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# Vascular Smooth Muscle as a Therapeutic Target in Disease Pathology

Andrew W. Holt and David A. Tulis

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## Abstract

Our circulatory system is composed of numerous elements that are responsible for transport of blood and delivery of essential nutrients and gases to vital downstream tissues. Among these components that make up our circulation is vascular smooth muscle (VSM), the primary muscular and contractile element of blood vessels and regulator of many blood vessel functions. This is of particular importance as cardiovascular disease (CVD), the number one killer of individuals in America and worldwide, is primarily vascular in origin. Logically, identifying and characterizing feasible targets that could control CVD are highly appealing and much desired. With this in mind and given its centrality in control of vascular physiology, VSM has gained wide attention as a plausible target to combat elements of CVD. This book chapter focuses on VSM as a potential therapeutic target against CVD and will provide overview of vascular anatomy and physiology and brief discussions about the pivotal roles of VSM in CVD pathology, the influence of abnormal blood flow mechanics and hemodynamics in CVD, neural control of VSM and the vasculature, and possible novel cellular and molecular signaling targets that could be used to control and/or minimize CVD. This chapter hopes to serve as a valuable resource for basic and applied scientists as well as clinicians interested in understanding the crucial roles that VSM plays in vessel physiology and pathology.

**Keywords:** Cardiovascular disease, cell signaling, cyclic nucleotides, hemodynamics, kinases, shear stress, vascular smooth muscle

## 1. Introduction

Our circulatory system is comprised of a vast network of cellular elements and organs that includes the heart, lungs, and vasculature composed of arteries, veins, and lymphatics. These integrated factors serve essential roles in controlling flow of blood and lymph and in the transport and delivery of essential nutrients, nutritive and vasoactive factors, and hormones and gases. Vascular smooth muscle (VSM), the primary functional constituent of blood vessels, serves critical regulatory roles of vessel relaxation and contraction to ensure adequate tissue blood flow and to maintain proper localized arterial blood pressures and perfusion of downstream tissues. These processes are elemental for normal vascular eutrophy and homeo-stasis and overall body health. Abnormalities in VSM anatomy and physiology, however, can contribute to a myriad of primary and secondary vessel pathologies. Moreover, adult blood vessels are normally contractile, quiescent, and static. However, under inimical conditions like those associated with cardiovascular disease (CVD), VSM cells undergo phenotypic alterations and revert to a growth-promoting, synthetic nature. In turn, this abnormal growth significantly contributes to the emerging cardiovascular disorder. These complications alone present significant health risks but also serve as confounding risk factors for associated cardiovascular complications including hypertension, hypercholesterolemia, diabetes, and metabolic syn-drome. In fact, recent statements by the American Heart Association [1] and the World Health Organization [2] point to dysfunctional VSM as a primary underpinning behind CVD. Logically, therapeutically targeting VSM for clinical interventions aimed at controlling and hopefully eradicating CVD is highly significant and essential.

The term "cardiovascular disease" defines a wide range of disorders, diseases, and conditions that deleteriously affect the heart and blood vessels. If the heart is the primary organ affected then this can include heart failure, various myopathies, arrhythmias and/or conduction delays, valve complications, myocarditis and/or pericarditis. If the disorder is vascular in origin, then problems could consist of occlusive plaque formation and atherosclerosis, arteriosclerosis, coronary and/or peripheral artery disease (PAD), aneurysm formation, and/or restenosis and remodeling. There are numerous forms and manifestations of CVD as well as broad prognoses based on form and severity of the disorder. Abnormal biology of the vessel wall constitutes a major element in the pathogenesis of most forms of CVD [1], and therefore the vessel wall and particularly VSM makes a prime target for further discovery and potential therapeutic utility against CVD.

The overall goal of this chapter is to present some of the more recent and novel theories surrounding the primary vessel wall constituent, VSM, its importance in CVD, and its promise as a therapeutic target. Discussion will include succinct overviews of normal vascular elements and physiology with a focus on VSM, some common forms of CVD and their pathologies, the role of VSM dysfunction in CVD etiology, the influence of flow alterations and hemodynamic forces in vessel physiology and pathology, neural control of VSM and the vascular network, unique molecular and cellular signaling pathways that may offer innovative and precise targets for therapy in VSM, current therapeutic paradigms, and treatment strategies used to combat VSM pathobiology ranging from homeopathic lifestyle modifications to pharmaco-

therapies to interventional vascular approaches. This chapter concludes with an overview of the therapeutic potential of VSM and its promise in the cardiovascular sciences. In all, this chapter promises to offer a valuable resource for basic, applied, and clinical scientists by providing a synopsis of the importance of VSM in normal vessel biology and vascular pathology and its utility as therapeutic target to combat CVD.

## 2. Overview of vascular anatomy and physiology

Upon leaving the heart, blood enters the vasculature which is comprised of diverse tissues and cell types. Conduit, large and small arteries, arterioles, capillaries, venules, veins, and lymphatics are basic elements of this system, each with unique characteristics and functions. The composition of the walls of each of these vessels also differs, based on size, location, and function, and this dictates the presence or absence of the three major vessel wall layers. Starting from the lumen that carries blood, the innermost blood vessel wall layer is the *tunica* (Latin for tunic, membrane, coat) *intima*, and this is formed by squamous vascular endothelial cells (VECs), a layer of connective tissue termed internal elastic lamina, and a basement membrane containing laminin, heparan sulphate proteoglycans and collagen, and often the glycosaminoglycan hyaluronan. The *tunica intima* provides a key, selectively permeable interface between flowing blood (which contains abundant growth factors and thrombogenic platelets) in the lumen and the highly thrombogenic components of the subintimal vessel wall. This important intimal layer is also largely responsible for controlling inflammation and medial VSM proliferation and migration, and these intimal VECs operate by eliciting crucial signals that, in communication with underlying VSM, help control blood vessel function. Hence, this layer is of utmost importance in maintaining normal vascular homeostasis. Interestingly, the endothelium of the *tunica intima* is continuous with the endocardium of the heart and is the only layer present in all blood vessels of all sizes and anatomical locations.

The thick and muscular middle layer of blood vessels is called *tunica media* and this is composed mainly of helical, spindle-shaped mononuclear VSM cells. The media also contains sparse macrophages and fibroblasts along with an interstitial matrix consisting of collagens; glycoproteins such as tenascin, vitronectin, and fibronectin; chondroitin sulphate proteoglycans including versican; and elastic laminae. The *tunica media* provides structural support to blood vessels and is the primary functional element that controls vascular constriction and dilation (and hence, blood flow) based on metabolic needs of the downstream tissues. VSM within the *tunica media* (in communication with intimal VECs) is predominantly responsible for maintenance of normal vascular physiology, yet dysfunction of medial VSM cells (and VECs) is a major contributor to the pathogenesis of CVD (discussed later). The outermost layer of blood vessels is termed *tunica externa* or adventitia, which is separated from the medial wall by the external elastic lamina and is made up of sparse VSM and nerve cells, fibroblasts, fat cells, and abundant connective tissue including elastin, collagen, and glycosaminoglycans that provide structural support as the extracellular matrix (ECM). In larger caliber vessels, the adventitia also contains unique small blood vessels termed *vasa vasorum* that feed nutrients to the thick muscular vessel wall (when the vessel wall cannot get adequate oxygen and nutrition simply

by diffusion from luminal blood). A schematic of blood vessel anatomy including these three critical layers is shown in Figure 1.
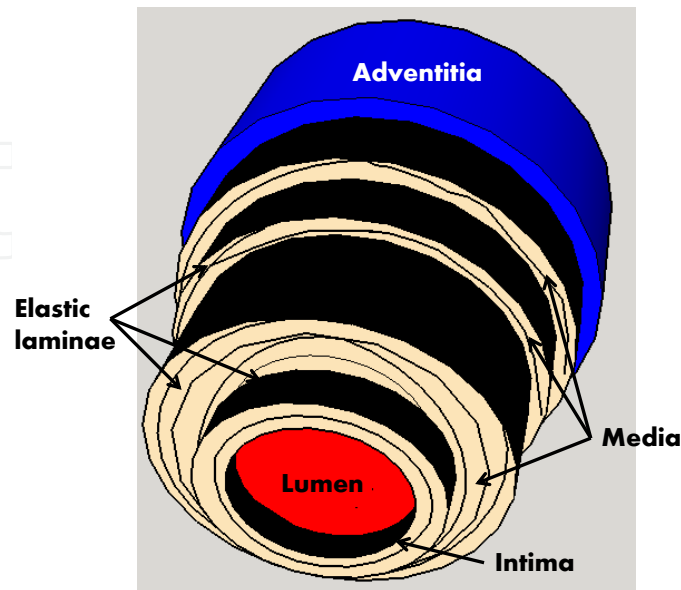


**Figure 1. Blood vessel anatomy.** The three primary circumferential layers of blood vessels include the innermost endo-thelium-rich *tunica intima*, the VSM-containing *tunica media*, and the outermost layer the *tunica externa* or adventitia. Elastic laminae exist between these layers as well as within the medial wall. These layers serve critical functions in maintaining normal blood flow and in providing key nutrients and gases to downstream tissues as well as in the re-moval of toxic by-products of metabolism. Dysfunction in their physiological abilities, however, can contribute to sig-nificant vessel disorders including uncontrolled growth which is foundational for the development of CVD.

Physiologically, blood vessels hold a critical place in our circulation between the heart, downstream tissues, and the lungs. As such, a major function of blood vessels is to provide blood flow complete with delivery of vital nutrients and gases (oxygen) to these essential tissues and removal of used metabolic by-products and gases (carbon dioxide) targeted for elimination via the lungs. Blood vessels also operate as highways for the distribution of secreted hormones and other endocrine or paracrine factors as well as white blood cells and/or platelets to their sites of action. Blood vessels also aid in the broader distribution of water, solutes, and heat throughout the system. In VSM-specific fashion, blood vessels have ability to regulate luminal caliber and hence, the amount of blood flow they carry, based on local and immediate metabolic needs of downstream tissues. This is of obvious importance for proper vascular function and tissue homeostasis and eutrophy. Blood vessels perform this critical function through abilities to constrict (vasoconstriction) and relax (vasodilation) through mechanisms fully dependent on functional medial VSM. Several other physiological aspects of blood vessels involve their critical roles in growth adaptations during wound healing or following surgical interventions or during vascular adaptations of exercise. These normal blood vessel growth responses can involve angiogenesis (formation of new blood vessels from existing vessels), vasculogenesis (de novo formation of new blood vessels), arborization or branching of existing vessels, and/or collateralization to provide a new blood supply to an existing vascular bed.
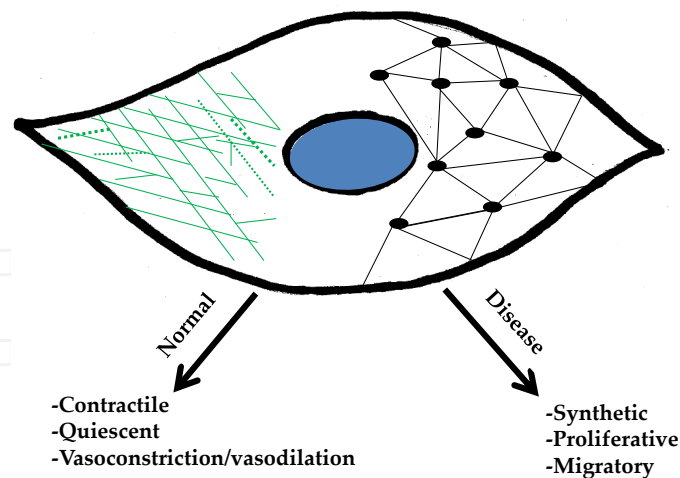
## 3. Vascular smooth muscle

As mentioned, the primary structural, muscular, and functional unit of a blood vessel is the medial wall comprised primarily of VSM. In adults, medial wall VSM cells are normally highly differentiated and display a contractile phenotype (with abundant expression of contractile proteins) which enables their abilities to contract and relax. By virtue of vasoconstriction and vasodilation, VSM directly regulates lumen caliber and thus, arterial and venous tone and vascular resistance. These, in turn, control distribution of blood flow in tissue-specific fashion throughout the body. Mechanistically, VSM cells contain many thin actin filaments and relatively few thick myosin filaments, which are arranged along the long axis of these cells. In this arrangement, using the sliding filament theory and numerous states of actin and myosin cross-bridge phosphorylation, VSM utilizes slow force development to produce prolonged force maintenance with low energy utilization, thus helping in the maintenance of tetanic vascular (myogenic) tone and blood flow control. Thus, vasoconstriction and vasodilation represent major physiological functions of contractile VSM.

Under homeostatic unstimulated conditions, adult medial VSM cells are also predominantly quiescent and lack significant growth, "resting" in the nonproliferative $G_0$ phase of the cell cycle. In this differentiated state, VSM cells have low turnover and minimal proliferative or synthetic activities and are dedicated to their primary function of vasoconstriction/dilation. These cells, however, show considerable plasticity and are able to phenotypically switch between quiescent and synthetic phenotypes in response to local stimuli. So, following activation from a variety of agonists, circulating factors, and/or hormones or cytokines or as a result of injury or the onset of disease, these VSM cells dedifferentiate into noncontractile and synthetic cells with reduced expression of contractile proteins and increased capacity to proliferate, migrate, and produce ECM components. During this dedifferentiation process, the cytoskeleton becomes highly organized with defined F-actin filaments, nuclear hypertrophy, and enlarged Golgi. Along with these morphological changes these dedifferentiated cells become highly sensitive to stimulation by mitogenic and chemotactic factors including angiotensin II, platelet-derived growth factor, fibroblast growth factor, interleukins, and tumor necrosis factor alpha. These processes and vasoactive signals allow VSM cells to contribute significantly to the emerging vascular dysfunction through heightened proliferation, migration, and synthetic properties foundational to CVD. This conversion to a growth-promoting and synthetic embryonic phenotype represents a key event in the genesis and evolution of numerous cardiovascular pathologies including atherosclerosis, restenosis following bypass grafting or stent deployment, or aneurysmal disease as elements of CVD. Figure 2 shows a schematic of the major functions of a VSM cell under healthy or pathologic conditions.

## 4. Cardiovascular disease

As discussed, abnormal VSM growth is elemental for development and maintenance of CVD, which has historically ranked as the number one cause of morbidity and mortality in the United

**Figure 2. Vascular smooth muscle cell function.** VSM cells are normally quiescent and operate via vasoconstriction/vasorelaxation to control local blood flows and pressures and downstream tissue perfusion. However, VSM dysfunction leads to dedifferentiation to an embryonic phenotype, and under these pathologic conditions VSM loses its contractile characteristics and becomes synthetic, proliferative, and migratory, serving as basis for many forms of CVD. Also shown in this schematic are cellular features of thick and thin filaments, dense plaques, and the cytoskeleton.

States and worldwide for many years [1,2]. Despite major advances in our knowledge of the numerous contributing mechanisms for CVD and potential therapeutic strategies against CVD, estimates suggest CVD-related deaths will continue to rise over the next 20 years or so [1,2]. Certainly, notwithstanding these significant basic science and clinical advances, failure in adequate clinical control of CVD highlights its complexity and points to the need for continued study of the underlying mechanisms of and potential routes for control of CVD.

Of the many forms of CVD perhaps the most common is an occlusive disorder termed atherosclerosis. Atherosclerosis is a gradual and progressive disease that involves combined influences of heightened inflammatory status, locally dysfunctional metabolism, abnormal vascular wall growth and remodeling, and the buildup of an occlusive plaque. As discussed, the involvement of uncontrolled VSM growth is a basic foundation of the evolution phase of atherosclerosis, and dedifferentiation of VSM cells into a growth-promoting and synthetic phenotype contributes largely to the emerging and growing plaque. This process of enhanced synthesis and proliferation of medial VSM cells can be slow and progressive and can occur over many decades and may not elicit observable symptoms. This pathology often eventuates in a stenotic plaque, either stable or complicated, which can then become jeopardized and lead to lumen obstruction and diminution of blood flow with clear repercussions of reduced oxygen and nutrient delivery to downstream tissues and diminished removal of toxic metabolites and gases. The plaque can also rupture and send microthrombi into the downstream circulation which can lodge in smaller blood vessels. If atherosclerosis occurs in the peripheral circulation, then these processes could manifest as tissue necrosis with perhaps loss of the affected tissue or limb. Of heightened significance, if this pathology occurs in the cerebral or myocardial circulation, this could result in a cerebral or myocardial infarct (stroke or heart attack) with critical and life-threatening implications.

## 5. Blood flow, vascular hemodynamics and neural control

The cardiovascular system is a closed circuit comprised of the heart and an elaborate network of vessels in the systemic and pulmonary circulations. Biophysical forces and factors that govern transportation of essential gases and metabolic fuels and nutrients as well as waste products of metabolism are termed "hemodynamics" and are largely determined by local tissue and cellular supply and demand balance. Hemodynamics, especially in the systemic circulation, are regulated by intrinsic (ability of local tissues to regulate their blood supply) and extrinsic (reliance on neural mechanisms to distribute blood flow at the tissue level) neural control systems. These complex regulatory mechanisms rely on the ability of blood vessels to properly respond to chemical cues in order to efficiently orchestrate where and how much blood is being delivered and removed from various vascular beds. Dysfunctional intrinsic and extrinsic neural control systems compromise efficient oxygen and nutrient delivery to tissues and manifests in a variety of disease pathologies such as PAD, stroke, and/or heart attack.

As oxygenated blood leaves the left ventricle of the heart, it enters the aortic arch and exerts biophysical forces on the arterial walls. Driving pressure, transmural pressure, and hydrostatic pressure are all important forces that regulate blood flow and two major components of these forces are tensile stress and shear stress. Tensile stress is the perpendicular force exerted by flowing blood on the vessel wall and represents forces due to blood pressure [3]. In comparison, fluid shear stress is the force parallel or tangential to the vessel wall which corresponds to the frictional force of the blood in contact with the intimal endothelium [4]. These combined forces are critical for stimulating both intrinsic and extrinsic neural control mechanisms in the vasculature.

### 5.1. Autonomic nervous system and the vasculature

The autonomic nervous system is comprised of the complementary yet distinct sympathetic (SNS) and parasympathetic (PSNS) nervous systems. Under normal physiological conditions, these systems perform divergent regulatory functions on the vasculature. In general, the SNS typically acts to constrict blood vessels, effectively reducing blood flow, while the PSNS generally dilates blood vessels to increase flow. Autonomic dysfunction in the context of abnormally high blood pressure is typically the result of an overly active SNS. This dysfunction produces a constant neuronal discharge of sympathetic neurons resulting in tonic vasoconstriction of the VSM [5].

The SNS contains preganglionic sympathetic neurons in the ventral horn of the thoracolumbar spinal cord which project their axons just outside of the sympathetic trunk and synapse with postganglionic sympathetic neurons near its target organ. At the initial synapse, depolarization of the presynaptic neuron triggers tethering, docking, and fusion of vesicles containing the neurotransmitter Acetylcholine (Ach) to the axon hillock. Once Ach is released from the presynaptic neuron, it binds to an ionotropic, nicotinic, cholinergic receptor on the postganglionic neuron, resulting in depolarization on the postsynaptic cell. In the context of carotid artery blood flow, the postganglionic neuron projects onto, and synapses with, medial VSM cells. At the postganglionic synapse, the neurotransmitter that is released is primarily norepi-

nephrine (NE). In similar fashion, vesicles containing NE are released at the postganglionic synapse, yet they bind to metabotropic, adrenergic receptors on the VSM cells. VSM contains both alpha 1 and beta 2 adrenergic receptors. NE will preferentially bind to the excitatory alpha 1 receptors causing vasoconstriction and reduced blood flow. Since it is well documented that the carotid artery is tonically constricted, it is only after inhibition of this signal that the SNS is able to be turned off by the PSNS, thereby resulting in carotid artery dilation and enhanced cerebral blood flow. Essentially, the vasomotor area of the medulla is sending excitatory, efferent output to the thoracolumbar section of the spinal cord which is innervating and contracting carotid VSM. This action is executed until the vasomotor area receives an inhibitory signal from the Nucleus Tractus Solitarii (NTS), at which point the SNS is inhibited while the PSNS is activated.

In comparison to the constitutively active SNS, the PSNS is alternatively activated by the carotid sinus when the carotid artery pressure increases. This means that as luminal pressure exerts increased force on the vessel wall, the carotid sinus increases its firing frequency and amplitude, activating the PSNS and effectively inhibiting SNS tonic discharge. The purpose of its activation is to dilate the artery in an attempt to normalize or depress the increased pressure that is currently acting on the vessel walls. The PSNS projects its preganglionic neurons directly from the brainstem and/or sacral spinal cord towards, and in close proximity to, its target organ(s). At this synapse, the preganglionic neuron releases Ach in a similar fashion as that described for the SNS. The ensuing depolarization of the postganglionic neuron reaches the carotid artery and releases Ach again, yet the mechanisms of action at this second synapse are different than what occurs at the initial synapse. Here, Ach binds to beta 2, metabotropic, muscarinic receptors located on the endothelium adjacent to the VSM cells in the medial layer. After binding to the appropriate G-protein coupled receptor on the endothelium, a signaling cascade is initiated that results in an influx of intracellular calcium and the synthesis of nitric oxide (NO) from endothelial nitric oxide synthase (eNOS). NO can then stimulate broad and multifaceted actions on downstream processes including those associated with cyclic nucleotide signaling (discussed below).

## 5.2. Intrinsic neural control mechanisms

Arterial tensile stress elicits myogenic tone and primarily modulates mechanoreceptor feedback found in baroreceptors within conduit arteries (discussed below). Myogenic tone is the inherent and tonic contractile response of VSM to increased luminal pressures and is a critical component for determining vessel wall hypertrophy according to LaPlace's equation: T = r x P (T = tension; r = vessel radius; and P = pressure), whereby if vessel caliber or diameter remains constant, any increase in intravascular pressure will elicit an increase in wall tension. Since these two factors are proportional, the vessel wall thickens in order to help offset any experienced increase in wall tension. In the high pressure environment of the systemic vasculature failure to adapt can result in increased matrix metalloproteinase (MMP) activity, causing vessel wall thinning and ensuing aneurysmal formation [6]. The other key force in regulating blood flow is fluid shear stress. This tangential force is exerted on intimal endothelium and elicits VSM relaxation through canonical dilatory (largely NO-mediated) pathways.

The mechanical stimulation of intimal VECs by luminal blood flow activates nonselective cation channels (TRP channels) within the cellular plasma membrane [7]. The resulting increase in intracellular calcium facilitates activation of eNOS and yields NO as a by-product of L-arginine to L-citrulline conversion [8]. As a diatomic gas, NO freely diffuses across the VEC membrane and travels in paracrine fashion to abluminal VSM cells. Here, NO binds to the heme moiety on soluble guanylate cyclase (sGC) and dephosphorylates guanosine triphophosphate (GTP) to elicit pyrophosphate (PPi) and cyclic GMP. Two cyclic GMP molecules can then bind to the regulatory subunit of the serine/threonine (Ser/Thr) protein kinase G (PKG), which in turn phosphorylates a vast number of intracellular proteins to include ion channels, phospholamban, myosin light chain phosphatase, and other phosphorylatable targets including vasodilator-stimulated serum phosphoprotein (VASP), a topic of interest discussed later in this chapter. Ultimately, PKG activation results in the resequestration of calcium within the sarcoplasmic reticulum and induces VSM relaxation resulting in vessel dilation and increased blood flow.

## 5.3. Extrinsic neural control mechanisms

Of all our organs that receive systemic blood flow, the cerebral circulation is of critical importance. According to the American Heart Association, stroke is a leading cause of death and the leading cause of adult disability in the United States [1]. Ischemic stroke, hemorrhagic stroke, and transient ischemic attacks all result from failure of oxygenated blood to reach the brain. In order to maintain appropriate cerebral blood pressure and constant brain perfusion, numerous cardiovascular and neural control mechanisms exist that act cooperatively to ensure adequate and appropriate cerebral circulation. The carotid sinus baroreceptor reflex is partly responsible for maintaining and regulating blood flow to the brain through local and systemic regulation of blood pressures. The carotid sinus innervates the internal carotid artery just distal to the bifurcation of the common carotid artery. Here, the sensory fibers of the carotid sinus extend into the medial VSM layer of the internal carotid artery. These sensory nerve endings sense stretch (via geometric alteration of resident TRP channels) within the blood vessel and follow the baroreceptor reflex arc. The source of this stretch is typically caused by an increase in intraluminal pressure, which forces the vessel to expand in diameter in order to contain an elevated blood volume within a defined enclosed space (the vessel walls). These volume and pressure changes are also reflected as increased transmural pressure that is exerted by the freely flowing blood on the vessel wall and incidentally on the nerve terminals of the carotid sinus. Following stimulation, an excitatory signal is generated by an influx of cations entering the nerve terminal and the generation of a depolarization event. This afferent signal travels up the sinus nerve and connects to the glossopharyngeal nerve, which proceeds and terminates at the NTS in the brainstem. Once the signal has reached the brainstem, a series of excitatory or inhibitory interneurons communicate and are either stimulated or inhibited, which will differentially activate or inhibit one of the two divisions of the autonomic nervous system previously described. However, under abnormal conditions in the cerebral vasculature or carotid arteries, these control mechanisms may become compromised, thereby resulting in dysfunctional baroreceptor reflex mechanisms and compromised cerebral blood flow [5,9]. On

a broader scale, any obstruction or dysfunction at any point along this reflex arc may be instrumental in the pathogenesis of stroke and/or CVD as a whole [10].

## 5.4. Physiological blood flow profiles

Four types of physiological blood flow dominate the circulation: 1) pulsatile, 2) oscillatory, 3) laminar, and 4) turbulent [4,11]. Pulsatile and oscillatory blood flows are similar and result from periodic fluctuations in the upstream pumping of the heart in relation to downstream "vacuum" or pulling forces generated by the respiratory and skeletal muscles as well as changing metabolic demands. Since balance between laminar and turbulent flow is important for moderation of flow-mediated stresses and the development of CVD, ensuing discussion focuses on these two forms of blood flow. Continuous (unidirectional) laminar blood flow represents uninterrupted flow that occurs at or near the capillary level and is frequently studied in cell culture perfusion systems or computer modeling simulations. Laminar flow is characterized by layered blood flow in the absence of detectable blood velocity fluctuations or turbulence (e.g., eddies, whorls, and flow reversals). By convention, blood flow is laminar when Reynold's number (Re), a ratio of inertial to viscous forces of a solution, is below 2000, where Re=2rvp/n with r = radius, v = velocity, p = density, and n = viscosity. Re exceeds 3000 under conditions of turbulent flow. When Re is high, it means that the inertial forces outweigh the viscous forces of the liquid. Because stenotic vessels create a pressure drop distal to the site of occlusion, blood velocity increases at these sites and creates audible turbulence with extremely high Re values. Turbulent blood flow, then, is associated with changes in the layered context of flow as seen in laminar settings and is correlated with increased stresses on the vessel wall. Turbulent blood flow is also caused by branching or arborization of blood vessels (at bifurcations of arteries, for example) or by lesions or plaque located along the luminal wall which creates flow obstructions. Any local vascular region with a calculated Re > 3000 is associated with elevated risk for developing vascular occlusions (plaque) at that site.

Considering the importance of laminar flow in maintaining proper vascular homeostasis and turbulent or erratic blood flow in contributing to vascular dysfunction underlying CVD, we began studies aimed at investigating the influence of altered blood flow and flow-mediated shear stress on VSM migration. Using a newly developed in vitro flow apparatus with controlled fluid viscosity (ViscoLab4000) and a newly developed in vitro wound healing and migration assay (Ibidi microchannel VI.4), preexposure of rat VSM cells to 4 hours of elevated shear stress (10 dyn/cm$^2$) significantly reduced serum-stimulated migration compared to static controls (SC) with no observable changes in cell morphology through 16 hours (Figure 3). These early findings support our notion that elevated fluid shear stress protects against enhanced VSM cell migration as occurs in the setting of CVD.

These results demonstrate the importance of homeostatic levels of fluid shear in maintaining normal vessel function and are commensurate with other studies that have shown this time point and level of fluid shear stress to be physiologically relevant in reducing migration using non-in-situ migration assays [12,13]. It is important to accept the findings of these previous studies with some prudence since removing cells from their perfusion location and seeding them elsewhere can disrupt focal adhesions and other cytoskeletal arrangements formed in
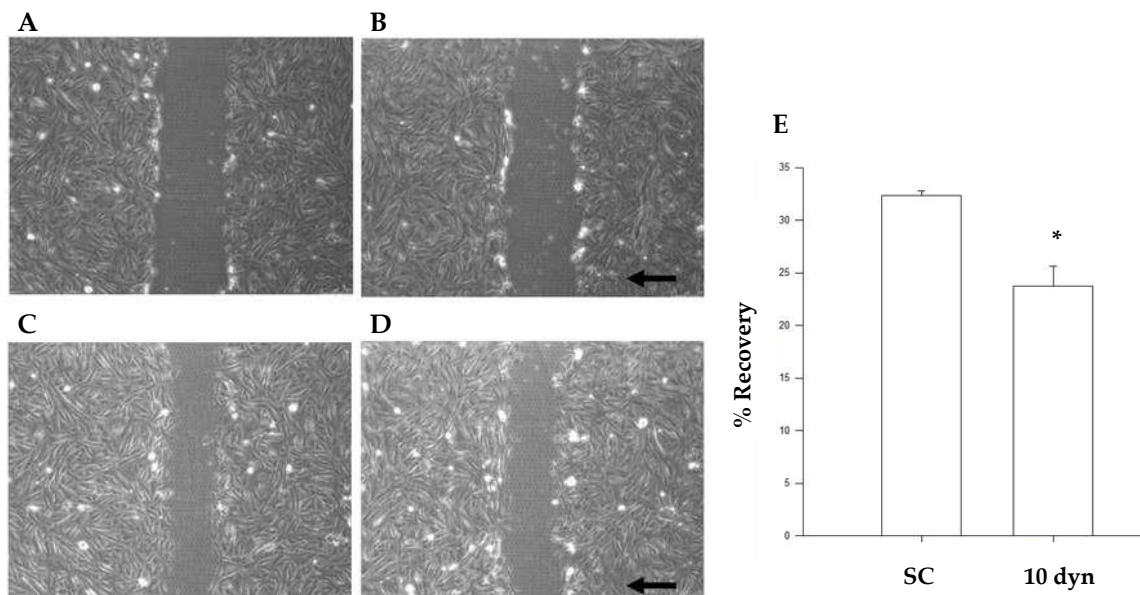
**Figure 3. Fluid shear stress reduces migration of VSM cells.** Using a newly developed laser capture microdissection (LCM)-assisted wounding assay to estimate cell migration, rat primary VSM cells exposed to increased shear stress show significantly reduced ability to migrate in response to serum compared to static control (SC) cells. Photomicrographs show confluent VSM cells exposed to a wounding scrape injury and treated with static flow (SC) or elevated flow (shear stress 10 dyn/cm$^2$) for 4 hours with continual exposure through 16 hours. **A** and **B** are photos taken after 4 hours pretreatment of 0 (SC) or 10 dyn/cm$^2$, and **C** and **D** represent photos taken after continual exposure for 16 hours. Arrows shown in **B** and **D** represent direction of flow. **E** shows measurements of percent recovery of the wound width normalized to time 0 for SC and flow-exposed cells.

response to fluid flow and that are necessary for proper cellular and cytoskeletal dynamics. Due to this shortcoming, we developed this novel wound-healing assay that utilizes an ultraviolet laser and LCM microscopy (Zeiss Palm Laser LCM) to precisely control and effectively denude adherent VSM cells from the perfusion channel substrate following exposure to fluid shear stress in a more physiological setting (without the aforementioned adverse confounding effects found in many traditional migration assays). This new technique allows the researcher to perfuse and injure cells in the same location without the deleterious effects of trypsinization and disruption of normal cellular architecture (including, notably, focal adhesions and cytoskeletal components). Channels are promptly washed with complete media prior to imaging at time zero. Migration time points are carried out up to 16 hours in order to prevent proliferation effects on wound closure rates. To further increase the accuracy and throughput of our migration measurements, we will use the motorized stage of the confocal microscope that is equipped with X, Y, Z coordinate control and on-stage incubator to perform simultaneous time-lapse analysis on multiple wounds.

As mentioned, one of the main functions of a healthy endothelial layer is to provide a key interface between blood flowing in the lumen and the underlying subintimal layer. When this functional endothelium becomes jeopardized, increased fluid shear stress experienced by the underlying medial VSM cells negatively contributes to arterial dysfunction. It has been shown that increased continuous laminar flow can improve re-endothelialization and attenuate VSM cell migration; however, in certain pathologies increased pulsatile and turbulent flow sensed

by exposed VSM and the presence of physical obstructions such as intimal plaque promotes VSM cell proliferation and migration as underpinnings of deleterious vessel growth and remodeling [14,15]. This pathogenic feed-forward mechanism has poor implications for proper vessel function and tissue perfusion.

## 6. Vascular endothelium

In addition to the essential VSM and autonomic neural mechanisms, a functional endothelium is equally important for appropriate blood flow control and modulation. VEC dysfunction is increasingly accepted as a common trait of nearly all forms of CVD and is often the initial insult in CVD pathogenesis. Though environmental and genetic factors consistently contribute to these disease states as well, the onset of vascular endothelial dysfunction can also be caused by smoking, hypertension, and/or diabetes [1]. Contrary to this evidence, Horvath and colleagues argue that arterial stiffness is not directly related to a properly functioning endo-thelium [9]. This is true in the sense that the mechanical elasticity of the vessel may compress or expand due to pressure changes alone, but a functional endothelium is nonetheless important for its contribution to baroreceptor function and is often a target for CVD prevention. It is well described that reduced NO bioavailability within the vasculature has a direct influence on VEC dysfunction [5]. Nonetheless, in the absence of an intact or functional endothelium, the VSM layer is still capable of receiving NO from alternate NOS isoforms independent of the endothelial layer. For example, inducible nitric oxide synthase (iNOS) either from circulating cells or platelets or from local adventitial nerves can provide VSM with bioavailable NO independent of endothelial contribution. Also, since iNOS is located within VSM it can intrinsically synthesize NO, in turn facilitating vasorelaxation in autocrine fashion. It should be noted though that the canonical pathway of vasodilation begins with VEC activation as described.

In sum, hemodynamics and intrinsic and extrinsic neural control of blood flow including sensory input to the carotid sinus and the ensuing baroreflex pathway are essential, yet can become compromised under many disease states. The causes of this dysfunction are not entirely understood, but research suggests that there is a direct link between autonomic output and neural integrity and vascular function/dysfunction and the establishment and/or mainte-nance of CVD. Thus, the preservation of the baroreceptor reflex within the carotid sinus as well as intrinsic/extrinsic neural control mechanisms are essential for ensuring adequate arterial blood supply to the brain in CVD prophylaxis.

## 7. Treatment strategies

Several treatment strategies currently exist to control and/or minimize symptoms associated with CVD. First, often the most prudent forms of CVD therapy involve lifestyle modifications, and many of these are based on known risk factors for CVD. Specific lifestyle choices may

already be in place in certain individuals in preventive/prophylactic measures and some of them may not be relevant to every CVD patient. Nonetheless, several examples of lifestyle modifications in either preventive fashion or following CVD diagnosis include appropriate weight control (keeping within the recommended BMI limits), particularly for increased waist-level fat or central fat (i.e., midline adiposity), appropriate control of diabetes and hypertension (if previously diagnosed), smoking cessation, low to moderate consumption of alcohol, an active lifestyle complete with regular moderate aerobic exercise, adequate and fitful sleep, and proper and well-balanced diet and nutrition [16]. In conjunction with these healthy lifestyle choices, the next line of defense against CVD often involves pharmacotherapy, also either already in place via prophylaxis (such as one-a-day baby aspirin, multivitamins including folate and B-complex) or prescribed against a form of CVD. Common pharmacotherapeutic strategies include antihypertension medication (beta-blockers, angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, diuretics), anticholesterol medications (statins), calcium signaling blockers, and agents to maintain proper insulin/glucose balance for diabetic patients. Antiplatelet therapies are also commonly prescribed for CVD patients. Following or concomitant with appropriate drug therapies can be surgical interventions to alleviate the problem. This can be of a variety of interventions including coronary artery or peripheral artery bypass grafting, transluminal angioplasty, and/or stent placement and deployment, depending on the nature of the complication. With many of these interventions, unfortunately, iatrogenesis or adverse side effects often occur, and the ensuing results can be even more significant than the original disorder.

## 8. Molecular and cellular signaling

Mechanistically, a wide variety of molecular and cellular signaling pathways and signal transduction processes have been theorized as serving key regulatory roles in the dedifferentiation and phenotypic conversion of VSM cells and subsequent vessel wall remodeling that leads to CVD. Among these, cyclic nucleotides and their predominantly kinase-driven downstream pathways represent significant regulatory factors in determining vascular physiology and pathology. Cyclic nucleotide signaling consists of purine and pyrimidine cyclic monophosphates, many of which have characteristics that allow them to serve as biologically important second messengers. The family of purine cyclic nucleotides consist of the well-known adenosine 3′,5′-cyclic monophosphate (cyclic AMP) and guanosine 3′,5′-cyclic monophosphate (cyclic GMP) and the lesser known inosine 3′,5′-cyclic monophosphate (cyclic IMP) and xanthosine 3′,5′-cyclic monophosphate (cyclic XMP). These signals operate primarily through protein kinase-driven phosphorylation events on downstream targets to exert functional control over a wide variety of cellular processes in a myriad of mammalian tissues. In the cardiovascular system, cyclic AMP and cyclic GMP are ubiquitous and are established as critical second messengers with abilities to regulate crucial homeostatic and pathophysiological functions. There also exists the less characterized family of cyclic pyrimidine nucleotides cytidine 3′,5′-cyclic monophosphate (cyclic CMP), uridine 3′,5′-cyclic monophosphate (cyclic UMP), and thymidine 3′,5′-cyclic monophosphate (cyclic TMP); however, their

physiological/pathophysiological importance in VSM and/or during CVD is not well understood.

As mentioned, several molecules exist in this wide family of cyclic nucleotides that are considered to be true second messengers. A second messenger is defined as being generated by a first-messenger-regulated enzyme, being activated by targeted effector proteins, exerting defined biological effects, being degraded by specific inactivation mechanisms, and whose effects can be duplicated by membrane-permeable analogues and/or bacterial nucleotidyl cyclase toxins. [17] Accordingly, both cyclic AMP and cyclic GMP are considered the traditional second messengers, being generated by membrane-bound and/or soluble cyclase enzymes, exerting many effects via intracellular kinases and/or nonkinase targets such as ion channels, have broad biological effects, are inactivated by a family of phosphodiesterases (PDEs), and whose effects can be mimicked by specific analogues or toxins. Also, new theories suggest that the pyrimidines cyclic CMP and cyclic UMP are in fact emerging second messengers in that they also fulfill these criteria [17,18].

The most recognized cyclic nucleotide second messengers are cyclic AMP and cyclic GMP which are synthesized and operate in similar mechanistic fashion. Cyclic AMP is generated through several different avenues including adenylate cyclase (AC) stimulation by direct agonists or following beta-stimulation or through G protein-coupled receptor activation. Following stimulation of AC, adenosine triphosphate (ATP) is dephosphorylated to produce cyclic AMP and PPi. Similarly, following activation of GC through natriuretic peptides (activating particulate GC) or from the gaseous ligands (NO) or carbon monoxide (CO) which activate soluble GC, GTP is dephosphorylated to yield cyclic GMP and PPi. Cyclic AMP and cyclic GMP operate largely through downstream phosphorylation events on Ser/Thr or tyrosine (Tyr) residues on target proteins and serve diverse roles in normal vascular physiology and homeostasis and during the pathogenesis of CVD. The preferred target proteins for cyclic AMP and cyclic GMP are protein kinase A (PKA) and PKG, respectively [19]. Much of the work from our lab over the past few years has focused on cyclic AMP and cyclic GMP and their abilities to target downstream phosphorylatable substrates mainly through PKA and PKG to elicit functional control over a variety of physiological and pathophysiological parameters elemental to CVD. Some of our latest studies have identified the Ser/Thr kinases PKA, PKG, protein kinase C (PKC), and the metabolic gauge AMP-activated protein kinase (AMPK) as biologically important regulators of VSM proliferation, migration, and chemotaxis; ECM and MMP balance; and cellular apoptosis or necrosis using a variety of experimental approaches and commercially available rodent VSM cells, primary rodent VSM cells, and human primary VSM cells. We have observed capacity of Ser/Thr-specific protein phosphatases (PPs) in moderating these kinase activities and in maintaining proper phosphorylative balance. We have also witnessed abilities of the Ser/Thr PPs to elicit control over VSM growth independent of direct kinase involvement. Additionally, many of these studies performed in cultured cells have been validated in whole animal models of injury-induced VSM growth, and these findings have verified biological ability of these cyclic nucleotide systems to operate in a whole body setting. Lastly, we have solidified these observations obtained in rodent models by recapitulating them in human primary coronary artery VSM preparations, thus

adding translational relevance to these basic science findings. Certainly, the capacity of cyclic AMP and cyclic GMP and their phosphorylatable kinase and PPi targets to control deleterious growth of VSM is significant and of particular importance for VSM-specific CVD. These observations provide important new perspectives on vessel wall biology and cell signaling and also highlight potential new targets that could be used to combat CAD and PAD and associated vascular occlusive disorders. Figure 4 depicts chemical structures of cyclic AMP and cyclic GMP, respectively.
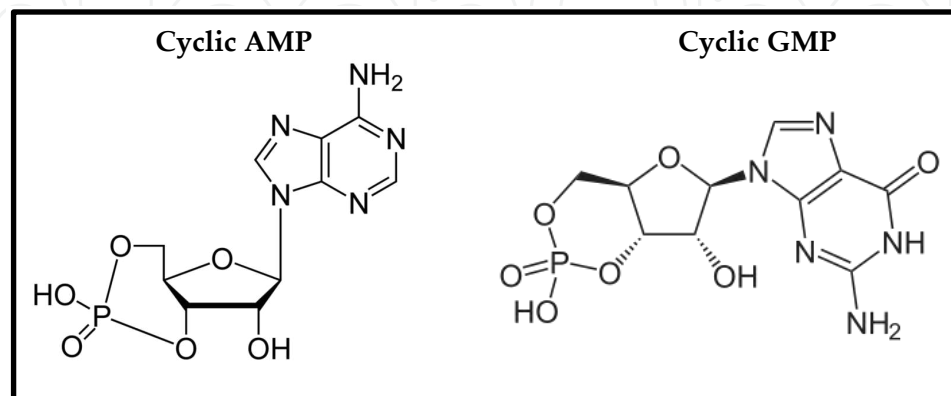


**Figure 4.** Chemical structures of cyclic AMP and cyclic GMP, respectively.

## 9. Vasodilator-stimulated phosphoprotein

Following generation of cyclic AMP and cyclic GMP and activation of their respective kinases PKA and PKG (among others, as discussed below), these kinases then target a variety of proteins and other factors to elicit functional consequences. Among these numerous bioactive targets is vasodilator-stimulated phosphoprotein or VASP. VASP belongs to the Ena/VASP Homology (EVH) family of cytoskeletal proteins that play essential roles in cellular dynamics and function. These proteins are involved in intracellular signaling processes and regulate "outside-in" communication via integrin–ECM interactions. VASP is comprised of an N-terminal EVH1 domain (targets focal adhesion and membrane domains), a proline-rich mid-region that binds to Src-homology 3 (SH3) domains, and WW domain-containing proteins that aid in Ser/Thr binding, and a C-terminal EVH2 domain that mediates tetramerization and actin/focal adhesion binding. Figure 5 shows a schematic of the primary VASP sequence including essential EVH1 and EVH2 domains.
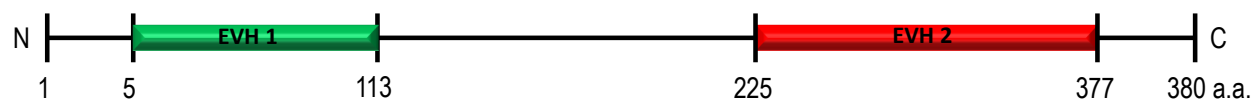


**Figure 5.** Schematic for the primary sequence of VASP complete with an N-terminal EVH1 domain (that targets focal adhesion/membrane domains) and a C-terminal EVH2 domain that targets focal adhesion/actin binding. (Adapted from [20])

VASP as a Ser/Thr-containing protein was originally characterized as a substrate for cyclic nucleotide-directed kinase-mediated phosphorylation signals, with cyclic AMP-driven PKA preferentially phosphorylating $VASP_{Ser157}$ and cyclic GMP-directed PKG primarily acting on $VASP_{Ser239}$ [21-24]. To date, at least four distinct Ser/Thr phosphorylation sites have been identified on VASP: $Ser_{157}$ and $Ser_{239}$ as well as $Thr_{278}$ and $Ser_{322}$ [25-27]. Interestingly, despite this original characterization of cyclic nucleotide-directed kinases "selectively" phosphorylating discrete Ser/Thr residues on downstream substrates including VASP, emerging reports from our lab and others have documented crosstalk and promiscuity of these and other Ser/Thr kinases to phosphorylate discrete residues on VASP. Recent observations from our laboratory in commercial [28] and primary VSM cells [19,29-31] as well unpublished findings in human primary coronary VSM cells document capacities of cyclic AMP and cyclic GMP to not only phosphorylate their respective canonical targets PKA and PKG but to also have abilities to phosphorylate other kinases as well. In these studies, findings reveal that cyclic AMP, stimulated via direct or indirect means, phosphorylates its accepted target PKA but also phosphorylates the preferred PKG target $VASP_{Ser239}$. Similarly, cyclic GMP, also activated directly or indirectly, phosphorylates its accepted target $VASP_{Ser239}$ as well as the supposed PKA substrate $VASP_{Ser157}$. We have also documented abilities of both cyclic AMP and cyclic GMP to stimulate members of the diverse PKC family [30]. Recently we observed that AMP kinase, another Ser/Thr enzyme, not only phosphorylates its canonical target $VASP_{Thr278}$ [32] but also has capacity to phosphorylate the preferred PKA target $VASP_{Ser157}$, yet interestingly without observable effects on $VASP_{Ser239}$ [33,34]. Recently it was reported that PKD, a downstream product of PKC and a Ser/Thr kinase involved in ECM receptor-mediated signal transduction pathways, phosphorylates both its reported $VASP_{Ser322}$ site as well as the PKA $VASP_{Ser157}$ site [35]. Considering this crosstalk among these kinases and the target VASP, it is important to note that while Ser/Thr and Tyr kinases can target a specific residue, these enzymes are also attracted to flanking residues alongside the known phosphoacceptor site. By this virtue, the catalytic cleft of the kinase interacts not only with its preferred Ser, Thr, and/or Tyr phosphoacceptor residue but also with their flanking regions, thereby binding to similar recognition sequences among similar substrate family members and in turn reducing their specificity for a particular residue. This kinase crosstalk then affords broad impact of upstream kinase signaling but also clouds precision of downstream targeting. Regardless of this observed promiscuity, these critical cyclic nucleotide/kinase/VASP signaling cascades elicit a vast array of significant biological effects in VSM and are of critical importance in vascular physiology and pathology.

Functionally, VASP operates as an anticapping protein and assists in the down-regulation of platelet adhesion molecules, promotes endothelial barrier protection, and assists in vessel structural integrity [36,37]. VASP was recently shown to protect against vascular inflammation and insulin resistance following exposure to high-fat diet [36]. In cancer research, VASP is implicated as a possible pharmacotherapeutic target aimed at controlling aberrant cancer cell migration [35,36]. In those studies, protection by VASP is attributed to its multiple phosphorylation residues (namely $Ser_{157}$, $Ser_{239}$, $Thr_{278}$, and $Ser_{322}$) that significantly and differentially impact its function as an actin-binding protein. Interestingly, like cancer cells, VSM cells rely on reorganization of their actin cytoskeleton as their primary mechanism of movement [38-40],
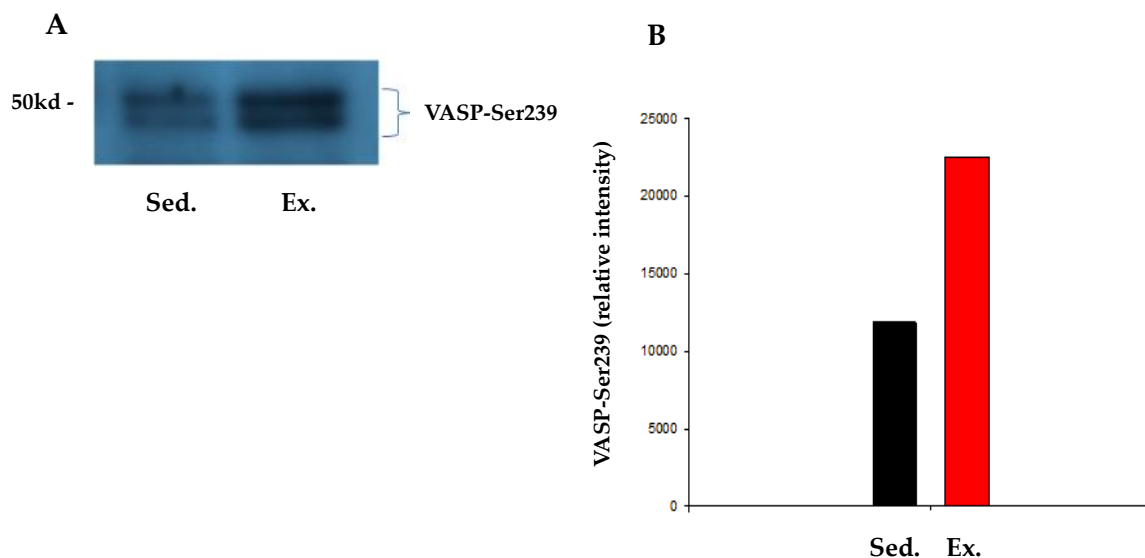
**Figure 6.** Exercise increases $VASP_{Ser239}$ in rat thoracic aorta (TA) segments following a 10 day treadmill running protocol. **A**: Tissue homogenates of TA sections were pooled from 4 animals per group (sedentary (Sed.) or exercised (Ex.)) and immunoprecipitation (IP) for $VASP_{Ser239}$ was performed. IP fractions were then subjected to gel electrophoresis followed by ECL Western blotting for $VASP_{Ser239}$. **B** represents results from densitometry for $VASP_{Ser239}$ from TA homogenates in Sed. or Ex. cohorts. Preliminary results show that exercise-induced shear stress approximately doubles $VASP_{Ser239}$ expression in rat TA sections compared to sedentary controls. These early results suggest that shear stress acts to reduce VSM migration via $VASP_{Ser239}$, which may serve as a mechanism underlying beneficial effects of exercise against CVD.

and so VASP serves as a plausible target for control of migration in the context of CVD. In our lab, we have recently examined site-specific phosphorylated VASP as a controller of VSM migration and proliferation in commercial and primary VSM cells [19,28-31]. In summary, results suggest that $VASP_{Ser239}$ acts primarily to control cell migration, yet $VASP_{Ser157}$ serves to regulate cell proliferation, both key components in CVD pathogenesis. Also, by virtue of its name, VASP is inherently involved in regulation of hemodynamics via its upstream activators (cyclic AMP/cyclic GMP/kinases) and downstream effectors. In the context of an endothelial-compromised (diseased) blood vessel with fenestration of the intimal–medial barrier and basement membrane, underlying VSM cells become exposed to elevated levels of blood flow and associated mechanical forces. In this regard, recent guidance from the American Heart Association [1] states physical activity as the first-line homeopathic therapy at ameliorating the symptoms associated with PAD, a main form of CVD. While it is known that exercise is beneficial for the circulatory system via formation of collaterals and arterialization of existing capillaries, our question of how hemodynamic forces alleviate PAD remains unanswered [41, 42]. Since exercise was recently shown to stimulate phosphorylated VASP [43], VASP is likely linked to beneficial outcomes observed following exercise. With this in mind, using a 10 day treadmill exercise regimen, preliminary observations show that hyperemic fluid shear stress has capacity to increase $VASP_{Ser239}$ in rat aorta segments compared to sedentary controls (Figure 6). This dilatory response can increase circumferential hoop stress and in the presence of an increased pressure head, consequently increase fluid shear stress. This effect can drastically alter the actin cytoskeleton within the cells of the exposed neointima [44,45] and perhaps then

serve to mitigate abnormal cellular migration and growth associated with CVD. Indeed, these early findings offer support for VASP and phosphorylated VASP species, as crucial cytoskeletal and signal transduction proteins, as capable of controlling deleterious vascular growth integral for CVD.

Indeed, VASP holds great promise regarding its ability to control VSM growth underlying vascular disorders but represents just one of the many end-targets of cyclic nucleotide-driven cellular signals. Vascular cyclic AMP and cyclic GMP and their Ser/Thr kinases also have capacity to exert regulatory control over synthetic TGF-beta signaling [19] as well as other cell-to-cell and focal adhesion/cytoskeletal proteins including gap junctional connexins [46,47], paxillin [34,48], G-actin and F-actin rations [34], and FAK [28,34]. We have also reported on capacities of these signals to regulate ECM-degrading MMPs as a potential route of action in VSM [34,49]. The broad influence of cyclic nucleotide signaling and its many end-target proteins including VASP and associated cytoskeletal components in VSM presents an attractive and biologically feasible therapeutic strategy aimed against basic elements of CVD and many forms of vascular dysfunction.

## 10. Summary and conclusions

The paramount importance of CVD on morbidity and mortality in the United States and globally is clear, yet inefficiencies in the clinical translation of basic science findings show that the underlying mechanisms of CVD are still not completely known and fully effective therapies against CVD are still needed. Indeed, despite ample basic and clinical research, CVD prevalence is estimated to increase 10% and the economic burden of CVD is projected to triple within the next 20 years with latest estimates that >40% of the adult US population will have some form of CVD by the year 2030 [50]. In our incessant search for possible targets to help control and possibly eliminate CVD, the pivotal influence of VSM growth in playing key roles in CVD pathogenesis is clear. We and others have focused our efforts on identifying and characterizing unique elements behind VSM biology and signaling under healthy as well as pathologic conditions, and findings to date reveal significant insights into many biochemical, molecular, and cellular elements foundational to CVD with perhaps that of cyclic nucleotide signaling at the forefront. Given its ubiquitous nature and multifaceted diverse functions, cyclic AMP and cyclic GMP and their downstream kinases and targets including VASP represent key elements capable of controlling deleterious vascular growth that serves as a basis for CVD. Only through persistent basic science and clinical investigation do we hope to fully understand these crucial factors that hold great promise in our seemingly never-ending struggle to combat and control CVD.

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## Author details

Andrew W. Holt and David A.  Tulis

*Address all correspondence to: tulisd@ecu.edu

Department of Physiology, Brody School of Medicine, East Carolina University, Greenville, North Carolina, USA

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