REGULATION OF PLACENTAL GROWTH FACTOR

BY FLUID SHEAR STRESS IN THE VESSEL WALL

By

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Abstract: Arteriogenesis is the process by which mature arteries form from collateral arterioles after upstream arterial stenosis or occlusion. During collateral remodeling, monocytes are recruited to the vessel wall. Placental growth factor (PLGF) is a potent stimulator of arteriogenesis via monocyte recruitment. Although fluid shear stress (FSS) is thought to be the primary signal for arteriogenesis, its role in regulating PLGF expression is unknown. However, PLGF is increased in collaterals by upstream arterial ligation. Therefore, we hypothesized that PLGF expression is regulated by FSS. To test this hypothesis, we created an *in vitro* model of coronary vessels, consisting of human coronary artery endothelial cells (HCAEC) and human coronary artery smooth muscle cells (HCASMC) cocultured on porous Transwell inserts. We used a cone and plate apparatus to expose the HCAEC of the coculture to different FSS waveforms. We also employed an *ex vivo* model consisting of isolated mesenteric arterioles perfused at various flow rates. Flow or FSS significantly increased PLGF expression at both the mRNA and protein level, and this effect was dependent on the duration and magnitude of FSS, confirming our hypothesis. PLGF was predominantly produced by HCAEC, but HCASMC also played a role in modulating HCAEC PLGF expression and response to FSS. Next, we set out to identify the signaling pathways mediating the effects of FSS on PLGF. Hydrogen peroxide and NADPH oxidase 4 were required for FSS to upregulate PLGF. Similarly, heme oxygenase 1 activity was also required for FSS to affect PLGF expression. Heme oxygenase 1 also mediated the effects of hypoxia on PLGF, and our studies suggest the existence of a common pathway between these two stimuli (FSS and hypoxia). This study builds on several recent studies using various redox knockout mice to study vascular remodeling. It further emphasizes the importance of PLGF for arteriogenesis and presents a novel possible mechanism by which arteriogenesis progresses.

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CHAPTER I

INTRODUCTION

Ischemic cardiovascular disease (CVD) is the leading cause of morbidity and mortality in western and developing countries, and even optimistic projections predict it to remain so for the foreseeable future [1]. Ischemic CVD results from narrowing of the coronary arteries due to buildup of atherosclerotic plaque in the vessel wall.

Arteriogenesis is the process by which mature arteries form from collateral arterioles after upstream arterial stenosis or occlusion. Collateral arterioles are interconnecting, narrow-diameter vessels that carry little blood under normal conditions due to their high resistance. Arteriogenesis can potentially reestablish flow to sites downstream of an occlusion, preventing ischemic injury. Thus, pharmacological stimulation or enhancement of arteriogenesis would provide a non-invasive treatment for ischemic CVD. For such a treatment to be safe and effective, comprehensive understanding of the mechanisms of arteriogenesis is required. Although arteriogenesis does occur in regions of tissue hypoxia, It more often occurs upstream of the ischemic region [2]. Flow-limiting occlusions cause an increase in the pressure gradient across the collaterals, which results in increased fluid shear stress (FSS) on the collateral wall[3]. FSS is thought to be the primary signal for arteriogenesis.

Placental growth factor (PLGF) is a potent arteriogenic factor (more so than the related protein VEGF-A, which mainly induces capillary proliferation). PLGF knockout in mice has little effect on vascular development, but results in impaired collateral growth in adults[4]. Ligation of the femoral artery in rats to induce collateral growth results in increased PLGF expression[5], and overexpression of PLGF in mice results in increased vascularization[6]. Furthermore, PLGF selectively increases the size of vessels with a diameter of 96-136 μm, the size range of collateral arterioles[7]. These findings demonstrate the key role of PLGF in arteriogenesis.

During collateral remodeling, monocytes are recruited to the vessel wall. The importance of monocytes to arteriogenesis was demonstrated in mutant mice with severely reduced circulating monocyte levels. These mice have impaired collateral remodeling in the ischemic hindlimb[8]. Interestingly, monocyte recruitment is diminished in PLGF gene knockout mice[9], and Pipp et al. have shown that the arteriogenic properties of PLGF are dependent on monocytes[10]. PLGF also enhances the mitogenic effects of VEGF on endothelial cells, and is required for VEGF to induce smooth muscle proliferation[4, 11].

Although the work cited above provides convincing evidence that PLGF plays a central role in arteriogenesis, the regulatory mechanisms controlling PLGF expression are poorly understood, especially in the vascular wall. The profound effect that shear stress has on regulating arteriogenesis has led us to hypothesize that PLGF expression in vascular cells is regulated by FSS.

To test our hypothesis we established two models of the vessel wall. The first model consisted of human primary coronary artery endothelial cells and smooth muscle cells cultured on either side a porous membrane insert. FSS was applied to the endothelial cells using a cone driven by an electrical stepping motor. In the second model mesenteric arterioles were mounted on glass cannula and perfused by altering the upstream and downstream pressure. We then determined the effects of physiologically relevant FSS waveforms on PLGF expression and characterized signaling pathways downstream of FSS that mediate its effects on PLGF.

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CHAPTER II

REVIEW OF LITERATURE

Ischemic Heart disease (IHD) is the leading cause of morbidity and mortality in western and developing countries, and even optimistic projections predict it to remain so for the foreseeable future[1]. The leading cause of IHD is obstructive atherosclerosis[2]. Atherosclerosis is an occlusive vascular disease characterized by abnormal inward growth of arteries along with accumulation of lipids (mainly cholesterol) inside these lesions.

Vessels consist of three overlaid layers from the inside outwards these are the tunica intima, tunica media, and tunica adventitia. The inner most layer, the intima, consists of a cobblestone monolayer of endothelial cells (EC), around which, is a fine meshwork of extracellular matrix (ECM) proteins forming a basement membrane (Fig. 1). Around this ECM component is yet another thicker fenestrated ECM layer called the internal elastic lamina, which delineates the divide between tunica intima and tunica media. The tunica media is mostly composed of smooth muscle cells (SMCs) arranged circumferentially and ECM proteins. This muscular layer is thicker in arteries that withstand high arterial pressures compared to the lower venous pressure of the veins. In larger vessels, another thick fenestrated ECM layer, the external elastic lamina, covers the outer layer of the tunica media. The tunica adventitia is mostly comprised of collagen fibers, fibroblasts, and macrophages. In larger vessels with thick media, diffusion of oxygen and nutrients across the intima is insufficient. As a result the adventitia of these vessels contains small arterioles called the vasa vasorum that supply the outer layer of the media with oxygen and nutrients.

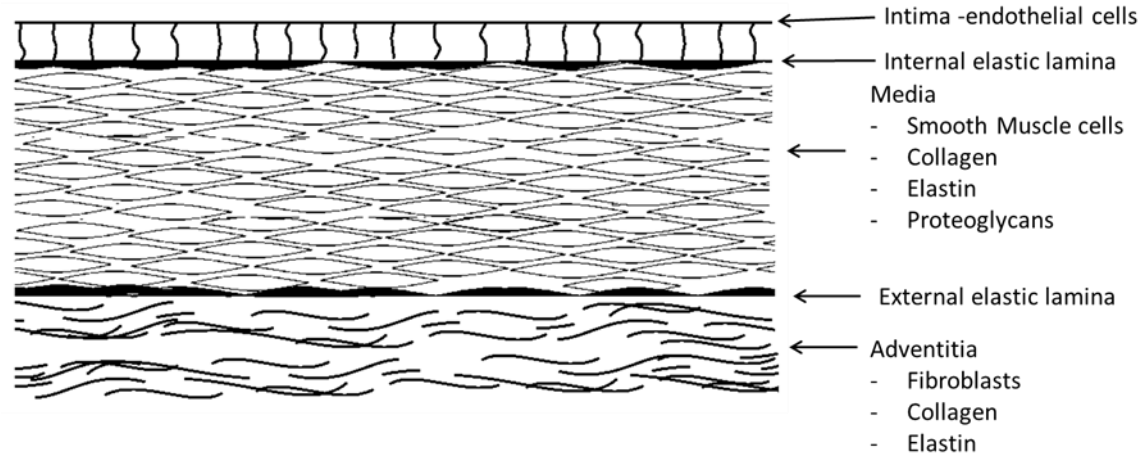

Figure 1. Structure of the arterial wall. This illustration shows one side of the arterial wall the innermost layer (intima) is at the top while the outermost layer (adventitia) is at the bottom.

Atherosclerosis

During atherosclerosis repetitive injury of the endothelium leads to endothelial dysfunction. This causes increased permeability of the endothelium and the adhesion of leukocytes and platelets. Concurrently, low-density lipoprotein LDL accumulates in the sub-endothelial basement membrane (Fig. 2). The release of enzymes such as myeloperoxidase and lipoxygenases by leukocytes, along with increased reactive oxygen species results in the oxidation of LDL, which in turn triggers the upregulation of growth factors, adhesion molecules and cytokines in the endothelium. These cause an increase in the inflammatory state of the vessel and further recruit leukocytes. Monocytes recruited to the area differentiate into macrophages after extravasation. These macrophages phagocytose the oxidized LDL. Macrophages that accumulate oxidized LDL become foam cells and release further cytokines. Initially, a non-protruding fatty streak develops consisting of few foam cells where the majority of lipids are intracellular. As more oxidized lipids accumulate, further aggravation of the inflammatory process ensues and increased release of growth factors and cytokines results in the increased motility and proliferation of SMC. This proliferative synthetic phenotype of SMCs produces

excessive amounts of collagen. This leads to the thickening of the intima. The lesion now intrudes into the lumen, resulting in narrowing of the lumen and disturbed flow. The turbulent flow further exasperates endothelial dysfunction, leading to further accumulation of oxidized LDL. The increased involvement of SMC, along with extracellular accumulation of oxidized LDL and necrotic cell debris results in the progression of the fatty streak into an atherosclerotic plaque.

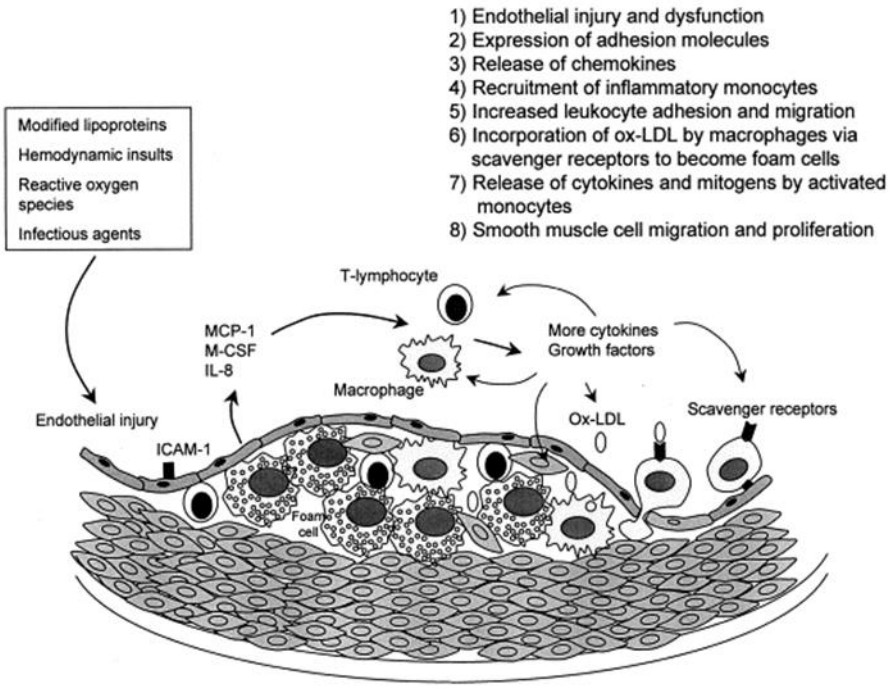

Figure 2. Illustration of the progression of atherosclerosis. Modified from Yu and Rifai 2000 *Clinical Biochemistry*, 33 (8); 601 - 610

The plaque continues to accumulate lipids, foam cells, SMC, collagen and inflammatory cells. This along with the cytotoxic effects of oxidized LDL induces apoptosis in SMCs and degradation of the fibrous cap. These processes result in structural weakening, leading to a plaque vulnerable to rupture. Plaque constituents are highly thrombogenic, rupture releases these materials into the blood stream resulting in thrombosis of the vessel and ischemia of the downstream tissue. The thrombus may result in incomplete occlusion, in which case the thrombus

is organized and included into the plaque further increasing the size and intrusion of the plaque. Atherosclerotic plaque also may fragment releasing fibrous lipid emboli that cause damage further downstream in the circulation. Finally, as lesions increase in size they weaken the vessel wall, which may lead to aneurysm and rupture of the vessel.

Atherosclerosis shows a tendency to develop in areas of disturbed flow, such as bifurcations and curvatures. Indeed, turbulent flow causes endothelial dysfunction *in vitro*[12]. Hyperlipidemia is also an important factor in the development of atherosclerosis, not only for the obvious increase in the lipids that accumulate in lesions. Increased circulating lipids also lead to endothelial dysfunction and increased production of reactive oxygen species.

In the coronary setting atherosclerosis can have deleterious consequences. In the early stages of stenosis, chest pain as the result of ischemia known as angina pectoris is brought on by exertion know as effort angina. As the lesion continues to grow it may rupture resulting in thrombosis and myocardial infarction or if incompletely occluded progresses into increasing chest pain at rest, termed crescendo angina. At this point, continued growth of the lesion results in increased ischemic damage to the myocardium, leading to hypertrophy as more damage accumulates. Decompensation and ultimately heart failure ensue.

Treatment of fully developed symptomatic atherosclerotic plaque currently requires surgical intervention. The two most common procedures are coronary angioplasty and coronary artery bypass graft (CABG) surgery. Coronary angioplasty involves the use of a catheter to position a balloon next to the atherosclerotic lesion. The balloon is then inflated, pushing the lesion into the vessel wall and increasing the luminal surface area. The balloon is then deflated and removed. Another common procedure is to implant a mesh tube known as a stent to support the vessel wall. Stents are more cost effective and less invasive than CABG surgery[13]. Unfortunately, in-stent stenosis or "restenosis" occurs in 36% of patients[5]. Restenosis is the

narrowing of a vessel at the site of stent implantation the mechanisms of which are varied. Excessive expansion of the stent can cause injury to several layers of the vessel, which exposes several of the factors that initially drive atherosclerosis and is correlated with lesion regeneration[14]. Vessel injury results in the exposure of tissue factor which promotes thrombus formation[15]. SMC invade the thrombus and deposit ECM proteins leading to the reorganization and incorporation into the vessel wall[16]. Furthermore, the injured vessel releases several pro inflammatory molecules that promote the recruitment of leukocytes further driving the remodeling and intimal thickening[17]. The application of drug eluting stents has shown great promise in reducing in-stent stenosis. These stents release a drug locally to inhibit the processes that lead to in-stent restenosis. Drug eluting stents result in improved out come with an average reduction of 10% in stent restenosis [5,10].

Coronary artery bypass graft surgery is the autologous transplantation of a vessel to the coronary circulation in order to bypass a stenotic artery. This procedure is invasive and requires opening the chest cavity and stopping the heart. Commonly harvested vessels are the internal mammary artery (IMA) and the saphenous vein (SV). CABG is an expensive and invasive procedure with several possible complications including neurocognitive decline as a result of cerebral damage[18]. Patency of the grafted vessel after surgery is a key prognostic indicator. IMA grafts show significantly increased patency compared to the SV grafts 10 years after surgery (95% vs. 60%, respectively)[19]. This increased stenosis of the SV grafts is the result of intimal thickening arising from SMC proliferation [13,14]. Evidence suggests the stimulating force behind this proliferation is the difference in hemodynamic forces between venous and arterial branches [13-16]. Increased stenosis of the SV graft requires intervention by SV graft angioplasty. This procedure is similar to coronary angioplasty but also includes several additional complications. Veins are slower at lipolysis [20]and have a higher rate of liposynthesis[21]. This results in SV graft lesions being more diffuse and friable than arterial plaque[22-25]. SV graft lesions also have a higher

content of foam and inflammatory cells [25], increasing the chance of complications caused by the release of lipid emboli and lesion debris into the bloodstream [26]. Furthermore, LSV grafts exhibit a higher frequency of in stent stenosis than native arteries [27].

Arteriogenesis

Collateral vessels are small interconnecting arterioles that connect tissues perfused by two different conducting arteries. These narrow diameter vessels carry little blood due to their high resistance. When a conducting artery is occluded, the pressure downstream of the occlusion is greatly reduced. The resultant pressure gradient increases flow through the collateral vessels to the lower pressure area. As this flow is maintained, an increase in the diameter of the collateral vessels is observed (Fig. 3). This increase is due to active remodeling of the vessel and not merely vasodilatation[28]. Additionally, these expanded vessels are stable, functional, and in the coronary circulation ameliorate some of the effects of myocardial ischemia[29, 30]. This outward remodeling of preexisting collateral vessels into mature patent conductance arteries was termed arteriogenesis [31]. This process is not to be confused with angiogenesis, which is the sprouting of endothelial cells from preexisting vessels to form new capillaries[32]. Arteriogenesis progresses through three stages. The first stage, initiation, involves the release of chemokines, upregulation of adhesion molecules and changes in the ECM. This is followed by the growth phase, in which recruitment of monocytes results in significant SMC migration and proliferation and continued change in the ECM. An increase in the area of the lumen and length of the vessel is observed. Finally, maturation or stabilization of the vessel occurs in which the SMCs return to a contractile rather than synthetic phenotype.

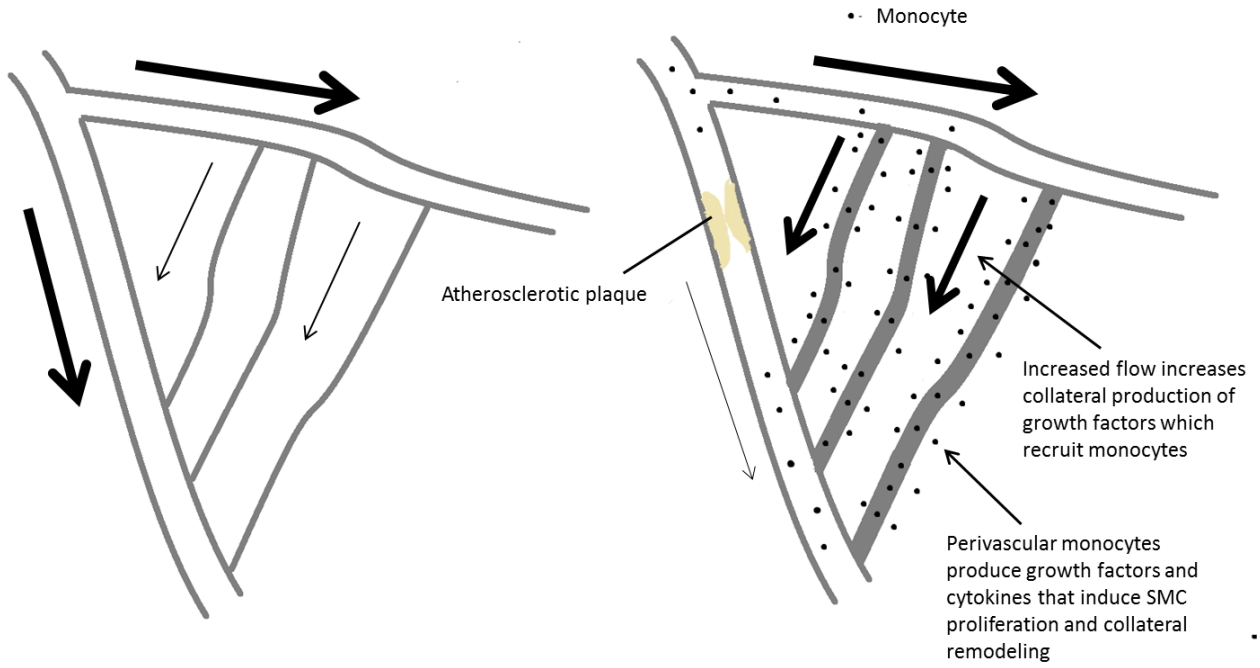

Figure 3. Illustration of arteriogenesis.

Initiating collateral growth

Identifying the initiating stimulus of collateral growth and characterizing how it affects the vessel wall is essential to develop a therapeutic compound that stimulates arteriogenesis. The two major factors that change during CAD are discussed below.

Tissue ischemia

Occlusion of an artery results in low perfusion of the downstream tissue, as oxygen is depleted from the tissue, hypoxia ensues, resulting in the stabilization of the nuclear factor hypoxia inducible factor-1 (HIF-1)[30,31]. HIF-1 upregulates vascular endothelial growth factor VEGF[33]. HIF-1 also activates members of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) family[34]. NF-κB regulates several inflammatory responses[35, 36] which are conducive to vascular remodeling.

Most of the arteriogenic effects of hypoxia are attributed to the upregulation of VEGF, which is a potent angiogenic factor. VEGF was first described as vascular permeability factor. A protein identified in the fluid accumulated in the interstitial space of tumors. This protein increased the permeability of vessels[37]. VEGF was then identified as a heparin binding secreted endothelial mitogen that induced angiogenesis *in vivo*[38] and induced angiogenesis in tumors [39]. VEGF binds to the receptors VEGFR-1, VEGFR-2, VEGFR-3, and neuropilin-1[40-43]. VEGF deficient mice develop with abnormal vasculature that leads to embryonic lethality[44, 45]. There are several isoforms of VEGF; A, B, C, D, E and Placental growth factor (PLGF). VEGF-A has multiple splice variants with varying degrees of expression [45].

Administering VEGF after occlusion of the left circumflex coronary artery in canine hearts resulted in an increase in the flow restoration compare to untreated dogs[46]. In the porcine heart occlusion of the left circumflex coronary artery resulted in a marked increase in VEGF expression in the myocardium[47]. Treatment with VEGF resulted in a significant increase in flow restoration compared to untreated controls. However, VEGF treatment also causes excessive hypotension[48]. Both VEGF treatment and VEGF gene transfection increases collateral growth in the peripheral circulation of rabbits following femoral artery ligation and causes collateral growth and flow recovery[49, 50]. A marked increase in the density of the capillary beds of the calf were also noted[49, 51]. In contrast to these findings, VEGF treatment in the canine heart after left circumflex coronary artery ligation had no effect on flow restoration or collateral size[52]. Additionally, the more troubling finding that VEGF caused accelerated intimal thickening at sites of vascular injury was reported[53]. Similarly, adenoviral induced over expression of VEGF had no arteriogenic effect in either the porcine coronary circulation after coronary occlusion or the rabbit hind limb after femoral artery occlusion[52]. These inconsistencies in the effects of VEGF may be attributable to varying surgical protocols, VEGF administration, or possibly, a temporal factor such as how long after the procedure was VEGF treatment commenced.

The arteriogenic effect of VEGF may be the result of its ability to induce upregulation of integrin expression on the surface of the endothelium [53]. Integrins play a critical role in both sensing arteriogenic stimuli and coordinating the remodeling process (see below). Similarly, VEGF signaling increases VE-cadherin phosphorylation[54]. VE-cadherin is important in mechanotransduction and endothelial permeability. VEGF also causes an increase in matrix metalloproteinase (MMPs)[54] expression. MMPs are instrumental in the remodeling of ECM during collateral growth (see below). Additionally, VEGF induces vasodilatation[55] by increasing nitric oxide (NO) production[56, 57]. NO production is important in the regulation of the collateral growth process (see below). One of the most important events of arteriogenesis is the recruitment and activation of monocytes at the site of remodeling (see below). Monocytes express VEGFR-1 and VEGF can activate monocytes by binding this receptor, inducing chemotaxis[58].

During femoral artery occlusion, angiogenesis does occur in the ischemic tissue and results in increased capillary bed density; this however does not bridge normoxic and ischemic regions of the organ. Additionally, once hypoxia subsides, the increase in density is reversed[59]. Collateral vessels branching off downstream of the occlusion i.e. lacking a pressure gradient do not expand as much as those across the pressure gradient[61]. The mild increase in vessel diameter observed is possibly the result of arteriogenic factors released upstream. The primary increase in conductance after femoral artery ligation is observed in the circulation branching off proximal to the occlusion and distal to the ischemic tissue, spatial separating the growth process and hypoxia[60, 61]. Furthermore, the majority of angiogenesis occurs in the areas of ischemia distal to the occlusion[62].

Hemodynamic forces

Stenosis of a vessel results in a decrease in pressure downstream of the occlusion. This increases the pressure gradient across any other vessel perfusing the area. According to the

Hagen–Poiseuille equation, flow rate is directly proportional to pressure gradient (Eq. 1). The resultant increase in flow rate causes an increase in fluid shear stress at the vessel wall. Fluid shear stress at the vessel wall is the result of frictional forces between the flowing blood and the luminal surface of the endothelium (Eq. 2). Pressure also causes circumferential wall tension in the vessel wall, which is defined by the law of Laplace as the product of the pressure and the internal radius of the vessel divided by the thickness of the vessel wall (Eq. 3). This tension acts mostly on the tunica media of the vessel particularly the SMCs of the vessel wall.

Hagen-Poiseuille $\left(\Delta P = \frac{8\mu LQ}{\pi r^4}\right)$ (Eq. 1)

Shear stress $\left(\tau = \frac{4Q\mu}{\pi r^3}\right)$ (Eq. 2)

Laplace $\left(\sigma_\theta = \frac{P \times r}{t}\right)$ (Eq. 3)

Where ΔP is pressure gradient, τ= shear stress, σ_θ= circumferential stress, μ is dynamic viscosity, Q= Flow rate, L= length of vessel, r= internal radius of vessel, and t= vessel thickness.

A fundamental event in arteriogenesis is the mobilization and proliferation of SMCs. Located in the media, SMCs are physically shielded from changes in shear stress, and can only directly sense changes in circumferential wall tension or stretch. It is important to note that stretch is a function of absolute pressure and not pressure gradient, which increases due to a drop in

downstream pressure not an increase in pressure. However, increased flow has a vasodilatory effect, via increased NO production in the endothelium. Vasodilation causes an increase in internal radius and a decrease in wall thickness of the vessel. This leads to an increase in stretch. Stretch causes changes in the expression of several mediators and inhibitors of arteriogenesis. In cultured human smooth muscle cells and endothelial cells cyclic stretch causes an increase in monocyte chemo-attractant protein-1 (MCP-1)[63], an important pro-arteriogenic factor[10]. In the rabbit aorta, stretch modulates the activation of focal adhesion kinase (FAK) in an integrin dependent fashion that is unique from the effects induced by shear stress[67]. FAK is a key participant in integrin signaling which plays a central role in several processes of arteriogenesis. Stretch also causes an increase in VEGF in vascular SMC in lambs[64].

The role shear stress plays in arteriogenesis is has been demonstrated in several studies. Ligation of intestinal arteries of rats results in a 2.5 fold increase in shear stress in the vessels collateral to the ligation. These vessels exhibit a significant increase in size one week after ligation[3]. Pipp *et al* demonstrate the role of increased fluid shear stress in arteriogenesis in pigs and rabbits by shunting the femoral artery post occlusion to the femoral vein, resulting in 2.3 fold increase in flow compared to the control that was occluded with no shunt. Collateral growth was 2.4 fold higher in the shunted group compared to control[65]. Shear stress is a function of the third power or radius. As a result, it decreases precipitately as the vessel expands. Further emphasizing the role of shear stress, collateral growth halts at roughly 40% maximal conductance pre-occlusion[53, 66] artificially increasing shear stress by arteriovenous shunt resulted in rapid collateral growth and complete restoration of flow. Continued shunting caused further collateral growth that surpasses pre-occlusion conductance[72].Individual infusion with several potent angiogenic growth factors only yields a fraction of the restored growth yielded by artificially increased fluid shear stress[67].

Mechanosensing and mechanotransduction

Although the exact mechanisms by which fluid shear stress is sensed and transduced are not entirely known, there are several candidates involved. Owing to the complex interactions and myriad of events orchestrated, it is highly unlikely that a single pathway is responsible for mechanotransduction. A reoccurring cast of soluble mediators and signaling pathways in both arteriogenesis and atherosclerosis implies a multi factorial mechanism, which may be tuned depending on the nature of endothelial fluid shear stress. Postulated contributors to fluid shear stress sensing include the glycocalyx, tyrosine kinase receptors, Ion channels, G protein receptors, integrins, adhesion molecules, cadherins, caveolae, and the cytoskeleton.

Ion channels expressed on the EC plasma membranes such as potassium, transient receptor potential (TRP) and chloride channels are activated by increased shear stress [68-70], causing changes in the polarization of the cell membrane. Shear stress also activates $P2X_4$ purinoreceptors, a member of the P2X purinoreceptors family of the ATP-gated cation channels [77], resulting in an influx of Calcium ions into the cell [71]. $P2X_4$ purinoreceptor activation is the result of endogenous ATP release [72] and is dose dependent on shear stress applied [73]. Knockout of $P2X_4$ in mice results in diminished shear induced calcium influx[74]. Functionally these knockouts exhibited reduced NO production, hypertension, poor vasodilatation and impaired vascular remodeling. Suggesting a role for $P2X_4$ purinoreceptors in the shear induced release of NO. The influx of calcium ions in response to shear stress has been demonstrated to originate in caveolae [75], where the shear induced release of ATP has also been shown to localize[76].

Caveolae are 50 – 100 nm flask shaped invaginations of the plasma membrane. Caveolae contain a myriad of receptors, ion channels and protein kinases, all involved in signaling [77]. They also contain large quantities of endothelial nitric oxide synthase (eNOS) which converts L-arginine and oxygen to L-citrulline and NO, under shear stress, caveolae release significant

amounts of NO [78]. Caveolae are a form of lipid raft which are domains of the plasma membrane with increased concentration of cholesterol and sphingolipids. This results in increased order and decreased fluidity of the domain. In caveolae, multiple caveolin-1 molecules bind to cholesterol in these lipid rafts, they then form homo-oligomers, which further organize changing the structure of the lipid raft into a Caveolae. Caveolin-1 further binds filamin anchoring the caveolae to actin and the cytoskeleton[79]. In the endothelium of caveolin-1 knockout mice there is a complete absence of caveolae on the surface of the cell. Furthermore, these mice exhibit putative (eNOS) activity resulting in excessive vasodilation [80, 81]. These mice also exhibited impaired vascular remodeling in response to chronically decreased flow[82].

The endothelial glycocalyx consists of a layer of glycoproteins and proteoglycans localized to the luminal extracellular side of the vascular endothelial plasma membrane. Proteoglycans consist of a protein core bound to glycosaminoglycans (GAG). GAGs are variable length repeats of distinct disaccharides forming a linear heteropolysaccharide [83]. These polysaccharides impart a highly negative charge to the proteoglycan that facilitates association with an array of cationic molecules circulating in the plasma [84]. They achieve this high negativity by sulfation (an exception being hyaluronic acid which gains its charge from carboxyl groups[85]). Different disaccharide combinations and linkages result in different classes of GAGs. Common GAGs in the vasculature are heparin sulfate, chondroitin sulfate, dermatan sulfate and hyaluronic acid[86]. Heparin sulfate is the most common GAG in the endothelial glycocalyx constituting 50 – 90% of all GAGs[87]. GAGs have been the subject of great focus due to their signaling abilities[88]. The thickness of the glycocalyx is highly sensitive to its environment, such as ion concentration and pH [89, 90]. This is largely due to changes in the conformation of the coiled GAG chains in response to electrostatic interactions [91]. Hyaluronic acid interweaves other GAG chains interacting with chondroitin sulfate and the hyaluronic acid receptor CD44 [92] which is also localized to the caveolae where they have been shown to interact with calcium channels [93].

Hyaluronidase treatment of canine vessels prior to exposure to steady flow shear stress resulted in a 5 fold decrease in NO production compared to control [94]. Treatment of cultured bovine aortic endothelial cells with heparinase III abolishes shear induced NO production [95]. Interestingly, treatment with chondroitinase [96] has no significant effect on the shear induced NO production in Bovine aortic endothelial cells. The shear induced reorganization of the cytoskeleton in rat ECs exposed to shear stress *in vitro* is attenuated when the cells are cultured in serum free media [97], which results in the collapse of the glycocalyx. One possible mechanism of glycocalyx mechanotransduction is the outward extension of helical sulphonated GAGs. [91] This creates a form of dragline extended from the core protein. As shear stress increases it causes a conformational change in the coil exposing anionic moieties that bind sodium ions, this binding results in a conformational change of the protein core that conveys the shear signal to the cytoskeleton [98] where it can be transmitted to both cell-matrix and cell-cell adhesion points.

Cell-matrix adhesions are mostly mediated by integrins. Integrins' mechanical role in cell adhesion coupled to their cell signaling capabilities suggest a possible role in mechanotransduction. Meyer et al. [113] used an activating antibody for integrin β and the integrin ligand RGD conjugated to ferromagnetic beads along with a magnetic twisting device to torque integrins to test their individual mechanotransductory potential in cultured bovine endothelial cells. Binding of the ligand or activating antibody resulted in a mild increase in cyclic adenosine monophosphate (cAMP). Upon application of shear stress the concentration of cAMP increased by three fold compared to control. They further demonstrated functional significance of this increase by quantifying the nuclear translocation of the catalytic subunit of protein kinase A (PKAc) and the activation of the transcription factor cAMP response element binding protein (CREB). Similar to cAMP, activation of integrin resulted in a small increase in nuclear PKAc. Applying torque to the integrins resulted in a dose dependent increase in nuclear PKAc up to threefold of control. The same pattern was observed with phosphorylation of CREB. This shear-

induced increase in PKA signaling was attenuated after treatment with Guanosine 5′-[β-thio] diphosphate trilithium salt a general Gα inhibitor suggesting that integrins transduce mechanical signals through G-proteins. Ligand dependent activation of the integrin but not binding to actin filaments was also demonstrated to be necessary for the shear-induced increase in PKAc signaling. Suggesting that integrins-PKAc signal originates from the integrins and doesn't require an external signal to be transmitted through the cytoskeleton.

Shear stress also results in ERK activation [114]. This activation is inhibited by the transfection of a dominant negative mutant of FAK [115]. Transfection with a dominant negative mutant of c-Src also inhibited the shear induced activation of ERK [114] this suggesting the involvement of integrins in the shear induced activation of the MAPK/ERK pathway. Integrins also activate TRPV4 channels when exposed to shear stress increasing calcium influx[99]. Furthermore, blocking of integrin binding to the ECM results in attenuation of the shear stress induced vasodilatation in porcine coronary arterioles [117]. Shear stress has been shown to activate VEGFR-2 independent of its ligand[100], this activation has been suggested to be integrin dependent and mediated by casitas b-lineage lymphoma (cbl) [101]. Additionally, integrins mediate the stimulation of NF-κB leading to an increase in intercellular cell adhesion molecule-1 (ICAM-1)[102]. Finally, activation of integrins by shear stress results in the reorganization of the endothelial cytoskeleton[103]. Integrins clearly play a role in mechanotransduction but the global effects integrin activation has on the cell, merits the discussion of other adhesion molecules connected to the cytoskeleton.

Several members of the immunoglobulin superfamily contribute to cell adhesion. The glycoprotein platelet endothelial cell adhesion molecule -1 (PECAM-1) is a member of the immunoglobulin superfamily with 6 immunoglobulin domains in its extracellular region. The cytoplasmic region of PECAM-1 is capable of binding the cytoskeleton by β-catenin and plakoglobin[104]. On the extracellular side PECAM-1 homophilically binds PECAM-1 on other

cells in a calcium independent manner. PECAM-1 lacks enzymatic activity but two tyrosines in the cytoplasmic region are phosphorylated when PECAM-1 is stimulated[105]. This usually involves the Src family of tyrosine kinases. The phosphotyrosines then bind Src homology 2 (SH-2) domains on several intracellular signaling molecules. PECAM-1 is mostly expressed on the surface of endothelial cells, platelets and leukocytes. PECAM-1 is a negative regulator of leukocytes and platelets[106-109]. It also plays a role in the adhesion and migration of leukocytes. In the endothelium PECAM-1 is predominantly localized to cell-cell junctions. When bovine aortic endothelial cells are exposed to shear stress PECAM-1 is rapidly phosphorylated[110, 111]. SHP-2 then binds the phosphorylated PECAM-1[112, 113]. GRB2-associated binding protein-1 (Gab1) co-localizes with PECAM-1/SHP-2[114]. Gab1 is an adaptor protein interacts with SHP-2 to activate ERK[115], suggesting a role for PECAM-1 in the shear induced activation of ERK. Indeed, Tzima et al. provide compelling evidence for the role of PECAM-1 as a mechanosensor within a larger mechanosensing complex.

While most cell-matrix adhesions consist of integrins, the cadherin family of adhesion molecules mediates most cell-cell adhesions. Cadherins are calcium dependent adhesion molecules. They are single pass transmembrane proteins, the N-terminal extracellular region of the protein contains tandem repeats of a motif called the cadherin domain. These 110 residue domains are rigid units, while the inter-domain areas are flexible. Calcium ions bind the inter domain regions significantly increasing their rigidity. This is the source of cadherin's calcium dependence. There are 4 subfamilies of cadherins. Two subfamilies indirectly bind actin filaments at the cytoplasmic regions, these are the type I or "classical" cadherins which have 5 cadherin domain repeats the furthest of which has a conserved signal tryptophan. The type II cadherins are similar to type I, except for two conserved tryptophans on the distal cadherin domain. Also similar to type I cadherins are the desmosomal cadherins which bind intermediate filaments at the cytoplasmic regions. The final subfamily is the protocadherins which are further divided into

protocadherin α, protocadherin β, and protocadherin γ based on different cytoplasmic regions. These cadherins have variable length extracellular regions. One of the type I cadherins is peculiar in that it consists of only an extra cellular region covalently bonded to GPI and is called T-cadherin (truncated cadherin). Most cadherins are homophilic and generally only bind the same or a very similar cadherin on the cell surface of the opposing cell, this homophilic specificity is one of the mechanisms by which cells form homogenous tissues. Binding of cadherins occurs at the distal cadherin domain repeat where a knob and a pocket form which meets its counterpart from the opposing cell where the knob inserts into the pocket of the counterpart and vice versa. Cadherins are anchored to the cytoskeleton. Type I and II cadherins bind actin filaments through p120, β-catenin, and vinculin while desmosomal cadherins bind intermediate filaments through plakoglobin, plakophilin, and desmoplakin. These interactions with the cytoskeleton are essential to cadherins signaling capabilities.

In the vascular endothelium desmosomal cadherins are essential to maintain cell-cell adhesions. Furthermore, vascular endothelial (VE)-cadherin has been implicated in a signaling complex with VEGFR-2 and β-catenin mediates a shear stress induced activation of Akt[100]. Tzima et al. proposed an expansion of this complex to include PECAM-1[116] and suggest it as a possible mechanism for the shear induced activation of integrins[116]. They report that in cultured endothelial cells exposed to flow both VE-cadherin null cells and PECAM-1 null cells show diminished activation of integrins. It was also demonstrated that VE-cadherin was necessary for the flow-induced increase in phosphatidylinositol 3 kinase (PI3K) activity, and that PECAM-1 was required for the increase in both PI3K and Src following shear stress. They further demonstrated that VE-cadherin activation of PI3k was ligand independent and mediated by VEGFR-2. Finally, they also showed that the direct application of shear stress to PECAM-1 causes the ligand independent activation of VEGFR-2 via Src and is dependent on VE cadherin.

One proposed model of the cytoskeleton that facilitates the conduction of mechanical forces across the cell is the tensegrity model proposed by Donald Ingber[117]. Tensegrity is an architectural principle for the design of bodies that are in constant tension in such a way that endows integrity, thus the name: tensegrity. The core concept of tensegrity is three or more non-touching rods or struts under constant compression bound together by pre-stressed tendons that define the body spatially. Tensegrity structures are geodesic because the tensioned tendons take the shortest path between on node and another making them the optimal geometry for baring that specific load. Furthermore, free standing tensegrity structures will rearrange while maintaining compression and tension when exposed to changes in mechanical forces to maintain optimal load bearing. In the cell model suggests microtubules as the compression struts, while actin and intermediate filaments act as tendons to form the cellular tensegrity.

Inflammation and arteriogenesis

The inflammatory nature of arteriogenesis is outlined by the upregulation of several inflammatory mediators in vessels after increased shear stress. These include adhesion molecules such as E- and P-selectins, intercellular ICAM-1 and vascular cell adhesion molecule (VCAM-1)[133-136] and the chemokines such as MCP-1, CCL3, and CCL7 [118]. These molecules play an important role in the recruitment and activation of leukocytes (in particular monocytes). Rabbits treated with MCP-1 exhibit increased femoral collateral growth after femoral artery occlusion compared to untreated controls[119] this increase was attenuated by blocking ICAM-1[120] suggesting that monocyte adhesion is required for the observed increase. Mice lacking MCP-1 expression show diminished collateral growth in response to femoral artery ligation[121]. Surprisingly, Mice lacking the receptor for MCP-1 (CCR2) have been reported to exhibit both normal and diminished flow restoration after femoral ligation[122, 123].

Evidence for the role of monocytes in arteriogenesis was demonstrated in osteopetrotic mice with significantly diminished monocytes[8]. These mice exhibited significantly decreased restoration of perfusion compare to controls. Monocytopenia induced in mice using 5-fluorouracil (5-FU) also leads to reduced restoration of flow[124]. Conversely, increased monocytes due to rebound from 5-FUtreatment or supplement increased percentage of restored flow[124]. Interestingly, neutrophils are reported to also invade the collaterals early in the remodeling process [142]. This early neutrophil population may serve to prepare the tissue for monocyte invasion [143]. Monocytes exact an arteriogenic effect by releasing growth factors and cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and basic fibroblast growth factor (bFGF)[144,145]. IL-1β induces a phenotypic shift of SMCs from contractile to inflammatory mediated by NF-κB [146] which is a necessary event in the remodeling process. TNF-α on the other hand induces SMC migration [147]. TNF-α knockout mice exhibit significantly decreased flow restoration after femoral artery occlusion compared to control [125]. bFGF is a mitogen for both endothelial and smooth muscle cells[126]. bFGF infusion of rats with femoral artery occlusion resulted in increased restoration of flow to the calf muscle compared to placebo control [127]. bFGF gene treatment significantly improved cardiac function during chronic myocardial ischemia in pigs[128]. This was accompanied by a significant 3.4 fold increase in vessel thickness compared to control. Furthermore, in a canine model for ischemic heart disease intracoronary administration of bFGF induced a 40% increase in collateral flow compared to control after surgical occlusion of the left circumflex coronary artery[126]. This increase was demonstrated to have a spatial and temporal component as neither intravenous nor pericardial administration affected collateral flow. Single administration of bFGF on the first day of occlusion had no effect on collateral flow [129]. This correlates with the appearance of monocytes in the collaterals 24 hours after occlusion[153]. In SMCs bFGF causes an increase in ERK1/2 phosphorylation mediated by MEK that results in an increase in the expression of the transcription factor early growth response-1 (Egr-1) [130]. Egr-1 promotes cell survival and proliferation [155,156]. Egr-1 is upregulated during vascular injury [2] and

by mechanical stress [131] including shear stress [159]. Egr-1 induces the expression of platelet-derived growth factor [160,161] and insulin like growth factor-2 (IGF-2) [162]. Egr-1 knockout rats demonstrate diminished collateral development compared to controls after femoral excision. Inversely, adenoviral induced over expression of egr-1 resulted in increased collateral development after femoral excision [132]. In the mouse adenoviral over expression of egr-1 resulted in increased restoration after femoral ligation [133]. Egr-1 also regulates the expression of Transforming growth factor-β1 (TGF- β1) [165] which has been shown to upregulate the expression of FGFR-1 [134] which, has been proposed has the rate limiting step in bFGF signaling [134].

Granulocyte-macrophage colony stimulating factor (GM-CSF) stimulates the differentiation of hematopoietic stem cells into monocytes, which play a key role in arteriogenesis. Increased shear stress causes a dose dependent increase in GM-CSF expression in cultured ECs[135]. GM-CSF causes an increase in the expression of the pro-arteriogenic factors MCP-1 and MMP12 in macrophages[136]. GM-CSF treatment after femoral artery ligation in rabbits increases macrophage recruitment to growing collaterals, flow restoration, and collateral vessel size[136, 137]. GM-CSF had a synergistic effect with MCP-1 increasing flow restoration beyond either factor on its own[138]. These findings suggest GM-CSF may be a candidate pro-arteriogenic factor released by the endothelium to increase monocyte recruitment. In a rat model for stroke GM-CSF significantly increased the size of the posterior cerebral artery after ligation of both vertebral arteries and the left carotid artery[139 171]. GM-CSF had the same effect in mice after ligation of the left carotid artery [140].

Nitric oxide

Nitric oxide (NO) is a soluble gaseous free radical produced by the nitric oxide synthase (NOS) family of proteins. NO plays an important role as a messenger in several physiological situations and is used as a cytotoxic agent by macrophages during inflammation. In the vasculature shear stress increases the production of NO by endothelial NOS (eNOS) in ECs. NO diffuses into SMCs, where it increases the activity of guanylate cyclase increasing the secondary messenger cGMP which in turn activates PKG which phosphorylates several downstream proteins resulting in SMC relaxation and vasodilatation. Inducing an increase in NO production by L-arginine dietary supplementation resulted in an increase in flow restoration after femoral artery excision in rabbits[141]. eNOS knockout mice on the other hand, exhibited diminished flow recovery after femoral artery excision[141]. In the dog myocardium repeated occlusion of the left anterior descending coronary artery resulted in growth of the coronary collaterals[142]. This growth was attenuated by the administration of L-NG-nitroarginine methyl ester (L-NAME) a nonspecific inhibitor of NOS. The arteriogenic effects of bFGF in rats after femoral artery ligation were reduced to vehicle control levels after L-NAME treatment[143]. eNOS is localized to the endothelium which experiences a six fold increase in expression[144]. Exercise accelerates arteriogenesis after femoral ligation and further increases eNOS expression[5]. L-NAME also inhibited collateral growth augmentation by exercise after femoral artery ligation in rats[145]. Interestingly, L-NAME did not inhibit exercise induced angiogenesis[145]. These varying effects of NO on arteriogenesis and angiogenesis were further demonstrated in rats overexpressing eNOS by adenoviral transfection. After femoral artery ligation, rats overexpressing eNOS exhibited increased collateral size and flow restoration but normal capillary density compared to control[146]. As stated earlier, eNOS knockout mice exhibit diminished arteriogenesis after femoral artery occlusion[141]. However, collateral growth was demonstrated to "catch up" with wild type mice later in the arteriogenic process[146]. Mice were treated with L-NAME either immediately after

femoral artery ligation or 3 days post-surgery. Both groups showed significant decreases in flow recovery compared to control[147], emphasizing the importance of NO during the entirety of arteriogenesis. Inhibition of inducible NOS (iNOS) in eNOS knockout mice resulting in severely decreased collateral growth after femoral artery ligation compared to control[148] suggesting a transition from eNOS to iNOS dependent NO production as arteriogenesis progresses.

PLGF

In 1991 Maglione et al. described the cloning of a 877 kb cDNA segment isolated from human placenta coding for a 149 residue protein. They demonstrated that this protein is a *N*-glycosylated secreted dimeric protein covalently bonded by disulfide bonds. The protein also exhibited strong sequence similarity (53%) to vascular endothelial growth factor (VEGF), and was aptly named placental growth factor (PLGF)[149]. The human PLGF gene is located on chromosome 14q24[150]. Alternative splicing of PLGF's seven exons results in four different isoforms, PLGF 1–4[150-153]. An insertion of 21 basic amino acids in PLGF-2 and PLGF-4 acts as a heparin-binding domain. PLGF-1 and PLGF-3 lack this domain[150, 151, 153]. This highly basic insertion also allows PLGF-2 to bind to neuropilin-1 and -2[154].

X–ray crystallography revealed that PLGF consists of two α-helices and seven β-sheets. Two disulfide bonds form between four cysteine residues on β3 and β7 to form a ring which is penetrated by a disulfide bond between two cysteines on β1 and β4[155]. The resultant structure is known as a cysteine knot motif[156]. Two more cysteines form two disulfide bonds with two cysteines on another symmetrically opposite PLGF monomer to form a dimer[155]. PLGF specifically binds VEGR-1 but not VEGFR-2[157]. VEGFR-1[40] and VEGFR-2[42] along with VEGFR-3[193] form the VEGF receptor family which are a member of type III tyrosine kinases. They consists of a seven immunoglobulin domain extra cellular segment[158], a single transmembrane domain, and a cytoplasmic tyrosine kinase segment containing a kinase insert domain[40]. For VEGFR-1 binding depends on the N- terminus α-helix and the β3-β4 loop of the

protein[159], while domains 2 and 3 of the extracellular immunoglobulin domains are responsible for binding on the receptor[160]. Like other tyrosine kinase receptors, only one monomer of the PLGF dimer binds VEGFR-1 while the second monomer binds a second VEGFR-1 molecule resulting in a VEGFR-1 dimer[160]. The juxtaposed tyrosine kinase domains on each receptor then transphosphorylate tyrosine residues on the opposite receptor[161]. The phosphotyrosines act as recognition sites for Src homology domains and phosphotyrosine binding domains of signaling molecules[162].

Although PLGF exclusively binds VEGFR-1, VEGF-A[40] and VEGF-B[163] are also capable of binding VEGFR-1. Initially, PLGF was thought to only act by releasing VEGF from VEGFR-1, which in turn would activate VEGFR-2. This was due to the weak kinase activity of VEGFR-1. Knockout of the VEGFR-1 receptor resulted in poor survival due to disorganized embryonic vasculature[164] but mice expressing a mutant VEGFR-1 with inactive kinase showed normal development[201]. This suggested that VEGFR-1 acts as a decoy receptor for VEGF. Since then, PLGF has been shown to exact effects independent of VEGF and VEGFR-2 via multiple signaling pathways. PLGF activates the mitogen activated protein kinase (MAPK) pathway in a VEGFR-1 mediated fashion in multiple cancerous cell lines[165-167]. Monocytes predominantly expressing VEGFR-1 are activated by PLGF despite no VEGFR-2 receptors present[168]. Inhibitors of MAPK kinase (MEK) and phosphoinositide 3′ kinase (PI3K) abrogate the effects of PLGF on monocytes[169]. PLGF signaling in smooth muscle cells involves Janus kinase (JAK) and Signal Transducer and Activator of Transcription III (STAT3) to induce proliferation[170]. The involvement of these signal pathways outlines PLGF's role in cell survival and proliferation. Furthermore, PLGF binding to VEGFR-1 results in a distinctive pattern of transphosphorylation compared to VEGF implying different downstream effects[171]. Indeed, analysis of microarray data collected from human umbilical vein endothelial cells shows treatment with PLGF or VEGF-A induce distinctive transcription patterns that are non-overlapping[172]. Additionally, VEGFR-1

activation by PLGF leads to crosstalk between VEGFR-1 and VEGFR-2 that amplifies VEGFR-2's downstream signal [171] ultimately leading to enhanced VEGF signaling[4].

Arguments against VEGFR-1 signaling are the result of a VEGFR-1 selective VEGF mutant failing to elicit a mitogenic effect or significantly activate extracellular signal-regulated kinase (ERK 1/2) or PI3K[173]. These findings may be the result of VEGFR-1's ligand dependent tyrosine phosphorylation patterns[171].

PLGF naturally dimerizes with VEGF in cells expressing both growth factors[174]. Due to the inability of PLGF to bind VEGFR-2 the VEGF/PLGF heterodimer is capable of dimerizing VEGFR-1 with VEGFR-1 or VEGFR-2 but not VEGFR-2 with VEGFR-2 [175]. The mitogenic effects of this heterodimer are a point of controversy in the literature. The VEGF/PLGF heterodimer exhibits a ~30 fold decrease in mitogenic effect compared to VEGF in endothelial cells[176]. Studies reporting angiostatic effects of the heterodimer attribute it to a decrease in the amount VEGF available to form homodimers[177-179]. Tarallo *et al.* demonstrate that the heterodimer exhibits decreased angiogenesis in favor of arteriogenesis (as indicated by decreased sprouting and increased caliber of vessels)[175], these effects are attenuated when a non VEGFR-1 binding PLGF mutant is used inhibiting any receptor dimerization[175]. Indeed, the studies in ref. 32 & 34 overexpress PLGF-1 in murine neoplasms despite the mouse only expressing PLGF-2 natively[180].

During pregnancy, a pathological condition called preeclampsia may develop which is marked by maternal endothelial dysfunction, hypertension, proteinuria, and hepatic and renal damage. One hypothesis suggests that insufficient placental perfusion causes the placenta to release vasoactive mediators that cause the endothelial dysfunction[218]. Maynard et al. found a 4-fold increase in soluble VEGFR-1 (sVEGFR-1) in patients presenting preeclampsia compared to control patients[181]. sVEGFR-1 is a secreted non-membrane bound isoform of VEGFR-1, and acts as a competing receptor[220]. Preeclampsia patients also exhibited decreased circulating PLGF and

VEGF[181]. PLGF plays an important role in preeclampsia. Decreased urinary PLGF is associated with preeclampsia and is suggested as a possible predictive marker of the condition[182]. PLGF induces the vasodilatation of uterine arteries of rats[183]. Mice over expressing sVEGFR-1 by adenoviral transfection exhibit symptoms of preeclampsia. Recombinant PLGF administration relieves these symptoms[184].

The vasculature of neoplasms and its extensiveness is directly related to patient survival [218,224]. Overexpression of PLGF results in increased perfusion and growth of implanted tumors in mice[225,226]. This effect is in part due to increased survival and proliferation of both ECs and macrophages[225,226], whereas knockout of the VEGFR-1 receptor on monocytes attenuates the effects of PLGF overexpression [227]. Conversely, inhibition of PLGF by antibodies results in diminished perfusion and growth of tumors from several origins [185, 186]. PLGF antibodies inhibit tumor growth by suppressing the expansion of arterioles rather than affecting the capillary network [186]. PLGF also directly affects neoplastic cells increasing motility and invasion of breast cancer cells [167, 187]. PLGF increases the growth of acute lymphoblastic leukemia cells[188]. These effects of PLGF are mediated by increased ERK1/2 phosphorylation [187, 189].

Most of PLGF's roles in disease are the result of its effects on blood vessels. Interestingly, PLGF knockout in mice is non-lethal and embryos develop normally[4]. These mice however, exhibit poor arteriogenesis after femoral ligation [4]. PLGF treatment improves the poor arteriogenesis[10]. While treatment of wild type mice with PLGF after femoral ligation improved flow, restoration 3 fold compared to control[11]. Transgenic mice overexpressing PLGF exhibit enlarged, tortuous vessels, with increased density and branching [6]. These studies support PLGFs role an arteriogenic factor. The effects of PLGF are monocyte dependent. After femoral ligation in rabbits, treatment with PLGF resulted in a significant increase in femoral collateral growth compared to control or VEGF-E treatment[10]. This growth however is attenuated in rabbits depleted of monocytes[10]. Emphasizing the role of bone marrow derived cells in PLGF mediated

arteriogenesis, wild type bone marrow transplantation into PLGF knockout mice ameliorates poor arteriogenesis[9]. Concurrent treatment of mice after femoral artery ligation with PLGF and VEGF resulted in greater collateral growth than either PLGF or VEGF on their own[190]. In agreement with these findings, PLGF enhances the effects of VEGF on ECs and is necessary for VEGF induced SMC proliferation[191, 192]. PLGF also decreases VEGF induced endothelium permeability by increases adherens junctions [193]. In rats, direct injection of PLGF in to coronary arteries improved cardiac function and a thicker myocardium in the penumbra after left anterior descending artery ligation compared to vehicle control [241]. Similarly, adenoviral transfection of PLGF after ligation of the left anterior descending coronary in mice resulted in enlargement of coronary vessel accompanied by improved cardiac function compared to control[194].

Effects of disease on arteriogenesis

Before pharmacological stimulation of arteriogenesis by any agent is considered it is important to understand the pathological context within which it must act. Metabolic syndrome and type II diabetes mellitus are more prevalent in patients with poor coronary collateral development after coronary obstruction compared to patients with good coronary collateral development[195]. Metabolic syndrome consists of a group of symptoms that are detrimental to cardiovascular health. These include hypertension, dyslipidemia, central obesity and insulin resistance [244,245]. Based on multivariate analysis, hyperglycemia is statistically the most detrimental risk factor to collateral growth[196]. The endothelium of hyperglycemic patients is dysfunctional. The increased circulating glucose leads to protein glycation (non-enzymatic glycosylation of proteins at arginine and lysine residues) resulting in advanced glycation end products (AGE). During the process, hydrogen peroxide is released, increasing oxidative stress. The AGEs themselves accumulate in the basement membrane of the vascular endothelium, where they contribute to endothelial dysfunction. Hyperglycemia diminished collateral growth in the canine myocardium after repetitive coronary occlusion, compared to control dogs[197]. Similarly,

myocardial ischemia was induced by repetitive coronary occlusion in obese (but not insulin resistant) rats. These rats showed no coronary growth, in contrast to their lean littermates that achieved 81% flow restoration [248]. In the obese rats, treatment with VEGF failed to improve collateral growth. However, concurrent treatment with VEGF and superoxide dismutase (SOD, an enzyme that breaks down superoxide to oxygen and hydrogen peroxide, which is further broken down by catalase) resulted in a significant increase in flow restoration[248]. Similarly, treatment with antioxidants resulted in improved collateral growth in the same model [249]. Knockout of SOD in mice results in impaired femoral collateral growth and flow recovery after femoral artery ligation compared to control. These animals also exhibited diminished bone marrow derived cells in the collateral area [198]. NADPH oxidase is an enzyme complex that catalyzes the formation of superoxide. $gp91^{phox}$ is a subunit of NAPH oxidase which along with $p22^{phox}$ is responsible for production of superoxide. $gp91^{phox}$ deficient mice were treated with streptozotocin (STZ) (a toxin with enhanced cytotoxicity towards beta cells) to simulate type I diabetes mellitus. After femoral ligation, collateral growth was increased in these animals compared to wild type STZ treated mice and comparable to wild type untreated, suggesting superoxide is an anti-arteriogenic factor [199]. However, poor femoral collateral growth was reported in untreated $gp91^{phox}$ knockout mice compared to wild type after femoral artery ligation[200]. This dual effect was further investigated in rat treated with either diphenyleneiodonium, an inhibitor of NADPH oxidase, or diethyldithiocarbamate, an inhibitor of SOD. Surprisingly, both treatments resulted in decreased coronary collateral growth after repetitive occlusion. Furthermore, p38 MAP kinase was demonstrated to be activated by repetitive occlusion, and this effect was diminished by either treatment[201]. The authors proposed that there is a narrow range within which oxidative stress is pro-arteriogenic, and that deviations in either direction reduce collateral growth. However, another conclusion could be drawn, considering the nature of the treatments used in the study above. Both treatments, despite attempting to increase or decrease oxidative stress, cause a

decrease in H_2O_2. The pro-arteriogenic factor PLGF is up regulated by H_2O_2 in SMCs [202]. Similarly, Egr-1 expression is increased after H_2O_2 treatment [255,256].

Patients with advanced diabetes mellitus exhibited decreased peripheral vasodilation after either heating the limb or administration of sodium nitroprusside[203]. These patients also exhibited reduced eNOS expression. Similarly, eNOS mRNA and protein are significantly decreased in the endothelium of a rat model of type I diabetes compared to wild type control [204]. Treating mice with STZ resulted in a decrease in NO production, and was demonstrated to be the result of decreased Akt expression[205]. Interestingly, type I diabetic mice have decreased eNOS expression compared to type II diabetic mice, and both models were less than control[206]. Femoral artery ligation caused no change in eNOS expression in type I diabetic mice, but resulted in a drop in eNOS expression in Type II diabetic mice to type I diabetic levels [206], suggesting that although differences between the type of diabetes and endothelial dysfunction exist, they are equally impaired with regard to arteriogenesis.

Monocytes play an important part in arteriogenesis. Several studies demonstrate that monocyte recruitment in diabetes mellitus is impaired, and that pharmacologically increasing monocyte recruitment could improve arteriogenesis. Diabetic patients with critical limb ischemia were treated with recombinant GM-CSF, which resulted in improved perfusion of the ischemic limb compared to placebo control. This was accompanied with a tenfold increase in bone marrow derived cells[207]. Rabbits treated with the cytotoxic glucose analogue alloxan to simulate type I diabetes exhibit diminished flow restoration following femoral artery ligation. Monocytes from the alloxan treated mice demonstrated reduced motility when treated with VEGF-A or MCP-1[208]. Monocytes from diabetic patients likewise exhibit reduced VEGFR-1 mediated motility compared to control. This effect however was not due to decreased ligand binding or tyrosine kinase activity[209]; diabetic patients were also found to express significantly less VEGFR-1 in the myocardium, compared to non-diabetic patients[210]. Furthermore, diabetic patient monocytes

exhibit increased baseline phosphorylation of signaling proteins downstream of VEGFR-1 such as Akt, MAPK P38, and ERK1/2[211]. These monocytes also exhibit increased expression of the AGE receptor[212]. Furthermore, high concentrations of glucose cause cultured ECs to significantly increase MCP-1 expression[213]. One possibility for reduced monocyte activation is that the increased inflammatory state in diabetes mellitus "drowns out" the pro-arteriogenic signal mediated by VEGFR-1 or other pathways.

The remodeling of the ECM is absolutely necessary for the progression of arteriogenesis. The effects of diabetes mellitus on the vascular ECM are largely unknown, although some studies have focused on this topic. Mice treated with STZ exhibited poor femoral collateral growth after femoral ligation compared to untreated controls. This diminished growth was improved by treatment with aminoguanidine, which inhibits AGE formation [214]. Interestingly, these mice showed no change in MMP-2, MMP-3 or MMP-13 expression. However, MMPs are expressed as zymogens, and STZ treated mice did exhibit decreased protease activity, which was again improved by aminoguanidine treatment [214]. Similarly, a mouse model for type II diabetes mellitus arising from spontaneous mutation of the leptin receptor exhibited reduced MMP-2, MMP-12 and MMP-16 mRNA, compared to wild type control[215]. In contrast, mice treated with STZ were found to express excessive MMP-2 and MMP-9 and reduced TIMP-1 after femoral artery excision compared to control [216]. However, no data were provided in that study regarding the functionality of these increased MMPs. These studies suggest that ECM remodeling via MMPs is impaired during diabetes mellitus, although further investigation is needed to understand the mechanisms of this inhibition.

Inward remodeling vs. outward remodeling

Atherogenesis and arteriogenesis show several similarities, and a real risk of stimulating arteriogenesis is aggravating pre-existing plaque or driving the formation of new plaque. This is

in part due to the dual effects many arteriogenic factors have. An understanding of how these similar growth factors and cytokines are coordinated to produce either lifesaving remodeling or lethal pathology is essential for the development of safe and effective pro-arteriogenic therapies.

A key event in both arteriogenesis and atherosclerosis is a phenotypic shift of SMCs, along with SMC migration and proliferation. FGF2 stimulates SMC growth and is necessary for arteriogenesis. Acidic fibroblast growth factor (FGF1) also shows arteriogenic potential. Prolonged delivery of FGF1 to ischemic porcine hearts caused an increase in cardiac function and greater perfusion of the ischemic zone[27]. Intra-arterial transfection with the FGF1 gene following femoral artery ligation resulted in increased collateral development in rabbits[217]. FGF1 and FGF2 also exhibit pro-atherosclerotic effects. Both growth factors' expression is increased in human atherosclerotic lesions and in restenotic lesions[218]. Rats treated with bFGF after carotid artery denudation exhibited a 2.4-fold increase in intimal hyperplasia[219], while transfection of porcine arteries with the aFGF gene leads to intimal hyperplasia[220]. The role of inflammation in both arteriogenesis and atherosclerosis is well established[274,275]. Essential for the progression of both remodeling processes is the recruitment of monocytes. Flow restoration was significantly improved with MCP-1 treatment after femoral artery ligation in minipigs[221]. Nicotine treatment increases the restoration of flow after femoral artery occlusion in rabbits. This increase is comparable to that found with FGF2 treatment. Furthermore, nicotine causes upregulation of ICAM-1 and MCP-1 expression in endothelial cells, while increasing CD11a and CD11b expression in monocytes. As expected, an increase in monocyte adhesion and migration is also observed[222]. Human atherosclerotic lesions stain positive for MCP-1[223]. Hyperlipidemic mice were treated with nicotine via drinking water for 20 weeks. This resulted in a marked increase of atherosclerotic lesion size[224]. The role of PLGF in arteriogenesis has been extensively studied and PLGF has even been suggested as possible treatment for ischemic heart disease. This however, must be weighed against the finding that in patients exhibiting multi-vessel coronary

atherosclerotic lesions, a reduction in circulating sVEGFR-1 was observed compared to patients with a single lesion or no lesions[225]. This reduction would most likely lead to increased PLGF bioavailability. Indeed, adenovirally induced overexpression of PLGF increased intimal thickening in both high cholesterol- and normal diet-fed rabbits compared to control[281], while PLGF knockout in hyperlipidemic mice resulted in diminished plaque area and macrophage content compared to hyperlipidemic mice normally expressing PLGF[281]. These results were repeated when hyperlipidemic mice were treated with PLGF antibodies[282]. Interestingly, this dual effect is also observed for endogenous angiogenesis inhibitors such as endostatin, which demonstrates both anti-arteriogenic[226] and anti-atherogenic [284] effects. This athero/arteriogenic relationship extends further than single mediators to whole animals. Two commonly used mouse strains are C57BL/6 and Balb/c. These two strains display different degrees of response to femoral artery ligation. Limbs of C57BL/6 mice are briefly hypoxic, but arteriogenesis progresses rapidly after ligation. In contrast, Balb/c mice develop severe limb hypoxia, and flow recovery time is increased compared to C57BL/6 mice. On the other hand, Balb/c mice resist diet-induced atherosclerosis, while C57BL/6 mice are susceptible to diet-induced atherosclerosis. Mice express high-density lipoprotein (HDL) as their predominant lipoprotein[227]. HDL reduces monocyte recruitment [285]. C57BL/6 mice express lower HDL than Balb/c mice[228], which may be a possible explanation for the strain differences observed. In a more clinical aspect, the degree of coronary collateral development assessed by angiography is positively correlated with the development of in-stent stenosis after coronary angioplasty[229].

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CHAPTER III

ARTERIOGENIC FLUID SHEAR STRESS UPREGULATES PLACENTAL GROWTH FACTOR IN THE VESSEL WALL VIA A NADPH OXIDASE 4 DEPENDENT MECHANISM

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Abstract

Placental growth factor (PLGF), a potent stimulator of arteriogenesis, is upregulated during outward remodeling. Increased fluid shear stress (FSS) is a key physiological stimulus for arteriogenesis. However, the role of FSS in regulating PLGF expression is unknown. To test the hypothesis that fluid FSS can regulate PLGF expression in vascular cells, and to identify signaling pathways involved, human coronary artery endothelial (HCAEC) and smooth muscle (HCASMC) cells were cultured on either side of porous Transwell inserts. HCAEC were then exposed to pulsatile FSS of 0.07 Pa ("normal," mimicking flow through quiescent collaterals), 1.24 Pa ("high," mimicking increased flow in remodeling collaterals) or 0.00 Pa ("static") for 2 h. High FSS increased secreted PLGF protein ~1.4-fold compared to static control ($n=5$, $p<0.01$), while normal FSS had no significant effect on PLGF. Similarly, high flow stimulated PLGF mRNA expression ~2-fold in isolated mouse mesenteric arterioles. PLGF knockdown using siRNA revealed that HCAEC were the primary source of PLGF in cocultures ($n=5$, $p<0.01$). Both H_2O_2 and NO production were increased by FSS, compared to static control ($n=5$, $p<0.05$). L-NG-nitroarginine methyl ester (100 μM) had no significant effect on the FSS induced increase in PLGF. In contrast, both catalase (500 U/mL) and diphenyleneiodonium (DPI, 5 μM) attenuated the effects of FSS on PLGF protein in cocultures. DPI also blocked the effect of high flow to upregulate PLGF mRNA in isolated arterioles. Further studies identified Nox4 as a source of ROS for this pathway. We conclude that fluid FSS regulates PLGF expression, and that this regulation is dependent on Nox4 and ROS signaling.

Keywords: arteriogenesis, collateral circulation, hemodynamics, endothelium, vascular endothelial growth factors

New and Noteworthy

Here we provide the first direct demonstration that the arteriogenic mediator placental growth factor (PLGF) is regulated by elevated fluid shear stress (a physiological stimulus for collateral artery remodeling) in both vascular cell cocultures and isolated perfused arterioles. We further identify Nox4 and H_2O_2 as downstream mediators of this response.

Introduction

Coronary artery disease (CAD) is the leading cause of morbidity and mortality in both western and developing countries, and even optimistic projections predict it to remain so for the foreseeable future. Coronary artery disease is characterized by stenosis of the coronary arteries due to atherosclerosis, resulting in decreased perfusion of downstream tissue. The decreased pressure downstream of the occlusion drives blood through small preexisting collateral vessels that bridge the area of low pressure with areas of higher pressure. With time, these collateral vessels remodel outward, increasing their conductive capacity and the perfusion of the ischemic tissue in a process called arteriogenesis. In CAD patients, the degree of collateral development correlates directly with positive outcome[130]. Unfortunately, collateral development is highly variable in humans[230]. Thus, new pharmacological interventions to induce arteriogenesis could prove life-saving for those CAD patients who have limited native ability to remodel collaterals. However, attempts to induce arteriogenesis by administration of growth factors such as VEGF-A have yielded disappointing results, indicating that a better understanding of the mechanistic basis of arteriogenesis is needed[231, 232].

Increased flow demand on collateral vessels downstream of arterial stenosis or occlusion results in an increase in fluid shear stress (FSS) on the arterial wall. The role of FSS as a key initiating stimulus for collateral remodeling has been extensively demonstrated[3, 65, 233, 234]. FSS is

inversely proportional to the cube of vessel diameter, and rapidly decreases as the vessel wall expands. However, prolonged increased FSS achieved by arteriovenous shunt results in continued enlargement of collateral vessels[65, 67, 235, 236]. Recently, compelling clinical evidence for the role of FSS in arteriogenesis was provided by Gloekler *et al*[237]. Diastolic coronary flow was pharmacologically increased in patients with CAD for six months, resulting in a significant increase in coronary flow index (CFI) compared to placebo control. The treatment group also demonstrated a decrease in the area of the ischemic zone, as indicated by ECG ST segment shift[237].

Placental growth factor (PLGF) is a member of the vascular endothelial growth factor (VEGF) family. PLGF is a potent stimulator of collateral growth, inducing more collateral vessels and greater flow recovery in mouse and rabbit hindlimb after femoral artery ligation than VEGF-A[10, 11]. Evidence from $PLGF^{-/-}$ mice suggests that PLGF is required for normal collateral remodeling, as re-establishment of hindlimb perfusion after femoral artery ligation is severely delayed in these mice[4]. On the other hand, overexpression of PLGF causes a marked increase in vessel size[6] and increased resistance to myocardial infarction[194, 225] in mice. Clinically, higher levels of PLGF predict improved patient outcome following acute myocardial infarction, as demonstrated by increased left ventricular ejection fraction[238].

The mechanism by which PLGF stimulates collateral remodeling is thought to rely on recruitment of monocytes to the vessel wall. These monocytes in turn orchestrate the remodeling process[239]. Although some insights have been gained into the downstream effects of PLGF as mediated by monocytes, the mechanisms regulating PLGF expression in the vessel wall during collateral remodeling remain largely unknown. The importance of both PLGF and FSS in collateral remodeling led us to hypothesize that FSS may regulate the expression of PLGF in the vessel wall. This hypothesis is supported by our previous observation that PLGF mRNA expression is sharply increased in hindlimb collateral arterioles of both sedentary and exercise-

trained rats immediately following acute femoral artery ligation, then decreases gradually as remodeling progresses[5]. Resting blood flow was sufficient to support tissue metabolic needs in sedentary but not exercising rats, yet PLGF was upregulated similarly in both groups, suggesting that elevated FSS can upregulate PLGF even in the absence of tissue hypoxia. The present study used an *in vitro* endothelial cell/smooth muscle cell coculture (EC/SMC) model of the vessel wall and isolated perfused mouse mesenteric arterioles to directly characterize the effects of FSS on PLGF expression, and to identify key signaling pathways mediating upregulation of PLGF by shear.

Methods

Reagents. All reagents were purchased from Sigma-Aldrich unless otherwise specified.

Perfused arterioles. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee at Oklahoma State University. Experiments were conducted on isolated second order mesenteric arterioles (≈120-180 μm) of 6-8 week-old C57BL/6J male mice (Jackson Laboratories). Mice were anesthetized with isoflurane delivered by a vaporizer and the heart was excised. The entire mesentery with the superior mesenteric artery and vein was dissected and washed with 4 ºC PBS. Second order mesenteric arterioles were isolated from the mesenteric tissue. The isolated artery was transferred to a vessel chamber at 4ºC (Living Systems Instrumentation). The chamber contained a pair of glass micropipettes and was filled with physiological saline solution (PSS; 142 mM NaCl, 4.7 mM KCl, 1.7 mM $MgSO_4$, 0.5 mM EDTA, 2.79 mM $CaCl_2$, 10 mM HEPES, 1.18 mM KH_2PO_4, pH 7.4). After cannulation of the proximal (upstream) end of the vessel, the intraluminal pressure was gradually raised (less than 20 mmHg) to clear the lumen of clotted blood. Once cleared, the distal (downstream) end of

the vessel was also cannulated. Time from euthanasia to complete cannulation was under 60 minutes. The temperature of the bath was then raised to 37ºC and pressure was gradually raised to 60 mmHg (~10 mmHg/10 minutes). The pressure increase was achieved by gradually raising two reservoirs connected by silicone tubing to each cannula. Perfusion buffer consisted of 1% bovine serum albumin in PSS. Once equilibrated at 60 mmHg, the longitudinal pressure gradient was increased from zero to 20 mmHg ("control") or 50 mmHg ("pro-arteriogenic") [63]. This was achieved by lowering the distal reservoir and raising the proximal reservoir, allowing for the average intraluminal pressure to be maintained at 60 mmHg. The control flow rate was ~75 μL/min, and the "pro-arteriogenic" flow rate was ~170 μL/min. Vessels were then perfused for 2h. Function of the vessel wall was determined at the end of perfusion by assessing the vasoconstrictive response to epinephrine and vasodilator response to acetylcholine, based on changes in vessel diameter measured by video micrometer.

Cell culture. Human coronary artery endothelial cells (HCAEC) and human coronary artery smooth muscle cells (HCASMC) were purchased from Lonza. For HCASMC, donors included a 12 year old male, a 56 year old female, and a 30 year old male, while HCAEC donors were a 21 year old male and a 30 year old male. HCAEC were cultured in EBM-2 basal media supplemented with EGM-2 MV SingleQuot factors (HCAEC complete media, Lonza). HCASMC were cultured in SMBM basal media supplemented with SmGM-2 SingleQuot factors (HCASMC complete media, Lonza). All cells were grown in a humidified incubator in 5% CO_2 at 37°C. Cells were used between passage five and six for all experiments. Serum reduced media for experiments to be performed in room air was prepared by diluting the appropriate complete media in low glucose DMEM (Hyclone, Fisher) at a ratio of 2:3 yielding a 2% serum media, and supplementing with 15 mmol/L HEPES. Phenol red free media was used for experiments requiring fluorescent assays.

Co-culture model. To model the vessel wall *in vitro*, porous Transwell inserts (Corning Costar, 0.4 μm pore size) were used as described in the literature[240]. Inserts were inverted and the bottom surface was coated with 0.1% gelatin in DMEM, then were placed in a humidified incubator in 5% CO_2 at 37°C for 1 h. HCASMC (10^4 cells/cm^2) were then seeded onto the inverted insert, and inserts were returned to the incubator overnight. The following day, the inserts were placed into 6 well plates containing SmGM-2 media and incubated for an additional 24 h. The top surface of the insert was then coated with 0.1% gelatin in DMEM and incubated for 1 h. HCAEC (25,000/cm^2) were then seeded on the top surface of the insert. EGM-2 MV was added to the insert and the system was again incubated overnight. When possible, HCASMC and HCAEC donors were matched. Confluence of the co-cultures was confirmed by Hoffman modulation contrast microscopy (Olympus IX71). Lastly, the confluent co-cultures were incubated in serum reduced media for 24 h prior to experiments. For HCAEC mono-culture experiments, no HCASMC were seeded on the bottom of the insert, but cultures were otherwise processed as described above.

Shear stress exposure. Only the HCAEC layer of the co-culture was directly exposed to FSS. FSS was applied using a cone and plate viscometer shearing system. The system consisted of four ultra-high molecular weight polyethylene 0.5° cones connected to microstepper motors (Optimal Engineering Systems, Inc.) linked to a controller unit (Optimal Engineering Systems, Inc.) (Fig. 1 A). Cones were placed into the four peripheral inserts of the 6-well plate, while the central two inserts were not exposed to FSS and were used as a static control. Co-cultures were then exposed to one of two pulsatile FSS waveforms, based on previously published information [241]. The "normal" waveform had time-averaged FSS of 0.07 Pa (Fig. 1B), and modeled flow through a quiescent collateral vessel downstream of a patent coronary artery. The "high" waveform had time-averaged FSS of 1.24 Pa (Fig. 1C), and modeled flow through an actively remodeling collateral vessel downstream of a coronary artery with 60% stenosis. Shear

experiments were performed on a laboratory benchtop in HEPES-buffered media with temperature maintained at 37°C. Cocultures were exposed to FSS for 2 h. Culture media and/or cell lysates were collected for analysis at various time points from pre-shear to 0-24 h post-shear.

siRNA knockdown experiments. HCAEC were seeded into 6-well plates at a density of 210,000 cells/well. After 24 h, cells were transfected with either PLGF siRNA (sense, 5'-AGGUGGAAGUGGUACCCUU-3', overhang dTdT; antisense, 5'-AAGGGUACCACUUCCACCU-3', overhang, dCdT), predesigned Nox4 siRNA (Silencer Select; s27013) or negative control siRNA (Silencer no.1 siRNA; scRNA), all purchased from Invitrogen. Prior to addition to cells, 5 nM of siRNA was precomplexed with lipofectamine RNAiMAX transfection reagent (Invitrogen) in Opti-MEM media (Gibco) for 20 minutes. Cells were exposed to transfection media (DMEM + 10% FBS containing precomplexed siRNA) for 6 h, after which cells were trypsinized and seeded onto the upper surface of inserts precoated with 0.1% gelatin. The lower surface of the inserts had been previously seeded with wild type HCASMC, as described above. Co-cultures were incubated overnight in reduced serum media as described above before exposure to shear stress. In a separate group of co-cultures, HCASMC were transfected similarly to HCAEC, after seeding onto the lower surface of inserts. At the end of the transfection period, untreated HCAEC were then seeded onto the upper surface as above. Cell specificity and efficacy of target mRNA knockdown was determined by real time PCR.

PLGF ELISA. Media samples were collected from sheared cells and their corresponding static controls, treated with protease inhibitor cocktail (1 mM PMSF, 1 mM Na_3VO_4, 1 µg/ml leupeptin, 1 mM benzamidine-HCl, 1µg/ml aprotinin, 1µg/ml pepstatin A) and stored at -80°C until further processing. PLGF was measured using the DuoSet ELISA development kit (R&D Systems) according to manufacturer's protocol. All samples were assayed in duplicate. Data were

normalized to total protein concentration, as determined by BCA assay (Pierce). All assay plates were read on a Biotek Synergy HT plate reader.

Real time PCR. After exposure to shear stress and media collection, cells were rinsed gently with PBS (HyClone) and trypsinized (TrypLE Express, Gibco). Collected cells were then resuspended in 1% β-mercaptoethanol in RLT lysis buffer (Qiagen) and frozen at -80°C for later processing. Cannulated vessels were placed in RLT lysis buffer immediately after perfusion and sonicated on ice using a Model D 100 Sonic Dismembrator (Fisher). Total RNA was isolated using RNeasy mini columns (Qiagen) following manufacturer's directions. Total RNA quantity and quality were determined spectrophotometrically using a Take3 Micro-Volume Plate in a Synergy HT plate reader (Biotek). Reverse transcription was carried out using the QuantiTect reverse transcription kit (Qiagen) following manufacturer's instructions. Real time PCR was performed on an ABI 7500 Fast instrument (Applied Biosystems) using PerfeCTa SYBR Green FastMix, Low ROX (Quanta Biosystems). Relative abundance of target mRNA was determined using the comparative Ct method and the following primer pairs: human PLGF forward 5'-CCTACGTGGAGCTGACGTTCT-3'; reverse 5'-TCCTTTCCGGCTTCA TCTTCT-3', human Nox1 forward 5'-TTGGAGCAGGAATTGGGGTC-3'; reverse 5'-AATGCTGCATGACCAACCTTT -3', Human Nox2 forward 5'-GGGAACTGGGCTGTGAATGA -3'; reverse 5'-CCAGTGCTGACCCAAGAAGT-3', Human Nox4 forward 5'-GGGGTTAAACACCTCTGCCT-3'; reverse 5'-ACACAATCCTAGCCCCAACA-3', Mouse PLGF forward 5'-CTGCTGGGAACAACTCAACAGA-3'; reverse 5'GCGACCCCACACTTCGTT-3'. Gene expression was normalized to β-actin, as amplified with the following primers: human β-actin forward, 5'-TGCCGACAGGATGCAGAAG-3'; human β-actin reverse, 5'-CTCAGGAGGAGCAATGATCTTGAT-3'; mouse β-actin forward, 5'-AGTTCGCCATGGATGACGAT-3'; mouse β-actin reverse, 5'-TGCCGGAGCCGTTGTC-3'.

The relative stability of β-actin after treatment was determined using the comparative Ct method with GAPDH as the housekeeping gene (human GAPDH forward 5'-GAAGGTGAAGGTCGGAGTC-3'; human GAPDH reverse 5'-GAAGATGGTGATGGGATTTC -3'). No significant change in β-actin was detected.

Hydrogen Peroxide Assay. The effect of shear stress on the H_2O_2 concentration in co-culture media was assessed using the Amplex Red Hydrogen Peroxide Assay kit (Invitrogen). Samples were collected in dark tubes, kept protected from light, and immediately frozen at -80°C until analysis. Further processing of samples was carried out on ice in reduced light conditions according to manufacturer's directions.

Nitrate/Nitrite Assay. Total nitrate and nitrite concentration was determined in samples of culture media from sheared and static control cocultures as an index of total NO production, , using the Nitrate/Nitrite Fluorometric Assay kit (Cayman Chemical).

Statistical analyses. All data are presented as mean ± SEM. Experiments were replicated at least five times. Data were analyzed by either two way repeated measures ANOVA or one way ANOVA, as appropriate. Both were followed by Tukey's range test. The level of significance was set at $p<0.05$.

Results

Increased fluid shear stress upregulates PLGF expression.

To characterize the effects of FSS on PLGF expression, cocultures were exposed to either normal or high FSS for 1, 2, or 4 hours. Media was sampled immediately before FSS exposure and 0, 4, 8, 12, and 24 h after FSS exposure. Exposure to either FSS waveform for 1 h had no significant effect on PLGF protein levels in media (compared to static control) at any time point up to 24 h (Fig. 2A). However, 2 h of high FSS significantly increased PLGF protein levels

in media starting 8 h after shear exposure, compared to static control conditions. PLGF protein levels continued to increase up to 24 h post-shear (Fig. 2B) and returned to static control levels by 36 h post-shear (data not shown). In contrast to the effect of high FSS, 2 h treatment with normal FSS had no significant effect on PLGF protein (Fig. 2B). Exposure to 4 h of FSS coincided with increased cell detachment, as subjectively determined by contrast microscopy (data not shown). Therefore, 2 h exposure was selected for further experiments.

To confirm that PLGF is regulated by FSS in intact vessels, we perfused mouse second order mesenteric arterioles using either a 50 mmHg longitudinal pressure gradient (high flow) or a 20 mmHg longitudinal pressure gradient (normal flow). PLGF mRNA was significantly increased after 2 h of perfusion at 50 mmHg, compared to control arterioles perfused at 20 mmHg (2.37 ± 0.36 fold of control) (Fig. 2C).

Smooth muscle cells increase endothelial cells' responsiveness to fluid shear stress.

We next sought to determine which cell type in the coculture was responsible for the increased PLGF protein expression in response to FSS by analyzing PLGF mRNA in HCAEC and HCASMC at 0, 4, 10 and 24 h post-FSS exposure. Neither level of FSS had a significant effect on PLGF mRNA in HCASMC (Fig. 3A). High FSS, but not normal FSS, produced an immediate and significant increase in PLGF mRNA expression in HCAEC, which was maintained until 4 h post-shear (Fig. 3B).

To further characterize the role of HCAEC in PLGF production by cocultures, PLGF was knocked down in either HCAEC or HCASMC using siRNA. PLGF knockdown was confirmed by RT-PCR (Fig. 4B, 4D). PLGF knockdown in HCAEC did not affect PLGF mRNA in cocultured HCASMC, or vice versa (not shown). Total PLGF expression was drastically and significantly decreased in cocultures with siPLGF treated HCAEC and abolished the effect of FSS to increase PLGF protein at 24 h post-shear exposure (Fig. 4A). Surprisingly, siPLGF

treatment of HCASMC resulted in a significant increase in PLGF protein concentration in coculture media, compared to media from cocultures containing untreated or scrambled siRNA treated HCASMC (Fig. 4C). siPLGF treatment of HCASMC did not prevent the FSS-induced increase in PLGF protein, in contrast to siPLGF treatment of HCAEC.

To assess the effect of communication between HCAEC and HCASMC on PLGF production by EC, we next compared HCAEC monocultures to HCAEC/HCASMC cocultures. HCAEC monocultures had significantly lower baseline PLGF protein levels in media, compared to HCAEC/HCASMC cocultures (Fig. 5A). In addition, the sensitivity to FSS intensity (normal vs. high) appeared to be lost in HCAEC monocultures, since exposure of HCAEC monocultures to both normal FSS and high FSS resulted in a significant and similar increase in PLGF protein. Furthermore, the effect of FSS in monocultures was transient and was significant only at 8 h post-shear (Fig. 5B), yielding a time course of PLGF expression that was dissimilar to our observations in cocultures. These data suggest that although HCAEC are clearly the primary source of PLGF in the coculture model, HCASMC nevertheless play a role in modulating both shear-induced and baseline expression of PLGF.

Expression of PLGF induced by fluid shear stress is dependent on NADPH oxidase.

We next investigated possible signaling mechanisms mediating upregulation of PLGF by FSS. The soluble mediator NO was evaluated first, based on its well-known role in mediating FSS-dependent responses in EC. Total nitrite/nitrate concentrations in coculture media were determined as a measure of NO production. High FSS, but not normal FSS, significantly increased total nitrite/nitrate (1.43 ± 0.14 fold of static control) immediately after exposure (Fig. 6A). To assess the role of NO in the regulation of PLGF expression, cocultures were treated with the NO synthase (NOS) inhibitor L-NG-nitroarginine methyl ester (L-NAME, 100 μM) 30 min prior to FSS exposure. Media was then sampled for PLGF protein levels immediately prior to

FSS exposure and 24 h post-shear (the time point at which maximal effect of FSS on PLGF protein levels in untreated cells was observed; Fig. 2B). Inhibition of NOS by L-NAME had no significant effect on the FSS-induced increase in PLGF protein (Fig. 6B).

We next tested the effect of FSS on H_2O_2. Both FSS waveforms caused a significant increase in H_2O_2 immediately after exposure (normal, 1.43 ± 0.16, high, 1.35 ± 0.14 fold of static control) (Fig. 7A). Treatment with the H_2O_2 scavenger catalase (500 U/mL) 30 min prior to FSS exposure abolished the effect of high FSS on PLGF protein (Fig. 7B), suggesting that H_2O_2 is a key mediator of this response. The effects of catalase were mimicked by the flavoenzyme inhibitor diphenyleneiodonium chloride (DPI, 5 μM) (Fig. 7C). Treatment with DPI (5 μM) also prevented the increase in PLGF mRNA in arterioles perfused at 50 mmHg compared to 20 mmHg (Fig. 7D).

Nox4 is necessary for the fluid shear stress-induced increase in PLGF and H_2O_2 in vascular cocultures.

We next investigated the role of the ROS-producing NAD(P)H oxidases (Nox) as a possible source of H_2O_2 for this pathway. Real time PCR was performed on mRNA isolated from static cocultures to determine the relative abundance of mRNA for Nox 1, 2 and 4, three isoforms previously implicated in the outward remodeling of coronary vessels (Table 1). In both HCASMC and HCAEC, the predominant isoform was Nox4. We next assessed Nox4 mRNA levels in cocultures at 0, 4, 10 and 24 h after 2 h of FSS exposure. In HCAEC, Nox4 mRNA was significantly increased 4 h after exposure to high FSS (1.42 ± 0.14 fold of static control) and returned to control levels by 10 h (Fig 8A). High FSS significantly increased HCASMC Nox4 mRNA 24 h after exposure (1.60 ± 0.23 fold of control) (Fig 8B). Normal FSS had no significant effect on Nox4 mRNA in either cell type.

Lastly, in order to determine whether Nox4 plays a role in the FSS induced increase in PLGF, we knocked down Nox4 in either HCAEC or HCASMC using siRNA. Knockdown was verified by RT-PCR (Fig. 9B, 9D). Nox4 knockdown in HCAEC did not affect Nox4 mRNA in cocultured HCASMC, or vice versa (not shown). Knockdown of Nox4 in HCASMC had no significant effect on basal levels of PLGF protein and did not affect the response of PLGF to high FSS (Fig. 9A). Knockdown of Nox4 in HCAEC abolished the effects of high FSS on PLGF protein levels without significantly affecting basal PLGF protein levels (Fig. 9C). Finally, knockdown of Nox4 in HCAEC prevented the increase in H_2O_2 observed immediately after high FSS in untreated cells (Fig. 9E).

Discussion

Our findings demonstrate that PLGF is regulated by FSS both in a vascular cell coculture model and in intact vessels, and that this response is sensitive to the magnitude and duration of FSS. We identified endothelial cells as the primary cell type producing PLGF in the coculture model; however, we also found that endothelial PLGF production is positively influenced by the presence of smooth muscle cells. Investigation of the mechanisms involved in FSS-induced PLGF expression revealed that this pathway is dependent on H_2O_2, and identified NADPH oxidase 4 (Nox4) as a likely source of H_2O_2 in this context. Further definition of the molecular mechanism by which FSS regulates PLGF may identify new therapeutic targets to either promote or inhibit PLGF expression and arteriogenesis, depending on the context (e.g. ischemic cardiovascular disease vs. cancer).

Although the cone and plate viscometer used to generate shear stress in the coculture system allows accurate control of the angular velocity of the cone (and thus FSS) in our model, exposure times longer than 2 h were prohibited by detachment of the endothelial cells. The

possibility that cessation of elevated FSS at the end of the exposure period affects PLGF expression (in addition to, or instead of, initiation of elevated FSS) cannot be completely excluded. However, the dependence of the PLGF protein response on both the duration and magnitude of FSS exposure, and the immediate effect of FSS on PLGF mRNA in both cocultures and perfused vessels, suggests that upregulation of PLGF is primarily due to initiation of elevated FSS.

In intact vessels, EC and SMC interact physically and also communicate through secreted mediators to maintain normal vessel function. The coculture model used in this study provides a close approximation of this situation by allowing for both physical and biochemical interaction. Our data show that PLGF is primarily produced by HCAEC in our model, consistent with our previously published findings[242]. Indeed, knockdown of PLGF in HCAEC decreased baseline media PLGF concentrations to less than 10% of the level in untreated cocultures. Nevertheless, HCASMC appear to play a major role in modulating PLGF levels in the coculture system. HCAEC/HCASMC cocultures secreted ~2-fold more PLGF than HCAEC monocultures. This effect cannot be explained by a simple additive effect of HCASMC PLGF production on total PLGF levels, since HCAEC produce ~two orders of magnitude more PLGF than HCASMC[242]. Furthermore, knockdown of PLGF in HCASMC cocultures yielded the seemingly contradictory result of increased PLGF media levels. One possible explanation for these observations is that HCASMC-produced PLGF may exert negative feedback on HCAEC PLGF production, whereas other factors generated by HCASMC have a stimulatory effect. Consistent with our findings for PLGF, several other growth factors have been reported to increase in endothelial cell/smooth muscle cell cocultures compared to endothelial monocultures, including VEGF-A, PDGF-AA, PDGF-BB, MCP-1 and TGF-β_1[243, 244]. Furthermore, coculture of EC with SMC leads to an increase in SMC differentiation[245], EC elongation, and EC permeability[246], demonstrating the importance of EC/SMC interaction on signaling and function in both cell types. In keeping with

these results in static cocultures and monocultures, we found the effect of FSS on PLGF to be transient and diminished in HCAEC monocultures, as compared to cocultures. These data suggest that HCAEC/HCASMC interaction and signaling is essential for both baseline PLGF expression and its upregulation by the physiological stimulus of increased FSS. Dysfunction in either of these vascular cell types may therefore lead to abnormal PLGF production and signaling.

In the vasculature, FSS increases the production of NO by endothelial nitric oxide synthase (eNOS). eNOS is localized to the endothelium and is increased six-fold during arteriogenesis[247]. Exercise, which has been reported to accelerate arteriogenesis after femoral ligation, also increases eNOS expression[5]. Furthermore, treatment with L-NAME inhibits collateral growth augmentation by exercise after femoral artery ligation in rats[145], and mice treated with L-NAME either immediately after femoral artery ligation or 3 d post-operatively show significant decreases in flow recovery compared to control[147]. eNOS knockout mice also exhibit diminished flow recovery after femoral artery excision[141], and the arteriogenic effect of bFGF in rats is dependent on NOS activity[143]. We verified that the high FSS condition used in this study was sufficient to increase NO production, as expected. Despite NO being an important arteriogenic factor[141, 145, 248], inhibition of NO production with L-NAME had no effect on the FSS-induced expression of PLGF in our model system. As noted above, eNOS knockout mice exhibit diminished arteriogenesis after femoral artery occlusion[141]. However, collateral growth in these mice has been demonstrated to "catch up" with that in wild type mice later in the arteriogenic process[146]. It should be noted that our model exposes the endothelium to FSS without changes in other parameters relevant to arteriogenesis (stretch, hypoxia). Stretch has been shown to induce PLGF expression in human bronchial airway epithelial cells in a NO dependent fashion[249]. Thus, although NO does not appear to be required for the upregulation of PLGF by FSS, the potential role of stretch-induced NO production in modulating PLGF expression in vascular cells during arteriogenesis remains to be investigated. Likewise, our group previously demonstrated

upregulation of PLGF protein in HCAEC by hypoxia [250], and potential interactions between FSS and hypoxia in regulation of PLGF also remain to be characterized.

The role of ROS in arteriogenesis has been clearly demonstrated in numerous studies evaluating the effects of antioxidants on collateral growth. Oxidative stress exerts a strong inhibitory effect on collateral remodeling. For instance, induction of oxidative stress by knockout of endothelial specific superoxide dismutase (SOD, which dismutates $O_2^{\bullet-}$, the superoxide radical anion, to H_2O_2) impairs flow recovery in mouse ischemic hindlimb[198]. Interestingly, suppression of ROS generation is also inhibitory. In the dog, the increase in coronary blood flow induced by repeated coronary occlusion is inhibited by the antioxidant N-acetylcysteine (NAC)[251]. Similarly, in mice the antioxidants NAC, ebselen and Tempol either delay or inhibit blood flow recovery in the hind limb following femoral artery ligation[198-200]. These seemingly contradictory observations have been made even within studies; in the rat heart, oxidative stress produced by treatment with the SOD inhibitor diethyldithiocarbamate (DETC) inhibits the increase in coronary collateral blood flow following repeated coronary occlusion in the rat heart, but a similar degree of inhibition is also observed when ROS generation was suppressed with either DPI or the Nox inhibitor apocynin [201]. These contradictory results suggest that arteriogenesis requires an optimal ROS concentration. An alternative interpretation is that H_2O_2 may be an important regulator of arteriogenesis. Nox inhibition results in a decreased production of both H_2O_2 and $O_2^{\bullet-}$, whereas inhibiting SOD would also result in decreased H_2O_2, but increased $O_2^{\bullet-}$. Taken together, these data suggest a potential positive role for H_2O_2 in regulating arteriogenesis.

In the vasculature, the predominant sources of ROS are Nox, xanthine oxidases, uncoupled eNOS, and the mitochondrial electron transport chain. Of these, Nox are the only family of enzymes that produce ROS as their primary function, rather than as a byproduct [252]. Nox-derived ROS activates several pro-arteriogenic signaling pathways, including Akt, MAPK, Src and PKC[253, 254]. Consistent with these findings, various Nox isoforms are also implicated in

collateral remodeling *in vivo* [200, 255-257]. Furthermore, FSS increases both Nox expression and Nox-derived ROS generation [258-260], and Nox isoforms colocalize with the mechanosensing machinery of the cell[261, 262]. We report that the Nox inhibitor DPI abrogates the effects of FSS on PLGF expression in both vascular cell cocultures and intact perfused vessels. In our *in vitro* model, we found Nox4 to be the predominant Nox isoform, consistent with previous reports for both endothelial[263] smooth muscle cells[264]. Nox 4, unlike other Nox isoforms, is constitutively active and only requires association with p22phox for its activity; furthermore, it uniquely produces mostly H_2O_2, rather than O2 [265]. Interestingly, Nox4 activity does not result in peroxynitrite production and NO inactivation[266], both of which are linked to vascular Nox activation [267, 268]. Indeed, endothelial-specific Nox4 overexpression in mice results in increased H_2O_2 production and vasodilation, along with lower systemic blood pressure[269]. These mice also exhibit accelerated flow recovery following induction of hindlimb ischemia [257]. Furthermore, similarly to PLGF knockout mice, mice lacking Nox4 fail to recover hindlimb perfusion after femoral artery ligation [256]. In this study, we found both endothelial and smooth muscle Nox4 mRNA to be increased by high FSS in our coculture system. siRNA knockdown of Nox4 in HCAEC attenuated the effects of shear stress on both PLGF and peroxide, whereas Nox4 knockdown in HCASMC did not alter the effects on FSS on PLGF expression, suggesting that endothelial Nox4 plays an important role in the regulation of PLGF by FSS and ROS.

In this study, we report that pro-arteriogenic FSS increases PLGF protein levels by ~40% compared to static control. Although the magnitude of the effect is not large, studies in humans suggest that a PLGF increase in this range is likely to be physiologically significant. Patients categorized as having "rich" coronary collaterals (Rentrop classification 2 and 3) were found to have 40% higher plasma PLGF levels compared to patients with "poor" coronary collaterals (Rentrop classification 0 and 1)[270]. These clinical observations suggest that the increase observed in our human *in vitro* model could have a significant effect on human coronary collateral circulation *in vivo*.

Our findings that FSS-stimulated ROS production is a key signal for PLGF expression raise questions about the growing use of antioxidants as a prophylactic measure in healthy individuals. While antioxidants may be beneficial in individuals with oxidative stress (by returning ROS to physiological levels), our data and that of others supporting the "redox window" hypothesis suggest that overuse of antioxidants could potentially inhibit beneficial collateral remodeling. It is therefore of interest to determine the effects of impaired oxidative balance on shear-induced PLGF expression.

In summary, we provide the first demonstration that the important arteriogenic growth factor PLGF is upregulated in cocultured vascular cells and isolated vessels by the key arteriogenic stimulus of FSS. We further show that this response is mediated by a novel signaling pathway involving H_2O_2 and Nox4, and that it is influenced by EC/SMC interaction. These findings have important clinical implications, as a better understanding of the molecular mechanisms regulating arteriogenesis may suggest new therapies for ischemic cardiovascular disease.

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Disclosures

None.

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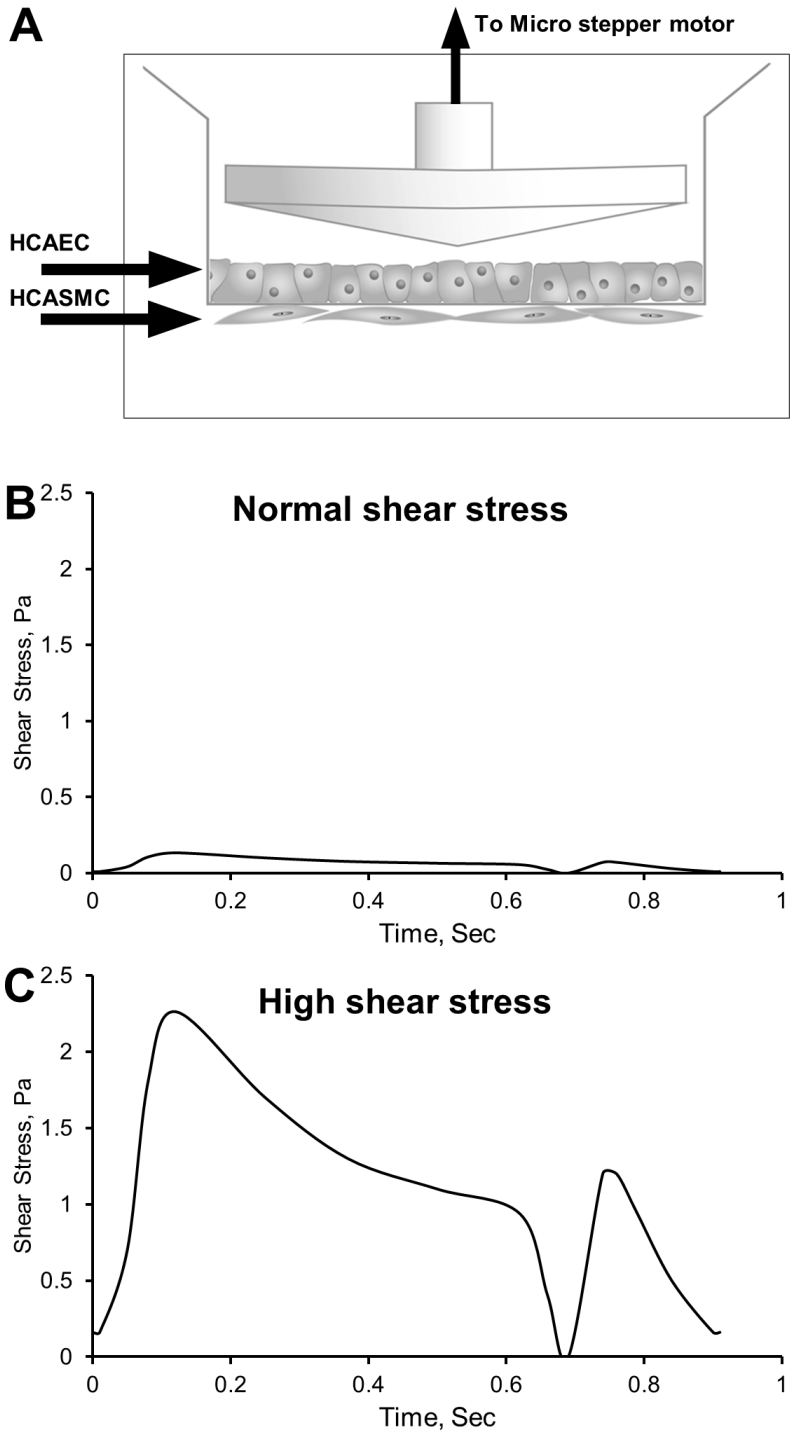

Figure 1. (A) Illustration of coculture system with cone in place. Transwell inserts were placed inside 6 well plates. HCAEC were seeded on the top side of the insert, while HCASMC were seeded on the underside. The pore size (0.4 µm) prevented either cell type from migrating

completely through the membrane, but was readily permeable to soluble factors and allowed some direct cell-cell contact. (B) and (C) Shear stress wave forms used in this study, shown over 1 cardiac cycle (0.9 sec). (B) Waveform representing shear stress in a collateral arising from a coronary artery free of stenosis. (C) Waveform representing shear stress in a collateral arising from a coronary artery with 60% stenosis.

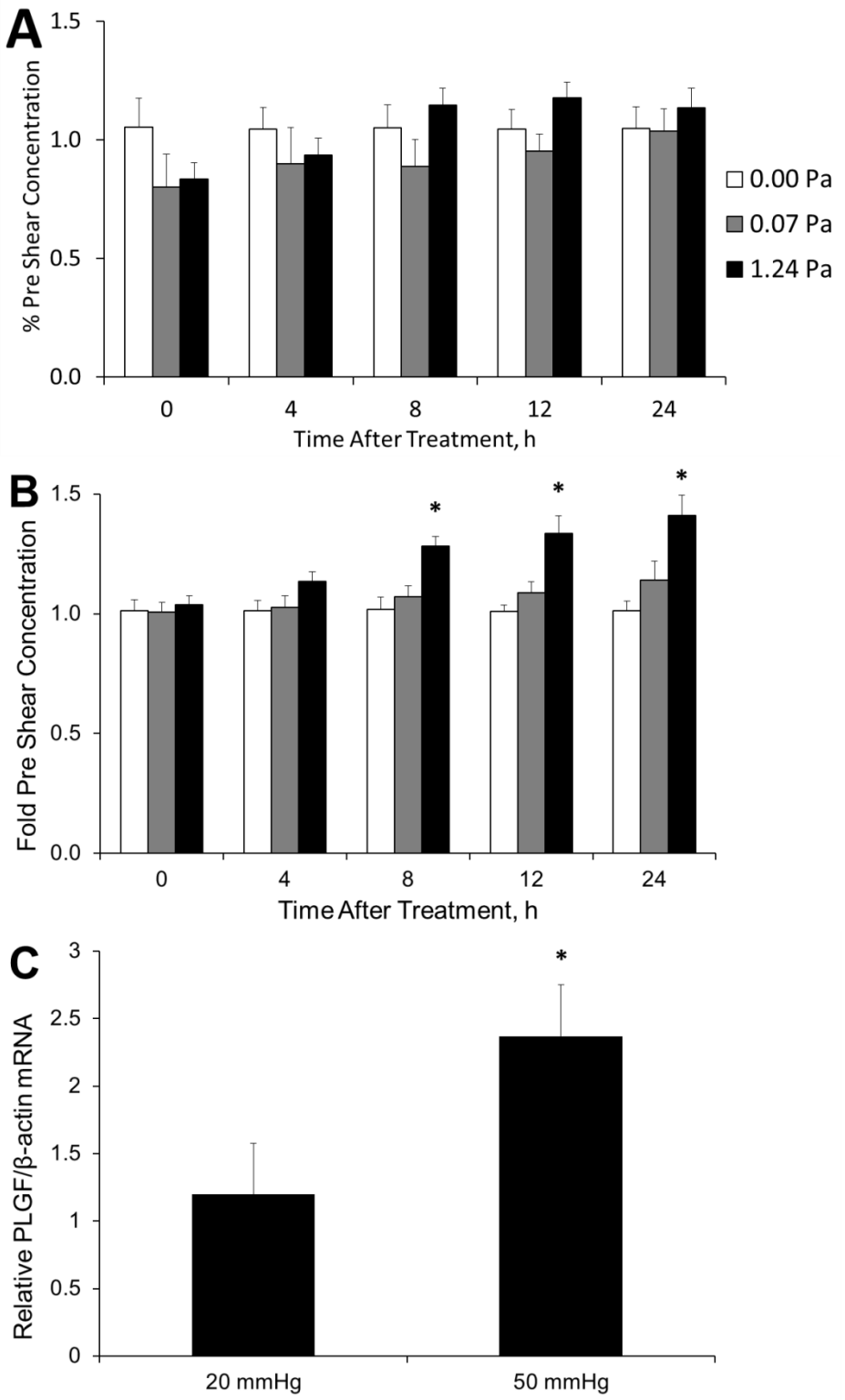

Figure 2. Effect of fluid shear stress exposure on PLGF expression in the vessel wall. (A) Cocultures were exposed to static control conditions (no FSS), normal shear stress (0.07 Pa) or

high FSS (1.24 Pa). PLGF protein in medium was assessed prior to shear exposure and up to 24 h post-shear. (A) Neither waveform had a significant effect on PLGF protein levels after 1h of exposure (n=5). (B) 2h exposure to high FSS, but not normal FSS, induced a significant increase in PLGF protein compared to static control starting 8h after shear exposure which remained evident up to 24 h (*n=5, $p<0.001$). (C) Mesentery arterioles (~150 μm ID) were cannulated and perfused with 1% BSA-PSS for 2h. Perfusion with a pressure gradient of 50mmHg significantly increased PLGF mRNA, compared to perfusion with a gradient of 20 mmHg (*n=5, $p<0.05$).

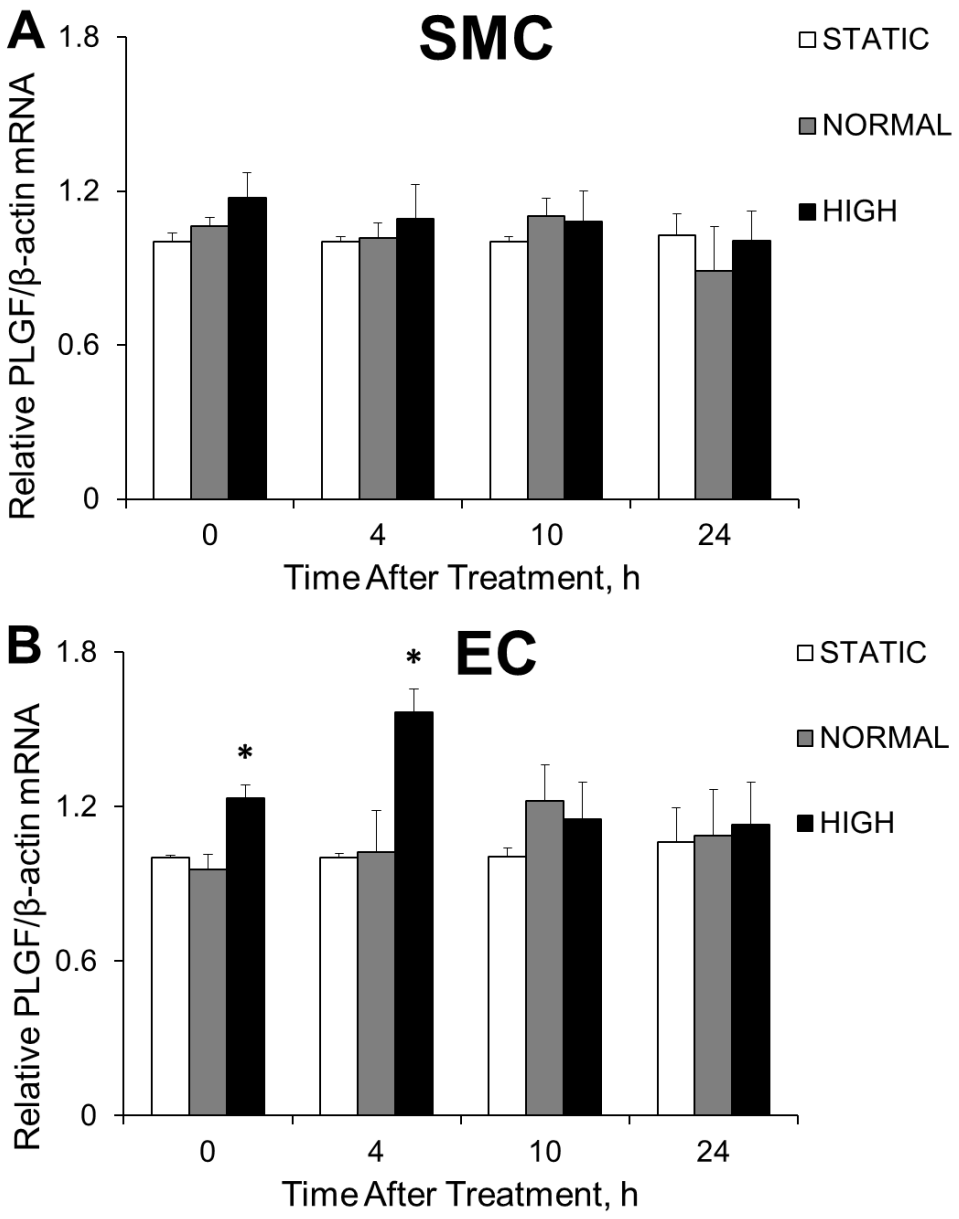

Figure 3. Fluid shear stress primarily affects PLGF expression in HCAEC. (**A**) HCASMC showed no significant change in PLGF gene expression, regardless of the level of shear exposure received by the coculture (n=5). HCASMC were not directly exposed to FSS. (B) HCAEC demonstrated an immediate increase in PLGF gene expression in response to high FSS which was maintained for up to 4 h after shear exposure (*n=5, $p<0.001$). Normal FSS had no significant effect on PLGF mRNA in HCAEC (n=5).

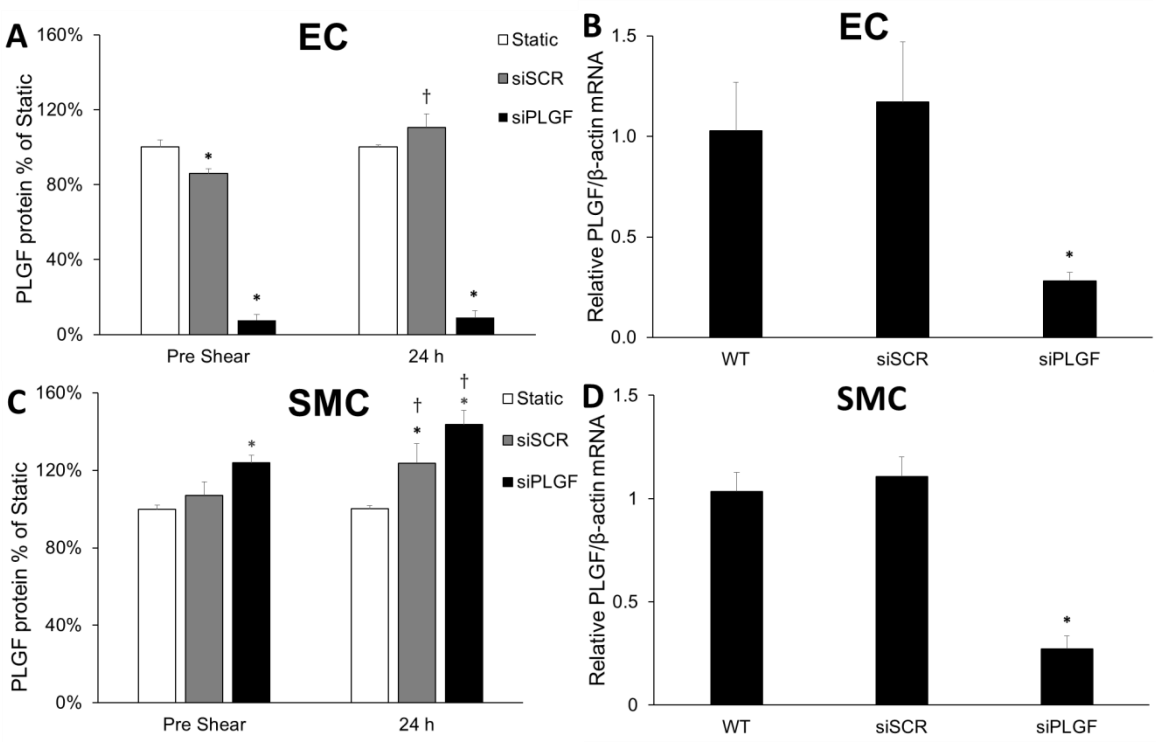

Figure 4. PLGF is predominantly produced by HCAEC. PLGF expression was knocked down using siRNA in HCAEC (A-B) or HCASMC (C-D). Media was sampled immediately prior to exposure to 2 h of high FSS and 24 h after. In cocultures containing siPLGF-treated HCAEC (A), PLGF was significantly decreased compared to levels in untreated cocultures prior to shear (*n=5, $p<0.001$), and failed to increase in response to FSS (*n=5, $p<0.001$). Cocultures with siSCR treated HCAEC showed a slight but significant decrease in pre-shear PLGF levels compared to untreated cells (*n=5, $p<0.001$); however, exposure to high FSS significantly increased PLGF as expected (n=5, † $p<0.05$). (B) Knockdown of PLGF in HCAEC was confirmed by real time PCR (*n=5, $p<0.01$). (C) PLGF knockdown in HCASMC resulted in a significant increase in total PLGF protein in the media compared to untreated cocultures prior to shearing (*n=5, $p<0.001$). PLGF was further increased by exposure to high FSS in both siSCR and siPLGF treated groups, as compared to static untreated controls (*n=5, $p<0.001$) and the corresponding pre-shear values for each treatment condition (†n=5, $p<0.05$). (D) Real time PCR confirmed PLGF knockdown in HCASMC (*n=5, $p<0.01$).

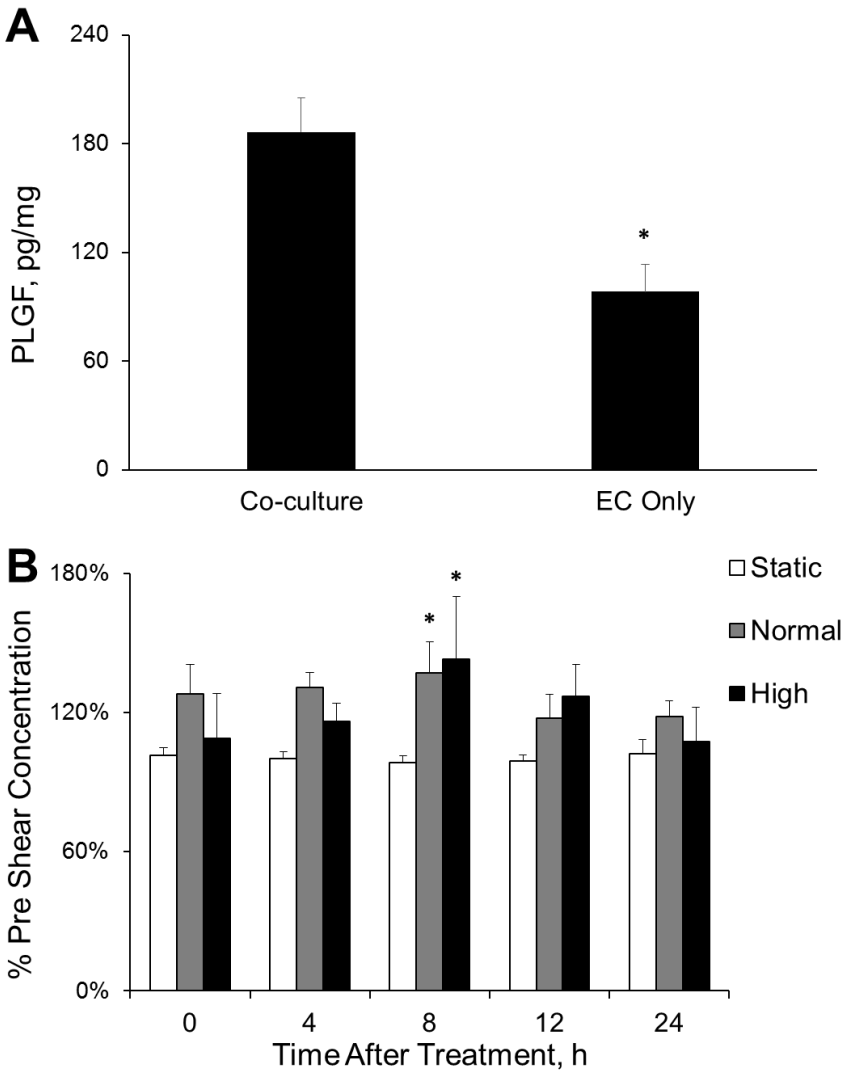

Figure 5. Effect of FSS exposure on PLGF production in HCAEC monoculture. (A) The baseline (unstimulated) level of PLGF protein was significantly lower in medium of HCAEC monocultures than in medium from HCAEC/HCASMC cocultures (*n=5, $p<0.001$). (B) HCAEC monocultures were exposed to static conditions, normal FSS, or high FSS for 2h as done for cocultures. Culture media was sampled pre- and post-shear. Both high FSS and normal FSS induced a significant increase in PLGF protein, but only at 8 h post-shear (*n=5, $p<0.05$). Thus, both the time course and intensity dependence of the effect were altered in the absence of HCASMC.

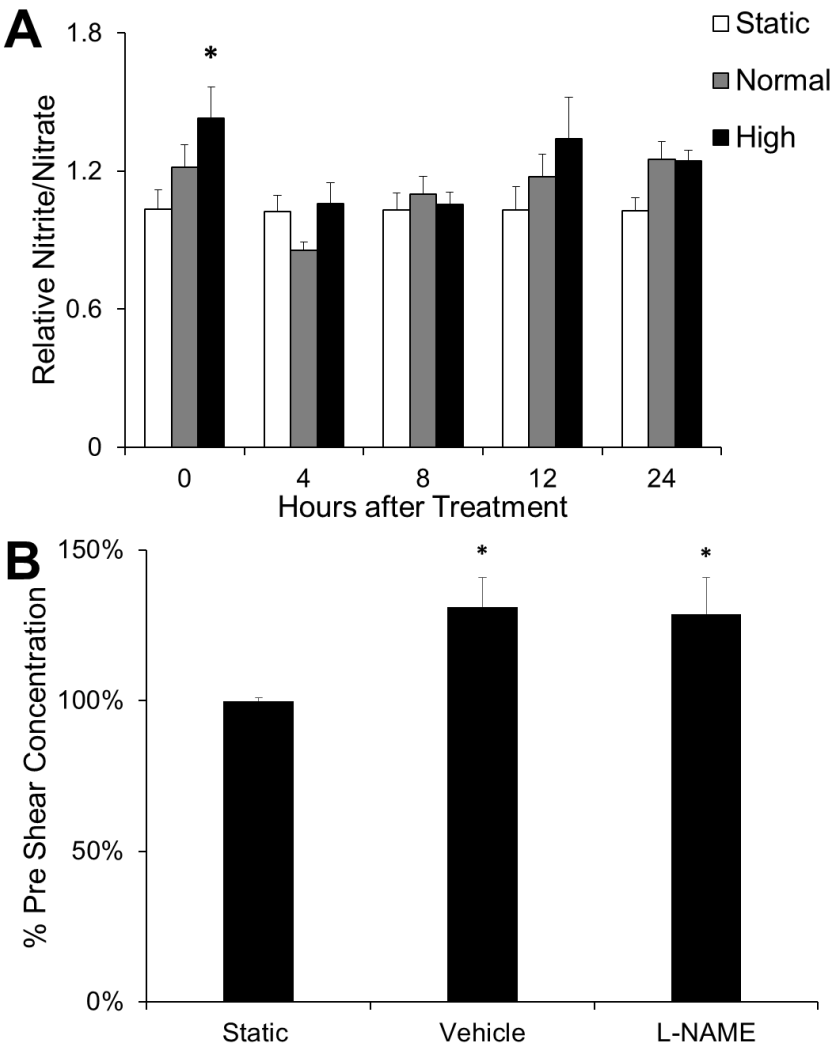

Figure 6. Fluid shear stress increases nitric oxide production but is not required for the FSS-induced increase in PLGF protein. (A) High FSS, but not normal FSS, significantly increased nitrite/nitrate levels immediately after shear exposure, compared to static control (*n=5, $p<0.01$). (B) The nitric oxide synthase inhibitor L-NAME (100 μmol/L) had no effect on the shear induced increase in PLGF protein (*n=5; $p<0.05$).

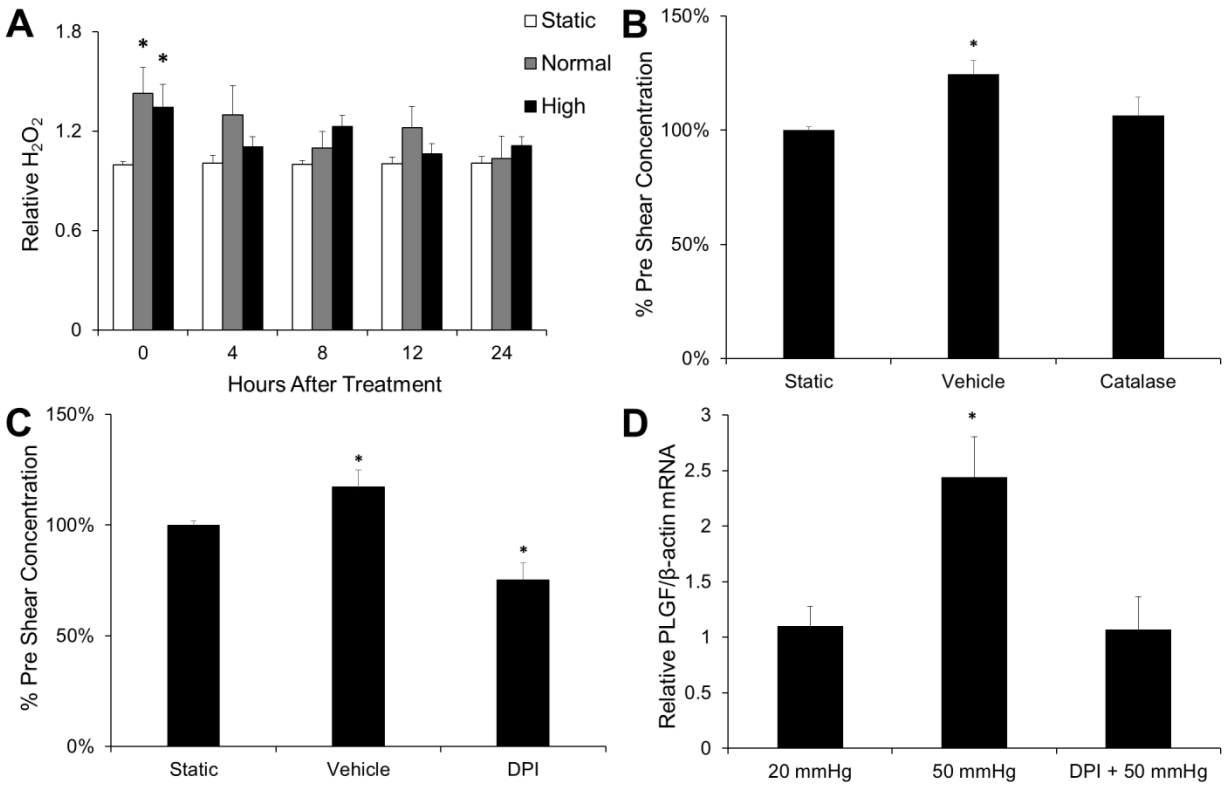

Figure 7. Hydrogen peroxide is required for FSS to upregulate PLGF expression. (A) Both high and normal FSS significantly increased H_2O_2 immediately after shear exposure, compared to static control in cocultures (*n=5, $p<0.05$). (B) Catalase (500 U/mL) blocked the effect of FSS to upregulate PLGF (*n=5; $p<0.01$). (C) DPI (5 μmol/L) also prevented the increase in PLGF protein in response to FSS, and further decreased PLGF protein below static control levels (*n=5; $p<0.01$). (D) DPI (5 μmol/L) also inhibited the flow induced increase in PLGF mRNA in cannulated arterioles. (20 mmHg n=6, 50 mmHg n=5, DPI + 50 mmHg n=4, $p<0.01$).

Table 1. Relative Nox isoform/β-actin mRNA.

	Nox1	Nox2	Nox4
HCAEC	1.00 ± 0.09	0.30 ± 0.05	1164.14 ± 60.43*†
HCASMC	1.17 ± 0.11	0.97 ± 0.14	15.92 ± 2.57*†

Values are shown as means ± SEM. Real time PCR revealed Nox4 to be the predominant isoform of Nox expressed in cocultures. (n=5, * *vs.* Nox1 $p<0.01$, † *vs.* Nox2 $p<0.01$).

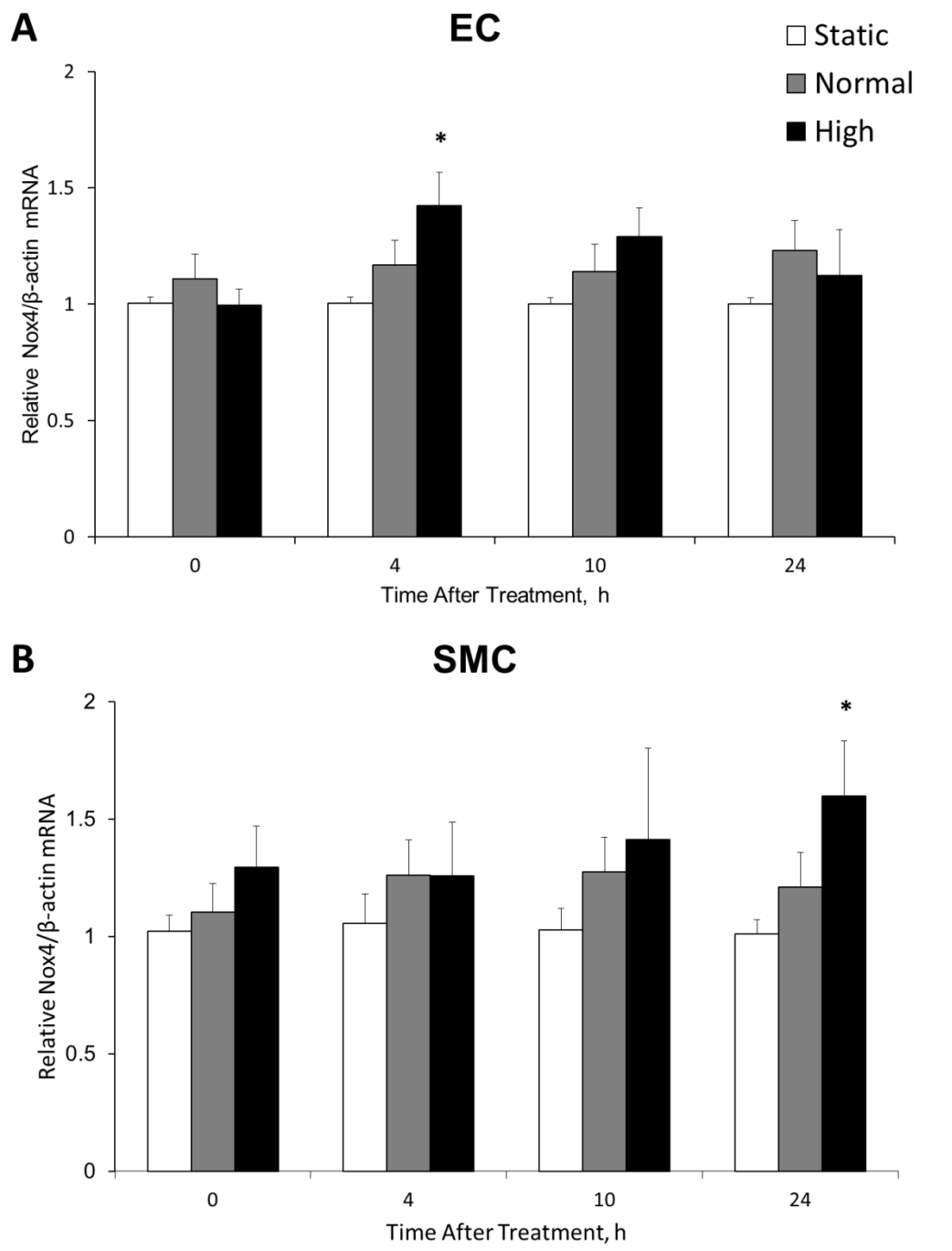

Figure 8. Nox4 is upregulated by shear stress. (A) In HCAEC, high FSS significantly increased Nox4 mRNA compared to static control while normal FSS had no significant effect on Nox4 mRNA. (*n=5, $p<0.01$) (B) No significant effect of FSS on Nox4 mRNA was observed in HCASMC (n=5).

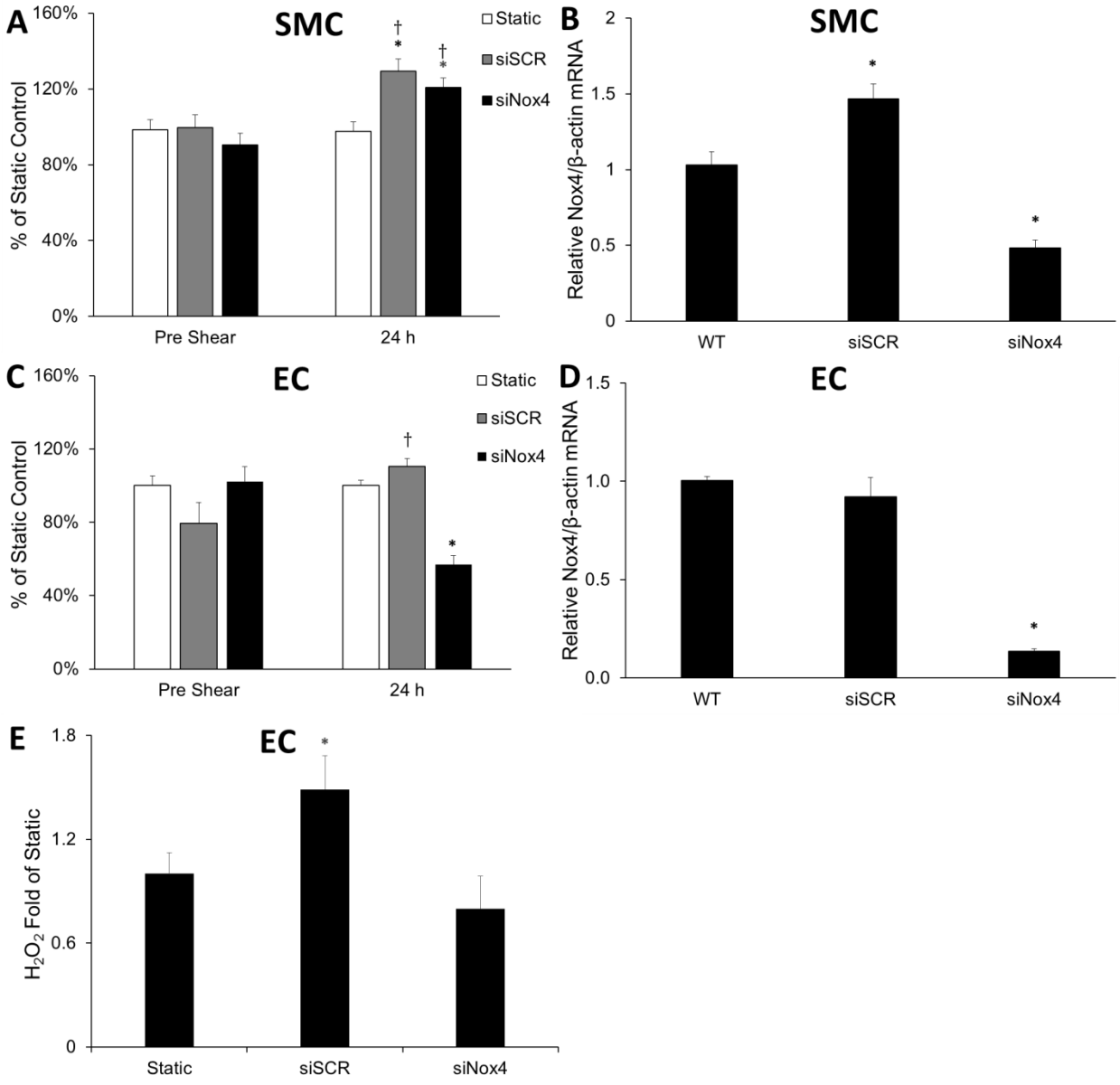

Figure 9. Endothelial Nox4 is the source of FSS induced hydrogen peroxide. (A) Knockdown of Nox4 in HCASMC in cocultures had no significant effect on the FSS-induced increase in PLGF protein (n=5, *vs.* static * $p<0.01$, *vs.* pre-shear † $p<0.05$). (B) Knockdown of Nox4 in HCASMC was confirmed by real time PCR (n=5, * $p<0.01$). (C) High FSS increased PLGF protein levels in HCAEC compared to pre-shear levels (n=5, † $p<0.05$). Knockdown of Nox4 in HCAEC in cocultures inhibited the FSS-induced increase in PLGF protein and significantly decreased PLGF

below static control. (n=5, * $p<0.01$). (D) Real time PCR confirmed knockdown of Nox4 in HCAEC (n=5, * $p<0.01$) (E) knockdown of NOX in HCAEC also attenuated the effects of high FSS on hydrogen peroxide, identifying endothelial Nox4 as a key source of FSS induced hydrogen peroxide production.

CHAPTER IV

FLUID SHEAR STRESS AND HYPOXIA REGULATE PLGF EXPRESSION VIA ALTERED IRON HOMEOSTASIS

Nabil A. Rashdan and Pamela G. Lloyd

Abstract

Increased fluid shear stress (FSS) is a key initiating stimulus for arteriogenesis, the outward remodeling of collateral arterioles in response to upstream stenosis or occlusion, and placental growth factor (PLGF) is an important arteriogenic mediator. We previously showed that elevated FSS increases PLGF in a reactive oxygen species (ROS)-dependent fashion both *in vitro* and *ex vivo*. Heme oxygenase 1 (HO-1) is a cytoprotective enzyme that is upregulated by stress and has arteriogenic effects. In the current study, we used a coculture model of human coronary artery endothelial cells (EC) and smooth muscle cells (SMC) to test the hypothesis that HO-1 mediates the effects of FSS on PLGF. Cocultures were exposed to arteriogenic FSS as we previously described. We also exposed mesenteric arterioles *ex vivo* to either high or low flow. HO-1 mRNA was increased by conditions of increased flow and shear stress in both cocultures (~1.5 fold) and vessels (~15 fold). Both inhibition of HO-1 with zinc protoporphyrin (30 μM) and HO-1 knockdown abolished the effect of increased flow on PLGF. Conversely, induction of HO-1 with hemin (2.5-40 μM) increased PLGF ~1.6 fold. Cocultures were then treated with a CO donor (CORM-A1), biliverdin, or ferric ammonium citrate (FAC). Only FAC (10-200 μg/ml) increased PLGF (~2-fold). Paradoxically, the iron chelators 1,10-phenanthroline (100 μM) and deferoxamine (100 μM) also significantly increased secreted PLGF compared to DMSO vehicle control (~1.3 fold; $n=5$, $p<0.01$). Finally, we demonstrated that hypoxia increases PLGF expression in an endothelial HO-1 dependent fashion. We conclude that the regulation of PLGF by FSS is mediated by a HO-1 dependent mechanism, and that HO-1 is a common mediator of both hypoxia and FSS effects on PLGF by decreasing intracellular labile iron and suggest a role for iron sensitive transcription factors.

Keywords: arteriogenesis, collateral circulation, hemodynamics, endothelium, vascular endothelial growth factors, heme oxygenase

Introduction

Coronary artery disease (CAD) is a major cause of death worldwide[271, 272]. The primary predictor of survival for CAD patients is the number of preexisting collateral vessels (arterial – arterial anastomoses) and the degree to which they have remodeled outward to increase their flow capacity[130, 273]. This outward remodeling occurs in response to increased flow through the vessels, which is generated by an increase in the pressure gradient across the vessels due to decreased downstream pressure in the occluded branch. This remodeling process is termed arteriogenesis. Pharmacological stimulation of arteriogenesis has been a long sought after goal, because of the potential of arteriogenesis to reduce mortality and morbidity in CAD. However, early attempts to induce arteriogenesis via administration of single exogenous growth factors were marred with failure[274], and it has become clear that a deeper understanding of the myriad of signaling events contributing to arteriogenesis is necessary to develop safe and effective pro-arteriogenic treatments.

Placental growth factor (PLGF) is a member of the vascular endothelial growth factor (VEGF) family. PLGF is a potent arteriogenic agent, even more so than VEGF-A[10, 11]. PLGF exclusively binds fms-like tyrosine kinase-1 (VEGFR-1) and elicits distinct downstream signaling events than those induced by VEGF-A binding of VEGFR-1[161]. PLGF exacts its arteriogenic effect by recruitment of monocytes (which only express VEGFR-1) to the vascular wall[10, 168, 275]. PLGF knockout mice exhibit a blunted arteriogenic response to hindlimb ischemia [10], whereas PLGF protein levels in coronary artery plasma are positively correlated with improved patient outcome following myocardial infarction[238].

Heme oxygenase (HO) catabolizes heme into equimolar quantities of CO, divalent iron, and biliverdin. There are two isoforms of HO; an inducible isoform (HO-1) and a non-inducible, constitutively expressed isoform (HO-2). HO-2 is constitutively expressed in the testes[276] and the

brain[277]. HO-1, on the other hand, is strongly induced in response to cellular stresses such as increased reactive oxygen species (ROS) and radiation. There are only two reported cases of HO-1 deficiency in humans. These patients exhibited severe growth retardation and endothelial dysfunction, along with abnormal hemostasis and an increased susceptibility to oxidative stress[278-280]. HO-1 knockout mice present with similar growth abnormalities and appear to be in a chronic inflammatory state[281]. Arteriogenesis is diminished with advanced age[282] and this effect may be related to abnormal HO-1 expression and/or signaling, since induction of HO-1 in aged rats with blunted arteriogenic potential restores outward vascular remodeling to levels comparable with young rats[283].

We previously reported that exposure to arteriogenic FSS increases PLGF protein and mRNA both *in vitro* in a coculture model of the vessel wall and *ex vivo* in isolated mouse mesenteric arterioles. We also reported that this increase is dependent on FSS-induced production of hydrogen peroxide by endothelial NADPH oxidase 4 (Nox4). It is established that FSS also increases activation and expression of HO-1 in endothelial cells [258, 284, 285], and that these effects are dependent on ROS produced by Nox isoforms [258, 284]. Similarly, HO-1 is upregulated in hindlimb skeletal muscle following femoral artery ligation [256, 286] in a Nox-dependent manner[256]. A possible link between HO-1 and PLGF is suggested by the observation that PLGF and HO-1 expression are both significantly increased in hindlimb skeletal muscle early after femoral artery ligation[118]. Furthermore, HO-1 haploinsufficiency in mice causes a decrease in PLGF expression and reduces the extent of revascularization following induction of hindlimb ischemia [287]. Lastly, HO-1 knockout increases the expression of anti-arteriogenic soluble VEGFR-1[288]. Therefore, we hypothesized that the effects of FSS on PLGF are mediated by HO-1. We tested this hypothesis in an endothelial cell/smooth muscle cell coculture model and in isolated mouse mesenteric arterioles in order to characterize the role of HO-1 in FSS-mediated regulation of PLGF.

Methods

Reagents. All reagents were purchased from Sigma-Aldrich unless otherwise specified.

Perfused arterioles. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee at Oklahoma State University. Experiments were conducted on isolated second order mesenteric arterioles (≈120-180 μm) of 6-8 week old C57BL/6J male mice (Jackson Laboratories). Mice were deeply anesthetized with isoflurane delivered by a vaporizer and the heart was excised. The entire mesentery, with the superior mesenteric artery and vein, was dissected free and washed with 4ºC PBS. Second order mesenteric arterioles were isolated from the mesenteric tissue. The isolated arterioles were transferred to a vessel chamber at 4ºC (Living Systems Instrumentation). The chamber contained a pair of glass micropipettes and was filled with physiological saline solution (PSS) (142 mM NaCl, 4.7 mM KCl, 1.7 mM $MgSO_4$, 0.5 mM EDTA, 2.79 mM $CaCl_2$, 10 mM HEPES, 1.18 mM KH_2PO_4, pH 7.4). After cannulation of the proximal (upstream) end of the vessel, the intraluminal pressure was gradually raised (less than 20 mmHg) to clear the lumen of clotted blood. Once cleared, the distal (downstream) end of the vessel was also cannulated. Time from euthanasia to complete cannulation was under 60 minutes. The temperature of the bath was then raised to 37ºC and pressure was gradually raised to 60 mmHg (~10 mmHg/10 minutes). The pressure increase was achieved by gradually raising two reservoirs connected by silicone tubing to each cannula. Perfusion buffer consisted of 1% bovine serum albumin in PSS. Once equilibrated at 60 mmHg, the longitudinal pressure gradient was increased from zero to 20 mmHg ("control") or 50 mmHg ("pro-arteriogenic") [63]. This was achieved by lowering the distal reservoir and raising the proximal reservoir, allowing for the average intraluminal pressure to be maintained at 60 mmHg. The control flow rate was ~75 μL/min, and the "pro-arteriogenic" flow rate was ~170 μL/min. Vessels were then perfused for 2h. Function of the vessel wall was determined at the end of

perfusion by assessing the vasoconstrictive response to epinephrine and the vasodilator response to acetylcholine, as assessed by changes in vessel diameter measured by video micrometer.

Cell culture. Human coronary artery endothelial cells (HCAEC) and human coronary artery smooth muscle cells (HCASMC) were purchased from Lonza. For HCASMC, donors included a 12 year old male, a 56 year old female, and a 30 year old male, while HCAEC donors were a 21 year old male and a 30 year old male. HCAEC were cultured in EBM-2 basal media supplemented with EGM-2MV SingleQuot factors (HCAEC complete media, Lonza). HCASMC were cultured in SMBM basal media supplemented with SmGM-2 SingleQuot factors (HCASMC complete media, Lonza). All cells were grown in a humidified incubator in 5% CO_2 at 37°C. Cells were used at passage five or six for all experiments. Serum reduced media for experiments to be performed in room air was prepared by diluting the appropriate complete media in low glucose DMEM (Hyclone, Fisher) at a ratio of 2:3 yielding a 2% serum media, and supplementing with 15 mmol/L HEPES.

Co-culture model. To model the vessel wall *in vitro,* porous Transwell inserts (Corning Costar, 0.4 μm pore size) as we previously described [289]. Inserts were inverted and the bottom surface was coated with 0.1% gelatin in DMEM, then were placed in a humidified incubator in 5% CO_2 at 37°C for 1 h. HCASMC (10^4 cells/cm^2) were then seeded onto the inverted insert, and inserts were returned to the incubator overnight. The following day, the inserts were placed into 6 well plates containing SmGM-2 media and incubated for an additional 24 h. The top surface of the insert was then coated with 0.1% gelatin in DMEM and incubated for 1 h. HCAEC (25,000/cm^2) were then seeded on the top surface of the insert. EGM-2MV was added to the insert and the system was again incubated overnight. When possible, HCASMC and HCAEC donors were matched. Confluence of the co-cultures was confirmed by Hoffman modulation contrast microscopy (Olympus IX71). Lastly, the confluent co-cultures were incubated in serum reduced media for 24 h prior to experiments. For HCAEC mono-culture experiments, no

HCASMC were seeded on the bottom of the insert, but cultures were otherwise processed as described above.

Shear stress exposure. Only the HCAEC layer of the co-culture was directly exposed to FSS. FSS was applied using a cone and plate viscometer shearing system as we previously described[289]. Co-cultures were then exposed to a pulsatile FSS waveform that had a time-averaged FSS of 1.24 Pa. This waveform is based on previously published information [241]. Shear experiments were performed on a laboratory benchtop in HEPES-buffered media with temperature maintained at 37°C. Cocultures were exposed to FSS for 2 h. Culture media and/or cell lysates were collected for analysis at various time points, from pre-shear up to 24 h post-shear.

Hypoxia exposure. Cells were treated as above and placed in a pre-warmed humidified cell culture chamber (Stem Cell Technologies). The chamber was then flushed with a mixture of 94% nitrogen, 5% carbon dioxide, and 1% oxygen. The chamber was then placed in a 37°C incubator for the indicated time.

siRNA knockdown experiments. HCAEC were seeded into 6-well plates at a density of 210,000 cells/well. After 24 h, cells were transfected with either predesigned HO-1 siRNA (Silencer Select; s194530) or negative control siRNA (Silencer no.1 siRNA; scRNA), all purchased from Invitrogen. Prior to addition to cells, 5 nM of siRNA was precomplexed with lipofectamine RNAiMAX transfection reagent (Invitrogen) in Opti-MEM media (Gibco) for 20 min. Cells were exposed to transfection media (DMEM + 10% FBS containing precomplexed siRNA) for 6 h, after which cells were trypsinized and seeded onto the upper surface of inserts precoated with 0.1% gelatin. The lower surface of the inserts had been previously seeded with wild type HCASMC, as described above. Co-cultures were incubated overnight in reduced serum media as described above before exposure to shear stress. In a separate group of co-cultures,

HCASMC were transfected similarly to HCAEC, after seeding onto the lower surface of inserts. At the end of the transfection period, untreated HCAEC were then seeded onto the upper surface as above. Cell specificity and efficacy of target mRNA knockdown was determined by real time PCR.

PLGF ELISA. Media samples were collected from treated cells and their corresponding controls, treated with protease inhibitor cocktail (1 mM PMSF, 1 mM Na_3VO_4, 1 µg/ml leupeptin, 1 mM benzamidine-HCl, 1 µg/ml aprotinin, 1 µg/ml pepstatin A) and stored at -80°C until further processing. Media samples were collected prior to treatment and 24h after treatment (The time at which FSS had the greatest effect on PLGF protein[289]) PLGF was measured using the DuoSet ELISA development kit (R&D Systems) according to manufacturer's protocol. All samples were assayed in duplicate. Data were normalized to total protein concentration, as determined by BCA assay (Pierce). All assay plates were read on a Biotek Synergy HT plate reader.

Real time PCR. After exposure to shear stress and media collection, cells were rinsed gently with PBS (HyClone) and trypsinized (TrypLE Express, Gibco). Collected cells were then resuspended in 1% β-mercaptoethanol in RLT lysis buffer (Qiagen) and frozen at -80°C for later processing. Cannulated vessels were placed in RLT lysis buffer immediately after perfusion and sonicated on ice using a Model D 100 Sonic Dismembrator (Fisher). Total RNA was isolated using RNeasy mini columns (Qiagen) following manufacturer's directions. Total RNA quantity and quality were determined spectrophotometrically using a Take3 Micro-Volume Plate in a Synergy HT plate reader (Biotek). Reverse transcription was carried out using the QuantiTect reverse transcription kit (Qiagen) following manufacturer's instructions. Real time PCR was performed on an ABI 7500 Fast instrument (Applied Biosystems) using PerfeCTa SYBR Green FastMix, Low ROX (Quanta Biosystems). Relative abundance of target mRNA was determined using the comparative Ct method and the following primer pairs: human PLGF forward 5'-CCTACGTGGAGCTGACGTTCT-3'; reverse 5'-TCCTTTCCGGCTTCA TCTTCT-3', : human

HO-1 forward 5'-CTGCGTTCCTGCTCAACATC-3'; reverse 5'-GGCAGAATCTTGCACTTTGTTG-3', Mouse PLGF forward 5'-CTGCTGGGAACAACTCAACAGA-3'; reverse 5'-GCGACCCCACACTTCGTT-3', Mouse HO-1 forward 5'- TCGTGCTCGAATGAACACTCTG-3'; reverse 5' - AGCTCCTCAAACAGCTCAATGT -3'. Gene expression was normalized to β-actin, as amplified with the following primers: human β-actin forward, 5'-TGCCGACAGGATGCAGAAG-3'; reverse, 5'-CTCAGGAGGAGCAATGATCTTGAT-3'; mouse β-actin forward, 5'-AGTTCGCCATGGATGACGAT-3'; reverse, 5'-TGCCGGAGCCGTTGTC-3'. Relative gene expression was quantified using the ΔΔCt method.

Statistical analyses. All data are presented as mean ± SEM. Experiments were replicated at least five times. Data were analyzed by either two-way repeated measures ANOVA or one-way ANOVA, as appropriate. Both were followed by Tukey's range test. The level of significance was set at $p<0.05$.

Results

To determine if HO-1 plays a role in the vascular response to shear stress, we first determined the relative mRNA levels of HO-1 in our models following experimental treatment. In endothelial cells (Fig. 1A), shear stress significantly increased HO-1 mRNA immediately after exposure (1.40 ± 0.08 fold of static control), and this increase remained evident 4 h after exposure (1.54 ± 0.18 fold of static). By 10 h after exposure, HO-1 mRNA was not significantly different from control. In smooth muscle cells (Fig. 1B), HO-1 mRNA was significantly increased 4h after exposure to shear stress (1.29 ±0.11 fold of static), and remained elevated 10 h post exposure (1.41 ± 0.13 fold of static). Mouse mesenteric arterioles (Fig. 1C) exposed to elevated flow (generated by a 50 mmHg pressure gradient) expressed significantly higher levels of HO-1 mRNA compared to vessels subjected to normal flow (generated by a 20mmHg pressure gradient) (14.43 ± 4.48 fold of 20 mmHg).

We previously reported that FSS increases PLGF expression both in our coculture model and in perfused vessels[289]. To determine whether HO-1 is a component of the signaling pathway mediating this response, we first tested the effect of zinc protoporphyrin IX (ZnPP), a HO-1 inhibitor, on the FSS-induced increase in PLGF expression. ZnPP (30 μM) blocked the effect of FSS on PLGF, and even decreased PLGF protein below static control levels (Fig. 2A). Similarly, in perfused vessels ZnPP (30 μM) prevented the effect of increased flow on PLGF mRNA (Fig. 2B). To determine the relative importance of endothelial cell vs smooth muscle cell HO-1 activity in mediating the effects of FSS on PLGF expression, HO-1 was separately knocked down in each cell type in the coculture model using siRNA (Fig. 3). HO-1 knockdown in endothelial cells prevented the FSS-induced expression of PLGF (Fig. 3A). In contrast, knockdown of HO-1 in smooth muscle cells did not affect the FSS-mediated increase in PLGF (Fig 3B). Knockdown in either cell type was confirmed by real time PCR (Fig 3C, 3D). HO-1 knockdown in endothelial cells did not affect HO-1 mRNA in cocultured smooth muscle cells, or vice versa (Fig. 3E, 3F). In agreement with these results, stimulating HO-1 with hemin chloride (2.5-40 μM) significantly increased PLGF protein levels in coculture media after 24 h (Fig. 4A).

HO-1 activity generates three products (biliverdin, carbon monoxide, and free iron). We next tested whether one or more of these molecules could upregulate PLGF expression in vascular cell cocultures. Treatment with 100 μM biliverdin (which is also a negative feedback inhibitor of HO-1) significantly decreased PLGF protein (0.70 ± 0.06 fold of control) after 24 h (Fig. 4B). To determine whether CO modulates PLGF expression, we treated cocultures with a CO-releasing molecule (CORM-A1, 0-400 μM) for 24h (Fig. 4C). Similarly to biliverdin, CORM-A1 significantly decreased secreted PLGF at the 200 μM (0.90 ± 0.03 fold of control) and 400 μM (0.86 ± 0.02 fold of control) doses. Lastly, we tested the effect of iron on PLGF expression. Divalent iron in the form of ferrous ammonium sulfate (FAS, 10-200 μM) had no significant effect on PLGF expression after 24 h (Fig 4D). In contrast, trivalent iron in the form of

ferric ammonium citrate (FAC, 10-200 μg/ml) mimicked the effects of hemin chloride and significantly increased PLGF levels after 24 h (Fig. 4E).

Surprisingly, the iron chelators 1,10-phenanthroline (100 μM) and deferoxamine (DFO, 100 μM) significantly increased secreted PLGF compared to DMSO vehicle control (1.28 ± 0.08 fold of DMSO and 1.32 ± 0.06 fold of DMSO, respectively; Fig. 5). DFO is a hypoxia mimetic agent. Therefore, and considering the close physical proximity of arteriogenesis to regions of tissue hypoxia in the coronary circulation, we next assessed the effects of hypoxia on PLGF expression in the coculture model. Cocultures containing either scRNA or siHO-1 treated endothelial cells were incubated in 1% O_2 for 24 h. Hypoxia exposure significantly increased PLGF protein concentrations in culture media of negative control treated cocultures relative to the normoxic control group (Fig. 6A) but not in the siHO-1 treated group, demonstrating that this increase was dependent on endothelial HO-1 expression (Fig. 6A). HO-1 knockdown was confirmed by real time PCR. Interestingly, hypoxia significantly decreased HO-1 expression in endothelial cells (Fig. 6B, 0.53 ± 0.05 fold of normoxic control). In smooth muscle cells (Fig. 6C), hypoxia significantly induced HO-1 expression in both the scRNA (4.40 ± 0.42 fold of control) and siHO-1 (5.50 ± 0.52 fold of control) treated groups. In contrast to these findings, incubating cocultures for the shorter period of 2 h in 1% O_2 potently induced HO-1 expression in both endothelial cells (Fig. 7A, 1.53 ± 0.11 fold of control) and smooth muscle cells (Fig. 7B, 4.85 ± 0.68 fold of control). FAC (100 μg/mL, 2 h) induced HO-1 mRNA in both endothelial cells (Fig. 7A, 1.79 ± 0.08 fold of control) and smooth muscle cells (Fig 7B, 11.67 ± 2.54 fold of control).

Discussion

In this study we demonstrated that FSS increases HO-1 expression both in an *in vitro* coculture model of the cell wall and *ex vivo* in intact vessels. We further showed that the FSS mediated increase in PLGF expression which we previously reported is dependent on HO-1 activity, and identified endothelial cell HO-1 activity as necessary for this response. FSS-independent activation of HO-1 (by hemin) was sufficient to induce PLGF expression. Treatment of vascular cell cocultures with the three products of HO-1 activity identified iron as a possible mediator of the effects of HO-1 on PLGF expression. Paradoxically, chelation of iron by either deferoxamine or 1,10-phenanthroline resulted in a similar increase in PLGF expression. Finally, we demonstrated that hypoxia also induces PLGF expression, and that this induction was dependent on endothelial HO-1 expression, indicating that both FSS and hypoxia can upregulate PLGF via a common pathway.

Blood flow recovery in the ischemic hindlimb of HO-1 knockout mice is significantly impaired[290] and inhibition of HO-1 activity following hindlimb ischemia in mice results in poor flow recovery and diminished recruitment of circulating cells to the ischemic hindlimb[286]. Consistent with these observations, we found that inhibition of HO-1 activity attenuates the effects of shear stress on PLGF. PLGF stimulates collateral remodeling by recruitment of monocytes[10] and other circulating cells to the vessel wall[291]. Therefore, our findings suggest a novel mechanism for the above-described effects of HO-1 inhibition on arteriogenesis in rodent models. Conversely to the effects of HO-1 inhibition, stimulation of HO-1 with cobalt protoporphyrin IX following myocardial infarction has been shown to result in improved outcome and greater neovascularization in rats[292]. Likewise, overexpression of HO-1 in both mice and rats results in improved re-establishment of blood flow following hindlimb ischemia [293, 294]. Similarly, overexpression of PLGF has been shown to improve cardiac performance and vascularization following myocardial infarction in mice[194].

We report that arteriogenic FSS increases HO-1 expression in endothelial cells and smooth muscle cells, consistent with a previous report that laminar FSS induces HO-1 in endothelial cells[295]. Increased flow induced HO-1 more robustly in intact vessels than did increased shear in our *in vitro* model. The coculture model much more closely approximates the vessel wall than do monocultures of vascular cells[296]. However, there are limitations to the model, including the lack of exposure of the cells to cyclic tangential or circumferential stretch. Furthermore, the model lacks the complexity of the *in vivo* extracellular matrix, including the extensive glycocalyx of intact vessels (which is important in the mechanosensing machinery of the vessel wall)[96, 297-299]. Despite these shortcomings, the coculture model offers practical advantages with regard to cell type specificity for both genetic manipulation and assays, which are not easily achieved in whole tissue.

HO-1 catabolizes heme into three products: biliverdin, CO, and divalent iron. Biliverdin is subsequently metabolized into bilirubin by biliverdin reductase. Biliverdin exhibits strong antioxidant effects[300-302] and has been demonstrated to inhibit ROS-induced angiogenesis in tumor cells[303]. Furthermore, biliverdin inhibits neointimal thickening following vascular injury in rats[304]. These beneficial effects have been attributed to biliverdin's antioxidant properties. We previously demonstrated that the effects of shear stress on PLGF expression are mediated by hydrogen peroxide produced by endothelial NADPH oxidase 4. Bilirubin is also a feedback inhibitor of HO-1[305]. Consistent with its antioxidant action and its ability to inhibit HO-1, we found that biliverdin inhibited rather than enhanced PLGF expression in static cocultures.

Several studies have demonstrated a cytoprotective role of HO-1[306, 307]. The cytoprotective effects of HO-1 have been attributed to CO. Rats pretreated with the CO donor methylene chloride before myocardial infarction developed more intermediate and large collateral arteries in the infarct area[292]. Furthermore, CO has been reported to induce VEGF expression in endothelial cells[308] and smooth muscle cells[309]. Despite these arteriogenic effects, the CO donor

CORM-A2 did not induce PLGF expression in our coculture model, but slightly but significantly reduced it instead. CO has also been shown to have antioxidant properties[310-312]. These properties may act to reduce PLGF expression.

HO-1 activity results in the release of labile iron, which is cytotoxic. Increased labile iron leads to oxidative stress within the cell through Fenton reactions. As a protective response against pro-oxidant Fenton reactions, increased labile iron induces an increase in ferritin translation[313] and iron sequestration. Labile iron also induces expression of an iron efflux pump colocalized with HO-1[314]. Ferroportin 1, an iron exporter, is also upregulated by labile iron generated by HO-1[315]. Indeed, HO-1 knockout in mice and HO-1 deficiency in humans is linked to anemia, due to accumulation of iron in tissues and decreased iron recycling[278, 280, 316], whereas overexpression of HO-1 is associated with increased cellular iron efflux and decreased influx [317]. Both hypoxia and FAC have also been reported to increase ferritin synthesis[318] and ferroportin expression[319, 320]. Taken together, these data suggest that an increase in HO-1 expression/activity would be expected to result in a decrease in labile or "chelatable" iron. In agreement with this idea, we found that chelating iron with either deferoxamine or 1,10-phenanthroline induced rather than inhibited PLGF expression. In agreement with this observation, others have reported that treatment with DFO results in enhanced flow recovery following hindlimb ischemia in mice [321] and rabbits[322].

Following myocardial infarction, collateral vessel remodeling occurs close to and within tissues that are ischemic (in contrast to peripheral artery disease, in which remodeling often occurs at a significant distance from the most ischemic regions). One of the hallmarks of ischemia is the stabilization and activation of hypoxia inducible factor (HIF). HIF1α is capable of increasing several arteriogenic cytokines, and its expression is increased during collateral development[323-325]. Interestingly, several single nucleotide polymorphisms that result in reduced HIF1α activity are enriched in patients with poor coronary collateralization[326, 327]. Furthermore,

monocytes collected from patients with either good or poor collateralization (based on Rentrop score) found a strong positive correlation between HIF1α expression and collateral development[328]. We previously reported that hypoxia increases PLGF expression in endothelial monocultures[250]. Likewise, in the present study, incubating vascular cell cocultures in 1% O_2 for 24 h resulted in a significant increase in secreted PLGF. Knockdown of endothelial HO-1 resulted in an attenuation of this effect, but real time PCR revealed HO-1 mRNA to be significantly decreased in the negative control group compared to the normoxic control group. Interestingly, 2 h exposure to hypoxia significantly increased HO-1 mRNA. This finding suggests that HO-1 may play an early role in the regulation of PLGF by hypoxia.

In conclusion, we demonstrate that the key arteriogenic factor PLGF is regulated by HO-1 during in response to the physiological stimuli of FSS and hypoxia, both of which are considered to be important signals for collateral development in the coronary circulation. This study builds on the increased interest in HO-1 and its metabolites in vascular remodeling by shedding light into a novel possible mechanism by which HO-1 exerts its arteriogenic effects.

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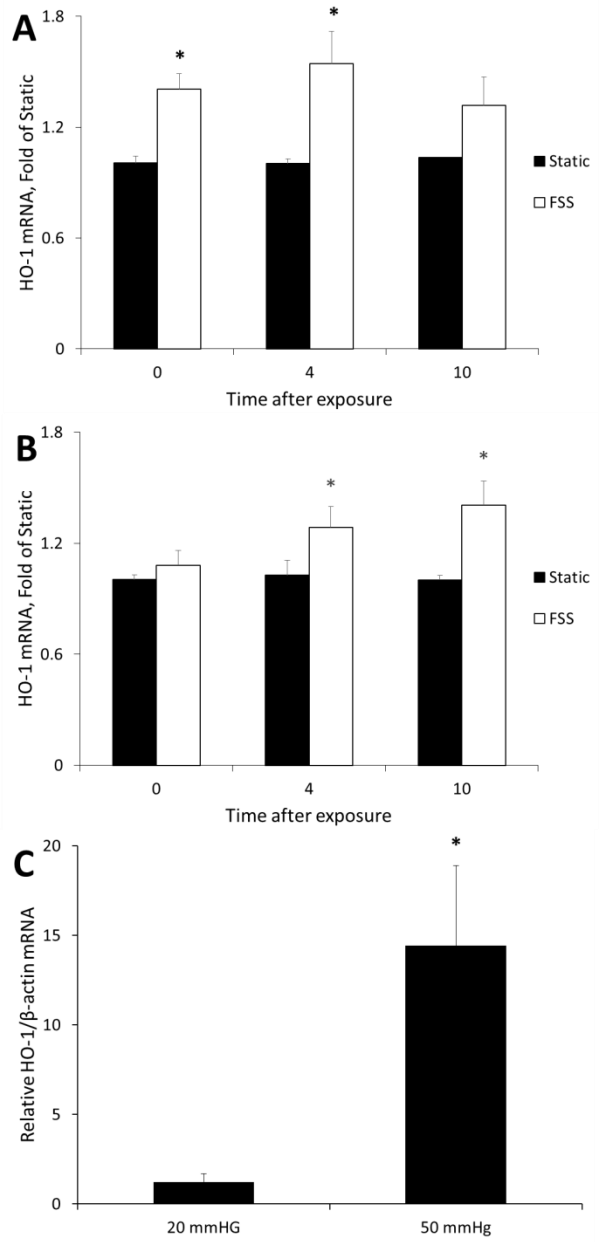

Figure. 1 FSS increases HO-1 expression. Endothelial/smooth muscle cocultures were exposed to FSS for 2h and samples collected immediately after exposure, 4h later and 10h later. (A) In endothelial cells FSS increased HO-1 mRNA compared to static control at 0h (1.40 ± 0.08) and at 4h (1.54 ± 0.18) but not at 10h (n=5, $p<0.05$). (B) In smooth muscle cells cocultured with

endothelial cells exposed to FSS HO-1 mRNA was increased at 4h (1.29 ±0.11 fold of static) and 10h (1.41 ± 0.13 fold of static) (n=5, $p<0.05$). (C) In mesentery arterioles perfusion at a pressure gradient of 50 mmHg increased HO-1 mRNA compared to 20 mmHg (14.43 ± 4.48) (n=5, $p<0.05$).

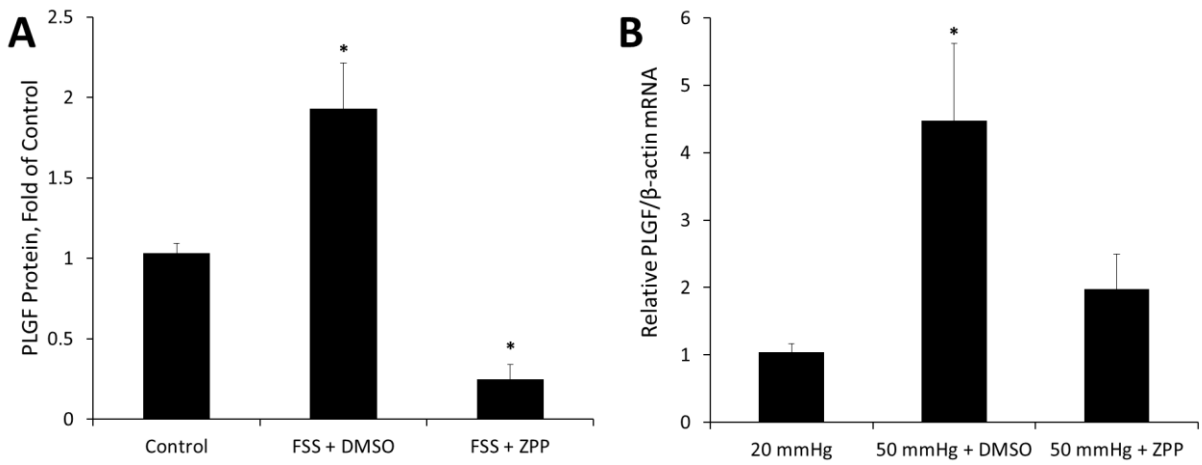

Figure. 2 HO-1 activity is necessary for FSS to increase PLGF expression. (A) In cocultures exposed to FSS ZPP (30 μM) inhibited the increase in secreted PLGF observed in coculture exposed to FSS and treated with DMSO (n= 5, $p<0.05$). (B) In mesentery arterioles perfusion at a pressure gradient of 50 mmHg for 2h increased PLGF mRNA compared to 20 mmHg (4.47 ± 1.15) this increase was blunted by ZPP (30 μM) (n=5, $p<0.05$).

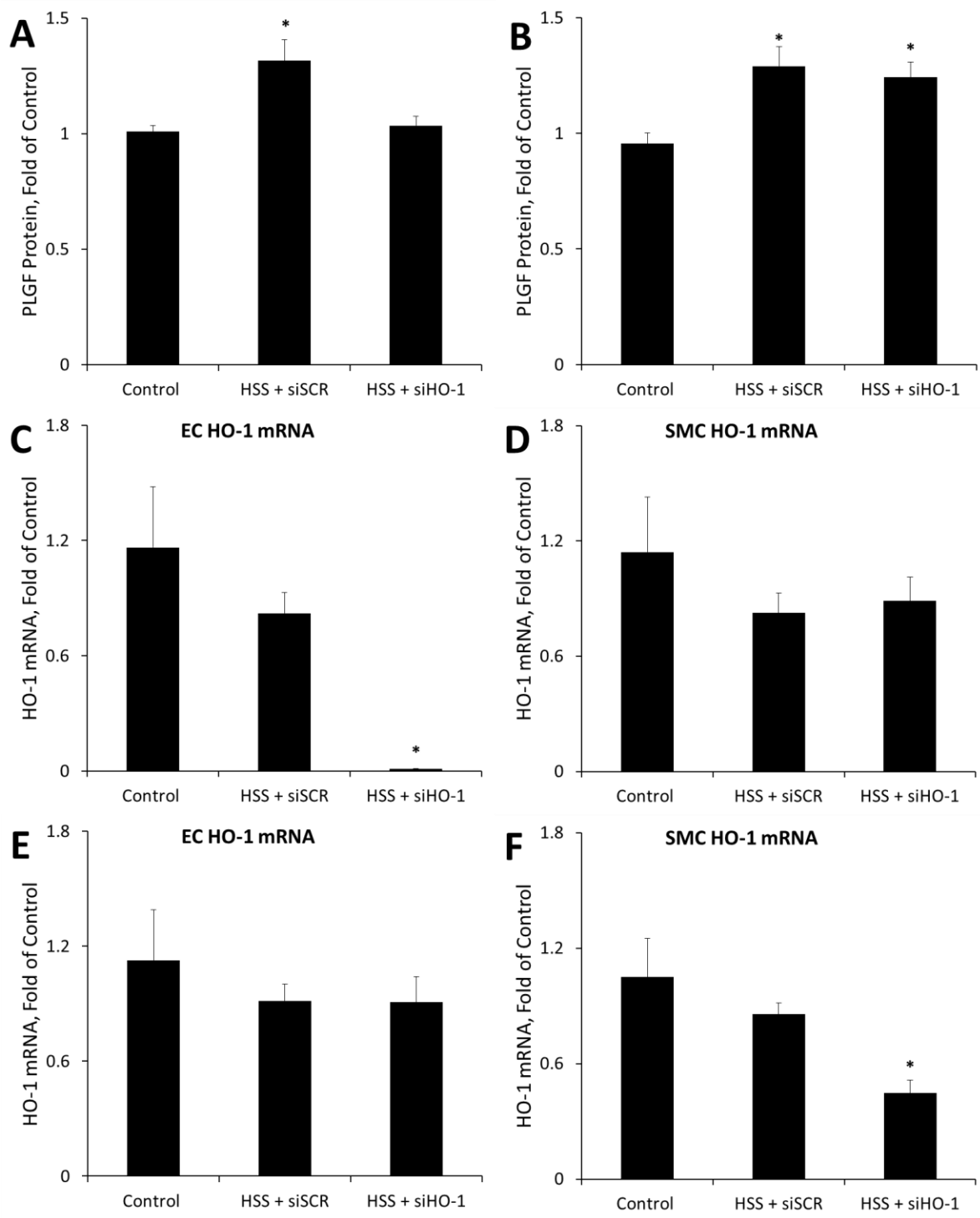

Figure 3. Endothelial HO-1 is necessary for FSS to increase PLGF expression. siRNA knockdown was performed on either endothelial cell or smooth muscle cells of cocultures. (A) knockdown of HO-1 in endothelial cells attenuated the effects of FSS on PLGF (n=5, $p<0.05$).

(B) FSS increased secreted PLGF in Knockdown of HO-1 in smooth muscle cells did not alter FSS effects on secreted PLGF (n=5, $p<0.05$). (C) HO-1 knockdown in endothelial cells significantly decreased HO-1 mRNA (n=5, $p<0.05$). (D and E) HO-1 knockdown in either cell type had no effect on HO-1 expression in the corresponding cocultured cells smooth muscle cells had not effect on HO-1 expression in endothelial cells (n=5). (C) HO-1 knockdown in smooth muscle cells significantly decreased HO-1 mRNA (n=5, $p<0.05$).

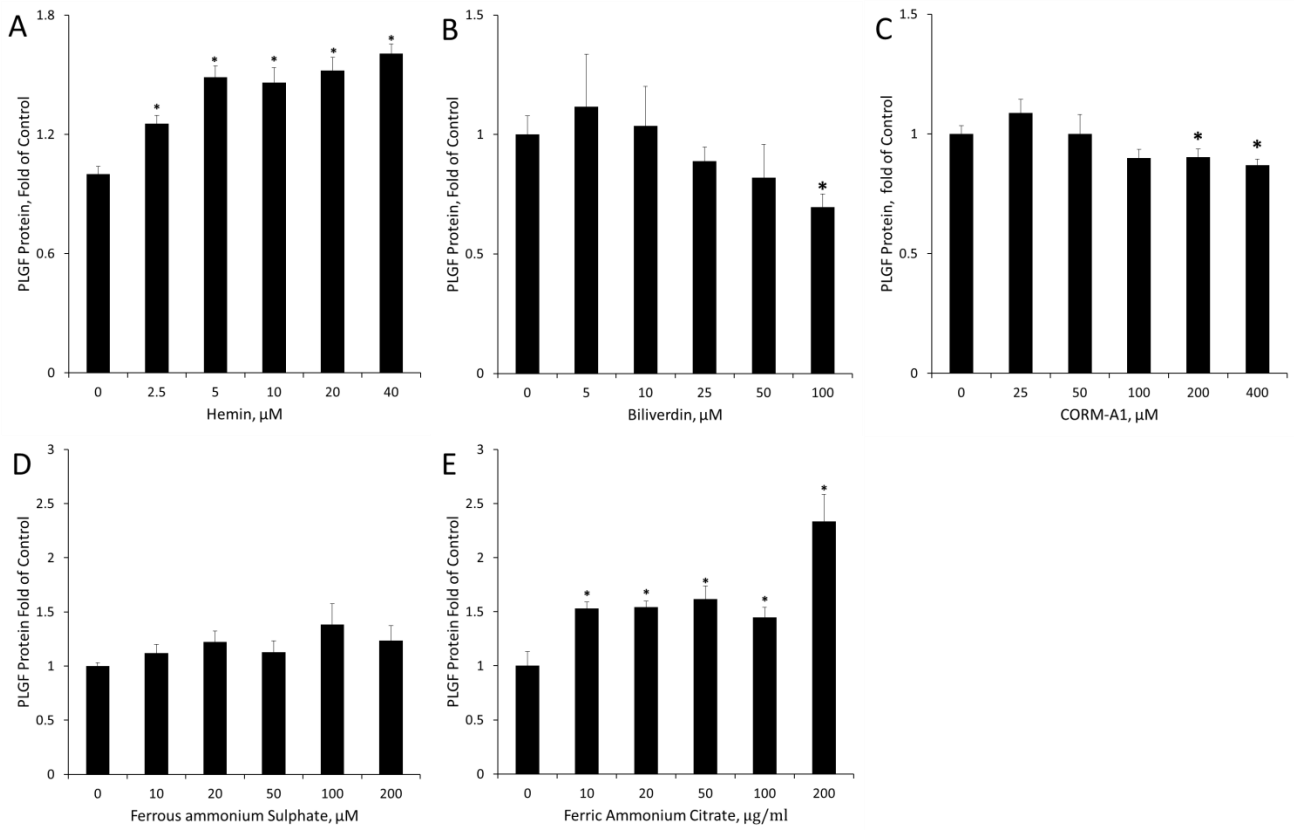

Figure 4. HO-1 activity increases secreted PLGF. Cocultures were treated for 24h with (A) hemin an inducer of HO-1 activity significantly increased secreted PLGF at all concentrations used (n=5, $p<0.05$). (B) Biliverdin significantly decreased secreted PLGF at 100 µM 0.70 ± 0.06 (n=5, $p<0.05$). (C) CORM-A1 significantly decreased secreted PLGF at 200 µM 0.90 ± 0.03 and 400 µM 0.86 ± 0.02 (n=5, $p<0.05$). (D) FAS had no significant effect on secreted PLGF. (E) FAC similar to hemin significantly increased secreted PLGF at all concentrations (n=5, $p<0.05$).

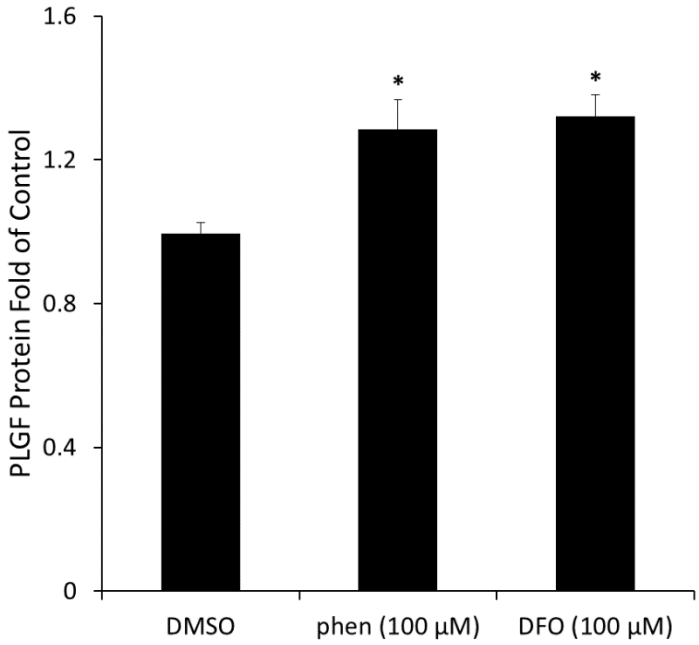

Figure 5. Iron chelation increases secreted PLGF. Treating the Cocultures with either the divalent iron chelators 1,10-phenanthroline 1.28 ± 0.08 (phen) (100 µM) or the trivalent iron chelator deferoxamine 1.32 ± 0.06 (DFO) (100 µM) significantly increased secreted PLGF compared to DMSO (n=5, $p<0.05$).

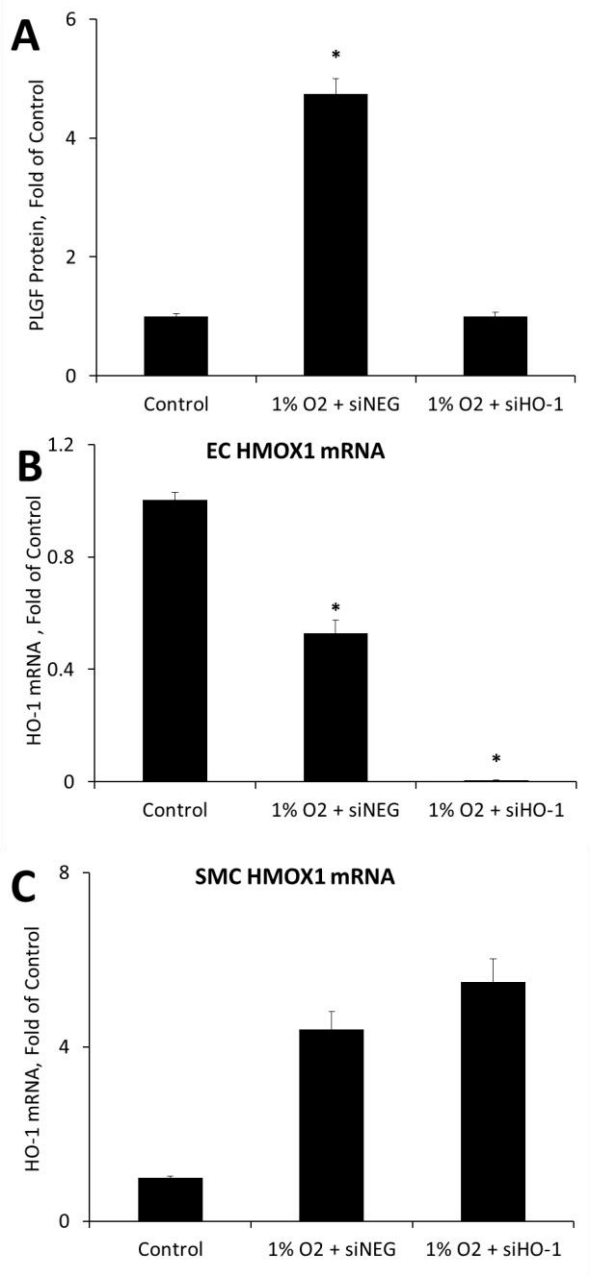

Figure 6. Hypoxia increases secreted PLGF and is dependent on Endothelial HO-1.

Cocultures were incubated in 1% O_2 for 24h. (A) hypoxia significantly increased secreted PLGF after 24h (4.74 ± 0.25) this effect was attenuated by HO-1 knockdown in the endothelial cells. (n=5, $P<0.05$) (B) In endothelial cells hypoxia significantly reduced HO-1 mRNA after 24h (0.53 ± 0.05). siRNA knockdown of HO-1 reduced HO-1 mRNA further (0.01 ± 0.00). (n=5, $P<0.05$)

(C) Hypoxia significantly increased HO-1 mRNA in smooth muscle cells (4.40 ± 0.42) and was not affected by HO-1 knockdown in endothelial cells (5.50 ± 0.52) (n=5, $P<0.05$).

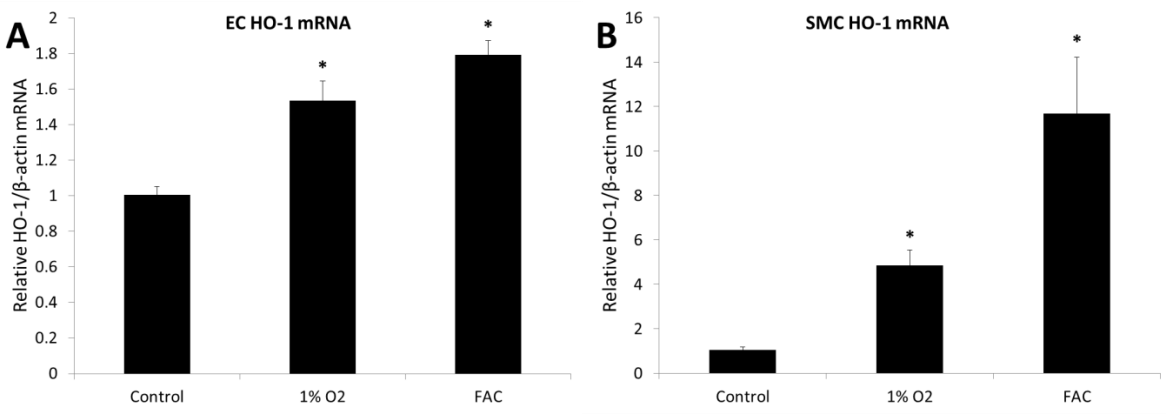

Figure 7. Both FAC and Hypoxia increase HO-1 mRNA after 2h. Treating cocultures with either 1% O_2 or FAC (100 µg/ml) for 2h resulted in an significant increase in HO-1 in (A) endothelial cells (1.53 ± 0.11) and (1.79 ± 0.08) respectively (n=-5, $p<0.05$). (B) in smooth muscle cells both hypoxia (4.85 ± 0.68 fold of control) and FAC (11.67 ± 2.54) significantly increased HO-1 mRNA (n=5 $p<0.05$).

CHAPTER V

AUTOCRINE-PARACRINE INTERACTIONS OF VEGF-A AND PLGF IN VASCULAR CELL COCULTURES

Nabil A. Rashdan and Pamela G. Lloyd

Abstract

Placental growth factor (PLGF) and vascular endothelial growth factor-A (VEGF-A) are important regulators of both physiological and pathological vascular remodeling. We previously reported that monocultured human coronary EC primarily express PLGF, while monocultured human coronary smooth muscle cells (SMC) primarily express VEGF-A. However, in the vasculature, EC and SMC are in close proximity to each other. Thus, in this study we sought to 1) determine whether this cell specific expression pattern is maintained when EC and SMC are cocultured, and 2) test the hypothesis that EC VEGF-A and/or PLGF production influences SMC VEGF-A and/or PLGF expression (and vice versa). EC and SMC were cultured on either side of a porous Transwell insert and media PLGF and VEGF-A levels were measured by ELISA. We confirmed that the cell type specific PLGF and VEGF-A expression we observed in monocultures remains evident in the coculture model. Interestingly, coculture of EC and SMC increased media PLGF relative to EC monoculture (38.38 ± 4.17 pg/µg total protein vs 63.78 ± 1.82 pg/µg total protein; n=5, $p<0.01$), but decreased media VEGF-A compared to SMC monoculture(38.90 ± 2.98 pg/µg total protein vs 228.13 ± 9.57 pg/µg total protein; n=5, $p<0.001$). Both VEGF-A and VEGF-E (VEGFR-2 specific Ligand) increased PLGF in EC monolayers (1.30 ± 0.06 fold of control; n=5, $p<0.01$). In SMC monolayers, the VEGFR-1 ligand PLGF reduced media VEGF-A(0.81 ± 0.04 fold of control; n=5 $p<0.01$), while the VEGFR-2 ligand VEGF-E had no significant effect on VEGF-A. Knockdown of PLGF in SMC increased VEGF-A production by SMC (1.63 ± 0.05 fold; n=5, $p<0.01$) , while knockdown of VEGF-A in EC decreased PLGF production by EC (0.54 ± 0.02 fold of control; n=5 $p<0.001$). Coculture conditions increased VEGFR-2 levels on the surface of EC, but decreased VEGFR-1 levels on the surface of SMC. Inhibition of VEGFR-2 tyrosine kinase activity decreased PLGF and increased VEGF in EC and in cocultures, but had no effect these parameters in SMC. We conclude that PLGF and VEGF-A

exert both paracrine and autocrine regulatory effects on each other and that these effects are partially mediated by VEGFR-2.

Introduction

Despite advances in pharmacological interventions, ischemic cardiovascular disease remains a leading cause of death in the United States[1] and is likely to remain so with the increased incidence of metabolic syndrome and diabetes, which favor the development of ischemic cardiovascular disease. In the coronary circulation, as atherosclerosis progresses, the development of a flow limiting stenosis leads to a drop in blood pressure downstream of the stenosed region. This decrease in pressure results in an increased pressure gradient across any anastomoses that connect the area of low pressure to an area of higher pressure, leading to increased flow through these vessels. If the increased flow persists, these collateral vessels remodel in an outward direction, such that their caliber and wall thickness increase to accommodate the higher flow rate. Collateral remodeling can reestablish flow to tissue downstream of the occlusion and alleviate ischemia. In the coronary circulation, the presence of native collaterals and the extent to which these remodel following an ischemic event is a direct predictor of survival[2, 3]. There is significant variability in the development of coronary collaterals in the human heart[4] and disease states such as type II diabetes are associated with a poor remodeling response. Therefore, treatments that could enhance collateral remodeling would be very useful clinically and could increase the survival of patients with coronary artery disease[5]. A more in-depth understanding of the mechanisms that govern collateral remodeling is needed before the development of pharmaceutical agents capable of stimulating it becomes possible.

Vascular endothelial growth factor-A (VEGF-A) is the archetypal member of the VEGF family of growth factors and is a ligand for the tyrosine kinase VEGF receptors 1 and 2 (VEGFR1, VEGFR2) and neuropilin-1[6-9]. VEGF-A plays an important role in the development of

the vasculature during embryogenesis and during both physiological and pathological vascular remodeling later in life [10]. Mice underexpressing VEGF-A exhibit a decrease in the number of preexisting collateral vessels, whereas mice overexpressing VEGF-A have a higher density of preexisting collateral vessels[11]. As a result, the former model demonstrates diminished arteriogenesis in response to hindlimb ischemia, while the latter demonstrates increased arteriogenesis. Placental growth factor (PLGF) is another member of the VEGF family, which only signals through VEGFR1[12, 13]. PLGF expression is necessary for collateral remodeling, and mice lacking PLGF exhibit impaired reestablishment of flow following femoral ligation[14]. PLGF is chemotactic for monocytes, which express VEGFR1 but not VEGFR2[15]. It is through the recruitment of monocytes that PLGF stimulates collateral remodeling[14]. Interestingly, PLGF binding to VEGFR1 elicits a downstream response distinct from the response that occurs when VEGF-A binds to VEGFR1[16], and despite the similarity in the structure of the two cytokines, they are not interchangeable and exhibit different physiological effects [14, 16-18]. Following femoral ligation in mice, exogenous VEGF-A stimulates sprouting of capillaries and increases branching of collateral vessels in the hindlimb; PLGF, on the other hand, stimulates enlargement of preexisting collaterals [14, 17]. In clinical trials, therapies focusing on one particular growth factor have fallen short of their intended goals [3]. Collateral remodeling is a complex process involving multiple chemokines and cytokines, and an understanding of the interactions between these factors is required in order to reveal the underlying mechanisms governing the remodeling process.

We previously reported that endothelial cells are the predominant source of PLGF in the vessel wall and that smooth muscle cells are the predominant source of VEGF-A[19]. Recently, we demonstrated that increased fluid shear stress increases PLGF expression in an endothelial cell/vascular smooth muscle cell coculture model[20]. We also showed that coculturing endothelial cells with smooth cells significantly increases the concentration of PLGF in media, compared to

media from endothelial monocultures, suggesting that PLGF production is influenced by cell-cell interactions. In this study, we sought to determine the autocrine and paracrine effects of PLGF and VEGF-A on each other, and to determine whether VEGF receptor expression is also modulated by coculture conditions.

Materials and Methods

Reagents

Human recombinant VEGF-A and PLGF were purchased from R&D Systems (Minneapolis, MN). VEGF-E was purchased from Prospec (East Brunswick, NJ). The VEGFR-2 tyrosine kinase inhibitor SU 1498 was purchased from Cayman Chemicals (Ann Arbor, MI). All other reagents were purchased from Sigma Aldrich (St. Louis, MO) unless stated otherwise.

Cell culture

Primary human coronary artery endothelial cells (HCAEC) and smooth muscle cells (HCASMC) were purchased from Lonza (Walkersville, MD). Cells were grown in 5% CO_2 in a humidified incubator at 37°C. HCASMC were grown in smooth muscle basal medium (SmBM, Lonza) supplemented with a smooth muscle growth medium kit (SmGm2, Lonza). The cell donors were a 56 year old female and a 30 year old male. HCAEC were grown in endothelial basal medium (EBM, Lonza) supplemented with an endothelial growth medium kit (EGM-2MV, Lonza). The cell donors were a 21 year old male and a 30 year old male. All experiments were carried out between passage 4 and 6.

Endothelial cell/smooth muscle cell cocultures were established as we previously described[20]. Briefly, Corning costar (Corning, NY) 24mm TRANSWELL® polyester membrane inserts with a 0.4 μM pore size were inverted and coated with 0.1% Gelatin diluted in growth

medium. Inserts were incubated for 1 h at 37°C in a humidified incubator. Gelatin was aspirated off the insert and HCASMC were seeded onto the inverted inserts (~10^5cells/insert) and incubated for 4 hours in 5% CO_2 at 37°C in a humidified incubator. Inserts were then inverted into 6 well plates containing growth medium and incubated for 48 h. 0.1% Gelatin diluted in growth medium was added to the apical side of the membrane and incubated as above for 1 h following the gelatin was aspirated and HCAEC ($2x10^5$cells/insert) seeded on the apical side of the insert and incubated as above for 24 h. cocultures were subjectively checked for confluence. All experiments monoculture and coculture were carried out at 80% - 90% confluence. Prior to experiments culture medium was replaced for 2% serum medium for 24 h. 2% Serum medium was made by diluting the corresponding medium in Dulbecco's Modified Eagle's Medium (DMEM) Hyclone (Pittsburgh, PA).

Real Time PCR

Cells were rinsed with 37°C PBS (Hyclone), then incubated in TrypLE Express (Gibco, Waltham, MA) for 5 minutes at 37°C. Following cell detachment, 2 volumes of complete medium were added to the cells and the cell suspension was centrifuged at 300g for 5 minutes. Total RNA was isolated from the pellet using RNeasy Mini columns (Qiagen, Valencia, CA) per manufacturer's instructions. The concentration and quality of the RNA was determined using a Take 3 plate in a Synergy HT multimode plate reader (BioTek, Winooski, VT). Both genomic DNA removal and cDNA synthesis were carried out using the QuantiTect reverse transcription kit (Qiagen). Real time PCR reactions were carried out in a ABI 7500 Fast instrument (Applied Biosystems, Carlsbad, CA) using PerfeCTa SYBR Green FastMix, Low ROX (Quanta Biosystems, Gaithersburg, MD). Primers were designed using Primer Express software and custom-synthesized by Invitrogen (Carlsbad, CA). Primer sequences were as previously described[19, 21]. To avoid confounding effects of housekeeping gene selection when making comparisons between different cell types, PLGF and VEGF-A mRNA expression was normalized

to the geometric average of the housekeeping genes β-actin and GAPDH, and relative gene expression was quantified using the ΔΔCt method.

ELISA

Secreted PLGF and VEGF-A concentrations in culture media were determined using the human PLGF and VEGF-A DuoSet ELISA development kits (R&D Systems, Minneapolis, MN) according to manufacturer's protocol. Data were normalized to total protein concentration, as determined by BCA assay (Pierce, Rockford, IL). Samples were treated with protease inhibitor cocktail (1 mM PMSF, 1 mM Na3VO4, 1 µg/ml leupeptin, 1 mM benzamidine-HCl, 1µg/ml aprotinin, 1µg/ml pepstatin A) prior to analysis.

Flow cytometry

To measure the cell surface expression of VEGFR1 and VEGFR2, cells were collected as above and resuspended in PBS containing 1% BSA, then incubated for 45 minutes at room temperature in the dark with a 1:500 dilution of both Dylight 650-conjugated rabbit anti-human VEGFR1 polyclonal antibody (NB100527, Novus Biologicals, San Diego, CA) and Dylight 488-conjugated rabbit anti-human VEGFR2 polyclonal antibody (NB1002382G, Novus Biologicals). After incubation, labeled cells were resuspended in 1% BSA-PBS and the mean fluorescence intensity (MFI) was determined by flow cytometry (Accuri C6, Ann Arbor, MI).

Transfection

Cells were transfected using Lipofectamine RNAiMAX (Invitrogen) following manufacturer's instructions. Five nM siRNA was precomplexed with Lipofectamine RNAiMAX for 20 min in Opti-MEM media (Gibco), after which 4 volumes of DMEM (Hyclone) containing 10% FBS (Gibco) were added, making complete transfection medium. siRNA consisting of the following sequences was used to knock down PLGF: sense, 5'-AGGUGGAAGUGGUACCCUU-

3', overhang dTdT; antisense, 5'-AAGGGUACCACUUCCACCU-3', overhang, dCdT (Invitrogen). For VEGF-A knockdown, a predesigned siRNA was used (Silencer Select s460, Invitrogen). Silencer Select negative control no.1 siRNA (Invitrogen) was used as a negative control. Cells were seeded into 6 well plates and grown to 90% confluence, after which cells were rinsed and the medium replaced with transfection medium. After 6 h in transfection medium, the cells were switched to 2% serum medium for a further 24h. For cell specific knockdown in cocultures, HCASMC were transfected directly on the insert prior to addition of HCAEC, whereas HCAEC were trypsinized immediately after transfection and seeded onto the apical side of the insert. Effectiveness of the siRNA treatment was determined by real time PCR, as outlined above.

Statistical analysis

All data are presented as mean ± SEM. Experiments were replicated at least five times. Data were analyzed by either student's T-test, or one-way ANOVA followed by Tukey's range test, as appropriate. The level of significance was set at $p<0.05$.

Results

Consistent with our previously reported results[19], we found that monocultured HCAEC predominantly expressed PLGF (PLGF, 38.38 ± 4.17 pg/µg total protein; VEGF-A, 5.28 ± 1.73 pg/µg total protein), whereas monocultured HCASMC predominantly expressed VEGF-A (PLGF, below detection limit; VEGF-A, 228.13 ± 9.57 pg/µg total protein; Fig.1A and B). Interestingly, coculture of HCAEC and HCASMC significantly increased the PLGF protein concentration in media (63.78 ± 1.82 pg/µg total protein), compared to media from HCAEC monocultures (Fig. 1A, $p<0.01$). The opposite effect of coculture was observed for VEGF-A, which was significantly lower in coculture media (38.90 ± 2.98 pg/µg total protein) compared to media from HCASMC monocultures (Fig. 1B, $p<0.001$). Consistent with the protein analysis,

PLGF mRNA was significantly higher under coculture conditions in both HCAEC (2.14 ± 0.26 fold of monoculture; $p<0.01$ Fig. 1C) and HCASMC (1.78 ± 0.10 fold of monoculture, $p<0.01$; Fig. 1D). Coculture also significantly increased VEGF mRNA in HCAEC (1.67 ± 0.22 fold of monoculture) (Fig. 1E, $p<0.05$), but had no effect on VEGF-A mRNA in HCASMC (Fig. 1F).

We hypothesized that HCASMC-derived VEGF-A could contribute to the increased production of PLGF by HCAEC in cocultures. Therefore, we next tested the effect of supplementing HCAEC monocultures with exogenous VEGF-A (100 ng/mL). VEGF-A significantly increased the concentration of PLGF in culture media (1.72 ± 0.05 fold of control) (Fig. 2A). Treatment of HCAEC with the VEGFR-2 specific ligand VEGF-E (100 ng/mL) (Fig. 2) also significantly increase secreted PLGF (1.30 ± 0.06 fold of control; $p<0.01$). Likewise, we hypothesized that HCAEC-derived PLGF could influence VEGF-A production by SMC. Treatment of HCASMC with PLGF, a VEGFR-1 specific ligand (100 ng/mL) significantly reduced media VEGF-A (0.81 ± 0.04 fold of control; $p<0.01$). In contrast, the VEGFR-2 ligand VEGF-E (100 ng/mL) had no significant effect on HCASMC media VEGF-A (Fig. 2B).

We next tested the effect of knocking down endogenous VEGF-A and PLGF on the levels of these cytokines. In HCAEC monocultures, knockdown of VEGF-A significantly decreased media PLGF (0.54 ± 0.02 fold of control; $p<0.001$; Fig. 3A). Supplementing media with VEGF-A (100 ng/mL) concurrently with VEGF knockdown rescued media PLGF, and led to a significant increase in media PLGF compared to control (1.28 ± 0.03 fold of control; $p<0.001$; Fig. 3A). However, knockdown of VEGF-A in HCAEC had no significant effect on PLGF mRNA (Fig. 3B). VEGF-A knockdown was confirmed by real time PCR (Fig. 3C). In HCASMC monocultures, knockdown of PLGF significantly increased media VEGF-A, compared to control (1.63 ± 0.05 fold; $p<0.01$; Fig. 4A). PLGF knockdown was confirmed by real time PCR (Fig. 4B) PLGF knockdown also significantly increased VEGF-A mRNA (3.84 ± 0.83 fold of control; $n=5$, $p<0.05$; Fig. 4C).

VEGFR-2 activation (with VEGF-E) increases PLGF production by HCAEC monocultures, and PLGF levels are also increased by HCAEC/HCASMC coculture. In contrast, VEGFR-1 activation (with PLGF) decreases VEGF-A production by HCASMC, and VEGF-A levels are also decreased by HCAEC/HCASMC coculture. Therefore, we hypothesized that coculture could be affecting the surface expression of VEGFR-1 and VEGFR-2. In HCAEC, consistent with the results reported above, coculture significantly increased surface VEGFR-2 expression (Fig. 5B; 1.36 ± 0.07 fold MFI; $p<0.05$), but had no significant effect on VEGFR-1 surface expression (Fig. 5A). In HCASMC, also consistent with the above-described results, coculture significantly decreased VEGFR-1 surface expression (Fig. 5C; 0.74 ± 0.02 fold MFI; $p<0.001$), with no significant effect on surface VEGFR-2 expression (Fig. 5D).

To further assess the role of VEGFR-2 in regulating PLGF and VEGF-A, we treated both monocultures and cocultures with the VEGFR-2 specific tyrosine kinase inhibitor SU1498 (10 nM). Inhibition of VEGFR-2 tyrosine kinase activity significantly decreased media PLGF protein in HCAEC (Fig. 6A; 0.60 ± 0.02 fold of HCAEC monoculture; $p<0.001$) and in cocultures (4.81 ± 0.22 vs 1.80 ± 0.09 fold of HCAEC monoculture), in agreement with our other findings suggesting a positive role for VEGFR-2 signaling in regulation of PLGF. PLGF was not detected in SMC monoculture media either with or without SU1498 treatment (Fig. 6A). Interestingly, SU1498 also significantly increased media VEGF-A in HCAEC (Fig. 6B; 3.15 ± 0.39 fold of HCAEC monoculture; $p<0.05$), and to a lesser extent in cocultures. Consistent with our findings suggesting that VEGFR-1, but not VEGFR-2, contributes to regulation of VEGF-A in HCASMC, SU1498 had no significant effect on VEGF-A in HCASMC monoculture media (Fig. 6B).

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Discussion

Coculturing HCAEC with HCASMC resulted in a significant increase in PLGF protein in culture media (as compared to media from HCAEC monocultures). HCAEC produced much more PLGF than HCASMC, consistent with previous reports by our group[20]. Along with increased PLGF protein, coculture resulted in a significant increase in PLGF mRNA in both HCAEC and HCASMC, suggesting that the increase in PLGF protein with coculture is at least partly due to increased gene transcription. These findings suggest that it is likely that PLGF production by endothelial cells *in vivo* is underestimated by *in vitro* studies of endothelial cell monocultures. Furthermore, PLGF levels may be reduced in vessels in which EC/SMC contact and/or communication is dysfunctional due to disease or injury, relative to expression in healthy quiescent vessels.

Coculture of HCAEC and HCASMC resulted in a significant reduction in VEGF-A protein in media. This effect occurred without a significant effect of coculture on VEGF mRNA in HCASMC, and with a significant increase in VEGF mRNA in HCAEC. The most likely explanation for the reduction in VEGF-A protein without a corresponding effect on VEGF-A mRNA in HCASMC (the primary source of VEGF-A in the coculture model) is that VEGF-A is being rapidly removed from the media by endothelial cell VEGF receptor binding and uptake. Indeed, we previously demonstrated that VEGF-A decreases in the presence of endothelial cells[19], while others have demonstrated active uptake (endocytosis) of VEGF-A by endothelial cells via VEGFR-2[22] and other pathways which lead to nuclear accumulation[23, 24]. In agreement with this possibility, we found that coculture resulted in an increase in HCAEC cell surface VEGFR-2 levels. Another mechanism that may contribute to the effects observed is the post transcriptional regulation of VEGF expression. Coculturing endothelial cells and smooth muscle cells has been demonstrated to result in the trafficking of miRNA from endothelial cells to smooth muscle cells via micro-particles[25, 26]. These miRNA include miR-145 which has been shown to inhibit VEGF

translation[27]. Although VEGF-A mRNA increased with coculture in HCAEC, HCAEC are a relatively minor source of VEGF-A in the coculture system and thus this effect on overall VEGF-A protein levels was negligible.

Exogenous VEGF-A has been shown to upregulate PLGF production in several primary endothelial cell lines [28, 29]. In the present study, we found that exogenous VEGF-A and VEGF-E, similarly increased PLGF production in HCAEC. VEGF-E exclusively binds VEGFR-2, while VEGF-A binds both VEGFR-1 and VEGFR-2. These data suggest the effects VEGF-A are mediated by VEGFR-2. Knockdown of VEGF-A resulted in a significant reduction in media PLGF. This is consistent with the *in vivo* reduction of PLGF expression that has been reported in mice treated with VEGF-A neutralizing antibodies[29]. The concurrent treatment with exogenous VEGF-A and knockdown of VEGF-A not only rescued the reduction in media PLGF but significantly increased media PLGF demonstrating the effects of knockdown are specific to a lack of VEGF-A.

Treating HCASMC with exogenous PLGF resulted in a significant decrease in VEGF-A protein in culture media, whereas knockdown of PLGF in HCASMC significantly increased both VEGF-A protein and mRNA. PLGF exclusively signals through VEGFR-1[12, 13]. Homozygous VEGFR-1 knockout is embryonically lethal in mice, and disorganized vasculature resulting from uncontrolled endothelial overgrowth is the primary defect causing lethality[30]. This suggests that PLGF is a negative regulator of endothelial proliferation through VEGFR-1 signaling. PLGF has been demonstrated to induce vascular normalization and decrease sprouting[31], whereas blocking VEGFR-1 results in increased angiogenesis in adipose tissue[32]. Similarly, several studies have demonstrated the inhibition of VEGF-A induced angiogenesis by PLGF[33-37]. Consistent with these findings, we demonstrate that both exogenous and endogenous PLGF acts as negative regulator of VEGF-A in HCASMC.

Blocking VEGFR-2 *in vivo* attenuates the VEGF-A induced increase in PLGF[38]. In keeping with these previously reported results, we found that inhibiting VEGFR-2 tyrosine kinase activity with the small molecule inhibitor SU1498 decreased PLGF protein in both HCAEC monocultures and in cocultures. Interestingly, VEGF-A protein was increased by SU1498 in both HCAEC monocultures and in cocultures. No significant effect of VEGFR-2 inhibition on PLGF or VEGF-A expression was observed in HCASMC. This cell type specific effect may be related to the fact that VEGF-induced PLGF expression is dependent on ERK phosphorylation[38], and VEGFR-2 activation has differential effects on ERK in EC and SMC. Studies with the VEGFR-2 inhibitor SU1498 demonstrated that VEGFR-2 inhibition decreases phospho-ERK activity in human aortic endothelial cells [39], but not in human aortic smooth muscle cells[39]. In addition, our results suggest that VEGFR-1 plays a more important role in regulating VEGF-A production by HCASMC than does VEGFR-2.

We observed that HCASMC cocultured with HCAEC exhibited decreased cell surface VEGFR-1 expression, compared to HCASMC monocultures. This finding is similar to results reported in models of vascular injury. Smooth muscle VEGFR-1 is increased in rat carotid arteries following balloon injury [40]. Likewise, wire injury of mouse carotid arteries increases smooth muscle VEGFR-1 expression[41]. Our data and these reports are consistent with an inhibitory effect of EC/SMC communication on VEGFR-1 expression.

Coculturing HCAEC with HCASMC significantly increased VEGFR-2 in HCAEC. Several studies have demonstrated the role of VEGFR-2 in mechanosensing of fluid shear stress. VEGFR-2 tyrosine kinase activity is required for fluid shear stress induced activation of eNOS and vasodilation[42]. Likewise, fluid shear stress increases VEGFR-2 phosphorylation, and inhibiting VEGFR-2 tyrosine kinase activity attenuates the anti-apoptotic effects of fluid shear stress[43]. Consistent with these findings, we previously reported that coculturing HCAEC with HCASMC results in an increase in sensitivity to fluid shear stress.

In conclusion, the results of this study demonstrate that crosstalk between vascular smooth muscle cells and endothelial cells regulates the expression of both PLGF and VEGF-A. These findings suggest that cocultures of these two cell types better recapitulate the signaling environment of stable quiescent the coculture model a useful tool in studying vascular physiology. Considering that the regulation of PLGF and VEGF-A has overarching implications for arteriogenesis, atherosclerosis, and tumor angiogenesis, greater insight into the molecular and cellular interactions of these factors vessels than does monocultures, making in the vessel wall will contribute greatly to the understanding of these pathophysiological conditions.

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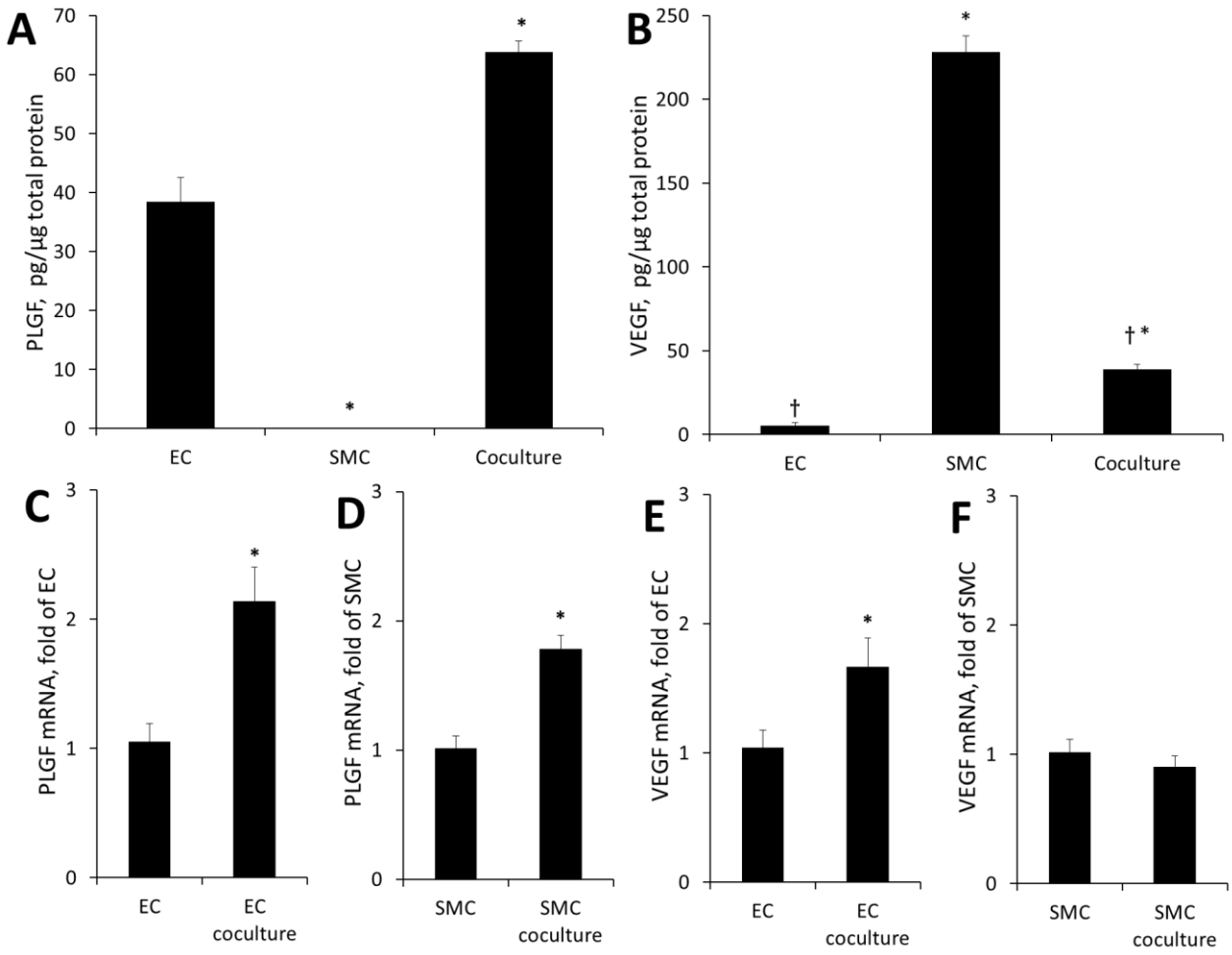

Figure 1. PLGF and VEGF expression in EC and SMC monoculture and coculture. (A) PLGF protein is predominantly produced by EC and is significantly increased when EC and SMC are cocultured (n=5, *p<0.001 compared to EC monoculture). (B) VEGF-A protein is mostly produced by SMC, and is decreased when EC and SMC are cocultured (n=5, *p<0.001 compared to EC monoculture, †p<0.001 compared to SMC monoculture). Coculture significantly increased PLGF mRNA in both EC (C) and SMC (D). Coculture significantly increased VEGF-A mRNA in EC (E), but not SMC (F) (n=5, *p<0.001 compared to monoculture).

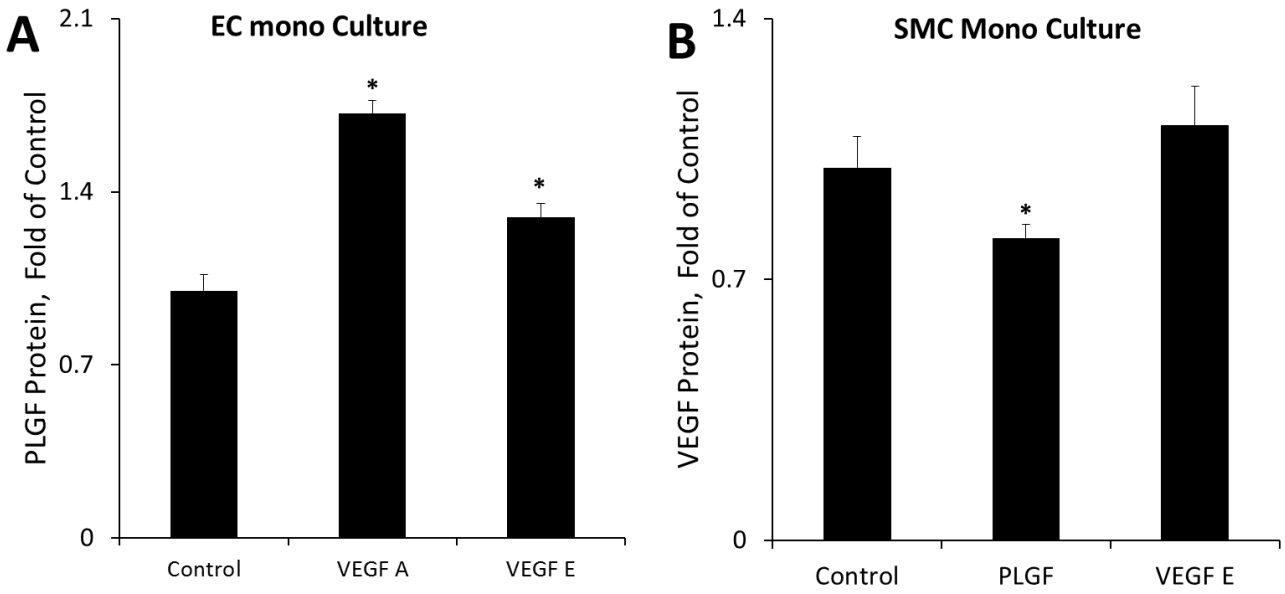

Figure 2. Effect of exogenous VEGFR ligands on VEGF-A and PLGF production in monocultures. (A) VEGF-A (100 ng/ml) significantly increased PLGF protein in media compared to control (1.72 ± 0.05 fold of control). The VEGFR-2 specific ligand VEGF-E (100 ng/mL) also significantly increased PLGF protein in HCAEC culture media (1.30 ± 0.06 fold of control). (B) The VEGFR-1 ligand PLGF (100 ng/mL) significantly decreased VEGF-A protein in HCASMC culture media (0.81 ± 0.04 fold of control), whereas the VEGFR-2 ligand VEGF-E had no effect (n=5, * $p<0.05$).

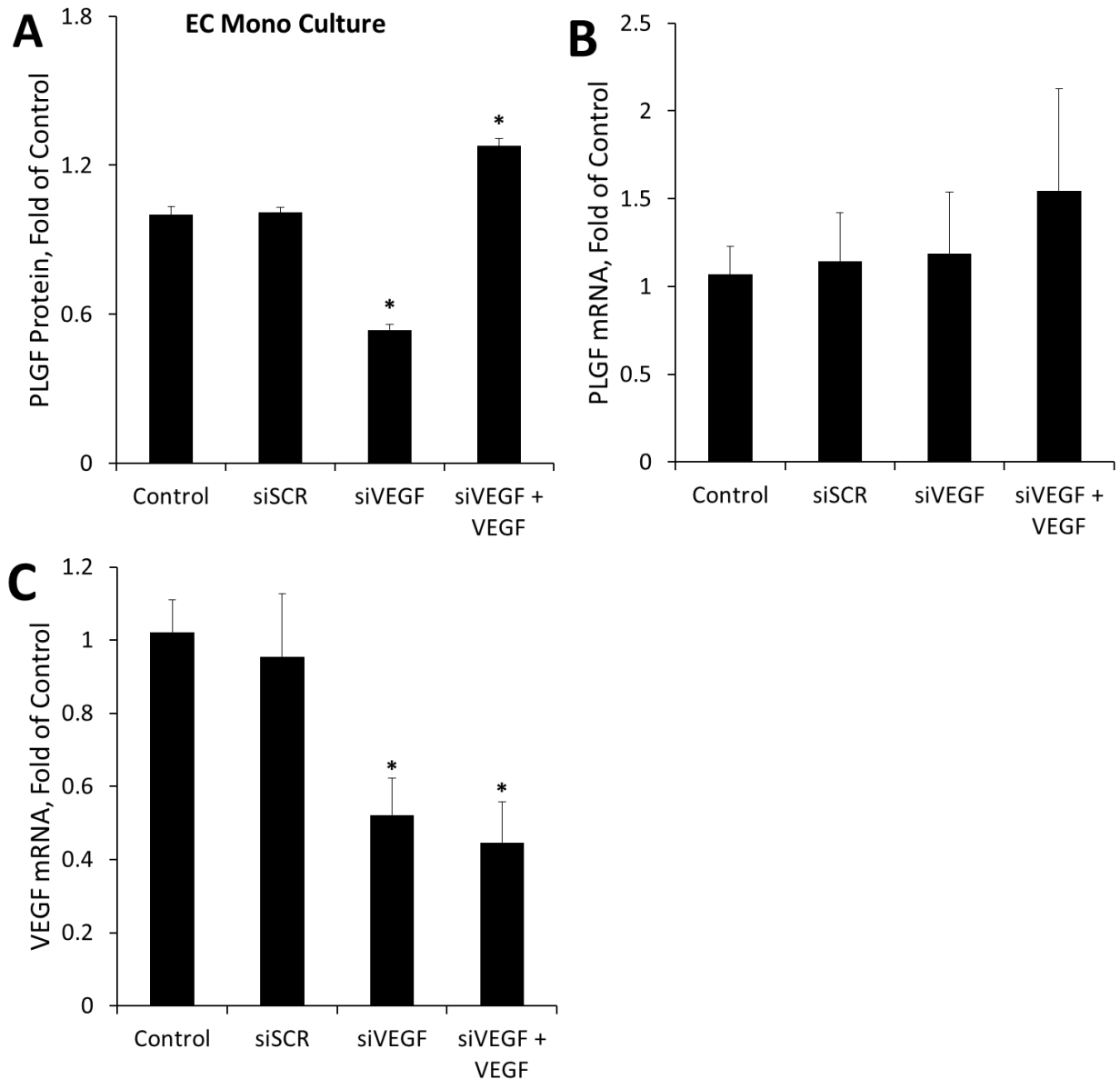

Figure 3. Effect of knockdown of VEGF-A in HCAEC monoculture (A) Knockdown of VEGF-A in HCAEC monoculture significantly decreased PLGF protein. Concurrent treatment with exogenous VEGF-A rescued PLGF protein expression and led to an increase relative to untreated cells. (B) In contrast to its effect on PLGF protein, knockdown of VEGF-A in HCAEC monocultures had no significant effect on PLGF mRNA expression. (C) siVEGF reduced VEGF-A mRNA levels to ~50% of control in HCAEC monocultures (n=5, *$p<0.05$ compared to both control and siSCR).

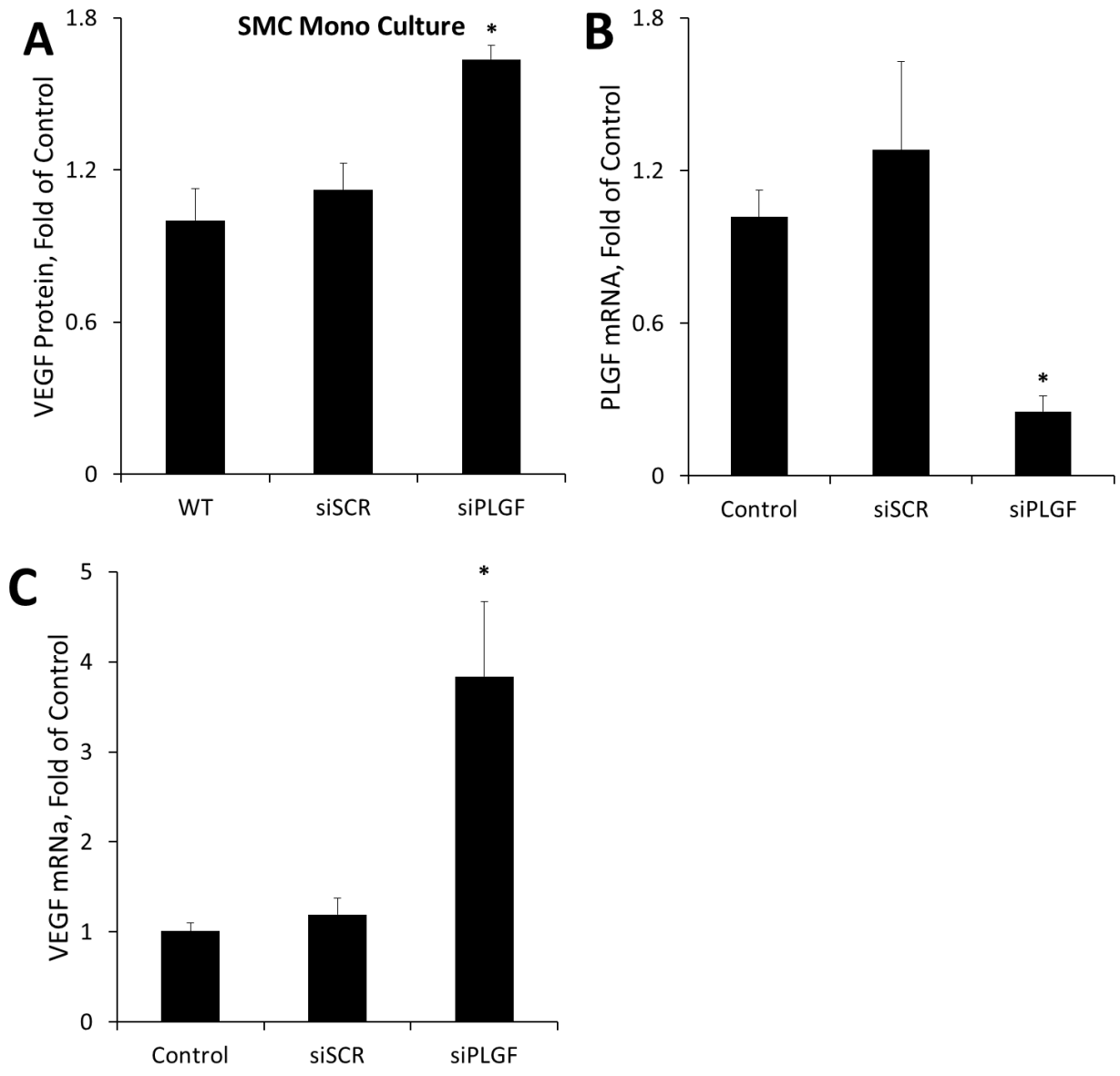

Figure 4. Effect of knockdown of PLGF in HCASMC monoculture. (A) Knockdown of PLGF in HCASMC monocultures increased VEGF-A protein in culture media. (B) siPLGF decreased PLGF mRNA to ~25% of control in HCASMC monocultures (n=5, *p<0.05 compared to both control and siSCR). (C) PLGF knockdown in HCASMC monoculture significantly increased VEGF-A mRNA (n=5, *p<0.05 compared to both control and siSCR).

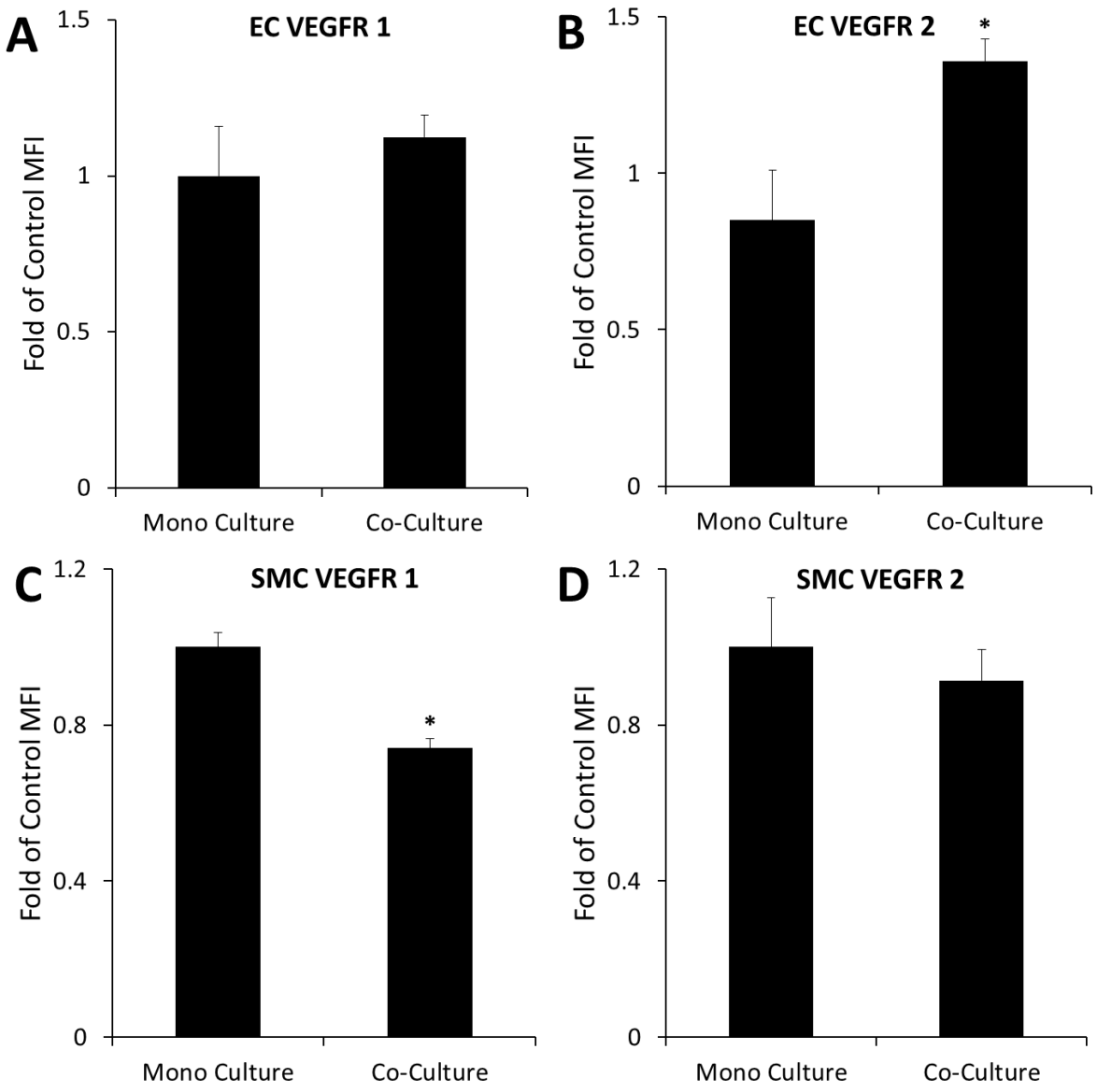

Figure 5. VEGFR-1 and VEGFR-2 expression in EC and SMC monoculture and coculture. (A) For VEGFR-1 no difference in MFI was detected between EC in monoculture and coculture. (B) MFI was significantly increased for VEGFR-2 in cocultured EC compare to monoculture EC. (C) SMC VEGFR-1 was significantly decreased in coculture compare to monoculture. (D) no significant change MFI was detected between SMC monoculture and coculture (n=5, *$p<0.05$ compared to monoculture)

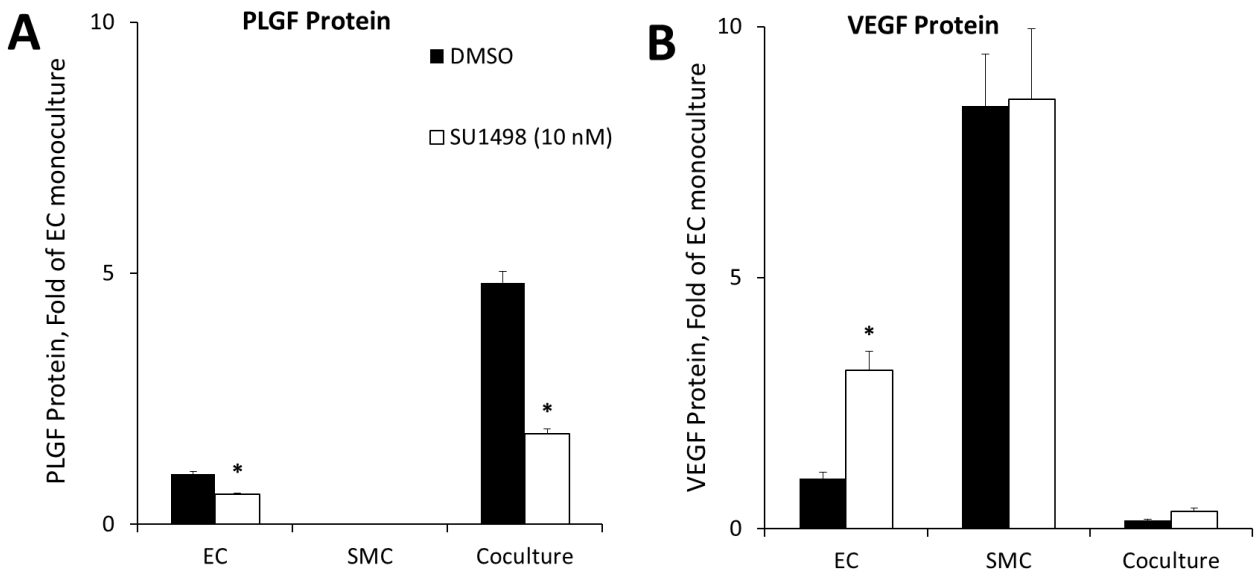

Figure 6. SU1498 inhibits PLGF expression and induces VEGF expression. (A) SU1498 significantly reduced PLGF protein in the media of both EC monocultures and cocultures. PLGF was below the limit of detection for SMC monocultures. (B) VEGF protein was significantly increased in SU1498 treated EC and coculture culture media. No significant difference was observed in SMC monocultures (n=5, *$p<0.05$ compared to DMSO)

CHAPTER VI

CONCLUSION

The overall objective of this study was to delineate the mechanisms regulating PLGF expression in the vascular wall during coronary arteriogenesis. Our central hypothesis was that PLGF expression in the vessel wall is regulated by fluid shear stress. The rationale for the proposed research is that both fluid shear stress and PLGF are integral to collateral growth and an understanding of the mechanisms by which fluid shear stress regulates PLGF would provide insight into growth factor regulation in general. To test this central hypothesis two specific aims were pursued aims:

1. Characterize the effects of increased fluid shear stress on PLGF expression in isolated perfused vessels and in a vascular smooth muscle cell/vascular endothelial cell co-culture system.
2. Characterize the pathway by which increased fluid shear stress regulates PLGF expression.
3. Characterize the effects of coculture on PLGF and VEGF expression.

The main findings of this study are that fluid shear stress does indeed regulate PLGF expression. These effects of fluid shear stress are mediated by hydrogen peroxide that is most likely Nox4 derived. HO-1 activity was also shown to be necessary for fluid shear stress to affect PLGF expression. We also demonstrated that PLGF and VEGF affect each other's expression and are differentially regulated in coculture. In this study we identified key regulators of arteriogenesis and demonstrate their role in the regulation of PLGF under flow conditions. The overarching findings described above further emphasize PLGF as a keystone of arteriogenesis.

FSS upregulated PLGF expression in both intact perfused vessels and in the vascular cell coculture model, we also demonstrate FSS-induced PLGF expression is dependent on H2O2.. In support of this finding and the role for H2O2 in arteriogenesis, increasing O2•− and decreasing H2O2 by knocking out endothelial SOD results in diminished collateral remodeling in the mouse ischemic hindlimb1. Antioxidant treatment which decreases both O2•− and H2O2 also decreases blood flow recovery after femoral artery ligation1-3. Furthermore, exogenous H2O2 increases PLGF expression in HCASMC4, 5. Nox are the only family of enzymes that are dedicated to the production of ROS 6. Inhibition of Nox causes a reduction in collateral remodeling in follow femoral artery ligation in mice 7 and several in vivo models have demonstrate a role for Nox in the remodeling process 3, 8-10. We found Nox4 expression to be increased by FSS agreement with previous studies11-13, intracellularly Nox4 is associated with the proteins implicated in sensing fluid shear stress14, 15. Nox 4 predominantly produces H2O2 and is constitutively active 16. In agreement with previous data we found Nox4 to be the predominant isoform in our coculture model17, 18. Flow recovery in Mice over expressing Nox4 in the endothelium following hindlimb ischemia is more rapid than wild type controls 10. Conversely, Nox4 knockout mice show poor recovery of hindlimb perfusion after femoral artery ligation 9. Knockdown of Nox4 in the endothelial cells of our coculture model attenuated the effects of

shear stress on both PLGF and H2O2, this suggest Nox4 was the source of FSS induced H2O2 which is necessary for FSS to regulate PLGF expression.

We next set out to identify pathways downstream of Nox4 that contribute to the regulation of PLGF by FSS. Schroder et. al. suggested that Nox4 produced H2O2 induces the nuclear accumulation of NF-E2-related factor 2 (Nrf2) and increased HO-1 expression who's activity facilitates the remodeling process9. The Nrf2 pathway is the major antioxidant response to oxidative stress 19. During oxidative stress Nrf2 is freed from keap1 suppression and allowed to translocate to the nucleus where it binds to antioxidant response element (ARE) domains in the promoter region of multiple antioxidant enzymes including heme oxygenase 1 (HO-1). Interestingly, Nrf2 knockout results in improved blood flow recovery in mice following femoral artery ligation20, 21 this is likely to be the result of a decrease in Nrf2's anti-inflammatory effects. Contradictory to the hypothesis that Nrf2 plays a beneficial role in collateral remodeling. HO-1 on the other hand is up regulated in the hindlimb following femoral artery ligation9, 22. The expression of PLGF and HO-1 are both significantly increased in mouse hind limb skeletal muscle early on following femoral artery ligation23. FSS induces HO-1 activity and expression in endothelial cells and is dependent on Nox derived ROS 11, 24, 25. While HO-1 haploinsufficiency in mice causes a decrease in PLGF expression and a reduction in revascularization following hindlimb ischemia 26. We report that FSS increases HO-1 mRNA and that inhibition of HO-1 attenuates the effects of FSS on PLGF. Furthermore, Knockdown of HO-1 in the endothelial cells of the coculture model resulted in abrogation of the effect of FSS on PLGF. Consistent with these findings and with the role of PLGF in recruiting monocytes to the vessel wall27 inhibiting HO-1 in vivo reduces recruitment of circulating cell to the vessel wall along with decreased collateral remodeling following femoral ligation22.

HO-1 catabolizes heme into three products biliverdin, CO, and divalent iron. Biliverdin and CO both caused a decrease in PLGF expression in cocultures. One explanation of these

findings is that both these metabolites have antioxidant properties28-30 31-33. This would result in a decrease in H_2O_2 which in turn would be expected to decrease PLGF expression.

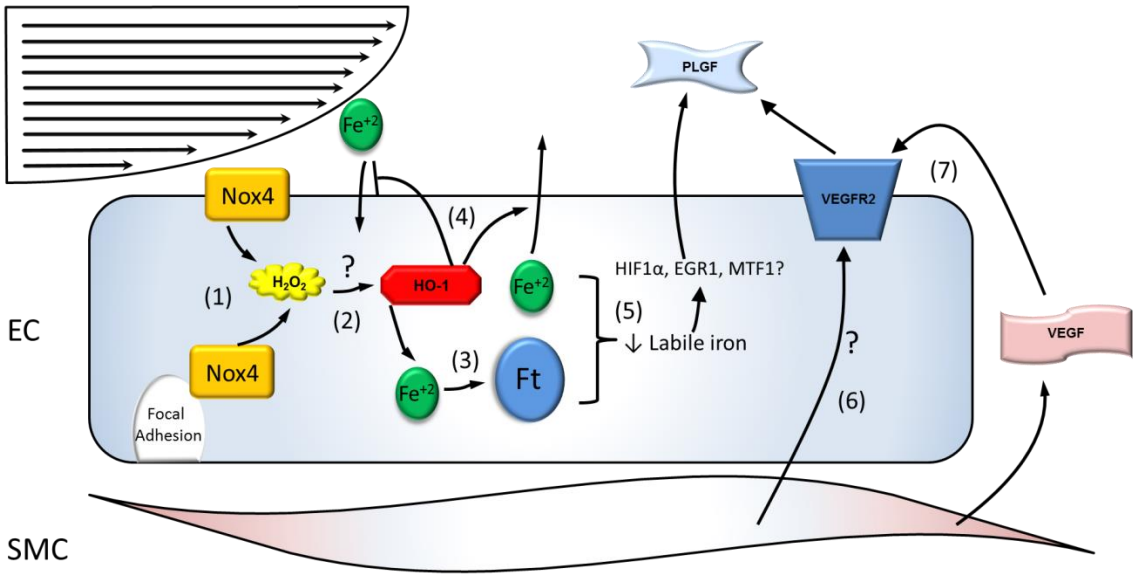

Figure 1. Diagram of proposed signaling pathway. (1) FSS increases NOX4 activity and H_2O_2 production. (2) H_2O_2 induces HO-1 by an unknown mechanism. (3) HO-1 activity releases Fe^{+2}, which induces Ft, which in turn sequesters iron (4). HO-1 activity also increases iron efflux and decreases iron influx (5); these effects result in an overall decrease in labile iron. We propose that this decrease upregulates PLGF by activating one or more of several possible iron sensitive transcription factors. (6) SMC induce an increase in EC VEGFR2 by an unknown mechanism. We hypothesize that this effect increases the sensitivity of EC to FSS, promoting upregulation of PLGF by FSS. (7) VEGF from SMC increases EC PLGF expression. (EC; endothelial cells, SMC; smooth muscle cells, NOX4; NADPH oxidase 4, HO-1; heme oxygenase 1, Ft; ferritin, VEGFR2; VEGF receptor 2).

HO-1 activity results in the release of labile iron. Labile iron can cause oxidative stress by taking part in Fenton reactions and is tightly regulated in all mammalian cells. The primary method is by sequestering iron in ferritin. HO-1 activity also induces an increase in ferritin

translation34. HO-1 Activity and increased labile iron have been linked to increased iron efflux 35-37. In general HO-1 activity can be considered to decrease the amount labile intracellular iron by increased efflux and sequestration in ferritin. This decreased iron has been previously demonstrated to induce several pro arteriogenic growth factors such as HIF-1α, EGR-1, and MTF-1 38-40 of which EGR-141 and MTF-142 have been demonstrated to be transcription factors of PLGF. We next provided evidence for a decreased labile iron pool increasing PLGF expression by chelating iron with either deferoxamine or 1,10-phenanthroline. Both increased PLGF expression. While others report that treatment with DFO results in enhanced flow recovery following hindlimb ischemia compared to vehicle treated control in mice 43 and in rabbits44. Due to similarities between the effects of fluid shear stress and hypoxia we determined how hypoxia affects PLGF expression. Expectedly we found it to also increase PLGF expression we also found endothelial HO-1 expression to be necessary for hypoxia to affect PLGF expression. Considering the central role of PLGF in arteriogenesis these data suggest HO-1 as common pathway for FSS and hypoxia to induce arteriogenesis.

We report that PLGF expression is significantly increased in coculture compared to HCAEC monoculture. We also report that HCASMC predominant produce VEGF-A. We then determined the autocrine and paracrine effects of VEGF-A on PLGF expression and concluded that VEGF-A is a positive regulator of PLGF in agreement with previous studies 45, 46. These finding would suggest that HCASMC induce an increase in HCAEC PLGF production via VEGF-A. Indeed, we found that treating cocultures with a VEGFR-2 inhibitor significantly reduced secreted PLGF. We also found cocultured endothelial cells to be more sensitive to FSS than monocultured cells. VEGFR-2 is considered to play a role in the mechanosensing of FSS47, 48. We found cocultured HCAEC to express significantly higher VEFGR-2 which would likely increase their sensitivity to FSS. Our finding suggest that there is significant variation between of vascular cells cultured separately or together.

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APPENDIX

The following appendix contains preliminary data from experiments that were carried out in studies that were either not pursued further, or were on going at the time of writing.

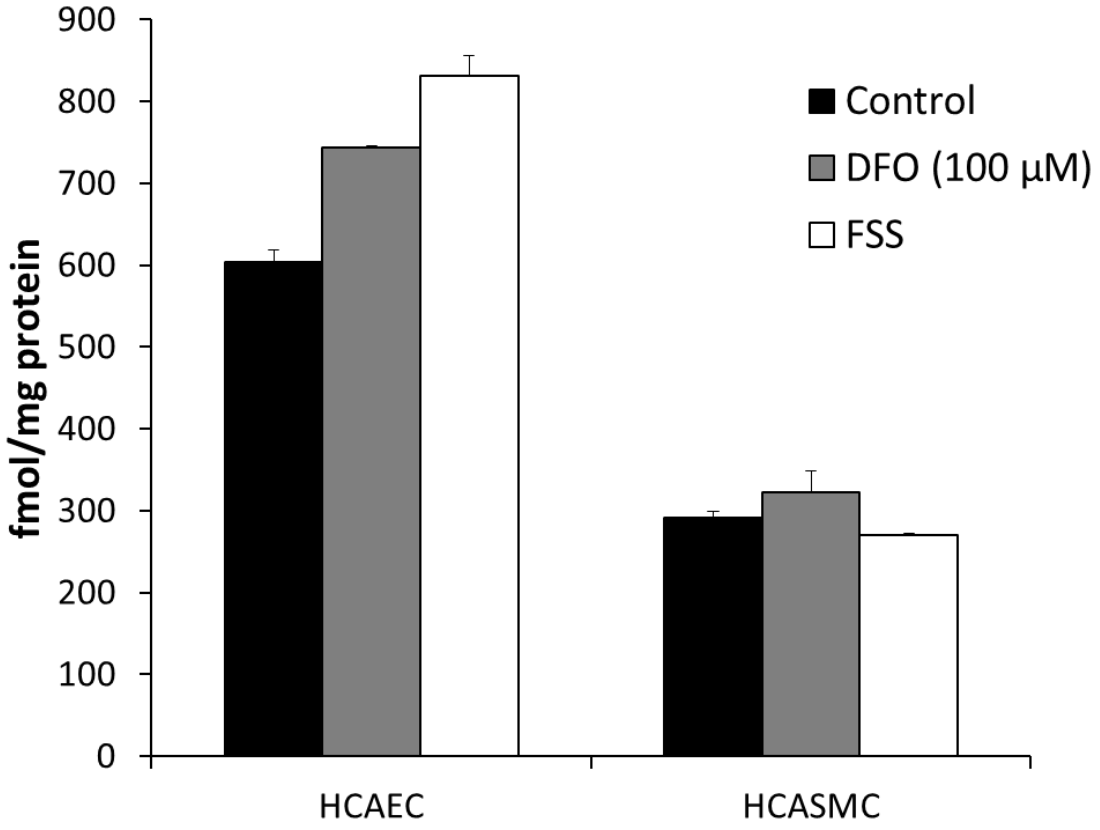

Figure 1. Shear stress increases iron response protein binding to RNA iron response elements. In endothelial cells cocultured with smooth muscle cells FSS induced an increase in IRP binding similar to that of iron chelation with DFO (100 μM). FSS had no effect on IRP binding in smooth muscle cells. These data support the hypothesis that FSS induces a reduction in the labile pool of iron within endothelial cells.

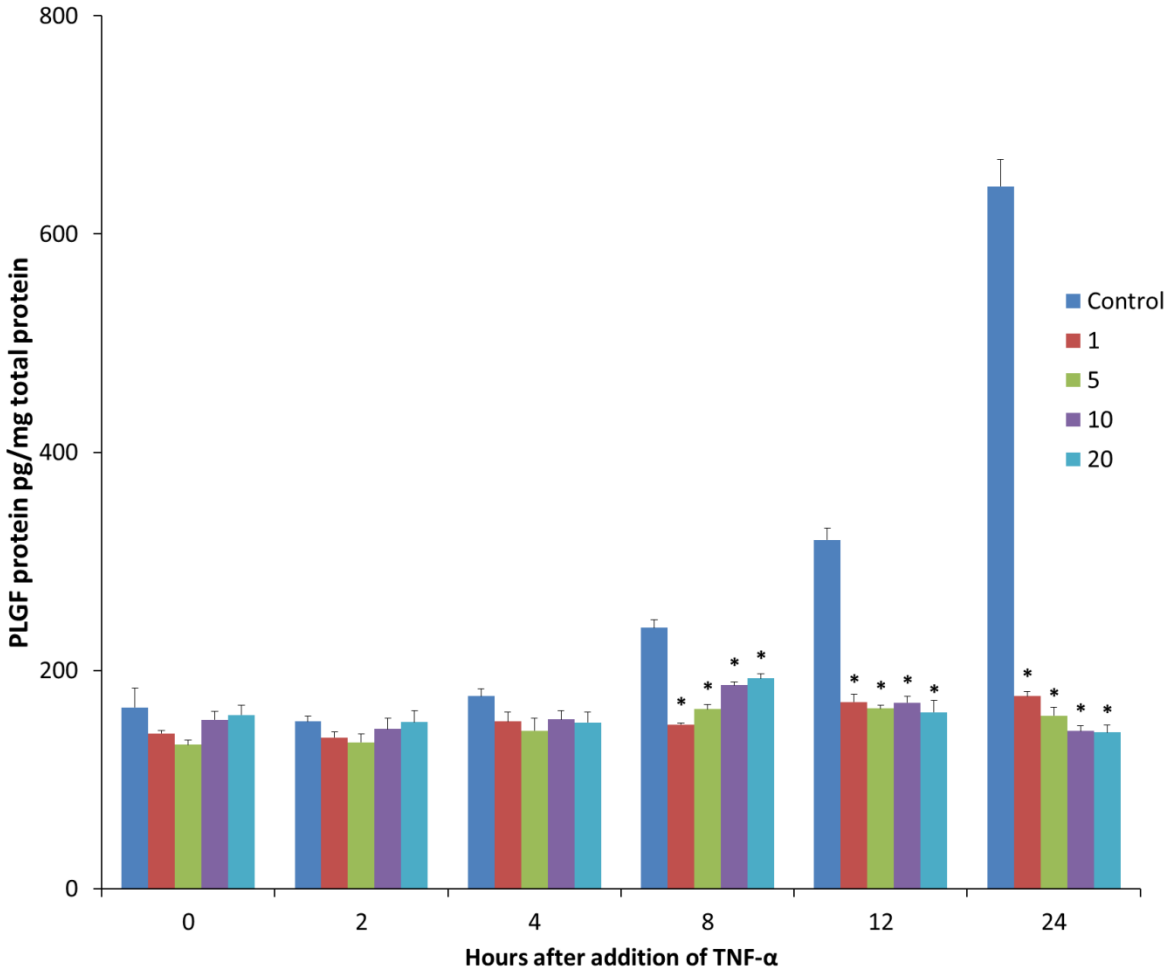

Figure 2. Effects of TNF-α on PLGF expression in endothelial monoculture. Endothelial monocultures were treated with 1-20 ng/ml TNF-α media was collected at the indicated times and assayed for PLGF protein as described above data was normalized to media total protein. All concentrations of TNF-α used inhibited PLGF production by endothelial cells (n=3 *p<0.001).

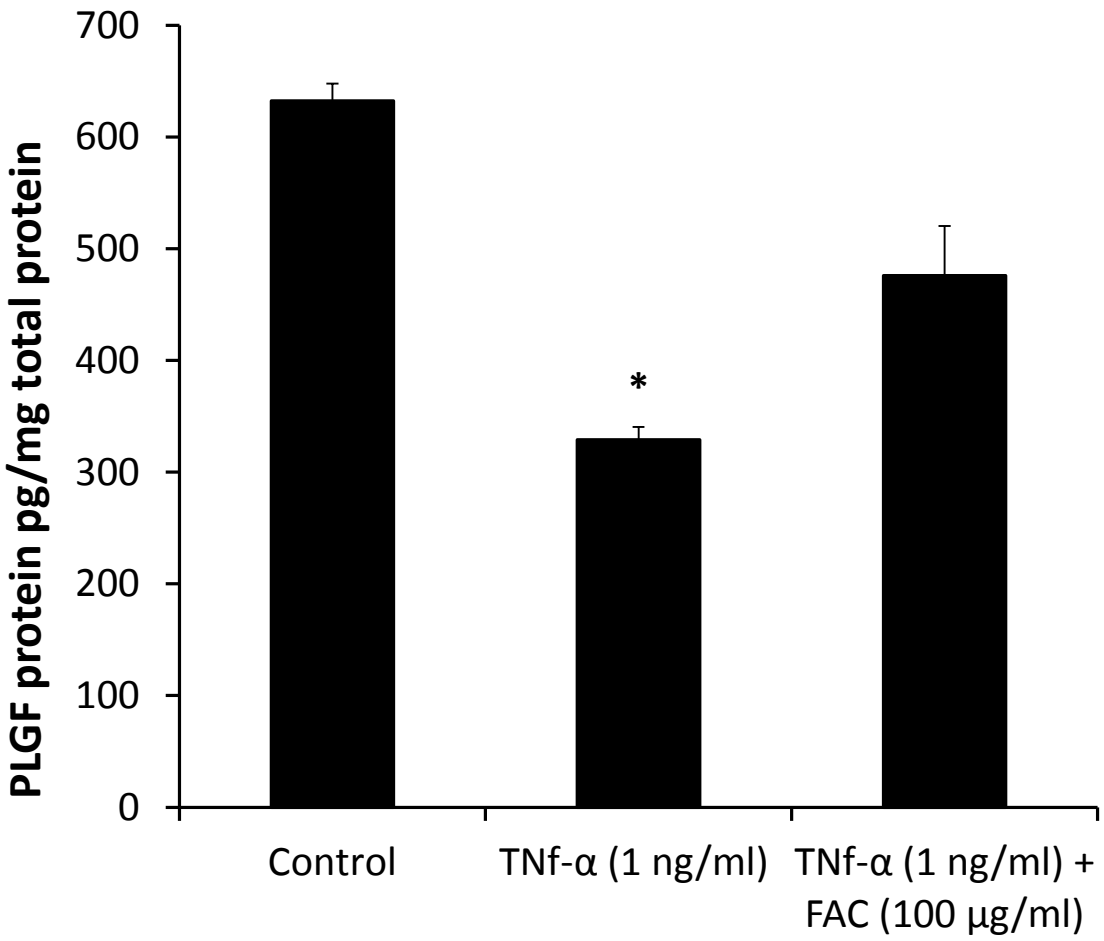

Figure 3. FAC partially restores PLGF expression during TNF-α treatment. Endothelial monocultures were treated as above with TNF-α in a separate group FAC was also added to the culture media cells were incubated for 24h at which time media was collected and analyzed for PLGF protein (n=5, * $p<0.05$).

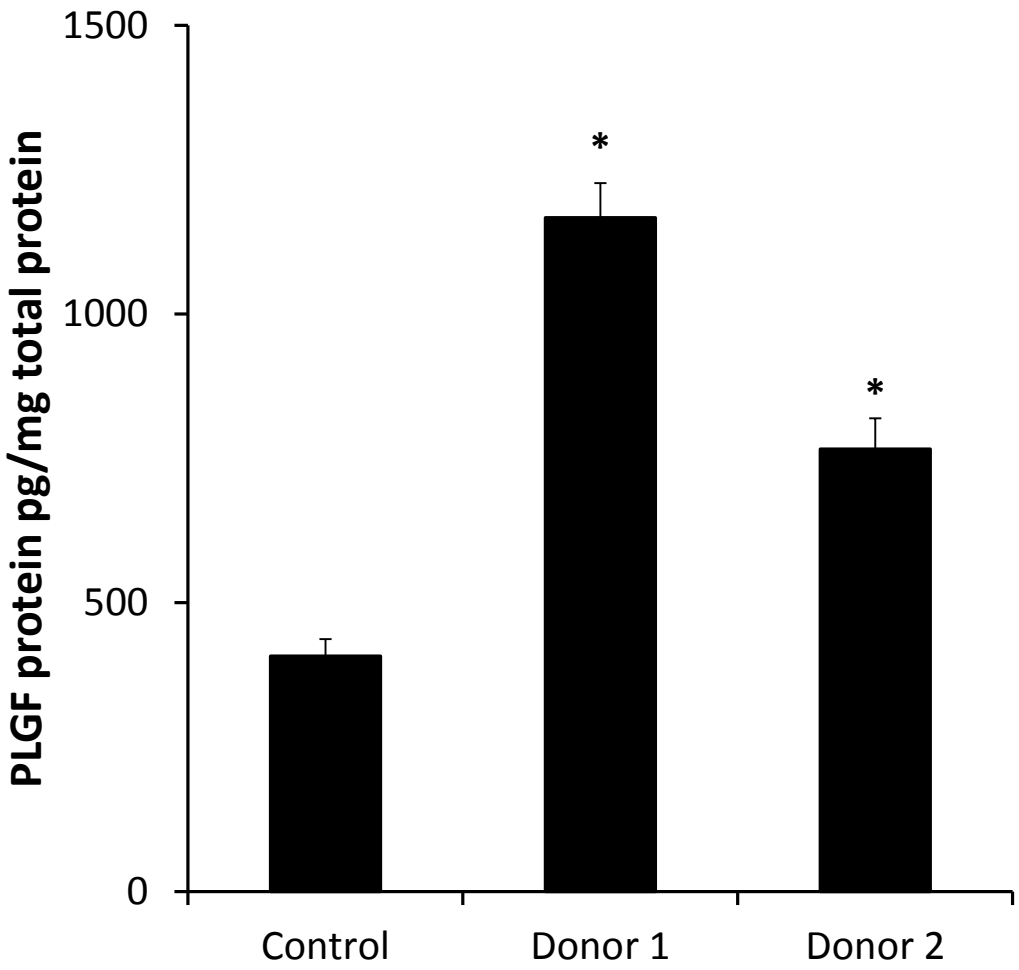

Figure 4. PLGF expression is up regulated in endothelial cells sourced from donors diagnosed with type II diabetes ante mortem. Cells were grown to confluence in 75 cm^2 flasks at which time cells were trypsinized and seeded at a density of $3x10^5$ cells/well. Cell cultures were grown to confluence in 6 well plates. Culture media was replaced for reduced serum media for 24h and samples collected and analyzed for PLGF protein (n=5, * $p<0.001$).

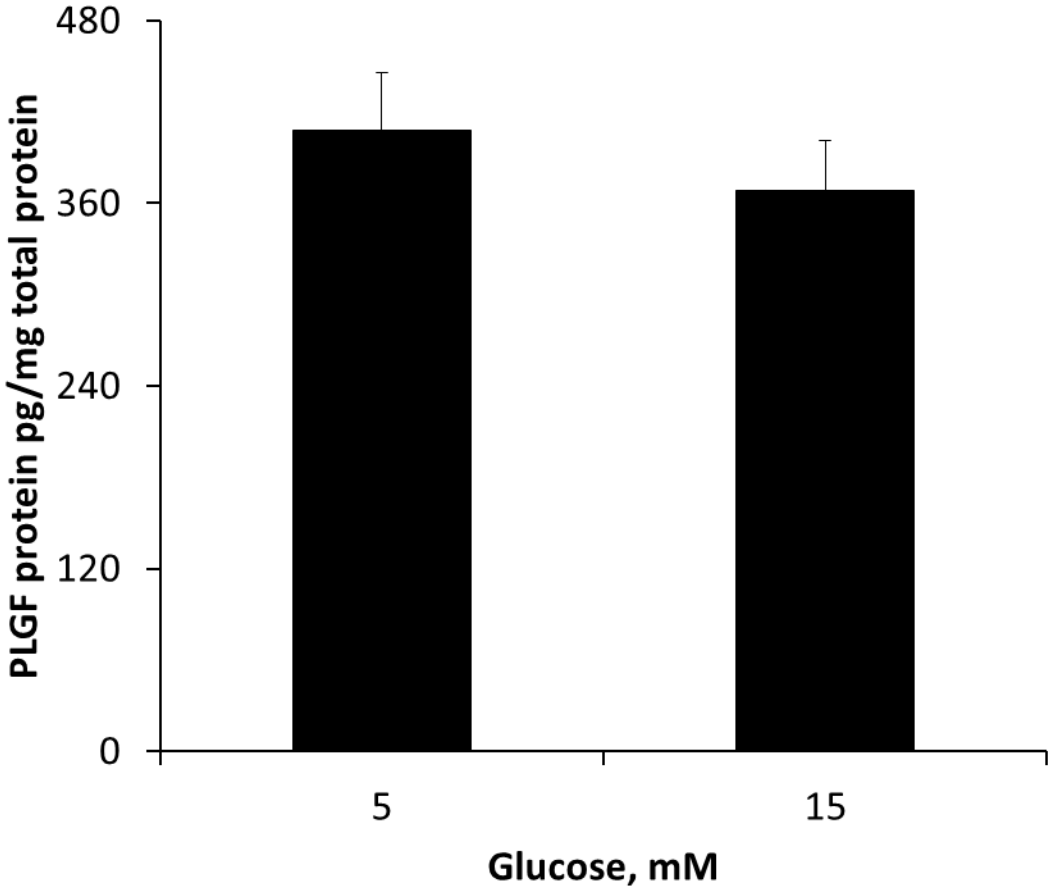

Figure 5. Effect of glucose concentration on PLGF in endothelial cells. Control donor endothelial cells were cultured as above. Media was supplemented with glucose to a final concentration of 15 mM in serum reduced media for 24h. 15 mM glucose had no significant effect on PLGF protein concentration in culture media (n=5).

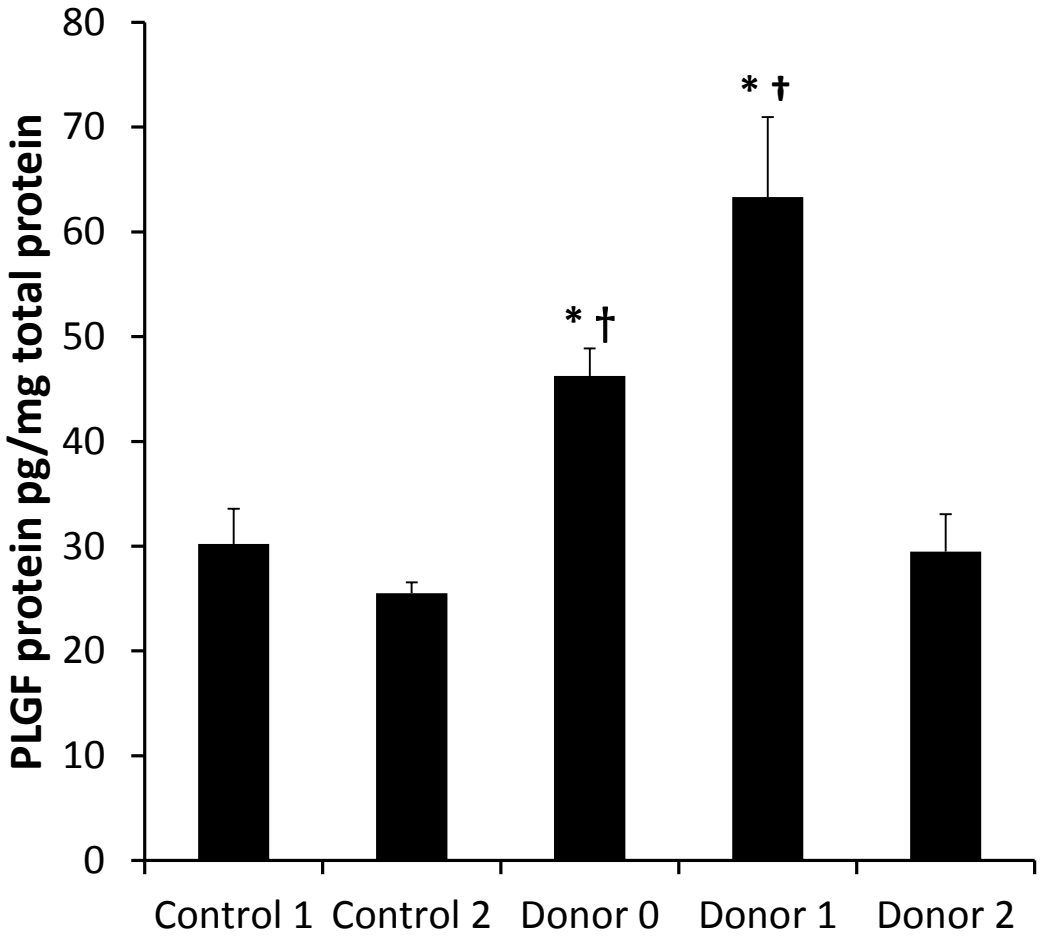

Figure 6. PLGF expression is up regulated in smooth muscle cells sourced from donors diagnosed with type II diabetes ante mortem. Samples were prepared as described above for endothelial cells. Donors 0 and 1 showed significantly higher PLGF protein in their culture media, compared to both control donors (n=4, * $p<0.01$ vs. control 1, † $p<0.01$ vs. control 2).

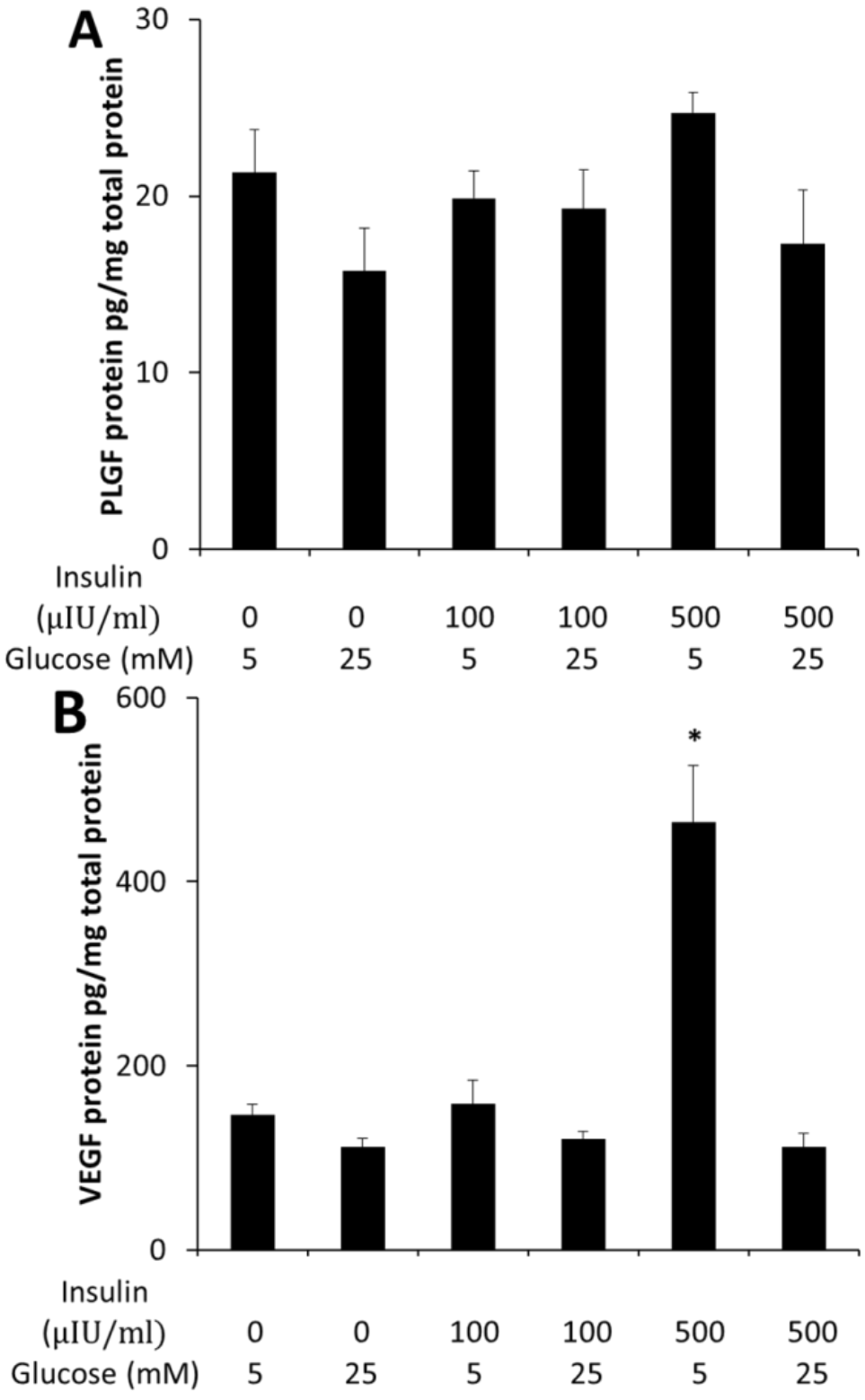

Figure 7. Donor Control 1 cells were cultured as outlined above in 6 well plates. Culture media was supplemented with the treatments outline above and incubated for 24h (n=3, *p<0.05).

VITA

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