Mechanotransduction of Shear Stress by the Endothelium

9

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Abstract

The ability of endothelial cells to convert forces from blood flow to biochemical signals underlies vascular health and disease. While the mechanisms of endothelial cell mechanotransduction are still an area of ongoing research, a picture is emerging that encompasses the temporal and spatial complexity of blood flow patterns and the highly heterogeneous and dynamic mechanical properties of endothelial cells. In this framework, cell sense blood flow-induced shear stress through specialized structures such as the glycocalyx, membrane microdomains, focal adhesions and adherens junctions, where forces are converted to biochemical signaling cascades via alterations in protein conformations and associations. The result of these processes are the production of vasodilators and the activation of genetic transcription factors that lead to changes in endothelium permeability, adhesiveness to circulating leukocytes and platelets, and changes in vascular diameter. Therefore, understanding the mechanobiology of endothelial cells is at the heart of promoting vascular health and predicting, diagnosing, treating, and preventing vascular disease.

Keywords

Endothelial cells • Mechanotransduction • Mechanobiology • Membrane • Cytoskeleton

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K. Tanishita, K. Yamamoto (eds.), *Vascular Engineering*,
DOI 10.1007/978-4-431-54801-0_9

9.1 Introduction

Sensation and transduction of forces by endothelial cells are part of their inherent role as permeability barriers and regulators of blood flow in arteries, capillaries, and veins. Because of their proximity to blood flow, they are continuously exposed to fluid flow-induced mechanical forces that exhibit a wide range of spatial and temporal scales and directions. Endothelial cells (ECs) form junctions to create the endothelium, a one-cell layer thick, highly regulated, semipermeable barrier to blood-borne solutes. It is now recognized that the interactions between the endothelium and hemodynamic forces, such as shear stress, are largely responsible for regulating the endothelium's barrier function, either through modulation of the space between cells, changes in mitotic turnover rates of endothelial cells, or by affecting the endothelium's adhesiveness to circulating leukocytes, which can migrate into the sub-endothelial intima. Such mechanotransduction involves the sensation of force by several endothelial cell structures. These include a surface glycocalyx layer composed of glycosaminoglycans, glycolipids, and glycoproteins, a plasma membrane composed of a multi-phase lipid bilayer and integral and peripheral membrane proteins, a four-component cytoskeleton (fodrin, actin filaments, microtubules, intermediate filaments), focal adhesion complexes responsible for cell signaling and adhesion to the basement membrane, and cell (gap and tight) junction proteins responsible for cell-cell communication and adhesion. These structures transmit forces to proteins and lipids, which become biochemically active and transduce these mechanical signals into chemical pathways in the cell leading to formation of vasodilators and vasoconstrictors, activated transcription factors, or alterations in cell shape and endothelium permeability.

The key questions about mechanotransduction are as follows. What components of force are sensed by the cell? What are the molecular sensors that transmit force to transducers in the cell? Is transduction through physical links that transmit force from one molecular partner to another, or does force work through existing receptors, which transmit biochemical signals largely through diffusive pathways? To what extent are the force transduction partners regulated by the very force they sense? And what are the main factors produced at the terminal end of the force transduction pathways? Answers to these questions require a detailed evaluation of vascular and cellular structures and inherent engineering analysis of flow dynamics in areas experiencing vascular disease. Second, molecular biological investigations are needed that elucidate molecular force sensors and their interconnections and dynamic rearrangements in response to force. Third, it will be necessary to understand, from an engineering perspective, what components of force are sensed, and what the mechanical properties of these sensors are and how they can respond to force magnitude, direction, frequency, and duration. Finally, it is important to determine the identity of final soluble products produced as a result of mechanotransduction and how these products can lead to vascular health and disease.

The purpose of this chapter is to systematically explore these questions and provide the most plausible answers to them. As in any emerging field of molecular

biology, there remains much controversy over the precise identity of molecular partners and pathways. In response to this, there have been some excellent reviews on the specific biochemical pathways that are activated by force. For example, shear stress elicits calcium increase in cells (Ando and Yamamoto 2013; Yamamoto and Ando 2010) and increases in transcription factor phosphorylation (Davies 2009; Li et al. 2005; Nigro et al. 2011). Nevertheless, there remains controversy about the precise mechanical pathways linking force and changes in biology (Huang et al. 2004). This chapter begins with an analysis of the main components of blood flow (stress and transport) that are sensed by the endothelium and then describes the mechanical linkages responsible for mechanotransduction and their dynamics. It is hoped that understanding these frameworks in the context of the role of shear stress in endothelium function will assist in the development of strategies to ameliorate vascular diseases that find their origins at the interface of engineering and biology.

9.2 Overview of Mechanotransduction and Organization of This Chapter

Mechanotransduction has been measured on temporal scales that range from seconds to hours of exposure to flow. Rapid responses include potassium channel activation (Boycott et al. 2013), intracellular calcium increase (Yamamoto et al. 2011), G-protein activation (Chachisvilis et al. 2006; Gudi et al. 1998), stimulation of mitogen-activated protein (MAP) kinases (Jalali et al. 1998), and cell junction responses (Tzima et al. 2005). A typical example of mechanotransduction occurs when forces that act on the apical surface are transmitted by the cytoskeleton to other locations where signaling can occur. For example, force can be applied to the apical surface of cells and integral membrane proteins and transmitted via the cytoskeleton to focal adhesions, intercellular junctions, cellular organelles, and the nuclear membrane. Such structures have been shown to remodel in response to force and to transduce force into biochemical signaling. Other predominant molecular players in mechanotransduction include membrane ion channels, cilia, tight and gap junction complexes, and nuclear tethered nesprins (nuclear envelope spectrin repeat proteins), each sharing the capability to sense, transduce, and adapt to force. Furthermore, studies have shown that EC function is not only affected by force but also by temporal and spatial gradients in force (Blackman et al. 2000; Butler et al. 2000, 2002; Frangos et al. 1996). Thus, the temporal qualities of force application need to be matched to the dynamics of sensing, transducing, and adaptation of the transducer elements in cells.

Most information about mechanotransduction arises from the intersection of engineering and molecular biology. Forces can be applied to cell cultures followed by assays of population of cells, single cells and single molecules, and assays that evaluate whole tissue and organ morphology. Many approaches to understanding the mechanical origins of vascular disease use flow chambers to impart

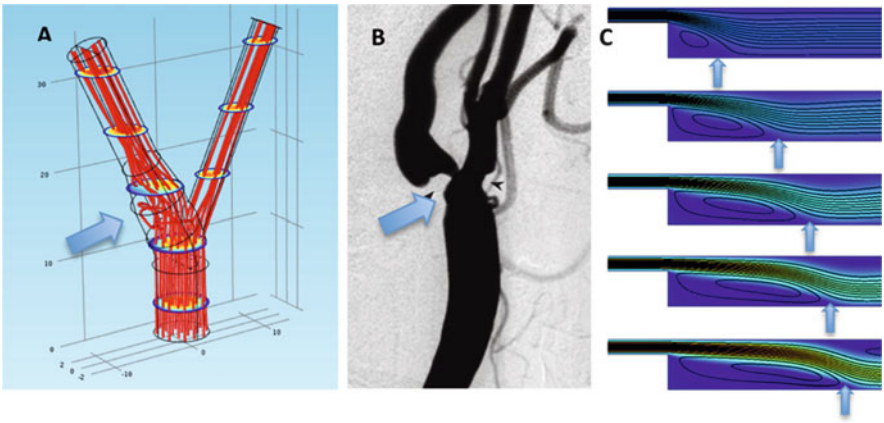

Fig. 9.1 (**a**) Streamlines and relative velocity magnitudes in the carotid bifurcation. (**b**) *In vivo* angiogram of stenosis (*arrows*) at areas predicted to experience disturbed laminar flow *in vivo* (compare with (a)) (Malek 1999). (**c**) In vitro, shear stress effects on endothelium can be studied by plating cells on the floor of flow chambers and subjecting them to fluid shear stresses that differ in spatial and temporal components (flow speeds and dimensions taken from (Haidekker et al. 2001) and solved for using COMSOL multiphysics simulation of the Navier-Stokes equations). *Arrow* indicates traveling location of the separation point as a function of time (frames are 0.1 s apart). To the *left of the arrow*, flow recirculates; to the *right* it increases to the free stream velocity

physiological levels of shear stress onto cells (e.g. see Fig. 9.1). Alternatively, some studies describe cell-generated traction forces, or consider the forces between the extracellular matrix and the endothelial cell. To begin to decipher mechanotransduction events, one must identify, characterize, and develop an engineering framework toward understanding how mechanical forces contribute to cell biology. One purpose of this chapter is to review these frameworks.

We begin this chapter with an overview of the shear effects on endothelial cells in atheroprone and atheroprotected areas of the vasculature. We then outline the frameworks for mechanotransduction of shear stress and discuss three of the main structural mechanisms by which force is transduced into biochemical signaling, namely, the glycocalyx, membrane, and cytoskeleton. We then discuss the possible mechanisms of force-induced changes in biochemical signaling and conclude with an example that focuses on force-induced nitric oxide production. When possible and helpful, we have created figures using finite element analysis of the Navier-Stokes equations, which describe the relationship between force and fluid dynamics, and equations of solid mechanics relating time-dependent force and cellular deformation. These engineering equations have been applied to realistic *in vitro* and *in vivo* geometries in order to quantitatively illustrate the relationship between cellular mechanotransduction and blood flow magnitudes and direction.

9.3 Shear Stress Is Associated with Vascular Health and Disease

9.3.1 Low Oscillatory Shear Is Atherogenic While High Unidirectional Shear Is Atheroprotective

In its most basic form, shear stress, τ, is the product of the blood velocity gradient (increase in axial velocity, u, as one moves away from a wall a distance y) and the blood viscosity, μ:

$$\tau = \mu \frac{\partial u}{\partial y}. \tag{9.1}$$

It is a frictional force in the direction of blood flow, tangent to the wall. Shear stress arises from blood flow, the temporal and spatial velocities ($\boldsymbol{u}$) of which are captured in the Navier-Stokes equations:

$$\rho \frac{\partial \boldsymbol{u}}{\partial t} + \rho \boldsymbol{u} \cdot \nabla \boldsymbol{u} = \; - \; \nabla P + \mu \nabla^2 \boldsymbol{u}. \tag{9.2}$$

In this form t is time, ρ is density, μ is viscosity, P is pressure, and $\boldsymbol{u}$ the velocity vector in three dimensions. The equation arises from equating the forces of pressure and shear with the acceleration of a differential element of fluid. In large blood vessels, inertial forces of blood flow dominate, while in small blood vessels frictional forces dominate. In general, this equation cannot be solved analytically. Rather, this equation can be implemented in complex geometries characteristic of branching blood vessels using finite element analysis, a numerical technique in which the complex flow field is divided into small elements on which the equation is linearized. The full flow field is then constructed by combining contributions from the small elements. Such simulations are indispensible in understanding the directional and temporal features of flow that are most likely responsible for the development of atherosclerosis via mechanotransduction by the endothelium. For example, in Fig. 9.1a, the flow streamlines and velocities were derived from solving the Navier-Stokes equations in a realistic geometry for the carotid bifurcation and a realistic pressure waveform.

These forces from fluid flow act on the blood vessel wall and induce deformation of the endothelial cells there. Further, flow chambers can be constructed in which the temporal and spatial gradient match the *in vivo* conditions. While it is beyond the scope of this chapter to describe the details of mechanical properties of cells, which is still an area of intense debate, evidence suggests that cells react to forces by deforming in a time-dependent manner, suggesting, in a continuum formulation, that they are viscoelastic, meaning that their deformation is proportional both to the magnitude of shear stress applied and to its rate of change. A recent study suggests that cells deform as viscoelastic liquids (Dangaria and Butler 2007), which can be described by Jeffrey's liquid model:

$$\boldsymbol{\tau} + \lambda_1 \frac{\partial \boldsymbol{\tau}}{\partial t} = \eta\left(\dot{\gamma} + \lambda_2 \frac{\partial \dot{\gamma}}{\partial t}\right). \tag{9.3}$$

where $\boldsymbol{\tau}$ is the stress tensor, η is the shear viscosity describing the resistance to shear strain, and $\dot{\gamma}$ is the shear rate describing the time-dependent deformation of the cell (in two dimensions, $\dot{\gamma} = \frac{\partial u}{\partial y} + \frac{\partial v}{\partial x}$, with u and v being the deformations in the x and y directions, respectively). This equation allows for deformation that is proportional to the stress as well as deformation that depends on the rate of change of stress, captured by two time constants λ_1 and λ_2. In one form of this equation, the cell can be described as a dashpot in parallel to a Maxwell element (in series dashpot and spring). Deformation of a cell in response to shear stress is small (on the order of 50–100 nm) and time dependent with a time constant on the order of 1–4 s (Fig. 9.2). This result suggests that small deformations lead to mechanotransduction

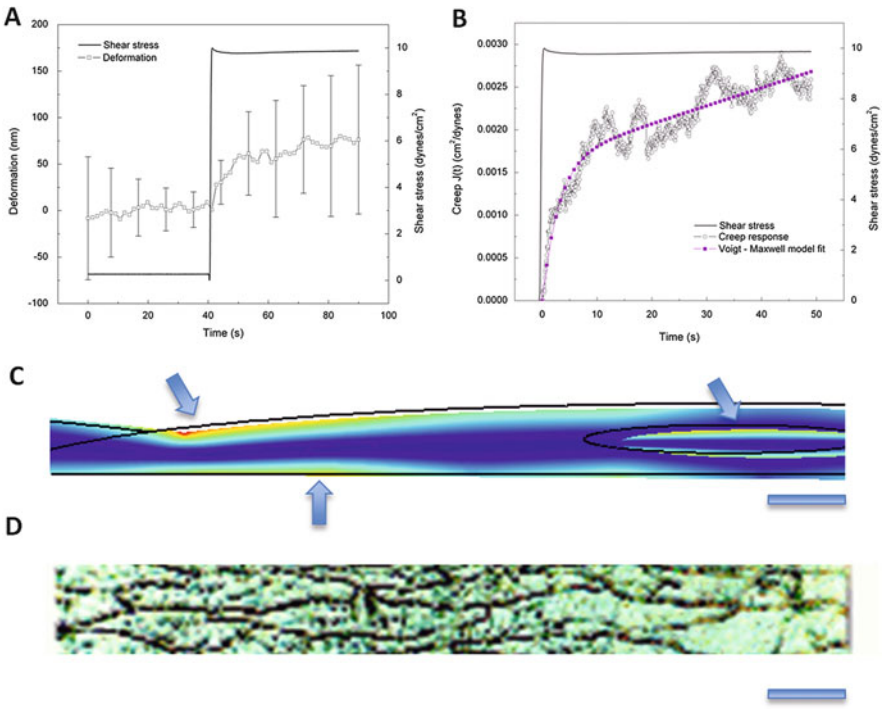

Fig. 9.2 (**a**) Shear stress results in time-dependent deformation of endothelial cells. (Dangaria and Butler 2007). (**b**) Creep in response to a step change in shear can be fit with a model for a viscoelastic liquid. (**c**) A simple viscoelastic model of a cell with a 2 s relaxation time was subjected to 1 Pa of surface shear. Deformation was on the order of 10 s of nanometers. *Arrows* denote areas of stress concentration in the membrane (*colors* represent relative von Mises stresses), at cell junctions, focal adhesions, and the nucleus. Bar = 10 μm. (**d**) Silver nitrate staining of endothelial cell borders in small arterioles (rat cremaster). Alignment of long axis of endothelial cell is in line with the prevailing flow direction. Bar = 20 μm

events and that the cell is potentially sensitive to temporal strains that are on the same time constant as flow pulsatility.

Studies that correlate flow patterns with vascular disease have determined that shear stress is the main determinant of whether the endothelium is atherogenic or atheroprotective (reviewed in (Cecchi et al. 2011)). Key features of blood flow that determine this distinction are shear magnitude and the temporal and spatial nature of shear stress. When low and oscillatory, shear stress has flow reversal that is associated with endothelial cells that are cobblestone in appearance, have elevated surface expression of leukocyte adhesion proteins such as vascular cell adhesion molecule-1 (VCAM-1) (Feaver et al. 2013), and increased production of monocyte chemotactic protein-1 (MCP-1) (Shyy et al. 1994) . The endothelium in these areas is leakier and more adhesive than similar endothelium found in areas of the vasculature that experience high unidirectional shear stress, even if it is also pulsatile. Such adhesiveness, leakiness, and cobblestone shape are strongly correlated with atherosclerotic lesion formation. In these areas, the temporal nature of the shear arises from the pumping action of the blood and elasticity of blood vessels, which behave as windkessels with phase angles between stretch and shear pulsatility that are greater than zero (Tada et al. 2007). In addition, when shear changes magnitude over short distances, spatial shear gradients exist which induce forces on cells that may be significantly different than forces on neighboring cells (DePaola et al. 1992). Regarding flow direction, areas experiencing oscillatory shear with flow reversal will see flow direction changes over the time of a heartbeat and an average shear around zero pascals.

Areas that experience atherogenic shear include large blood vessels with large curvature such as the aortic arch, coronary arteries, the carotid sinus, superficial femoral arteries near the adductor canal, femoral arteries, and aortic bifurcation, aneurisms, and areas downstream of stenoses. In addition, vascular devices intended to aid in improving vascular function and patency can induce alterations in blood flow that are atherogenic in, for example, stents (Koskinas et al. 2012) and vascular grafts (Kassab and Navia 2006). Thus, interventions often intended to ameliorate the effects of atherosclerosis can lead to relapses of the disease. Areas devoid of atherogenic shear include straight parts of the aorta, and in the microcirculation, where pulsatility and, hence, temporal flow reversal are absent.

When shear stress is unidirectional and high, pro-inflammatory mediators are downregulated (reviewed in (Davies 2009)). Such reactions to prevailing flow suggest that there are homeostatic mechanisms by which cells attempt to achieve an intracellular and intercellular tension that is optimal for health-related singling pathways. Long-term high shear downregulates the transcription factors required for MCP-1 transcription. High unidirectional pulsatile shear up-regulates KLF-2 (Kruppel-like factor 2), a molecule that is a beneficial to endothelial cell survival. Differences in KLF-2 are also seen *in vivo* with high KLF-2 on the medial flow divider where high shear stresses are predicted. Similarly, MCP-1 was found in flow divider regions where lower and oscillatory shear would be expected. These *in vivo* results corroborate *in vitro* results that suggest that areas experiencing low and

oscillatory shear are atherogenic, while high shear protects the endothelium and artery wall.

Methods to determine shear dynamics *in vivo* include non-invasive computational fluid dynamics (Giddens et al. 1993), which can provide detailed shear profiles even in the face of pulsatile flow-induced distention. Ultrasound techniques include pulsed Doppler and magnetic resonance imaging phase contrast velocity mapping. Alternatively ultrasound or MRI can be used to provide detailed anatomical representations of blood vessels. These images can be reconstructed using solid modeling software followed by image segmentation, meshing, and computational reconstruction (using finite element analysis) of the fluid-structure interactions. Such computational methods provide detailed stress profiles and they can also be used to calculate mass transfer of blood borne metabolites that might be atherogenic. Approaches to reconstruction of shear profiles based on anatomical data provide the possibility of patient-specific diagnosis and treatment (Cecchi et al. 2011).

The role of shear oscillations and shear stress gradients in vascular health and disease is covered extensively in other chapters of this book. In this chapter, we focus on the mechanism by which temporal and spatial shear gradients might induce mechanosensation and transduction in endothelial cells, the main cell type reacting to these flow dynamics.

While shear stress is one of the main aspects of blood flow governing susceptibility of the vasculature to atherosclerosis, to date there has been little progress on using this observation to prevent shear-induced atherogenesis, largely because it is difficult to conceive how one might alter shear *in vivo*. However, the fact that high shear is generally seen as atheroprotective means that regular exercise, which may increase blood flow rate temporarily, may help to mitigate the progression of the disease. The connection between a healthy vasculature and exercise is well known and covered in other chapters of this book. The role of high shear in prevention of stenosis could also help in surgical procedures such as vascular graft anastomosis where angle and suture strategies could be employed that minimize the occurrence of recirculation zones.

9.3.2 Evidence for Correlation Between Low Shear and Atherogenesis

One of the early studies demonstrating the correlation of low and oscillating shear stress was by Caro and co-workers (Caro et al. 1969). While others had noticed the focal nature of atherosclerotic lesions, many had assumed that mass transfer effects (such as platelets) or high shear stress-induced endothelial cell injury were responsible for the localization of disease. Caro et al. argued that high shear has a protective effect on the endothelium, a prescient observation that has been borne out in numerous subsequent studies. In high shear regions, the boundary layer is expected to be relatively thin while low shear areas would be marked by a thicker boundary layer. Thus, Caro hypothesized that atherosclerotic lesions should occur

where the boundary layer was thick, such as the outer walls (where the flow moves away) of daughter arteries. Observation of human superior mesenteric and renal arteries indeed showed that inner walls were generally spared lesions while the outer wall, immediately downstream from flow dividers, had evidence of lesions. From these observations, the authors proposed a new theory on atherosclerosis formation. They suggested that in areas of low shear, the shear rate is also low and this fact would minimize mass transfer away from these areas. The concomitant increase in local concentration of atherogenic blood borne substances (e.g. cholesterol) that would accumulate in these areas could contribute to the formation of lesions. From a clinical perspective, this suggests that exercise that increases heart rate may lead to the washing out these areas and thus explain its known health benefits (Caro et al. 1971).

To test further the role of hemodynamics in atherogenesis, Ku *et al*. developed realistic models of the carotid sinus lumen and measured flow velocities in 3D in areas of the bifurcation where cadaver specimens indicated atherosclerotic lesion formation and absence (Ku et al. 1985). In this study, the authors developed an oscillatory shear index that quantified how much of the instantaneous shear was in the direction of the prevailing shear of inner and outer walls where the midpoint was defined as the centerline of the sinus of the internal carotid. The outer wall (proximal internal carotid) was prone to atherosclerosis, while the inner wall was relatively protected. The formulation of this index recognized that not only low shear might be important but flow direction as well. Results show that the intimal thickness was positively correlated to the oscillatory shear index indicating that flow reversal at low shear stress is associated with intimal thickening.

To assess an *in vivo* link between vascular geometry, altered hemodynamics, endothelial wall shear stress and atherogenesis, Koskinas and colleagues performed an *in leporine* serial study using intravascular ultrasound to create 3D models of all major coronary arteries before and after induction of diabetes and hyperlipidemia, considered strong risk factors for atherosclerosis. They found that low wall shear stress promoted the formation of plaques (Koskinas et al. 2010). From a clinical perspective, these authors demonstrated *in vivo* correlation between low wall shear stress and expansive remodeling of the vessel wall.

However, not all studies point to a relationship between low and oscillatory shear stress and atherogenesis. Peiffer et al. conducted a systematic review of articles purporting to draw correlations between low and oscillatory shear stress and atheroma formation (Peiffer et al. 2013). Their study reviewed 27 articles out of an initial 406 that had in common the condition that lesions were formed only as a result of diet and flow patterns (not surgically induced) and that anatomical flow patterns were correlated to computational fluid dynamics simulations, so that calculations of wall shear stress came from hemodynamically accurate flows and in conjunction with true geometric properties. In this study, when looking at only the steady component of blood flow, nine of the 27 papers concluded that there was an inverse relationship between shear stress magnitude and wall thickening, confirming earlier hypotheses that low shear stress was atherogenic while high shear was atheroprotective. Even when considering shear averaged over the cardiac

cycle, three additional studies concluded that low shear co-localized with atherosclerosis. Other papers include oscillation by computing an oscillatory shear index. In theses studies, both high shear oscillations and low shear coincided with plaque location. Of course, shear oscillation and averaged wall shear do not account for all properties of flow that might be involved with plaque development. Shear angle and relative residence time of blood borne solutes may also be strongly correlated with plaques. While eleven articles supported the low and oscillatory shear theory of plaque progress, these studies used qualitative mapping and correlation with calculated shear to make their conclusions. Point-by-point comparisons in some cases failed to reach a correlation between low and oscillatory shear and intimal thickening. With respect to shear magnitude, a number of studies cited suggested that a wall shear threshold of 1–1.5 Pa range was associated with atherogenesis.

One possible solution to the controversy over low/oscillatory shear and other atherogenesis predictors is by Davies and co-workers who laid out a hypothesis suggesting that low shear leads endothelial cells to operate in a low level inflammatory state (Davies et al. 2013). ECs become susceptible to atherosclerosis in areas of low and oscillating shear where eddies concentrate undesirable metabolites such as reactive oxygen species (ROS) and where lower stress fails to elicit increases in anti-inflammatory genes. These phenotypes include cells with leaky junctions, higher levels of cell division and proliferation, and higher expression of receptors for monocytes. However, these phenomena are not sufficient for atherosclerosis without the existence of additional cardiovascular risk factors, such as hypercholesterolemia, hypertension, diabetes, and smoking. Thus, hemodynamic shear stress (a mechanical viewpoint) can be seen as priming the endothelium for atherosclerosis development, while mass transfer characteristic may contribute the actual development of atheroma. Pro-inflammatory events in these areas include suppressed endothelial cell nitric oxide synthase (eNOS) activity, increase NF-κB transport to the nucleus, decreased protective transcription factors KLF-2 and KLF-4, and reduced expression of the electrically conductive connexin 43. Interestingly, the increased in protein message causes overload in the endoplasmic reticulum (ER) of protein packaging, leading to an increase in misfolded proteins. These misfolded proteins cause an increase in upregulation of ER chaperones and folding enzymes to prevent aggregation of misfolded proteins. Thus is must be recognized that the link between mechanics and biology can be circuitous and highly indirect. Mechanotransduction often includes all of these pathways simultaneously, somewhat obscuring the cause and effects between mechanical force and changes in biological function.

9.3.3 Hemodynamics Influences Mass Transfer of Blood Borne Solutes

While is now generally accepted that shear forces play a dominant role in localization of atherosclerotic lesions, there has also been considerable number of studies on mass transport properties. For example, disturbed blood flow in areas of the

vasculature with abrupt geometric changes causes eddies, swirling flow, and reverse flow that can alter the degree of accumulation of blood borne lipids and blood cells, as well as the transport of substances produced by the endothelial cells themselves (e.g. ATP), leading to an autocrine activation of endogenous ATP receptors (Nollert et al. 1991; Yamamoto et al. 2003). Tarbell has reviewed the mechanism by which transport is important in atherosclerosis localization (Tarbell 2003). These mechanisms involve a reactive surface in which a species is transported to the endothelial cell surface and catalyzed by surface enzymes via Michaelis–Menten kinetics. In such a scenario, the shear rate (rather than shear stress) is responsible for modulating the local concentration of reactants, a principle modulator of the reaction rate. By comparing the mass transfer (characterized by a dimensionless Sherwood number) and the reaction rates (characterized by a Damkholer number), it is apparent that when $Da < Sh$ the process in reaction-limited (in other words, there are plenty of reactants and the rate is controlled by the reaction rate), whereas when $Da > Sh$, the reaction is transport-limited (reaction is controlled by the rate at which reactants are transported because the reaction proceeds much faster). Therefore, in the reactive surface model, flow rate, rather than force, controls the rate of reaction, particularly in areas where the flow is low and reversing.

The most important feature of transport is the fact that geometry of blood vessels creates unique patterns of blood flow. In the longer straight parts of aortae, the flow is unidirectional with an overall pulsatility governed by the heart rate. In these areas, Sh for small molecules such as O_2 and ATP is low relative to Da making the reaction fluid transport limited. Alternatively, larger molecules such as albumin and low density lipoprotein (LDL) are not transport limited. Such analysis suggests that transport may be an important factor in the localization of lesions if the mechanism of lesion formation requires small molecules. In support of a small molecule origin for atherosclerosis, hypoxia appears to be prevalent in recirculation zones and at areas of the vasculature that are predicted to have a low Sherwood number. Indeed, the areas of low shear often correlate with the areas of low concentration of small molecules such as oxygen and ATP. Thus, a vexing and outstanding problem with investigating the connection between mass transfer and focal nature of atherosclerosis is that the shear and mass transfer are linked.

Thus, the precise relationship between shear, mass transfer, and vascular disease remains uncertain (Ethier 2002). Attempts to separate the two generally focus on altering shear using viscosity-altering agents. Nollert and colleagues investigated the role of mass transfer and shear directly using cultured endothelial cells (Nollert et al. 1991). In their study, initiation of flow caused the increase in prostacylcin production, arachadonic acid incorporation, mRNA, and calcium. They then investigated the potential role of mass transfer using a computational model of ATP convection and diffusion because flow did not appear to increase calcium in the absence of ATP. Overall, the role of mass transfer in activation of cells via fluid flow may depend on the receptor pathway involved. Some pathways appear to be shear stress (mechanical) sensitive while other pathways are shear rate (convective flux) sensitive. Nevertheless, because shear is so important, at least in so far as it

acts to allow or inhibit inflammation of the endothelium, depending on its spatial and temporal features, much research has focused on engineering analysis of shear-induced deformation of endothelial cells and their subcellular structures. It is hoped that by such analysis, the identity of shear stress sensors and transducers can be found.

9.4 The Main Frameworks for Mechanotransduction Include Structural Transduction and Transduction Through Diffusive Pathways

9.4.1 Sensitivity of Cells to Force Dynamics Depends on the Dynamics of the Pathway Stimulated

Hoffman and colleagues reviewed studies to determine if mechanotransduction occurs through a switch mechanism (Hoffman et al. 2011). In this framework, mechanotransduction works through mechanotransmission, mechanosensing, and mechanoresponse. Since load-bearing structures are structurally dynamic, forces may elicit mechanoresponses through alterations in intrinsic rates of subcellular responses. By understanding in the rates of cellular reactions in cells, it may be possible to determine why cells are sensitive to multiple time scales of temporally changing mechanical stimuli. In a switch model, mechanotransduction begins with mechanotransmission, by which forces are transmitted directly or remotely to mechanosensitive elements. For example, force applied on the apical side of the cell exerts effects through focal adhesions on the basal side of the cell. In this case, the entire cell acts as a mechanotransmitter.

In mechanosensing, forces applied to a structure are accommodated by alterations in either the molecular assembly of that structure, or in the conversion of a protein from one conformational state to another. Proteins sample many energy minima and can be transformed from one to another by ligand binding. It is not clear yet whether forces can directly induce a protein to convert into an active conformation that is equivalent to one know to be induced by ligand binding (Lee et al. 2007), but it is known in many instances that methods to block protein function (e.g. antibodies, siRNA knockdown) are effective if blocking certain mechanotransduction events. As discussed later in this chapter, mechanosensing can occur with changes in conformation of a protein, whole protein unfolding, or alteration in association with other proteins.

Mechanoresponses are the cellular processes that are initiated after mechanosensation. Theses processes (such as calcium signaling, phosphorylation pathways, and transcription factor binding to DNA) may not be directly force sensitive, but they participate in the most dramatic elements of mechanotransduction. As discussed later, there remains some distinction between mechanotransduction in which all forces are carried through force bearing structures to the final mechanically induced response (e.g forces transmitted to the nucleus to affect transcription of DNA) and those mechanotransduction pathways that work through diffusive elements (e.g. MAPK signaling pathways

leading to activation of transcription factors). In switch-like models, there is often a direct connection of load bearing structures to the mechanosensor. A mechanosensor might be an ion channel, or a protein, whose force-induced conformational changes make it accessible to other adapter proteins (Bao et al. 2010; Lee et al. 2007). Further studies are necessary to demonstrate how such a mechanism could also depend on the temporal aspects of applied force, as have been shown to be important for many mechanoresponses.

Other models must incorporate reaction rates. In focal adhesions, where many force sensitive molecules reside, it has been found that integrins and actin are continually recycling and flow in response to force. The recycling has a time constant of seconds to minutes (Lele et al. 2006), while flow is on the order 0.1 μm/min. Therefore, observable dynamics of focal adhesions in response to force is on the order of minutes to hours. These multiple time constants make these structures differentially sensitive to the temporal features of the applied forces. Membranes have dynamic microdomains that assemble and disassemble on the order of milliseconds (Eggeling et al. 2009). Since stretch-induced ion channels can be activated by force on the order of milliseconds, one can see how there is a relationship between time course of molecular reorganization of a structure and the time courses of its mechanoactivation.

Because broken links cannot transmit force, the dynamic interaction with linking partners dictates the transmission of force to mechanosensors. Slip bonds have very short lifetimes while catch bonds have longer lifetimes and their binding strength increase with force. With respect to the whole cytoskeleton as a force transmitter, forces can cause fluidization of the cytoskeleton, therefore reducing its ability to transmit force. In other cases, reinforcement results in a nonlinear relationship between applied force and the force reaching mechanosensors. This interplay of reinforcement and fluidization can be manifested in viscoelastic behavior, which carries with it a source of sensitivity to a range of frequencies of force transmission.

Similarly, time scales of mechanosensing can dictate how temporally applied forces are interpreted by cells. When the mechanoresponse is dynamic, the feedback processes that alter mechanosensitive molecules and organization of mechanosensitive molecule complexes can result in responses that depend on the temporal nature of the applied force. For example, the formation of focal adhesions requires diffusion of integrins into the plaque, binding of integrins to extracellular matrix, reinforcement by intracellular binding of focal adhesion kinase and talin and actin. Subsequently, in mature focal adhesions, Zyxin binds to plaques that are in need of repair after force application by recruiting α-actinin leading to actin polymerization and repair. Therefore if cyclic strain was applied to these plaques, mechanosignals that occurred on the same temporal scale as the frequency of force application would experience strains that ranged from positive to negative.

As a rule of thumb, when forces are applied statically, such as the average forward component of unidirectional shear stress, the mechanoreceptor is likely to be a stronger bond with a long life and can sense force by undergoing a conformational change to a new functional shape. In contrast, with rapidly changing forces, such as the pulsatile component of blood flow, mechanosensing is likely to arise from weak linkages between proteins or weakly folded proteins. Regarding flow/

glycocalyx/caveoli/eNOS signaling pathway, research remains as to which parts are sensitive to statically applied forces and which parts are sensitive to cyclic shear applications.

9.5 Shear Stress Acts on the Apical Surface and Is Transmitted to the Cell Interior by Cellular Structures

9.5.1 Shear Stress First Acts on the Glycocalyx

The glycocalyx is a highly glycosylated structure on the surface of almost all cells (Reitsma et al. 2007). In endothelial cells, it has been visualized by electron microscopy and fluorescence confocal microscopy (Lipowsky et al. 2011) and is likely to exhibit a thickness of between 100 nm and 0.5 μm . However, it is thicker in large arteries where it grows up to as much as 4.5 μm in the carotid artery, compared to 0.5 μm in capillaries. Its net composition is a result of dynamic equilibrium between the formation of new molecules and flow-mediated washout of old molecules (Lipowsky et al. 2011). Interspersed with the glycocalyx are membrane-anchored glycoproteins. The selectins are the most studied because of their role in adhesion to circulating leukocytes. Importantly, they tend to be significantly shorter than the proteoglycans and thus are buried in the glycocalyx. Thus the glycocalyx needs to be digested away in order for leukocytes to adhere to the glycoproteins (Lipowsky et al. 2011). While there is significant evidence that glycocalyx proteins are involved in shear stress sensing, there is little evidence that the selectins or other glycoproteins are.

The glycocalyx is composed of acidic oligosaccharides anchored to the membrane via glycoproteins (Pahakis et al. 2007). In addition, there are unanchored polyanionic constituents. Overall the structure is slightly negatively charged and therefore significantly hydrated. In this structure, heparin sulfate (HS), chondroitin sulfate (CS) and hyaluronic acid (HA) link with membrane-bound glypicans and syndecans. Syndecans (1, 2, and 4) are large transmembrane proteins decorated with HS, to which they have a high affinity, with a lower binding affinity to CS. As transmembrane proteins, syndecans enable transduction of forces from outside the cell to the inside. Glypicans, in contrast, form GAG attachments close to the surface of the cells and are decorated exclusively with heparin sulfate. Interestingly they are associated with glycophoinositol (GPI) anchors, which are, in turn, associated with cholesterol-rich microdomains termed caveolae and rafts, suggesting that known involvement of the membrane with shear-induced signaling may be through glypicans and rafts. HA weaves through the glycocalyx and does not associate directly with glypicans or syndecans. Rather, HA links with membrane-bound CD44 receptor that localizes in caveolae.

The syndecans, glypicans, HS, CS, and HA make up a highly ordered periodic structure on the cell surface. To study this structure, Squire and colleagues stained the glycocalyx of frog mesentery endothelial cells using a variety of staining techniques followed by electron microscopy (Squire et al. 2001). They found that the glycocalyx had a quasi-regular meshwork with a characteristic periodic spacing

of 20 nm. Further, these periodic structures were bundled and anchored to the cell surface at attachment points spaced on the order of 100 nm apart. This spacing is similar to known spectrin (fodrin) periodicity of around 100 nm suggesting that the bundles were anchored to the actin/fodrin cortical cytoskeleton through transmembrane proteins. Thus, forces transmitted to the glycocalyx may be mechanically transduced directly to the underlying cytoskeleton. Importantly, with respect to mechanotransduction, this arrangement appears to be sufficient to significantly attenuate flow within the glycocalyx suggesting that fluid shear is transduced through the solid portion of the glycocalyx rather than through fluid shear stress per se. Their data also suggested that the thickness obtained from rapid freezing and staining is on the order of 100–200 nm. Arkill and colleagues followed up on the observation of periodic spacing of 20 nm observed in frog mesentery capillaries (Arkill et al. 2011). In this study, they tested for the ubiquity of this spacing in mammalian tissue including choroid, renal tubules, glomerulus, and psoas muscle. Each of these tissues revealed a 20 nm spacing as was observed in frog, suggesting a universality of patterned spacing. Interestingly the 100 nm spacing of bundles was not always observed with some spacing being larger. This suggests the 20 nm spacing may represent the minimal spacing possible.

With respect to mechanotransduction, Ebong and colleagues investigated the roles of HS proteoglycan in shear-induced eNOS activation and cytoskeletal remodeling (Ebong et al. 2014). They specifically focused on the protein anchors used by heparin sulfate. By knocking down glypican but maintaining the syndecan anchor, they showed that shear induced cytoskeletal remodeling but failed to elicit eNOS activation. This result suggests that eNOS is immediately activated by virtue of its association (perhaps indirectly through caveolin) with GPI-anchored protein glypican. Similarly, when syndecans were knocked down and glypicans were intact, shear failed to induce remodeling, but was able to activate eNOS. This suggests that syndecans, by virtue of their direct linkage to the cytoskeleton, transmit shear to the cytoskeleton in a decentralized mechanotransduction model. In contrast, the glypican anchor is more intimately tied with eNOS activation suggesting that it plays as a role in focal mechanotransduction.

To complement observations that the glycocalyx is involved in mechanotransduction, a number of studies have attempted to quantify the forces in the glycocalyx and forces that arise as a result of its interaction with the membrane and cytoskeleton. Secomb modeled the endothelial cell surface layer as flow through vertical strands and predicted a significant attenuation of fluid flow (and hence shear) in the glycocalyx (Secomb et al. 2001). Weinbaum and colleagues idealized the glycocalyx as stalks of core proteins of varying density and length, consistent with the Squire model (Squire et al. 2001). In this model, shear stress acts on the tips of the core proteins and deflects them (Weinbaum et al. 2003). Most of the flow is attenuated before it actually reaches the cell membrane. The drag on a single core filament was calculated to be on the order of 7×10^{-4} pN for a shear of 10 dyn/cm^2. This force is likely to be insufficient to cause major rearrangement of the underlying cytoskeleton or to alter the conformation of attached core proteins. However, the drag on a collection of core glycocalyx strands anchored to a single core protein may cause the core protein to experience a

force of 1.9×10^{-2} pN, sufficient to result in about 6 nm displacements. With a mechanical advantage afforded by the long strands, this displacement could be as much as 17 nm, which may be enough for mechanical activation. It is not clear, however, if cells respond to apical surface deformation or internal strain or stress of mechanosensors deep in the cell. Following Secomb's and Weinbaum's models, Ferko and colleagues estimated flow through the glycocalyx on a model of the cell that was constructed directly from fluorescence images (Ferko et al. 2006, 2007). Using finite element evaluation of a Brinkman layer whose dimensions and porosity were estimated from the Weinbaum model, they demonstrated significant transmission of stress to the cell membrane surface as well as to basal regions of the cell and nucleus. Thus the forces integrated from drag on the glycocalyx may lead to increased force production deeper in the cell, at focal adhesions, nucleus and cell junctions.

9.5.2 The Glycocalyx Is Dynamic and Spatially Non-Uniform

Complicating the role of the glycocalyx as a mechanotransducer is the fact that it is spatially non-uniform and compositionally dynamic. For example, Forbes-Dewey and colleagues have demonstrated that HS redistributes in the face of shear stress (Giantsos-Adams et al. 2013). In addition, enzymatic degradation caused the release of GAGs while repopulation of GAGs was shear stress dependent. Shear accelerated repopulation of GAGs and proteoglycans to 12 h compared to 20 h for static cells. These data further suggest that the atheroprotective effects of shear may be related to its ability to simulate cells to produce glycocalyx. Moreover, this remodeling of the glycocalyx was necessary for realignment of cells in the flow direction. The authors further developed a kinetics model to suggest that shear induced an increase in exocytosis of HS. Such studies are consistent with *in situ* imaging of the endothelial cell surface layer in which the inflammation-induced shedding of the glycocalyx could lead to regrowth that is shear stress dependent (Lipowsky et al. 2011). Using high-speed confocal intravital microscopy, Lipowsky and colleagues demonstrated that inflammation in the microvasculature is associated with glycocalyx shedding (Lipowsky et al. 2011). In this study, FMLP was used to induce inflammation. This work suggests that a link between atherosclerosis, shear and mechanotransduction may be through inflammation such that inflammation causes shedding, which causes a reduction in mechanosensation leading to further reduction in production of vascular protective molecules such as NO.

Other recent studies suggest that shear stress can induce alterations in the structure of the glycocalyx (Gouverneur et al. 2006) where exposure of endothelial cells to 24 h of atheroprotective-like shear of 10 dyn/cm^2 (1 Pa) led to increased incorporation of glycosaminoglycans in the glycocalyx. This production helps improve the integrity of the glycocalyx and may explain why high shear regions are protected from atherosclerosis. It is possible that increased GAGs make the shear-eNOS pathway more likely to be activated than in areas where shear is low and GAG synthesis is inhibited.

With respect to how the glycocalyx may transmit forces from flow to the cell, Zeng and Tarbell demonstrated that glycocalyx anchors, membrane rafts and the actin cytoskeleton may constitute a mechanosome (group of molecules that, together, form a mechanosensitive structure) (Zeng and Tarbell 2014). They showed that shear stress caused reorganization of each of these components in concert with each other. Consistent with their architecture, glypicans, which are peripheral membrane bound proteins free to move in the plane of the membrane, associate with GM-1 (lipid raft marker) and may play a role in the transport of heparin sulfate to the junctions of cells after 30 min of shear stress. Conversely, syndecans, which are transmembrane and bound to the cytoskeleton, associate with HS and CS but remain fixed during the 30 min of shear stress. After 24 h of shear stress, new caveolae were produced and glypicans-associated lipid raft redistribution contributed to a uniform distribution of HS and CS on the cell surface. This study points to the role of lateral diffusion and dynamic production of surface mechanoreceptors in the mechanotransduction of shear stress.

9.5.3 Forces from Shear Stress can Result in Force Transmission to the Membrane and the Membrane Microdomains, Rafts and Caveoli

The cellular plasma membrane is made up of a 5-nm thick lipid core that houses various carbohydrates and proteins that are in either the outer leaflet, the inner leaflet or transmembrane proteins. The core is made up of two phospholipid monolayers which are stabilized by hydrophobic and van der Waals interactions. The multiple combinations of lipid hydrophilic head groups and hydrophobic acyl chains permit the inclusion of various molecules such as glycerophospholipids, sphingolipids, glycolipids and sterols. In addition, complex lipids are not distributed randomly in the membrane but form lipid domains, where sphingolipids and cholesterol are segregated in dipalmitoyl phosphatidyl choline-rich membrane areas. These domains are involved in the trafficking and sorting of specific proteins, as well as in signal transduction processes. Therefore, there is a link between the composition of lipid membrane domains and their biological and functional properties. However, the molecular basis and implications of these links to cellular function are not yet fully understood.

Membrane rafts are small ~50 nm groups of liquid-ordered domain lipids. Small rafts are able to form larger stabilized platforms by means of protein-protein and protein-lipid interactions. Membrane rafts may also function as general signaling compartments, allowing the clustering of proteins, such as integrins, that regulate cytoskeletal organization. The composition of these domains, besides being rich in sterol- and sphingolipids, is also associated with the inclusion of gangliosides. Gangliosides are complex glycolipids that have a strong amphiphilic character because of their large saccharidic head groups and the double-tailed hydrophobic moiety. They are particularly abundant in the plasma membrane, where they are inserted into the external leaflet, with a hydrophobic ceramide moiety and with the oligosaccharide chain protruding into the extracellular space. The lipid moiety of

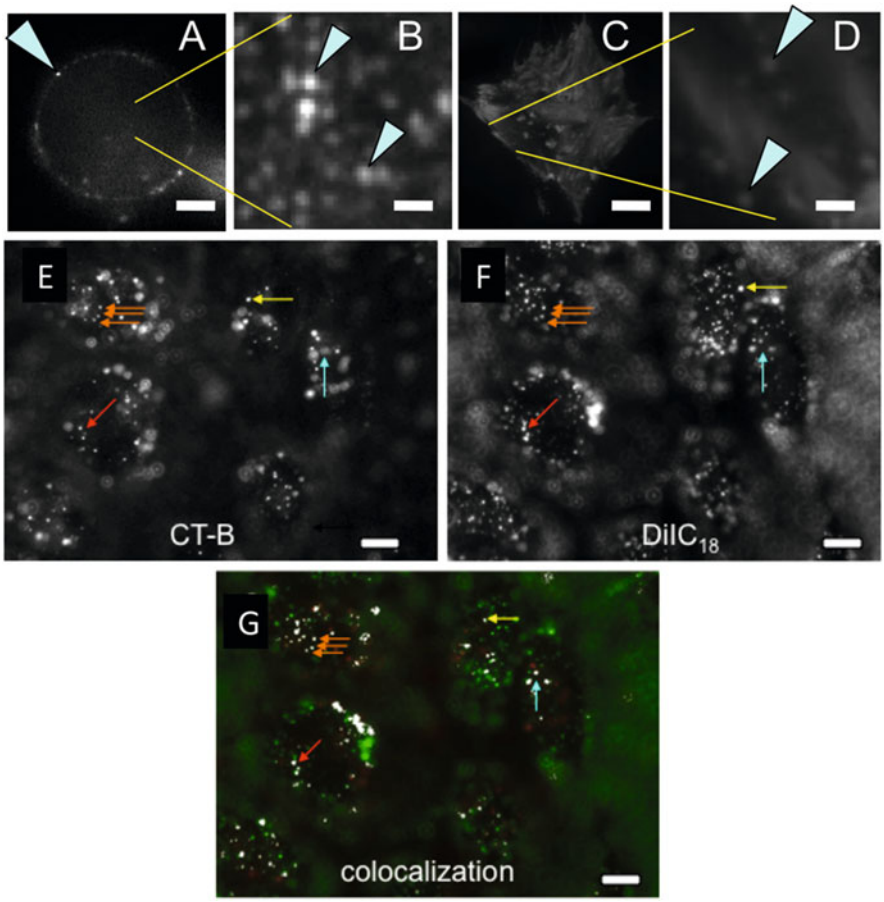

Fig. 9.3 (**a**) Phase separation in giant unilamellar vesicles (Diameter ~ 50 μm). (**b**) Close-up of domains seen in (a). Morphological similarities to domains seen in (c). (**c**) Total internal reflection fluorescence image of DiI-C_{18} stained cells. Higher intensity indicates localization within 100 nm of coverslip (focal adhesion). (**d**) Liquid ordered phase doamins. (**e**–**g**). Colocalization of DiI C_{18} with cholera toxin B (CT-B) indicates presence of ordered domains. Bars in (a, c, e, f, g) = 10 μm; Bar in (b, d) = 1 μm

gangliosides, shared with all sphingolipids, is called ceramide. The common name of this is sphingosine and is connected to a fatty acid by an amide linkage.

There are different oligosacharide chains associated with various gangliosides. The oligosaccharides depicted lack the membrane-anchoring ceramide, which would be attached to the terminal Glc in the GM1 ganglioside. The GM1 is the most abundant ganglioside and is commonly used to identify membrane raft using fluorescence (see Fig. 9.3) by taking advantage of the strong affinity between GM1 and cholera toxin subunit B.

Caveolae are dynamic structures that can fuse or leave the membrane through endocytosis in order to regulate their density (reviewed in (Parton and del Pozo 2013)) in response to membrane stresses. As such they are ideal candidates for mechanoreceptors of surface shear stress. Caveolae are cholesterol-rich microdomains that are curved by virtue of their association with caveolin, a major structural and scaffolding protein, as well as a newly discovered cavins, which associate with caveolin and assist in curvature generation. These 60–80 nm invaginations are composed of ~150 caveolin molecules that help form the membrane into a cup shape that is open on the exoplasmic side of the membrane. While caveolins have been implicated in mechanosensation of shear stress, there is currently very little research on cavins and even less on whether these molecules participate in mechanosensation. Caveolin molecules are located on the interfacial leaflet with a characteristic hairpin turn that integrates into one leaflet. Cavins appear to stabilize caveolin oligomers after trafficking to the plasma membrane. Importantly, the lipid make-up (e.g. cholesterol) ensures that caveoli are liquid-ordered microdomains. Potentially important for mechanosensing, when order is disrupted or the architecture of caveoli is forced into a flattened state, caveoli disassemble via caveolin-1 and cavin 1 disassembly. Cholesterol depletion causes similar dissociation. Thus, this ease of disassembly may make caveoli a metastable structure capable of sensing minor membrane forces.

Also important for mechanosensing is PIP_2 (phophatidylinositol-4,5-biphophate), which is enriched around the caveolar opening (Parton and del Pozo 2013). This lipid has long been associated with shear sensing in that conversion of PIP_2 to IP_3 leads to binding of IP_3 to receptors on the ER and opening of calcium channels (Park et al. 1998). The resultant release of calcium to the cytoplasm appears to be necessary for the production of nitric oxide via the calcium-calmodulin system. Cytoskeletal modulation of caveolin organization via actin has been demonstrated (Parton and del Pozo 2013). On the cell surface, actin appears to bind to caveolin via PKCα. In addition, caveolae are known to associate with actin and can be organized in long straight lines along stress fibers. As such, experiments that degrade stress fibers, likely alter the organization and structure of caveolae.

A main mechanism of mechanosensing may be caveolae flattening, which can arise from surface shear or through cell stretch as in capillaries under high transmural pressure (Schmid-Schönbein et al. 1995). The excess membrane afforded by caveolae results in increase in the pool of membrane needed during mechanical activation. This process can protect against membrane lysis. Whether membrane flattening is the means by which caveolin in caveolae is activated, is not known directly. However, it is known that caveolin can be phosphorylated in response to membrane stress and that this depends on β_1 integrin activation and src-kinase activity. Src-kinases are known to be involved in mechanosensing. Such phosphorylated caveolin can regulate Rho-dependent actomyosin contraction. As reviewed by Parton and del Pozo (2013), one possible mechanism of

mechanosensing is when cells are rich in caveolin-1, stretch causes caveolae to flatten and cavin 1 then dissociates from caveolin 1. Without cavin 1, p190RHOGAP can be sequestered in the liquid-disordered parts of the membrane leading to loss of Rho inhibition by p190RHOGAP. When Rho is free to be active, it leads to actomyosin contraction, ECM remodeling, and integrin-dependent changes in focal adhesion assembly. Conversely, when p190RHOGAP is sequestered in the liquid-ordered parts of membrane, it is more likely to inhibit Rho leading to the preservation of actomyosin contraction.

G-proteins have long been suspected as being involved in mechanotransduction of shear stress. Oh and Schnitzer found that G-proteins reside in caveolin on the cell surface and are activated there (Oh and Schnitzer 2001). Specifically Gq proteins are associated with caveolae, and Gi and Gs proteins are associated with lipid rafts. This distribution may explain the differential activation of these proteins by shear noted by Frangos and colleagues (Bao et al. 2000). Rizzo and colleagues showed that flow activates endothelial nitric oxide synthase in caveolae (Rizzo et al. 1998). This activation was associated with dissociation of caveolin and calmodulin. Interestingly this finding is consistent with the role of the calcium-calmodulin system known to participate in shear-induced nitric oxide synthesis (Isshiki and Anderson 1999). Rizzo and colleagues exposed endothelial cells to shear stress and noted increased caveolin at the cell surface. The density of caveolae was enhanced sixfold after shear exposure. Importantly, it was only after shear that the density of caveolae resembled the i*n vivo* density and distribution suggesting that chronically loaded cells are more *in vivo* like. Importantly, flow-conditioned cells exhibited higher density of caveolae at cell borders, confirming earlier reports of functional activation at cell-cell junctions (Rizzo et al. 2003). Park and colleagues showed that caveolin participates in the mechanotransduction of shear stress and extracellular signal regulated kinase (ERK) phosphorylation. In this study, the group developed a caveolin antibody that prevented shear-induced activation of ERK, suggesting that caveolin 1 participates in the pathway between shear and ERK activation (Park et al. 2000). Interesting, this antibody bound to oligomerization domains of the caveolin and prevented its oligomerization. Thus clustering of caveolin may play a role in its ability to transduce shear into ERK activation.

Yamamoto and colleagues demonstrated that shear stress can elicit ATP release and that this release coincides with the location of caveolae (Yamamoto et al. 2011). It is thought that this ATP binds to cell surface purinergic receptors to initiate increases in cytosolic calcium, a known contributor to shear-induced nitric oxide release. Importantly, ATP release occurred exactly at locations where caveolin 1 was enriched, suggesting that local autocrine mechanism may play a role in shear-induced calcium signaling. Together with research on glycocalyx, it is possible that glycocalyx connection to proteins in caveolae may lead to increased ATP production followed by calcium influx and eNOS activation. Removal of cholesterol attenuated shear-induced ATP release. Furthermore, the location of initiation of calcium waves coincided with the location of caveolin at cell junctions

and ATP release was largely localized to the cell edges. These temporal and spatial correlations suggests that there may be a convergence of research on force transmission from the cell surface to the cell edges, PECAM-1, membrane order, ATP release, calcium increases, and eNOS activation.

Research on the association of mechanotransduction events with rafts is less prominent, owing to the fact that rafts have no real structural protein associated with them and they are very dynamic (Eggeling et al. 2009). The association of the glycocalyx with rafts via glipicans and GM-1 gangliosides suggest that they could be an important mechanotransducer. Recently, Fuentes and colleagues showed that rafts are the first participants in adhesion to the extracellular matrix via β_1 integrins and fibronectin (Fuentes and Butler 2012). In addition, Wang and colleagues noted that mechanically activated src activity appeared to originate from Lyn- rich microdomains, commonly thought to be rafts (Lu et al. 2008).

9.5.4 Stresses on Membrane are Non-Uniform and Increase in the Upstream Direction

Satcher et al. noted that the undulating cell shape characteristic of endothelial cells produces surface shear stresses that are non uniform (Satcher et al. 1992). Later, Barbee and colleagues measured the exact surface undulations using atomic force microscopy and showed that shear stress gradients occur over the surface of single cells (Barbee 2002). In order to understand the relationship between local and distributed models of mechanotransduction, Ferko and colleagues developed a multi-structural model of sheared endothelial cells that included focal adhesions, the nucleus and glycocalyx (Ferko et al. 2007). They found that focal adhesions caused large stress concentrations arising from having sudden changes in membrane support by the extracellular matrix (see Fig. 9.4). In addition, larger stress concentrations occurred around the cell nucleus upon shear because of the juxtaposition of low and high moduli organelles. These results suggest that the forces arising from the application of shear stress to the cell surface can be significantly amplified in part because of the unique architecture of the endothelial cell. This model was made possible because of the development of methods for accurate delineation of surface topography of the cell from fluorescence and confocal microscopy (Ferko et al. 2006).

Despite the fact that shear distribution is not uniform, there have been only a few papers that identify that this shear distribution correlates to mechanotransduction events. For example, Fung and Liu developed a model of the endothelial cells that attempted to predict the forces occurring on the membrane from shear stress (Fung and Liu 1993). They found that the stresses can accumulate appreciably as one moves upstream. This result arises because membrane stresses result from an integration of all the stress felt downstream. This accumulation of stress on the upstream side of the cell may explain why Butler and colleague found a shear-induced increase in fluidity on the upstream side of the cell and not on the downstream side (Butler et al. 2001). Recently, such subcellular stress distributions

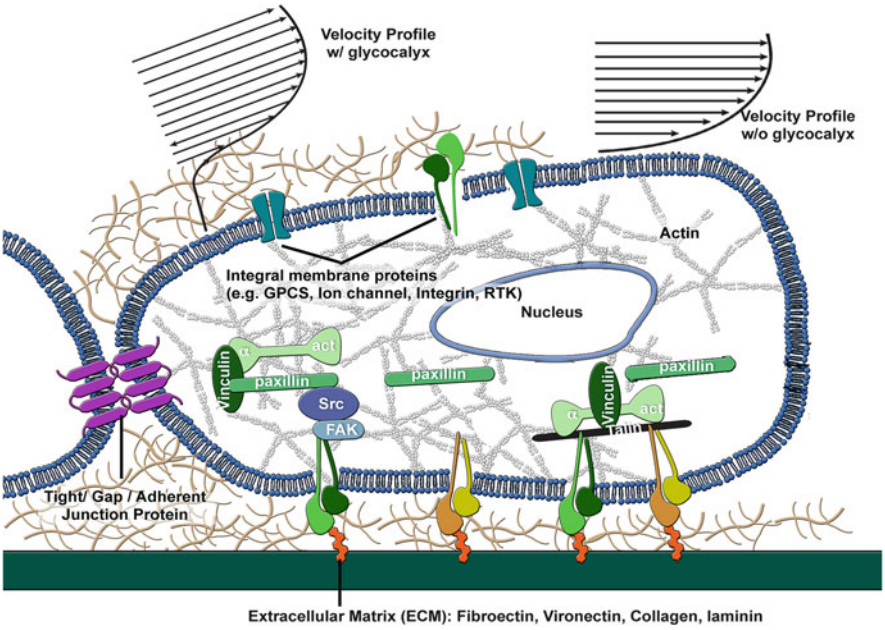

Fig. 9.4 *Top*: Shear stress impacts the glycocalyx or membrane. The forces are transmitted via the cytoskeleton to cell junctions, focal adhesions, nucleus and other organelles. Molecular complexes in these locations transduce force to soluble signals such as transcription factors or vasodilators and constrictors (Figure adapted from (Kamm and Mofrad 2009)). *Bottom*: The range of forces is in pN (result of about 1 Pa shear stress) and cause deformations on the order of 10 s of nm. This deformation results in focal strains at focal adhesions and near the nucleus (Bottom figure adapted from (Ferko et al. 2007))

have been modeled quantitatively. These results suggest that shear stress can result in high stresses at focal adhesions and cell junctions, an emerging mechanosensitive organelle (Bagi et al. 2005; dela Paz et al. 2014; Fujiwara et al. 2001; Tzima et al. 2005) in which PECAM-1 may constitute part of a mechanosome.

9.5.5 Forces in the Membrane Result in Alterations of Lipid Mobility

Butler and colleagues discovered that shear-induced stresses are likely to be non uniform as indicated by changes in the diffusion of lipids in response to applied surface shear stress (Butler et al. 2001). Accordingly, the increases in membrane fluidity on the upstream side of the cell corresponded to increased positive shear stress gradients as predicted by Barbee and colleagues (Barbee 2002). In this study, the authors used fluorescence recovery after photobleaching (FRAP) with a single scanning laser in the direction of flow to catch, within 2 ms, the bleach event and recovery of an upstream part of the cell and downstream nearly simultaneously.

Newly derived FRAP equations allowed the calculation of diffusion coefficients. These diffusion coefficients of DiI indicated changes in the membrane fluidity upon shear stress. Recently, Yamamoto and colleagues were able to validate these results and show that fluidity increased differentially in liquid-ordered domains (Yamamoto and Ando 2013).

Tabouillot and colleagues investigate shear-induced fluidity changes of membrane domains by staining the lipid domains differentially with long chain DiI (18 carbon stains rafts and caveolae) and short chain dyes (staining liquid disordered parts of the membrane using DiI C_{12}) (Tabouillot et al. 2011). In these studies, they used time correlated single photon counting (TCSPC) to determine changes due to the application of shear stress, of the fluorescence lifetime, diffusion coefficients (FCS) and number of molecules arising from a small confocal volume that intersected a small section of the membrane (system described in (Gullapalli et al. 2007)). They found that shear induced a decrease in lipid order (as assessed by a decrease in fluorescence lifetime) in the liquid-disordered part of the membrane that was rapid and transient (over a few 10s of seconds) while shear induced a later but sustained decrease order of liquid-ordered membranes. This suggests that membrane microdomains are differentially sensitive to applied stresses, which is consistent with the notion that these domains have different moduli, with liquid-ordered domains (cholesterol rich) being stiffer then liquid-disordered domains (Shamitko-Klingensmith et al. 2012). In addition to changes in lipid order, number of molecules decreased with application of shear. This decrease can be explained by a flattening of the cell membrane with shear. Such flattening may be important stimulus for the disassembly of caveolin, dissociation of cavin, sequestering of p190RHOGAP, loss of inhibition of RHO and actin-myosin contraction and mechanosensing (Parton and del Pozo 2013).

9.5.6 Forces Can Act on the Cytoskeleton, Which Has Static Structural Integrity as Well as Dynamic Reorganization

Wang and colleagues highlighted the importance of tensegrity structures, which are a combination of elastic and compressive elements that combine to give the cell its structure (Wang et al. 2009). When a tensegrity structure is deformed by an applied force it reaches a new equilibrium in which forces are transferred to other structures. Such force transfer could be a way to rapidly transmit force to distant parts of the cell where it is transduced into mechanoresponses. This framework for mechanotransduction explains some of the possible force-at-a-distance phenomenon observed in cells, but it does not include diffusive factors that were converted upstream and diffusion of activators downstream. While the focus was on structures that maintain structural integrity during force application, it must be recognized that forces can cause changes in this structural integrity (e.g. fluidization). In addition, forces applied at the membrane are indeed dissipated, but this dissipation implies energy transfer to the lipid molecules, which could manifest itself into reorganization and attendant force-dependent signaling.

In the tensegrity model, structural elements maintain their integrity because their load bearing mechanisms are stable for longer periods than the turnover time of individual monomers. In order for the tensegrity model to transmit stress it must be under pre-stress; isometric tension generated by contractile elements acting against compressive elements. Compressive elements are normally thought to be microtubules, but they can also include the extracellular matrix as well as organelles such as mitochondria and nucleoli capable of minimal deformation under compressive stress. While the tensegrity concept explains rapid transduction, many processes known to be involved in mechanotransduction are not as rapid as that. For example, MAPK phosphorylation peaks at around 5 min, about the same time scale as agonist-induced phosphorylation, suggesting that the mechanically induced pathway shares some similar molecular partners as the agonist induced one.

However, the cytoskeleton is not static but dynamically remodels. Noria and colleagues looked directly at the remodeling occurring as a result of sustained application of physiological fluid shear stress (Noria et al. 2004). These authors confirmed existing evidence that shear stress causes elongation of actin stress fibers in the direction of applied shear stress. Interestingly, by microinjecting monomeric fluorescent actin, they showed that in response to shear, actin polymerization originated at the location of an existing stress fiber end with neither the upstream or downstream part preferred. This insertion of monomeric actin into focal adhesions was independent of the classic zyxin/vasodilator-stimulated phosphoprotein (VASP) complex.

The overall cell deformability rests largely on its cytoskeleton, a crowded array of filamentous structures (Bursac et al. 2005). The cytoskeleton is comprised of actin filaments, intermediate filaments and microtubules, while other structural proteins (such as fodrin) are also present in endothelial cells. Bursac and colleagues pointed out that in glassy rheology systems, the crowding causes metastable states in which the dynamic (e.g. diffusion of an entrapped bead) is locally constrained. Periodically, the system can jump from one metastable state to another. Over time, the system samples many of these states and evolves through multiple microconfigurations with increases in their stability. This process is called aging and can be reversed by applying a highly dynamic force (strong fast oscillations) that returns an aged system back to its original state. Importantly, the group also found natural ATP-dependent oscillations in cells. This feature is radically different from passive glassy systems. ATP may provide fuel for cells so that they sample many different metastable states. Similarly applied force can drive cells to settle into different metastable states. This group focused on the role of the cytoskeleton, which has fairly accessible (slow) dynamics. It remains unexplored whether the membrane or other dynamic parts of the cell exhibit similar glassy rheology, and whether other force-dependent states arise from self assembly and reorganization.

Evidence for shear-induced transition of one structural state to another has come from using fluorescence recovery after photobleaching (FRAP). Osborn and colleagues demonstrated that net cytoskeletal depolymerization is high and that

shear stress causes immediate (within minutes) remodeling after shear application (Osborn et al. 2006). Such depolymerization could arise from the loss of myosin II or other crosslinking resulting in fluidization. Danagaria and Butler found that shear stress elicits rapid softening of the cell cytoplasm as assessed by rheological investigations of endogenous vesicles (Dangaria and Butler 2007). Interestingly even though shear was maintained, the softening response was reversed by 4 min. When shear was removed the cell became stiffer. In addition, creep tests to step shear indicated that, on the time scale tested, ECs behaved like a viscoelastic fluid with a deformation time scale of about 3 s (Fig. 9.2). Thus physiological shear with pulsatility in an atheroprone area is likely to lead to continuous adaptation of cell shape.

Krishnan et al. noted that the cell can employ different strategies to modulate intrinsic mechanics in response to applied force (Krishnan et al. 2009). Whereas it has been long known that, on longer time scale, force application induces reorganization of the cytoskeleton such that the cell, in general, becomes stiffer; it is now recognized that on short time scales, the cell can fluidize and become softer. Fluidization from stretch is thought to happen because of passive breaking of crosslinks in the cytoskeleton, whereas reinforcement occurs after alteration in mechanosensitive transduction processes. Reinforcement can prepare a cell for responses to large forces, or fluidization could be employed to rapidly relieve stress in response to applied force. Which of these cases predominates is an active area of research. By combining cell stretch and traction force microscopy, Krishnan et al. used cell-mapping rheometry to investigate the time course of fluidization versus reinforcement after applied stretch. Cells were stretched with varying degrees of anisotropy for 4 s. The degree of stretch ranged from 2.5 to 10 % corresponding to stretches that might occur by large changes in pressure of blood vessels. Immediately after the stretch maneuver, the traction force of the cell was monitored. It was observed that stretch caused an immediate reduction in traction (which was thought to arise from fluidization of the cytoskeleton) that was sustained for about 100 s, after which reinforcement mechanisms took over. This fluidization did not depend on isotropy, presumably because the cells were grown with no preferred direction in the cytoskeleton.

The authors concluded that the mechanism of stretch-induced fluidization was because of many weak dynamic bonds that are responsible for overall integrity of the cytoskeleton. These bonds can be rapidly broken and reformed giving the cell the ability to respond to indiscriminate non-specific forces from the environment. Again, shear forces appear to induce similar fluidization (Dangaria and Butler 2007), but it is not clear that shear-induced fluidization is due to the breaking of weak bonds or due to signaling pathways that result in changes in crosslinking.

9.5.7 Forces Can Be Transmitted to Focal Adhesions and Cell-Cell Junctions

Focal adhesions are assembled via sequential transport of proteins via diffusion through the membrane or from the cytosol (Broday 2000) and are converted to a mechanotransducing organelle through reinforcement of simple adhesion plaques to stable adhesions. The lipid around focal adhesions is highly ordered (Gaus et al. 2006) suggesting that, with respect to the membrane, the focal adhesion is a lipid-ordered membrane domain similar to caveolae and lipid rafts. Kanchanawong and colleagues recently studied the ultrastructure of focal adhesions (Kanchanawong et al. 2010). They found that focal adhesions have a complex yet ordered interaction with the cytoskeleton, particularly in the reinforcement phase after adhesion. Focal adhesions are comprised of an integrin extracellular domain that selectively binds extracellular matrix proteins (laminin, fibronectin, collagen, etc.) depending on the type of integrin. The integrins span the plasma membrane to bind to an internal integrin-signaling domain composed of talin and paxillin. The force transduction domain is thought to attach to these proteins and be comprised of vinculin and vasodilator-stimulated phosphoprotein (VASP). Next, the actin regulatory domain governs the insertion and remodeling of actin into the focal adhesion via α-actinin. Finally, actin and myosin form stress fibers that enable the application of force to the focal adhesions. Therefore, focal adhesions can exert forces on the ECM surrounding the cell and feel force through reactions to ECM deformation.

Forces can be transmitted to the endothelial cell junctions via actin filaments (Dejana 2004; Tzima et al. 2005). Cell junctions consist of tight junctions, which are largely responsible for transcellular permeability and are composed of claudins (e.g. occuldins), gap junctions (connexins), responsible for cell-to-cell communication via ion transport, and adherens junctions (e.g E-cadherins), which participate with claudins to regulate endothelium permeability. An additional important mechanotransducing molecule is PECAM-1, which participates in eNOS signaling upon force application (Bagi et al. 2005; Fujiwara et al. 2001)

An important question remains whether forces from shear stresses are sufficient to alter molecular conformation or alter changes in assembly of protein complexes. Recently, Dabagh et al. (2014) developed a computational model that incorporated each of the main structural elements responsible for mechano-transmission. These included the glycocalyx, membrane, actin cytoskeleton, adherens junctions, focal adhesions, the nuclei, and neighboring cells. The group estimated forces on focal adhesions and adherens junctions and found them to be on the order of 8 pN, sufficient for mechanoactivation. While the precise binding partners that might feel these forces are still unknown, this model provided some of the first experimentally validated stress profiles of cells undergoing physiological shear stress. The results argue for both centralized models of mechanotransduction, in which forces act directly on specific organelles, such as the glycocalyx and cell membrane, and a decentralized model in which forces are transmitted to remote sites and transduced into biochemical signaling.

9.6 Answering the Questions

This chapter began with key questions about mechanotransduction. We answer these questions using a concrete example of shear-induced nitric oxide production. It should be recognized that the answers to these questions would be different for different mechanotransduction pathways. In addition, the answers presented here are by no means final and require significant amount of additional research. Nevertheless, these answers can help the reader form a new framework for thinking about mechanotransduction in such a way that, perhaps in the future, knowledge of mechanotransduction can be used to develop novel diagnostic tools and clinical strategies to treat cardiovascular disease.

9.6.1 Integrin Ligation May Play a Role in Assembly of Mechanosomes

A widely accepted paradigm is that shear stress imparts forces on the EC surface glycocalyx, which transmits these forces to cellular structures whose molecular activity or association is altered by force. This perturbation leads to Ca^{2+} influx and release from cellular stores. Ca^{2+} is one ingredient needed to activate calcium-calmodulin, which is required for the conversion of L-arginine to nitric oxide (NO) by EC nitric oxide synthase (eNOS). This release of NO dilates blood vessels in the microvasculature and inhibits platelet aggregation in blood. Recent studies have demonstrated that the ubiquitous mechanosensitive Ca^{2+} channel, transient receptor potential vanilloid-4 (TRPV4), is involved in the mechanosensation of shear stress in ECs and responsible for shear-induced Ca^{2+} influx (Hartmannsgruber et al. 2007). In addition, it is part of a mechanosome consisting of spectrin, protein 4.1, Lyn (a member of the Src family of protein tyrosine kinases), and β_1 integrins, suggesting that mechanically gated TRPV4 channel conductance may be related to cytoskeleton-membrane adhesion (spectrin-protein 4.1), membrane rafts (Lyn), and focal adhesion (FA) assembly (β_1 integrins) (Fig. 9.5).

There is evidence that assembly of such mechanosomes may depend on lipid membrane properties in that the very first constituents of this mechanosome, namely rafts and β_1 integrins, must diffuse to these sites of adhesion to extracellular matrix proteins in order to coordinate mechanotransduction (e.g. talin activation). We hypothesize that membrane lipid raft recruitment to locations where β_1 integrins have ligated ECM ligands, as indicated by recruitment of the ganglioside GM-1, enable further (β_3) integrin clustering. Evidence supporting these ideas comes from the measurement of raft transport to nascent adhesion plaques and assays for integrin activation by imaging talin (Fuentes et al. 2011; Fuentes and Butler 2012).

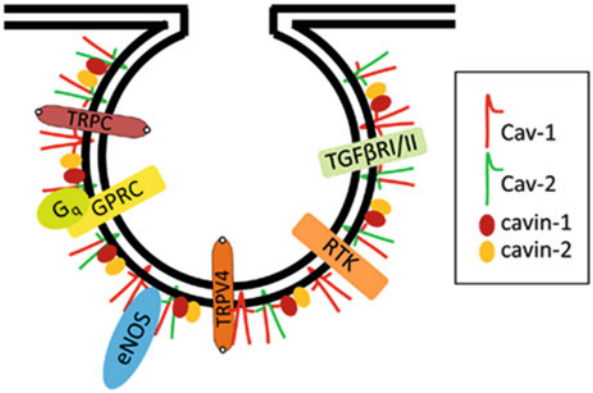

Fig. 9.5 Caveolae structure. Association of TRPV4 with eNOS and other caveolar associated proteins suggests a possible mechanotransduction mechanism in which forces applied to the cell surface and the reaction forces from actin association with caveolae cause the disruption of association of caveolin with TRPV4 and eNOS. This release allows these proteins to be active thus increasing Ca^{2+} influx into the cell as well as increased eNOS activity. TRPC, transient receptor potential cation channel; GPRC, G-protein couple receptor; eNOS, endothelial cell nitric oxide synthase; TRPV4, transient receptor potential cation channel, subfamily V, member 4; RTK, receptor tyrosine kinase; TGF, transforming (or tumor) growth factor (Figure adapted from Sowa (2012))

9.6.2 What Components of Force Are Sensed by the Cell?

Cells respond to shear stress temporal aspects. Feaver and colleagues recently described the role of other harmonics of blood flow that may impact endothelial cell mechanotransduction (Feaver et al. 2013). Although blood flow dynamics are often simplified as averages stresses (e.g. 0 ~ 2 Pa) with a superimposed oscillatory stress (with amplitude of about 0.5–1 Pa), wall shear stress actually has a number of other harmonics associated with it. These frequencies come from the rhythmic pumping off blood described by the PQRST wave of an ECG, longitudinal pulsatile waves arising from windkessel mechanics of the vascular wall, sharp closing of valves, and the impact of stochastic forces arising from blood cell interaction with the blood vessel wall. While it is difficult to capture all of these temporal variations, Feaver succeeded in capturing some of the complex stress frequency harmonics by analyzing the effects of 0th (average shear) and 1st (temporal) harmonic. Atheroprone hemodynamics were considered as low pulsatile multidirectional shear such as those that occur in the carotid sinus. These stresses up-regulated genes involved in inflammation that depended on NF-κB activity and genes which this transcription factor regulates (e.g. VCAM-1, fibronectin, E-Selectin, MCP-1, IL-8). Such activation of NF-κB was inhibited when PECAM-1 was knocked down using siRNA suggesting that PECAM-1 may be a mechanosensor. Conversely, high shear up-regulated anti-inflammatory genes such as eNOS, KLF-2, KLF-4, and BCL2-like protein A1. Analysis of all higher order frequency modes indicated that

the first harmonic was largely responsible for atheroprotection via down regulation of NF-κB.

Cells may also respond to spatial shear stress gradients (DePaola et al. 1992). In these experiments, there is a large change in shear stress from one point on a cell to another or from one cell to a neighboring cell. In areas of flow recirculation, these gradients can be very large, with as much as a few dynes per cm^2 over a few microns. Such gradients could induce differential forces between one part of the cell and another part thus causing stretching between these points. Similarly, if shear on one cell was different than a neighboring cell, then resultant forces could span junctions between cells. Consistent with this idea, connexins were specifically activated when shear stress gradients were present (Chadjichristos and Kwak 2007; Johnson and Nerem 2009). In addition, consistent with activation in junctions, PECAM-1 has emerged as an important sensor of force at the cell surface (Bagi et al. 2005; Fujiwara et al. 2001; Tzima et al. 2005)

9.6.3 What Are the Molecular Sensors That Transmit Force to Transducers in the Cell?

Ueki showed that shear stress elicits shear strain in endothelial cells with a measured shear modulus of 231 Pa (Ueki et al. 2010). Such a low modulus is consistent with other measurements of shear-induced deformation by Dangaria (Dangaria and Butler 2007). In this experiment, the group used high speed laser line scanning confocal microscopy to take a rapid axial section through a cell in the direction of shearing. By focusing only on one slice, they were able to capture cellular deformation at 3–4 frames per second. Using image correlation methods, they found that shear stress deformed the cells in the direction of shear. This strain was found to be dependent on actin as cytochalasin D increased the shear strain (and decreased the modulus) dramatically. Such studies indicate that the cytoskeleton and internal organelles are mechanically linked and that shear stress can be transmitted to remote locations almost everywhere in the cell.

As a discrete example, direct mechanical linkages exist between the cell surface and organelles where mechanosensitive processes are known to happen (Maniotis 1997). Maniotis developed an assay based on micropipette manipulation of an ECM coated bead and showed that there exists strong cytoskeletal linkages between the cell surface and the nucleus (Maniotis et al. 1997). While they used deformations that were much larger and faster than *in vivo* deformations by shear, the work showed that force can act at a distance in cells. Later work showed that such a phenomenon might be relevant in shearing of endothelial cells (Helmke et al. 2001). While there has not been definitive proof that forces can directly cause changes in transcription in the nucleus, there is growing evidence for such linkages (N. Wang et al. 2009).

Alternatively, force could act directly on the cell membrane and cause clustering of mechanosensitive proteins. Rafts coalesce with force due to enhanced hydrophobic mismatch between liquid-ordered (Lo) and liquid-disordered (Ld) membrane

domains. Mismatch of the hydrophobic thickness of various lipids in the membrane bilayer drives aggregation of lipid domains (Baumgart et al. 2003) which, in turn, facilitates segregation or aggregation of membrane proteins (Ayuyan and Cohen 2008; Botelho et al. 2006; Periole et al. 2007). Membrane tension induces raft clustering (Ayuyan and Cohen 2008; Heinrich et al. 2010) with a time course on the order of seconds. These studies demonstrate that rafts are poised to coalesce at physiological temperatures (Lingwood et al. 2008) or with minor alterations in the force landscape (Garcia-Saez et al. 2007).

9.6.4 Is Transduction Through Physical Links That Transmit Force from One Molecular Partner to Another, or Does Force Work Through Existing Receptors That Transmit Biochemical Signals Largely Through Diffusive Pathways?

Mechanotransduction appears to occur in specialized molecular complexes and organelles. While diffusion may carry some mechanotransduction players into active phosphorylation pathways (Dimova et al. 2000; Jalali et al. 1998; Lu et al. 2008; Wang et al. 2005), it appears that many mechanical systems are hard-wired for mechanotransduction (Na et al. 2008). One example of a protein complex involved in shear stress induced nitric oxide production is the mechanosome composed of spectrin and protein 4.1. This membrane-associated complex regulates the activity of transient receptor potential vanilloid-4 (TRPV4), a ubiquitous mechanosensitive Ca^{2+} channel. This activation is specifically regulated by spectrin and actin, and the deletion of protein 4.1 binding domain to TRP channels prevents TRP activation, consistent with early observations linking spectrin, actin, and protein 4.1 (Leto et al. 1986). TRPV4 and the spectrin skeleton are associated with TRPV4-mediated Ca^{2+} conductance in ECs as disruption of the spectrin-protein 4.1 interaction abolishes TRPV4 activity (Wu et al. 2001). In addition, the activation of TRPV4 channels by osmotic stretch depends on interaction with β_1 integrins and Lyn (a src-family kinase and a lipid raft protein) (Alessandri-Haber et al. 2008; Xu et al. 2003). Thus there appears to be a correlative, and possibly mechanistic, link between spectrin, protein 4.1, membrane rafts, integrins, TRPV4 activation, and Ca^{2+} entry through the plasma membrane. Thus, it is likely that TRPV4 is part of a mechanosome (Bidwell and Pavalko 2010) consisting of spectrin, protein 4.1, Lyn, and β_1 integrins. In support of this concept, the Ingber group showed that when TRPV4 was associated with β_1 integrins, forces applied to β_1 integrins result in ultra-rapid (within 4 msec) activation of Ca^{2+} influx through TRPV4 channels (Matthews et al. 2010), suggesting that TRPV4 may be directly mechanosensitive (Christensen and Corey 2007). The TRPV4 channels were specifically activated by mechanical strain applied to β_1 integrins and not by deformation of the lipid bilayer or submembranous cortical cytoskeleton alone. Thus, at least the β_1 integrin component of the proposed mechanosome is necessary for mechanical activation of TRPV4. It is also known that TRPV4 channels are involved in EC mechanosensing of shear stress (Hartmannsgruber et al. 2007;

O'Neil and Heller 2005; Wu et al. 2007), Ca^{2+} influx (Nilius et al. 2003), and nitric oxide (NO) generation (Kohler et al. 2006). Thus, we propose that EC mechanosensing of shear stress derives from coordination between spectrin, protein 4.1, β_1 integrin, Lyn, and TRPV4.

9.6.5 To What Extent Are the Force Transduction Partners Regulated by the Very Force They Sense?

A fruitful direction of research would be measurements of EC surface dynamics and force-dependent kinetics of lipids, integrin activation, and reinforcement (Fuentes and Butler 2012) that precede the formation of the TRPV4 mechanosome, in order to delineate the mechanical origins of EC dysfunction that underly hypertension and atherosclerosis. Previously, this chapter described how the glycocalyx and cytoskeleton remodel in the face of shear stress. Similarly, focal adhesion proteins are in constant state of flux (Lele et al. 2006). For example, new integrin ligation and clustering are major events in vascular tone regulation and shear-induced gene expression. Jalali et al. showed that shear stress caused an increase in new ligand binding of β_1 integrins in and around focal adhesions (FAs) of endothelial cells (ECs) plated on fibronectin and an increase in ligand binding of β_3 integrins in ECs plated on vitronectin (Jalali et al. 2001). In *ex vivo* arteriolar preparations, activation of the vitronectin receptor, $\alpha_v\beta_3$-integrin, and fibronectin receptor, $\alpha_5\beta_1$-integrin, induced coronary arteriolar dilation by stimulating endothelial production of cyclooxygenase-derived prostaglandins (Hein et al. 2001), which dilate blood vessels (Butler et al. 2000; Frame et al. 2007). Thus dynamic integrin-matrix interactions at FAs are required to initiate the signaling pathway leading to shear stress-induced vasodilation and blood pressure regulation.

Furthermore, integrin association with focal adhesions is modulated by force via diffusion of lipid rafts (Fuentes and Butler 2012). Lipid rafts are 10–200 nm cholesterol- and sphingomyelin-enriched liquid-ordered (Lo) membrane domains that are involved in signaling and nucleate actin polymerization [reviewed in (Levitan and Gooch 2007)] by concentrating phophatidylinositol 4,5 biphosphate (PIP_2) (Kwik et al. 2003). FAs are cholesterol rich microdomains, as are caveolae and rafts (del Pozo et al. 2004) and β_1 integrins are required for raft formation (Singh et al. 2010) and signaling through Rac-1 (del Pozo et al. 2004). Wang and colleagues found that Src-activation colocalized with Lyn, a raft marker (Lu et al. 2008) supporting an emerging picture of rafts as dynamic nanodomains that cluster the necessary critical mass of receptors (van Zanten et al. 2009) for downstream signaling of important pathways such as mitogen-activated protein kinases (MAPK) (Rotblat et al. 2010) with time scales of formation of 20 ms and length scales of 10 s of nanometers (Eggeling et al. 2009). The dynamic formation and dissolution of rafts may be related to membrane bending fluctuations which facilitate spectrin-protein 4.1 association and protein sorting (Heinrich et al. 2010).

9.6.6 What Are the Main Factors Produced at the Terminal End of Force Transduction Pathways?

The mechanism of eNOS activation is connected to its subcellular location. eNOS converts l-arginine to L-citruline and nitric oxide. NO, in turn, acts as a free radical scavenger, relaxes blood vessels, and inhibits platelet aggregation, apoptosis, and can inhibit the binding of monocytes to endothelium. Therefore, it is considered as an important atheroprotective molecule. eNOS activity is connected with the transcription factor NF-κB in that NFKB binds to the eNOS promoter and initiates the generation of eNOS. Since NO in turn can downregulate NF-κB activity, this may comprise an important negative feedback system for controlling NO bioavailability. Regarding localization in the cell, eNOS is thought to reside in caveolin and is inhibited by its association with caveolin-1 (Fleming and Busse 1999; Rizzo et al. 1998; Yamamoto et al. 2011). It is also been found to associate in an inhibitory fashion to PECAM-1. When shear is initiated, these associations are temporarily lost and eNOS is then available for the generation of NO. So an intriguing mechanism of mechanotransduction becomes simply that force pulls on the eNOS-caveolin-1-PECAM-1 complex and changes the affinity of molecules for each other thus making eNOS available for NO production. Once separated from PECAM-1 or caveolin 1, eNOS is free to catalyze the conversion of l-arginine to l-citruline and NO through Akt-mediated phosphorylation of eNOS.

9.7 Conclusion

Shear stress impacts cells via transport of macromolecules to the cell surface and through transport of blood momentum in the form of normal and shear stresses. These mechanical features of blood flow are translated into biochemical signals by mechanotransduction. All in all, conversion of force to biological changes occurs through mechanotransmission of forces to mechanosensors, which deliver forces to mechanotransducers, where force is converted to biochemical signaling. The nature of this process depends on a coordination of temporal and spatial aspects of the fluid momentum transfer, mechanical properties of transducing partners, the dynamics of cellular remodeling of these transmitting elements, the biochemical rate constants of the transducers, and the transport (active or diffusive) of the downstream factors that ultimately manifest themselves as changes in vascular biology. A thorough understanding of this process will require increased research from experts in mathematics, computational analysis, statistics, and molecular biology in order to develop predictive models of the endothelium capable of assessing the predilection of the vasculature to disease. Such a platform would provide a valuable tool to clinicians in their decisions on what types of interventions to employ when confronted with vascular disease. Importantly, it may also provide insight into strategies to prevent endothelial dysfunction and resulting atherosclerosis.

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