbulence implies random movement of elements in the flow field. Extreme or abrupt changes in geometry distal to severe stenoses, around projecting edges, or about other obstacles in the flow stream may cause focal turbulence in the bulk of the vessel. A laminar layer of fluid exists on the boundary of flow along the wall, making it less likely that any turbulence in the blood will come in direct contact with endothelial cells on the vessel wall. Not surprisingly, arterial regions immediately distal to severe stenoses, where turbulence can occur, are not prone to plaque localization (19, 69).

2.3. Steady and unsteady fluid shear

a. Mean positive fluid shear stress

The nature and magnitude of shear stress at a given location within the vasculature plays an important role in the long-term health of the blood vessel. Blood flow patterns throughout the vasculature are not uniform. The hemodynamic forces exerted upon a vascular endothelial cell at any point within the vasculature is a direct function of the vessel geometry in that region. Within non-obstructed linear regions of the vasculature, or along the inner flow-divider wall of a bifurcation, blood flows in ordered laminar patterns. If the time-averaged fluctua-

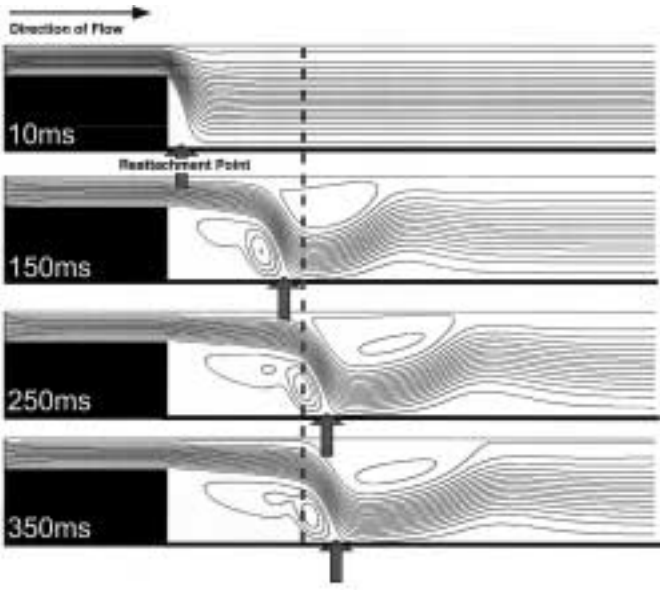

Figure 4: The spatial migration of the recirculation zone during the sudden onset of flow. Flow lines are given for fluid flow through a sudden expansion flow chamber. The recirculation eddy grows, and the reattachment point (arrow) moves downstream. Cells at any given location beneath the developing eddy (dashed line) experince strong changes of shear stress (i.e. temporal gradients) as the recirculation zone passes over them.

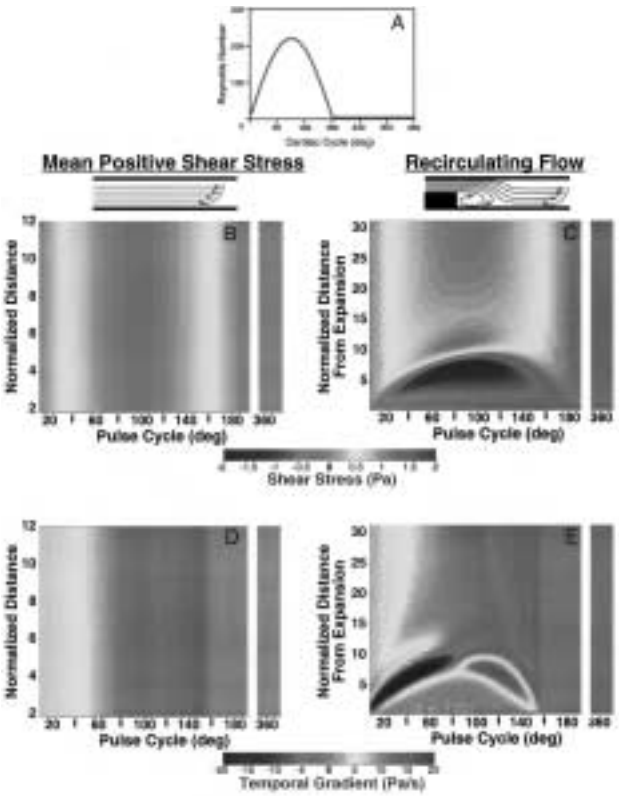

Figure 5: Spatial and temporal changes of wall shear stress throughout a simplified cardiac cycle in regions of mean positive shear stress and recirculating flow. A simplified model of the cardiac cycle (A) was used to model shear stress (B, C) and temporal gradients (D, E) along the bottom plate of two different *in vitro* flow chambers. A parallel plate flow chamber (PPFC) was used to model mean positive shear stress (left panels). A sudden expansion flow chamber (SEFC) was used to model recirculation flow (right panels). Except for the 1:2 sudden expansion in the flow path of the SEFC, the geometry was the same in both chambers. Flow was identical in both chambers. Red colors indicate a positive shear stress, while a blue tint represents negative shear stress. In the SEFC, the migration of the recirculation zone throughout the cardiac cycle (E) significantly magnifies the peak temporal gradient compared to the temporal gradient generated in the PPFC (D). Within recirculation zones, both maximal spatial and temporal gradients overlap each other (C, E). *Note: 1Pa = 10 dyne/cm*^{2}.

tions in shear stress are measured within these regions, the mean average shear stress is positive (Figure 6A.).

Although some earlier studies indicated that acute levels of high shear stress may lead to endothelial surface degeneration and erosion (45, 78), most lines of evidence are consistent with the view that a chronic exposure of endothelial cells to high levels of shear stress with little temporal fluctuations promotes an atheroprotective phenotype (reviewed in 27 and 118). MPSS promotes release of atheroprotective factors from endothelial cells that inhibit coagulation, migration of leukocytes, and smooth muscle proliferation. Most importantly, MPSS may be critical for endothelial cell survival. A number of investigators have demonstrated that MPSS is required for optimal regeneration of an injured endothelium (2, 80, 122). While MPSS may be necessary for endothelial cell integrity, MPSS also seems to inhibit proliferation. Endothelial turnover in regions of MPSS, or when cultured under flow, is extremely low (80, 127). Not surprisingly, endothelial cell geometry and surface topography is also influenced by the magnitude and localization of hemodynamic forces acting at the endothelial surface. Endothelial cells located within regions of positive shear stress, are aligned with their longitudinal axis parallel to the direction of blood flow (35, 41). This orientation streamlines the endothelial cell, and effectively decreases drag resistance (10, 11). Thus, it appears MPSS acts as an endothelial cell survival factor rather than a *growth* factor (118).

b. Unsteady fluid shear

The hemodynamic forces exerted upon the endothelium are much more complex where flow dynamics are altered by a change in shape, or a curvature of the vessel. The unsteady shear stress

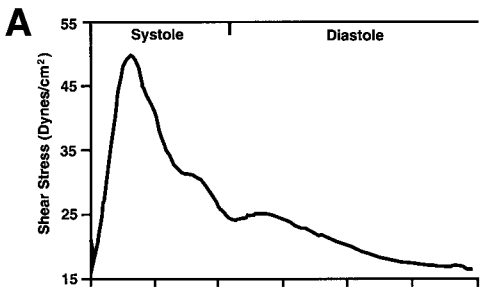

Figure 6A: Changes in wall shear stress throughout the course of the cardiac cycle at two locations within the carotid bifurcation. (A) Shear stress along the inner flow-divider wall of the bifurcation. Reproduced with permission from Ku *et al.* 1985.

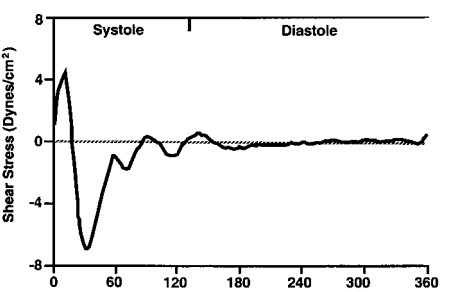

Figure 6B: Changes in wall shear stress throughout the course of the cardiac cycle at two locations within the carotid bifurcation. (B) Shear stress within the zone of recirculating flow along the outer lateral wall of the bifurcation. Reproduced with permission from Ku *et al.* 1985.

profiles generated in these regions significantly differ from shear stress profiles generated within regions of MPSS. Within regions of recirculating flow, such as the outer lateral wall of the carotid bifurcation, the time-averaged fluctuations in shear stress measured throughout the cardiac cycle are low approaching zero (Figure 6B.). Variations in shear stress are greatest during the systole phase of the cardiac cycle, and result from the spatial migration of the recirculation zone.

The morphology of endothelial cells within regions of recirculating flow is also significantly different from cells located within regions of MPSS (35, 41). Cells in these low mean shear stress regions are not aligned, and are characterized by a rounded shape, an increased proliferation rate, and increased permeability. (77, 79, 102, 103). The lack of streamlining in the macroscopic topography of the luminal endothelial surface in rounded and non-aligned cells may expose the cells to high spatial shear stress gradients (27). Increased endothelial turnover in regions of recirculating flow has long been implicated in the process of atherogenesis (23, 127). A number of studies have demonstrated enhanced macromolecular permeability of aortic endothelial cells during mitosis (22, 82). The low shear stress surrounding the stagnation point of the flow reattachment site may allow prolonged residence times for circulating pro-inflammatory cells to adhere to the endothelial monolayer of the vessel (52). The vascular endothelium serves as a dynamic interface between circulating blood elements and the interstitial tissues, disruption of its permeability characteristics may permit the localized influx of circulating low-density lipoproteins and other pro-inflammatory macromolecules into the artery wall (66).

The strong correlation of localized plaque formation with regions of recirculating flow has lead to the general hypothesis that low mean shear stress and oscillatory flow with flow reversal stimulate a cascade of cellular events that leads to endothelial cell dysfunction and atherosclerotic plaque formation (51, 72, 129). Conversely, high levels of MPSS have been hypothesized to stimulate cellular responses that are essential for endothelial cell function and are atheroprotective. Although most *in vitro* studies strongly support this hypothesis, other studies have demonstrated that endothelial cells are sensitive not only to the absolute magnitude of shear stress, but also to gradients in shear stress generated within regions of recirculating flow (10, 32, 60, 119, 125). The *in vivo* role of temporal and spatial gradients of shear stress play in the pathogenesis of atherosclerosis remains unclear. Some studies link atherogenesis to the large temporal gradients in shear due to the change of shear direction (72, 94, 100). Other studies relate this to different spatial distributions of mean wall shear stress (63, 71).

A number of *in vitro* studies have specifically investigated the pro-atherosclerotic effect of

spatial gradients in shear stress on endothelial cells. DePaola *and al.* and Truskey *and al.* have developed two similar *in vitro* models that simulate *in vivo* spatial patterns of flow separation, recirculation and reattachment (32, 119). By creating a sudden asymmetric expansion in the flow path of perfusing media, these models generate a large spatial gradient in shear stress over a relatively small region of a cultured endothelial monolayer. This high gradient is caused by flow separation: Near to the expansion step, flow recirculates in an eddy, while further downstream, the flow reforms to the regular parabolic profile. In between, there is a point of flow reattachment where shear stress is zero (stagnation point). When the flow of perfusing media is held constant, a stable recirculating flow pattern is generated over the same spatial region of a cultured monolayer. Utilizing these *in vitro* models of recirculating flow, several studies have demonstrated that sustained exposure (≥24hrs) to a highly localized and stable spatial shear stress gradient induces a pro-atherosclerotic endothelial cell proliferation-migration-loss cycle at the point of maximal spatial gradient (the reattachment point)(23, 32, 117, 119). Furthermore, the spatial regulation of gap junction connexin43 has also been observed with the same fluid shear stress model (33).

While it is true that these model systems generate large spatial gradients when flow is fully established, recirculating flow undergoes a distinct developmental phase of several hundred milliseconds (38, 60). During the developmental phase, the recirculation zone migrates forward in the direction of flow until flow is fully established. If flow is pulsatile, the recirculation zone repeatedly migrates back and forth across the same spatial region of the monolayer (Figure 4). As such, large temporal gradients are also produced over the same spatial region of the recirculation zone if the onset of flow is sudden or pulsatile. White and Haidekker have developed a technique to eliminate the temporal component during the onset of flow in this model (125, 60). A negligible temporal gradient can be achieved in this model if the onset of flow is slowly ramped up over time (>30sec), rather than a sudden onset of flow. Both onset flow profiles generate the same spatial gradient in shear stress when flow is fully developed. Endothelial monolayers exposed to spatial and temporal gradients (4hrs) were compared to those exposed only to spatial gradients. A pro-atherosclerotic mitogenic response was observed at the reattachment point (which is also the point of maximal temporal gradient) only when the endothelial monolayer was exposed to a temporal gradient at the onset of flow. Spatial gradients in shear stress devoid of the temporal component were found to affect endothelial proliferation no differently than steady uniform shear stress.

Given the highly transient nature of the temporal gradient, and that both maximal temporal and spatial gradients overlap each other, these studies suggest that the induction of atherogenic phenotypes in the sudden asymmetric expansion model seen in previous studies (23, 32, 117, 119), may be due to temporal, rather than spatial gradients of shear stress. However, because the dynamics of flow initiation were not expressly specified in the previous studies (23, 32, 117, 119), and longer exposures to recirculating flow and different asymmetric expansion geometries were used, it is difficult to make direct comparisons. Notwithstanding, temporal gradients in the absence of spatial gradients have also been shown to induce pro-atherogenic phenotypes (7, 8, 9, 18), and as such, either type of gradient may play a role in the pathogenesis of atherosclerosis.

III. The atheroprotective role of steady fluid shear

Mean positive shear stress predominates throughout much of the vasculature. Within regions of MPSS, endothelial cells are exposed to shear stresses on the order of 10 to 30 dyne/cm^2 (27). By

sensing and integrating hemodynamic stimuli, the endothelium in these regions plays a critical role in the maintenance of circulatory and blood vessel integrity, and vascular homeostasis. Although the biophysical mechanism by which endothelial cells sense hemodynamic forces, and transduced them into biochemical signals is still unclear, the cellular response to these forces is better understood. Shear-induced endothelial cell modulation of the biological processes related to vascular homeostasis include: Regulation of plasma lipoproteins uptake and metabolism, adhesion and transmigration of leukocytes into the vessel wall, and the release of prothrombotic / antithrombotic factors, smooth muscle growth factors, inhibition of endothelial proliferation, and the release of vasoactive substances (118). Three of the most important atheroprotective roles of MPSS will be reviewed.

3.1 Nitric oxide (NO)

In 1980, Furchgott and Zawadzki (47) first demonstrated relaxation of vascular smooth muscle to ACH was dependent on the integrity of the endothelium. The endothelium derived relaxing factor was eventually identified as the free radical gas nitric oxide (NO). In the endothelium, the amino acid L-arginine is converted to L-citrulline and NO by the endothelial isoform of NO synthase (eNOS). Since the time flow-induced NO release was first reported (109), NO has emerged as the key mediator of the atheroprotective effects of MPSS. NO is a pluripotent molecule. In addition to its role as a vasodilator, NO has been reported to play a role in nearly every major flow-induced atheroprotective mechanisms: The inhibition of platelet aggregation and leukocyte binding to the endothelium, the inhibition of vascular smooth muscle tone and mitogenesis, and the alteration of lipoprotein metabolism (4).

A major atheroprotective role of flow-induced NO release is the inhibition of leukocyte adhesion through inhibiting the expression of adhesive molecules (ICAM-1, VCAM-1 and MCP-1). The NO-dependent downregulation of VCAM-1 expression is mediated via a redox-sensitive pathway (30, 70). The inhibition of endothelial-derived NO promotes early monocyte infiltration of the arterial wall (84). So important is the NO inhibition of monocyte infiltration, a second NOS isoform can also act to inhibit infiltration. Any condition leading to a decrease in flow-induced NO release, the inflammatory-elicited expression of inducible-NOS may lead to a compensatory production of NO. The pivotal role of endothelial NO in protecting against atherosclerotic lesion development is further emphasized in the vascular response to injury. Defects in endothelium-dependent vasodilation are positively linked to fatty streak infiltration. eNOS knockout mice develop typical atherosclerotic lesions in response to adventitial vessel wall injury whereas wild-type mice do not (95).

Since endothelium-derived NO was first identified and characterized, shear stress has been established as the most potent regulatory factor of eNOS activity (13, 75) and gene expression (97, 98). Conversely, low levels of shear stress and turbulent flow fail to stimulate NO release or gene expression (98). Although the precise flow-induced mechanisms activating the enzyme remain to be elucidated, eNOS activity is regulated by both Ca^{2+}/calmodulin-dependent and independent mechanisms. eNOS activity is dependent on the binding of a Ca^{2+}/calmodulin cofactor. The activated eNOS-calmodulin complex synthesizes NO until the intracellular free Ca^{2+} concentration decreases to a point where the calmodulin dissociates and an inhibitory eNOS-caveolin-1 complex forms. Caveolin-1 is a transmembrane protein that is located in small invaginations in the endothelial plasma membrane. The direct interaction of eNOS with caveolin-1 leads to an inhibitory effect under static or low flow conditions (90). The caveolin-1 inhibition of eNOS activity is completely reversible with additional influxes of free Ca^{2+}. *In vitro*

studies have shown a biphasic production of NO in response to flow. The sudden onset of flow induces a burst of NO production. This process is both calcium- and G-protein-dependent. In contrast, the steady shear stress that follows induces a sustained release of NO, at a rate of 10% of the initial peak of NO production, and is both calcium– and G-protein-independent (75). Moreover, the initial and transient production of NO is directly related to the rate of change in shear stress rather than to its absolute magnitude, whereas the sustained release of NO is directly related to the level of MPSS (75). Hence, shear-induced NO production does not seem to be a single response modulated over time, but rather a superposition of two independent mechanochemical pathways (43). Indeed, although the precise flow-induced mechanisms activating the enzyme remain to be elucidated, it is now widely accepted that both a Ca^{2+}/calmodulin-dependent and -independent signalling cascades regulate eNOS activity.

The classic Ca^{2+}-dependent mechanism involves the binding of a cofactor, the calmodulin-Ca^{2+} complex, to eNOS. In the absence of calcium, the calmodulin does not bind the enzyme, hence inhibiting NO synthesis. The existence of the Ca^{2+}-independent signaling cascade, which regulates eNOS activity in response to shear stress, was first reported in our laboratory (75). It was subsequently confirmed in endothelial rings and porcine endothelial cells (5). More recently, the stimulation of the phosphatidylinositol-3 kinase (PI(3)K) and the serine / threonine kinase Akt by various stimuli including shear stress and agonists (such as the vascular endothelial growth factor) has been shown to elicit a double serine phosphorylation of eNOS. One of these phosphorylation events (on serine 1177) enhances the eNOS activity and seems to change its sensitivity to calcium, allowing a maintained NO release at resting concentrations of calcium (37, 46, 91). These results provide a mechanism explaining to some extent the calcium-independent steady eNOS activation. However, it is unclear how the shear stress effect on the cell plasma membrane results in the recruitment and activation of PI(3)K and Akt. The "receptor" initializing this activation and the "scaffold" recruiting PI(3)K to the membrane is still unidentified. Moreover, the molecular events that confer to eNOS the ability to respond discriminately to MPSS and unsteady flow is still not understood.

The intracellular location of eNOS, and, more specifically, its targeting to the plasma membrane, most likely play a prominent role in the regulation of shear-induced eNOS activation. eNOS lacks any hydrophobic transmembrane domain, but it is dually acetylated by both N-myristoylation and cysteine palmytoylation. N-myristoylation is necessary for its membrane association and targeting into the Golgi complex. It also appears necessary for efficient NO production (112). Palmitoylation, while it does not affect the enzyme overall membrane affinity or catalytic activity, appears to allow an optimal release of NO, in response to an ionomycin stimulation (83). It also appears to target eNOS to specific membrane domains, the caveolae (48, 114).

Caveolae possess a distinctive lipid composition, particularly rich in cholesterol and sphingolipids. As a result they appear as liquid-ordered membrane domains (an intermediate between a fluid and liquid crystalline molecular state), which are well known for their TritonX-insolubility, in contrast to the major part of the plasma membrane, which is a fluid compartment. The main structural proteins of those microdomains are caveolins. Several independent co-immunoprecipitation and domain-mapping studies have demonstrated a direct interaction of eNOS with caveolin-1 scaffolding domain (residues 82-101), which results in a markedly attenuated enzyme activity (49, 50, 68, 89, 90). Thus, caveolin appears to bind eNOS on its scaffolding domain, maintaining this enzyme in an inactive conformation. Although eNOS has been reported to be regulated by the competitive interaction of caveolin-1 and calmodulin (90), it appears that caveolin may rather attenuate eNOS activity by binding to eNOS

reductase domain, hence slowing the electron transport necessary to the enzyme activity (50). More generally, caveolin can be considered as a molecular "velcro" which binds many signalling proteins in their inactive conformation and thus organizes some "preassembled signalling complexes" at the plasma membrane (101).

The location of eNOS in caveolae and its interaction with caveolin therefore provide a compartmentalization of eNOS with other signalling proteins, including: G-proteins α subunits, Src tyrosine kinases, arginine subunits transporter CAT-1, PKC isoforms, Ha-Ras, and some agonists receptors (like B2 bradykinin receptor) (34, 81, 88, 101). Since many of these proteins have been shown to take part in the signalling pathways subsequent to temporal gradient stimulation, eNOS targeting to caveolae may facilitate, or improve the efficacy of the coupling between temporal gradient stimulation and eNOS activation. Furthermore, it places the enzyme at the interface between two membrane domains of different fluidities, an area prone to feel any sudden change in membrane tension consecutive to an impulse in shear stress.

However, the intracellular localization of eNOS differs in different endothelial cell types (3), as do the specific endothelial proteins expressed from one vascular bed to the next (106). Recent immunofluorescence and immunoprecipitation studies, using freshly isolated quiescent and confluent human umbilical vascular endothelial cells (HUVECs), have demonstrated that eNOS colocalizes with platelet endothelial cells adhesion molecule (PECAM-1) at the cell-cell junction, rather than with caveolin in caveolae (39). This observation is confirmed by histology obtained from rat aorta (39) as well as for different other endothelia (3). Furthermore, exposing HUVECs to a 0.5s impulse of 12 dynes/cm^2 resulted in the transient dissociation of the eNOS-PECAM complex and was accompanied by a 2.5 fold augmentation in cGMP production (39). Therefore, the eNOS-PECAM-1 complex seems to be involved in the modulation of eNOS activity by the sudden onset of temporal gradients. In contrast, the application of a ramping steady shear stress did not trigger any dissociation of the eNOS-PECAM-1 complex. This suggests that this complex is not involved in eNOS activation by a steady flow. Since it has recently been suggested that PECAM-1 may function as an inhibitory receptor, interacting with activating receptors via its SHP-2 binding domain (96), we propose that PECAM may interact with eNOS in a similar way than caveolin does, hence providing a compartmentalization of eNOS with other signalling proteins. Furthermore, while the relative tautness of the membrane at this location and the stiffness of the abundant cytoskeleton make it a site not readily activated by MPSS or temporal gradients, shear stress may cause an important strain in this area, inducing a local increase in membrane fluidity. Thus interactions of eNOS with PECAM-1, like with caveolin-1, will place the enzyme at the interface between two membrane domains of different fluidities. Therefore, we suggest that both molecules could play similar functions and that their differential distribution among the different endothelial cell types may regulate both eNOS fine sublocalization and activation by MPSS in those cells.

Whether eNOS is activated by a steady or unsteady shear could be mainly dependent on its location. The slow and constant NO release induced by MPSS being sensed and responded to by eNOS molecules broadly distributed along the plasma membrane. Rapid and intense NO production initiated by a sudden change in shear (temporal gradients) would be sensed and responded to by eNOS molecules concentrated in specific locations inside the membrane (caveolae and cell-cell junctions). In both cases, the plasma membrane itself would be the primary mechanotransducer.

3.2 Prostacyclin (PGI2)

Flow-induced release of prostacyclin (PGI2) from the endothelium plays a dual atheroprotective role in the vasculature. PGI2 acts as an endothelium-derived vasodilator that relaxes the underlying vascular smooth muscle through the activation of adenylate cyclase and the subsequent initiation of a cAMP signalling cascade. PGI2 also acts as a powerful antithrombotic agent. PGI2 was the first inhibitor of platelet aggregation shown to be released from endothelial cells by exposure to shear stress (42, 54). The *in vitro* release of prostacyclin from endothelial cells is enhanced when steady flow patters are spatially uniform but contain temporal fluctuations (pulsatile) compared to steady flow that is temporally and spatially uniform (42). Flow-induced release of PGI2 is biphasic. After an initial rapid release, production slowly declines over several hours before recovering to maintain a steady release rate (16). The first phase of rapid release is tightly linked to calcium mobilization. *In vitro*, production of flow-induced PGI2 is significantly inhibited when cultured endothelium cells are exposed to an inhibitor of intracellular Ca^{2+} mobilization, or cyclopiazonic acid (an endolasmic reticulum Ca+-ATPase inhibitor) (17, 61). The second phase is directly related to the magnitude of shear stress, and an exogenous source of the arachidonic acid (a precursor to PGI2 synthesis). The mechanisms responsible for the longer second phase of PGI2 synthesis have yet to be completely elucidated, but may be calcium independent, and related to an upregulation of PGI2 synthase (16).

3.3 Inhibition of endothelial cell proliferation

Throughout most of the vasculature, endothelial turnover is extremely low (23, 127). However, significantly elevated rates of localized endothelial proliferation are observed within arterial bifurcations prone to atherosclerosis. Given that increased cell division may enhance endothelial permeability (22, 82), the integrity of the endothelial monolayer may be achieved and maintained by restricting endothelial proliferation. Although the inhibition of endothelial cell proliferation has been positively linked to MPSS greater than 5 dyne/cm^2 in magnitude (1, 80), the molecular mechanisms of inhibition are still somewhat unclear (28). The inhibition of proliferation due to shear stress is associated with the suppression of cell transition from the G1 to S phase of the cell cycle. MPSS induces cell cycle arrest by up-regulating the cyclin-dependent kinase inhibitor $p21^{sdi1/cip1/waf1}$ (1). By inhibiting Cyclin-dependent kinase (cdk2 and cdk4), MPSS prevents the phosphorylation of retinoblastoma protein (pRB) which is the key regulatory point in the transition from the G1 to S phase of the cell cycle. This regulatory mechanism suggests that low mean shear stress (< 5 dyne/cm^2), such as occurs *in vivo* regions of recirculating flow, favors G_1 to S phase transition and hence cell proliferation through release of p21 suppression of cdk activity. Furthermore, NO has also been shown to increase expression of both $p21^{cip1/waf1}$ (another member in the family of cdk inhibitors) and tumor suppressor protein p53. Both proteins also inhibit S phase transition and suppress proliferation (65, 67). The initiating links between the mechanical force and p21 induction have yet to be determined.

IV. The pro-atherogenic role of unsteady fluid shear

In many aspects, endothelial function within regions of recirculating flow and / or low mean shear, is the antithesis of endothelial function in regions of MPSS. Spatially defined hemodynamic patterns of recirculating flow and / or low mean shear are postulated to underlie the focal origin of

plaque formation by inducing a small group of endothelial cells toward a pro-atherosclerotic phenotype through differential mechanosignalling, transcription and protein expression.

4.1 Oxidative stress

The redox state of a cell reflects a balance between processes that promote either oxidative or reductive pathways in the cell. The oxidative state is an important functional parameter that modulates a wide variety of endothelial functions: Gene expression, activity of signalling pathways and paracrine factors, apoptosis, and cell growth. The most significant source of intracellular oxidative stress is the superoxide free radical ($O_2^{\bullet -}$). All mammalian cells generate superoxide anions. The endothelium generates substantial amounts of $O_2^{\bullet -}$. The mechanism of production has not been extensively characterized (93, 108).

The enzymatic source(s) of shear stimulated $O_2^{\bullet -}$ has remained elusive. Macrophage-derived foam cells represent the natural candidate as a major contributor to $O_2^{\bullet -}$ oxidative stress. Foam cells overexpress the NADH / NADPH oxidase enzyme complex, which is the largest producer characterized to date. However, both the endothelium and the vascular smooth muscle contain membrane-bound NADH / NADPH oxidases that can also generate $O_2^{\bullet -}$. A study of enzyme activity in semi-crude cell suspensions points to NADH oxidase as the major source of reactive oxygen species in vascular cells (31). Notwithstanding, a variety of other cellular enzymes, including lipoxygenase, xanthine / xanthine oxidase, cyclooxygenase (COX), represent potential $O_2^{\bullet -}$ generating systems. In particular, COX has been shown to be activated by peroxynitrite (124). It has been suggested that peroxynitrite may influence COX activity via nitration of tyrosine residues within the enzyme. Even the activation of eNOS, albeit in the presence of suboptimal concentrations of the substrate L-arginine or the cofactor tetrahydrobiopterin, can lead to the production of $O_2^{\bullet -}$ (102, 26). Therefore, the identification of the shear induced enzymatic source(s) endothelial cell $O_2^{\bullet -}$ holds substantial promise as a potential alternative clinical target for the treatment of atherosclerosis.

a. Nitric oxide and the induction of pro-inflammatory molecules

Mean positive shear stress induced NO has been shown to inhibit the expression of redox sensitive pro-atherosclerotic gene products monocyte chemotactic protein-1 (MCP-1) and vascular cell adhesion molecule-1 (VCAM-1) (120, 121), and stimulates the expression of the antioxidant defense enzyme Cu / Zn superoxide dismutase (SOD) (31, 36). These observations suggest that MPSS induced NO plays a protective role in the regulation of endothelial cell redox sensitive gene products by reducing overall intracellular oxidative stress. Conversely, the response of endothelial cell subjected to recirculating flow seems to result with the increase of intracellular oxidative stress. Prior to the development of atherosclerotic plaques in these regions, availability of locally produced endothelial derived NO is reduced, and localized production of $O_2^{\bullet -}$ increases (40). The increase in localized $O_2^{\bullet -}$ has been postulated to contribute to atherogenesis by a variety of mechanisms including the oxidation of LDLs, the downregulation of SOD, and the stimulation of redox sensitive transcription factors NFkB and *egr* –1 to upregulate VCAM-1 expression (120). Although, both MPSS and oscillatory shear have been shown to be initially pro-oxidant, prolonged exposure to MPSS has been reported to reduce intracellular oxidative stress via the upregulation of SOD expression (31). This suggests that endothelial cell $O_2^{\bullet -}$ generation is specifically sensitive to the onset of shear. Indeed, endothelial cell exposed to temporal gradients in shear has been expressly shown to upregulate redox sensitive pro-atherosclerotic genes PDGF-A and MCP-1 (7, 8). It is not known however, if recirculating flow directly stimulates endothelial

cell $O_2^{\bullet -}$ production, or if other shear profiles can also stimulate $O_2^{\bullet -}$ production. It is plausible that prolonged MPSS continues to stimulate endothelial cell $O_2^{\bullet -}$ production, but $O_2^{\bullet -}$ generation is masked by virtue of increased intracellular SOD expression.

Superoxide is also the chemical inactivator of NO. The $O_2^{\bullet -}$ inactivation of NO to form the potent oxidant peroxynitrite ($ONOO^-$) may represent another important mechanism of vascular dysfunction and early atherogenesis (92). In vascular regions exposed to recirculating flow, the balance of NO and $O_2^{\bullet -}$ in the vascular wall is likely to be perturbed in favor of NO inactivation and thus, reduced NO functional availability. It is unlikely however, that NO availability is reduced by directly scavenging $O_2^{\bullet -}$ due to the lack of substantially elevated levels of peroxynitrite (120). Although other factors such as hypercholesterolemia and elevated levels of LDLs significantly contribute to the pathogenesis of atherosclerosis (99), these factors may still be secondary or additive to localized shear induced $O_2^{\bullet -}$ production.

The adhesion and migration of moncytes and leukocytes into the blood vessel wall are regulated by the secretion of chemotactic factors and the expression of cell-surface molecules. VCAM-1 mediates adhesion of monocytes to the endothelium, ICAM-1 binds to β2-integrins on various inflammatory macromolecules, while MCP-1 is a key chemotactic peptide involved in monocyte recruitment. The redox regulation of these molecules is underscored by the fact that the expression of ICAM-1 and VCAM-1 can be prevented by the angiotensin II antagonist irbesartan (84).

b. Angiotensin II

One particularly important aspect in the regulation of oxidative stress in both endothelia and vascular smooth muscle cells, is that $O_2^{\bullet -}$ production can be stimulated by angiotensin II (55). Angiotensin II is an important vascular smooth muscle growth factor, receptor-dependent vasoconstrictor, and may also be anti-apoptotic (14). Angiotensin II induces $O_2^{\bullet -}$ release in both endothelia and vascular smooth muscle cells via activation of membrane-bound NADH / NADPH oxidase, an effect that is mediated by both angiotensin II-1 and angiotensin II-2 receptors (130). The activation by angiotensin II is specific. None of the plausible metabolites of angiotensin II (angiotensin III, IV, or [1-7]) are as efficacious in promoting $O_2^{\bullet -}$ generating activity (104). Moreover, NADH / NADPH -dependent $O_2^{\bullet -}$ production via angiotensin II is concentration dependent (76). Shear stress also regulates tissue levels of angiotensin II by virtue of changes in angiotensin converting enzyme (ACE) expression. Prolonged exposure to MPSS significantly reduced ACE mRNA and activity (107). Clinically, ACE-inhibitors have proven aid in restoration of impaired endothelial function in patients with minimal atherosclerosis and mild hyperlipidaemia (87).

4.2 Endothelin-1 (ET-1)

Vascular smooth muscle cells also play a major role in the progression encroachment of the atherosclerotic lesion into the vessel lumen. In the advanced stages of atherogenesis, increased smooth muscle cell proliferation and increased vasomotor tone contribute to the lumenal narrowing that is characteristic of progressive atherosclerosis. Although more than 20 receptor specific growth factors can stimulate smooth muscle proliferation (24), endothelium derived endothelin-1 (ET-1) is thought to play the most significant role in flow-induced atherogenesis. ET-1 is a 21-amino acid peptide that acts as a powerful, and long-lasting vasoconstrictor and a smooth muscle cell mitogen (128). The release of ET-1 is inhibited by NO (20). A number of investigators have reported an apparent critical threshold

value of shear stress required to stimulate or inhibit ET-1 release (73, 85). ET-1 release is inhibited at shear stress values greater than 6 dyne/cm^2, whereas ET-1 release is stimulated by low mean shear stress levels less than 5 dyne/cm^2 (73). Oscillatory shear stress (with a mean average shear stress value of 2 dyne/cm^2) has also been shown to stimulate both ET-1 release and upregulate ET-1 mRNA levels (131). Antithetically, oscillatory shear stress simultaneously inhibited NO release and downregulated eNOS mRNA levels in the same cultured endothelial monolayers.

V. Conclusion

When considering the role of steady and unsteady shear stress in the pathogenesis of atherosclerosis, it is important to bear in mind that atherosclerosis is a multifactorial disease that involves many circulating blood elements, hemodynamic forces, and a complex cascade of molecular events within the endothelium and the arterial wall. While hemodynamic forces may play an important role in the nonrandom localization of atherosclerotic lesions, the mechanochemical mechanism(s) by which hemodynamic forces are sensed and transduced into a chemical signal is still unclear. Many of the biochemical transduction pathways have been characterized, the primary mechanoreceptor(s), however, remain unknown. It is our hypothesis that hydrodynamic shear destabilizes the plasma membrane, leading to a decrease in membrane microviscosity, or more precisely, an increase in membrane free volume. Mechanochemical transduction is proposed to occur when membrane-associated signalling proteins are activated by the increase intramolecular mobility (12). A number of studies have implicated a role of heterotrimeric G proteins in the mediation of cellular responses to fluid shear stress and stretch (15, 43, 64, 74). Studies from our lab demonstrate that heterotrimeric G proteins are rapidly activated by hydrodynamic shear, representing the earliest known biochemical response to mechanical stimulation presented (56). Furthermore, both fluid shear stress and membrane fluidizing agents activate these G proteins in the absence of classical G protein coupled receptors (57). Using fluorescent molecular rotors it was recently shown that hydrodynamic shear increases membrane free volume (58, 59). Taken together, these results demonstrate that hydrodynamic shear stress stimulates cellular responses by increasing membrane fluidity and activating heterotrimeric G proteins. Still to be determined are the mechanisms by which endothelial cells differentiate between MPSS and temporal gradients in shear stress. Given that these two opposing hemodynamic profiles appear to stimulate different and opposing signal transduction pathways, the elucidation of this biomechanical mechanism would represent an important step in understanding the pathogenesis of atherosclerosis.

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References list

1. Akimoto S, Mitsumata M, Sasaguri T, Yoshida Y(2000) Laminar Shear Stress Inhibits Vascular Endothelial Cell Proliferation by Inducing Cyclin-dependent Kinase Inhibitor p21$^{Sdi1/Cip1/Waf1}$. Cir. Res. 86:185-90.

2. Albuquerque ML, Waters CM, Savla U, Schnaper HW, Flozak AS (2000) Shear Stress Enhances Human Endothelial Cell Wound Closure *in vitro*. Am. J. Physiol. Heart. Circ. Physiol. 279:H293-302.
3. Andries LJ, Brutsaert DL, Sys SU (1998) Nonuniformity of Endothelial Constitutive Nitric Oxide Synthase Distribution in Cardiac Endothelium. Circ. Res. 82:195-203.
4. Arnal JF, Dinh-Xuan AT, Pueyo M, Darblade B, Rami J (1999) Endothelium-derived Nitric Oxide and Vascular Physiology and Pathology. Cell. Mol. Life Sci. 55:1078-87.
5. Ayajiki K, Kindermann M, Hecker M, Fleming I, Busse R (1996) Intracellular pH and Tyrosine Phosphorylation but not Calcium Determine Shear Stress-induced Nitric Oxide Production in Native Endothelial Cells. Circ. Res. 78:750-58.
6. Bakker SJ, Gans RO(2000) About the Role of Shear Stress in Atherogenesis. Cardiovasc. Res. 45:270-72.
7. Bao X, Lu C, Frangos JA (1999) Temporal Gradient in Shear but not Steady Shear Stress Induces PDGF-A and MCP-1 Expression in Endothelial Cells: Role of NO, NF Kappa B, and egr-1. Arterioscler. Thromb. Vasc. Biol. 19:996-1003.
8. Bao X, Clark CB, Frangos JA (2000) Temporal Gradient in Shear-induced Signaling Pathway-Involvement of MAP Kinase, c-fos and Connexin-43. Am J Physiol Heart Circ Physiol. 278:H1598-H1605.
9. Bao X, Lu C, Frangos JA (2001) Mechanism of Temporal Gradients in Shear-induced ERK1/2 Activation and Proliferation in Endothelial Cells. (2001) Am. J. Physiol. Heart. Circ. Physiol. 281:H????-????
10. Barbee KA, Davies PF, Lal R (1994) Shear Stress-induced Reorganization of the Surface Topography of Living Endothelial Cells Imaged by Atomic Force Microscopy. Circ. Res. 74:163-71.
11. Barbee KA, Mundel T, Lal R, Davies PF (1995) Subcellular Distribution of Shear Stress at the Surface of Flow-aligned and Nonaligned Endothelial Monolayers. Am. J. Physiol. 268:H1765-72.
12. Beece D, Eisenstein L, Frauenfelder H, Good D, Marden MC, Reinisch L, Reynolds AH, Sorensen LB, Yue KT (1980) Solvent Viscosity and Protein Dynamics. Biochemistry, 19:5147-57.
13. Berk BC, Corson MA, Peterson TE, Tseng H (1995) Protein Kinases as Mediators of Fluid Shear Stress Stimulated Signal Transduction in Endothelial Cells: A Hypothesis for Calcium-dependent and Calcium-independent Events Activated by Flow. J. Biomech. 28:1439-50.
14. Berk BC, Duff JL, Marrero MB, Bernstein KE Angiotensin II Signal Transduction in Vascular Smooth Muscle. In: Sowers JR, ed. Endocrinology of the Vasculature. Totowa, New Jersey: Humana Press; 1996:187-204.
15. Berthiaume F, Frangos JA (1992) Flow-induced Prostacyclin Production is Mediated by a Pertussis Toxin-sensitive G-Protein. FEBS Letters. 30:277-9.
16. Berthiaume F, Frangos JA (1994) Flow Effects on Endothelial Cell Signal Transduction, Function, and Mediator Release. In: Flow-dependent Regulation of Vascular Function, edited by Bevan J, Kaley G, and Rubanyi G. New York: Oxford Univ. Press.
17. Bhagyalakshmi A, Frangos JA (1989) Mechanism of Shear-induced Prostacyclin Production in Endothelial Cells. Biochem. Biophys. Res. Commun. 158:31-7.
18. Blackman BR, Thibault LE, Barbee KA. Selective Modulation of Endothelial Cell [Ca2+]i Response to Flow by the Onset Rate of Shear Stress. J. Biomech. Eng. 2000 Jun;122(3):274-82.
19. Bomberger RA, Zarins CK, Taylor KE, Glagov S (1980) Effect of Hypotension on Atherogenesis and Aortic Wall Composition. J. Surg. Res. 28:402-9.
20. Boulanger C, Luscher TF (1990) Release of Endothelin from the Porcine Aorta. Inhibition by Endothelium-derived Nitric Oxide. J. Clin. Invest. 85:587-90.
21. Britten MB, Zeiher AM, Schachinger V (1999) Clinical Importance of Coronary Endothelial Vasodilator Dysfunction and Therapeutic Options. J Intern Med. 245:315-27.
22. Caplan BA, Schwartz CJ (1973) Increased Endothelial Cell Turnover in Areas of *in vivo* Evans Blue Uptake in the Pig Aorta. Atherosclerosis 17:401-12.
23. Chiu JJ, Wang DL, Chien S, Skalak R, Usami S (1998) Effects of Disturbed Flow on Endothelial Cellls. J Biomech Eng 120:2-8.
24. Corson MA, Berk BC (1993) Growth Factors and the Vessel Wall. Heart Dis. Stroke. 2:166-70.
25. Corson MA, James NL, Latta SE, Nerem RM, Berk BC, Harrison DG (1996) Phosphorylation of Endothelial Nitric Oxide Synthase in Response to Fluid Shear Stress. Circ. Res. 79:984-91.
26. Cosentino F, Katusic ZS (1995) Tetrahydrobiopterin and Dysfunction of Endothelial Nitric Oxide Synthase in Coronary Arteries. Circulation. 91:139-44.
27. Davies PF (1995) Flow-mediated Endothelial Mechanotransduction. Physiol. Rev. 75:519-560.
28. Davies PF (2000) Spatial Hemodynamics, the Endothelium. and Focal Atherogenesis: A Cell Cycle Link? Circ. Res. 86:114-6.

29. DeBakey ME, Lawrie GM, Glaeser DH (1985) Patterns of Atherosclerosis and Their Surgical Significance. Ann. Surg. 201:115-31.
30. De Caterina R, Libby P, Peng HB, Thannickal VJ, Rajavashisth TB, Gimbrone MA Jr, Shin WS, Liao JK(1995) Nitric Oxide Decreases Cytokine-induced Endothelial Activation. Nitric Oxide Selectively Reduces Endothelial Expression of Adhesion Molecules and Proinflammatory Cytokines. J. Clin. Invest. 96:60-8.
31. De Keulenaer GW, Chappell DC, Ishizaka N, Nerem RM, Alexander RW, Griendling KK (1998) Oscillatory and Steady Laminar Shear Stress Differentially Affect Human Endothelial Redox State: Role of a Superoxide-producing NADH Oxidase. Circ. Res. 82:1094-101.
32. DePaola N, Gimbrone MA, Davies PF, Dewey CF (1992) Vascular Endothelium Responds to Fluid Shear Stress Gradients Arterioscler Thromb 12:1254-57.
33. DePaola N, Davies PF, Pritchard WF, Florez L, Harbeck N, Polacek DC (1999) Spatial and Temporal Regulation of Gap Junction Connexin43 in Vascular Endothelial Cells Exposed to Controlled Disturbed Flows *in vitro*. Proc. Natl. Acad. Sci. U.S.A. 96:3154-9.
34. de Weerd WF, Leeb-Lundberg LM (1997) Bradykinin Sequesters B2 Bradykinin Receptors and the Receptor-coupled Galpha Subunits Galphaq and Galphai in Caveolae in DDT1 MF-2 Smooth Muscle Cells. J. Biol. Chem. 272:17858-66.
35. Dewey CF Jr, Bussolari SR, Gimbrone MA Jr, Davies PF (1981) The Dynamic Response of Vascular Endothelial Cells to Fluid Shear Stress. J. Biomech. Eng. 103:177-85.
36. Dimmeler S, Hermann C, Galle J, Zeiher AM (1999) Upregulation of Superoxide Dismutase and Nitric Oxide Synthase Mediates the Apoptosis-suppressive Effects of Shear Stress on Endothelial Cells. Arterioscler. Thromb. Vasc. Biol. 19:656-64.
37. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM (1999) Activation of Nitric Oxide Synthase in Endothelial Cells by Akt-dependent Phosphorylation. Nature 399:601-5.
38. Durst F, Pereira JCF (1988) Time-dependent Laminar Backward-facing Step Flow in a Two-dimensional Duct. Journal of Fluids Engineering. 110:289-96.
39. Dusserre N, L'Heureux N, Frangos JA. PECAM-1 (CD31) Interacts with Nitric-oxide Synthase in Human Endothelial Cells. Implication for Flow-induced Nitric-oxide Synthase Activation (in submission).
40. Finkel T (1998) Oxygen Radicals and Signaling. Curr. Opin. Cell. Biol. 10:248-53.
41. Flaherty JT, Pierce JE, Ferrans VJ, Patel DJ, Tucker WK, Fry DL (1972) Endothelial Nuclear Patterns in the Canine Arterial Tree with Particular Reference to Hemodynamic Events. Circ. Res. 30:23-33.
42. Frangos JA, Eskin SG, McIntire LY, Ives CL (1985) Flow Effects on Prostacyclin Production by Cultured Human Endothelial Cells. Science 227:1477-9.
43. Frangos JA, Huang TY, Clark CB (1996) Steady Shear and Step Changes in Shear Stimulate Endothelium via Independent Mechanisms-superposition of Transient and Sustained Nitric Oxide Production. Biochem. Biophys. Res. Commun. 224:660-5.
44. Frangos SG, Gahtan V, Sumpio B (1999) Localization of Atherosclerosis: Role of Hemodynamics. Arch. Surg. 134:1142-9.
45. Fry DL (1968) Acute Vascular Endothelial Changes Associated with Incresed Blood Velocity Gradients. Circ. Res. 22:165-92.
46. Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, Sessa WC (1999) Regulation of Endothelium-derived Nitric Oxide Production by the Protein Kinase Akt. Nature 399 6736 597-601 [published erratum appears in Nature 1999 Aug 19;400(6746):792].
47. Furchgott RF, Zawadzki JV (1980) The Obligatory Role of Endothelial Cells in the Relaxation of Arterial Smooth Muscle by Acetylcholine. Nature. 288:373-6.
48. Garcia-Cardena G, Oh P, Liu J, Schnitzer JE, Sessa WC (1996) Targeting of Nitric Oxide Synthase to Endothelial Cell Caveolae via Palmitoylation: Implications for Nitric Oxide Signalling. Proc. Natl. Acad. Sci U.S.A. 93:6448-5643.
49. Garcia-Cardena G, Martasek P, Masters BS, Skidd PM, Couet J, Li S, Lisanti MP, Sessa WC (1997)Dissecting the Interaction between Nitric Oxide Synthase (NOS) and Caveolin. Functional Significance of the Nos Caveolin Binding Domain *in vivo*. J. Biol. Chem. 272:25437-4340.
50. Ghosh S, Gachhui R, Crooks C, Wu C, Lisanti MP, Stuehr DJ (1998) Interaction Between Caveolin-1 and the Reductase Domain of Endothelial Nnitric-oxide Synthase. Consequences for Catalysis. J. Biol. Chem. 273:22267-71.
51. Gibson CM, Diaz L, Kandarpa K, Sacks FM, Pasternak RC, Sandor T, Feldman C, Stone PH (1993) Relation of Vessel Wall Shear Stress to Atherosclerosis Progression in Human Coronary Arteries. Arterioscler. Thromb. 13:310-5.
52. Glagov S, Zarins C, Giddens DP, Ku DN (1988) Hemodynamics and Atherosclerosis : Insights and Perspectives Gained from Studies of Human Arteries. Arch. Pathol. Lab. Med. 112:1018-31.

53. Govers R, Rabelink TJ. Cellular Regulation of Endothelial Nitric Oxide Synthase. (2001) Am. J. Physiol. Renal. Physiol. 280:F193-F206.
54. Grabowski EF, Jaffe EA, Weksler BB (1985) Prostacyclin Production by Cultured Endothelial Cell Monolayers Exposed to Step Increases in Shear Stress. J. Lab. Clin. Med. 105:36-43.
55. Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW (1994) Angiotensin II Stimulates NADH and NADPH Oxidase Activity in Cultured Vascular Smooth Muscle Cells. Circ. Res. 74:1141-8.
56. Gudi SRP, Clark CB, Frangos JA (1996) Fluid Flow Rapidly Activates G Proteins in Human Endothelial Cells. Circulation Research, 79:834-9.
57. Gudi SRP, Nolan JP, Frangos JA (1998) Modulation of GTPase Activity of Reconstituted G Proteins by Fluid Shear Stress and Phospholipid Composition. Proc. Natl. Acad. Sci. U.S.A. 95:2515-9.
58. Haidekker M, L'Heureux N, Frangos JA (2000) Fluid Shear Stress Increases Membrane Fluidity In Endothelial Cells: A Study with DCVJ Fluorescence. Am J Physiol, 278: H1401-6.
59. Haidekker M, Ling T, Anglo M,, Stevens HY, Frangos JA, Theodorakis EA (2000) New Fluorescent Probes for the Measurement of Cell Membrane Viscosity. Chemistry and Biology 8: 123-31.
60. Haidekker M, White CR L'Heureux N, Frangos JA (2001) Analysis of Temporal Shear Stgress Gradients during the Onset Phase of Flow over a Backward-facing Step. Implications on Endothelia Cell Proliferation. In press at J. Biomech. Eng.
61. Hanada T, Hashimoto M, Nosaka S, Sasaki T, Nakayama K, Masumura S, Yamauchi M, Tamura K (2000) Shear Stress Enhances Prostacyclin Release from Endocardial Endothelial Cells. Life Sci. 66:215-20.
62. Harrison DG (1997) Cellular and Molecular Mechanisms of Endothelial Cell Dysfunction. J. Clin. Invest. 100:2153-7.
63. Hazel AL, Pedley TJ Alteration of Mean Wall Shear Stress Near an Ooscillation Stagnation Point. J. Biomech. Eng. 1988;120:227-37.
64. Hsieh HJ, Li NQ, Frangos JA (1992) Shear-induced Platelet-derived Growth Factor Gene Expression in Human Endothelial Cells is Mediated by Protein Kinase-C. J. Cellular Physiology, 150:552-8.
65. Hsieh TC, Juan G, Darzynkiewicz Z, Wu JM (1999) Resveratrol Increases Nitric Oxide Synthase, Induces Accumulation of p53 and p21(WAF1 / CIP1), and Suppresses Cultured Bovine Pulmonary Artery Endothelial Cell Proliferation by Perturbing Progression Through S and G2. Cancer Res. 59:2596-601.
66. Hunt SC, Hopkins PN, Williams RR (1996) Genetics and Mechanisms. In: Atherosclerosis and Coronary Artery Disease. Fuster V, Ross R, Topol EJ. eds. Philadelphia: Lippincott-Raven 209-35.
67. Ishida A, Sasaguri T, Kosaka C, Nojima H, Ogata J (1997) Induction of the Cyclin-dependent kinase Inhibitor p21(Sdi1 / Cip1 / Waf1) by Nitric Oxide-generating Vasodilator in Vascular Smooth Muscle Cells. J. Biol. Chem. 272:10050-7.
68. Ju H, Zou R, Venema VJ, Venema RC (1997) Direct Interaction of Endothelial Nitric Oxide Synthase and Caveolin-1 Inhibits Synthase Activity. J. Biol. Chem. 272:18522-5.
69. Khalifa AM, Giddens DP (1981) Characterization and Evolution Poststenotic Flow Disturbances. J. Biomech. 14:279-96.
70. Khan BV, Harrison DG, Olbrych MT, Alexander RW, Medford RM (1996) Nitric Oxide Regulates Vascular Cell Adhesion Molecule 1 Gene Expression and Redox-sensitive Transcriptional Events in Human Vascular Endothelial Cells. Proc. Natl. Acad. Sci. U.S.A. 93:9114-9.
71. Kleinstreuer C, Lei M, Archie JP Flow Input Waveform Effects on the Temporal and Spatial Wall Shear Stress Gradients in a Femoral Graft-artery Connector. J. Biomech. Eng. 1996;118:506-10.
72. Ku DN, Giddens DP, Zarins CK, Glagov S (1985) Pulsatile Flow and Atherosclerosis in the Human Carotid Bifurcation. Positive Correlation Between Plaque Location and Low Oscillation Shear Stress. Arteriosclerosis. 5:293-302.
73. Kuchan MJ, Frangos JA (1993) Shear Stress Regulates Endothelin-1 Release via Protein Kinase C and cGMP in Cultured Endothelial Cells. Am. J. Physiol. 264:H150-6.
74. Kuchan MJ, Jo H, Frangos JA (1994) Role of G Proteins in Shear Stress-Mediated Nitric Oxide Production by Endothelial Cells. American Journal of Physiology, 267:C753-8.
75. Kuchan MJ, Frangos JA (1994) Role of Calcium and Calmodulin in Flow-induced Nitric Oxide Production in Endothelial Cells. Am. J. Physiol. 266:C628-36.
76. Lang D, Mosfer SI, Shakesby A, Donaldson F, Lewis MJ (2000) Coronary Microvascular Endothelial Cell Redox State in Left Ventricular Hypertrophy : The Role of Angiotensin II. Circ. Res. 86:463-9.
77. Langille BL, Adamson SL (1981) Relationship Between Blood Flow Direction and Endothelial Cell Orientation at Arterial Branch Sites in Rabbits and Mice. Circ. Res. 48:481-8.

78. Langille LB (1984) Integrity of Arterial Endothelium Following Acute Exposure to High Shear Stress. Biorheology. 21:333-46.
79. Levesque MJ, Liepsch D, Moravec S, Nerem RM (1986) Correlation of Endothelial Cell Shape and Wall Shear Stress in a Stenosed Dog Aorta. Arteriosclerosis. 6:220-9.
80. Levesque MJ, Nerem RM, Sprague EA (1990) Vascular Endothelial Cell Proliferation in Culture and the Influence of Flow. Biomaterials. 11:702-7.
81. Li S, Couet J, Lisanti MP (1996) Src Tyrosine Kinases, Galpha Subunits, and H-Ras Share a Common Membrane-anchored Scaffolding Protein, Caveolin. Caveolin Binding Negatively Regulates the Auto-activation of Src Tyrosine Kinases. J. Biol. Chem. 271:29182-90.
82. Lin SJ, Jan KM, Schuessler G, Weinbaum S, Chien S (1988) Enhanced Macromolecular Permeability of Aortic Endothelial Cells in Association with Mitosis. Arteriosclerosis. 73:223-32.
83. Liu, J,Garcia-Cardena G, Sessa WC (1996) Palmitoylation of Endothelial Nitric Oxide Synthase is Necessary for Optimal Stimulated Release of Nitric Oxide: Implications for Caveolae Localization. Biochemistry. 35:13277-81.
84. Luvara G, Pueyo ME, Philippe M, Mandet C, Savoie F, Henrion D, Michel JB (1998) Chronic Blockade of NO Synthase Activity Induces a Proinflammatory Phenotype in the Arterial Wall: Prevention by Angiotensin II Antagonism. Arterioscler. Thromb. Vasc. Biol. 1998 18:1408-16.
85. Malek A, Izumo S (1992) Physiological Fluid Shear Stress Causes Downregulation of Endothelin-1 mRNA in Bovine Aortic Endothelium. Am. J. Physiol. 263:C389-96.
86. Malek AM, Izumo S (1994) Molecular Aspects of Signal Transduction of Shear Stress in the Endothelial Cell. J. Hypertens. 12:989-99.
87. Mancini GB, Henry GC, Macaya C, O'Neill BJ, Pucillo AL, Carere RG, Wargovich TJ, Mudra H, Luscher TF, Klibaner MI, Haber HE, Uprichard AC, Pepine CJ, Pitt B (1996) Angiotensin-converting Enzyme Inhibition with Quinapril Improves Endothelial Vasomotor Dysfunction in Patients with Coronary Artery Disease. The TREND (Trial on Reversing ENdothelial Dysfunction) Study. Circulation. 94:258-65.
88. McDonald KK, Zharikov S, Block ER, Kilberg MS (1997) A Caveolar Complex Between the Cationic Amino Acid Transporter 1 and Endothelial Nitric-oxide Synthase May Explain the "Arginine Paradox". J. Biol. Chem. 272:31213-6.
89. Michel JB, Feron O, Sase K, Prabhakar P, Michel T (1997a) Caveolin Versus Calmodulin. Counterbalancing Allosteric Modulators of Endothelial Nitric Oxide Synthase. J. Biol. Chem. 272:25907-12.
90. Michel JB., Feron O, Sacks D, Michel T (1997b) Reciprocal Regulation of Endothelial Nitric Oxide Synthase by Ca2+- Calmodulin and Caveolin. J. Bio.l Chem. 272:15583-6.
91. Michell BJ, Griffiths JE, Mitchelhill KI, Rodriguez-Crespo I, Tiganis T, Bozinovski S, de Montellano PR, Kemp BE, Pearson RB (1999) The Akt Kinase Signals Directly to Endothelial Nitric Oxide Synthase. Curr Biol 9:845-8.
92. Miller FJ Jr, Gutterman DD, Rios CD, Heistad DD, Davidson BL (1998) Superoxide Production in Vascular Smooth Muscle Contributes to Oxidative Stress and Impaired Relaxation in Atherosclerosis. Circ. Res. 82:1298-305.
93. Mohazzab KM, Kaminski PM, Wolin MS (1994) NADH Oxidoreductase is a Major Source of Superoxide Anion in Bovine Coronary Artery Endothelium. Am. J. Physiol. 266:H2568-72.
94. Moore JE, Xu C, Glagov S, Zarins CK, Ku DN. Fluid Wall Shear Stress Measurements in a Model of the Human Abdominal Aorta: Oscillatory Behavior and Relationship to Atherosclerosis. Atherosclerosis 1994;110:225-40.
95. Moroi M, Zhang L, Yasuda T, Virmani R, Gold HK, Fishman MC, Huang PL (1998) Interaction of Genetic Deficiency of Endothelial Nitric Oxide, Gender, and Pregnancy in Vascular Response to Injury in Mice. .J Clin. Invest. 101:1225-32.
96. Newman PJ (1999) Switched at Birth: a New Family for PECAM-1. J. Clin. Invest. 103:5-9.
97. Nishida K, Harrison DG, Navas JP, Fisher AA, Dockery SP, Uematsu M, Nerem RM, Alexander RW, Murphy TJ (1992) Molecular Cloning and Characterization of the Constitutive Bovine Aortic Endothelial Cell Nitric Oxide Synthase. J. Clin. Invest. 90:2092-6.
98. Noris M, Morigi M, Donadelli R, Aiello S, Foppolo M, Todeschini M, Orisio S, Remuzzi G, Remuzzi A (1995) Nitric Oxide Synthesis by Cultured Endothelial Cells Is Modulated by Flow Conditions. Circ. Res. 76:536-43.
99. Ohara Y, Peterson TE, Harrison DG (1993) Hypercholesterolemia Increases Endothelial Superoxide Anion Production. J. Clin. Invest. 91:2546-51.
100. Ojha M. Wall Shear Stress Temporal Gradient and Anastomotic Intimal Hyperplasia. Cir. Res. 1994;74:1227-31.

101. Okamoto T, Schlegel A, Scherer PE, Lisanti MP (1998) Caveolins, a Family of Scaffolding Proteins for Organizing "Preassembled Signaling Complexes" at the Plasma Membrane. J. Biol. Chem. 273:5419-22.
102. Okano M, Yoshida Y (1992) Endothelial Cell Morphometry of Atherosclerotic Lesions and Flow Profiles at Aortic Bifurcations in Cholesterol Fed Rabbits. J. Biomech. Eng. 114:301-8.
103. Okano M, Yoshida Y (1993) Influence of Shear Stress on Endothelial Cell Shapes and Junction Complexes at Flow Dividers of Aortic Bifurcations in Cholesterol-fed Rabbits. Front. Med. Biol. Eng. 5:95-120.
104. Pagano PJ, Chanock SJ, Siwik DA, Colucci WS, Clark JK(1998) Angiotensin II Induces p67phox mRNA Expression and NADPH Oxidase Superoxide Generation in Rabbit Aortic Adventitial Fibroblasts. Hypertension. 32:331-7.
105. Pou S, Pou WS, Bredt DS, Snyder SH, Rosen GM (1992) Generation of Superoxide by Purified Brain Nitric Oxide Synthase. J. Biol. Chem. 267:24173-6.
106. Rajotte D, Arap W, Hagedorn M, Koivunen E, Pasqualini R, Ruoslahti E (1998) Molecular Heterogeneity of the Vascular Endothelium Revealed by *in vivo* Phage Display. J. Clin. Invest.102:430-7.
107. Rieder MJ, Carmona R, Krieger JE, Pritchard KA Jr, Greene AS (1997) Suppression of Angiotensin-converting Enzyme Expression and Activity by Shear Stress. Circ. Res. 80:312-9.
108. Rosen GM, Freeman BA. Detection of Superoxide Generated by Endothelial Cells. (1984) Proch. Natl. Acad. Sci. U.S.A. 81:7269-73.
109. Rubanyi GM, Romero JC, Vanhoutte PM. Flow-induced Release of Endothelium-derived Relaxing Factor. (1986) Am. J. Physiol. 250:H1145-9.
110. Rubanyi GM. The Role of Endothelium in Cardiovascular Homeostasis and Diseases (1993) J. Cardiovasc. Pharmacol. 4:S1-S14.
111. Schwarz G, Callewaert G, Droogmans G, Nilius B (1992) Shear Stress-induced Calcium Transients in Endothelial Cells from Human Umbilical Cord Veins. J. Physiol. 458:527-38.
112. Sessa WC., Garcia-Cardena G, Liu J, Keh A, Pollock JS, Bradley J, Thiru S, Braverman IM, Desai KM (1995) The Golgi Association of Endothelial Nitric Oxide Synthase Is Necessary for the Efficient Synthesis of Nitric Oxide. J. Biol. Chem. 270:17641-4.
113. Shaaban AM, Duerinckx AJ (2000) Wall Shear Stress and Early Atherosclerosis: a Review. A.J.R. Am. J. Roentgenol. 174:1657-65.
114. Shaul P. Smart WEJ, Robinson LJ, German Z, Yuhanna IS, YingY, Anderson RG, Michel T (1996) Acylation Targets Emdothelial Nitric-oxide Synthase to Plasmalemmal Caveolae. J. Biol. Chem. 271:6518-22.
115. Singh GK, Mathews TJ, Clarke SC, Yannicos T, Smith BL (1995) Annual Summary of Births, Marriages, Divorces, and Deaths: United States, 1994. Mon. Vital. Stat. Rep. 43:1-37.
116. Svindland A. (1983) The Localization of Sudanophilic and Fibrous Plaques in the Main Left Coronary Bifurcation. Atherosclerosis 48:139-45.
117. Tardy Y, Resnick N, Nagel T, Gimbrone MA, Dewey CF (1997) Shear Stress Gradients Remodel Endothelial Monolayers *in vitro* via a Cell Proliferation-migration-loss Cycle. Arterioscler. Thromb. Vasc. Biol. 17:3102-6.
118. Traub O, Berk BC (1998) Laminar Shear Stress: Mechanisms by which Endothelial Cells Transduce an Atheroprotective Force. Arterioscler. Thromb. Vasc Biol. 18:677-85.
119. Truskey GA, Barber KM, Robey TC, Olivier LA, Combs MP (1995) Characterization of a Sudden Expansion Flow Chamber to Study the Response of Endothelium to Flow Recirculation. J. Biomech. Eng. 117:203-10.
120. Tsao PS, Buitrago R, Chan JR, Cooke JP (1996) Fluid Flow Inhibits Endothelial Adhesiveness. Nitric Oxide and Transcriptional Regulation of VCAM-1. Circulation 94:1682-9.
121. Tsao PS, Wang B, Buitrago R, Shyy JY, Cooke JP (1997) Nitric Oxide Regulates Monocyte Chemotactic Protein-1. Circulation 96:934-40.
122. Vyalov S, Langille BL, Gotlieb AI (1996) Decreased Blood Flow Rate Disrupts Endothelial Repair *in vivo*. Am. J. Pathol. 149:2107-18.
123. Wang DM, Tarbell JM (1995) Modeling Interstitial Flow in an Artery Wall Allows Estimation of Wall Shear Stress on Smooth Muscle Cells. J. Biomech. Eng. 117:358-63.
124. Wang W, Diamond SL (1997) Does Elevated Nitric Oxide Production Enhance the Release of Prostacyclin from Shear Stressed Aortic Endothelial Cells? Biochem. Biophys. Res. Commun. 233:748-51.
125. White CR, Haidekker M, Bao X, Frangos JA (2001) Temporal Gradients in Shear Stress, but not Spatial Gradients or Steady Shear, Induce Endothelial Cell Proliferation. Circulation 103:2508-13.

126. Wissler RW (1994) New Insights into the Pathogenesis of Atherosclerosis as Revealed by PDAY. Pathobiological Determinants of Atherosclerosis in Youth. Atherosclerosis. 1994;108 Suppl:S3-20.
127. Wright HP. Mitosis Patterns in Aortic Endothelium. Atherosclerosis. 1972;15:93-100.
128. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T(1988) A Novel Potent Vasoconstrictor Peptide Produced by Vascular Endothelial Cells. Nature. 332:411-5.
129. Zarins CK, Giddens DP, Bharadvaj BK, Sottiurai VS, Mabon RF, Glagov S (1983) Carotid Bifurcation Atherosclerosis. Quantitative Correlation of Plaque Localization with Flow Velocity Profiles and Wall Shear Stress. Circ. Res. 53:502-14.
130. Zhang H, Schmeisser A, Garlichs CD, Plotze K, Damme U, Mugge A, Daniel WG (1999) Angiotensin II-induced Superoxide Anion Generation in Human Vascular Endothelial Cells: Role of Membrane-bound NADH -/ NADPH-oxidases. Cardiovasc. Res. 44:215-22.
131. Ziegler T, Bouzourene K, Harrison VJ, Brunner HR, Hayoz D (1998) Influence of oscillatory and Unidirectional Flow Environments on the Expression of Endothelin and Nitric Oxide Synthase in Cultured Endothelial Cells. Arterioscler. Thromb. Vasc. Biol. 18:686-92.