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eNOS and TMEM184A in Heparin Signaling effects on Vascular Cells

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12B1/18H6	Anti-Heparin Receptor Monoclonal Antibodies
A7r5s	Cloned Rat Vascular Smooth Muscle Cells
AKT	Protein kinase B
BAOECs	Bovine Aortic Endothelial Cells
Co-IP	Co-immunoprecipitation
Elk1	ETS domain-containing protein Elk1
eNOS	Endothelial Nitric Oxide
ERC	Endocytic Recycling Compartment
ERK	Extracellular Signal-Regulated Kinase
FAK	Focal adhesion kinase
GFP	Green Fluorescent Protein
HeBS	Hepes-Buffered Saline
kDa	Kilodaltons
PDGF	Platelet-Derived Growth Factor
PKA	Protein Kinase A
PKG	cGMP-dependent protein kinase
MAPK	Mitogen-Activated Protein Kinase
MKP-1	MAPK Phosphatase-1 (DUSP1)
NO	Nitric Oxide
ROCK	Rho-associated protein kinase
TGN	Trans-Golgi-Network

TMEM184A	Transmembrane Protein 184A
VAMP	Vesicle associated membrane proteins
VE-cadherin	Vascular Endothelial Cadherin
VEGF	Vascular Endothelial Growth Factor
VSMC	Vascular Smooth Muscle Cell

ABSTRACT

Heparin, which is a glycosaminoglycan, was originally found and used as an anti-coagulant. But it has also been implicated in binding to vascular cells, down-regulating their proliferation and inflammation responses at least through MAPK pathways [1], [2] by employing the PKG pathway [3]. Previous studies have indicated that heparin increases eNOS(endothelial nitric oxide synthase) activity in bovine endothelial cells. However, the precise mechanism has not been fully elucidated.

Immunoprecipitation in endothelial cells by using monoclonal heparin binding blocking antibodies identified a protein as a gene product of TMEM184A, to be a putative heparin receptor [4].

In this study, we investigated the impact of heparin treatment on eNOS's activation and the role of eNOS in heparin signaling. The results indicate that heparin induced phosphorylation and activation of eNOS. eNOS was internalize to perinuclear region in a TMEM184A dependent manner in response to heparin. We also examined how heparin treatment lead to phosphorylation of eNOS and confirmed that TMEM184A and Ca^{2+} were required to mediate heparin elicited eNOS phosphorylation. Integrin and TRPV4 are demonstrated to be potentially involved in this activation process on eNOS.

Chapter 1: Introduction

1.1 Heparin and its signaling

Heparin, which is a glycosaminoglycan, was originally found and used as an anti-coagulant. But it has also been implicated in binding to vascular cells, down-regulating their proliferation and inflammation responses at least through MAPK pathways [1], [2] by employing the PKG pathway [18]. However, detailed and comprehensive mechanisms for such functions of heparin remain unclear.

In vivo data suggest that low-anticoagulant heparin can inhibit adhesion of leukocytes to endothelium [5] and the binding is a key response to inflammation. Proteolytic enzymes and toxic oxygen radicals are released following excessive leukocyte activation and consequently lead to vascular and tissue damage.

P-selectin is mainly expressed on activated endothelial cells and responsible for loose association between neutrophils and the endothelium. This interaction is effectively inhibited by heparin [6]. When neutrophils were incubated with heparin or low anticoagulant derivatives, heparin was able to inhibit adhesion of these cells to endothelial cell monolayers that are induced to produce P-selectin [7].

The ability of heparin to bind chemokines, cytokines and other mediators released from inflammatory cells is also considered to contribute to heparin's anti-inflammatory effects because such binding inhibits these pro-inflammatory molecules [8] from binding with the respective receptors.

The anti-inflammatory effects of heparin are also observed on cultured vascular cells. Heparin is observed to bind endothelial cells and inhibit inflammation via NF-κB and stress kinases pathways [12]. NF-κB regulates many genes that increase production of

inflammatory and immune response genes, including interleukin-6 and 8, ICAM-1, L- and P-selectins [10]. Heparin has been thought to bind to and be endocytosed into the cytosolic compartment of endothelial cells and VSMCs [11] and prevent NF-κB from translocating to nucleus [12].

Heparin is also found to bind [13] and reduce the growth of VSMCs [14], decrease MAPK signaling [15] and thereby inhibits early proto-oncogene, AP-1, promoters controlling gene expression, and the G1 phase of the cell cycle. Heparin treatment reduces expression of c-fos and c-myc in serum, PDGF, or bFGF activated cells [13] and down-regulates cyclin dependent kinase 2 activity by up-regulating levels of p27kip1 [16]. Heparin also promotes synthesis of MKP-1 in VSMCs that regulates activity of ERK [17].

Published data indicate that protein kinase G signaling is at least partially involved in heparin's anti-proliferation effects on VSMCs. PKG inhibitors down-regulate heparin-induced ERK, Elk-1 inactivation, MKP-1 synthesis, and eliminate heparins' effect on bromodeoxyuridine incorporation. Knocking down PKG also decreases heparin responses in activated VSMCs [18]. Moreover, the cGMP level is increased transiently after heparin treatment [18].

Immunoprecipitation in endothelial cells by using monoclonal heparin binding blocking antibodies identified a protein as a gene product of TMEM184A, to be a putative heparin receptor[4].

1.2 Heparin signaling and eNOS

1.2.1 Introduction on eNOS

The messenger molecule nitric oxide (NO) is important in regulating cardiovascular physiology including vasodilators, antioxidants, and platelet inhibitors. NOSs are enzymes that are responsible for biosynthesis of NO and L-citrulline from L-arginine. Endothelial nitric oxide synthase (eNOS) is the predominant NOS isoform in synthesizing most of vascular nitric oxide (NO), which is an upstream factor that can induce PKG signaling events. eNOS has been implicated in heparin signaling events and consequently protecting vascular functions [19].

There are 3 well-characterized NOS isoforms that can be differentiated by their subcellular localization and regulatory mechanisms, the eNOS (endothelial), nNOS (neuronal), and iNOS (inducible) isoforms.

nNOS (neuronal) is mostly expressed in peripheral and central neurons and in various other cell types. eNOS (endothelial) is largely present in endothelial cells and also expressed in vascular smooth muscle cells (VSCMs), cardiac myocytes and blood platelets. eNOS and nNOS are constitutively expressed while their activity is controlled by calcium levels, phosphorylation and through other mechanisms. eNOS is the predominant NOS isoform in synthesizing most of vascular nitric oxide (NO), which is an upstream factor that can induce PKG signaling events. Heparin has been implicated in regulating eNOS5 and consequently protecting vascular functions [19].

1.2.2 Heparin and eNOS

Numerous studies support the hypothesis that heparin and its derivatives can regulate eNOS and can thereby protect endothelial function. Both heparin and its non-anti-

coagulant derivative, N-acetyl heparin, dose-dependently increase eNOS activity by promoting citrulline and nitric oxide metabolite formation in BAOECs [20]. In vivo experiments suggest that heparin can increase NOS activity and cGMP levels in lung at both three days and three weeks [19].

Moreover, eNOS might be mediating heparin's signaling because it is a upstream factor of PKG, and PKG has been shown to be involved in heparin signaling [18]. Activation of eNOS leads to production of NO which stimulates soluble guanylate cyclase (sGC) and that sGC results in accumulation of cGMP which leads to activation of the PKG [21]. Therefore, heparin treatment might affect PKG and cGMP through regulation of eNOS. However, heparin has also been reported to suppress Akt at least in some cell types. Treatment with heparin leads to significant reduction in cell viability and a significant decrease in phosphorylated Akt at 24 hrs in SCC cell lines [22]. Akt is a serine/threonine protein kinase that plays important roles in regulating many biological processes, including proliferation, cell growth and apoptosis. Akt is able to phosphorylate eNOS at ser 1177 to activate it. There have also been studies showing that heparin can compete with endogenous heparan sulfate and disrupt interactions between PECAM-1 and Gaq/11 [23] and thereby may decrease the activity of Akt. According to these results, heparin might also down-regulate eNOS by decreasing its phosphorylation at p1177 at some time after treatment in some cell types.

1.2.3 Introduction on eNOS Regulation

eNOS is regulated through various cellular events including post-translational modification, calcium wave stimulation, association or dissociation with its binding proteins. The pathways are illustrated in Figure 1 [24].

Intracellular Ca^{2+} increases can cause calmodulin binding to nNOS and eNOS and hence facilitate electron transfer from the reductase domain to the oxygenase domain of the NOS to synthesize NO. NO consequently activates soluble guanylate cyclase (sGC) and downstream signaling pathways. eNOS has vasoprotective and anti-atheroslerosis functions contributing to controlling vascular tone, angiogenesis, and other effects.

Internal electron transfer is required for the synthesis of NO by eNOS. The binding between Ca^{2+} and calmodulin regulates internal electron transfer through redox-linked conformational changes. CaM binds to eNOS at increased intracellular Ca^{2+} concentration [25] and couples electron transfer between the reductase domain and the oxygenase domain [26].

eNOS can be regulated through multiple post- translational modifications including acylation, nitrosylation, and phosphorylation which can dynamically regulate not only activity but also its localization and its interaction with other cellular elements.

A major principle of post-translational regulation of eNOS activity is through phosphorylation, multi-site phosphorylation, and dephosphorylation at key serine and threonine residues in eNOS. Stimulatory phosphorylation loci include Ser1177 [27], Ser 617, and Ser 635, while inhibitory sites are Thr 495 and Ser 116 [28].

Most of the phosphorylation events control activity of eNOS by affecting affinity between calmodulin and eNOS. For example, phosphorylation at Ser 1177 in eNOS

inhibits dissociation of calmodulin from eNOS and enhances intermodule electron transfer of eNOS by unlocking the FMN domain [28].

Another stimulatory phosphorylation at Ser 617 makes eNOS sensitive to calmodulin binding [29]. Phosphorylation at Thr 495, which is mediated by PKC and AMPK, weakens the association between calmodulin and eNOS to reduce activity of eNOS [30]. Protein phosphatase 2A or phosphatase 1 appears to dephosphorylate Thr 495 and enhance binding of calmodulin to eNOS and derepress acitivity of it [31].

1.2.4 Life Cycle and Signaling Pathways of eNOS.

Under physiological conditions, p-Ser 1177 of eNOS is phosphorylated in response to shear stress, hormones and autocoids such as bradykinin [32]. Insulin and adipokines pathways also regulate phosphorylation of Ser1177 [33].

A large number of kinases, as Akt (protein kinase B), CaM kinaseII (calcium/calmodolun-dependent protein kinase ii), and AMPK (AMP-activated protein kinase) catalyze phosphorylation at ser 1177 of eNOS. Besides kinases, phosphatases such as calcineurin and PP1 play important role in manipulating phosphorylation [34]. PKA dependent agonists and basal stimuli such as shear stress activate PKA and induce Phosphorylation at Ser 635 [28].VEGF promotes dephosphorylation at Ser116 and upregulates activity of eNOS [19].

eNOS can also be regulated by numerous eNOS binding proteins including caveolins. Caveolins are caveolar membrane proteins that have transmembrane domains and reversible C-terminal palmitoylation. In endothelial cells, caveolin-1 and caveolin-2 are prevalent [35]. The membrane association and oligomerization of caveolins determines

the structure of caveolin. Caveolin also serves as a scaffold for eNOS and other proteins to form signaling complexes.

Although not necessary for localizing eNOS to caveolae, caveolin binds with and inhibits the activity of eNOS. Caveolin not only hinders caveolar-targeted receptor signaling but also blocks calmodulin binding sites in eNOS [35]. Dissociation from caveolin association is a vital early step in activating eNOS. Intracellular calcium, calmodulin and shear stress can promote detaching eNOS from cav-1.

Caveolins are increasingly characterized as contributing to assembly and regulation of signaling effectors such as VEGF receptors, PI3K, c-Src kinase, estrogen receptors, etc. Many of these effectors are tightly associated with eNOS regulation [36].

eNOS can also be associated with and regulated by GPCRs. Many GPCRs are linked to Gαi or Gαq that stimulate phospholipase C (PLC) that result in mobilization of calcium through IP3 mediated signaling events and thereby activate eNOS [37]. These GPCRs respond to ligands such as bradykinin, histamine, acetylcholine, S1P, ADP/ATP, and thrombin[38]. Subsequent to GPCR stimulation, calcium waves are started initially at caveolae, which is confirmed by high resolution microscopy [39].

Another eNOS activating mechanism besides intracellular calcium mobilization, the PI3K/Akt cascade also assists in VEGF-induced eNOS activation. GPCR mediated activation of PI3K produces PIP3 (phosphatidylinositol (3,4,5)-trisphosphate) from PIP2 (phosphatidylinositol (4,5)-bisphosphate). Akt is recruited to plasma membrane by PIP3 and phosphorylated by phosphoinositide dependent kinase. Phosphorylation of Akt

allows it to be stabilized in the active conformation and translocated to the cytoplasm or nucleus to phosphorylate its targets, including eNOS [38].

In addition, GPCR is found to transactivate RTKs and integrins by Gα or Gβy to activate PI3K/Akt pathways [40]. Ca^{2+} waves activate eNOS by helping calmodulin to bind eNOS, similarly, after activation, Akt can phosphorylate eNOS at ser 1177, which enhances affinity between eNOS and calmodulin and promotes electron transfer and upregulates activity of eNOS [30].

In response to shear stress, Ca^{2+} activated PI3K/Akt pathway leads to phosphorylation of eNOS at Ser 1177 and consequently contributes to maintaining basal vascular tone [28].

Under quiescent conditions, eNOS is anchored to caveolae by co-translational myristoylation and post-translational palmitoylation [41]. Caveolin binds to eNOS to confer tonic inhibition [42]. Stimulations can alter post-translational modifications on eNOS [28] to increase its activity. Simultaneously, Ca^{2+} /calmodulin and Hsp90 replace caveolin to bind with and activate eNOS [43].

A slower process of inactivation and internalization of eNOS follows the rapid response to agonist stimulation and eNOS activation. eNOS is depalmitoylated to allow translocation of eNOS from caveolae to intracellular compartments including peri-nuclear region, Golgi, mitochondria and cytoskeleton. It has been reported that the destination of eNOS translocation differs according to different agonists. For example, acetylcholine prefers translocation to Golgi while platelet-activating factor relocalizes eNOS to the cytosol [44].

During translocation, eNOS is deactivated through several mechanisms, including inhibitory phosphorylation and dephosphorylation of stimulatory loci, uncoupling of eNOS from upstream signaling molecules and events, and moving away from the calcium wave. Re-nitrosylation also occurs when eNOS is internalized [45].

eNOS eventually relocalizes to the caveolar membrane by reassociation with caveolin and re-nitrosylation. Palmitoylation is involved in eNOS re-targeting to caveolae [46].

eNOS is responsible for synthesizing most of vascular NO. NO synthesized by eNOS is a key gaseous free radical that regulates a large number of cardiovascular and cellular events, such as endothelial cell migration, extracellular matrix degradation, proliferation, controlling vascular tone, angiogenesis, platelet function, mitogenesis, etc.

Besides NO, eNOS is also able to produce superoxide $O2\cdot-$, which reacts with NO to form peroxynitrite ($ONOO-$) with diminished levels of BH4 (eNOS uncoupling). This transformation of eNOS into an oxidative stress-promoting enzyme is found in cardiovascular diseases [47].

1.2.5 eNOS and Shear Stress

The steady, undisturbed laminar blood flow creates a constant shear stress in straight arteries along the EC surface. The exposure of EC to laminar shear stress leads to anti-atherogenic processes, which prevents lesions in the laminar flow prevalent straight arteries. Expression of anti-atherogenic genes including SOD [48], p53 [49], interleukin 1β-converting enzyme and capase-3 [48] are regulated in response to laminar shear stress. One of the best characterized and important shear stress responsive genes is eNOS, which catalyzes the production of NO while NO inhibits atherogenesis by repressing SMC

proliferation and migration, leukocyte adhesion and platelet aggregation. Shear stress is considered to be a critical physiological stimulus to activate eNOS [50]. In cultured ECs, 1 hour of laminar shear stress elevated eNOS mRNA expression [51]. It was also reported that high laminar shear stress increases eNOS mRNA following 2 and 4 hours treatment in cannulated and isolated porcine coronary arterioles [48]. It was revealed that shear stress results in phosphorylated Ser-1177 on eNOS through the PI3K-Akt pathway [28]. Upon increase in flow in intact resistance vessels, NO production was induced by short term Ca^{2+} dependent eNOS translocation and long term Ca^{2+} + independent phosphorylation through PI3K-Akt pathways [52]. Based on in vivo studies, high shear stress causes a 3-fold rise of eNOS in the Golgi complex area compared with undisturbed or low shear stress. Increased eNOS colocalizing with PECAM-1 is seen in responding to high shear and low shear stress compared with oscillatory shear stress [53].

As illustrated in Chapter 3, heparin signaling share some factors with laminar shear stress response.

1.3 TMEM184A as A Heparin Receptor

Heparin has been known to bind vascular cells and regulate their physiology's suppressing proliferation and inflammation. Heparin can bind and be taken up by endothelial cells [9], inhibiting their inflammatory responses, and can bind VSMC [13] while decreasing MAPK dependent cell proliferation [14].

Possible explanations have been proposed, including that heparin binding blocking proteins are mediating heparin's anti-proliferation and anti-inflammation effects.

There is a huge number of heparin binding proteins, including integrin [54], bFGF [55] and fibronectin [56]. Some studies report that heparin can be found in nucleus to regulate transcription factors [57]. A receptor mediated model is thus suggested for heparin response and its transport across the membrane while it is highly charged. A putative heparin receptor was found to support this idea. Monoclonal heparin binding blocking antibodies were used to immunoprecipitate a membrane protein [58], which was identified to be coded by TMEM184A gene4 according to the paper(part of which includes some of my work) published in 2016 by the lab [4].

Transmembrane protein 184A is a multi-pass transmembrane protein as predicted. It is not well characterized but a few studies found Sdmg1, its counterpart in male gonads, plays a role in membrane trafficking. In SK11 Sertoli cells, TMEM184A colocalizes with VAMP containing peri-nuclear and peripheral endosomes [59]. VAMP contributes to vesicle fusion in membrane transport [60], [61], [62]. And knocking down TMEM184A in sertoli cells results in membrane trafficking defects [59]. In addition, a potential C-terminal dileucine targeting motif of TMEM184A implies possible endosome/lysosome targeting functions. TMEM184A was also suggested to promote interaction between intra-lumenal cargo and lipid microdomains or cytosolic membrane trafficking proteins [59].

In addition, in vascular smooth muscle cells, the putative heparin receptor TMEM184A has been found to co-localize with caveolin-1, which is one of the most important eNOS associating proteins that regulates activity of eNOS [4].

1.3.1 Rab11: a GTPase involved in endosomal recycling

A large number of recycling membrane components avoid degradation in late endosomes and lysosomes by going through the endocytic recycling compartment (ERC). Rab11, which is distributed at membrane compartments including TGN (trans-Golgi-network) and ERC, is one of the proteins that known to specifically regulate transport from ERC to both the cell surface and TGN [63]. Transport from ERC can be blocked by deficiency in Rab11[63].

Integrin is one of the transmembrane proteins that can be trafficked from plasma membrane into endosomal compartments and recycled back to cell surface. Such trafficking and recycling regulated integrin's functions including controlling cell migration and proliferation. Various Rab GTPases regulate trafficking of integrin. Some integrins can be endocytosed through Rab21 mediated clathrin independent process [64]. Subsequently, Rab4 mediates the fast recycling from early endosomes back to plasma membrane while Rab11 mediates the slow recycling through the perinuclear recycling compartment [65].

The RAB11 family of GTPases is found in post-Golgi membranes, perinuclear recylcing endosomes. It is well known to regulate endocytosed cargos. It is also revealed to regulate vesicle exocytosis at plasma membrane [66]. Rab11 is commonly used as a marker for Perinuclear recycling compartment, which located in juxtanuclear region and needs Rab11 for its function and morphology. Many endocytosed recptors such as integrins are delivered here before recycling back to plasma membrane [67]. Rab11 is involved in building motor protein complexes [68] and transporting of recycling endosomes [69]. Based on the fact that TMEM184A co-localizes with eNOS at

perinuclear regions and it is highly suspected in membrane trafficking, it is reasonable to see if TMEM184A co-localize with Rab11 or work together in some way.

Activities and distributions of integrin can be regulated by its internalization and recycling. For example, a Rab11 dependent pathway can increase recycling of α5β1 integrin and triggers activation of ROCK and inactivation of cofilin [67]. As mentioned above, TMEM184A might interact with integrin and they might associate with each other in caveolae. If TMEM184A participates in recycling, it is possible that it regulates recycling of integrin or conversely.

1.3.2 Background on Integrin Mechanotransduction

Integrins are a family of cell surface glycoproteins composed of 18 alpha and 8 beta subunits that can be assembled to 24 combinations and 13 out of 24 are found in VSMCs (α1β1, α2β1, α3β1, α4β1, α5β1, α6β1, α7β1, α8β1, α9β1, αvβ1, αvβ3, αvβ5, αnd α6βv) [70]. Integrin binding to fibronectin promotes significant mitogenic responses in VSMCs while RGD peptides and antibodies to integrin αvβ3 or αvβ5 but not β1 abolish the mechanical strain induced mitogenic response in VSMCs [71]. These data show mechanical strain induced myogenic response requires specific integrin subunits in VSMCs.

The extracellular domain of the subunits binds to ECM proteins while the cytoplasmic domains associate with cytoskeletal proteins such as actin and signaling proteins to trigger cytoskeletal organization and signal transduction. The different integrin expression patterns determine the specific type of ECM proteins to which the cell can bind [72]. Integrin recognizes and binds to RGD motifs, which are present in many ECM

proteins including laminin and thrombospondin [73]. The specificity of integrin in binding to various matrix proteins is dictated by amino acids surrounding the RGD sequence [73].

Integrin mediates rapid and spatially bidirectional signaling between ECM and the intracellular environment. Integrins are clustered when bound by ligands and the clustering allows enhanced activity of integrin receptors [74].

Existing studies show that many integrin induced signaling pathways are also activated by shear stress. Integrin activation is mainly achieved by using antibodies against integrins or attaching cells to ECM proteins [75]. Such evidence supports that integrins are mediating mechanotransduction. Integrins are reported to be activated by shear stress. Shear stress leads to conformational change of integrin αvβ3 in BAOECs and results in higher affinity between integrin and its ligands confirmed by an integrin ligand mimetic Fab fragment [76]. Many shear stress activiated kinases as ERK and FAK are under control of integrins. FAK and Shc are known to manipulate MAPK activation by shear stress. When stimulated by growth factors, integrins co-localize with growth factor receptors and their associated signalling proteins as FAK, Src and cytoskeletal molecules at focal adhesion sites and regulate the downstream effectors such as AKT, ERK and JNK [77]. ERK is activated through paxillin, p130, CAS, Crk, C3G, Rap-1, and Raf which is dependent on intergin induced FAK/c-Src pathway.

1.3.3 Integrin and Galectin-3

The β1 integrin is implicated to be a binding protein for gal-3 (galectin-3 [78], a pro-angiogenic carbohydrate-binding protein and a tumor associated lectin. Gal-3 is found to

mediate VEGF and bFGF dependent angiogenic response [79] while up to 30 times as much of gal-3 can be found in the circulation of cancer patients [80]. Blocking the major gal-3 ligand integrin αvβ3 significantly hindered the galectin-3–induced angiogenesis while gal-3 elevated the clustering of integrin αvβ3 and activated FAK (focal adhesion kinase) [79].

Other membrane proteins are potential to be involved in TMEM184A signaling pathways. Gal-3, as a member of lectin and possess a conserved sequence within the CRD (carbohydrate recognition domain) that bind to B-galactoside structures. Chemically modified heparin derivatives inhibited gal-3-ligand binding and gal-3 dependent cancer cell-endothelial adhesion and angiogenesis [81]. Gal-3 has also been reported to promote cell migration through MAPK/ERK1/2 pathway [82].

1.3.4 VE-cadherin

VE-cadherin is implicated to be part of the mechanosensing protein complex on endothelial cells in sensing blood shear stress [83]. Onset of shear stress down-regulates tension across VE-cadherin and total cell-cell junctional tension [84]. Local cytoskeleton remodeling including actin and vinculin recruitment can be triggered by VE-cadherin mediated mechanotransduction [85]. There is known cross talk between the two transmembrane adhesion receptors, integrin and VE-cadherin, which share many downstream effectors and cytoskeletal proteins. There are lateral associations between integrin and VE-cadherin [86]. Both of the two receptors are able to increase expression of cyclin D1 through Rac to enhance cell proliferation [87].

1.3.5 Heparin Signaling and Mechanotransduction

Heparin and laminar shear stress have some similar anti-proliferative and anti-inflammatory responses. As heparin, laminar shear stress can also suppress proliferation of endothelial cells and VSMCs [88]. Chronic shear exposure decreases ERK activation whereas the Akt response is increased [89]. As mentioned earlier, heparin can down-regulate ERK's activity. And Akt might be upregulated by heparin since heparin treatment causes increase in cGMP3, which responds to Akt-eNOS signaling [21].

In addition, chronic laminar shear increases the number of caveolae by 45-48% above control and caveolin-1 translocates from Golgi to the luminal plasma membrane. Such regulation of caveolae might play significant role in endothelial mechanosensing [89]. TMEM184A has also been suggested to accelerate intra-lumenal cargo interaction with cytosolic membrane trafficking proteins or lipid microdomains [90]. The re-distribution of caveolin-1 might result in a change in activity and localization of eNOS, which is predicted to play a role in heparin signaling.

Shear stress itself can modulate glycocalyx and results in modification of membrane receptors. Shear stress can redistribute the glycocalyx on endothelial cells [91]. And heparan sulfate proteoglycan is required for shear stress induced NO production [23]. This evidence implies that heparin signaling might be linked with mechanosensing events of the shear stress response and TMEM184A might contribute to or be related to mechanotransduction processes by interacting with canonical players of mechanotransduction.

Among the members involved in mechanosensing, integrins might interact with heparin signaling and TMEM184A. As cell surface receptors, integrins mediate cell-cell and cell-

matrix adhesion. Integrins and the adhesions they form are critical in transducing mechanical signals through the cell membrane.

Many shear stress-activated kinases, including ERK and FAK, are under the regulation of integrin. ERK, which is known to be affected by heparin treatment, can be activated through an integrin dependent FAK/c-Src pathway. Shear stress is able to activate integrin and induce the downstream signaling through rapid activation of tyrosine kinases, including FAK in focal adhesions [49], which consequently activate ERK [80].

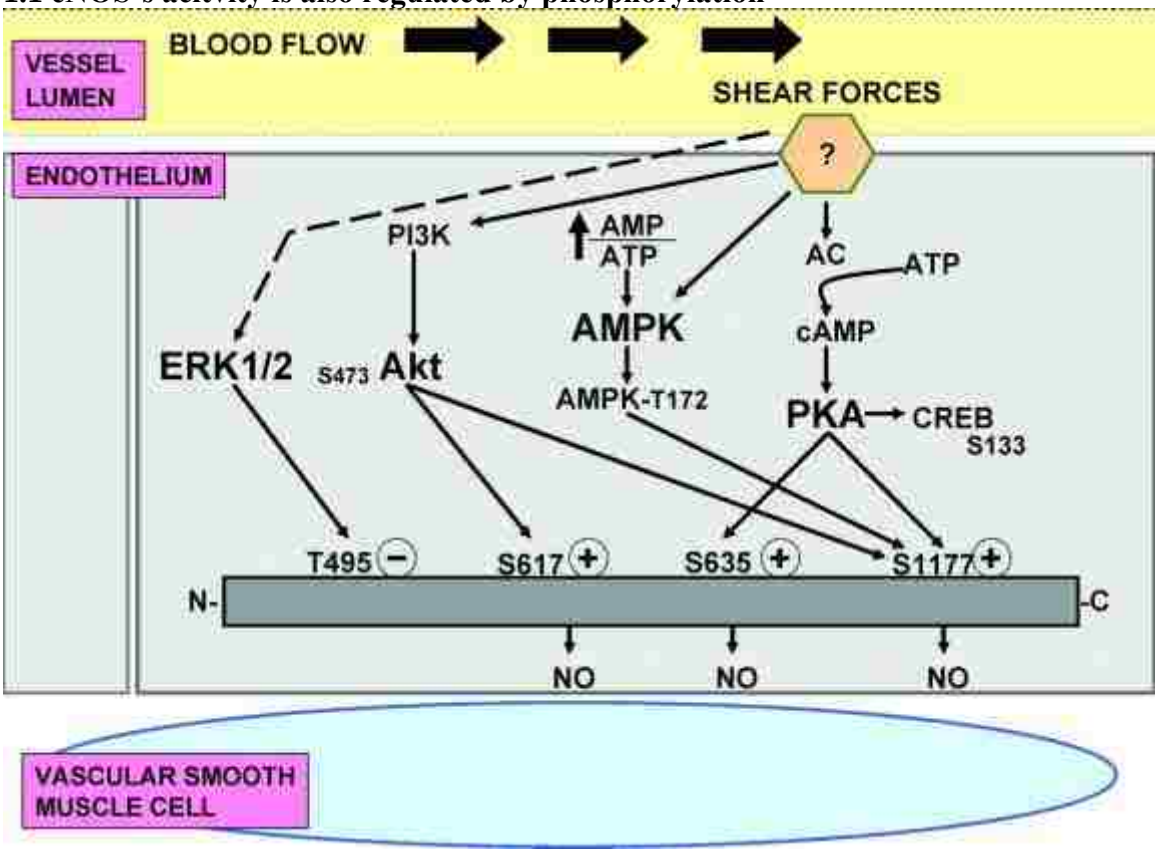
There have also been reports about links between heparin and integrin. The ectodomain of α5β1 integrin binds to heparin with high affinity (KD = 15.5 nM) [93]. Heparin also binds to αvβ3 integrin [93]. It is hypothesized that the direct binding between heparin/heparan sulfate and integrins is required for the localization of endostatin in endothelial cell lipid rafts [94]. Endostatin is a C-terminal fragment derived from type XVIII collagen. It is an angiogenesis inhibitor and found to block growth factors such as VEGF mediating pro-angiogenic action [95].

The stimulation of HSPG synthesis and sulfation pattern modification are found to be mediated by interaction between heparin and integrin while RGD peptides block such effects [96]. And this up-regulation is associated with phosphorylation of focal adhesion proteins, MAPK pathways, and NO pathways [96].

Based on this evidence, integrin might be involved in heparin signaling through a mechanosensing based mechanism such as cross activation of RTKs and their downstream signaling effectors or possibly through its binding with heparin to control localization of endostatin to suppress proliferation.

1.4: Figures

1.1 eNOS's acitvity is also regulated by phosphorylation



The figure shows the known eNOS is regulated by heparin. The figure is borrowed from a paper referenced [24].

Chapter 2: TMEM184A plays a role in heparin binding and uptake: Confirmation of the heparin receptor identity

2.1: Introduction

Heparin has been known to bind to and be internalized by vascular cells [9] and regulate their physiology as suppressing proliferation and inflammation [14]. Heparin can bind and be taken up by endothelial cells [9], inhibiting their inflammatory responses, and can bind VSMC [13] while decreasing MAPK dependent cell proliferation [14].

Possible explanations have been proposed, including that heparin binding blocking proteins are mediating heparin's anti-proliferation and anti-inflammation effects.

There is a huge number of heparin binding proteins, including integrin [54], bFGF [55] and fibronectin [54]. Some studies report that heparin can be found in the nucleus to regulate transcription factors [57]. A receptor mediated model was thus suggested by previous members of our lab and others to account for the heparin response and its transport across the membrane considering it is highly charged [2].

Much of the work described in this chapter was published in The Journal Of Biological Chemistry [4] and Journal of Visualized Experiments [97]. Earlier work from our lab also determined that monoclonal antibodies, which block heparin binding to endothelial cells [17], lead to similar heparin effects in vascular endothelial cells [58] and in vascular smooth muscle cells [17].

A former member of our lab, Raymond Pugh Ph.D., used the heparin binding blocking antibodies to isolate and purify the heparin receptor from BAOECs [4].

Raymond obtained protein sequence data by using MALDI-TOF(Matrix-Assisted Laser Desorption/Ionization – Time of Flight) Mass Spectrometry.through collaboration with previous member of our lab, Walter Patton Ph.D. Cleavage products of the peptides by carboxypeptidase Y were analyzed by MALDI-TOF to determine sequencing of the

peptide fragments [98]. Then bioinfomatic searches was used to analyze similarities to known proteins in the database. Identified by MALDI-TOF Mass Spectrometry, the protein product was revealed to be an uncharacterized membrane protein, coded by TMEM184A gene [4].

Subsequent research from the lab suggests that TMEM184A is involved in mediating the heparin responses in VSMCs (reported in Chapter 3) as it is required for heparin induced attenuation of PDGF-activated pElk-1 and synthesis of DUSP1 which regulates ERK's activity. To support the hypothesis that TMEM184A is interacting with heparin to trigger downstream signaling events, we determined to examine if TMEM184A binds with heparin and participates in heparin uptake.

We used Monoclonal heparin binding blocking antibodies to precipitate samples from BAOECs and A7r5s. The samples were recognized by commercial TMEM184A antibody to examine if the heparin binding blocking antibodies which mimic heparin is binding with TMEM184A.

2.2: Methods

2.2.1 Materials

Anti-TMEM184A (catalog no. sc292006, N-terminal domain, NTD, rabbit; catalog no. sc163460, internal domain, INT, goat) were from Santa Cruz Biotechnology (La Jolla, CA). Anti-TMEM184A (C-terminal domain, CTD, rabbit) was obtained from ProSci Inc. (Poway, CA). Secondary antibodies with fluorescent tags or Biotin-labeled

(donkey or bovine for goat primary antibodies, minimal cross-reactivity) were obtained from Jackson ImmunoResearch Laboratories, Inc. (West
Grove, PA). Extra-avidin-alkaline phosphatase TM, 5-bromo-4-chloro-3-indolyl phosphate, and nitro blue tetrazolium were obtained from Sigma.

2.2.2: Cell Culture

A7r5 rat smooth muscle cells were obtained from the ATCC (Manassas, VA). Bovine aortic endothelial cells (BAOECs), A7r5s were cultured, harvested and analyzed as described previously [4].

2.2.3: Western Blotting

2X sample buffer was added to cell sample buffer and boiled at 100^{o}C for 5 min. The protein samples were separated by SDS-PAGE. The blots were developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate and alkaline phosphatase system as described in our earlier paper [4].

2.2.4: Co-Immunoprecipitation

Confluent 150-mm dishes of BAOECs or A7r5 cells were washed 3 times with ice-cold PBS. 1ml ice-cold RIPAs buffer supplemented with two protease inhibitor cocktails (Sigma, catalog nos. P8340 and P2714) were added after PBS was drained. The cells were then agitated on rocker for 30 min at 4^{o}C. After incubation, the cells were scraped off the dishes and transferred to a cold microcentrifuge tube and centrifuged for 10 min at 10,000×g at 4^{o}C. The supernatant was incubated at 4^{o}C on a rocker overnight with

antibody targeting eNOS. Following that, 75 μl of equilibrated EZview red protein G affinity gel beads (Sigma) were added and incubated on a rocker at 4°C overnight. The beads were washed 3 times with the RIPA buffer with the protease inhibitors added. The beads in 200 μ l sample buffer were then boiled for 5 min and the supernatant was collected for running western blotting.

2.2.5: TMEM184A Knockdown

Stable transfection of A7r5 cells was accomplished by transfecting the TMEM184A shRNA construct which allows for puromycin selection and GFP expression as described in our earlier paper [4]. After electroporation, the cells were plated onto 100-mm dishes for 48 hours normal growth. Then minimum Eagle's medium containing 3 μ g/ml puromycin was changed every 2 days for 4 times. Surviving colonies were transferred to a 35-mm dish for 7 more days growth in puromycin selection medium. Cells were later examined for GFP and TMEM184A expression.

2.2.6: Indirect Immunofluorescence

Cells were fixed with ice-cold methanol (MeOH) for 5 min at 4°C, washed three times in PBS. The coverslips were incubated with primary antibodies at 4°C overnight. Following this incubation, coverslips were rinsed with PBS and incubated with proper secondary antibodies with lowest cross-reactivity conjugated to desired fluorofours for 45 min at 37 °C. Secondary antibody-only controls were carried out to exclude nonspecific staining. Cells were imaged using a Zeiss LSM 510 Meta microscope with a ×40 oil immersion

lens at room temperature. Staining for Erk and Elk-1 upon eNOS KD were also imaged using Nikon eclipse TE 2000-U fluorescence microscope (Nikon, Tokyo Japan) and the fluorescence intensity was relativized. Fluorescence intensity of the cell was determined using Image J. Statistical significance was determined by One-way ANOVA (ANalysis Of VAriance) with post-hoc Tukey HSD (Honestly Significant Difference).

2.2.7: GFP-TMEM184A Expression.

Vascular cells were trypsinized and pelleted from 100 mm dishes. Cell pellets were resuspended in 1ml of HeBS(Hepes-buffered saline) electroporation buffer. GFP-tagged TMEM184A construct was added to the cells at 20 µ g/ml. 1ml of the solution was placed in an electroporation cuvette. The cells in the cuvette were electroporated in electroporation chamber by 170V and then seeded into 30 mm dish.

2.2.8: Rhodamine-Heparin Binding and Uptake.

GFP-TMEM184A expressing A7r5s were incubated with 100 µ g/ml rhodamine heparin in dark at 37° C for 10 mins and then fixed by 4% PFA.

2.2.9: Live Cell Imaging

GFP-TMEM184A expressing A7r5s were grown in MatTek (Ashland, MA) 35-mm dishes for at least 24 hours before confocal microscope observation. After the cells were in focus, rhodamine heparin was added into cell culture.

2.3: Results

2.3.1: TMEM184A co-immunoprecipitated with anti-heparin receptor monoclonal antibodies

Monoclonal heparin binding blocking antibodies were used to precipitate samples from BAOECs and A7r5s. The samples will be recognized by commercial TMEM184A antibody to examine if the heparin binding blocking antibodies which mimic heparin is binding with TMEM184A.

Co-IP from BAOECs and A7r5s by the monoclonal heparin binding blocking antibodies and commercial antibodies against N-terminal of TMEM184A resulted in identical western blot staining with antibodies against internal region of TMEM184A (Figure 2.1).

2.3.2: TMEM184A is necessary for rhodamine-heparin uptake in A7r5s

Both wild-type A7r5 cells and TMEM184A stable knockdown A7r5s were transfected with the GFP-TMEM184A construct or not and incubated with rhodamine-heparin. Heparin uptake was analyzed in these types of cells. TMEM184A stable knockdown A7r5s has significantly lower rhodamine-heparin uptake than normal A7r5s. Transfection of GFP-TMEM184A into the stable knockdown cells recovers the uptake. Transfection of GFP-TMEM184A into the normal A7r5s results in elevated rhodamine heparin uptake (Figure 2.2AB).

2.3.3: TMEM184A co-translocate with Internalized heparin

A7r5s transfected with GFP-TEM184A were incubated with rhodamine heparin and observed immediately under confocal microscope by live imaging to investigate whether TMEM184A is participating in heparin uptake. The internalized rhodamine heparin was

enclosed by GFP-TMEM184A and these two proteins co-translocate as a vesicle (Figure 2.2 C).

2.3.4: TMEM184A stable knockdown significantly lowered the level of TMEM184A In A7r5 cells

The level of TMEM184A in TMEM184A stable knockdown cells were determined by examining the level of TMEM184A stained by antibody against N-terminus and GFP-shRNA expression. The result shows that TMEM184A was significantly reduced in the stable knockdown cells (Figure 2.3).

2.3.5: TMEM184A associates with Rab11

Anti TMEM184 antibodies were used to precipitate samples from BAOECs and A7r5s. The samples will be recognized by anti-Rab11 antibody. The results indicate that TMEM184A directly or indirectly associates with Rab11 (Figure 2.4).

2.4: Discussion

The results presented here provide evidence that heparin bind with and endocytosed with TMEM184A. By knocking down TMEM184A, the endocytosis of heparin is abolished. After re-expressing TMEM184A, the uptake of heparin is restored. The stable knockdown of TMEM184A allows for functional study of its role in heparin signaling. As illustrated in Chapter 3, TMEM184A knockdown abolished heparin's effects including regulation of eNOS activation. Data presented in this chapter and chapter 3

supports the fact that TMEM184A is the receptor of heparin and mediates heparin signaling including p-ERK, p-ELK down-regulation.

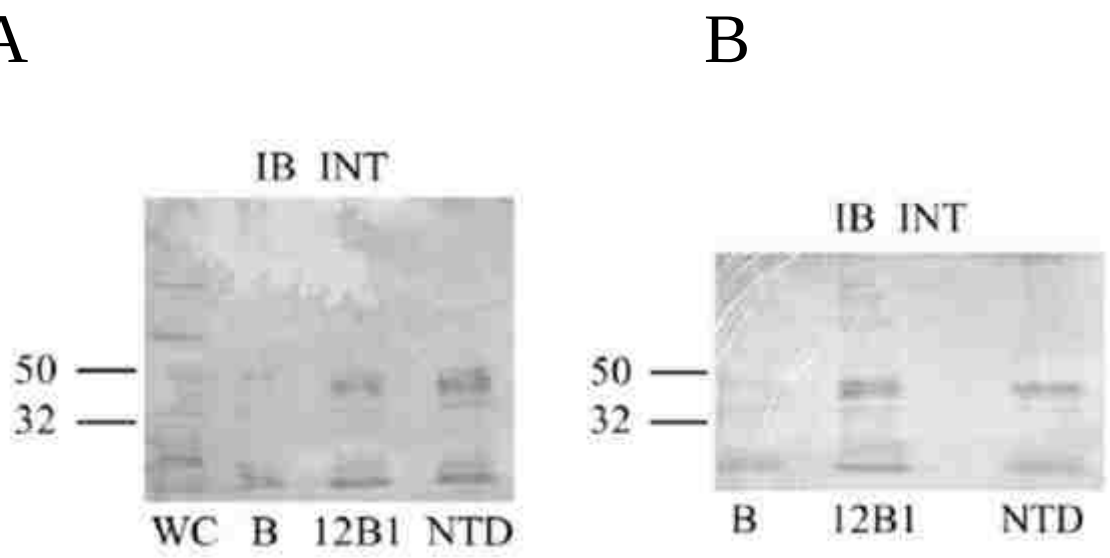
There are not many researches of TMEM184A published. TMEM184A's function is not known well. But some studies suggest TMEM184A might be involved in membrane trafficking. TMEM184A possesses a C-terminal potential dileucine targeting motif known for mediating endosome/lysosome targeting [59]. In SK11 Sertoli cells, knocking down TMEM184A, which localized to endosomes knock down resulted in membrane trafficking defects [59]. TMEM184A was also found to co-localize with various VAMP containing endosomes. TMEM184A was also proved to co-localize with cav-1 in BAOECs based on previous data of the lab [4]. Results in Chapter 3 showed that TMEM184A co-localize with eNOS at endosomal recycling compartment (Figure 3.3). And the distribution pattern that amount of TMEM184A accumulated at perinuclear region outweigh those at plasma membrane region and cytoplasm suggest that TMEM184A might undergo higher rates of internalization rather than recycling back to plasm membrane. Thus, it makes sense to explore the role of TMEM184A in membrane trafficking.

Results here show that TMEM184 associates with Rab11 (Figure 2.4). The RAB11 family of GTPases is found in post-Golgi membranes, perinuclear recylcing endosomes. It is well known in regulating endocytosed cargos. It has also revealed to regulate vesicle exocytosis at plasma membrane [66]. Rab11 is commonly used as a marker for Perinuclear recycling compartment, which located in juxtanuclear region and need Rab11 for its function and morphology. Many endocytosed recptors such as integrins are

delivered here before recycling back to plasma membrane [67]. Rab11 is involved in building motor protein complexes [68] and transporting of recycling endosomes [68]. Based on the fact that TMEM184A co-localizes with eNOS at perinuclear regions and it is highly suspected in membrane trafficking, it is reasonable to see if TMEM184A work together with Rab11. It is not clear if the TMEM184A at perinuclear region is under recycling between plasma membrane and ERC or is just synthesized and transported to Golgi. In order to answer this question, sub-cellular distribution GFP-TMEM184A can be monitored either by fixation at different time points or live cell imaging while blocking new protein synthesis with cycloheximide after 48hrs of expression. If TMEM184A is not involved in recycling, there should be a decrease of TMEM184A at perinuclear region after a period of time while significant level still can be seen on plasma membrane. Alternatively, the expressed TMEM184A can be tracked to see if those delivered to plasma membrane after expression will be internalized and go back to membrane again. If TMEM184A is proved to be part of the recycling process, further experiments are to be done to see if adding heparin is affecting the recycling process. Considering that fact that TMEM184A associates with integrin (Chapter 3). It is possible that TMEM184A assist in integrin recycling. Activities and distributions of integrin can be regulated by its internalization and recycling. For example, a Rab11 dependent pathway can increase recycling of α5β1 integrin and triggers activation of ROCK and inactivation of cofilin [67]. As mentioned above, TMEM184A might interact with integrin and they might associate with each other in caveolae. If TMEM184A participate in recycling, it is possible that it regulates recycling of integrin or conversely.

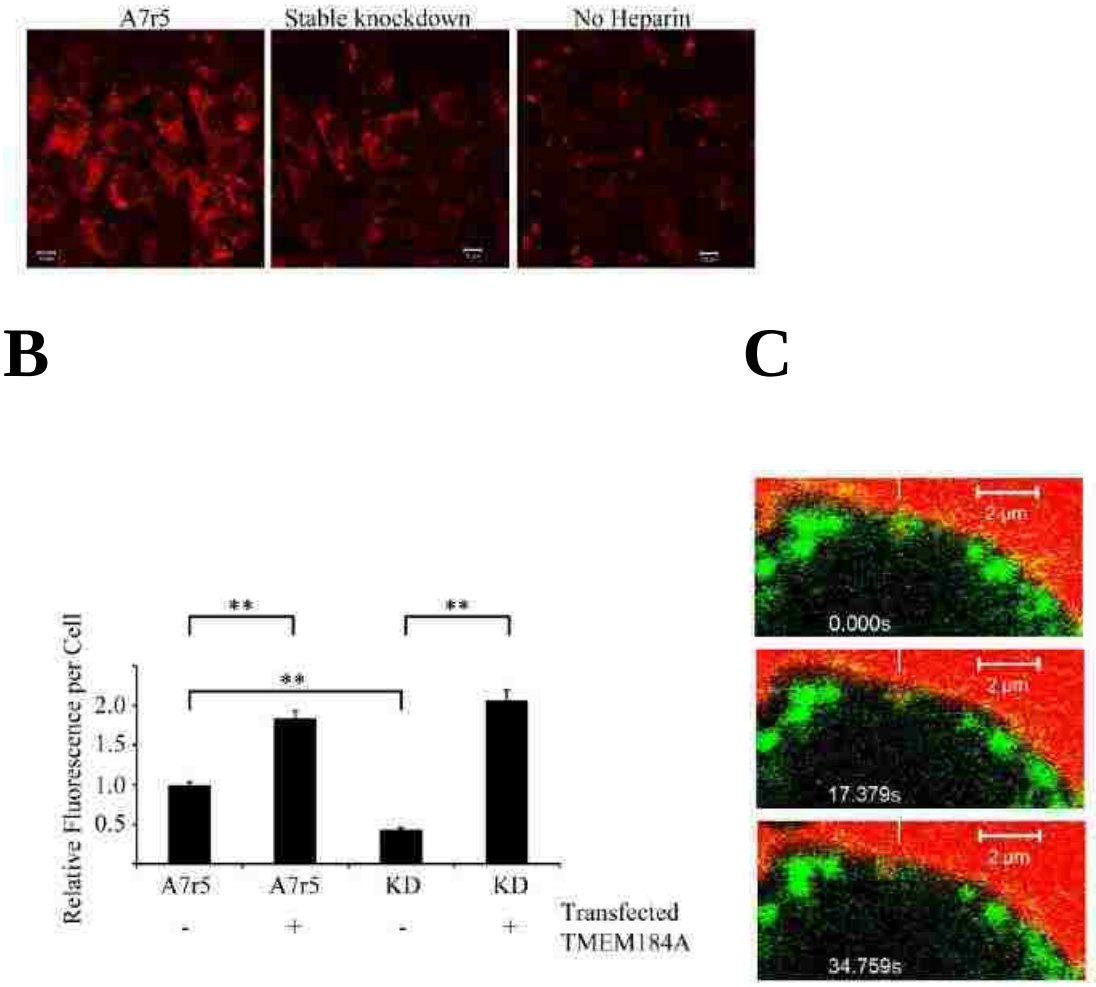
2.5: Figures

2.1 Immunoprecipitation of the heparin receptor detects TMEM184A.



A, BAOECs were harvested for immunoprecipitation (*IP*) as described under "Experimental Procedures." *A* and *B*, similar BAOEC lysates (*A*) and A7r5 lysates (*B*) were divided into identical fractions and incubated with 12B1, commercial NTD TMEM antibody, or beads alone (*B*). A whole cell (*WC*) sample was also used in *A*. Blots were developed using INT TMEM184A antibody and are representative of three repeats. Bands near the bottom of the blots are also seen with secondary antibodies only. All blots were developed and converted to grayscale for post-hoc analysis as described under "Experimental Procedures."

2.2 GFP-taggedTMEM184Acolocalizes with rhodamine-heparin



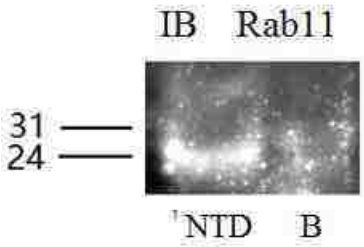
A, A7r5 cells and stable knockdowns for TMEM184A were treated with 100 µ g/ml rhodamine-heparin for 10 min and fixed with 4% PFA. Images are representative of duplicates from two separate experiments. *Scale bars*=10 µ m. *B*, A7r5 cells and stable knockdowns for TMEM184A (*KD*) were either transfected with the GFP-TMEM184A construct or not transfected and treated as in *B*. At least 50 cells/condition (in each of three experiments) were analyzed for heparin uptake. **, $p<0.0001$. *C*, GFP-TMEM184A-transfected A7r5 cells were incubated with 200 µ g/ml rhodamine-heparin, and cells were imaged immediately without fixing. Time-lapse confocal microscopy initiated at about 4 min after heparin addition identified colocalization of rhodamine-heparin (*white vertical line*) with a cluster of the GFP-TMEM 184A construct (scan zoom, 6.9; objective, X63/1.4 oil differential interference contrast). The reference *white bar* points to the initial location and is a reference for concurrent movement of both labels.

2.3 TMEM184A stable knockdown significantly lowered the level of TMEM184A In A7r5 cells

GFP-shRNA of TMEM184A **N-term of TMEM184A**

A7r5 stable knockdowns for TMEM184A were fixed with 4% PFA for 15min and stained with antibodies against N-term of TMEM184A and imaged under epifluorescence microscope.

2.4 Immunoprecipitation of TMEM184A detects Rab11



Immunoprecipitation by TMEM184 NTD antibodies in A7r5s (no treatment) show association between TMEM184A and Rab11.

Chapter 3: Heparin treatment regulates eNOS while eNOS plays a role in heparin signaling

3.1: Introduction

Heparin is known to bind vascular cells and regulate their physiology. For example, treatment of vascular cells with heparin can suppress proliferation and inflammation [4]. Heparin can bind to and be taken up by endothelial cells [9], inhibiting their inflammatory responses via decreasing NF-κB and stress kinases pathways [99], and can bind VSMC [13] while decreasing MAPK dependent cell proliferation [16]. Protein kinase G signaling is at least partially responsible for heparin's anti-proliferative effects on VSMCs [18]. A receptor-mediated model is hence suggested for the heparin responses and heparin transport across the membrane. A heparin receptor, TMEM184A, was recently identified and supports this idea [4].

Numerous studies provide evidence supporting the hypothesis that heparin and its derivatives can regulate eNOS, an important producer of NO, which leads to the activation of PKG. Both heparin and its non-anti-coagulant derivative, N-acetyl heparin, dose-dependently increase eNOS activity by promoting citrulline and nitric oxide metabolite formation in BAOECs [19]. In vivo experiments suggest that heparin can increase NOS activity and cGMP levels in lung at both three days and three weeks [19]. In cell lines including RAOSMCs and BAOECs, the heparin receptor TMEM184A has been found to co-localize with caveolin-1 [4], which is one of the most important eNOS associating proteins that regulates activity of eNOS [100]. Although not necessary for localizing eNOS to caveolae, caveolin binds with and inhibits the activity of eNOS [101]). Caveolin not only hinders caveolar-targeted receptor signaling but also blocks

calmodulin binding sites in eNOS [44]. Dissociation from caveolin is a vital early step in activating eNOS.

A principle post-translational regulation of eNOS activity is through multisite phosphorylation, and dephosphorylation at key serine and threonine residues in eNOS. Stimulatory phosphorylation loci include Ser 1177 (human, Ser1179 in bovine), Ser 617, and Ser 635, while inhibitory sites are Thr 495 and Ser 116 [102]. Various kinases such as Akt, CaM kinase and AMPK, shear stress, hormones and other stimuli can cause phosphorylation at Ser1177 of eNOS. It was revealed that shear stress, which shares similar anti-proliferative and anti-inflammatory responses with heparin in vascular cells [88] resulted in phosphorylated Ser-1177 on eNOS through the PI3K-Akt pathway [89]. An increase in flow in intact resistance vessels induces NO production by short term Ca^{2+} dependent eNOS translocation and long term Ca^{2+} independent phosphorylation through PI3K-Akt pathways [52]. PP2A and PP1 are phosphatases found to dephosphorylate Thr 495 and Ser p1177 [101] of eNOS. Heparin is reported to inhibit PP1 [103] Hence it is possible that heparin treatment inhibits PP1 or PP2A, and consequently upregulates level of Ser p1177 eNOS.

Heparin's anti-inflammatory and anti-proliferation signaling pathway has overlap with laminar shear stress response. Laminar shear stress and heparin can both reduce SMC proliferation and activity ERK1/2. And they can modulate the alignment and morphology in a similar way. Thus, heparin with its receptor TMEM184A may play roles in mechanotransduction processes by interacting with some classic members of mechanotransduction signaling system. We anticipated integrin which is vital in the

mechanosensing complex to be one of those members. Integrin is an upstream factor of eNOS and Erk1/2, which are regulated in response to heparin treatment.

There are facts indicating integrin might be involved. First integrin is vital in the mechanosensing complex and secondly it is an upstream factor of Akt, eNOS and ERK1/2 (which are important or potentially important in heparin signaling). In addition, integrins are present in both ECs and SMCs (heparin effect exists in both ECs and SMCs). There are also published results showing that up-regulation of HSPG synthesis induced by heparin in endothelial cells is dependent on the interaction of heparin with integrin while RGD peptide abolishes the effect [104]. This up-regulation is also associated with the phosphorylation of focal adhesion proteins and Ras/Raf/MEK/ERK MAP and Ca^{2+} /NO pathways which are downstream signaling factors of integrins confirmed by using inhibitors of Ras, MEK, NOS. Besides the relevance in signaling events between intergrin and TMEM184A, there are also many reports for interactions between integrin and caveolin. For example, β1 Integrin/caveolin-1 mechano-signaling complex respond to shear stress and lead to RhoA and actin Remodeling [105]. β1 integrin endocytosis is dependent on caveolin-1 [106]. There is also evidence suggesting that integrin controls localization of CEMMs [107] at the plasma membrane to regulate cellular processes [108]. Given the fact that TMEM184A co-localizes with caveolin-1 [4], it is possible that TMEM184A also interacts with integrin in caveolae or lipid rafts. Thus, the relationship between integrin, TMEM184A and heparin becomes good to study. Interaction between heparin and integrin has been reported to be necessary in various signaling events. Heparin is known to bind to αvβ3 integrin [93] and α5β1 integrin [94].

Antibodies to integrin αvβ3 or αvβ5 but not β1 abolish the mechanical strain induced mitogenic response in VSMCs [71]. Integrin αvβ3 is also a major galectin-3–binding protein while antibodies that blocks αvβ3 integrin's function significantly inhibited the galectin-3–induced angiogenesis. In SMCs, the occupancy of the heparin binding domain of αvβ3 ligand is necessary for proliferative effects of IGF-1 including phosphorylation of ERK [109]. These facts make αvβ3 integrin an interesting protein to investigate with regard to possible involvement in TMEM184A and heparin signaling.

It has been reported that association between some types of integrin and gal-3 induced angiogenic response while gal-3 promoted clustering of the integrins and activated FAK. And MAPK/ERK1/2 pathway is also mediating gal-3 signaling events.

In this study, we confirmed that heparin effects on Erk/Elk required eNOS's activity. Interaction between heparin and TMEM184A elicited activation of eNOS by increasing its Ser 1177 phosphorylation.

3.2: Methods

3.2.1: Cell Culture

A7r5 rat smooth muscle cells were obtained from the ATCC (Manassas, VA). Bovine aortic endothelial cells (BAOECs), TMEM184A knockdown A7r5s were cultured, harvested and analyzed as described previously [4].

3.2.2: Western Blotting

2X sample buffer was added to cell sample buffer and boiled at 100ºC for 5 min. The protein samples were separated by SDS-PAGE. The blots were developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate and alkaline phosphatase system as described in our earlier paper [4]. The blots in shown Figs. 5 employed secondary antibodies

conjugated to fluorophore (diluted 1:5000). And the bands were visualized by fluorescence detection using the Bio-Rad ChemiDoc MP system (catalog no. 170-8280).

3.2.3: Co-Immunoprecipitation

Confluent 150-mm dishes of BAOECs or A7r5 cells were washed 3 times with ice-cold PBS by three times. 1ml ice-cold RIPAs buffer supplemented with two protease inhibitor cocktails (Sigma, catalog nos. P8340 and P2714) as added after PBS was drained. The cells were then agitated on a rocker for 30 min at 4ºC. After incubation, the cells were scraped off the dishes and transferred to a cold microcentrifuge tube and centrifuged for 10 min at 10,000×g at 4ºC. The supernatant was incubated at 4ºC on a rocker overnight with antibody targeting eNOS identified here. Following that, 75 μl of equilibrated EZview red protein G affinity gel beads (Sigma) were added and incubated on a rocker at 4ºC overnight. The beads were washed 3 times with the RIPAs buffer with the protease inhibitors added by three times. The beads were then boiled in SDS PAGE sample buffer for 5 min and the supernatant was collected for running western blotting.

3.2.4: eNOS Knockdown

A7r5 cells were trypsinized, rinsed with PBS, suspended in HEPES-buffered saline, electroporated with 20 μg/ml of eNOS siRNA, control siRNA, or only electroporated with the preset Hela protocol, plated and grown on glass cover slips. The cells were treated and harvested after 48 hours post electroporation.

3.2.5: TMEM184A Knockdown

Stable transfection of A7r5 cells was accomplished by transfecting the TMEM184A shRNA construct as described in chapter 2.

3.2.6: Indirect Immunofluorescence

Cells were fixed with ice-cold methanol (MeOH) for 5 min at 4^{o}C, washed three times in PBS. The coverslips were incubated with primary antibodies against specific primary antibodies at 4^{o}C overnight. Following this incubation, coverslips were rinsed with PBS and incubated with proper secondary antibodies with lowest cross-reactivity conjugated to desired fluorofuors for 45 min at 37 °C. Secondary antibody-only controls were carried out to exclude nonspecific staining. Cells were imaged using a Zeiss LSM 510 Meta or Zeiss LSM 880 microscope with a ×40 oil immersion lens at room temperature. Staining for Erk and Elk-1 upon eNOS KD were also imaged using Nikon eclipse TE 2000-U fluorescence microscope (Nikon, Tokyo Japan) and the fluorescent intensity was relativized. Fluorescent intensity of the cell was determined using Image J. Statistical significance was determined by One-way ANOVA (ANalysis Of VAriance) with post-hoc Tukey HSD (Honestly Significant Difference).

3.2.7 Drug Treatment

Cells were treated with 50 μmol/L RGD for 30 min, KN-93 0.001 μmol/ml 30 min, GSK 2193874 15 μM 30 min, RN1734 10 μM 30 min, or EGTA 5 mmol/L 30 min. Cells were processed for IF and analyzed as above.

3.3: Results

3.3.1: Knockdown of eNOS Abolishes Heparin Responses in VSMCs

In order to determine whether eNOS is necessary in heparin effects in VSMCs, A7r5 cells and BAOECs were transfected with small interfering RNA (siRNA) for eNOS to down-regulate its expression. eNOS levels are significantly reduced in eNOS knocked down cells 48h post transfection compared to control siRNA transfected cells.

These results verified that eNOS play an important role in heparin response mediated by MAPK pathway in VSMCs (Figure 3.1).

3.3.2: Heparin Increases P-Ser 1177 eNOS in VSMCS Dependently of TMEM184A

To investigate how eNOS is involved in the heparin signaling pathway, we examined whether heparin regulates phosphorylation of eNOS at ser 1177, a pivotal stimulatory post-translation modification site and whether TMEM184A mediates such potential regulation.

A7r5s and stable knockdowns for TMEM184A were incubated with or without heparin at 37°C and then fixed at 3, 13 and 23 minutes, and subsequently stained with Phospho-

eNOS (Ser1177) Antibody. The results show that heparin treatment caused increased eNOS phosphorylation at Ser1177 as early as 3 min and retained at 13 min in A7r5s but not in stable TMEM184 stable knockdown A7r5 cells (Figure 3.2). The level of p-Ser 1177 eNOS reached a peak after the A7r5 cells were incubated with heparin for 3 min among the times as in Fig 2. After 23 min of heparin treatment, p-Ser1177 eNOS level was dropped approaching original basal level in A7r5 cells (Figure 3.2). Whereas in TMEM184A stable knockdown A7r5 cells, heparin did not induce elevation of p-Ser1177 eNOS after 3 min or 13 min treatment but slightly down-regulated p-Ser1177 eNOS after 23 min incubation (Figure 3.2).

3.3.3: eNOS Co-localizes with TMEM184A in Vascular Cells

TMEM184A has been implicated in membrane trafficking [90] and found to co-localize with caveolin-1 [4], suggesting that TMEM184A might play a role in regulation of activity or translocation of eNOS by interacting with eNOS. To confirm that TMEM184A is an eNOS binding partner, vascular cell proteins immunoprecipitated with eNOS antibodies and were analyzed by TMEM184A NTD antibodies. TMEM184A staining was observed by TMEM184A NTD antibodies instead of affinity beads alone (Figure 3.3A). Through immunofluorescent assays in which we co-stained the cells for TMEM184A and p-Ser 1177 eNOS with or without heparin treatment, it can be observed that an elevated amount of p-Ser 1177 eNOS resided together with TMEM184A at focal adhesion sites and on cell membrane after incubation with heparin for 3 min compared to that under quiescent status. After 13 min, the level of p-Ser 1177 eNOS and TMEM184A

on cell membrane was reduced but increased at peri-nuclear region. After 30 min, there is evident accumulation of co-localization of the two proteins at peri-nuclear region. However, the signal intensity of the two proteins in the cytosol and focal adhesion sites dropped close to basal level (Figure 3.3 B).

These results suggest that TMEM184A is an eNOS binding partner. It might serve to regulate the activity or translocation of p-eNOS from the region under cell surface to perinuclear compartments while TMEM184A is implicated to participate in membrane trafficking [59]. Besides binding to eNOS, TMEM184A was also found to co-localize with caveolin-1 previously [4] and it is required for heparin signaling [4]. It is possible that TMEM184A facilitates the dissociation from caveolin-1 and subsequent internalization of the activated eNOS after heparin treatment. Detaching from caveolin-1 is necessary for activation and the following internalization of p-Ser 1177 eNOS is required for the consequent deactivation and desensitization of eNOS.

3.3.4: Integrin involved eNOS activation requires TMEM184A

Heparin and laminar shear stress have some similar anti-proliferative and anti-inflammatory responses. Both heparin and laminar shear stress can suppress proliferation of endothelial cells and VSMCs [88], and down-regulate p-Erk. Integrin, which is involved in mechanosensing, is one of the heparin binding proteins [54]. Integrin is an important upstream receptor that can mediate phosphorylation of eNOS, which plays a role in heparin signaling. To explore the potential relationship between integrin and heparin induced eNOS activation, we examined the level of activated eNOS in both A7r5

cells and TMEM184A stable knock down A7r5s in response to RGD and heparin treatment. As shown in Figure 3.4, cells treated with heparin and RGD has lower eNOS phosphorylation than heparin alone. The level of p-Ser1177 eNOS was significantly increased in response to RGD treatment in A7r5 cells and heparin treatment diminished this increase, which may be because of potential competition between heparin and RGD in interacting with integrin. In TMEM184A stable knockdown cells, there was no evident effect of heparin or RGD. It is thus revealed that heparin can interfere with and TMEM184A is required in an RGD-integrin-eNOS pathway.

3.3.5: TMEM184A associates with integrin αV

Results from Figure 3.5 suggest that integrin is involved in heparin signaling pathway. There have also been reports about association between heparin and integrin. The ectodomain of α5β1 integrin binds to heparin with high affinity [93]. Heparin also binds to αvβ3 integrin [93]. To examine whether TMEM184A, which is the receptor of heparin, associates with integrin αV, we immunoprecipitated vascular cells proteins with antibodies against NTD of TMEM184A and analyzed with antibodies against integrin αV in a western blot. The result indicates that TMEM184A associates with integrin αV in vascular cells (Figure 3.5.).

3.3.6: Interactions between Ca^{2+} and heparin signaling pathway

Results from Figure 3.6 suggest that heparin activates eNOS. Ca^{2+} is important in activation of eNOS. eNOS activation requires eNOS-calmodulin binding which can be triggered by intracellular calcium transients [96]. In addition, Ca^{2+}/CaM complex interacts with and regulates a large number of proteins including CaM kinaseII

(calcium/calmodolun-dependent protein kinase ii). CaM kinaseII can catalyze phosphorylation at ser 1177 of eNOS, which activate eNOS by enhancing affinity between calmodulin and eNOS.

Heparin has been revealed to be a competitive antagonist of IP3 receptors [85]. Heparin is found to inhibit the inositol (1,4,5)-trisphosphate (IP3) receptor, which is a classic intracellular Ca^{2+} release channel [85] and a principle signaling protein involved in gap junction-mediated propagation of Ca^{2+} waves [85]. But it is reported that heparin treatment (125 IU) results in intracellular Ca^{2+} release, actives PLCγI and CaMKII, as well as inducing NO production [96] while PLCγI is known to be able to produce IP3 and induce Ca^{2+} release.

In order to investigate if heparin's activation, which we observed, on eNOS is dependent on Ca^{2+} level and activity of CaM kinaseII, we treated A7r5 cells with EGTA (Ca^{2+} chelator) (Figure 3.6) and KN-93 (CaM kinaseII) inhibitor (Figure 3.6) respectivelly. The results indicate that EGTA abolished heparin's effects on eNOS, which indicates that extracellular Ca2+ is necessary for the ability of heparin treatment to result in eNOS activation. Next, KN-93, a CaM kinase II inhibitor was employed (Figure 3.7) to determine whether CaM Kinase II activity was required for the heparin effect. The results from Figure 3.7 reveal that inhibiting CaM kinase II reduced the ability of heparin treatment to activate eNOS. This implies that CaM kinase II is involved in heparin-induced activation of eNOS but suggests that calcium has effects on eNOS activation that go beyond the activation of CaM Kinase II, consistent with the requirement of calcium for direct involvement in eNOS activity.

3.3.7: TRPV4's role in heparin signaling pathway

Results above suggest that Ca^{2+} is required in heparin induced activation of eNOS. Results from 3.3.6 indicates that the short term calcium wave is extracellular originated, potentially through a Ca^{2+} channel. we used GSK2193874 and RN1734, two inhibitors of a membrane Ca^{2} channel TRPV4, which has been reported to be involved in eNOS's activation in response to Ca^{2+} transients [110] (Fig 8.). Results showed that one of the inhibitors RN1734 reversed heparin's up-regulation of eNOS while another inhibitor GSK2193874 reduced such up-regulation significantly (Fig 8.). These results imply that Ca^{2} wave in response to heparin that activates eNOS is mediated or partially mediated through TRPV4.

3.3.8: Akt is not necessary in heparin activated eNOS

Results for Fig. 5 indicates that Akt is not activated upon heparin treatment. Thus we did not do further study to investigate if blocking Akt would affect heparin's activation on eNOS.

3.4: Discussion and future direction

We here demonstrated that eNOS has an essential role in heparin's anti-inflammatory responses in vascular smooth muscle cells. Our group previously reported that cGMP-dependent protein kinase (PKG), which is a downstream factor of eNOS is involved in heparin down-regulates MAPK activation in vascular smooth muscle cells [4][18]. Our previous data revealed that heparin's receptor TMEM184A co-localizes with caveolin-1

in Rat aortic smooth muscle cells and BAOECs [4], which is eNOS's binding partner. This further supports the hypothesis that eNOS potentially plays a role in heparin's anti-inflammatory signaling pathway. We reported here that by knocking down eNOS, heparin's inhibition on ERK and ELK-1 activation is abolished, which provides evidence for this hypothesis.

We showed that in response to heparin treatment, eNOS's activity and sub-cellular translocation is altered. Phosphorylation of eNOS on Ser1177 was induced by heparin treatment as early as 3 min and dropped to basal level after 30 min, which extend the finding by Kouretas et al. [111], who showed that heparin and its derivative, N-acetyl heparin, increase eNOS activity, determined by citrulline and nitric oxide (NO) metabolite formation.

We also demonstrated that eNOS activation upon heparin treatment is dependent on heparin receptor TMEM184A by knocking down TMEM184A.

Our work also reveals that phosphorylated eNOS accumulated at cell membrane and focal adhesion sites upon heparin treatment and subsequently co-translocated with TMEM184A to perinuclear region. This suggest that TMEM184A may be a eNOS binding partner. It might serve to regulate the activity or translocation of p-eNOS from the region under cell surface to perinuclear compartments while TMEM184A is implicated to participate in membrane trafficking in mouse germ cells [59]. Besides binding to eNOS, TMEM184A was also found to co-localize with caveolin-1 in Rat aortic smooth muscle cells and BAOECs previously [4] and it is required for heparin signaling responses in A7r5 cells [4]. It is possible that TMEM184A facilitates the

dissociation from caveolin-1 and subsequent internalization of the activated eNOS after heparin treatment. Detaching from caveolin-1 is necessary for activation and the following internalization of p-Ser 1177 eNOS is required for the consequent deactivation and desensitization of eNOS.

Given the result that activated eNOS accumulated at focal adhesion sites with TMEM184 after heparin treatment and the following facts we hypothesized that integrin might be involved in heparin, eNOS and TMEM184A signaling pathway.

We presented that TMEM184A co-translocated with eNOS in response to heparin while it was necessary in heparin's up-regulation of eNOS in A7r5 cells. We also note that TMEM184A co-localized with caveolin-1. However, these did not demonstrate how the transmembrane protein TMEM184A transmits the signal from heparin stimulation to intracellular factors. We hypothesized that there might be membrane proteins associated with TMEM184A to affect or induce heparin signaling. Several proteins including integrin, gal-3, VE-cadherin have potential to bind with TMEM184A and participate in heparin signaling as illustrated below. As mentioned above in background, heparin's anti-inflammatory and anti-proliferation signaling pathway share some players with laminar shear stress response. For example, laminar shear stress and heparin can both reduce smooth muscle cell proliferation and activity ERK1/2 (and Akt). And they modulate the alignment and morphology in similar way according to previous data of our lab. Thus, it is reasonable to predict heparin and its potential receptor TMEM184A have interactions with or play roles in mechanotransduction processes by interacting with some classic members of mechanotransduction signaling system.

There are facts indicating integrin might be one of those members. First integrin is vital in the mechanosensing complex and secondly it is an upstream factor of Akt, eNOS and ERK1/2. In addition, integrins are present in both ECs and SMCs. There are also published results showing that up-regulation of HSPG synthesis induced by heparin in endothelial cells is dependent on the interaction of heparin with integrin while RGD peptide abolishes the effect [96]. This up-regulation was also associated with the phosphorylation of focal adhesion proteins and Ras/Raf/MEK/ERK MAP and Ca^{2+} /NO pathways which are downstream signaling factors of integrins confirmed by using inhibitors of Ras, MEK, NOS. Besides the relevance in signaling events between integrin and TMEM184A, there are also many reports for interactions between integrin and caveolin. For example, β1 Integrin/caveolin-1 mechano-signaling complex responds to shear stress and lead to RhoA and actin remodeling in Bovine aortic endothelial cells [112]. β1 integrin endocytosis is dependent on caveolin- 1 in myofibroblasts [106]. There have been reports about association between heparin and integrin. The ectodomain of α5β1 integrin binds to heparin with high affinity in endothelial cells (KD = 15.5 nM) [93]. Heparin also binds to αvβ3 integrin in endothelial cells [93]. There is also evidence suggesting that integrin controls localization of CEMMs [107] at the plasma membrane of epithelial cells to regulate cellular processes [108]. Based on our previous data that TMEM184A co-localizes with caveolin-1 [4], it is possible that TMEM184A also interacts with integrin in caveolae or lipid rafts. Therefore, we hsypothesized that integrin might interact with TMEM184A to interfere with eNOS's binding with caveolin-1 in response to heparin.

Our work demonstrated that integrin was involved in heparin's regulation of eNOS while TMEM184A was required for the regulation. We also showed that TMEM184A co-localized with integrin αV. We also ran co-IP for integrin β1 and TMEM184A but such co-localization was not seen. Future research may determine the role of different types of integrin subunits. The results showed that RGD itself significantly activated eNOS however diminished heparin's activation on eNOS. This may be because RGD itself, as an integrin binding motif, may activate integrin as well as compete and impede heparin induced integrin's binding to its agonists. Heparin blocks RGD induced eNOS activation. eNOS is regulated through various cellular events including post-translational modification, calcium wave stimulation, association or dissociation with its binding proteins. Given that heparin elevated eNOS activity shortly after treatment, we hypothesized that calcium stimulation might be involved in eNOS activation by heparin. Intracellular Ca^{2+} increases can cause calmodulin binding to eNOS and hence facilitate electron transfer from the reductase domain to the oxygenase domain of the NOS to synthesize NO [26]. Besides facilitating electron transfer, Ca^{2+}/calmodulin complex can also activate CaM kinaseII to phosphorylate eNOS. We used CaM kinaseII inhibitor KN-93 and showed that CaM kinaseII may be partially involved in eNOS's activation by heparin because KN-93 decreased the activation but did not abolish it. We showed that EGTA inhibited heparin's activation of eNOS, which suggests that Ca^{2+} is essential for phosphorylation of eNOS upon heparin treatment by 10 min. However, it did not prove the source of Ca^{2+} that activates eNOS because EGTA only eliminated extracellular Ca^{2+} from the cell media to reduce Ca^{2+} concentration. But based on that fact that heparin

activates eNOS as early as 3min, we hypothesize that extracellular Ca^{2+} plays a role in short term activation of eNOS by heparin. We cannot exclude the possibility that eNOS was also activated by intracellular Ca^{2+} release in long term, which has been implicated in heparin induced signaling events. Considering that eNOS activation is fast in response to heparin treatment, we decided to explore transmembrane Ca^{2+} channels. We used two inhibitors GSK2193874 and RN1734 of TRPV4 instead of using only one inhibitor because the effect of the various TRPV4 are different according to reports. The results we obtained showed that RN1734 reversed heparin's effect on eNOS while another inhibitor GSK2193874 only reduced such up-regulation. Given that GSK2193874 is a potent antagonist of TRPV4 channels that reported to be not as effective as some of other antagonist [113]. We think the results still support that TRPV4 may play a role in heparin's activation on eNOS.

One of the eNOS activating mechanisms besides intracellular calcium mobilization, the PI3K/Akt cascade also assists in eNOS activation. Upon activation, Akt can phosphorylate eNOS at ser1177, which enhances affinity between eNOS and calmodulin and promotes electron transfer and upregulate activity of eNOS [30]. In response to shear stress, Ca^{2+} activated PI3K/Akt pathway leads to phosphorylation of eNOS at Ser1177 and consequently contributes to maintain basal vascular tone in endothelial cells [28]. We also performed experiments on Akt but there was no sound evidence to support Akt's function in heparin's up-regulation on eNOS.

It is hypothesized that the direct binding between heparin/heparan sulfate and integrins is required for the localization of endostatin in endothelial cell lipid rafts [93]. Endostatin is

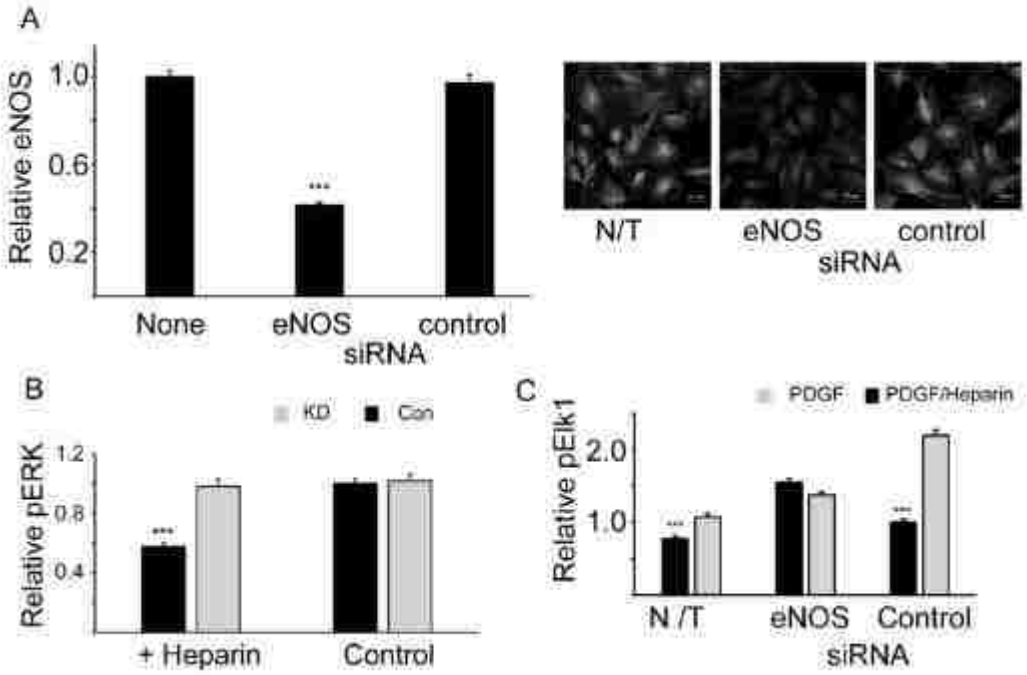
a C-terminal fragment derived from type XVIII collagen. It is an angiogenesis inhibitor and found to block growth factors such as VEGF mediating pro-angiogenic action [95]. The stimulation of HSPG synthesis and sulfation pattern modification are found to be mediated by interaction between heparin and integrin while RGD peptides block such effects in endothelial cells [96]. And this upregulation is associated with phosphorylation of focal adhesion proteins, MAPK pathways, and NO pathways in endothelial cells [96]. Based on this evidence, integrin might be involved in heparin signaling through a mechanosensing based mechanism such as cross activation of RTKs and their downstream signaling effectors or possibly through its binding with heparin to control localization of endostatin to suppress proliferation.

Several proteins including integrin, gal-3, VE-cadherin have potential to bind with TMEM184A and participate in heparin signaling as illustrated below. As mentioned above in background, heparin's anti-inflammatory and anti-proliferation signaling pathway share some players with laminar shear stress response. For example, laminar shear stress and heparin can both reduce SMC proliferation and activity ERK1/2 (and Akt). And they modulate the alignment and morphology in similar way according to previous data of our lab. As illustrated in Chapter 3, integrin plays a role in TMEM184A mediated heparin signaling in rat smooth muscle cells. Heparin and its receptor TMEM184A may also have interactions with or play roles in mechanotransduction processes by interacting with integrin, which is one of the classic members of mechanotransduction signaling system.

Another member of mechanosensor complex, VE-cadherin, also worth investigation. Earlier data from the lab indicates that TMEM184A directly or indirectly associates with VE-cadherin in BAOECs. VE-cadherin can serve as adaptor between PECAM and VEGFR and form a complex that lead to integrin activation and other events [114]. Besides αv, we also investigated β3's interaction with TMEM184A. Antibodies to integrin αvβ3 or αvβ5 but not β1 abolish the mechanical strain induced mitogenic response in VSMCs [74]. Integrin αvβ3 is also a major galectin-3–binding protein while antibodies that blocks αvβ3 integrin's function significantly inhibited the galectin-3–induced angiogenesis. In SMCs, the occupancy of the heparin binding domain of αvβ3 ligand is necessary for proliferative effects of IGF-1 including phosphorylation of ERK [115]. These facts suggest that αvβ3 integrin is an interesting protein to investigate regarding its involvement in TMEM184A and heparin signaling. It has been reported that association between some types of integrin and gal-3 induced angiogenic response while gal-3 promoted clustering of the integrins and activated FAK. And MAPK/ERK1/2 pathway is also mediating gal-3 signaling events. Thus, it worth hypothesizing that heparin treatment might disrupt or enhance binding between integrin and gal-3 since heparin can both bind to integrin and gal-3. The disruption might hinder or improve either integrin signaling or gal-3 signaling.

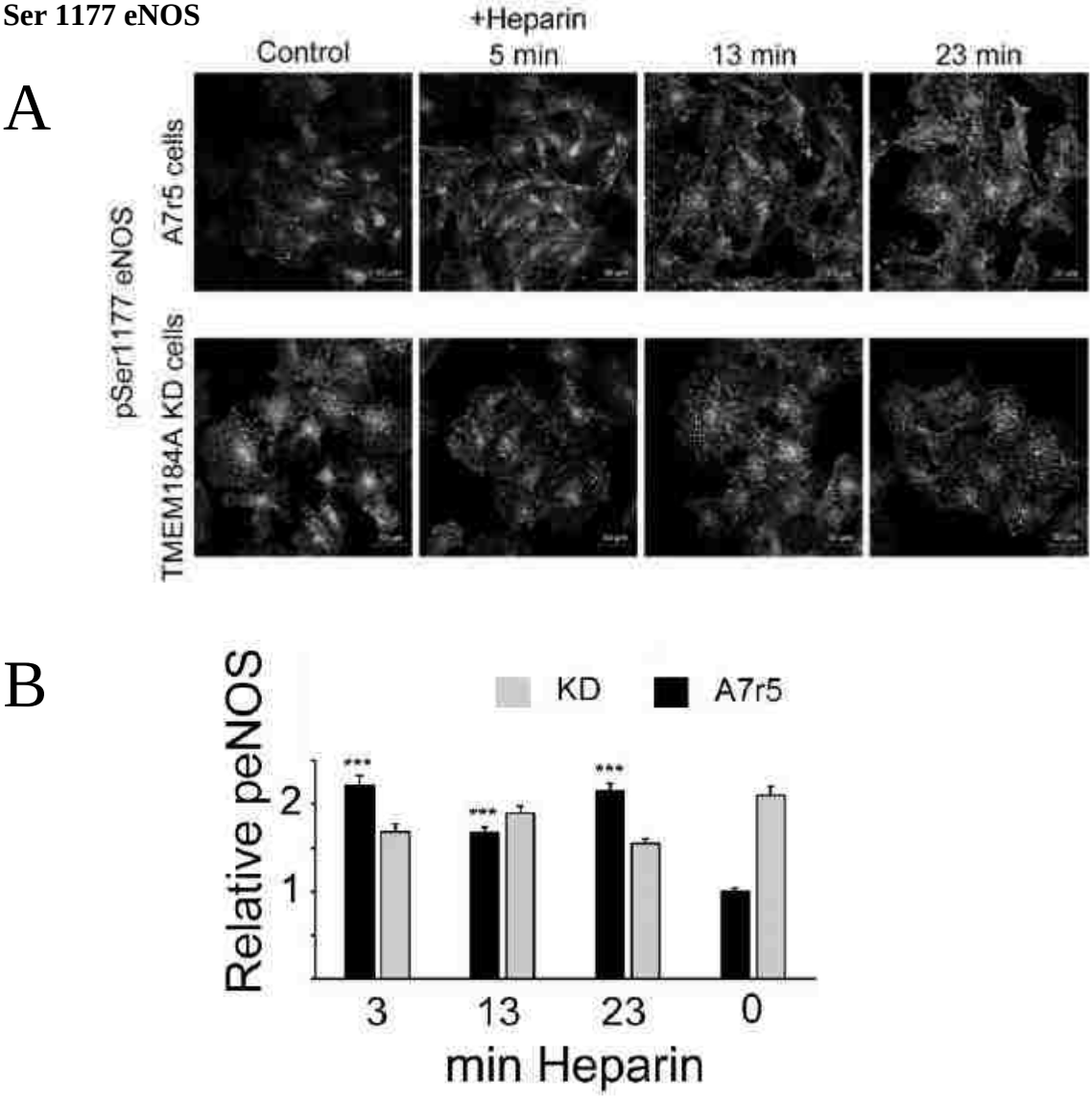
3.5: Figures

3.1 Knockdown of eNOS eliminates heparin responses in A7r5 cells.



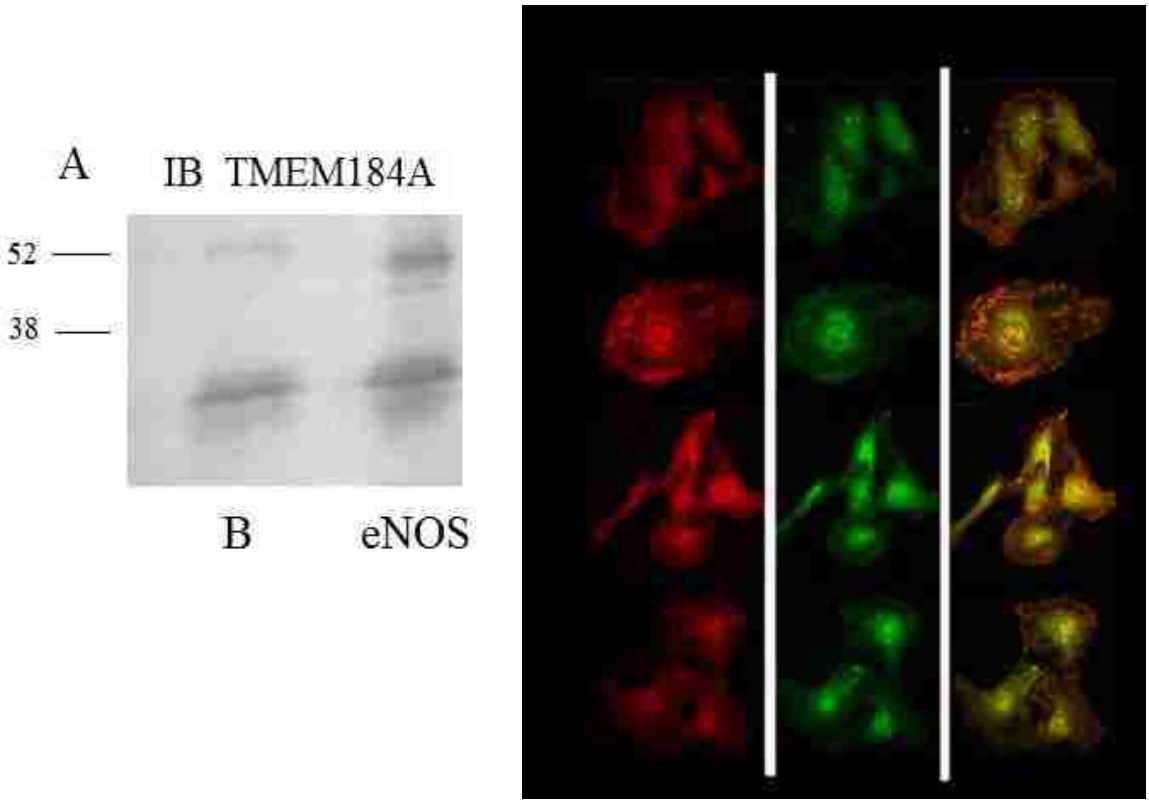
A. A7r5 cells transfected with siRNA against eNOS or control siRNA were treated with 200 μg/ml heparin for 10 min followed by fixation with ice-cold MeOH. Cells were stained for eNOS (sc-376751). *Scale bars = 50 μ*m. At least 100 cells in three separate experiments were analyzed for each condition. *** p<0.005 **B**. Identical cells were prepared as in A. Images of cells stained for pERK are representative of three independent experiments. *Scale bars = 10 μ*m. At least 50 cells per condition in each individual experiment were analyzed for phosphorylated Erk. **, $p < 0.0001$. **C.** A7r5 cells (NT), eNOS siRNA transfected A7r5 cells and control siRNA transfected A7r5 cells were treated with heparin for 10 min followed by PDGF stimulation. Cells were stained for pELK, ***, $p < 0.0001$ heparin/PDGF compared to PDGF. *Scale bars = 50 μ*m. The graph represents data from three independent experiments with at least 50 cells analyzed per condition in each experiment.

3.2 TMEM184A knockdown A7r5 cells do not show heparin-induced increase in p-Ser 1177 eNOS



A. A7r5 cells and stable TMEM184A A7r5 knockdown cells were treated with 200 μg/ml heparin for the indicated times followed by fixation and staining for p-eNOS. Images are representative of three independent experiments. *Scale bars = 50 μ*m. **B.** A7r5 cells and TMEM184A stable knockdown cells were treated with heparin as in A. At least 50 cells per condition (from three separate experiments) were evaluated for p-eNOS. ***, $p < 0.0001$.

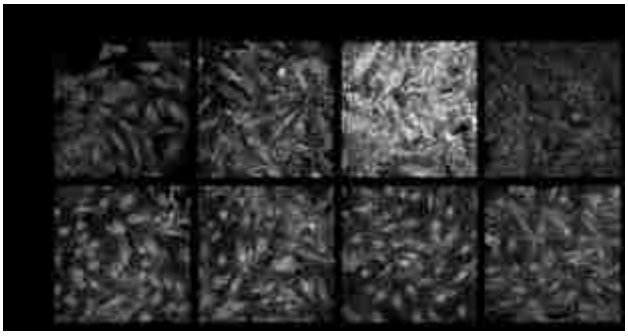
3.3 eNOS co-localize under quiescent state and co-translocate with TMEM184A after heparin treatment in VSMCs



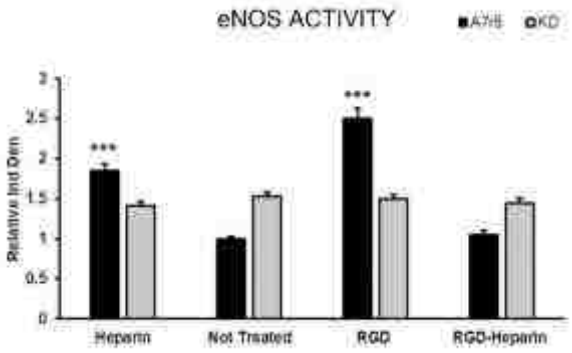
A, eNOS. Membrane protein samples of BAOEC lysates were incubated with the eNOS antibody, precipitated using EZview affinity beads, or beads alone(B) and developed using INT TMEM184A antibody. *IB*, immunoblot. The blot is representative of 2 separate experiments. *B*, A7r5 cells were incubated with 200 μg/ml heparin for the indicated times and fixed by 4% PFA. Images are representative of two separate experiments. *Scale bars = 10 μm.*

3.4 Heparin interferes with and TMEM184A mediates RGD-integrin-eNOS pathway

A

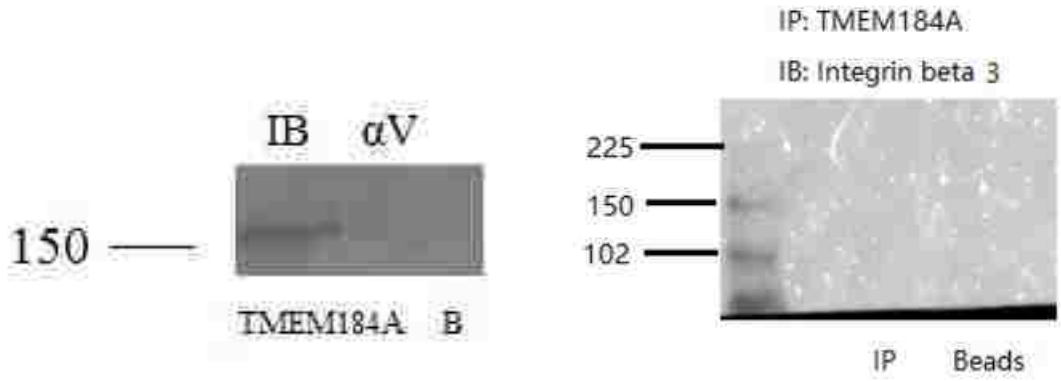


B



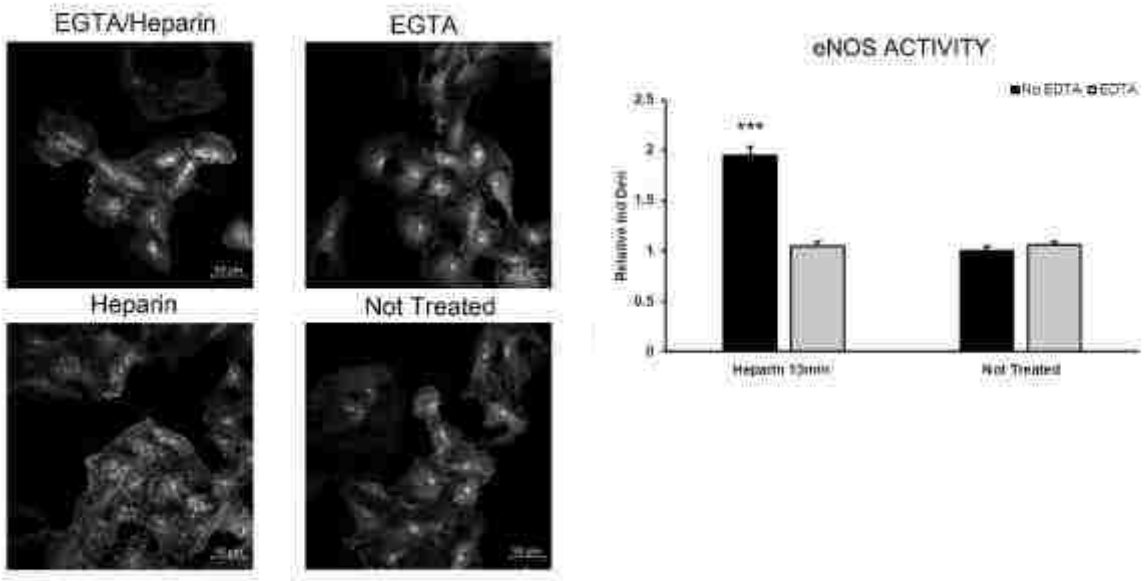
A, A7r5 cells and stable knockdown for TMEM184A A7r5 cells were treated with or without 200 μg/ml heparin only or after RGD pre-treatment followed by fixation with 4% PFA. Images are representative of three independent experiments. *Scale bars = 10 μm. B,* A7r5 cells were treated with heparin and RGD as in A. At least 50 cells per condition were examined in each repeat for phosphorylated eNOS. ***, $p < 0.0001$.

3.5 Immunoprecipitation of TMEM184A detects integrin alpha V but not beta 3 subunit



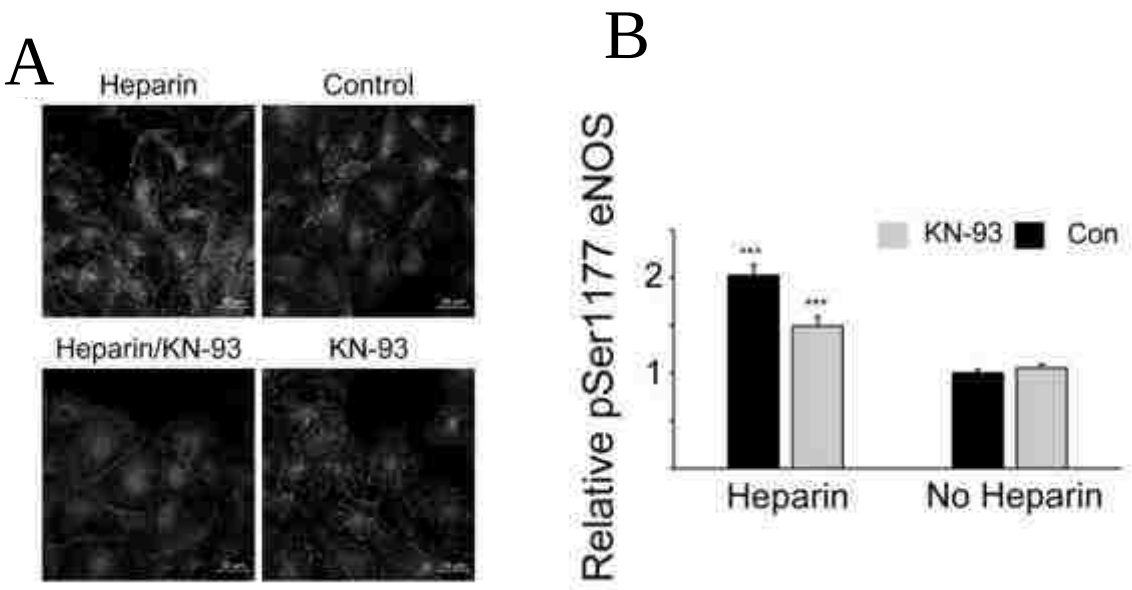
A7r5 cells were harvested for immunoprecipitation (*IP*). Membrane samples from cell lysates were incubated with TMEM184A, precipitated using EZview affinity beads, or beads alone(B) and developed using integrin αV and beta 3 antibody. *IB*, immunoblot. The blot is representative of 2 separate experiments.

3.6 EGTA abolished heparin induced activation of eNOS



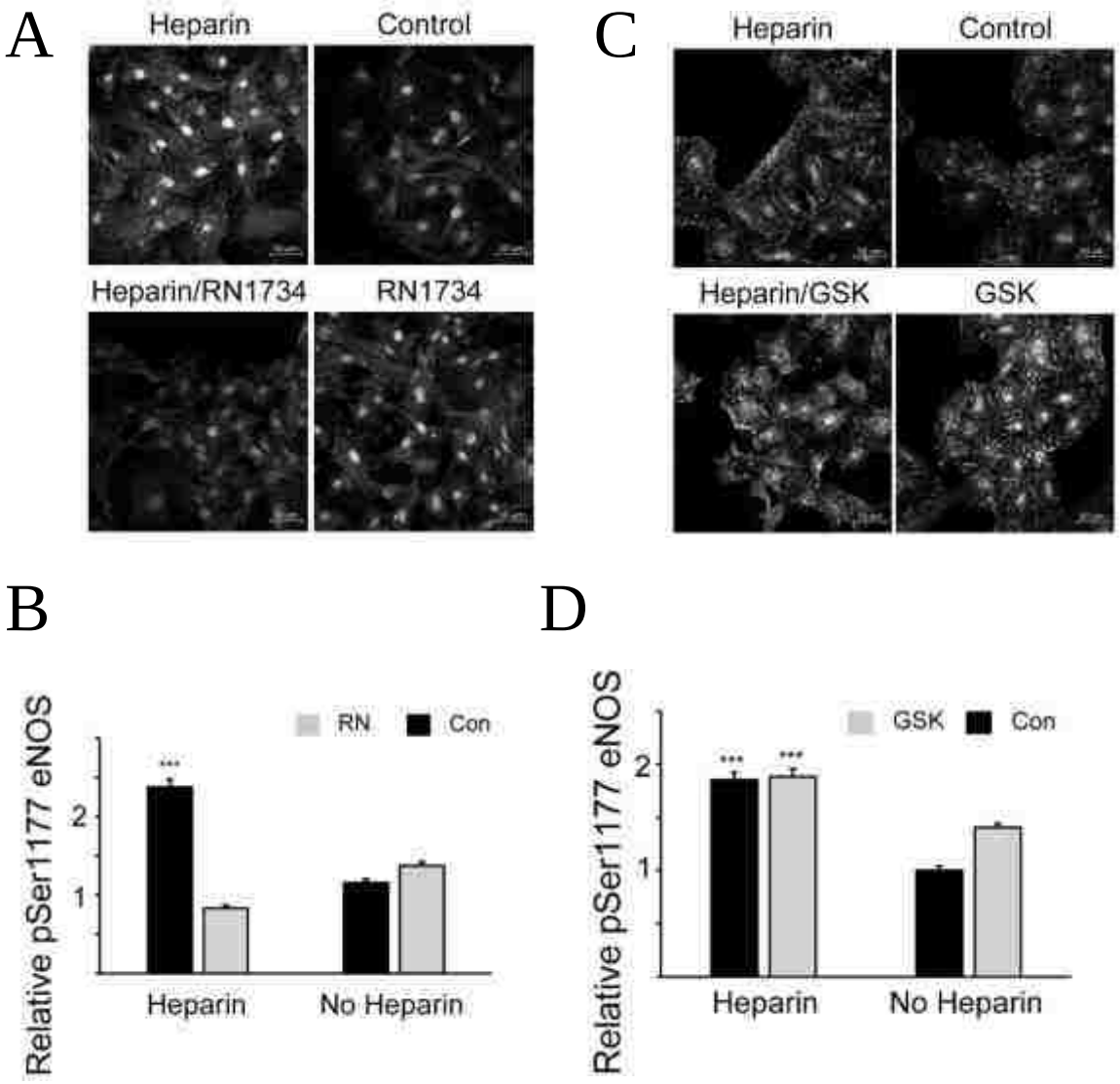
A, A7r5 cells preincubated with or without EGTA were treated with or without 200 μg/ml heparin followed by fixation with 4% PFA. Images are representative of three independent experiments. *Scale bars = 50 μm. B,* A7r5 cells were treated with heparin and EGTA as in A. At least 50 cells per condition were examined in each repeat for phosphorylated eNOS. ***, $p < 0.0001$.

3.7 KN-93 decreased heparin induced activation of eNOS



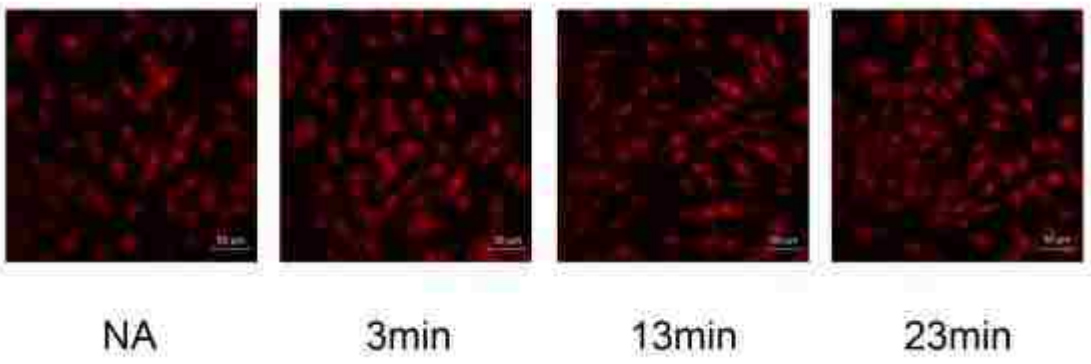
A, A7r5 cells preincubated with or without KN-93 were treated with or without 200 μg/ml heparin followed by fixation with 4% PFA. Images are representative of three independent experiments. *Scale bars = 50 μm. B,* A7r5 cells were treated with heparin and KN-93 as in A. At least 50 cells per condition were examined in each repeat for phosphorylated eNOS. ***, $p < 0.0001$.

3.8 TRPV4 inhibitors decreased heparin-induced activation of eNOS.



A and B., A7r5 cells preincubated for 30 min with or without RN1734 were treated with or without 200 μg/ml heparin followed by fixation and staining for p-eNOS. Images are representative of three independent experiments. *Scale bars = 50 μ*m. **C and D**. A7r5 cells were treated with GSK and heparin as in A and B. At least 50 cells per condition were examined for phosphorylated eNOS in each repeat. **, $p < 0.0001$, heparin vs. without heparin.

3.9 Heparin does not significantly change activity of Akt in A7r5 cells



A7r5 cells were treated with 200 μg/ml heparin followed by fixation with 4% PFA and staining for active Akt. Images are representative of three independent experiments. *Scale bars = 50* μm.

Chapter 4: Conclusions and Future Directions

4.1 Conclusions

We identified the heparin receptor TMEM184A by using Monoclonal heparin binding blocking antibodies. We also examined the hypothesis that eNOS has an essential role in heparin's anti-proliferation responses in VSMCs. Our group previously reported that cGMP-dependent protein kinase, which is a downstream factor of eNOS, is involved in heparin-induced down-regulation of MAPK activation in VSMCs [18]. Our finding that heparin's receptor, TMEM184A, co-localizes with caveolin-1, which is eNOS's binding partner, also supports the hypothesis that eNOS plays a role in heparin's anti-proliferative signaling pathway. We report here that by knocking down eNOS, inhibition of ERK and Elk-1 activation typically induced by heparin is abolished, a finding which provides evidence supporting this hypothesis.

To further support the hypothesis, we determined that in response to heparin treatment, eNOS's activity and sub-cellular translocation are altered. Phosphorylation of eNOS on Ser1177 was induced by heparin treatment as early as 3 min and dropped to basal levels after 30 min. These data are consistent with earlier results indicating that heparin treatment can preserve NOS activity in damaged tissue [20]. Crucially, we demonstrated that eNOS activation upon heparin treatment is dependent on heparin receptor TMEM184A by knocking down TMEM184A.

Our work also reveals that phosphorylated eNOS is initially found at cell membrane and focal adhesion sites upon heparin treatment and subsequently moved with TMEM184A to the peri-nuclear region. This result coupled with co-immunoprecipitation suggests that TMEM184A may be an eNOS binding partner that serves to regulate the activity or

translocation of p-eNOS from the regions adjacent to the cell surface to perinuclear compartments. These results are consistent with published evidence on TMEM184A, which implicated TMEM184A in membrane trafficking [59]. Besides binding to eNOS, TMEM184A also co-localizes with cav-1 and is required for heparin signaling responses [4]. It is possible that TMEM184A facilitates the dissociation from cav-1 and subsequent internalization of the activated eNOS after heparin treatment. Detachment from cav-1 is necessary for activation [116] and the following internalization of p-Ser1177 eNOS is required for the consequent deactivation and desensitization of eNOS.

There are many reports indicating interactions between integrin and cav-1. For example, β1 Integrin/cav-1 mechano-signaling complex responds to shear stress and leads to RhoA and actin remodeling in endothelial cells [105]. β1 integrin endocytosis is dependent on cav- 1 in myofibroblasts [106]. There have been reports of association between heparin and integrin. The ectodomain of α5β1 integrin binds to heparin with high affinity in endothelial cells (KD = 15.5 nM) [117]. Heparin also binds to αvβ3 integrin in endothelial cells [118]. There is also evidence suggesting that integrin controls localization of cholesterol-enriched membrane microdomains [96] at the plasma membrane of epithelial cells to regulate cellular processes [108]. Based on our previous data that TMEM184A co-localizes with cav-1 [4], it is possible that TMEM184A also interacts with integrin in caveolae or lipid rafts. Several proteins including integrin, gal-3, and VE-cadherin have a potential to also associate with TMEM184A and participate in heparin signaling. Heparin's anti-inflammatory and anti-proliferation signaling pathways share some players with laminar shear stress response. For example, laminar shear stress

and heparin can both reduce VSMC proliferation and ERK1/2 activity. Thus, it is reasonable to hypothesize that heparin and its receptor TMEM184A have interactions with or play roles in mechanotransduction processes by interacting with one or more members of mechanotransduction signaling system.

Integrin is vital in the mechanosensing complex and secondly it is an upstream factor of Akt, eNOS and ERK1/2. In addition, integrins are present in both ECs and VSMCs. There are also published results showing that up-regulation of HSPG synthesis induced by heparin in endothelial cells is dependent on the interaction of heparin with integrin while RGD peptide abolishes the effect [96]. This up-regulation is also associated with the phosphorylation of focal adhesion proteins and Ras/Raf/MEK/ERK MAPK and Ca^{2+} /NO pathways that are downstream signaling factors of integrins confirmed by using inhibitors of Ras, MEK, NOS. Therefore, we hypothesized that integrin might interact with TMEM184A to interfere with eNOS's binding with cav-1 in response to heparin. Our finding of activated eNOS in what appeared to be focal adhesions further supported the hypothesis of integrin involvement in the heparin/TMEM184A induced eNOS activation. Our results demonstrate support for this hypothesis. In addition to the localization of pSer 1177 eNOS at focal adhesions, immunoprecipitation with anti-TMEM184A antibodies pulled αV integrin along, though similar results were not seen using probes for β1 integrin. As expected, RGD treatment resulted in eNOS activation in a pattern similar to heparin-induced activation. However, such treatment with either heparin or RGD in TMEM184A knockdown cells did not result in eNOS activation. Most intriguingly, heparin treatment coupled with RGD did not result in eNOS activation.

Whether heparin interferes with RGD binding, or whether structural changes induced by either heparin or RGD cannot occur when both heparin and RGD are present, further studies are needed to determine the inter-relationships between these various players. Evidence suggests that the eNOS pathway in endothelial cells has important effects at focal adhesions including cGMP-dependent protein kinase regulation of migration and focal adhesion sites [119] and NO induced modulation of focal adhesions [120]. eNOS is regulated through various cellular events including post-translational modification, calcium wave stimulation, association or dissociation with its binding proteins. In addition, shear stress induces phosphorylation of endothelial eNOS through a PI3-kinase Akt pathway [89][77]. We evaluated the possibility that Akt was activated and then phosphorylated eNOS, but we found no evidence of changes in Akt activity in the period consistent with heparin effects (Fig 4.3.9). Given that heparin elevated eNOS activity shortly after treatment, we hypothesized that calcium stimulation might be involved in eNOS activation in response to heparin. Intracellular Ca^{2+} increases can cause calmodulin binding to eNOS and hence facilitate electron transfer from the reductase domain to the oxygenase domain of the NOS to synthesize NO [96]. Heparin treatment results in intracellular Ca^{2+} release, actives PLCγI and CaMKII, as well as inducing NO production [96] since PLCγI produces IP3 and induces Ca^{2+} release. Besides facilitating electron transfer, Ca^{2+}/calmodulin complexes can also activate CaMKII to phosphorylate eNOS. Initial studies found that EGTA inhibited heparin-induced eNOS activation, which suggests that Ca^{2+} is essential for heparin's effect on eNOS. EGTA eliminated extracellular Ca^{2+} from the cell media to reduce Ca^{2+} concentration suggesting that

extracellular Ca^{2+} is required for initial eNOS activation. We cannot exclude the possibility that eNOS was also activated by intracellular Ca^{2+} release in the longer term. We used CaMKII inhibitor KN-93 and showed that CaMKII is involved in eNOS's activation by heparin because KN-93 decreased, but did not abolish, the activation. It is likely that both calcium/calmodulin binding to eNOS and CaMKII phosphorylation are involved in the response.

Considering that eNOS activation is fast in response to heparin treatment, and eliminating extracellular Ca^{2+} blocked immediate heparin-induced responses, we decided to explore transmembrane Ca^{2+} channels. A TRPV4/ Ca^{2+}-mechanism of eNOS activation was recently identified in endothelial cells [110]. There is an ATP-dependent TRPV4 activation coupled with an eNOS feedback mechanism in the endothelial system that limits the extent of activation. Another recent study identified a role for TRPV4 in fluid shear stress in endothelial cells with a specific localization in small clusters at the basal membrane [121]. We used TRPV4 inhibitors GSK2193874 and RN1734. The results we obtained using RN1734, a very commonly employed inhibitor, showed a reversal of heparin's effect on eNOS, consistent with TRPV4 playing a role in heparin-induced eNOS activation. The inhibitor GSK2193874 only reduced such up-regulation, and resulted in increased basal eNOS phosphorylation. The reason for different responses to the two TRPV4 inhibitors is not clear, but could possibly be due to TRPV4 interacting proteins, differently influencing effects of the two inhibitors. Overall, our results support the idea that TRPV4 plays a role in heparin's activation of eNOS through facilitating calcium transients required for eNOS phosphorylation. Additional studies will be

required to determine how the heparin-TMEM184A-TRPV4 activation mechanism works, but this system is consistent with the rapid activation of eNOS identified in our studies. Immunoprecipitation in endothelial cells using heparin-receptor MAbs identified the target protein as TMEM184A [4]. In cell lines including RAOSMCs and BAOECs, the putative heparin receptor TMEM184A co-localizes with cav-1, which is one of the most important eNOS associating proteins that regulates activity of eNOS [4]. Here we report that heparin interactions with TMEM184A result in activation of eNOS through Ca^{2+}-dependent phosphorylation at Ser 1177. These results provide additional evidence linking mechano-signaling and heparin-induced signaling pathways. In addition, they suggest intriguing next steps in our exploration of heparin signaling mechanisms.

In addition to facilitating Ca^{2+}-induced phosphorylation, it is possible that after heparin treatment, TMEM184A acquires the ability to interfere with eNOS's binding with its binding partners such as cav-1 and subsequently activates eNOS. Alternatively, TMEM184A might alter eNOS's activity by regulating its sub-cellular translocation and life cycle since TMEM184A has been reported to be involved in membrane trafficking [59]. Additional studies will be necessary to examine the possible roles for TMEM184A in trafficking of eNOS. What is now clear is that heparin interactions with TMEM184A play a role in activation of eNOS through increases in intracellular Ca^{2+} and Ca^{2+}-induced phosphorylation of eNOS.

4.2 Signal Model

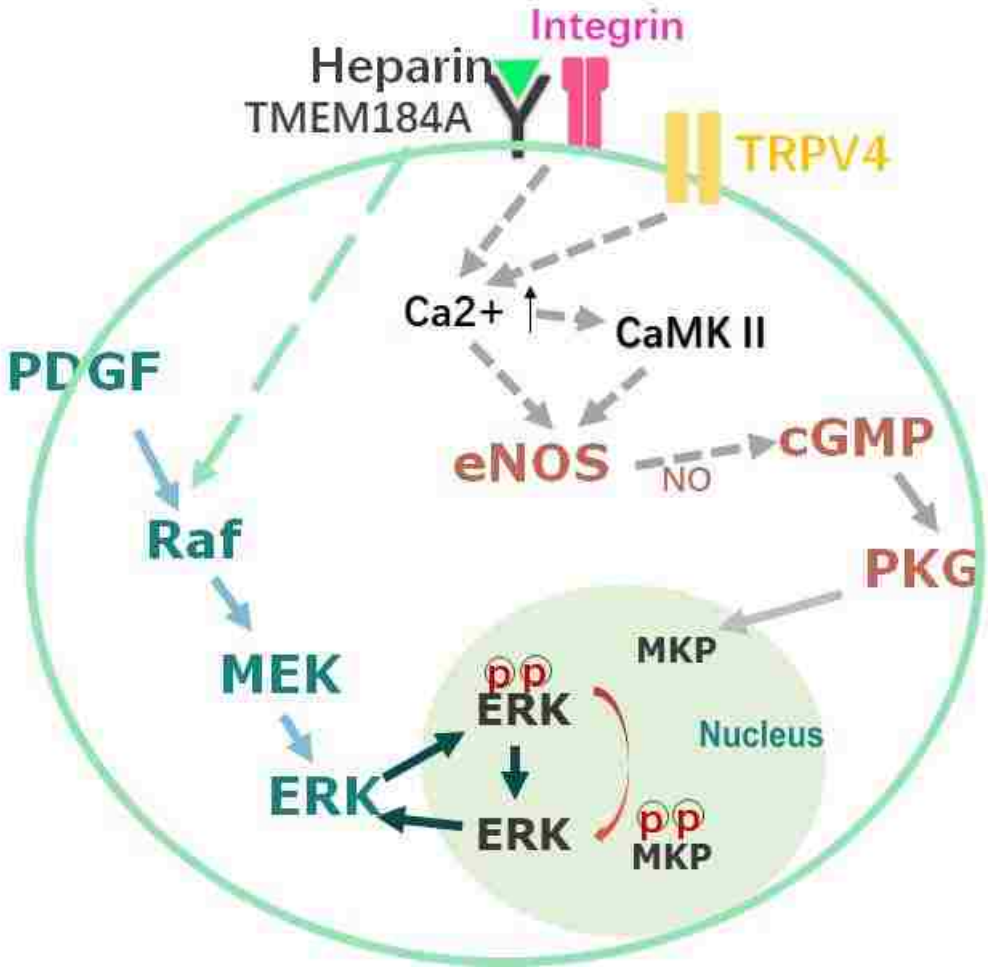


Figure 4.1 Predicted signal pathway for heparin-TMEM184A signaling pathway. The figure shows the signaling from heparin-TMEM184A binding to the activation of eNOS and decrease in p-ERK level.

4.3 Future Directions

4.3.1 Integrin in Heparin Signaling

Our work demonstrated that integrin was involved in heparin's regulation of eNOS while TMEM184A was required for the regulation. However, the detailed integrin related signaling events during the regulation process is not clear.

It is hypothesized that the direct binding between heparin/heparan sulfate and integrins is required for the localization of endostatin in endothelial cell lipid rafts ([118]. Endostatin is a C-terminal fragment derived from type XVIII collagen. It is an angiogenesis inhibitor and found to block growth factors such as VEFG mediating pro-angiogenic action [95]. The stimulation of HSPG synthesis and sulfation pattern modification are found to be mediated by interaction between heparin and integrin while RGD peptides block such effects [96]. And this upregulation is associated with phosphorylation of focal adhesion proteins, MAPK pathways, and NO pathways [96]. Based on this evidence, integrin might be involved in heparin signaling through a mechanosensing based mechanism such as cross activation of RTKs and their downstream signaling effectors or possibly through its binding with heparin to control localization of endostatin to suppress proliferation. Antibodies to integrin αvβ3 or αvβ5 but not β1 abolish the mechanical strain induced mitogenic response in VSMCs [122]. Integrin αvβ3 is also a major galectin-3–binding protein while antibodies that block αvβ3 integrin's function significantly inhibited the galectin-3–induced angiogenesis. In SMCs, the occupancy of the heparin binding domain of αvβ3 ligand is necessary for proliferative effects of IGF-1 including phosphorylation of ERK [115]. It has been reported that association between some types of integrin and gal-3 induced an angiogenic response while gal-3 promoted clustering of the integrins and activated FAK. And MAPK/ERK1/2 pathway is also mediating gal-3 signaling events. Thus, it worth hypothesizing that heparin treatment might disrupt or enhance binding between integrin and gal-3 since heparin can both bind to integrin and gal-3. The disruption might hinder or improve either integrin signaling or gal-3 signaling.

4.3.2 Rab11 and TMEM184A in Intracellular Recycling

The RAB11 family of GTPases is found in post-Golgi membranes, perinuclear recylcing endosomes. It is well known in regulating [123] endocytosed cargos. It is also revealed to regulate vesicle exocytosis at plasma membrane [123]. Rab11 is commonly used as a marker for Perinuclear recycling compartment, which located in juxtanuclear region and need Rab11 for its function and morphology. Many endocytosed recptors such as integrins are delivered here before recycling back to plasma membrane [67]. Rab11 is involved in building motor protein complexes [122] and transporting of recycling endosomes [68]. Based on the fact that TMEM184A co-localizes with eNOS at perinuclear regions and it is highly suspected in membrane trafficking, it is reasonable to see if TMEM184A co-localize or interacts with Rab11.

It is not clear if the TMEM184A at perinuclear region is under recycling between plasma membrane and ERC or is just synthesized and transported to Golgi. In order to answer this question, sub-cellular distribution GFP-TMEM184A can be monitored either by fixation at different time points or live cell imaging while blocking new protein synthesis with cycloheximide after 48 hrs of expression. If TMEM184A is not involved in recycling, there should be a decrease of TMEM184A at perinuclear region after a period of time while many of them still can be seen on plasma membrane. Alternatively, the expressed TMEM184A can be tracked to see if those delivered to plasma membrane after expression will be internalized and go back to membrane again. If TMEM184A is proved to be part of the recycling process, further experiments are to be done to see if adding heparin is affecting the recycling process.

Activities and distributions of integrin can be regulated by its internalization and recycling. For example, a Rab11 dependent pathway can increase recycling of α5β1 integrin and triggers activation of ROCK and inactivation of cofilin [67]. As mentioned above, TMEM184A might interact with integrin and they might associate with each other in caveolae. If TMEM184A participate in recycling, it is possible that it regulates recycling of integrin or conversely.

The work illustrated in this dissertation provide a potential model that TMEM184A might work with transmembrane proteins to transduce heparin signal into intracellular signaling pathways, triggering Ca^{2+} flows, activating eNOS and subsequently down regulates p-ERK and p-ELK. It further reveals TMEM184A's role in regulating heparin signaling based on what was reported earlier by our lab.

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PUBLICATIONS

1. Pugh, R., Slee, J.B., Farwell, S.L.N., Li, Y., Barthol, T., Patton, W.A. and Lowe-Krentz, L.J. 2016. Transmembrane protein 184A is a receptor required for vascular smooth muscle cell responses to heparin. J. Biol. Chem. 2016 Jan 14pii: jbc.M115.681122.

2. Farwell S, Slee J, Li Y, Lowe-Krentz L. Using a GFP-tagged TMEM184A Construct for Confirmation of Heparin Receptor Identity. Journal of Visualized Experiments. 2017 (120)

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1. Li Y, Lowe-Krentz L. Endothelial Nitrous Oxide Synthase activation is required for heparin receptor effects on vascular smooth muscle cells

CONFERENCES

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