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Blood Pressure Regulation VIII: Resistance Vessel Tone and Implications for a Pro-Atherogenic Conduit Artery Endothelial Cell Phenotype

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Abstract

Dysfunction of the endothelium is proposed as the primary initiator of atherosclerotic peripheral artery disease, which occurs mainly in medium to large-sized conduit arteries of the lower extremities (e.g., iliac, femoral, popliteal arteries). In this review article, we propose the novel concept that conduit artery endothelial cell phenotype is determined, in part, by microvascular tone in skeletal muscle resistance arteries through both changes in arterial blood pressure as well as upstream conduit artery shear stress patterns. First, we summarize the literature supporting the involvement of sympathetic nerve activity (SNA) and nitric oxide (NO) in the modulation of microvascular tone and arterial blood pressure. We then focus on the role of elevated blood pressure and shear stress profiles in modulating conduit artery endothelial cell phenotype. Last, we discuss findings from classic and emerging studies indicating that increased vascular resistance, as it occurs in the context of increased SNA and/or reduced NO bioavailability, is associated with greater oscillatory shear stress (e.g., increased retrograde shear) in upstream conduit arteries. The ideas put forth in this review set the stage for a new paradigm concerning the mechanistic link between increased microvascular tone and development of conduit artery endothelial dysfunction and thus increased risk for peripheral artery disease. Indeed, a vast amount of evidence supports the notion that excessive blood pressure and oscillatory shear stress are potent pro-atherogenic signals to the endothelium.

Introduction

Peripheral artery disease is characterized by the formation of atherosclerotic lesions typically localized in the medium to large-sized conduit arteries of the lower extremities (Ross *et al.*, 1984; Stary *et al.*, 1995; Kroger *et al.*, 1999; Moore, 2002). In the United States, peripheral artery disease affects approximately 8 to 12 million men and women over the age of 35 (Hirsch *et al.*, 2001), and it has been projected that by the year 2050 this debilitating disease will influence approximately 19 million Americans over the age of 65 (Becker *et al.*, 2002). The narrowing of the arteries produces progressive reductions in blood flow to the legs, often leading to pain on exertion, limited mobility, and diminished quality of life (Stewart *et al.*, 2002; McDermott *et al.*, 2008). Furthermore, patients with peripheral artery disease are at increased risk for mortality and cardiovascular events including

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Conflict of Interest None

myocardial infarction and stroke (Criqui *et al.*, 1992). Because disturbance of the endothelium is proposed as a primary etiology of atherosclerotic cardiovascular disease, intense research efforts have been and continue to be directed at understanding the physiological and molecular mechanisms that regulate the phenotype of endothelial cells in conduit arteries.

Herein, we propose the concept that conduit artery endothelial cell phenotype is influenced by alterations in vascular tone of downstream skeletal muscle resistance arteries. This proposal is founded on the idea that changes in microvascular tone produce alterations in systemic blood pressure and shear stress profiles in upstream conduit arteries, both of which are important signals for the regulation of endothelial cell phenotype (Laughlin *et al.*, 2008). In this regard, we discuss the evidence from classic and recent studies suggesting that alterations in vascular resistance and ensuing changes in systemic blood pressure and conduit artery shear stress profiles can modulate conduit artery endothelial cell phenotype. A particular emphasis is placed on the individual and interactive roles of sympathetic nerve activity (SNA) and endothelium-derived nitric oxide (NO) bioavailability in modulating vascular resistance in skeletal muscle. Accordingly, the overall purpose of this review is to make the case that increases in vascular tone in resistance arteries may importantly contribute to a pro-atherogenic endothelial cell phenotype in upstream conduit arteries through alterations in hemodynamic forces (i.e., arterial pressure and shear stress; Figure 1), a notion that has been largely underappreciated.

Role of SNA and NO in modulating vascular resistance and arterial blood pressure

SNA is well recognized for its critical role in maintaining vascular conductance and blood pressure during rest and periods of stress (Marshall *et al.*, 1961; Cowley *et al.*, 1973; Montastruc *et al.*, 1996; Coote, 2007). In order for sympathetic nerve impulses to change vascular tone there must be successful release of neurotransmitter(s) into the synaptic cleft (Boura & Green, 1959), alpha receptor binding (Youmans *et al.*, 1955), and resultant signal transduction within vascular smooth muscle (Waugh, 1962) to elicit a vasoconstrictor response (Vanhoutte *et al.*, 1981). This paradigm is widely accepted (Su, 1978; Burnstock, 1986; Hirst *et al.*, 1996), and most apparent, in response to large reflex-mediated increases in SNA (Davy *et al.*, 1998; Minson *et al.*, 2000; Ray & Monahan, 2002). Our group has recently performed a series of studies examining the ability of spontaneously occurring muscle SNA bursts to dynamically control skeletal muscle vascular tone on a beat-by-beat basis under normal resting conditions in healthy young men (Fairfax *et al.*, 2013). We adapted a spike triggered averaging method first described by Wallin and Nerhed (Wallin & Nerhed, 1982) and characterized beat-by-beat femoral and brachial vascular conductance (duplex Doppler Ultrasound) responses for 15 cardiac cycles following heartbeats associated with and without muscle SNA bursts (microneurography). The findings indicated that individual, spontaneously occurring muscle SNA bursts produced transient beat-by-beat decreases in femoral and brachial artery vascular conductance that are eliminated by alpha adrenergic blockade. Furthermore, natural variations in the amount of spontaneous muscle SNA (i.e., burst pattern and burst size) was strongly associated with the magnitude of sympathetic vasoconstriction. Perhaps more notably, cardiac cycles without muscle SNA bursts exhibited increases in vascular conductance, highlighting the importance of muscle SNA bursts in the maintenance of resting vascular tone in skeletal muscle. We also found that changes in femoral and brachial artery forearm vascular conductance occurred in temporal synchronicity with changes in total vascular conductance and mean blood pressure, indicating that beat-by-beat sympathetic vascular transduction in skeletal muscle plays an important role in the regulation of resting systemic cardiovascular hemodynamics.

Collectively, these data demonstrate the critical role of muscle SNA in the beat-to-beat control and maintenance of skeletal muscle resistance vessel tone and blood pressure (Figure 1).

Another important modulator of skeletal muscle resistance vessel tone and blood pressure is endothelium-derived NO. In 1980, Furchgott and Zawadzki (1980) demonstrated that the presence of vascular endothelial cells is obligatory for acetylcholine to induce relaxation in the isolated rabbit aorta. In particular, throughout a series of experiments the authors noted that the occasional loss of acetylcholine-induced relaxation of aortic rings was attributable to an “unintentional rubbing of its intimal surface against foreign surfaces during its preparation” (Furchgott & Zawadzki, 1980). This astute observation that acetylcholine-induced relaxation is endothelium mediated supported the existence of an endogenous factor initially named endothelium derived relaxing factor (EDRF), and subsequently identified as NO (Ignarro *et al.*, 1987; Palmer *et al.*, 1987). NO is produced by the enzyme NO synthase (NOS) during the oxidation of the amino acid L-arginine to L-citrulline. Three isoforms of NOS have been identified: the endothelial isoform (eNOS), neuronal isoform (nNOS), and macrophage or inducible isoform (iNOS). While eNOS and nNOS are normal constituents of healthy cells, iNOS is not typically expressed in vascular cells and its expression is mainly present in conditions of infection or inflammation (Vallance & Chan, 2001). The first evidence in humans that NO plays a central role in the regulation of vascular resistance was provided by Vallance, Collier and Moncada in 1989 (Vallance *et al.*, 1989). In this study, infusion of N^G monomethyl-L-arginine (L-NMMA), a NOS inhibitor, into the brachial artery of healthy subjects produced 50% reduction in basal blood flow (Vallance *et al.*, 1989). In addition, it has been shown that when the NOS inhibitor is administered systemically, subjects exhibit marked increases in blood pressure (Hansen *et al.*, 1994; Sander *et al.*, 1999; Young *et al.*, 2009). Further evidence that endothelium-derived NO bioavailability is critical for maintenance of blood pressure comes from eNOS knockout studies in rodents. To our knowledge, Shesely *et al.* (1996) were the first to demonstrate that mice lacking a functional eNOS gene exhibited increased blood pressure compared to the wild type mice, an observation that was later substantiated by Stauss *et al.* (1999). Together, it is evident that endothelium-derived NO is a potent vasodilator and its balance with various endothelium derived vasoconstrictor agents (e.g., endothelin 1) and SNA dictate overall vascular tone. Several studies have demonstrated an interaction between NO-mediated dilation and sympathetically-mediated constriction (Hijmering *et al.*, 2002). Likewise, we have recently observed that phenylephrine-induced contraction of isolated rat aortic rings is augmented after NOS inhibition and even further increased after mechanical denudation of the endothelium (Figure 2, unpublished data). These data suggest that basal NO can function as a “buffer” for sympathetically-mediated vasoconstriction. Importantly, lack of NO bioavailability in the resistance arteries and thus impaired microvascular endothelial function may increase the propensity to development of hypertension as well as overall risk for cardiovascular diseases (Panza *et al.*, 1990; Landmesser & Drexler, 2007; Schulz *et al.*, 2008). In this regard, in a 7-year follow up study in patients with essential hypertension, greater endothelium-dependent increases in forearm blood flow achieved with intra-brachial infusions of the NO-dependent vasodilator acetylcholine was associated with reduced cardiovascular events (Perticone *et al.*, 2001).

While the effects of NO on the peripheral vasculature have been extensively studied, less attention has been focused on the central actions of NO. Importantly, all NOS isoforms are present in brainstem centers involved in the regulation of SNA and blood pressure (Lawrence *et al.*, 1998; Paton *et al.*, 2006) and functional evidence indicates that NO is a key signaling molecule involved in the tonic restraint of sympathetic outflow from the brainstem (Sander *et al.*, 1995; Sander *et al.*, 1997; Sander *et al.*, 1999; Lepori *et al.*, 2001; Sartori *et al.*, 2005). Indeed, intracisternal injection (Togashi *et al.*, 1992; el Karib *et al.*,

1993), or direct injection of NOS inhibitors into the nucleus tractus solitarius (Harada *et al.*, 1993) or rostral ventrolateral medulla (Shapoval *et al.*, 1991; Zanzinger *et al.*, 1995), known sites of sympathetic regulation within the brainstem, has been shown to evoke acute increases in renal SNA and blood pressure. The extension of these findings in anesthetized preparations to conscious animals revealed that the initial hypertension caused by systemic NOS inhibition is not dependent on SNA, rather SNA contributes to the slower progressive increase and maintenance of the elevation in blood pressure (Sander *et al.*, 1997; Sander *et al.*, 1999). In fact, a minimum of thirty minutes may be necessary to detect the sympathetic component to NO-deficient hypertension induced by systemic NOS inhibition. This may reflect the time needed for NOS inhibitors to pass the blood brain barrier and override the baroreflex-mediated suppression of sympathetic nerves in response to the immediate blood pressure increase caused by inhibition of endothelium-dependent vasodilation in the peripheral vasculature (Traystman *et al.*, 1995). The temporal component to NO-deficiency induced activation of central sympathetic outflow was first noted by studying conscious rats with and without sympathectomy during systemic NOS inhibition with L-NAME (Sander *et al.*, 1997). The initial increase in blood pressure was similar in intact and sympathectomized rats whereas over time blood pressure continued to rise in intact rats but not in the sympathectomized rats indicating the importance of SNA to NO-deficient hypertension. These results have recently been followed up and supported by Augustyniak *et al.* using direct measures of renal SNA in conscious rats (Augustyniak *et al.*, 2006). Several human studies have extended these animal findings demonstrating a clear sympathetic contribution to NOS inhibition induced hypertension. Studies have indicated a dose dependence of the sympathetic response to systemic intravenous L-NMMA with low dose infusion not altering MSNA and higher dosages suppressing SNA (Hansen *et al.*, 1994; Owlya *et al.*, 1997; Lepori *et al.*, 1998). The lack of a sympathetic effect at the lower dosage in comparison to the sympathoinhibition evoked by an equal pressor response elicited by phenylephrine was taken as evidence for a sympathoexcitatory effect of NOS inhibition. Additionally, minimizing the L-NMMA induced increase in blood pressure with the co-infusion of nitroprusside caused muscle SNA to increase. Thus, prevention of the baroreflex-mediated suppression of muscle SNA unmasked a sympathoexcitatory effect of L-NMMA infusion. Furthermore, Sander *et al.* (1999) reported that although blood pressure was significantly elevated after infusion of the NOS inhibitors L-NMMA and L-NAME, the hypertension caused by L-NAME was much greater and continued to progressively increase up to 180 minutes post infusion. Moreover, alpha-adrenergic blockade with phentolamine to block the sympathetic contribution to the NOS inhibition-induced hypertension had very little effect when administered immediately after L-NAME infusion but reversed a substantial part of the increase in blood pressure seen 90 minutes after the infusion. These findings agree with the animal studies and indicate that inhibition of NO mediated endothelium-dependent vasodilation in the periphery is the primary mechanism underlying the initiation of the hypertensive response to NOS inhibition but the sympathetic nervous system plays an important role in the full expression and maintenance of the blood pressure raising effect. Indeed, we recently demonstrated that systemic experimental inhibition of NOS with L-NAME causes central sympathetic activation in healthy humans (Young *et al.*, 2009). In this study, we measured skin SNA because it is not under arterial baroreceptor control in normothermic conditions (Wallin *et al.*, 1975) and therefore, we could avoid the initial sympathetic inhibition via the arterial baroreflex due to the immediate rise in blood pressure with removal of NO mediated vasodilation in the periphery. Time and hypertensive (phenylephrine) control experiments were also performed with skin SNA only being increased significantly after L-NAME. Overall, there is abundant evidence from both animal and humans demonstrating that NO is a key signaling molecule involved in the regulation of central sympathetic outflow. Thus, the impact of NO deficiency, as occurs in many diseases,

would not only be the removal of peripheral NO but also, in combination, the removal of central NO and associated increases in SNA.

Role of elevated arterial blood pressure in modulating conduit artery endothelial cell phenotype

A growing body of evidence indicates that hypertension is a major and independent risk factor for coronary artery (Julius *et al.*, 1990; Stauss *et al.*, 1999) and peripheral artery disease (Vogt *et al.*, 1993; LaMorte *et al.*, 1995). It has been estimated that the risk of cardiovascular disease doubles with each blood pressure increment of 20/10 mm Hg, beginning at 115/75 mm Hg (Lewington *et al.*, 2002). Even in children and young adults, elevated blood pressure has been associated with atherosclerotic autopsy lesions, as well as with increased carotid intima-media thickness and decreased arterial compliance (Berenson *et al.*, 1998; McGill *et al.*, 2000; Li *et al.*, 2003; Raitakari *et al.*, 2003; Chen *et al.*, 2004; Juonala *et al.*, 2005). In this regard, data from the Young Finns Study indicate that elevated blood pressure in adolescence predicts impairment of brachial artery flow-mediated dilation (FMD) 21 years later in adulthood (Juonala *et al.*, 2006). This association is independent of other childhood and adulthood cardiovascular risk factors suggesting the important influence of elevated blood pressure in modulating vascular endothelial function.

In cross-sectional studies, blood pressure levels have also been shown to be inversely correlated with brachial artery FMD, and hypertensive patients exhibit blunted FMD responses (Celermajer *et al.*, 1994; Andrews *et al.*, 1997; Gokce *et al.*, 2001; Benjamin *et al.*, 2004; Corrado *et al.*, 2005). Further evidence that elevated blood pressure is deleterious to endothelial cells is derived from coarctation studies in animals (Lockette *et al.*, 1986; Miller *et al.*, 1987; Lai *et al.*, 1989; Bell & Bohr, 1991; Bell, 1993). Increased aortic pressure proximal to the coarctation increases its susceptibility to atherosclerosis, whereas reduced aortic pressure distal to the coarctation is protective of plaque formation (Lyon *et al.*, 1987). These findings are consistent with the fact that atherosclerosis preferentially develops in systemic arteries, where blood pressure is high, and not in pulmonary arteries and/or veins, where blood pressure is low (Barboriak *et al.*, 1974; Bedarida *et al.*, 1993; Thubrikar & Robicsek, 1995).

The pro-atherogenic effects of increased blood pressure are also supported by experiments in isolated intact arteries. In this context, we have observed in isolated, pressurized rat carotid arteries that increased intraluminal pressure for 4 hrs results in increased expression of adhesion molecules such ICAM-1 and VCAM-1 (Figure 3A, unpublished data). Similarly, Ungvari *et al.* (Ungvari *et al.*, 2003) exposed rat femoral arterial branches to normal and high intraluminal pressures for 30 min and found high pressure to impair endothelial function due to an overproduction of superoxide radicals.

With elevated blood pressure, endothelial cells are exposed to an increased transmural force and increased circumferential stretch, both of which are signals that can alter endothelial cell phenotype (Laughlin *et al.*, 2008). Numerous cell culture studies have investigated the specific effects of pressure and stretch on endothelial cells. For example, Hishikawa *et al.* (Hishikawa *et al.*, 1992; Hishikawa *et al.*, 1995) have shown that application of pressure on cultured endothelial cells inhibits the release of NO and increases the release of ET-1. On the other hand, the effects of stretch on endothelial cells have been investigated by applying cyclic stretch to endothelial cells cultured on elastic membrane mounted in a stretch device. Studies utilizing this preparation demonstrate that cyclic stretch increases endothelial expression of proatherogenic genes such as MCP-1, ET-1, ICAM-1, E-Selectin and PAI-1 (Wang *et al.*, 1995; Cheng *et al.*, 1996a; Cheng *et al.*, 1996b; Wung *et al.*, 1996; Cheng *et al.*, 1998). Importantly, current data suggest that this pro-atherogenic effect of cyclic stretch

in endothelial cells is mediated in part by the production of reactive oxygen species (Cheng *et al.*, 1998). The role of pressure-induced circumferential stretch in mediating atherosclerosis is also supported by *in vivo* data. In an elegant study, Tropea *et al.* (2000) reduced aortic wall motion in the hypertensive atherosclerosis-prone aorta proximal to a coarctation by externally wrapping a segment of the rabbit aorta and studied the effect on plaque formation. The authors found that localized removal of aortic wall motion in the lesion-prone hypertensive aorta resulted in a significant reduction of intimal plaque formation, thus suggesting that increased pulse pressure that leads to greater than normal wall cycle stretch stimulates development of atherosclerotic lesions (Tropea *et al.*, 2000). Data in humans also suggest that even acute increases in blood pressure can be detrimental to the endothelium of conduit arteries. For example, exposure of the brachial artery to a hydrostatic pressure gradient by vertically hanging the arm for 3 hrs markedly blunts the FMD response (Padilla *et al.*, 2009). Likewise, a transient increase in blood pressure with leg press exercise is also associated with a blunted brachial artery FMD response in normal healthy subjects (Jurva *et al.*, 2006). Together, these data derived from *in vitro* and *in vivo* experiments support the idea that elevated blood pressure is a pro-atherogenic stimulus to the arterial wall (Figure 1).

Role of shear stress profiles in modulating conduit artery endothelial cell phenotype

This section addresses the effects of altered shear stress profiles on vascular endothelial phenotype, highlighting (i) correlational evidence from human and animal studies that local shear patterns are important determinants of the heterogeneous distribution of atherosclerosis; (ii) *in vitro* data from cell culture models and isolated arteries demonstrating that low mean flow (shear) and oscillatory flow patterns (i.e., increased presence of retrograde flow and/or decreased presence of antegrade flow) produce shear stresses that are deleterious signals to the endothelium, (iii) studies from animal models demonstrating atherogenic effects of disturbed flow using *in vivo* experimental modulation of shear, and (iv) recent experimental studies in humans demonstrating that disturbed blood flow acutely causes endothelial dysfunction and injury in humans. For the purposes of this review, increases in oscillatory blood flow as a result of increases in retrograde flow and/or decreases in antegrade flow is referred to as disturbed flow.

In vivo correlational studies supporting a link between disturbed flow and endothelial cell phenotype

There is a long-standing correlation between *in vivo* local shear patterns and susceptibility to atherosclerotic disease in humans as well as in animal models of atherosclerosis. In their classic paper of 1969, Caro and colleagues (1969) examined the frequency of atherosclerotic lesions in low shear versus high shear regions of branch points in the coeliac, superior mesenteric, and the left renal and right renal arteries. They demonstrated that compared to regions of higher shear (i.e., inner wall of branch points), low shear regions (i.e., outer wall of daughter vessels) displayed a ~3-fold greater burden of atherosclerotic lesions. Having established the co-localization of lesions with sites of low shear, the authors put forth the theory that local hemodynamics play a causal role in the etiology of atherosclerotic disease (Caro *et al.*, 1969). Additionally, of historical significance, it is important to note that Caro *et al.* may have been the first to suggest that “physical exercise involving increase of cardiac output, and hence increased shear rate, might retard the development of atheroma” (Caro *et al.*, 1969). In further support of the association between local shear patterns and vascular phenotype, it was also shown that postmortem human carotid artery specimens displayed the greatest intima thickness and atherosclerotic burden at the outer wall of the vessel where mean shear is low and there is substantial flow reversal (Zarins *et al.*, 1983). Readers are

referred to an excellent historical review summarizing these and other early milestones of discovery regarding the relationship between shear stress and the development of atherosclerosis (Caro, 2009).

Animal studies investigating the site specificity of atherosclerosis have been largely confirmatory of the early human observations. Our group has made extensive use of porcine models for the study of atherosclerosis and related aspects of vascular biology (Laughlin *et al.*, 2008; McAllister *et al.*, 2008; Laughlin *et al.*, 2012), as the pig is widely considered to be among the best non-primate animal models for human cardiovascular research (Critser *et al.*, 2009). As seen in the human arterial tree, hemodynamic analyses have revealed that low and oscillatory shear regions occur in porcine coronary and peripheral conduit arteries at geometrically irregular sites (i.e. branch points, bifurcations and curvatures), and these regions are highly athero-susceptible (Greer *et al.*, 1966; Passerini *et al.*, 2004; Huo *et al.*, 2007; LaMack *et al.*, 2007; Davies *et al.*, 2010; Civelek *et al.*, 2011). In a comprehensive assessment of phenotypic heterogeneity across multiple porcine conduit arteries and veins, our group recently demonstrated that expression of anti-atherogenic mRNAs and proteins such as eNOS, KLF2 and KLF4 was lower in athero-susceptible arterial regions (e.g., the distal aorta and aortic arch) relative to athero-resistant sites (e.g., the proximal aorta and internal mammary artery). In addition, we also found that in the Rapacz pig model of familial hypercholesterolemia (Rapacz *et al.*, 1986), the distal portion of the aorta, which is exposed to disturbed blood flow, displays a ~3 fold greater extent of atherosclerotic burden compared to the proximal portion of descending aorta, which is subjected to more unidirectional flow (Figure 4, unpublished data).

***In vitro* evidence for disturbed flow-induced alterations in endothelial phenotype**

Endothelial cell culture experiments have elucidated a number of complex mechanotransduction and signaling mechanisms acting in concert to alter gene expression and function in response to directional changes in the shear stimulus (Johnson *et al.*, 2011). From these studies, it is clear that shear patterns characteristic of disturbed blood flow induce a pro atherogenic endothelial phenotype. Early studies established that oscillatory shear induces endothelial inflammation, activation, and abnormal alignment of endothelial cells (Davies *et al.*, 1986), whereas laminar shear attenuates inflammation-related events such as leukocyte-endothelial adhesion (Lawrence *et al.*, 1987). Recent studies also indicated that pro-atherogenic genes are upregulated in cultured endothelial cells exposed to shear patterns of *in vivo* atheroprone regions relative to cells exposed to patterns observed in protected regions (Brooks *et al.*, 2002; Hastings *et al.*, 2007; O’Keeffe *et al.*, 2009). Pro-atherogenic shear patterns increase production of NADPH oxidase- and mitochondria-derived reactive oxygen species, augments production of ET-1, and enhances expression of VCAM-1 and ICAM-1 (Hwang *et al.*, 2003a; Hwang *et al.*, 2003b; Dai *et al.*, 2004; O’Keeffe *et al.*, 2009; Conway *et al.*, 2010; Takabe *et al.*, 2011; Willett *et al.*, 2011), all critical early atherosclerotic markers.

Similar results have been observed in isolated artery studies. We show in isolated, pressurized rat carotid arteries that reduced shear stress (3 dyn/cm²) for 4 hrs results in increased expression of adhesion molecules such ICAM-1 and VCAM-1 (Figure 3B, unpublished data). In addition, pig carotid arteries exposed for three days to oscillatory shear displayed markedly reduced endothelium-dependent relaxation (Gambillara *et al.*, 2006). This functional effect of oscillatory shear was accompanied by reduced eNOS mRNA and protein expression in isolated arteries as well as reduced basal and bradykinin-stimulated eNOS activation in primary culture of porcine endothelial cells. Retrograde flow produced significant reductions in NO production in isolated porcine arteries as a result of enhanced superoxide production (Lu & Kassab, 2004), a well-established molecular mediator of

atherogenesis (Pacher *et al.*, 2007). The primary source of disturbed flow-induced reactive oxygen species production in isolated arteries appears to be NADPH oxidase (Lu & Kassab, 2004; Godbole *et al.*, 2009). Overall, the data from *in vitro* cell culture and isolated vessel preparations strongly and consistently support the notion that disturbed blood flow patterns promote a pro-atherogenic endothelial cell phenotype.

Experimental evidence for deleterious effects of disturbed flow *in vivo*: animal studies

The partial carotid artery ligation model has proven to be a useful experimental model for the *in vivo* study of disturbed blood flow in rodents (Korshunov & Berk, 2003; Nam *et al.*, 2009). In this procedure, all but one of the distal branches of the common carotid artery are ligated, leaving open either the occipital artery (Korshunov & Berk, 2003) or the superior thyroid artery (Nam *et al.*, 2009). As a result of this experimentally-induced increase in vascular resistance, the proximal portion of the carotid artery is chronically subjected to disturbed blood flow. Partial carotid ligation-induced disturbed flow produces substantial wall thickening with leukocyte infiltration and smooth muscle proliferation within 2 weeks in normal wild type mice (Korshunov & Berk, 2003) and endothelial dysfunction and advanced atherosclerosis in ApoE^{-/-} mice (Nam *et al.*, 2009). These functional changes are preceded by rapidly-induced molecular phenotypic changes, as the expression of some genes are altered in as little as 12 hrs, and expression of more than 500 genes is altered within 2 days following ligation (Ni *et al.*, 2010). Moreover, this model has provided substantial insights into the mechanistic underpinnings of disturbed blood flow-induced atherosclerosis. Leukocytes have been reported to rapidly accumulate in the arterial wall during initiation and progression of disturbed flow-induced atherosclerosis (Alberts-Grill *et al.*, 2012). There is also evidence that eNOS uncoupling (Li *et al.*, 2011), NADPH oxidase-derived reactive oxygen species (Nam *et al.*, 2009), fibronectin polymerization (Chiang *et al.*, 2009), IL-17 signaling (Madhur *et al.*, 2011), and adhesion molecules such as PECAM-1 (Chen & Tzima, 2009) all contribute to the development of the proatherogenic phenotype induced by partial carotid ligation in mice. Finally, experiments in which the partial ligation model was superimposed onto rodent models of obesity (Lee *et al.*, 2012) and renovascular hypertension (Jin *et al.*, 2012) suggest that the pro-atherogenic effects of disturbed flow are more pronounced in the presence of cardiovascular risk factors. This is an area that warrants additional research.

Experimental evidence for deleterious effects of disturbed flow *in vivo*: human studies

Surprisingly, whether disturbed blood flow influences the human vasculature *in vivo* has been investigated in only three experimental studies to date. In their seminal experiment, Thijssen *et al.* (2009) used 25, 50, and 75 mmHg distal forearm cuff occlusion pressures to elicit progressive increases in brachial artery retrograde flow and shear, which induced a dose-dependent reduction in brachial artery FMD. In a follow-up study, Johnson *et al.* (2013) found that the decline in FMD following a period of distal forearm cuff occlusion is prevented with vitamin C supplementation, suggesting that the detrimental influence of disturbed blood flow on endothelium-dependent dilation is mediated by an increase in oxidative stress. Together, these data provide experimental evidence to support that disturbances in blood flow produce conduit artery endothelial dysfunction *in vivo*. Our group recently provided insight into the cellular/molecular mechanisms underlying the impairment in endothelial function. In particular, we observed that disturbed blood flow acutely induces endothelial injury, as evidenced by local increases in the concentration of endothelial microparticles (EMPs) (Jenkins *et al.*, 2013). EMPs are small particles (< 1 μ m) that are released from endothelial cells into circulation due to endothelial cell activation, apoptosis, or injury. In our study, a 220 mmHg occlusion cuff was placed on the distal forearm (i.e., around the wrist) of 10 young, healthy men to create a localized region of disturbed blood flow in the proximal vasculature for 20 min (Figure 5A). This maneuver reduced mean and

antegrade blood flow and increased retrograde flow and oscillatory shear stress, indicative of disturbed blood flow. Relative to baseline, CD62E⁺ and CD31⁺/CD42b⁻ EMP levels increased substantially (Figure 5B and 5C). As these EMP populations are well-established markers of endothelial activation (CD62E⁺) and apoptosis (CD31⁺/CD42b⁻) (Chironi *et al.*, 2009; Dignat-George & Boulanger, 2011), these data provide novel evidence for disturbed blood flow-induced endothelial cell injury in humans (Jenkins *et al.*, 2013).

Role of SNA and NO in modulating vascular resistance and conduit artery shear stress profiles

The usual triphasic flow pattern observed in peripheral conduit arteries is characterized by a large antegrade flow during systole, followed by a brief episode of retrograde flow in early diastole and a subsequent phase of antegrade flow in mid and late diastole (McDonald, 1955; Blackshear *et al.*, 1979). However, the shape of the flow wave varies markedly between individuals, physiological states, and arterial locations. Considering the recognized impact of shear stress magnitude and patterns on endothelial cell phenotype, as discussed above, it appears fundamental to devote efforts towards elucidating factors that may influence flow profiles in peripheral conduit arteries. Previous (Baccelli *et al.*, 1985) and more recent (Thijssen *et al.*, 2009; Jenkins *et al.*, 2013; Johnson *et al.*, 2013) observations that inflating a pressure cuff on the forearm results in greater retrograde flow at the brachial artery strongly suggests that changes in downstream peripheral vascular resistance may contribute to alterations in upstream conduit artery flow patterns. Reciprocally, brachial artery retrograde flow is reduced or eliminated as a result of decreases in downstream resistance (Blackshear *et al.*, 1979; Tinken *et al.*, 2009; Simmons *et al.*, 2011), such as during prolonged exercise and forearm heating (Padilla *et al.*, 2011b; Simmons *et al.*, 2011). Based on these observations, we reasoned that factors altering vascular tone (e.g., SNA) may be prime candidates participating in the regulation of upstream oscillatory shear patterns in conduit arteries. In this regard, pilot work generated by our group provided anecdotal evidence that the abrupt and prominent increase in muscle SNA induced by a voluntary apnea was followed by enhanced retrograde flow at the brachial artery. This anecdotal observation, later confirmed by Millar *et al.* (Millar *et al.*, 2011), prompted us to systematically test the hypothesis that acute increases in muscle SNA and associated increases in vascular resistance would be accompanied by augmented conduit artery retrograde and oscillatory shear rate (Padilla *et al.*, 2010). Indeed, we found in young healthy subjects that during lower body negative pressure (LBNP), graded increases in muscle SNA resulted in progressive increases in retrograde and oscillatory shear, as well as reductions in mean shear (Padilla *et al.*, 2010). This link between muscle SNA and disturbed blood flow profiles is not only apparent when large reflex-mediated increases in muscle SNA are present. For example, we recently used spike-triggered averaging to examine the influence of spontaneously occurring muscle SNA bursts on femoral artery shear rate patterns. In a beat-by-beat basis, we found an increase in oscillatory shear following muscle SNA bursts, an effect that was absent after cardiac cycles without muscle SNA bursts (Figure 6, unpublished data). Furthermore, Casey *et al.* reported in young healthy subjects that endogenous norepinephrine release via intra-arterial infusion of tyramine results in an increase in brachial artery retrograde and oscillatory shear; whereas alpha-adrenergic blockade via infusion of phentolamine abolished retrograde and oscillatory shear (Casey *et al.*, 2012). Collectively, the available literature clearly supports a role of muscle SNA in influencing conduit artery shear rate profiles via increased downstream resistance vessel tone in resting humans (Figure 1).

Because muscle SNA increases with aging (Davy *et al.*, 1998; Hart *et al.*, 2011; Vianna *et al.*, 2012), it is possible that an increased alpha-adrenergic vasoconstriction in resistance

vessels contributes to the age-related differences in resting shear rate patterns in upstream conduit arteries (Young *et al.*, 2010; Padilla *et al.*, 2011a). This hypothesis was recently tested by Casey et al. (Casey *et al.*, 2012). In this study, LBNP increased retrograde and oscillatory shear in young, but not older adults, such that basal differences between young and older subjects were eliminated. Of note, during infusion of phentolamine, retrograde and oscillatory shear were abolished in young adults and markedly reduced, yet still present, in older adults. The observation that age-related differences in retrograde shear persisted despite removal of alpha-adrenergic vasoconstrictor tone suggested that additional factors beyond muscle SNA contribute to the age-related increases in conduit artery retrograde shear. In this regard, we found that NO synthase inhibition in the forearm circulation of young, but not older, subjects increased retrograde and oscillatory shear, such that differences between young and old were abolished (Padilla *et al.*, 2011a). Taken together, these findings indicate that increased alpha-adrenergic vasoconstriction and/or reduced NO bioavailability in the downstream resistance vasculature contribute to age-related differences in conduit artery shear stress profiles during rest.

Summary

In this review we put forth the idea that increased athero-susceptibility of the conduit arteries of the lower extremities may be determined by alterations in vascular tone of downstream skeletal muscle resistance arteries via changes in SNA and NO. This proposition is founded on classic and emerging studies indicating that increased microvascular tone is associated with elevated blood pressure and enhanced oscillatory shear stress in upstream conduit arteries. A vast amount of evidence supports the notion that excessive blood pressure and oscillatory shear stress are potent pro-atherogenic signals to the endothelium. Accordingly, we propose that increased blood pressure and conduit artery oscillatory shear stress resulting from increases in downstream vascular resistance underlies this increased athero-susceptibility of conduit arteries. That is, this review sets the stage for the emergence of a new paradigm concerning the mechanistic link between increased microvascular tone and development of conduit artery endothelial dysfunction and thus increased risk for peripheral artery disease. Importantly, as summarized in Figure 1, SNA and NO are viewed as critical modulators of vascular resistance and, consequently, important determinants of blood pressure and conduit artery shear stress profiles of the arteries perfusing the limbs. Future integrative research is needed to further understand not only the interaction between SNA and NO in the modulation of blood pressure and shear stress profiles, but also the interaction between blood pressure and shear stress in the regulation of conduit artery endothelial cell phenotype in health and disease. The effects of increased SNA on blood pressure suggest that increased SNA could contribute to the development of atherosclerosis in all atheroprone regions of the arterial tree. Also, although it is expected that the undesirable effects of SNA on shear patterns in skeletal muscle vascular beds may apply to other conduit arteries, we are not aware of any available data. Addressing this question requires additional research. Indeed, an integrated understanding of the physiological and molecular mechanisms underlying the progression towards a pro-atherogenic endothelial cell phenotype may stimulate development of novel therapeutic strategies aimed at prevention and treatment of peripheral artery disease.

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Figure 1.

Proposed links between increased resistance vessel tone in skeletal muscle and the development of atherosclerosis. SNA, sympathetic nerve activity; NO, nitric oxide.

Figure 2.

Phenylephrine-induced contraction of isolated rat aortic rings is augmented after NOS inhibition and even further increased after mechanical denudation of the endothelium. Aortic rings were incubated with *NG*-nitro-L-arginine methyl ester (L-NAME, an NOS inhibitor, 300 M) or vehicle control for 30 min before tension was assessed in response to a submaximal dose of phenylephrine (3×10^{-7} M). It should be noted that NOS inhibition alone did not increase basal tone of the aortic rings. Values are means $\pm$ SE. These are unpublished observations.

Figure 3.

(A) High intraluminal pressure and (B) low shear stress for 4 hrs increases expression of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) in isolated rat carotid arteries. Low pressure = 30 mmHg; high pressure = 80 mmHg; low shear stress = 3 dyn/cm², normal shear stress = 40 dyn/cm². For pressure experiments, shear stress was maintained at 40 dyn/cm². For shear stress experiments, pressure was maintained at 50 mmHg. Values are means $\pm$ SE. These are unpublished observations.

Figure 4.

Regional differences in atherosclerotic disease between proximal (characterized by low oscillatory shear index) and distal (characterized by high oscillatory shear index) regions of abdominal aorta in hypercholesterolemic pigs (n=22). Top images are representative photographs of abdominal aorta sections from one pig after vessel was longitudinally cut (lumen face up) and stained with Sudan IV. Values are means $\pm$ SE. OSI, oscillatory shear index. These are unpublished observations.

Figure 5.

(A) Experimental set up: (i) a distal occlusion cuff (220 mm Hg) produced a localized region of disturbed blood flow in the proximal vasculature; (ii) an indwelling venous catheter was placed for repeated blood sampling; (iii) brachial artery blood flow was measured using Doppler ultrasound; (iv) a proximal cuff (40 mm Hg) was applied to facilitate the trapping of any EMPs released during the experiment; (v) the contralateral arm was used as a control. Representative Doppler brachial artery blood velocity tracings are presented to illustrate the reduction in flow in the experimental arm. (B) CD62E+ and (C) CD31+/CD42b- EMPs released in the experimental arm, indicating that disturbed blood flow induced endothelial activation and apoptosis, respectively. Values are means $\pm$ SE. From Jenkins et al. (Jenkins *et al.*, 2013) with permission. CD62, also known as E-selectin; CD31, also known as PECAM-1; CD42b, also known as glycoprotein Ib.

Figure 6.

Signal averaged beat-by-beat percent changes in oscillatory shear index in the common femoral artery following cardiac cycles associated with and without spontaneous muscle SNA bursts in young healthy subjects (n=7) resting quietly in the supine position. Values are means $\pm$ SE. These are unpublished observations.