Differential Effects of Reduced Cyclic Stretch and Perturbed Shear Stress within the Arterial Wall

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PAR

Tyler THACHER

acceptée sur proposition du jury:

Prof. V. Hatzimanikatis, président du jury

Prof. N. Stergiopulos, Dr R. Fernandes Da Silva, directeurs de thèse

Prof. J. A. Hubbell, rapporteur

Prof. S. Lehoux, rapporteur

Dr P. Silacci, rapporteur

ÉCOLE POLYTECHNIQUE
FÉDÉRALE DE LAUSANNE

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Summary

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Due to the pulsatile nature of blood flow, arteries are constantly exposed to dynamic mechanical forces; the pulsatility continuously stretches the vessel wall and the flow creates a frictional force on the interior surface. These stresses, referred to as cyclic circumferential stretch and shear stress, are known to determine arterial structure and morphology; modulation of which leads to the progression of vascular diseases such as hypertension and atherosclerosis. Yet the individual contributions of cyclic stretch and shear stress, with regards to vascular disease, have yet to be revealed.

In this thesis I wish to identify the role of reduced cyclic stretch in the development of endothelial dysfunction and vascular remodeling, develop an experimental model for studying the autonomous effects of shear stress and cyclic stretch and how these two stimuli individually modulate markers of vascular disease in different regions of the vascular wall.

I will begin by introducing the different structural and cellular components of the vascular wall and their individual functions. From here I will introduce how hemodynamic forces transmitted to the vascular wall due to the pulsatile nature of blood flow play an essential role maintaining arterial health and function. And as such, how deviations from a physiologic hemodynamic range can have catastrophic implications for the vasculature. Next I will introduce how certain hemodynamic conditions can stimulate cellular dysfunction and how this relates to initiation and progression of vascular disease.

In the first paper, we set out to determine if reduction of cyclic stretch could be a factor which induces remodeling of the arterial wall. We found that reducing compliance caused a decrease in vascular smooth muscle function, as well as inducing switch in smooth muscle cell phenotype. Arteries exposed to a reduced cyclic stretch also exhibited increased matrix degradation and cellular proliferation than those allowed to stretch physiologically. These findings accent the importance of cyclic stretch in the maintenance of a differentiated and fully functional phenotype of vascular smooth muscle cells, as well as in the regulation of migratory properties, proliferation and matrix turnover in the vascular wall.

In the second paper we investigated how reduction of cyclic stretch influences endothelial dysfunction and modulation of nitric oxide bioavailability. We observed that reduced compliance significantly decreases the activity of the enzyme responsible for producing nitric oxide (eNOS). Overall production of reactive oxygen species were also increased by reducing compliance, which we were able to attribute to stimulation of the superoxide generating NAD(P)H oxidase. We found that experimentally reduced compliance also caused a significant decrease in endothelial function, as assessed with bradykinin dependent vascular relaxation. The results from this study point out how reduced arterial compliance interrupts the eNOS activation pathway and increases vascular levels of oxidative stress, which together could explain the measured decreases on endothelial functionality.

In the third article we used our experimental model to investigate how shear stress and cyclic stretch independently stimulate the vascular wall. We found that both oscillatory flow and reduced stretch are detrimental to endothelial function, whereas oscillatory flow alone, dominated total endogenous vascular wall superoxide anion production. Yet when superoxide anion production was analyzed in just the endothelial region we observed that it was

modulated more significantly by reduced cyclic stretch than by oscillatory shear, emphasizing an important distinction between shear and stretch mediated effects to the vascular wall. Analysis of eNOS and nitro-tyrosine, the by-product of superoxide anion and nitric oxide, proved that they too are more significantly negatively modulated by oscillatory flow, than by reduced stretch. The findings from this study point out how shear and stretch stimulate regions of the vascular wall differently, affecting NO bioavailability and contributing to vascular disease.

The goal of the fourth article is to further investigate how shear stress and cyclic stretch modulate markers of vascular remodelling in different regions of the arterial wall. We demonstrated that while total superoxide production, fibronectin expression and gelatinase activation are predominantly mediated by shear stress, their expression in the endothelial region is mediated by reduced cyclic stretch, which correlates well with results from total MMP-2 expression. By plotting intensity versus radius for these markers of vascular remodeling we are able to see that superoxide production and gelatinase activity follows trends indicating their expression is in part mediated by stress distributions through out the vascular wall, while fibronectin and p22-phox were much less or not at all. Most importantly these findings, when coupled with our results from tissue reactive studies, suggest that the arterial remodeling process triggered in the endothelial region due to reduced stretch causes the most significant changes in arterial smooth muscle function. Perturbed shear stress and reduced arterial compliance have both been implicated in the initiation and progression of vascular disease: this work provides a new perspective into how these stimuli are perceived through out the vascular wall.

To conclude, this body of work has shown that cyclic stretching due to the pulsatile nature of blood flow is an essential stimulus regulating arterial remodeling and endothelial viability. We have performed experiments permitting the autonomous effects of cyclic stretch and shear stress to be studied. Yet more importantly, we have shown how cyclic stretch and shear stress stimulate the vascular wall in different regions and compared this data to arterial functionality studies. These results have indicated that although reduced cyclic stretch may not stimulate the total expression of certain markers of vascular disease as much as an OSC shear stress, it does so in specific regions of the vascular wall which can in fact have a more detrimental effect on arterial function. As reduced arterial cyclic stretching is associated with the aging process, this work gives insight into the progression of vascular disease over the course of a person’s life time.

Keywords: Artery, circumferential stretch, shear stress, endothelial function, arterial remodeling

Résumé

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A cause de la nature pulsatile de l'écoulement sanguin, les artères sont constamment exposées à des forces mécaniques dynamiques, l'effet pulsatile déforme continuellement la paroi artérielle et le flux sanguin crée une force de friction sur la surface intérieure. Ces contraintes, qui se réfèrent aux déformations cyclique circonférentielle et aux contraintes de cisaillement, sont connues pour affecter la structure artérielle et morphologique; cette adaptation conduit à la progression de maladies vasculaires telles que l'hypertension et l'athérosclérose. Jusqu'alors, les contributions individuelles des déformations cycliques et contraintes de cisaillement concernant les maladies vasculaires n'ont pas été reportées.

Dans le cadre de cette thèse, je souhaite identifier le rôle d'une déformation cyclique réduite dans le développement du disfonctionnement endothélial et le remodelage vasculaire, développer un modèle expérimental afin d'étudier les effets indépendants des contraintes de cisaillement et de l'étirement cyclique et de la manière dont ces deux stimuli affectent individuellement les marqueurs des maladies vasculaires dans différentes régions de la paroi artérielle.

Je vais commencer par introduire les différents composants structurels et cellulaires de la paroi artérielle ainsi que leurs fonctions individuelles. Ensuite j'introduirai la manière dont les forces hémodynamiques, dues à la nature pulsatile de l'écoulement sanguin, sont transmises à la paroi artérielle et leurs rôles essentiels dans le maintien de la bonne santé et de la fonction artérielle. En tant que telles, les déviations d'une plage physiologique d'hémodynamique peuvent avoir des implications catastrophiques sur la vascularisation. Finalement, j'introduirai la manière dont une hémodynamique délétère stimule le dysfonctionnement cellulaire et comment cela se rapporte à la progression des maladies vasculaires.

Dans le premier papier, nous avions planifié de déterminer si la réduction des déformations cycliques pouvait être un facteur qui induit le remodelage de la paroi artérielle. Nous avons trouvé que la réduction de la compliance cause une diminution de la fonction des cellules musculaires lisses, de même qu'induit une permutation du phénotype des cellules musculaires lisses. Les artères exposées à une réduction des déformations cycliques présentaient également une plus grande augmentation de la dégradation de la matrice de même qu'une plus grande prolifération cellulaire par rapport à celles permises lors de déformations physiologiques. Ces résultats ont accentué l'importance des déformations cycliques dans le maintien d'un phénotype différencié et complètement fonctionnel des cellules musculaires lisses, de même que la régulation des propriétés migratoires, de la prolifération et du renouvellement de la matrice de la paroi artérielle.

Dans le second papier, nous avons étudié la manière dont la réduction de la déformation cyclique pouvait influencer le dysfonctionnement endothélial et l'adaptation de la biodisponibilité de l'oxyde nitrique. Nous avons observé que la réduction de la compliance diminuait significativement l'activité de l'enzyme responsable de la production de l'oxyde nitrique (eNOS). La production globale d'espèces réactives oxygénées était également augmentée par la réduction de la compliance, pour laquelle nous étions capables de l'attribuer à la stimulation de superoxyde générant l'oxydase de NAD(P)H. Nous avons trouvé qu'une réduction expérimentale de la compliance engendrait également une diminution significative de la fonction endothéliale, comme évalué avec la relaxation vasculaire dépendante de la bradykinine. Les résultats de cette étude montrent comment la réduction de la compliance artérielle interrompt le pathway d'activation de l'eNOS et augmente le niveau de contrainte

oxydative vasculaire, lesquels peuvent expliquer ensemble, la diminution mesurée de la fonctionnalité de l'endothélium.

Dans le troisième article, nous avons utilisé notre modèle expérimental pour étudier la manière dont les contraintes de cisaillement et les déformations cycliques stimulent chacune indépendamment la paroi artérielle. Nous avons trouvé qu'un flux oscillant et une réduction de la déformation sont tous les deux préjudiciables à la fonction endothéliale, alors qu'un flux oscillant seul, est le plus significatif dans la production totale d'anion superoxyde de la paroi artérielle. Jusqu'alors, lorsque la production d'anion de superoxyde était uniquement analysée dans la région endothéliale, nous observions que cela était adapté de manière plus significative par la réduction de la déformation cyclique que par le cisaillement oscillant, accentuant la distinction importante entre les effets engendrés du cisaillement et de la déformation sur la paroi artérielle. L'analyse de l'eNOS et de la nitro-tyrosine, le dérivé de l'anion du superoxyde et de l'oxyde nitrique, a prouvé qu'ils sont les deux négativement adaptés de manière plus significative par le flux oscillant que par la réduction de la déformation. Les résultats de cette étude montrent à quel point le cisaillement et la déformation stimulent différemment la paroi artérielle, affectant la biodisponibilité de NO et contribuant aux maladies vasculaires.

L'objectif du quatrième article était d'étudier davantage la manière dont les contraintes de cisaillement et les déformations cycliques adaptent les markers du remodelage vasculaire dans différentes régions de la paroi artérielle. Nous avons montré, que tandis que la production totale de superoxyde, l'expression de fibronectine et l'activation de la gélatinase étaient engendrées principalement par les contraintes de cisaillement, leurs expressions dans la région endothéliale l'étaient par la réduction des déformations cycliques, lesquels sont bien corrélés avec l'expression totale de MMP-2. En représentant l'intensité de ces markers vasculaires du remodelage par rapport au rayon, nous avons été capable de remarquer que la production de superoxyde et que l'activité de la gélatinase suivaient une tendance indiquant que leur expression était en partie engendrée par la répartition de contrainte au travers de la paroi artérielle, alors que la fibronectine et le p22-phox l'étaient beaucoup moins ou pas du tout. Encore plus important, ces résultats, lorsque couplés avec ceux des études des tissus réactifs suggèrent que le processus de remodelage artériel déclenché dans la région endothéliale, due à la réduction des déformations, cause le plus de changements significatifs de la fonction des cellules musculaires lisses. La perturbation de la contrainte de cisaillement de même que la réduction de la compliance artérielle ont les deux été impliqués dans l'initiation et la progression de maladie vasculaire: ce travail fournis une nouvelle perspective de la manière dont ces stimuli sont perçus à travers toute la paroi artérielle.

En guise de conclusion, ce travail a montré que les déformations cycliques, dues à la nature pulsatile de l'écoulement sanguin, sont un stimuli essentiel régulant le remodelage artériel et la viabilité de l'endothélium. Nous avons effectué des expériences permettant d'étudier l'effet indépendant des déformations cycliques et des contraintes de cisaillement. Encore plus important, nous avons montré de quelle manière les déformations cycliques et les contraintes de cisaillement stimulent la paroi artérielle dans différentes régions et comparé ces données à des études sur la fonctionnalité artérielle. Ces résultats ont indiqué que, bien que la réduction de la déformation cyclique ne puisse pas stimuler l'expression totale de certains markers de maladies vasculaires, autant qu'une contrainte de cisaillement oscillante, elle le peut dans des régions spécifiques de la paroi artérielle, laquelle peut en fait, avoir plus d'effets préjudiciables sur la fonction artérielle. Etant donné que la réduction de la déformation

artérielle est associée au vieillissement, ce travail donne un aperçu de la progression des maladies vasculaires au cours de la vie d'une personne.

Mots-clés: Artère, deformation cyclique, cisaillement, fonctionnement endothélial, remodelage vasculaire

Contents

Introduction

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Motivation

Cardiovascular diseases remain the leading cause of morbidity and mortality in the western world. One of the most prevalent involves plaque buildup on the inner wall of arterial vessels and is called atherosclerosis. Atherosclerosis is usually regarded as a systemic disease, having several well defined risk factors (hypertension, diabetes mellitus and cigarette smoking) implicated in its pathogenesis. The causes of atherosclerosis are multi-factorial; understanding them could help earlier detection or prevention of the disease. The regular occurrence of atherosclerotic plaques in regions of curvature and branch points, magnified by ailments such as high blood pressure, suggests hemodynamics, arterial wall mechanics and geometry play significant roles in plaque formation [1]. The purpose of this thesis is to investigate how two hemodynamic forces, shear stress and cyclic stretch, modulate molecular, cellular and vascular biologic processes linked to the initiation and progression of atherosclerosis plaque buildup.

Arterial structural and functional layers

Arteries are comprised of multiple layers of cells and connective tissue which provide the functional and structural components required to adapt to changing hemodynamic conditions and to support high pressures. The innermost layer of the arterial wall is called “intima” and is comprised of an endothelial monolayer adhered to a thin network of vascular smooth muscle cells (VSMC), elastin and collagen fibers. Endothelial cells provide the barrier which interacts with blood flow, sense hemodynamic changes and are responsible for the signaling required for long and short term adaptations in the arterial wall.

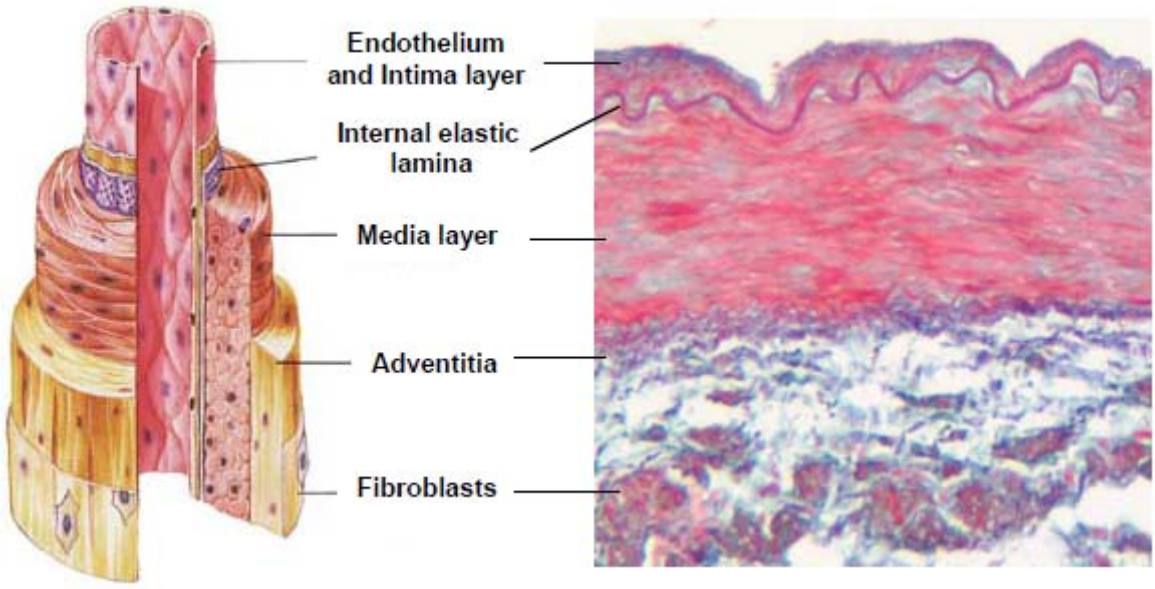

Figure 1: *Image depicting the different layers of a healthy artery*

The next layer of the vascular wall is called “media”, and is separated from the intima by the internal elastic membrane. This membrane acts as a filter, blocking the passage of macromolecules and cells while allowing the passage of nutrients and waste. But it also provides an important structural support to maintain healthy, fully differentiated endothelial cells. The medial layer is much thicker than the intimal layer and comprised of VSMC, distributed throughout a collagen and elastin network, thus providing the bulk of the structural

support of the arterial wall. In the beginning of the heart cycle, at lower pressures, it is mainly the VSMC tone and the elastic fibers which bear load. In this region the artery's deformation is essentially elastin, which helps to buffer pulsating blood flow. But later in the heart cycle, at higher pressures, it is the collagen fibers which bear the majority of the load. Separating the media from the next layer, the "adventitia" is the external elastic membrane, which like the internal elastic membrane is also comprised of mainly elastin fibers. The adventitia is a fibrotic connective tissue comprised of mainly of collagen, but also containing some elastin, fibroblasts and a small quantity of smooth muscle cells. The main purpose of this tissue is to tether the exterior of the artery to surrounding tissues within the body.

Arterial Function

Endothelial cell function

A monolayer of endothelial cells forms the primary barrier between blood flow and the arterial wall, and consequently is directly exposed to complex hemodynamic forces including shear stress and cyclic stretch. Endothelial cells are capable of perceiving and responding to changes in hemodynamic stimuli, thus mediating the expression of pro- or anti-atherogenic effects both on their surface and communicating these changes to the underlying smooth muscle. A defined characteristic of healthy, fully differentiated endothelial cells are that they align and elongate in the direction of flow [2]. One of the most important contributions of a functional endothelium is the ability to produce nitric oxide (NO). NO possesses strong anti-thrombotic, anti-adhesive and anti-inflammatory effects as well as regulating the contractile state of smooth muscle, thus adapting vascular diameter to compensate for changes in blood flow.

NO is a very reactive molecule and once formed has a half life of only a few seconds. Once formed, the fate of NO is either to diffuse into the blood stream and bind to passing red blood cells, diffuse into adjacent smooth muscle cells or react with superoxide anion. By binding to the heme moiety of hemoglobin in red blood cells, NO suppresses the coagulation cascade and is promptly broken down. In the smooth muscle, NO binds with the enzyme guanyl cyclase which promotes the de-phosphorylation of GTP to cGMP. This process decreases intracellular calcium concentrations, hyperpolarizes the VSMC membrane (via K+ channel activation) and activates MLC phosphatase, three mechanisms which cause a smooth muscle relaxation.

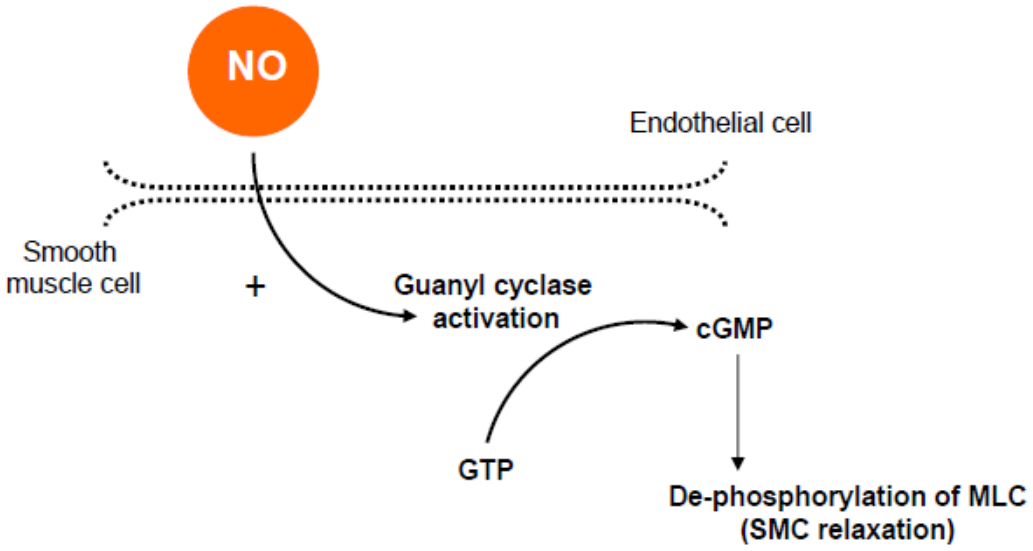

Figure 2: *NO mediated SMC relaxation*

NO is formed from the amino acid L-arginine by the enzymatic action of nitric oxide synthase (NOS). Enzyme activity can be induced by phosphorylation of NOS at Serine 1177 by activated Akt/PKB. Within the endothelial cell there are two types of NOS present, constitutive endothelial NOS (eNOS) and inducible NOS (iNOS). Under physiological conditions in blood vessels NO is continuously produced by eNOS, via mechanical and receptor mediated pathways. First, shearing forces acting on the vascular endothelium cause a release of intracellular calcium, thus activating the calcium-calmodulin dependent release of NO. Second, receptors for vasoactive substances such as acetylcholine and bradykinin, present on the surface of endothelial cells also stimulate the release of intracellular calcium and subsequently mediate NO production.

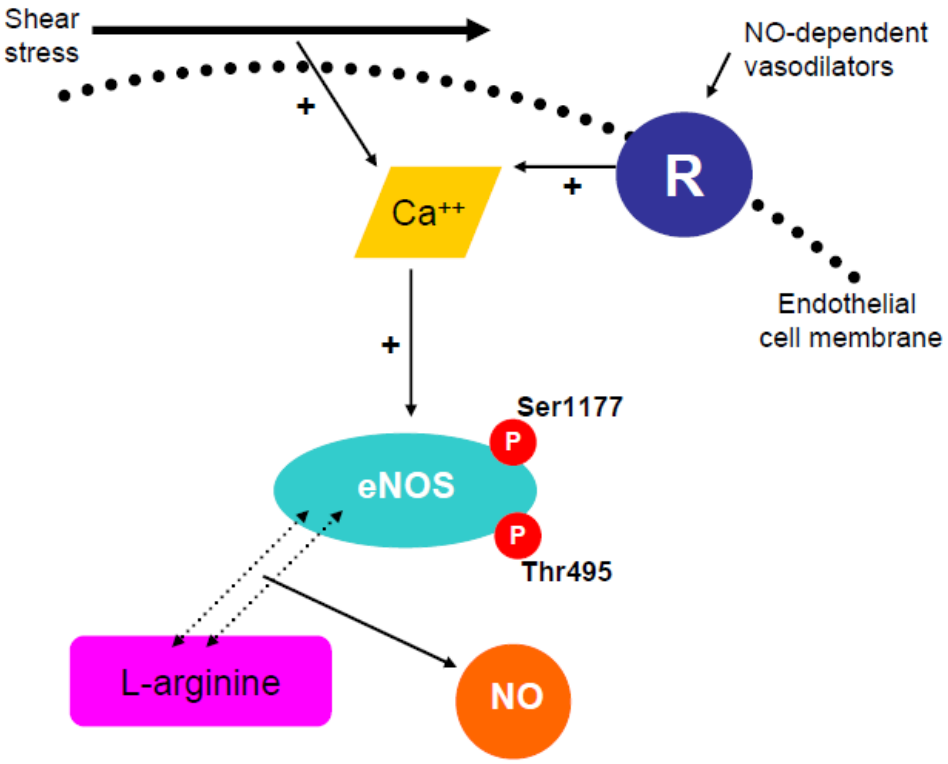

Figure 3: *eNOS signaling cascade*

The other isoform of endothelial NOS is iNOS. Its expression differs from eNOS, as it is calcium-independent. Under basal conditions the activity of iNOS is very low and is only stimulated by inflammatory cytokines and bacterial endotoxins. During inflammation the contribution of vascular NO via iNOS-mediated release can be upwards of 1000 times greater than that of eNOS.

Smooth muscle cell function

Smooth muscle cells are thought to respond primarily to mechanical stretching, but also receive biochemical signals from the endothelium. Under normal physiologic conditions well differentiated smooth muscle cells express a contractile phenotype allowing them to regulate their tone in response to mechanical and biochemical stimuli, thus regulating blood flow and dampening arterial pulsations. Smooth muscle cells expressing a non-contractile, or synthetic, phenotype lack the ability to adequately react to changing hemodynamic conditions and tend to be more proliferative, hence are thought to contribute to vascular disease [3].

Contractions in vascular smooth muscle can be initiated by a number of stimuli, including; mechanical, called a myogenic response; electrical, via depolarization of the VSMC membrane; and chemical, via stimuli such as norepinephrine, prostaglandin and endothelin-1 which bind to specific receptors on the VSMC. The different mechanisms of VSMC contraction are numerous, but all revolve around increasing intracellular calcium concentrations.

As intracellular calcium concentrations increase, either by influx through cell surface calcium ion channels or from internal stores (sarcoplasmic reticulum), the free calcium binds to a protein called calmodulin. In the presence of ATP, the calcium-calmodulin complex activates myosin light chain kinase, an enzyme which phosphorylates myosin light chains (MLC). The MLC phosphorylation induces the heads of the MLC to form a network with actin filaments, thus composing a contraction.

Two crucial structural proteins expressed in fully differentiated contractile smooth muscle cells are desmin and alpha-smooth muscle actin (alpha-SMA). Desmin plays a very important role connecting the cell nucleus to the cytoplasm, giving the cell structural integrity necessary for mechanical load bearing [4, 5]. There is also some evidence that desmin may connect certain components of smooth muscle to the extra cellular matrix, thus helping regulate contraction and movement [5]. Alpha-SMA also plays a central role in smooth muscle cell architecture, but as well provides the scaffold to which myosin proteins generate force to achieve smooth muscle contractions. Equally important, alpha-SMA provides the "hard-wiring" necessary to for the cell to support signal transduction [6].

Endothelial shear stress and cyclic mechanical stretch are key regulators of arterial function

We now know that within the vascular system the present mechanical forces are not simply artifacts of a beating heart ejecting blood through an elastic arterial system, but are stimuli essential to arterial function. Fluid shear stress and radial cyclic stretch of the arterial wall are understood to be two such stimuli.

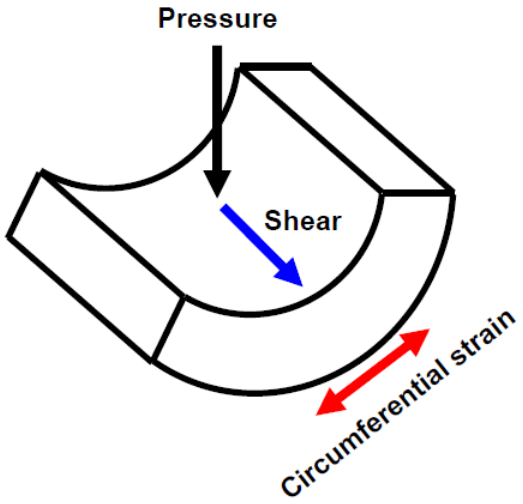

Figure 4: *Mechanical forces acting on the vascular wall*

Vascular cells have been shown to expect a narrow range of shear stress and cyclic stretch values, deviations of which are thought to initiate and propagate various vascular diseases such as atherosclerosis and hypertensive remodeling of the arterial wall.

Shear stress within the vasculature is the horizontal frictional force transmitted to the inner surface of the vascular wall by blood flow. Although a seemingly subtle detail, shear stress is recognized as an extremely important regulator of vascular function regulating arterial function, metabolism and defense against various diseases. The magnitude of shear stress (τ) can be estimated in straight vessels as being proportional to the blood flow (Q) and viscosity (μ) and inversely proportional to the third power of the internal radius of the vessel (r). More specifically it is defined by Poiseuille's law as:

$$\tau_{wall} = \frac{4\mu Q}{\pi r^3}$$

Experimental measurements have shown that magnitudes of shear stress in the human arterial tree generally range from 10 to 20 Dynes/cm^2 [7, 8]. But it has also been shown that there exists a huge variation in mean shear stress values between species which is inversely proportional to weight of the animal [9].

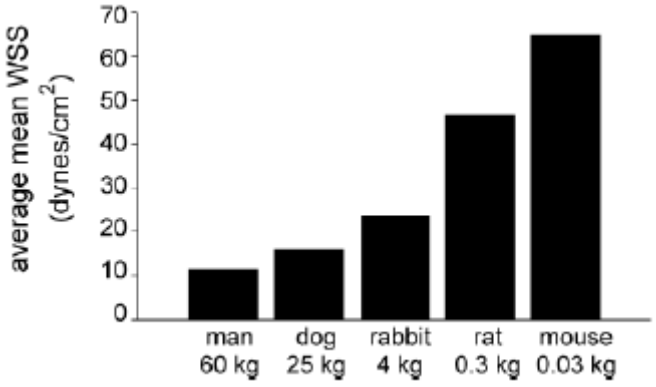

Figure 5: *Graph depicting how average shear stress scales to total weight of animal*

Exposing endothelial cells to shear stresses within this physiologic range encourages the production of anti-inflammatory, anti-thrombotic, anti-proliferative and vaso-dilative mediators. Combinations of these factors encourage a more robust arterial wall which is easily adaptive to hemodynamic changes. But the arterial system is a complicated network of branched and curving vessels creating areas of complex and regionally varying shear stress patterns. On the inner wall of a curving arterial vessel low shear stress patterns develop, while at branching points as the flow separates we see areas of low and/or re-circulating flow.

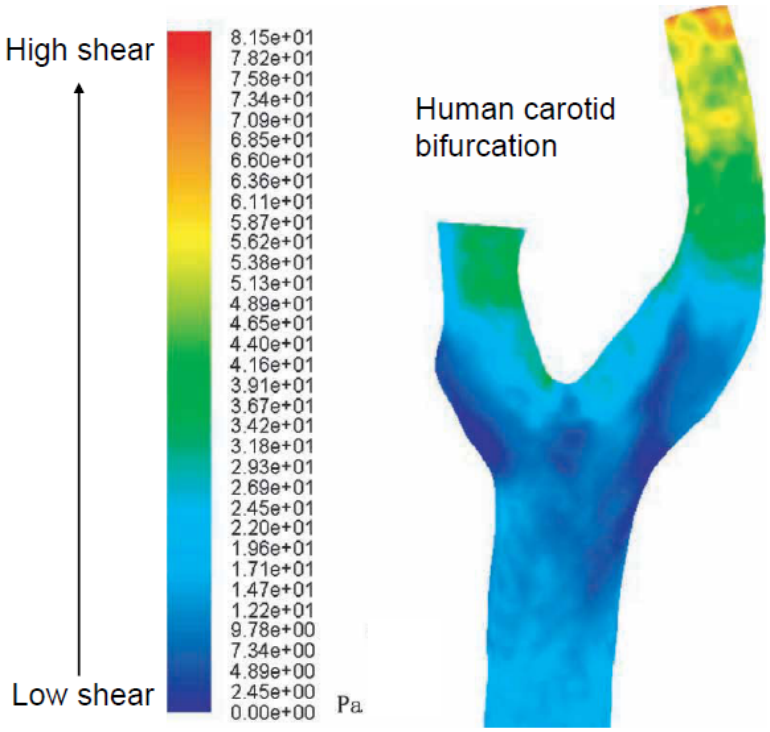

Figure 6: *Shear stress variations in a healthy human carotid*

Thus it is no coincidence that these are also the regions where atherosclerotic plaques develop, suggesting the link between regions of perturbed shear stress and arterial disease.

Throughout each heart cycle as blood is pushed through the arterial tree it is accompanied by a pulsating pressure wave thus coupling the average pressure of the arterial system with a cyclical stretching of the arterial wall. As the initial shock wave travels through the arterial system and arterial diameters become smaller, peripheral resistance increases thus sending a reflecting wave back in the opposite direction. A graph of pressure vs. time for a youthful human aorta is represented here.

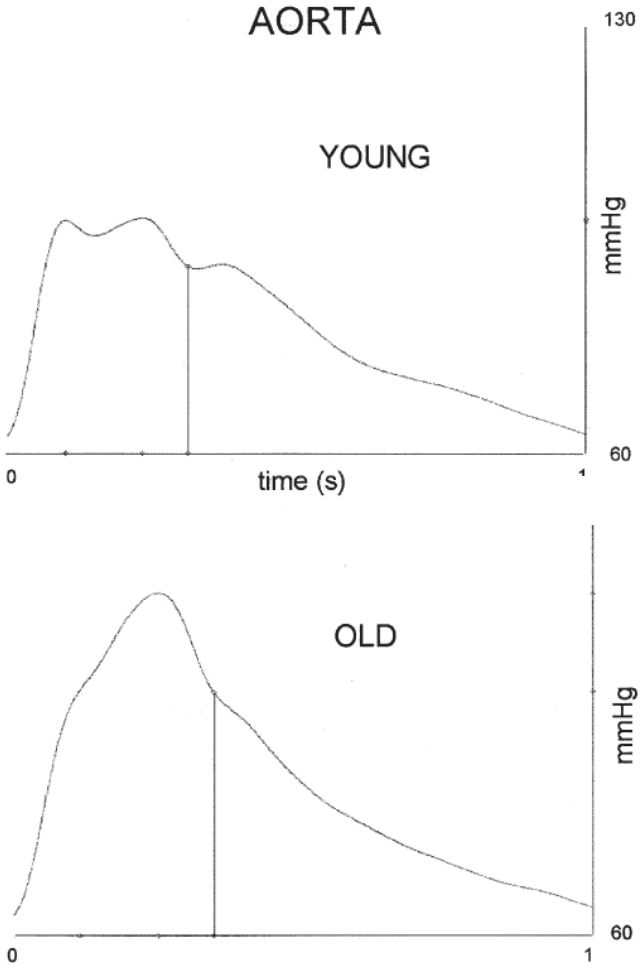

Figure 7: *Pressure wave versus time wave forms in a young and aged aorta*

As a person becomes older the arterial tree becomes stiffer, thus altering the pulse wave velocity and reflected wave forms. Increased stiffness is characteristically represented by an increased pulse wave velocity while changes in reflection can be recognized by the "late" systole and obvious changes in the diastole wave form.

The physiologic stretch of a healthy large arterial vessel over the course of one heart cycle corresponds to a change in diameter of 4 – 5%. Arterial stiffening, due to diseases such as hypertension, diabetes mellitus, or even simply aging are known to decrease the systemic compliance of the arterial tree thus reducing stretch. More focalized regions of arterial stiffening have been suggested to be modulated by hemodynamic forces [10] and involve interactions between the cellular and structural elements of the vascular wall [11].

Arterial Dysfunction

Oscillatory shear stress and reduced cyclic stretch contribute to vascular dysfunction
Hemodynamic variables, such as shear stress and stretch, outside of the normal range are most certainly linked to the initiation and progression of vascular disease, the most common of which is called atherosclerosis. Atherosclerosis is generally initiated when endothelial cells lose their wide range of healthy functions, which is marked by a general state of

inflammation, increased permeability and loss of several mechanisms to preserve their anti-coagulant surface. Progression of the disease is characterized by a focal, slow and progressive accumulation of cells, extracellular matrix and lipids in the intima region of medium to large sized conduit arteries.

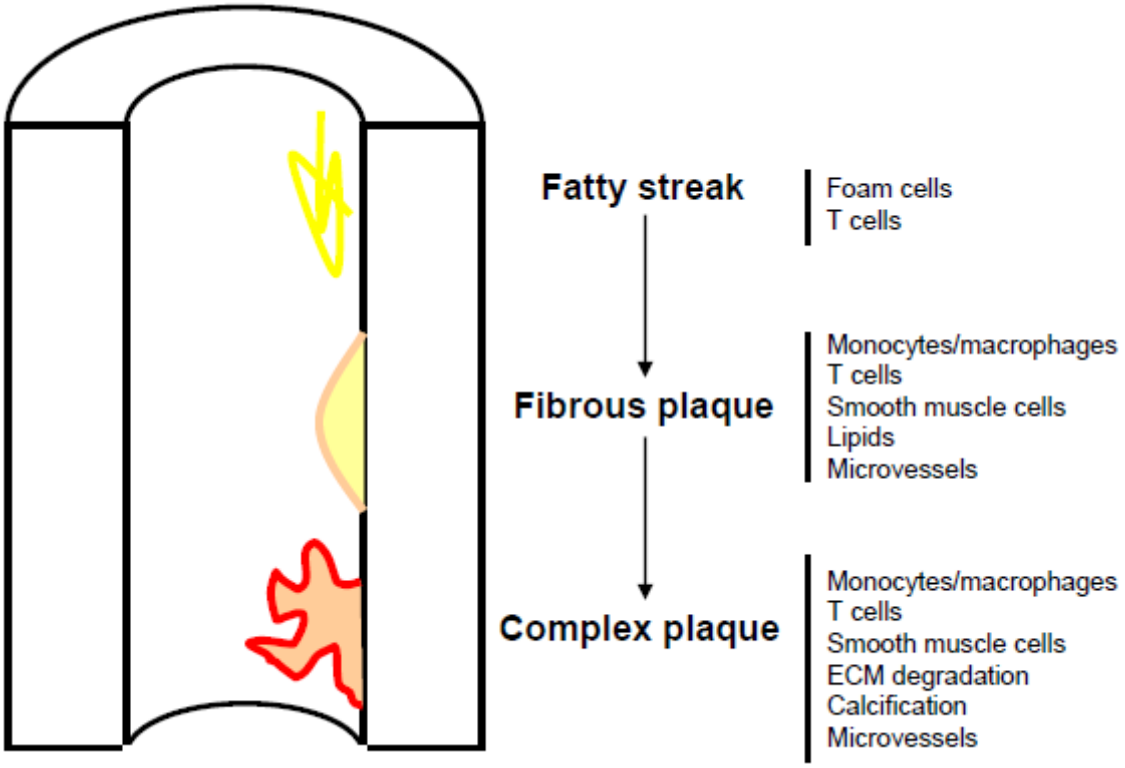

Figure 8: *Progression of atherosclerosis*

Previous works have shown that areas of the vasculature exposed to oscillatory flow and/or reduced cyclic stretch are at higher risk of developing endothelial dysfunction [11-16], intimal cellular proliferation and matrix accumulation [17, 18], than those exposed to a high unidirectional shear stress (HSS) and/or normal cyclic stretch.

Endothelial dysfunction and the progression of atherosclerosis

The underlying mechanisms of endothelial dysfunction are numerous and remain unclear, yet is generally agreed upon that the most dangerous revolve around a central point, that is diminished bioavailability of nitric oxide (NO)[19]. Thus, processes which upset the production or activation of eNOS or are capable of reacting with NO before the vasculature can benefit from its therapeutic properties are considered threatening. As mentioned previously, shear stress and cyclic stretch are known to be very important regulators of eNOS production and activation, thus affecting the production of NO. It is also suspected that mechanical forces play a role in the production of agents which have a high affinity for NO, thus lowering its bioavailability.

Reactive oxygen species and endothelial dysfunction

Reactive oxygen species (ROS) refer to a category of molecules containing an oxygen molecule with an unpaired electron. Within the vasculature this includes superoxide ($^{\cdot}O_2$-), hydrogen peroxide (H_2O_2) and hydroxyl anion (OH-). These molecules are produced in all vascular cell types (endothelial, smooth muscle and adventitial fibroblasts) during the

metabolism of O_2 and are biologically important for their oxidation/reduction potential. It is now recognized that ROS are important regulators of vascular function, modulating cell growth/death, migration, inflammation and extracellular matrix production. An imbalance in the redox state where pro-oxidants overwhelm the anti-oxidant ability results in what is called oxidative stress.

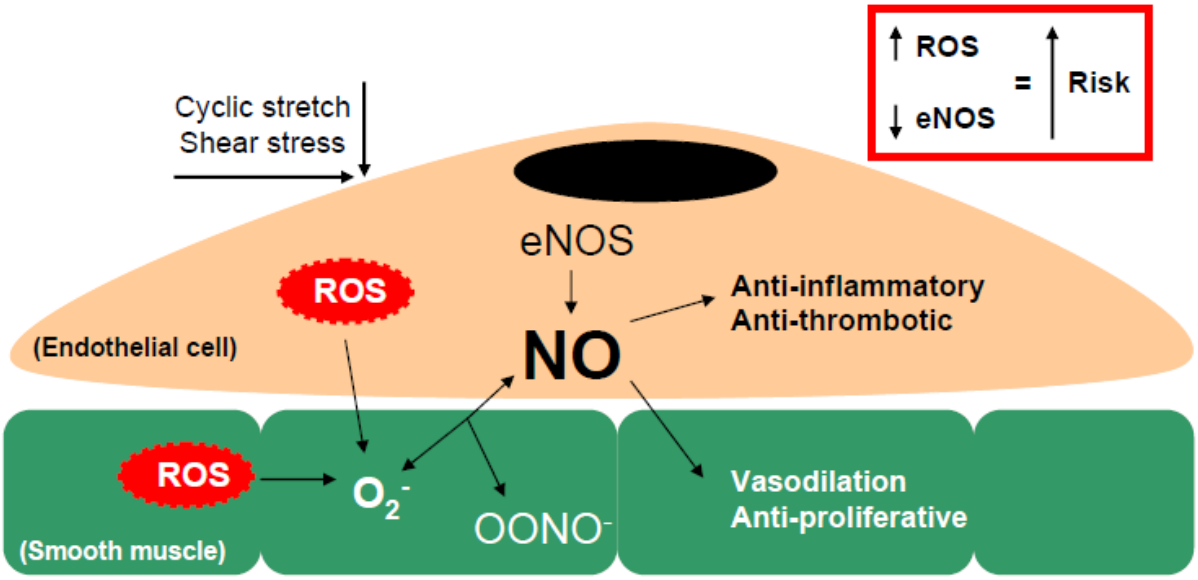

Figure 9: *NO and ROS interaction in vascular cells*

In vascular cells, ROS are primarily produced via the NAD(P)H oxidase, which is a membrane bound multi-subunit enzyme. In the presence of molecular oxygen the NAD(P)H oxidase catalyzes the production of $^{\cdot}O_2$-, donating one electron.

$2O_2 + NAD(P)H \rightarrow 2O_2^- + NAD(P)H + H^+$

The NAD(P)H oxidase comprises five subunits: p47phox, p67phox, p40phox, p22phox and gp91phox. In unstimulated cells, p40phox, p47phox and p67phox exist in the cytosol, where as p22phox and gp91phox are located in the cell membranes. Upon stimulation, p47phox becomes phosphorylated, the cystolic components form a complex which then migrates to the membrane where it assembles to form the activated NAD(P)H oxidase, capable of transferring electrons to O_2 leading to $^{\cdot}O_2$- generation.

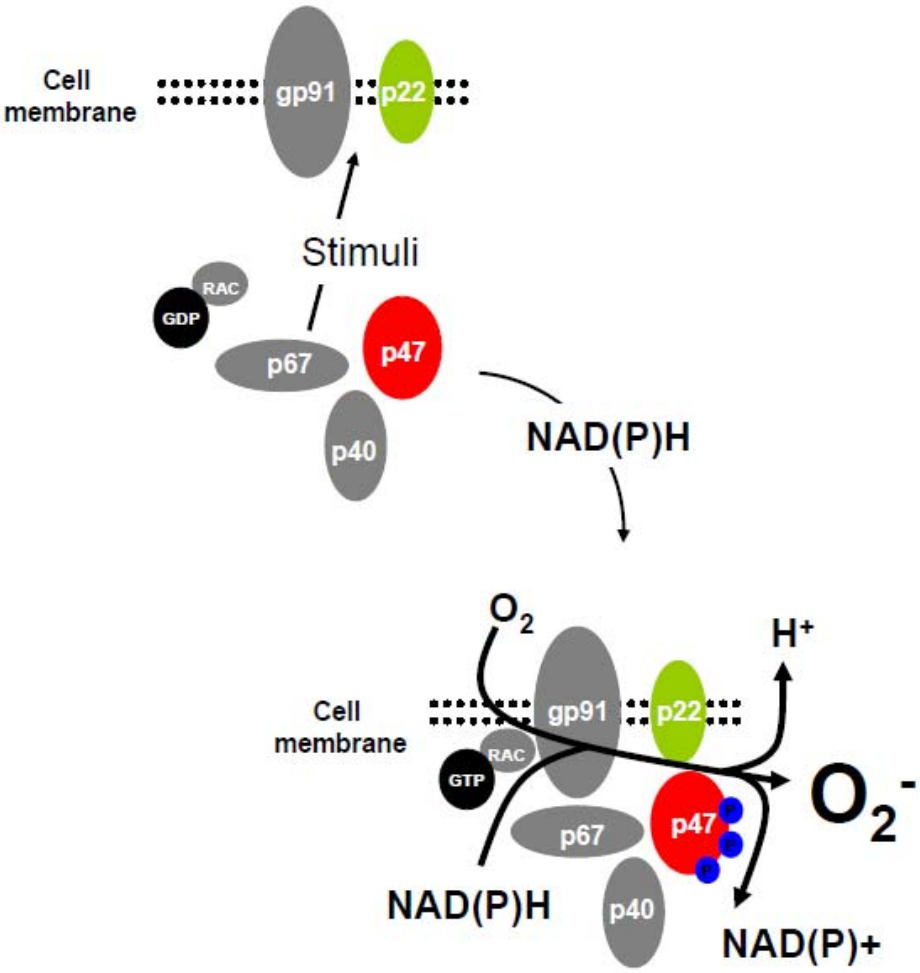

Figure 10: *NOX signaling cascade*

Vascular NAD(P)H oxidase has been shown to be regulated by many biochemical factors, such as cytokines, growth factors and vasoactive agents, but as well by physical factors such as shear stress and cyclic stretch [20, 21]. Having an unpaired electron makes ROS extremely reactive. In physiologic conditions, at neutral pH the favoured reaction of $^{\cdot}O_2$- is the dismutase reaction forming H_2O_2 [22]. But when produced in excess, significant amounts of $^{\cdot}O_2$- reacts with NO to form ONOO- (peroxynitrite) [23]. Peroxynitrite is also a very reactive molecule and potent oxidant, which can modify tyrosine residues in proteins to form a stable molecule, 3-nitro-tyrosine [24]. Thus, when vascular ROS reacts with NO it forms peroxynitrite (ONOO-) [24], removing NO [25] before it has the ability to perform it's therapeutic role in the vasculature.

Smooth muscle dysfunction and the progression of atherosclerosis

Vascular remodelling is an essential process allowing for adaptation and repair of the vascular wall which involves any sustained change in the shape, size and/or composition of a blood vessel. But improper remodelling, or absence of remodelling, is the underlying mechanism marking the progression of cardiovascular diseases such as atherosclerosis [26]. Mechanisms implicated in the initiation of chronic adaptive arterial remodeling are numerous, but recent evidence has implicated the production of reactive oxygen species (ROS) to be an important mediator of hypertensive vascular remodeling [27].

Vascular remodeling is a process which requires the breakdown and reorganization of extracellular matrix (ECM), allowing enhanced cellular migration and proliferation in the intima region. The most rigid component of the ECM in the vascular wall is collagen, thus any restructuring of the arterial wall is going to depend on breaking down and reorganizing collagen fibers. Matrix metalloproteinases (MMPs) are a large family of enzymes capable of digesting collagen, gelatin (denatured collagen) and reorganizing all different components of the ECM. MMPs can be produced by vascular cells [28] as well as in inflammatory cells such as macrophages [29]. In addition, activated macrophages are known to secrete cytokines shown to up-regulate MMP gene expression in vascular cells [30]. Within arterial wall the MMP-2 [31] and 9 are the primary MMPs responsible for remodeling, although it should be mentioned that there are 25 known MMP isoforms, some of which have also been implicated in arterial wall remodeling.

MMP are secreted inactive, with a proform domain which must be removed before the enzyme is active and capable of degrading ECM. Enzyme activation can occur through degradation by other proteases such as plasmin (PAI-1), or cell associated membrane type MMPs (MT-MMPs). Therefore the ability of MMPs to degrade ECM is regulated by both the expression and activation of the latent enzyme, and as well by interactions of tissue inhibitors of metalloproteinases (TIMPs) [32], which associate with activated MMP regulating their activity.

Another important mediator of vascular remodeling is fibronectin, a high molecular weight extracellular matrix glycoprotein present inside vascular cells. Fibronectin is involved in a number of processes, including adhesion, growth, migration and differentiation [33], essential for normal functioning cells. Cellular fibronectin can also be assembled into an insoluble fibrillar matrix according to a complex cell mediated process [34]. Matrix assembly begins when compact fibronectin dimers are secreted from cells, begin to bind to integrin receptors on the cell surface and form short fibrin fibrils between cells. Cells are believed to begin to move and stretch these fibrils, elongating them and exposing their binding sites, allowing for neighboring fibronectin fibrils to bind, thus making a network.

Oscillatory shear stress, vascular stiffness and plaque development

Although it is now commonplace to regard shear stress as a hemodynamic stimuli crucial to arterial viability, it was not always so. The ability to perform blood flow measurements were first developed by Deppe and Wetterer (1940) using a modified electromagnetic flow meter, creating the foundation which first allowed for the calculation of shear stress. Nearly thirty years passed before endothelial wall shear stress was implicated in the localization of atherosclerotic plaque development [35]. Later, sophisticated computational fluid dynamic models were developed and compared to autopsy based measurements showing that areas of low wall shear stress correlate well with plaque localization found at autopsy [36]. Further support indicating the role of endothelial wall shear and the development of atherosclerosis has been performed using in-vivo animal models [37]. More recent studies elucidating the molecular and cellular pathways by which shear stress leads to plaque development, as well as the distinction between stable and vulnerable plaques have allowed for further classification of risk factors responsible for acute coronary syndromes [38, 39]. Case and point: there is a clear correlation between atherosclerotic lesion development and regions experiencing low or oscillators shear. Yet blood flow is pulsatile by nature, how stretch is involved in this story is still misunderstood.

Increased arterial stiffening is a hallmark of the aging process and is accelerated by diseases such as diabetes, atherosclerosis and chronic kidney failure. Clinical markers used to diagnose arterial stiffness, such as pulse pressure and systolic hypertension typically increase in prevalence with age [40]. Morphologic changes in the arterial wall due to aging involve hyperplasia in the intimal region, but are most obviously represented in the media where there is progressive disorganization of elastin fibers and laminae. Loss of elastin functionality is associated with an increased production of collagenous material and/or deposition of calcium, creating a stiffer arterial wall. Detailed reports of how aging influences arterial wall structure and chemistry were first reported by Moritz and Oldt (1937) [41], although changes in arterial elasticity with age had been reported much earlier (Roy, 1880). It was not until much later that epidemiological evidence confirmed the negative implications of aging and elevated pulse pressure [42]. More recent studies have been focused on how changes in vascular wall stiffness modulates cell function and ECM remodeling [43] as well as activation of inflammatory pathways [44]. Thus it is clear that both reduction of stretch as well as oscillatory shear contribute to vascular disease, but how they work together as well as individually remains unclear.

Aims of the thesis

- To determine whether reduced cyclic stretch affects arterial smooth muscle cell function, phenotype and certain aspects of vascular remodeling.

- To determine if reduced cyclic stretch affects endothelial cell function, interrupts the production of NO and modulates superoxide anion production in the arterial wall.

- To understand the autonomous effects of stretch and shear stress with regards to endothelial function, NO and superoxide anion production, as well as how this relates to NO bioavailability.

- To study how cyclic stretch and shear stress modulate different aspects of vascular remodeling and superoxide anion production in different regions through out the vascular wall and relate this to smooth muscle functional changes.

The ex vivo set up and unique aspects of this study

There are no shortages of studies regarding how vascular shear stress and aging influence the development of arterial disease, though certain methodological aspects of previous works in this domain may be misleading. In-vivo experiments in this area, thus referring to research performed on animals has largely been conducted on rats. Yet, it has been shown that hemodynamic conditions in rats vary greatly from that of humans; wall shear stress values in the common carotid have been reported to average 50 dynes/cm^2, roughly 5 times higher than the average for a human, [9] and with cumulative exposure to cyclic stress approximately 25% that of humans [45]. Performing in-vivo experiments on larger animals, such as pigs, has proven to be too costly an endeavor for most labs. Another approach, which has been used extensively to study hemodynamic effects, employs in-vitro perfusion of endothelial cells. Although these setups benefit from having very precise control of experimental conditions, they generally lack an element of realism. Endothelial contact with ECM proteins and smooth muscle cells are generally not reproduced well in-vitro. Also, the variability of in-vitro experimental set ups and the vide variety of cell lines available have made comparisons between different in-vivo experiments very difficult.

An interesting compromise, which has been employed by some over the years, is to take native arterial tissue and perfuse it under artificially created, physiologic conditions ex-vivo.

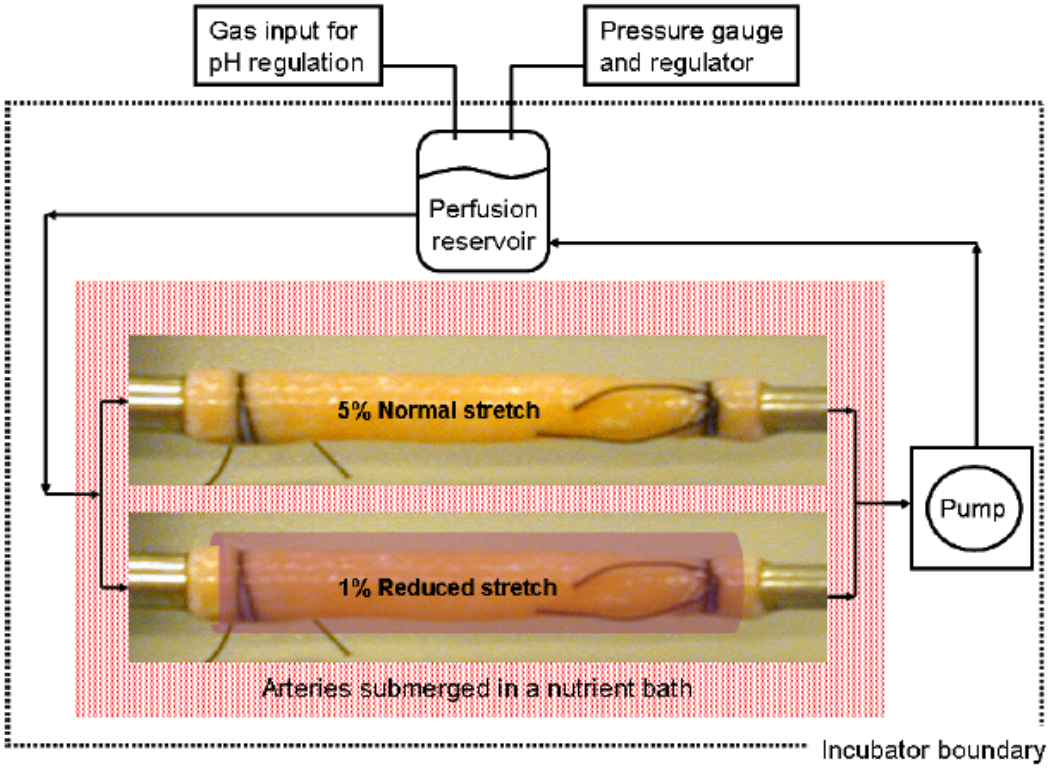

Figure 11: *Simplified diagram of ex-vivo set up*

Having total control of experimental conditions and being able to impose them on native arterial tissue gives the flexibility of an in-vitro set-up and the realism of in-vivo work. A realm previously unexplored using native porcine arterial segments was to uncouple the effects of shear stress and stretch, thus learning more about their individual contributions to arterial function and viability. Our experimental design, essentially creates a 2 x 2 matrix, modulating shear stress on one axis and stretch on the other, thus creating four separate experimental conditions.

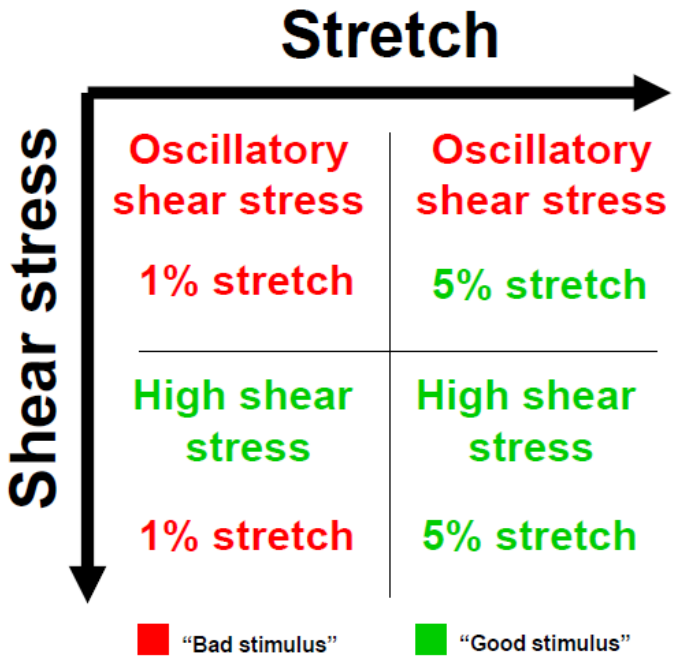

Figure 12: *Experimental outline*

Because so much work has been done in the past regarding the effects of shear stress to endothelial function and arterial remodeling, it was interesting to use shear stress as a sort of "bench mark" to compare to the effects of reduced stretch. By comparing the HSS 1% and OSC 5% experiments we can directly compare shear stress to stretch. A study such as this can also be used to study the evolution of arterial disease. The HSS 5% experiment can be considered a young healthy artery. At this point one could either consider the HSS 1% group, which mimics an aging artery, but located in a region not exposed to perturbed hemodynamics. Or one could consider the OSC 5% group, which mimics the hemodynamics at a bifurcation, but still maintaining the stretch of a young artery. Then moving to the OSC 1% group, this would mimic the same region, but of an aging person.

Another unique aspect of our study, was that we extensively used imaging and analysis software to evaluate certain aspects of endothelial function and arterial remodeling. If one assumes that the vascular wall is virtually incompressible [46], has a thickness h, radius R, and is inflated at a distending pressure P, the circumferential wall stress T, is found using Lamé's equation.

$$T = \frac{PR}{h}$$

Thus, Lamé's equation suggests that as the arterial wall is stretched by pulsatile blood flow, there exists a variation stress throughout the vascular wall. Thus we found it of interest to investigate whether changes in the distribution of stresses throughout the arterial wall correlated with the expression of markers related to arterial disease.

Summary of papers

Paper I

We found that reducing compliance caused a decreased in vascular smooth muscle function, as evaluated by a reduced contraction capacity induced by norepinephrine. This was associated with lower levels of α-smooth muscle-actin and desmin protein expression, thus indicating reduced stretch induced a change in smooth muscle cell phenotype. Arteries exposed to a reduced cyclic stretch exhibited higher levels of matrix-metalloproteinase-2 expression activity as well as an increase in Ki67 expression, suggesting that matrix degradation and cellular proliferation were initiated. Furthermore, expression of plasminogen activator inhibitor-1 was reduced in stiffened arteries when compared to control arteries. These findings of this study underline the importance of cyclic stretch in the maintenance of a differentiated and fully functional phenotype of vascular smooth muscle cells, as well as in the regulation of migratory properties, proliferation and matrix turnover in the vascular wall.

Paper II

In this study we found that the experimentally reduced compliance caused a significant decrease in endothelial function, as assessed with bradykinin dependent vascular relaxation. Reduced compliance significantly decreased the phosphorylation of Serine 1177 on eNOS, suggesting the activity of eNOS was decreased. Overall production of reactive oxygen species was also increased by reducing compliance. Finally, p22-phox and p47-phox, key players in the superoxide generating NAD(P)H oxidase were also up regulated by reduced compliance.
These findings point out how reduced arterial compliance increases risk of arterial disease by creating a less functional endothelium, interrupting the eNOS activation pathway and increasing vascular levels of oxidative stress.

Paper III

This study showed that both oscillatory flow and reduced stretch are detrimental to endothelial function, whereas oscillatory flow alone, dominated total endogenous vascular wall superoxide anion production. Yet when superoxide anion production was analyzed in just the endothelial region we observed that it was modulated more significantly by reduced cyclic stretch than by oscillatory shear, emphasizing an important distinction between shear and stretch mediated effects to the vascular wall. Western blotting analysis of eNOS and nitro-tyrosine proved that they too are more significantly negatively modulated by oscillatory flow, than by reduced stretch. These findings point out how shear and stretch stimulate regions of the vascular wall differently, affecting NO bioavailability and contributing to vascular disease.

Paper IV

This study showed that while total superoxide production, fibronectin expression and gelatinase activation were predominantly mediated by shear stress, their expression in the endothelial region was mediated by reduced cyclic stretch, which correlated well with results from total MMP-2 expression. By plotting intensity versus radius for these markers of vascular remodeling we were able to see that superoxide production and gelatinase activity followed trends indicating their expression was in part mediated by stress distributions through out the vascular wall, while fibronectin and p22-phox were much less or not at all. Most importantly these findings, when coupled with our results from tissue reactive studies, suggest that the arterial remodeling process triggered in the endothelial region due to reduced stretch causes the most significant changes in arterial smooth muscle function. Perturbed shear stress and reduced arterial compliance have both been implicated in the initiation and

progression of vascular disease: this work provides a new perspective into how these stimulus are perceived through out the vascular wall.

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Paper I

Effects of reduced cyclic stretch on vascular smooth muscle cell function of pig carotids perfused *ex vivo*

Veronica Gambillara*, Tyler Thacher*, Paolo Silacci, and Nikos Stergiopulos
*Both authors contributed equally

Laboratory of Hemodynamics and Cardiovascular Technology, Swiss Federal Institute of Technology, Lausanne

Abstract

With age arteries stiffen, reducing arterial compliance, leading to development of systolic hypertension and to a substantial increase in pulse pressure. An augmented pulse pressure can be used to predict the development of hypertension, which has been linked to several cardiovascular diseases such as, atherosclerosis and pathologies such as diabetes and renal dysfunction. In this study, we tested the hypothesis that reduced wall compliance induces pulse pressure-mediated changes in arterial wall metabolism and remodeling.

Porcine carotid arteries were perfused using an *ex vivo* arterial support system for 24 hours. Control arteries were exposed to a pulse shear stress (6 ± 3 dynes/cm^2) combined with a pulse pressure of 80 ± 10 mmHg, yielding a physiological cyclic stretch of 4-5 %. A reduced compliance group was also studied, in which arteries were wrapped with an external band, decreasing cyclic stretch to levels below 1%.

The experimentally reduced compliance caused a decreased contraction capacity induced by norepinephrine, which was associated with lower levels of α-smooth muscle-actin and desmin protein expression. Arteries exposed to a reduced cyclic stretch exhibited a higher level of matrix-metalloproteinase-2 expression activity as well as an increase in Ki67 expression, suggesting that matrix degradation and cellular proliferation was initiated. Furthermore, expression of plasminogen activator inhibitor-1 was reduced in stiffened arteries when compared to control arteries.

These findings underline the importance of cyclic stretch in the maintenance of a differentiated and fully functional phenotype of vascular smooth muscle cells, as well as in the regulation of migratory properties, proliferation and matrix turnover.

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Introduction

Arterial stiffening is considered an accurate marker of the aging process and is associated with several cardiovascular diseases such as hypertension, atherosclerosis and pathologies including diabetes and renal dysfunction[1, 2]. A consequence of arterial stiffening is an overall decrease in systemic compliance, augmenting vascular impedance, wave reflection and leads to an increase in systolic and pulse pressure. Augmented pulse pressure is now recognized as a strong predictor of coronary heart disease [3]. Another consequence of arterial stiffening is a decrease in arterial diameter pulsation over the heart cycle, or cyclic circumferential stretch.

Cyclic circumferential stretch is considered a potent biomechanical stimulus; reduction of which could alter smooth muscle cell (SMC) phenotype. Two proteins up-regulated during SMC differentiation are α-smooth muscle cell (SMC) actin[4], and desmin[5]. α-SMC actin is one of the most abundant proteins found in smooth muscle cells and is crucial to contractile function[6]. Desmin is a crucial component in muscle cell architecture and is highly expressed in fully differentiated smooth muscle cells. Together, α-SMC actin and desmin can be used to mark SMC differentiation providing insight on smooth muscle contractile characteristics with regards to phenotype.

Reduction of arterial compliance is also suspected to initiate certain cellular responses known to trigger the remodeling process, including cellular proliferation and migration rates[4, 7, 8], all processes which are involved in atherosclerosis and intimal wall thickening[9, 10] . Arterial remodeling is initiated by the breakdown of extracellular matrix, increasing the migratory capabilities of cells moving from the media to the intima. Matrix metalloproteinase's (MMP's) are a family of proteases capable of degrading extracellular matrix, upon activation. MMP-2 has been implicated as a key player in arterial remodeling[11]. MMP activation allowing for neointima development may be regulated by the plasmin system[12], namely plasminogen activator inhibitor-1 (PAI-1)[13]. Studying regulation of MMP-2 and PAI-1 in response to altered wall compliance may provide insight in to certain aspects of arterial remodeling.

The effects of cyclic stretch on the regulation of EC signaling or SMC tone were mostly studied on cells cultured, either on stretch chambers or in elastic tubes, where cell-cell and matrix-cell interactions are missing. A few *in vivo* studies have been reported where cyclic stretch has been suppressed by wrapping a stiff band around a vessel. However, total suppression of cyclic stretch by a non-extensible cuff is not physiological and does not permit the study of reduced cyclic stretch where, with the exception of calcified arteries, some residual diameter pulsation always persists. We have therefore designed experiments where arterial segments are perfused *ex vivo* under physiologically relevant pressure and flow conditions. Control arteries are subjected to either a physiological (5%) cyclic stretch or to a reduced cyclic stretch (1%). We addressed the short-term effects of reduced cyclic stretch on SMC in their native tissue environment in which cell-cell and cell- matrix interactions are preserved. We focused our study on the effects of reduced cyclic stretch on SMC phenotype, contractile function, migration, proliferation and induction of vascular remodeling.

Methods

Arterial groups

Porcine left internal carotid arteries were obtained at the local slaughterhouse shortly after sacrifice of the animal, which were 6-month-old pigs, weighing 120-150 kg. Adventitial tissue was removed and a 3.5 cm segment, 1 cm distal to the bifurcation was excised. After cleaning, the arterial segments were mounted onto the *ex vivo* arterial support system (EVASS, see description below). The segments were stretched longitudinally to 1.3 times of the un-stretched and un-pressurized length. To simulate decreased compliance, a silicon cuff of 6.0 mm or 8.0 mm (depending on outer diameter), was placed around the arterial segment. The reduction in circumferential cyclic stretch obtained with the cuff was roughly 80%, when compared to the un-cuffed arterial segment.

Arterial perfusion system

The *ex vivo* arterial perfusion system used in this study enables the perfusion of isolated arterial segments under precise control of perfusion pressure and flow. Details on EVASS have been given previously[14]. The arterial segments were perfused for 24 hours with M199-EBS (Amersham) containing 5% fetal calf serum. The medium was constantly gassed with 5% CO_2 and 95% Air. Perfusion flow was adapted to create a pulsatile unidirectional shear stress with a mean value of 6 dyne/cm^2, amplitude of 3 dyne/cm^2 and frequency of 1 second. Mean perfusion pressure was set to 80 mmHg, and pulse pressure amplitude was ±10 mmHg. Resulting strains were 4-5% for the un-cuffed segment, which is in the physiological range of pulsatile stretch for the porcine carotid, and less than 1% for the cuffed segment, simulating a less compliant arterial segment.

Something worth noting is that by calculating shear stress based on the diameter of a pressurized vessel under static conditions then introducing the pulsatile flow, one will actually achieve slightly higher shear stresses in the cuffed vessel when compared to the non cuffed segment. To minimize this affect we first measure the diameter, under pressurized, static flow conditions. Then we set the flow rates and calculate shear stress based on this initial value. Once we get an acceptable flow and achieve our desired cyclic stretch we then re-measure the diameter, taking its average value throughout the stretching cycle. We then use this new value to fine tune our flow rates and shear stress. We also re-check our flow through out the 24 hour perfusion, making fine adjustments, if necessary, to maintain the proper shear stress and cyclic stretching.

Smooth muscle cell analysis

Arterial rings were tested before and after the perfusion experiment to determine vasocontractility. Arterial rings were mounted in an organ chamber (EMKA Technology), equilibrated in Krebs solution at 37 °C, gassed with 95% O_2 and 5% CO_2. Resting tension was adjusted to 2 grams. Arterial rings were precontracted with 60 mmol/L KCl until a constant maximum contraction was reached. Then norepinephrine (NE) and prostaglandin F2-α (PG-F2α) dose-response curves were obtained varying NE and PG-F2α concentrations between 10^{-8} mol/L and 10^{-5} mol/L. Endothelial functionality was a criteria for inclusion in our study and was assessed using Bradykinin (Bk) (Results not shown).

Protein extraction and analysis

Protein expression and gelatinolytic activity were assessed using immunoblot and zymography techniques respectively, as described previously[14]. Protein was extracted from the samples with a Brij-35 lyses buffer (50 mmol/L Tris pH 7.5, 1mol/L NaCl, 2M Urea, 0.1% Brij-35, and 1 protease inhibitor cocktail (Roche)). 20 μg of protein was electrophoresed, after which the proteins were transferred to a nitrocellulose filter (Amersham). Filters were incubated with mouse anti-α-smooth muscle cell-actin antibody[15] (1:500, kind gift from the Gabbiani group in Geneva, Switzerland), mouse anti-desmin antibody (1:500, Dako), rabbit anti-plasminogen activator inhibitor -1 antibody (1:200, Santa Cruz Biotechnology), mouse anti-matrix metalloproteinase -2 antibody (1:500 Chemicon International), mouse anti- Glyseraldehyde-3-phosphate dehydrogenase antibody (1:500, VWR), followed by ECL-peroxidase labeled anti-mouse or anti-rabbit antibodies (1:1000, Amersham). Protein expressions were normalized to the Glyseraldehyde-3-phosphate dehydrogenase (GAPDH) protein expression. Matrix metalloproteinase 2 (MMP-2) activity was determined by gel zymography and expressed as the ratio of the active form over the total MMP-2 (latent and active form) for each sample.

Immunofluorescence and in situ zymography

At the end of the perfusion period, part of the arterial segment was rinsed with 0.9% NaCl, snap-frozen in OCT compound (Tissue-Tek) and stored at –80 °C for later analysis. For α-smooth muscle cell-actin (α-SMC-actin), desmin, Ki67, plasminogen activator inhibitor (PAI-1) staining, serial sections of 5 μm were cut, air-dried, and fixed in 100% acetone for 5 minutes at -20°C. Sections were incubated successively with 0.1% Triton X-100 in PBS for 10 minutes, then incubated for 60 minutes with α-SMC-actin (1:20, kind gift from the Gabbiani group in Geneva, Switzerland), desmin (1:50, Dako), Ki67 (1:40, Chemicom) or with rabbit anti-PAI-1 antibody (1:20, Santa Cruz Biotechnology) in 10% normal goat serum in PBS. The sections were then incubated with a rabbit anti-mouse IgG rabbit fluorescein-conjugate (1:100, Amersham) or a goat anti-mouse IgG rhodamine-conjugate (1:250, Amersham) as secondary antibody for 45 minutes. All steps were performed at room temperature. Sections were examined on a Zeiss Axiovert 135 microscope.

For the apoptosis detection, an *in situ* cell death detection kit was used (Roche). Then cryopreserved tissue sections were fixed and permeated. 50 μl of a TUNEL reaction mixture has been added on the sample and kept for 60 minutes at 37°C in the dark. After washing with PBS, samples were analyzed under a fluorescence microscope. Negative and positive samples have been performed to validate the experiment. To quantify the apoptotic cells, we normalized the positive TUNEL stained cells to the total number of cells counted through nucleus detection of DAPI in 4 different arteries for each group.

For *in situ* zymography, vessel sections were incubated at 37°C for 5 hours with a fluorogenic gelatin substrate (DQ gelatin, Molecular Probes) at a concentration of 25 μg/ml in zymography buffer. To support the hypothesis that increases in fluorescence were due to enhanced MMP activity, an inhibitor of such enzymes, 1- 10-phenanthroline, was also added to some arterial sections into the reaction buffer at a

final concentration of 50µM. Proteolytic activity was detected through green fluorescence (530 nm).

Statistics

Data are reported as means ± SD. Student's t-tests were performed to assess significant differences. A value of $P < 0.05$ was considered significant.

Results

SMC contraction capacity in response to cyclic stretch

Results were calculated by first normalizing the percent change in contraction upon addition of between 10^{-8} and 10^{-4} M concentrations of NE and PG-F2α to the contraction obtained with 60 mmol/L of KCl. This is protocol was performed for the freshly harvested arteries, normal and reduced stretch groups. The contractile data from each normal and reduced stretch experiment was then subtracted from the contractile data obtained from its corresponding freshly harvested segment. Therefore the dose response curves for NE and PG-F2α represent the average absolute increase in contractile response due to the perfusion conditions.

NE-derived contraction capacity was increased by 60% in arteries submitted to a normal cyclic stretch when compared to arteries exposed to a reduced cyclic stretch, but manifested itself only at higher concentrations.

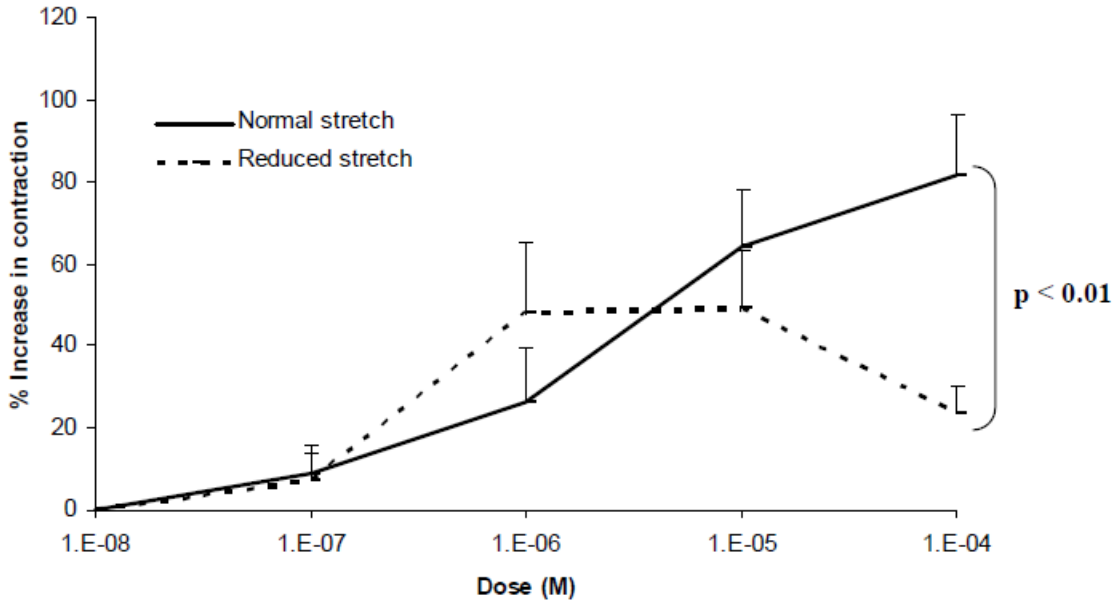


Figure 1a: *Norepinephrine (NE) dose-dependent constriction capacity in porcine carotid artery segments after 1 day of ex vivo perfusion with a normal cyclic stretch (4-5%) and a reduced stretch (less than 1%). Data are represented as mean ± SD, n=6, *P<0.01.*

PG-F2α, which followed the same trends as NE at lower concentrations but with a much lower over all contractile response, exhibited insignificant results.

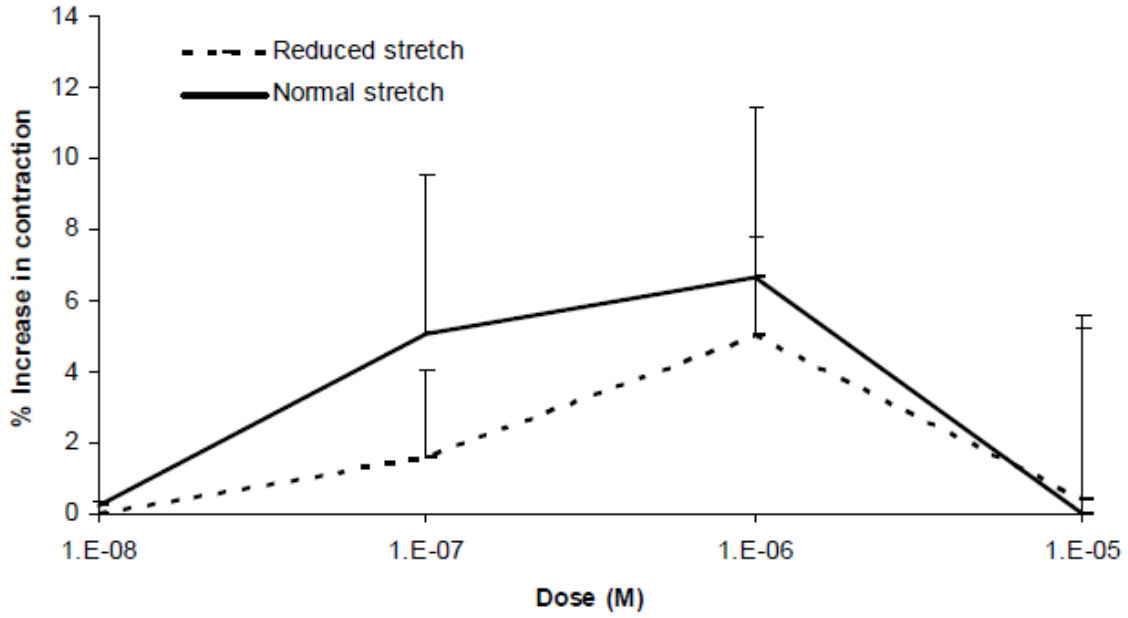
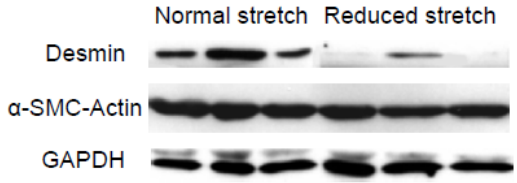


Figure 1b: *Prostaglandin F2-α (PG-F2α) dose-dependent constriction capacity in porcine carotid artery segments immediately after harvesting and after 1 day of ex vivo perfusion with a normal cyclic stretch (4-5%) and a reduced stretch (less than 1%). Data are expressed as mean ± SD, n=3.*

The largest difference between experimental conditions, representing where we see the largest difference in PG-F2α mediated contraction between our reduced and normal stretch cases, occurs at a dosage of 1.E-7 M (not significant). The largest absolute values of contraction occur at 1.E-6 (not significant). At the final point, 1.E-5, the values both approach zero, meaning that the average differences in contractile response due to our experiments do not differ from the control segment.

Influence of cyclic stretch on SMC phenotype

To investigate the effect of cyclic stretch on SMC phenotype, we assessed the protein expression of α-SMC-actin and desmin, both markers of SMC differentiation. A significant difference in SMC phenotype was observed between the arteries exposed to physiological and reduced cyclic stretch. Reducing cyclic stretch causes a decrease in the protein expression α-SMC-actin by 30% (A) and of desmin by 85% (B).



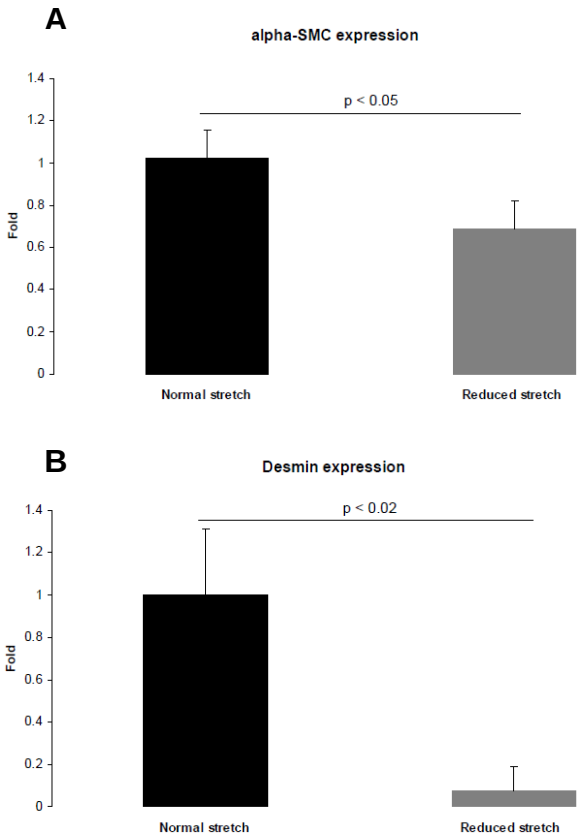


Figure2a: *Representative immunoblot showing α-SMC-actin and desmin protein bands in arteries exposed to normal and reduced cyclic stretch. Data are expressed as mean ± SD, n=6, *P<0.05.*

These results were further supported by immunofluorescence of arterial sections. In arteries submitted to a normal stretch (left), desmin expression could be easily detected in the media whereas in sections of vessel with reduced compliance very little desmin expression was found (right).

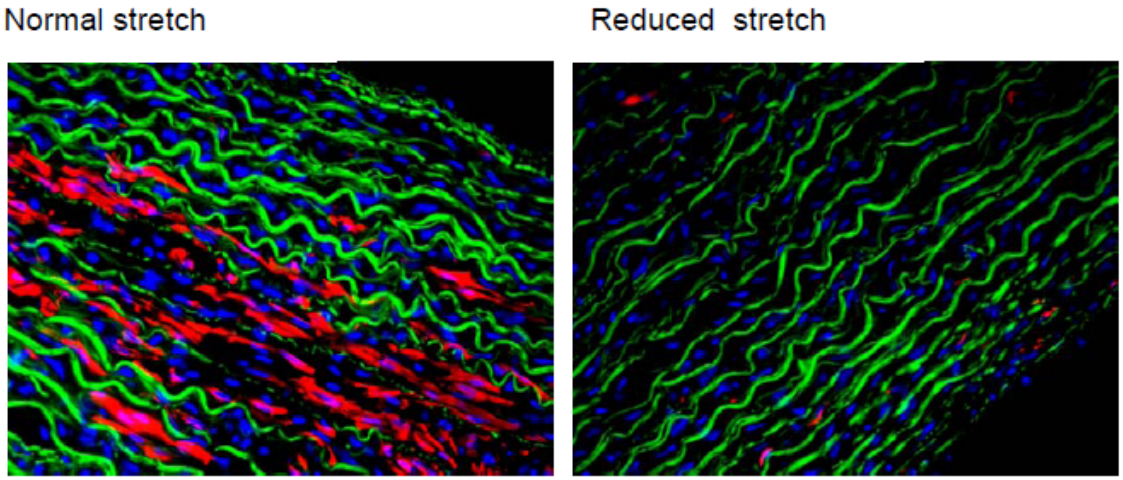


Figure 2b:*Desmin was localized by immunostaining on cross sections of arteries exposed to normal and reduced cyclic stretch. Desmin is seen in red, elastin in green and nuclei in blue. All images were taken at the same contrast and luminescence levels at 20x magnification, n=4.*

Reduced cyclic stretch on MMPs expression and activation

MMP-2 expression in arteries exposed for 24 hours to reduced cyclic stretch were 50% higher, when compared to those exposed to normal cyclic stretch.

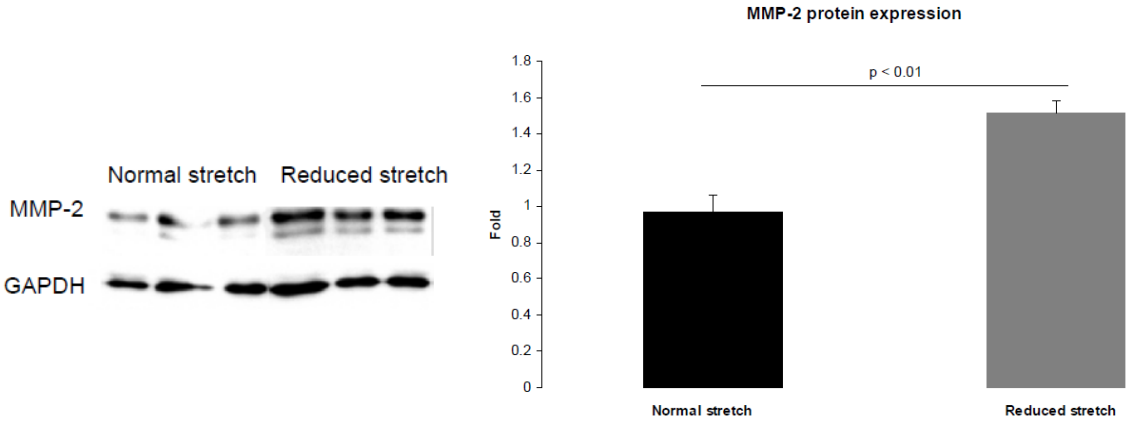


Figure 3a: *Effect of cyclic stretch on MMP-2 protein expression by immunoblotting. MMP-2 protein expression was normalized to total GAPDH expression. Data are expressed as mean ± SD, n=6, *P<0.05.*

Zymography assays also revealed a higher activation of MMP-2 in arteries exposed to a reduced stretch.

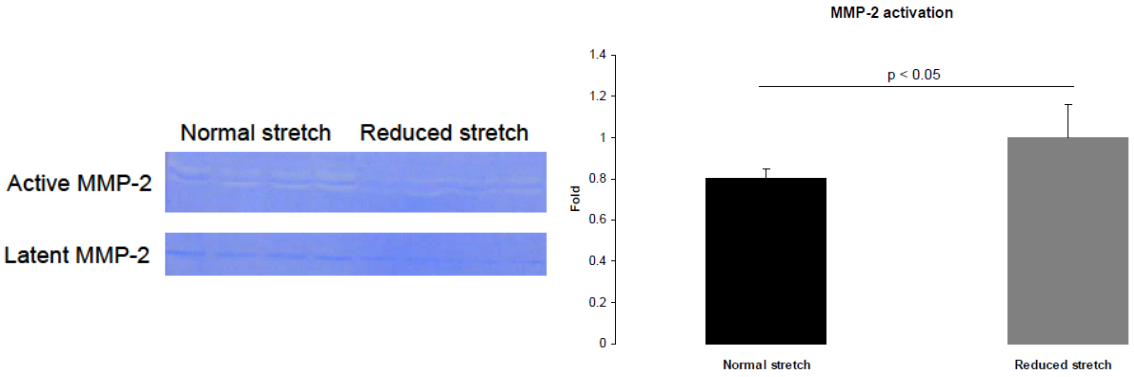


Figure 3b: *Effect of MMP-2 activity evaluated by zymography. Data are expressed as mean ± SD, n=4, *P<0.05.*

To analyze the distribution of gelatinolytic activity throughout the arterial wall, we performed an *in situ* zymography. The increased MMP expression observed in arteries exposed to reduced cyclic stretch correlated with the increase in gelatinolytic activity in the vessel wall (A). This gelatinolytic activity was mainly distributed in the tunica media and was observed in both groups. Localisation of gelatinolytic activity was reduced in the presence of the inhibitor, 1- 10-phenanthroline, proving the specificity of the assay (B).

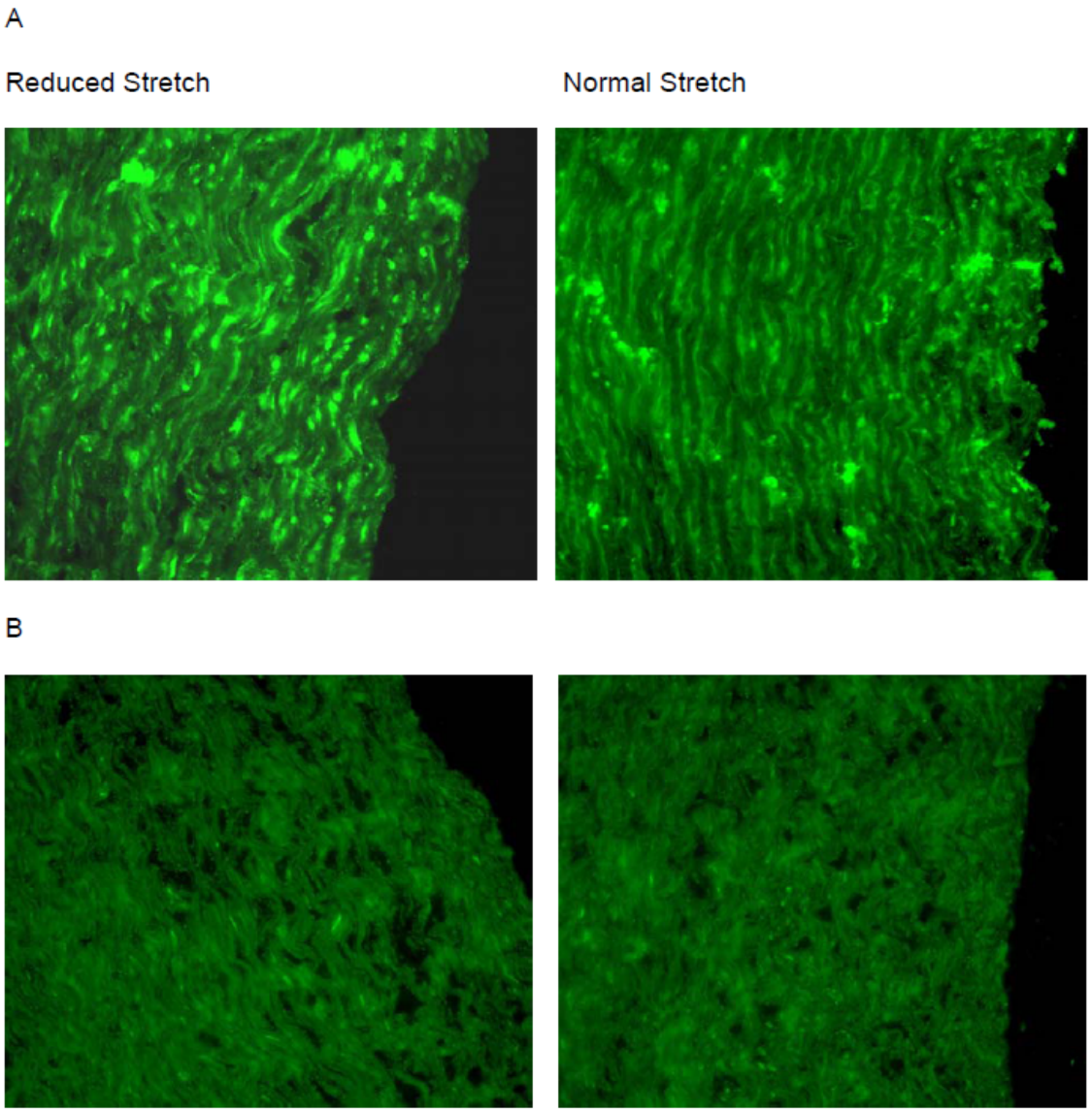


Figure 3c: *A) Effect of cyclic stretch on total MMP activation assessed by gelatinolytic activity. B) Proof of specificity of the assay using the inhibitor, 1-10-phenanthroline. All images taken at the same contrast and luminescence levels at 20x magnification n=4.*

Effect of cyclic stretch on PAI-1 protein expression and localization

The expression level of PAI-1 was higher in arteries exposed to normal cyclic stretching. Cuffed arteries presented a 20% decrease of PAI-1 expression after 1 day of perfusion.

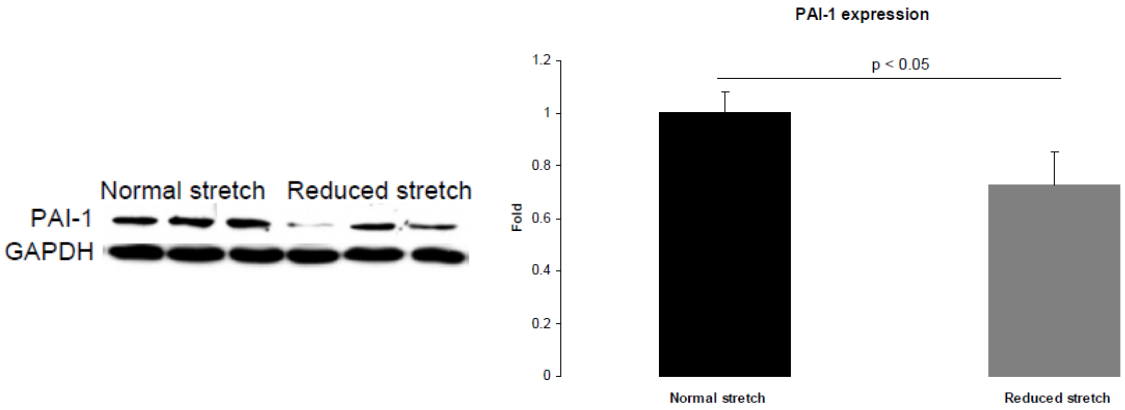


Figure 4a: *Representative immunoblot showing PAI-1 protein bands in arteries exposed to normal and reduced cyclic stretch. Data are expressed as mean ± SD, n=6, *P<0.05.*

Immunostaining analysis allowed localization of PAI-1 expression, mainly in the media, in arteries exposed to normal cyclic stretch (left). In contrast, for arteries exposed to a reduced cyclic stretch, PAI-1 protein was down regulated and expressed in the media and endothelium (right).

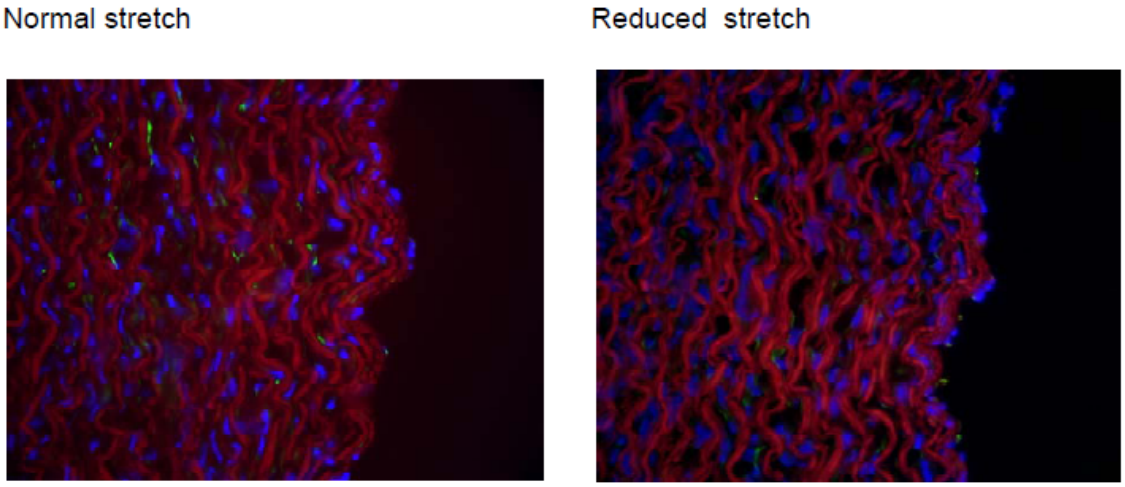


Figure 4b: *PAI-1 was visualized by immunostaining on cross sections of arteries exposed to normal and reduced cyclic stretch. PAI-1 is visualized in green, elastin in red, and nuclei in blue. All images are taken at the same contrast and luminescence levels at 20x magnification n=4.*

Effect of cyclic stretch on proliferation

To investigate the effect of reduced cyclic stretch on cellular proliferation we stained arteries exposed to normal and reduced cyclic stretch with Ki67. We noticed that proliferating cells were localized predominantly in the media (A). We observed a significant 2.2 fold increase in cellular proliferation when comparing our reduced stretch group to our normal stretch group (B).

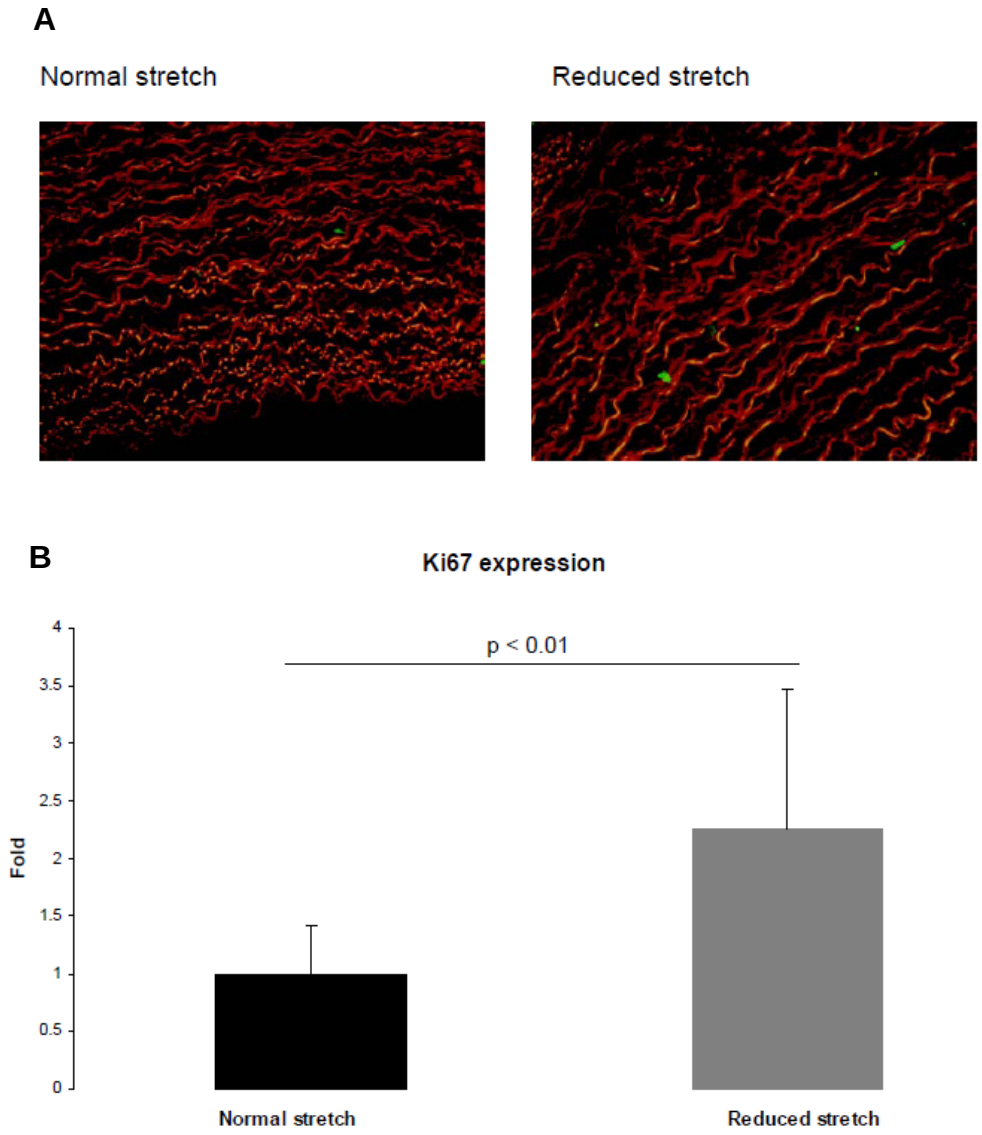


Figure 5: *A) Proliferation was visualized by staining arterial sections exposed to normal and reduced cyclic stretch with Ki67. Cells expressing Ki67 are seen in green and elastin in red. Images were taken at the same contrast and luminescence levels at 20x magnification n=4. B) The percentage of Ki67 positive cells per arterial section was calculated by dividing the number of positively stained cells (co-localized with a nucleus) by the total number of cells (n=4), *P<0.05.*

Effect of cyclic stretch on cell apoptosis

There was no statistical difference in apoptosis between the two arterial groups (figure 6B). Arteries exposed to normal cyclic stretch had 9± 3% cell death, whereas in arteries with artificially reduced cyclic stretch had 7± 3%, suggesting that reducing compliance does not induce higher apoptosis rates. We noticed that positive stained cells were principally present in regions closer to the intima in both groups (figure 6A).

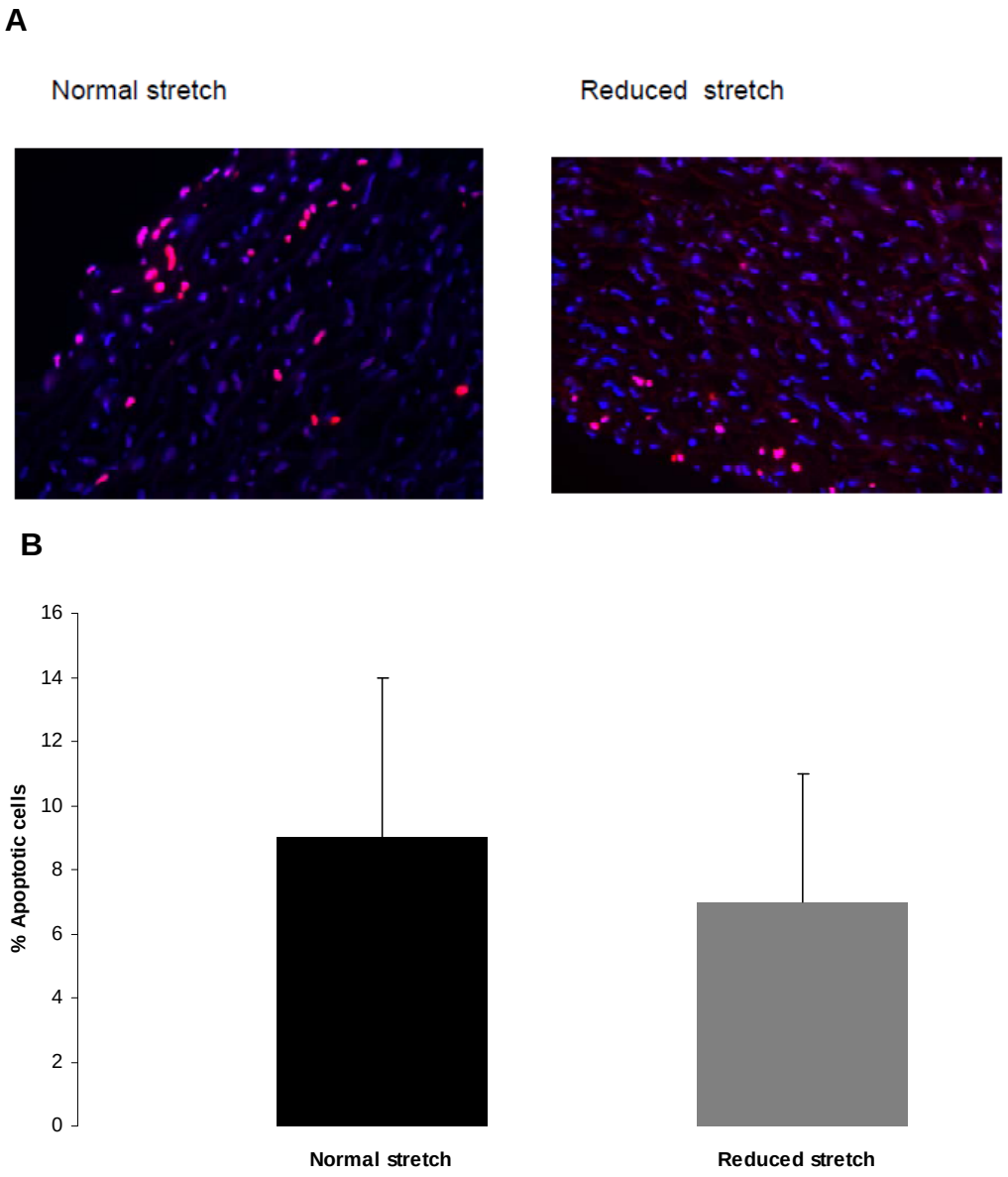


Figure 6: *A) Apoptosis was visualized through TUNEL reaction kit on cross sections of arteries exposed for 1 day to normal and reduced cyclic stretch. Apoptotic cells are visualized in red and nucleus in blue. All images were taken at the same contrast and luminescence levels at 20x magnification n=6. B) The percentage of apoptotic cells in arterial sections was calculated by dividing the number of positively stained cells by the total number of cells (n=6), *P<0.05.*

Discussion

Arterial stiffening decreases wall compliance, which in turn reduces cyclic stretch. Cyclic stretch is known to be a potent mechanical stimulus to EC and VSM cells. To study the effects of reduced cyclic stretch, we cuffed carotid arteries perfused under pulsatile pressure *ex vivo*. The experimentally reduced wall compliance induces changes in cellular responses and matrix turnover. Perfusion for 1 day with a reduced cyclic stretch leads to drastic decrease in contractile capacity mediated by NE, which was in order of 60%, when compared to the arteries perfused with a normal cyclic stretch. PG-F2α, which also induces a contractile response, although much smaller than NE, followed a similar trend to NE, yet yielded insignificant results. Contractile phenotype

markers such as α-SMC-actin and desmin were previously shown to decrease in arteries submitted to a reduced cyclic stretch. α-SMC-actin, the most abundant protein in the cytoskeleton, diminished in cuffed vessels by 30% and the desmin expression was almost completely abolished. Hence, suggesting that reduced cyclic stretch leads to changes in SMC phenotype, apparently from contractile to synthetic. These results confirm previous findings showing that cyclic stretch regulates the expression of some markers of SMC differentiation[4, 8]. In our experiments, arteries perfused under reduced cyclic stretch have shown a decreased contraction to NE when normalized to the maximal contraction induced by 60 mmol/L of KCl and. Given the fact that the maximal contraction induced by KCL did not differ between our two groups, we hypothesize that the decrease in contractile response to NE in cuffed arteries is linked to a change in phenotype.[16-18] Our hypothesis seems to be in accordance to Li et al., who demonstrated that human spindle-shape clones exhibit a higher expression of contractile phenotype markers and a stronger response to NE stimulation in comparison to epithelioid SMCs [16]. The conclusions we can draw from the PG-F2α data are more vague, but suggest that reducing cyclic stretch may also affect certain receptors responsible for SMC contraction.

Previously reported *in vitro* effects of mechanical strain on SMCs include increased cell proliferation and deposition of a matrix necessary for the organization and development of arteries, and later for vascular remodeling during atherosclerosis and hypertension[8] [19]. There is increasing evidence that changes in cyclic stretch lead to vascular wall remodeling, a process that involves degradation of the extra-cellular matrix (ECM) through stimulation of MMPs [20] [21, 22]. We observed an increase in MMP-2 expression in arteries submitted to a reduced cyclic stretch when compared to arteries submitted to a normal cyclic stretch. The activation of MMP-2 was also higher in the cuffed arteries. These results suggest that in arteries with artificially reduced cyclic stretch a vascular remodeling process is triggered, which may lead to structural changes similar to those observed in *in vivo* studies [23-25]. *In situ* zymography was used to assess global gelatinase activity and to evaluate overall matrix turnover. When compared to the normal stretch group these results confirm that under reduced cyclic stretch the matrix turnover cycle is being augmented.

Plasmin is a potent activator of most MMPs promoting the cleavage of the latent precursors to the active molecule and is regulated by plasminogen activator inhibitor-1 (PAI-1)[26]. Several groups have reported the involvement of MMPs and the fibrinolytic (plasmin/plasminogen) system as key factors in cell migration and tissue remodeling [13, 27]. We have shown a decrease in PAI-1 in arteries perfused with reduced cyclic stretch. Fluorescence data has revealed that PAI-1 was predominantly localized at the endothelium whereas in arteries perfused with normal cyclic stretch PAI-1 was primarily expressed by smooth muscle cells. Hence, a reduced circumferential cyclic stretch induces a simultaneous decrease of PAI-1 expression and an increase in MMP-2 activity, which combined with cell migration and proliferation is suspected to contribute to increased intimal thickening[19, 28]. In addition, as discussed before, in arteries with reduced cyclic stretch cells dedifferentiated into a more synthetic phenotype, know to have higher proliferation rates and mobile capacities[29, 30].

We found no difference in apoptosis rates between our normal and reduced stretch groups. This seems to partially contrast the findings of Courtman et al., who observed no changes in proliferation but marked an increase in apoptosis of smooth muscle cells in the media of infrarenal rabbit aortas, which had been cuffed to reduce cyclic stretch. Courtman et al. also observed significant atrophy and necrosis. The differences may be attributed to different design and time length of the two studies. Courtmann et al. performed long-term *in vivo* experiments and analyzed the consequences of the stiff external banding on vessel wall integrity after 6 weeks. Furthermore, their band was quite tight, reducing diameter by 25% and totally unloading arterial media from circumferential stresses[31]. Our study diminishes but does not totally abolish cyclic stretch, leaving a residual pulsation in the order of 1%, which is closer to the physiological pulsation in aged or stiffened conduit arteries. Also, our study describes changes in smooth muscle cell phenotype and migration potential after only 1 day of *ex vivo* perfusion.

In conclusion, the current study demonstrates that a decrease in cyclic stretch leads to changes in SMC phenotypes and to a concurrent decrease in NE-induced contraction capacity. Furthermore, the increase in MMP-2 associates with the decrease of PAI-1 as well as the increase in Ki67, suggesting that the decrease in cyclic stretch may promote wall remodeling, SMC proliferation and migration. Because reduction in cyclic stretch is often the result of a stiffened arterial tree, this work gives insights into possible consequences of arterial stiffening on large vessel wall physio-pathology.

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Paper II

Reduced cyclic stretch, endothelial dysfunction and oxidative stress: an *ex-vivo* model

Tyler N. Thacher [1], Veronica Gambillara, Rafaela F. da Silva [1,2,3], Paolo Silacci [4] and Nikos Stergiopulos [1]

[1] *Laboratory of Hemodynamics and Cardiovascular Technology, Swiss Federal Institute of Technology, Lausanne*
[2] *Department of Neurosurgery, Geneva University Hospital*
[3] *Department of Neurosciences, University Medical Center, University of Geneva Medical School*
[4]*Station de recherche Agroscope Liebefeld, Posieux, Switzerland*

Abstract

The objective of this study is to investigate if reduction of cyclic circumferential stretch will impair endothelial function and elevate basal levels of oxidative stress, both known risk factors linked to cardiovascular disease.

Ex vivo and *in vitro* models were used to perfuse porcine carotid arteries and porcine endothelial cells, respectively, for 24 hours. In both cases, one group was allowed to stretch naturally when exposed to a pulse shear stress (6 ± 3 dynes/cm^2) combined with a pulse pressure of 80 ± 10 mmHg, yielding a physiological cyclic stretch of 4-5 %. This group was compared to a reduced stretch group, achieved by wrapping the arterial segment with a silicon band, or seeding the endothelial cells inside less compliant tubes, decreasing cyclic stretch to 1 %.

The experimentally reduced compliance caused a significant decrease in bradykinin dependent vascular relaxation. Reduced compliance significantly decreased the phosphorylation of Serine 1177 on eNOS, suggesting the activity of eNOS was decreased. Overall production of reactive oxygen species was increased by reducing compliance, as visualized with dihydroethidium. Finally, p22-phox and p47-phox, key players in the superoxide generating NAD(P)H oxidase were also up regulated by reduced compliance.

These findings point out how reduced arterial compliance increases risk of arterial disease by creating a less functional endothelium, interrupting the eNOS activation pathway and increasing vascular levels of oxidative stress.

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Introduction

Reduction of arterial compliance typically correlates well with aging and is known to contribute to cardiovascular events such as coronary artery disease, and stroke [1, 2]. Reduction of systemic arterial compliance augments vascular impedance, wave reflection and leads to an increase in systolic and pulse pressure. Augmented pulse pressure is easily measured and is currently recognized as a strong predictor of coronary heart disease [3]. At a more local level, reduced arterial compliance manifests itself by reducing arterial pulsation in the radial direction over the course of the heart cycle, commonly referred to as reduced cyclic stretch.

We suspect reduction of cyclic circumferential stretch could increase the risk of cardiovascular disease by impairing endothelial functionality and elevating basal levels of oxidative stress, marked by the production of reactive oxygen species (ROS). Elevated levels of ROS are known to damage cardiovascular tissue and react with Nitric Oxide (NO), therefore lowering the bioavailability of NO [4] and increasing the risk of cardiovascular disease [5]. A useful marker of oxidative stress is dihydroethidium (DHE), which reacts will all ROS, providing a measure of total oxidative stress. The predominant system producing ROS in vascular smooth muscle and endothelial cells is the membrane bound NAD(P)H oxidase and is regulated by the expression of p22-phox[6] and p47-phox[7].

We also suspect that reduction of cyclic stretch could affect the production of NO, an essential regulator of vascular reactivity and tone, produced by the endothelium and regulated by the expression as well as activation of eNOS. Phosphorylation of eNOS Ser1177 is a hallmark of eNOS activation[8, 9]. By interrupting the phosphorylation of eNOS on Ser1177 one diminishes the amount of active vascular NO, increasing the risk of cardiovascular disease.

Studying the regulation of NO and oxidative stress in relation to altered arterial wall mechanics may provide insight into how reduced stretch affects endothelial functionality and its contribution cardiovascular disease.

Methods

Arterial groups

Left internal carotid arteries of 6 month old pigs weighing 120-150 kg were obtained from the local slaughter house (Bell SA, Cheseaux-sur-Lausanne, Switzerland) shortly after sacrifice. Adventitial tissue was removed and a 3.5 cm segment, 1 cm distal to the bifurcation was excised. The arterial segments were then mounted onto the *ex vivo* arterial support system (EVASS, see description below). The segments were stretched longitudinally to 1.3 times the un-stretched and un-pressurized length. To simulate decreased compliance, a silicon cuff (Statice Sante, Besançon France) of 6.0 mm or 8.0 mm

(depending on the outer diameter) and of 0.2 ± 0.05 mm thick was placed around the arterial segment.

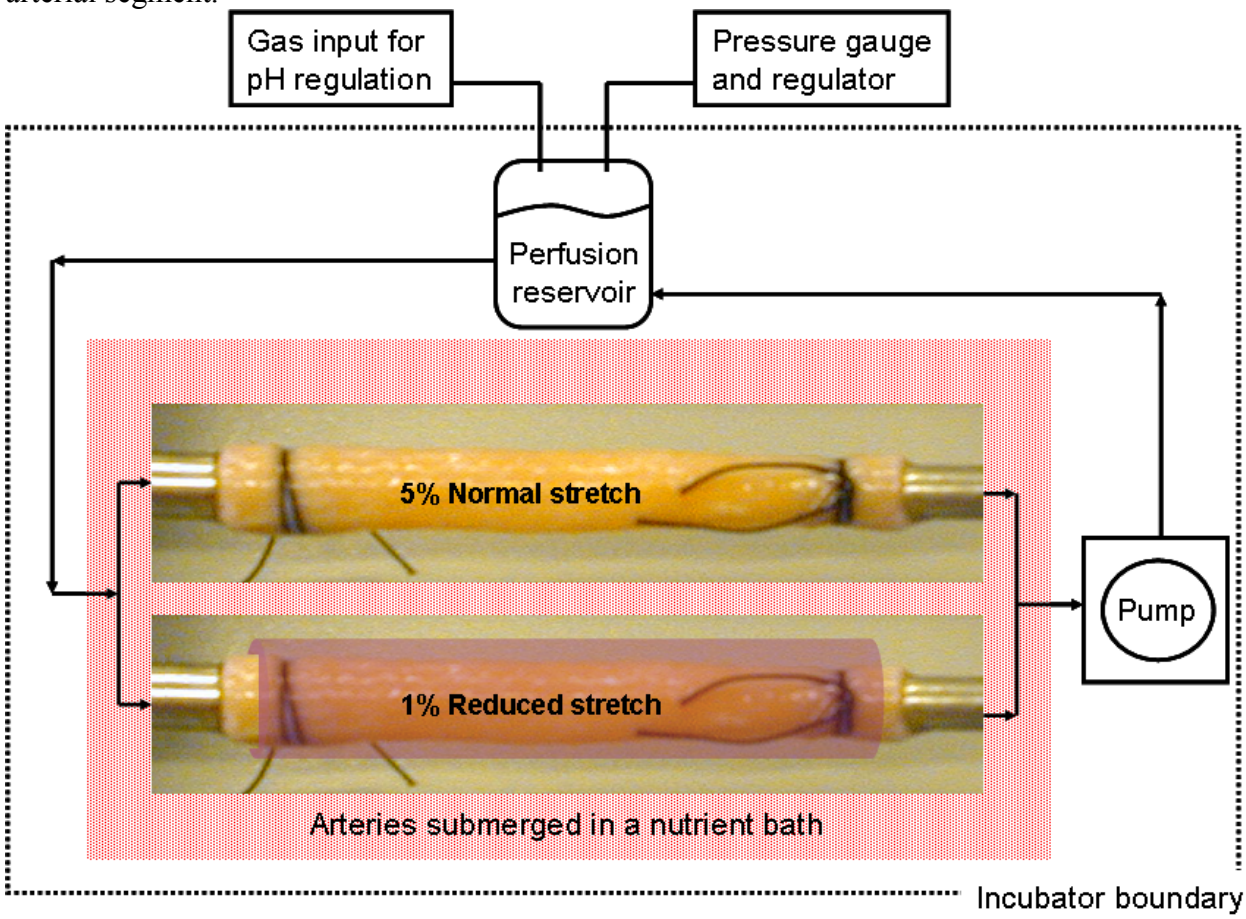


Figure 1: *Illustration depicting perfusion circuit and depicting perfusion circuit and showing normal and reduced stretch arteries.*

The reduction in circumferential cyclic stretch obtained with the cuff was roughly 80%, when compared to the un-cuffed arterial segment.

Ex vivo arterial perfusion system

The *ex vivo* arterial perfusion system used in this study enables the perfusion of isolated arterial segments under precise control of perfusion pressure and flow. Details on EVASS have been given previously [10-12]. The arterial segments were perfused for 24 hours with M199-EBS (Amersham) containing 5% fetal calf serum, 10mmol/L HEPES (Sigma), 20µg/ml Gentemicin (Gibco), 100 units/ml penicillin-streptomycin solution (Sigma) and 0.75 µg/ml Amphoterin B (Gibco). 8% medical grade Dextran (Sigma) was added to increase the viscosity of the medium to that of blood (~ 0.04 Ns/m^2). The medium was constantly infused with 5% CO_2 and 95% air. Perfusion flow was adapted to create a pulsatile unidirectional shear stress with a mean value of 6 $dyne/cm^2$, amplitude of 3 $dyne/cm^2$ and frequency of 1 second. Perfusion pressure was set to 80 mmHg with a pulse

pressure amplitude of ±10 mmHg. Resulting strains were 4-5% for the un-cuffed segment, which is in the physiological range of pulsatile stretch for the porcine carotid, and less than 1% for the cuffed segment, simulating a less compliant arterial segment.

Initially we feared that by introducing a cuff we would locally affect the hydrostatic pressure felt by the vascular cells, thus incorporating not only a reduction of stretch, but an increase in the local pressure, a phenomena we were not interested in addressing. But after careful pressure measurements using a catheter (Millar Mikro-Tip Catheter Transducers, model SPR-52A) inserted into the artery's lumen during perfusion, we noticed no significant elevation in pressure between the cuffed and un-cuffed experiments.

Something worth noting is that by calculating shear stress based on the diameter of a pressurized arterial segment under static conditions then introducing the pulsatile flow, one will actually achieve slightly higher shear stresses in the cuffed segment when compared to the un-cuffed segment. To minimize this affect we first measure the diameter, under pressurized, static flow conditions. Then we set the flow rates and calculate shear stress based on this initial value. Once we get an acceptable flow and achieve our desired cyclic stretch we then re-measure the diameter, taking its average value throughout the stretching cycle. We then use this new value to fine tune our flow rates and shear stress. We also re-check our flow through out the 24 hour perfusion, making fine adjustments, if necessary, to maintain the proper shear stress and cyclic stretching. Thus, any changes in shear stress due to cyclic stretching we may have after these precautions we consider negligible.

In vitro flow system

The *in vitro* flow system used in this study enables the perfusion of isolated porcine carotid endothelial cells seeded in silicon tubes (Statice Sante, Besançon France) under precise control of perfusion pressure and flow, as described previously [13]. The cells were perfused for 24 hours using the same medium cocktail, shear stress and pulsatile pressure as described above for the *ex-vivo* system. Cyclic stretch was controlled by using tubes of varying compliancy. Resulting strains were 4-5% for the more compliant tubes, which is in the physiological range of pulsatile stretch for the porcine carotid, and less than 1% for the less compliant tubes, simulating a less compliant arterial segment.

Endothelial cell functionality analysis

Arterial rings were tested before and after the perfusion experiment to determine the capacity to achieve endothelial mediated vasorelaxation. Arterial rings were mounted in an organ chamber (EMKA Technology), equilibrated in a Krebs solution at 37 ^{o}C, infused with 5% CO_2 and 95% O_2. Resting tension was adjusted to 2 grams. Arterial rings were pre-contracted with 80 mmol/L KCl until a constant maximum contraction was reached. Then a 10^{-6} mol/L dose of Norepinephrine was introduced, achieving a contraction 50% as large as that achieved with KCl. Next doses of Bradykinin from 10^{-11} to 10^{-8} mol/L were given, allowing us to calculate dose response curves for Bradykinin mediated relaxation. Dose response curves for Sodium nitroprusside (SNP) were also achieved.

Protein extraction and analysis

Protein expression was assessed using standard Western blot techniques. Protein was extracted from the samples with a Brij-35 lyses buffer (50 mmol/L Tris pH 7.5, 1mol/L NaCl, 2M Urea, 0.1% Brij-35, and 1 protease inhibitor cocktail (Roche)). 20 μg of protein was electrophoresed, after which the proteins were transferred to a nitrocellulose filter (Amersham). Filters were incubated with either mouse anti-eNOS (eNOS) (1:1000, BD Biosciences), rabbit anti-Ser^{1177} phospho-eNOS (eNOS Ser1177) (1:1000, Cell Signaling Technologies), goat anti-VE-cadherin (1:1000, Santa Cruz Biotechnology), rabbit anti-$p22^{phox}$ (p22-phox) (1:200, Santa Cruz Biotechnology), mouse anti-$p47^{phox}$ (p47-phox) (1:200, BD Transduction Laboratories), mouse anti-Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) (1:1000, Chemicon International) followed by ECL-peroxidase labeled anti-mouse, goat or rabbit secondary antibodies (1:5000, Amersham). Protein expression of eNOS and eNOS Ser1177 were normalized to the expression of VE-cadherin, while p47-phox and p22-phox were normalized to GAPDH.

Immunofluorescence and Dihydroethidium

After the perfusion a 5 mm segment of the artery was rinsed with 0.9% NaCl, snap-frozen in OCT compound (Tissue-Tek) and stored at -80 ^{o}C for further analysis. For p22-phox and p47-phox staining, sections of 5 μm were cut, air-dried and fixed in 100% acetone for 5 minutes at -20 ^{o}C. Sections were permeablized with 0.1% Triton X-100 in PBS for 10 minutes, then incubated for 60 minutes with rabbit anti-$p22^{phox}$ (p22-phox) (1:200, Santa Cruz Biotechnology) or mouse anti-$p47^{phox}$ (p47-phox) (1:200, BD Transduction Laboratories) in 10% normal goat serum in PBS. The sections were then incubated with Alexa Fluor 568 goat anti-rabbit IgG (Invitrogen) or Alexa Fluor 568 goat anti-mouse IgG (Invitrogen) as a secondary antibody for 45 minutes. All steps were performed at room temperature. Sections were examined on a Zeiss Axiovert 135 microscope at 20 times magnification.

For DHE detection 5 μm arterial cryosections (as described above) were incubated for 30 minutes at 37 ^{o}C with 5 μmol/L dihydroethidium (DHE) (FluoProbes) in PBS, then rinsed for 1 minute in PBS. Sections were examined on a Leica DM5500 at 20 times magnification and images were tiled together enabling visualization of the entire arterial section. Metamorph was used to calculate average intensity per area of the arterial section. All steps we performed in the dark.

Statistics

The data are reported as mean values ± standard deviation. Student's t-tests were applied to assess significant differences. A value of $P < 0.05$ was considered significant.

Results

Reduced compliance affects endothelial functionality

No significant changes between normal and reduced stretch groups were observed when contracted to KCl (data not shown) or SNP (data not shown). Endothelial cell functionality

was significantly higher in arteries exposed to a normal cyclic stretch when compared to arteries exposed to a reduced cyclic stretch, but manifested its self only at the highest concentration, 10^{-8}mol/l. At a concentration of 10^{-8}mol/l the endothelial cell functionality was 10% less in the reduced than normal stretch group.

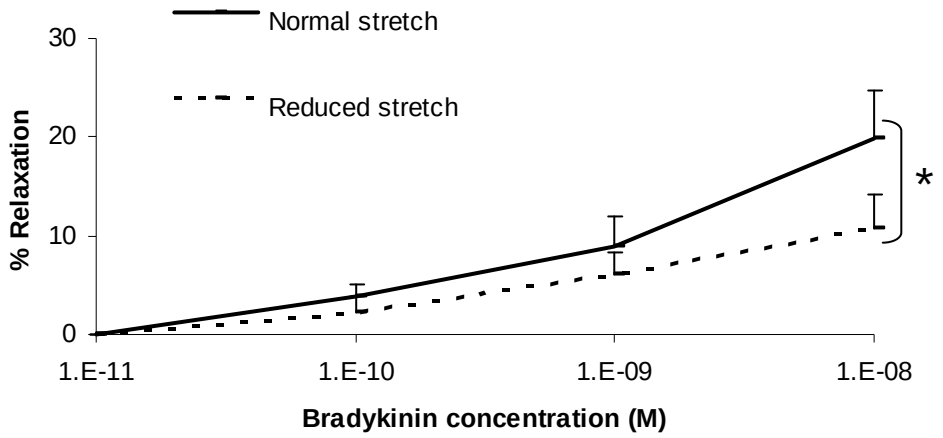


Figure 2: *Bradykinin (Bk) dose-dependent relaxation capacity of porcine carotid artery segments after 1 day of ex-vivo perfusion with a normal cyclic stretch (4-5%) and a reduced cyclic stretch (less than 1%). Data are represented as mean ± SD, n=6, (*P <0.05).*

Reduced compliance in relation to eNOS expression and phosphorylation

To investigate the mechanisms by which reducing arterial wall compliance impairs endothelial functionality in response bradykinin stimulation, we first analyzed eNOS protein expression by immunoblot in carotids exposed to normal and reduced cyclic stretch. We saw no significant change (data not shown). eNOS protein expression was normalized to an endothelial specific marker, VE-Cadherin, which has previously been shown not to be regulated by stretch (results not shown). To further explain the loss of endothelial dependent relaxation we looked at eNOS protein activation. To perform this we had to isolate porcine carotid endothelial cells and expose them to the same hemodynamic conditions as perfused arterial segments, of which endothelial cell protein content was too low for analysis. At the end of perfusion cells were exposed to bradykinin at a concentration of 10^{-6}mol/l for 30 seconds then total protein extracts were analyzed for eNOS Ser1177 phosphorylation, a crucial component of eNOS activation[8, 9]. We found that endothelial cells exposed to a normal stretch showed a 2 fold higher activation than those exposed to reduced stretch.

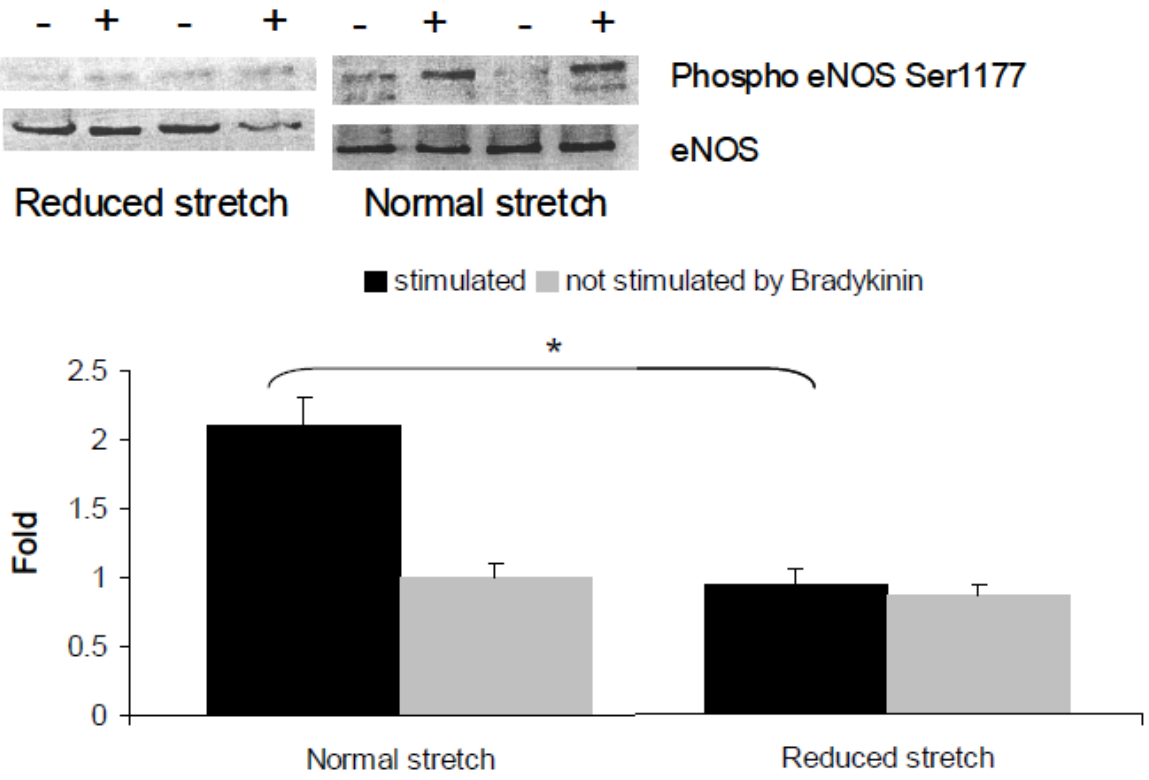


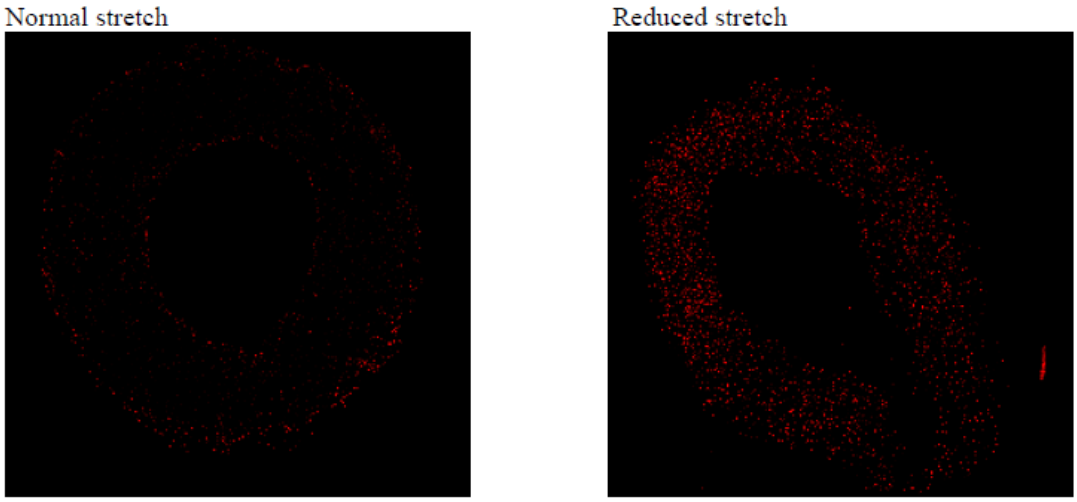
Figure 3: *Effect of reduced cyclic stretch on the phosphorylation of the Serine 1177 residue on eNOS as evaluated by immunoblot. Data are expressed as mean ± SD, n=6, (*P <0.05).*

This data supports our hypothesis that reduced compliance down regulates eNOS activation, effectively making NO less bioavailable and increasing the risk of cardiovascular disease.

Reduced compliance increases overall ROS production, quantified by DHE staining

To visualize overall ROS production we stained arterial cryosections with DHE (A). We saw an evenly distributed, significant 1.9 fold increase in the production of ethidium (B) in our reduced stretch samples, indicating an increase in ROS production stimulated by reduced compliance.

A



B

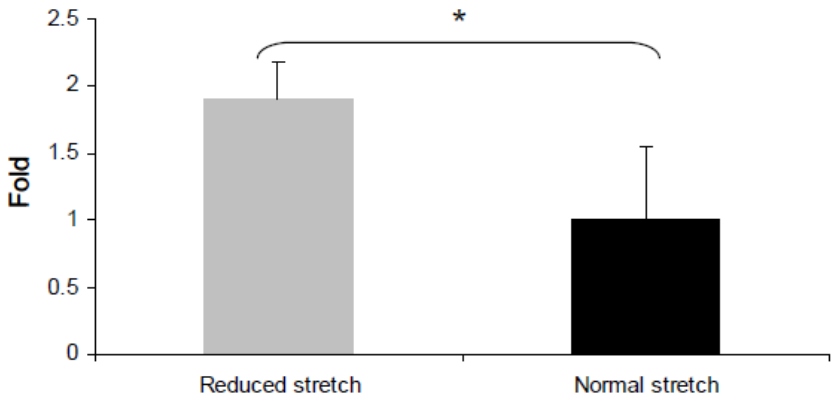


Figure 4: *A) Effect of reduced stretch on total production of reactive oxygen species (ROS) as evaluated with Dihydroethidium (DHE) staining on arterial cross sections exposed to normal and reduced cyclic stretch. All images evaluated on a Leica DM5500 at the same contrast and luminescence levels at 20x magnification. Images were then tiled together for whole image analysis. B) Quantification of DHE staining. Data are expressed as mean ± SD, n=3, (*P <0.05).*

Reduced compliance influences p22-phox and p47-phox, both key players in the ROS producing NAD(P)H oxidase complex

To investigate the influence of reduced cyclic stretch and the origin of the production of ROS we studied the membrane bound NAD(P)H oxidase complex, now considered as the predominant source of superoxide in the vascular wall [14]. Two key players in the NAD(P)H oxidase are p22-phox and p47-phox. By reducing arterial compliance we saw a significant, 2.5 fold increase in the expression of p22-phox when normalized to GAPDH.

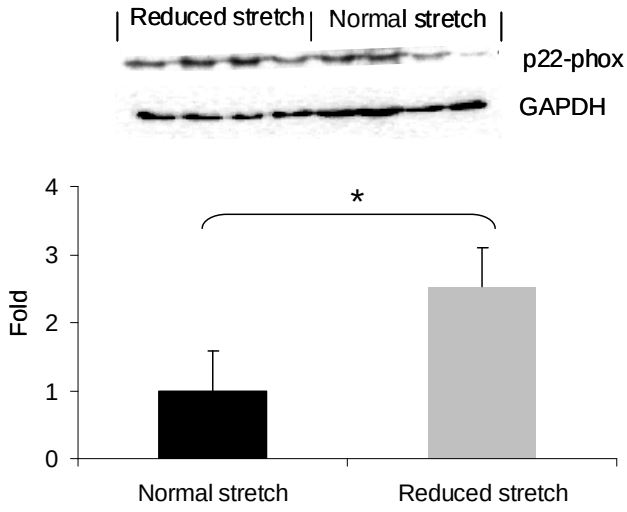


Figure 5: *Effect of reduced cyclic stretch on the expression of p22-phox as evaluated by immunoblot. Data are expressed as mean ± SD, n=6, (*P <0.05).*

We also saw a trend indicating a 1.9 fold increase in the expression of p47-phox when normalized to GAPDH (Fig. 6, $p<0.1$, $n = 6$), but the results were not significant.

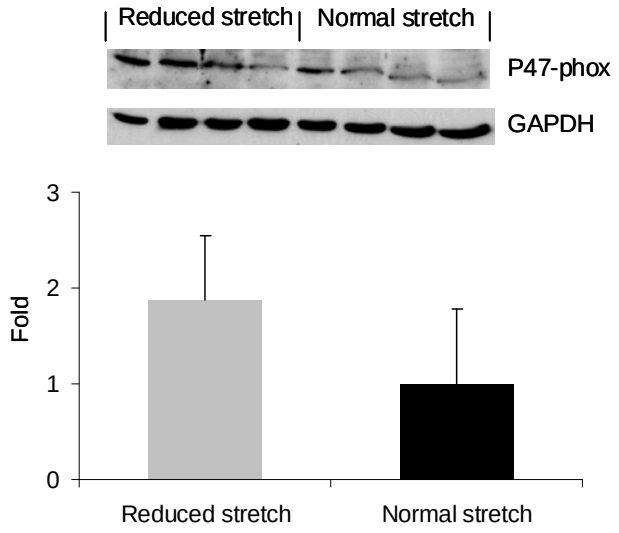


Figure 6: *Effect of reduced cyclic stretch on the expression of p47-phox as evaluated by immunoblot. Data are expressed as mean ± SD, n=6, (*P <0.1).*

Immunostaining of arterial sections with p22-phox revealed that activation due to reduced cyclic stretch was evenly activated throughout the arterial segment wall, especially noticeable on the endothelium.

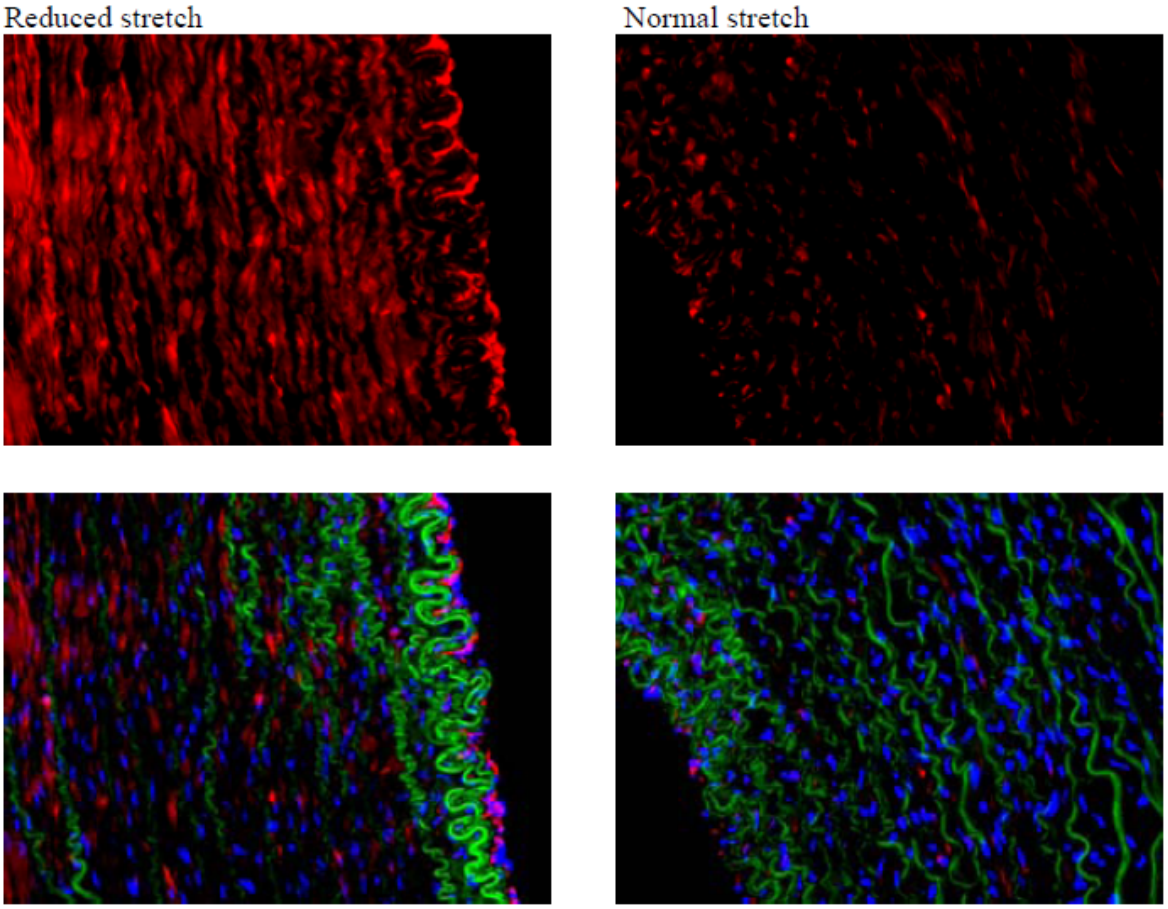


Figure 7: *p22-phox was visualized by immunostaining on cross sections of arteries exposed to normal and reduced cyclic stretch. Elastin is represented in green, nuclei are represented in blue, while p22-phox is in red. All images are taken at the same contrast and luminescence levels at 20x magnification, n=3.*

These results demonstrate that reduced arterial compliance directly influences the activation of the NAD(P)H oxidase in both vascular smooth muscle and endothelial cells, indicating an increased production of ROS, increasing the risk of cardiovascular disease.

Discussion

In this study we demonstrate that reducing the compliance of porcine carotid arteries exposed to physiologic perfusion conditions for 24 hours reduces endothelial functionality, affects eNOS activity and increases vascular oxidative stress. We saw an overall increase in ROS, as measured with DHE staining, as well as increases in p22-phox and p47-phox, key players in the superoxide generating NAD(P)H oxidase. These results indicate that cyclic stretch is an important regulator of vascular oxidative stress levels. While total eNOS protein expression was not modulated by reduced stretch, its phosphorylation at Ser1177 residue was decreased, hence inactivating endothelium derived NO. These findings suggest

that reduced arterial compliance regulates the expression of various factors at the vascular endothelium known to be related to cardiovascular disease.

Although numerous studies have related pulsatile flow [15, 16] and reduced compliance [17] to modulation of eNOS production and endothelial cell homeostasis [18-20], direct links between reliable models and functionality data are lacking. In our study we have been able to combine data from *in-vitro* and *ex-vivo* models and relate this to endothelial functionality. In both our *in-vitro* and *ex-vivo* experiments we retain precise control of shear stress, pressure and cyclic stretch performing experiments under identical perfusion conditions. Our *ex-vivo* perfusion system is more physiologically relevant, retaining cell-cell and cell-matrix interactions as well as allowing us to perform the functionality experiments. In our *in-vitro* system endothelial cells are seeded on the inner wall of compliant tubes, thus allowing us to harvest a much higher concentration of endothelial cell protein, permitting analysis otherwise impossible with our *ex-vivo* tissue samples.

To study the effects of reduced compliance we cuffed porcine carotid arterial segments perfused under physiologically relevant conditions, *ex-vivo* for 24 hours. The experimentally reduced compliance caused a significant decrease in endothelial derived relaxation capacity upon stimulation with bradykinin when compared to arteries allowed to stretch normally. This result indicates that on a functional level, reducing cyclic stretch impairs the production of endothelial derived NO and agrees with previous findings by Peng et al, who showed that reduced stretch affects eNOS activation, effectively diminishing the endothelium's ability to produce NO[18]. We saw no significant differences in the contraction achieved with KCl or relaxation achieved with SNP between normal or reduced stretch groups. These results verified that the changes in relaxation capacity observed with bradykinin were indeed due to the reduction of stretch, and not due to loss of smooth muscle function.

To investigate the origins linking reduced compliance and NO production we first measured total eNOS protein expression of our normal and reduced stretch groups of perfused arterial segments and saw no significant changes. This result agrees with previous work by Zeigler et al, who showed that total eNOS expression is not modulated by stretch in human umbilical vein endothelial cells perfused in an elastic tube model [21, 22]. At this point we suspected that reducing cyclic stretch may interrupt the activation pathway converting eNOS into NO. Yet the enzymatic activity of eNOS is affected by multiple factors such as, sub cellular localization, co-factors such as NAD(P)H, availability of substrate L-Arginine and phosphorylation of the eNOS Ser1177 residue [23]. We choose to look at the phosphorylation of eNOS Ser1177, but were unable to obtain highly enough concentrated endothelial cell protein from our *ex-vivo* model for detection. For this we moved to our *in-vitro* model and performed perfusions of porcine carotid endothelial cells seeded inside of silicon tubes of differing compliancy, allowing us to perfuse under identical conditions as the *ex-vivo* model and achieve the same cyclic stretch. From these experiments we found that for endothelial cells allowed to achieve normal stretch, eNOS Ser1177 was activated 2 fold higher than those experiencing a reduced stretch. This result underlines the importance of stretch with regards to the vascular endothelium.

Next we choose to investigate how stretch modulates the expression of ROS. It has become evident that basal levels of ROS are essential to vascular cell physiological function and contribute to the signaling required for vascular remodeling [24, 25]. Yet, significantly elevated levels of ROS have been shown to diminish vascular levels of NO, cause inflammation and even cell death [26]. There are many processes which produce ROS; two major players being the membrane bound NAD(P)H oxidase, found on the surface of endothelial and smooth muscle cells, as well as mitochondrial respiration, where ATP is produced by reducing molecular oxygen to water. For this reason we found it interesting to look at how total ROS production is regulated by reduced compliance. To visualize total ROS production we stained arterial cryosections with DHE, a cell permeable compound which forms ethidium upon reacting with $O^{\cdot}{}_2^{-}$. The ethidium then binds to DNA, which absorbs fluorescent light with a wavelength in the range of 500-530 nm and emits red florescent signal of wavelength 590 to 620 nm. We analyzed intensity of the emission of ethidium and divided it by the area of the arterial section to come up with a value of average intensity. We noticed that in our reduced stretch samples ROS was evenly distributed throughout the arterial wall, where as in our normal stretch samples it was predominantly localized near the endothelium and adventitia. Upon quantification we saw a significant 1.9 fold increase in the production of ethidium in our reduced stretch samples, indicating a significant increase in ROS production, stimulated by reduced compliance. It is known that ROS stimulate processes such as intracellular alkalinization, MAP kinase phosphorylation, tyrosine kinase activation, DNA synthesis and increased production of proto-onco-genes [27, 28], all stimuli known to initiate vascular remodeling. Previous work from our lab has shown that inhibiting the cyclic stretch of porcine carotid arterial segments perfused for 24 hours initiates vascular remodeling [29]; we suggests that increased ROS production could be one of the primary stimuli.

To further investigate the origins of ROS in response to reduced stretch, we focused on the superoxide generating NAD(P)H oxidase cascade, known to be a major source of reactive oxygen species in vascular cells [30]. To mark the activation of the NAD(P)H oxidase we measured the expression of two crucial components, namely, p22-phox and p47-phox. By reducing arterial compliance we saw a significant, 2.5 fold increases in the expression of p22-phox and a trend indicating a 1.9 fold increase in the expression of p47-phox. These results were further supported by immunostaining showing that increases in p22-phox were activated throughout the vascular wall and noticeably on the endothelium in our reduced stretch groups. This correlates well with our DHE images, suggesting that the majority of ROS produced by the vascular smooth muscle and endothelial cells are derived from the NAD(P)H oxidase. The measured increases in ROS correlate well with our hypothesis; reduced stretch causes increased ROS, which can scavenge endothelium derived NO lowering vascular bioavailability.

In conclusion, this study demonstrates that decreasing cyclic stretch leads to a significant decrease in the functionality of the endothelium caused by an interruption of eNOS Ser1177 phosphorylation, reducing the production of endothelial derived NO. We also measured significant increases in total ROS production via staining with DHE. This data

coupled with marked increases in p22-phox and p47-phox, both key players in the membrane bound superoxide producing NAD(P)H oxidase indicated that indeed, reducing an artery's ability to stretch naturally significantly increases the production of ROS. Because reduced arterial systemic compliance correlates with aging, this work gives insight into the links between reduced stretch, endothelial functionality and increased risk of cardiovascular disease over the course of a lifetime.

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Paper III

Autonomous effects of shear stress and cyclic circumferential stretch regarding endothelial dysfunction and oxidative stress: an *ex-vivo* arterial model

Tyler N. Thacher [1], Paolo Silacci [4], Nikos Stergiopulos [1]
and Rafaela F. da Silva [1,2,3]

[1] *Laboratory of Hemodynamics and Cardiovascular Technology, Swiss Federal Institute of Technology, Lausanne*
[2] *Department of Neurosurgery, Geneva University Hospital*
[3] *Department of Neurosciences, University Medical Center, University of Geneva Medical School*
[4]*Station de recherche Agroscope Liebefeld, Posieux, Switzerland*

Abstract

Cyclic circumferential stretch and shear stress caused by pulsatile blood flow work in concert, yet are very different stimuli capable of independently mediating endothelial function by modulating eNOS expression, oxidative stress (via production of superoxide anion) and NO bioavailability.

Porcine carotid arteries were perfused using an *ex-vivo* arterial support system for 72 hours. Groups we created by combining normal (5%) and reduced (1%) stretch with high shear (6±3 dynes/cm^2) and oscillatory shear (0.3±3 dynes/cm^2) stress while maintaining a pulse pressure of 80±10 mmHg.

Oscillatory flow and reduced stretch both proved detrimental to endothelial function, whereas oscillatory flow alone, dominated total endogenous vascular wall superoxide anion production. Yet when superoxide anion production was analyzed in just the endothelial region we observed that it was modulated more significantly by reduced cyclic stretch than by oscillatory shear, emphasizing an important distinction between shear and stretch mediated effects to the vascular wall. Western blotting analysis of eNOS and nitro-tyrosine proved that they too are more significantly negatively modulated by oscillatory flow, than by reduced stretch.

These findings point out how shear and stretch stimulate regions of the vascular wall differently, affecting NO bioavailability and contributing to vascular disease.

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Introduction

Within the vasculature endothelial cells are constantly exposed to dynamic mechanical forces generated by pulsatile blood flow. Two such stimuli known to modulate endothelial function are shear stress and cyclic circumferential strain. It has been documented that in areas of the vasculature exposed to low mean shear stress and cyclic reversal of flow direction (also called oscillatory flow, OSC) are at higher risk of developing endothelial dysfunction than those exposed to a high unidirectional shear stress (HSS) [1-4]. Similarly, it has also been suggested that reduction of arterial compliance may contribute endothelial dysfunction, since it has been linked to diseases such as atherosclerosis and hypertension [5, 6]. Yet, in most studies these two stimuli are simultaneously coupled *in-vivo*, making it very difficult to understand their individual contributions. Some attempts have been made to de-couple stretch and shear stress *in-vitro* by using different cell lines in a variety of stretch systems and flow chambers, straying from reality and making it hard to draw definitive conclusions. In this study we wish to find a compromise between the *in-vivo* and *in-vitro* work of the past by studying the independent effects of shear stress and cyclic stretch and how they contribute to endothelial dysfunction.

The underlying mechanisms of endothelial dysfunction are numerous and remain unclear, yet is generally agreed upon that the most dangerous revolve around a central point, that is diminished bioavailability of nitric oxide (NO)[7]. One mechanism involves the down regulation of endothelial NO synthase (eNOS) [8] expression, an enzyme produced by the endothelium which upon conversion of L-arginine to L-citrulline produces NO. Another mechanism thought to significantly affect NO bioavailability involves the up-regulation of vascular levels of reactive oxygen species (ROS). Elevated levels of ROS are known to damage vascular tissue as well as react with NO, forming peroxynitrite [9], thus removing NO [10] from the vasculature and contributing to pro-atherogenic conditions [11]. The predominant system producing ROS in vascular smooth muscle and endothelial cells is the membrane bound NAD(P)H oxidase (NOX) and is regulated by the expression of p22-phox [12] and p47-phox [13].

The goal of this study is to understand how shear stress and cyclic stretch individually mediate the expression of eNOS, the interaction of ROS and NO and if these factors contribute to endothelial dysfunction in porcine arterial tissue perfused *ex-vivo*.

Methods

Arterial groups

Left internal carotid arteries of 6 month old pigs weighing 120-150 kg were obtained from the local slaughter house (Bell SA, Cheseaux-sur-Lausanne, Switzerland). Adventitial tissue was removed and a 3.5 cm segment, 1 cm distal to the bifurcation was excised. The arterial segments were then mounted onto the *ex vivo* arterial support system (EVASS, see description below). The segments were stretched longitudinally to 1.3 times the un-stretched and un-pressurized length. To simulate decreased compliance, a silicon cuff (Statice Sante, Besançon France) of 6.0 mm or 8.0 mm (depending on the outer diameter) and of 0.2 ± 0.05 mm thick was placed around the arterial segment. The reduction in circumferential cyclic stretch obtained with the cuff was roughly 80%, when compared to the un-cuffed arterial segment.

Ex vivo arterial perfusion system

The *ex vivo* arterial perfusion system used in this study enables the perfusion of isolated arterial segments under precise control of perfusion pressure and flow. Details on EVASS have been given previously[[14]]. The arterial segments were perfused for 72 hours with a medium cocktail, as described previously [15]. Perfusion flow was adapted to create either a pulsatile unidirectional high shear stress with a mean value of 6 dyne/cm^2 and amplitude of 3 dyne/cm^2, or an oscillatory shear stress with a mean value of 0.3 dyne/cm^2 and amplitude of 3 dyne/cm^2, both with a frequency of 1 second. Perfusion pressure was set to 80 mmHg with pulse pressure amplitude of ±10 mmHg. Resulting strains were 4-5% for the un-cuffed segment, which is in the physiological range of pulsatile stretch for the porcine carotid, and less than 1% for the cuffed segment, simulating a less compliant arterial segment.

Endothelial cell functionality analysis

Arterial rings were tested before and after the perfusion experiment to determine the capacity to achieve endothelial mediated vasorelaxation. Arterial rings were mounted in an organ chamber (EMKA Technology), equilibrated in a Krebs solution at 37 ^oC, infused with 5% CO_2 and 95% O_2. Resting tension was adjusted to 2 grams. Arterial rings were pre-contracted with 80 mmol/L KCl until a constant maximum contraction was reached. Then a 10^{-6} mol/L dose of Norepinephrine was introduced, achieving a contraction 50% as large as that achieved with KCL. Next doses of Bradykinin (BK) from 10^{-11} to 10^{-8} mol/L were given and dose-response curves for BK mediated relaxation were calculated. Dose response curves for Sodium nitroprusside were also obtained.

Immunofluorescence and Dihydroethidium

After the perfusion, a 5 mm segment of the artery was rinsed with 0.9% NaCl, snap-frozen in OCT compound (Tissue-Tek) and stored at -80 ^oC for further analysis. For p22-phox and Nitro tyrosine staining, sections of 5 μm were cut, air-dried and fixed in 100% acetone for 5 minutes at -20 ^oC. Sections were permeablized with 0.1% Triton X-100 in PBS for 10 minutes, then incubated for 60 minutes with rabbit anti-p22^{phox} (p22-phox) (1:500, Santa Cruz Biotechnology) or mouse anti-nitro tyrosine (nitro tyrosine) (1:100, abcam) in 10% normal goat serum in PBS. The p22-phox sections were then incubated with Alexa Fluor 568 goat anti-rabbit IgG (Invitrogen) as a secondary antibody for 45 minutes. Sections were examined on a Zeiss Axiovert 135 microscope at 20 times magnification. For nitro tyrosine, sections were incubated with ECL-peroxidase labelled anti-mouse (1:1000, Amersham) as a secondary antibody and counterstained using a DAB substrate kit (Vector laboratories). Sections were examined on an Olympus AX 70 at 10 times magnification. All steps were performed at room temperature.

For DHE detection 5 μm arterial cryosections (as described above) were incubated for 30 minutes at 37 ^oC with 5 μmol/L dihydroethidium (DHE) (FluoProbes) in PBS, and then rinsed for 1 minute in PBS. Sections were examined on a Leica DM5500 at 20 times. All steps we performed in the dark.

Protein extraction and analysis

Protein expression was assessed using standard Western blot techniques. Protein was extracted from the samples with a Brij-35 lyses buffer (50 mmol/L Tris pH 7.5, 1mol/L NaCl, 2M Urea, 0.1% Brij-35, and 1 protease inhibitor cocktail (Roche)). 20 μg of protein was electrophoresed, after which the proteins were transferred to a nitrocellulose filter (Amersham). Filters were incubated with either mouse anti-eNOS (eNOS) (1:1000, BD Biosciences), mouse anti-nitro tyrosine (nitro tyrosine) (1:500, abcam), mouse anti-eNOS

(eNOS) (1:1000, BD Transduction Laboratories), goat anti-VE-cadherin (1:1000, Santa Cruz Biotechnology), rabbit anti-$p22^{phox}$ (p22-phox) (1:200, Santa Cruz Biotechnology), mouse anti-$p47^{phox}$ (p47-phox) (1:200, BD Transduction Laboratories), mouse anti-Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) (1:1000, Chemicon International) followed by ECL-peroxidase labelled anti-mouse, goat or rabbit secondary antibodies (1:5000, Amersham). Protein expression of eNOS was normalized to the expression of VE-cadherin, while nitro tyrosine, p47-phox and p22-phox were normalized to GAPDH.

Image analysis

Images were analyzed using Metamorph (Meta imaging series 7.0). For endothelial DHE calculations we used a WACOM graphic tablet to manually define the endothelial region.

Statistics

The data are reported as mean values ± standard deviation. Two-way analysis of variance (ANOVA) statistics were performed to assess significant differences between shear stress and cyclic stretch groups. When the interaction among groups was determined to be significant, post tests using the Bonferroni correction were used to assess their interaction. A value of $P < 0.05$ was considered significant.

Results

Vascular tissue reactivity studies

Endothelial function, as assessed at the 10-8 M dosage of BK, was significantly modulated by both hemodynamic stimuli shear stress and cyclic stretch. We observed that changing the flow from HSS to OSC, while maintaining a normal cyclic stretch in both cases, the function of the endothelium decreased by 84%. When we compared normal and reduced stretch groups where HSS was maintained for both, we observed an 87% down regulation in endothelial functionality.

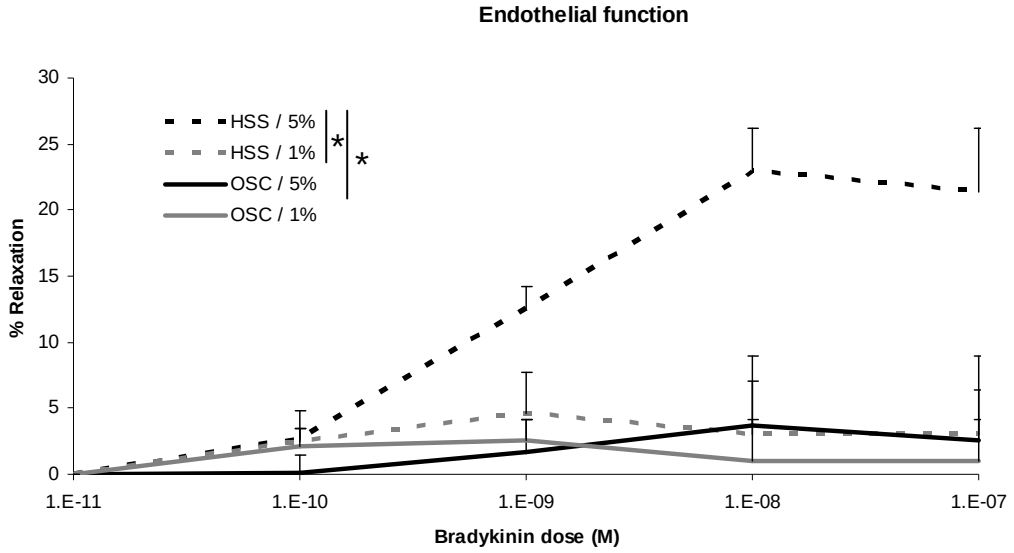

Figure 1: *Bradykinin (Bk) dose-dependent relaxation capacity of porcine carotid arterial segments after 72 hours of ex-vivo perfusion. Data are represented as mean ± SD, n=6, *P<0.05.*

Shear stress, but not cyclic stretch, modulates eNOS expression

Exposure of arteries to OSC significantly decreased the expression of eNOS as compared to HSS. This effect was observed regardless of normal or reduced stretch levels (32% and 28% decrease respectively).

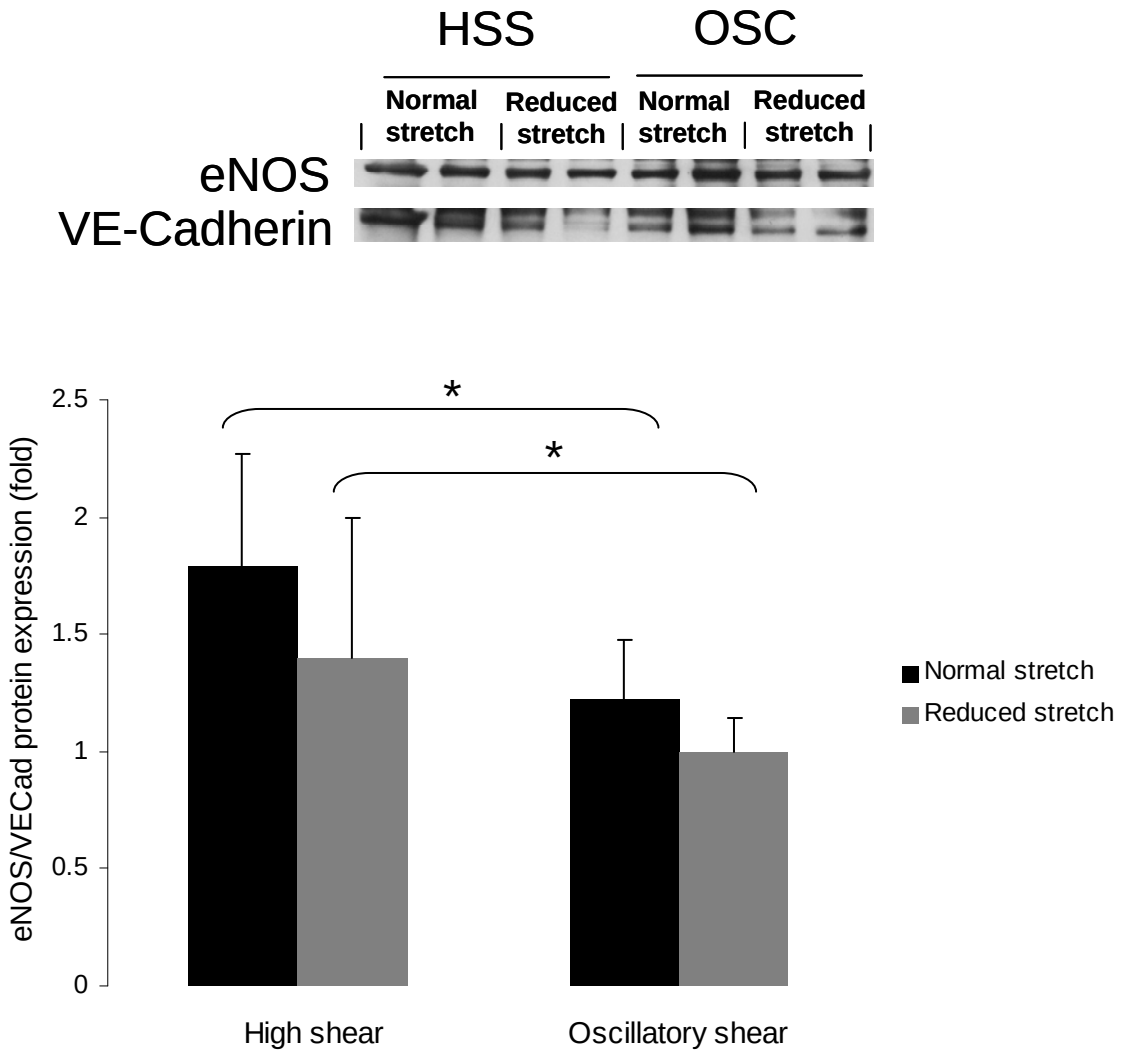

Figure 2a: *The effect of reduced cyclic stretch and oscillatory flow on eNOS expression as evaluated by immunoblot. Data are expressed as mean ± SD, n=6, *P <0.05.*

Seemingly independent of changes in shear stress eNOS expression seems to correlate with decreased cyclic stretch, however results did not reach significance. Results from western blot analysis were further supported by immunofluorescence of arterial sections. In arteries submitted to HSS eNOS expression was easily detected on the endothelium, whereas those submitted to OSC eNOS expression was barely visible.

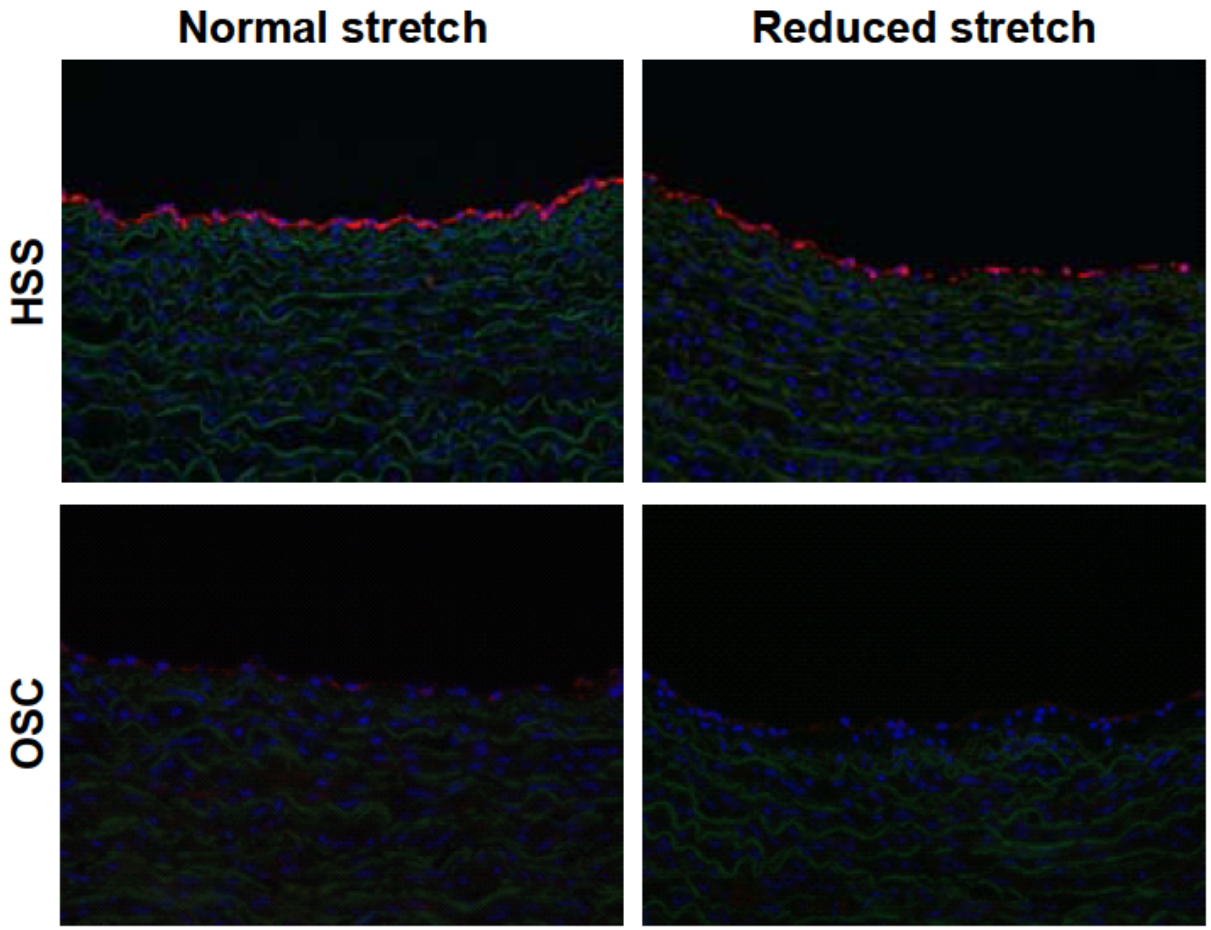

Figure 2b: *eNOS was visualized by immunostaining on arterial cross sections. Elastin is represented in green, nuclei are represented in blue and eNOS in red. All images were taken at the same contrast and luminescence levels at 20 times magnification.*

Cyclic stretch and shear stress distinctly regulate the production of superoxide anion

When looking at total vascular ROS production, throughout the endothelial, intimal and medial layers, we observed that ROS is significantly modulated by oscillatory shear stress. Arteries exposed to OSC at both normal and reduced stretch increased 2.1 and 2.3 fold the formation of ROS as compared to HSS (respectively).

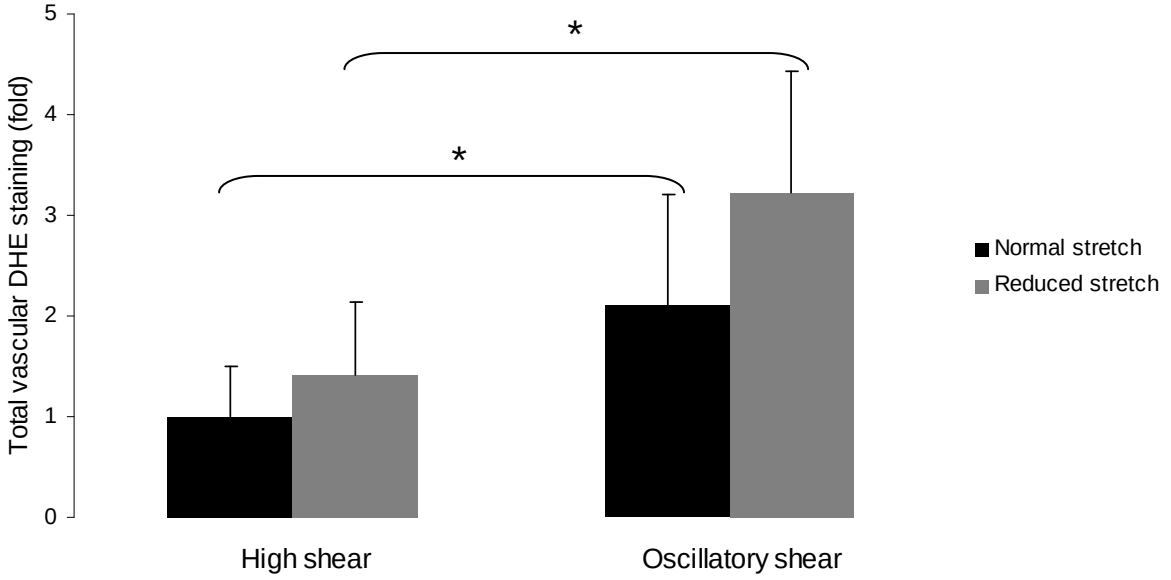

Figure 3a: *The effect of reduced cyclic stretch and oscillatory flow on total ROS expression, as evaluated with DHE staining. Data are expressed as mean ± SD, n=6, *P<0.001.*

Regarding stretch, we observed a trend indicating that reducing stretch increases ROS independent of shear stress, although these changes were not significant.

Interestingly, when focusing on ROS produced solely by endothelium cyclic stretch became the dominant hemodynamic stimuli. Indeed, ROS production is markedly increased from normal to reduced stretch for both HSS and OSC (2 and 1.5 fold increase respectively).

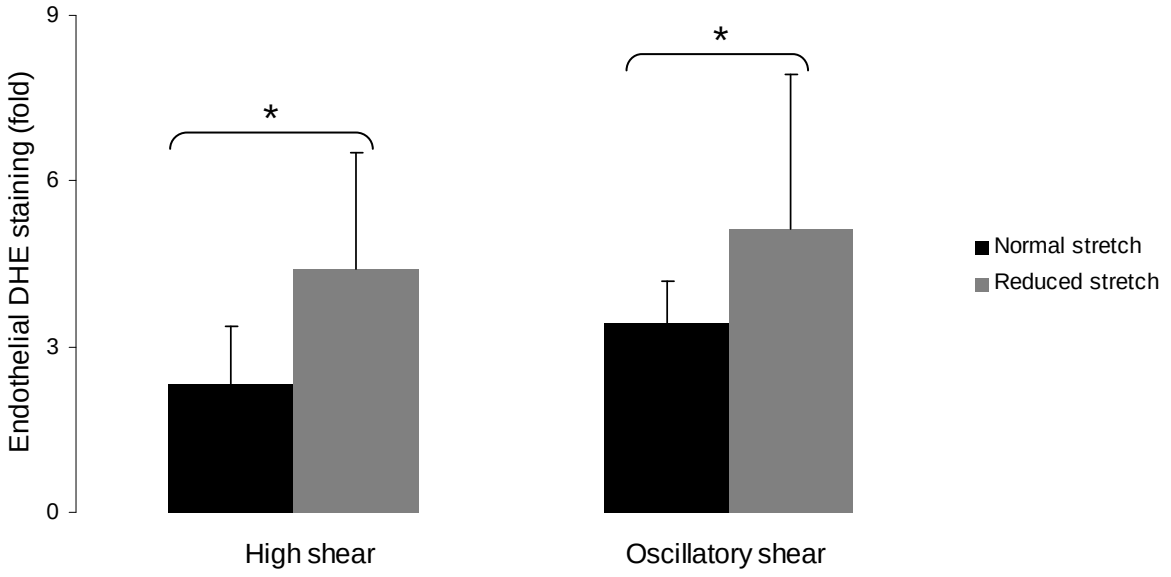

Figure 3b: *The effect of reduced cyclic stretch and oscillatory flow on endothelial ROS expression, as evaluated with DHE staining. Data are expressed as mean ± SD, n=6, *P<0.05.*

DHE images representative of those used for analysis have been included (below).

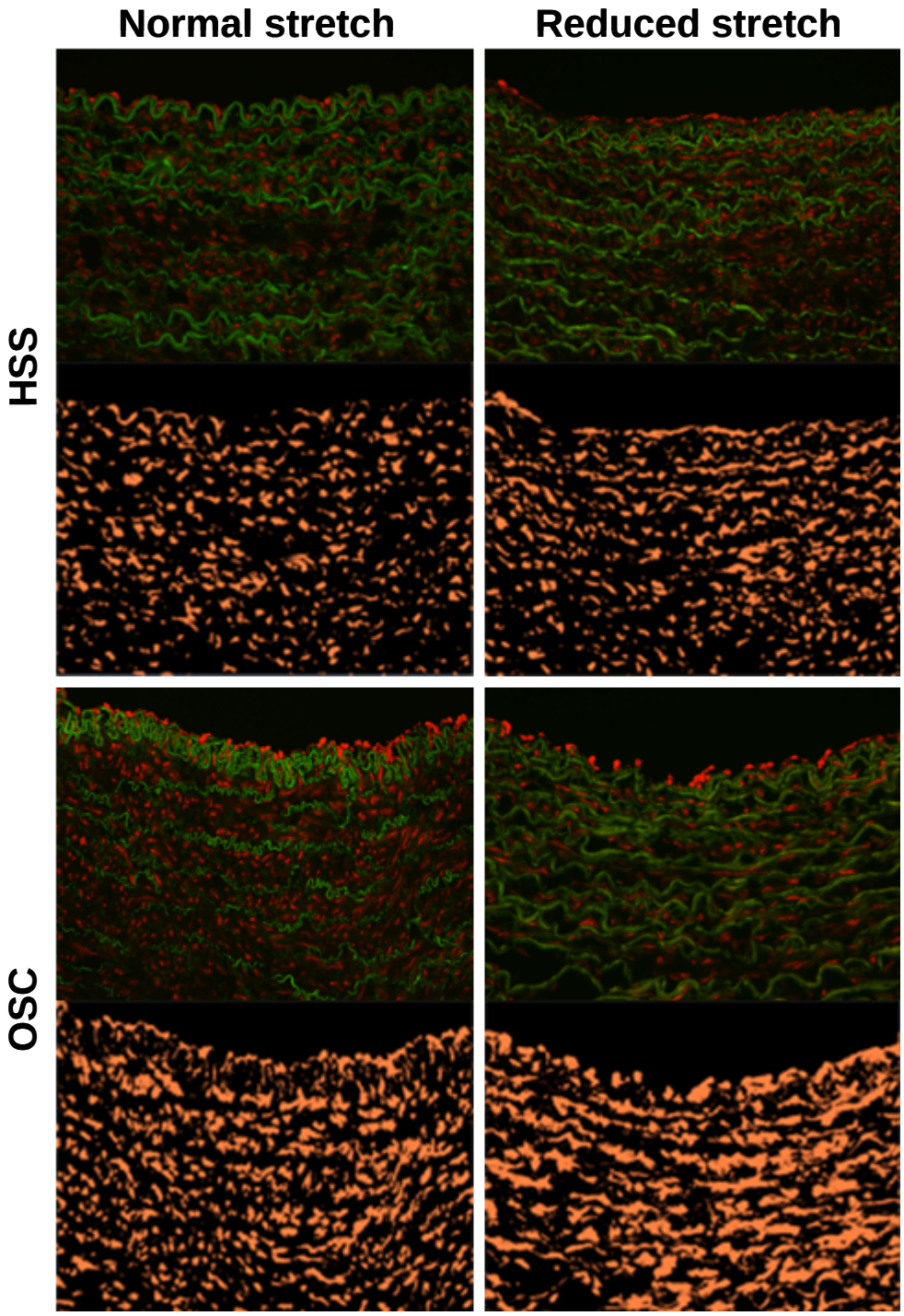

Figure 3c: *Sample DHE images showing originals on top (elastin in green, DHE in red) and image after being analyzed with a script written in Metamorph.*

Shear stress modulates the expression of p22-phox and p47-phox

Vascular ROS formation is predominantly mediated by the NOX system, which in turn is regulated by the production of p22-phox and p47-phox. At the same normal stretch condition, OSC increased the expression of p22-phox by 1.4 fold as compared to HSS. Increase on p22-phox expression by OSS was also seen when arteries were under reduced cyclic stretch condition (2.3 fold up- regulation as compared to HSS).

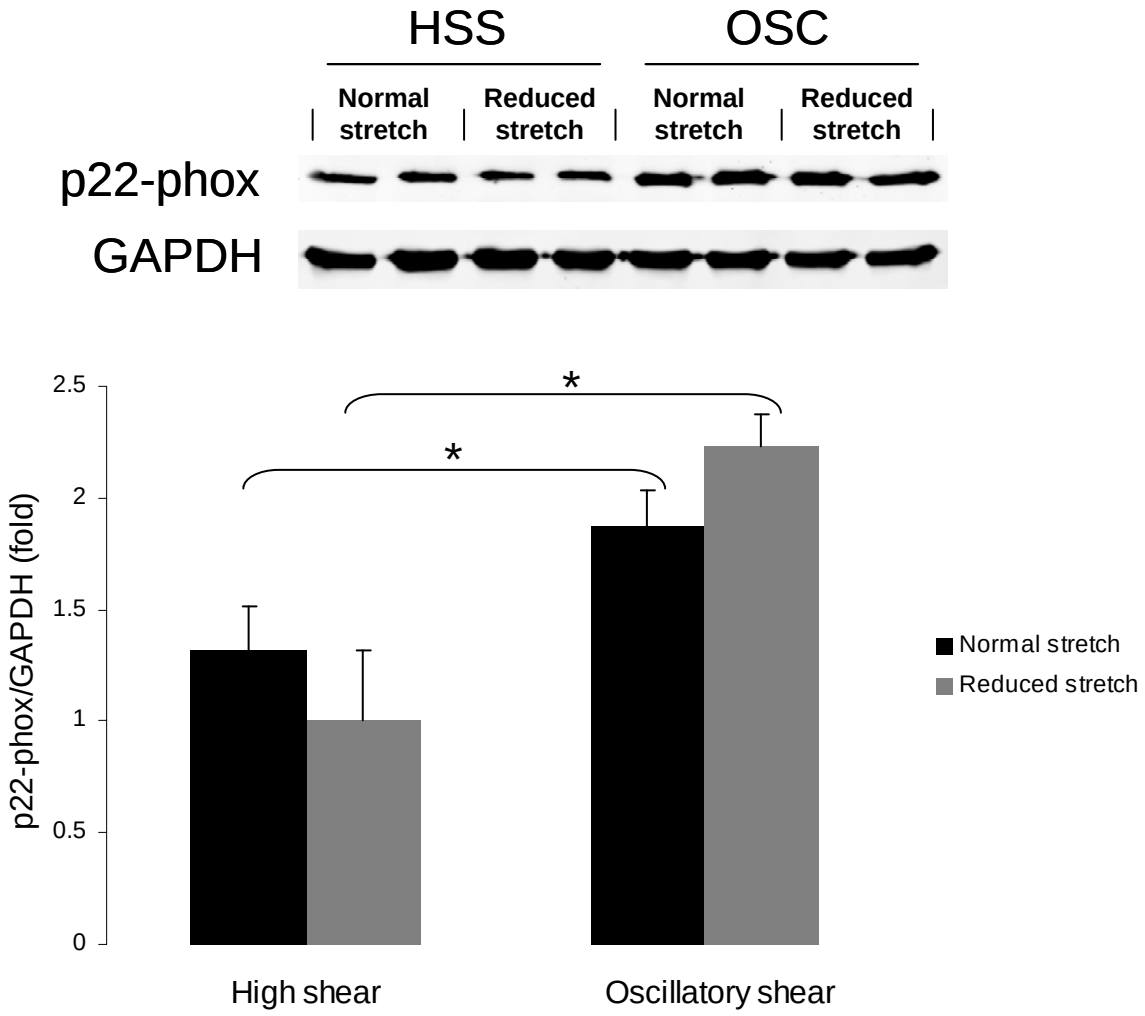

Figure 4a: *The effect of reduced cyclic stretch and oscillatory flow on p22-phox expression, as evaluated by immunoblot. Data are expressed as mean ± SD, n=6, *P<0.05.*

Assessment of p22-phox expression by immunostaining and analysis using Metamorph software allowed us to see the localization of p22-phox expression. We observe that reduced stretch tends to increase the expression of p22-phox evenly through out the arterial wall, whereas oscillatory flow predominantly focuses the expression of p22-phox on the endothelium and intimal layers.

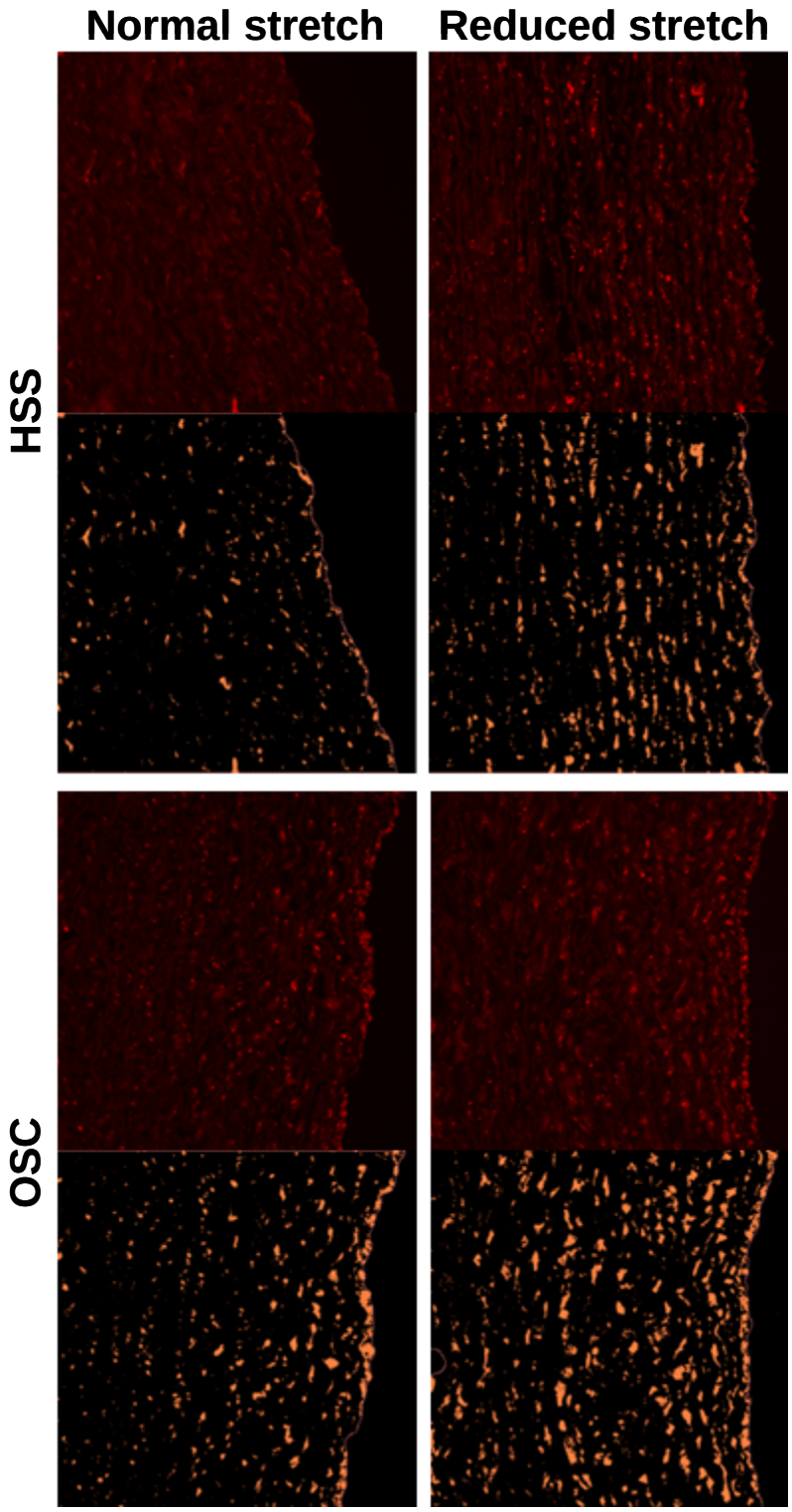

Figure 4b: *p22-phox was visualized in red by immunostaining on arterial cross sections. Images on top are originals from just the red channel, while those on the bottom were analyzed with a script written in Metamorph. All images were taken at the same contrast and luminescence levels at 20 times magnification.*

The expression of p47-phox was also increased by OSC as compared to HSS. However, in this case the expression was only seen on arteries perfused under normal stretch conditions.

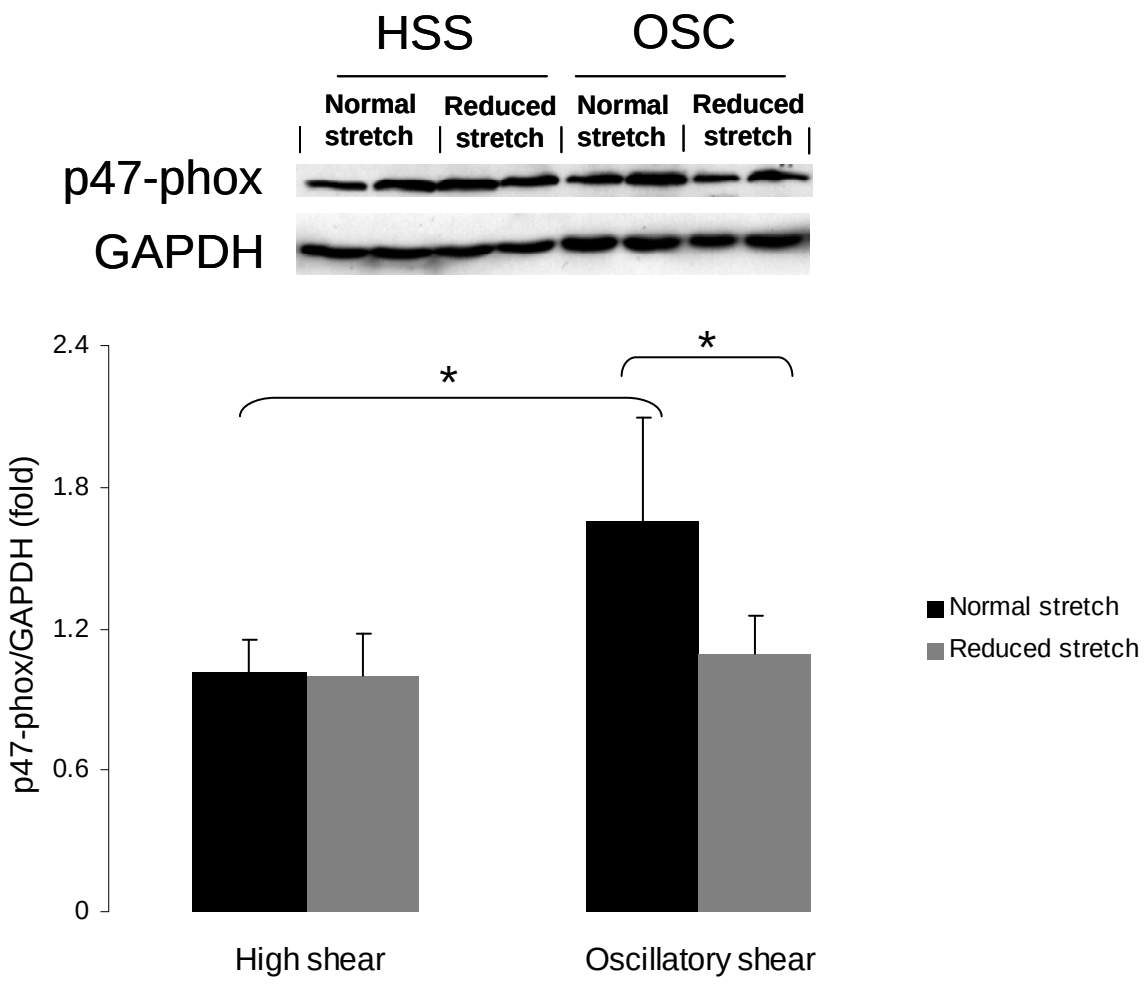

Figure 4c: *The effect of reduced cyclic stretch and oscillatory flow on p47-phox expression, as evaluated by immunoblot. Data are expressed as mean ± SD, n=6, *P<0.05.*

Shear stress mediates the formation of Nitro tyrosine

Increased formation of nitro tyrosine is directly related to the production of peroxynitrate, which is produced when ROS react with NO, thus lowering the vascular bioavailability of NO. Exposure of arteries to OSC under both normal and reduced stretch significantly increased the formation of nitro tyrosine as compared to HSS at same stretch conditions (1.4 and 1.65 fold up-regulation respectively).

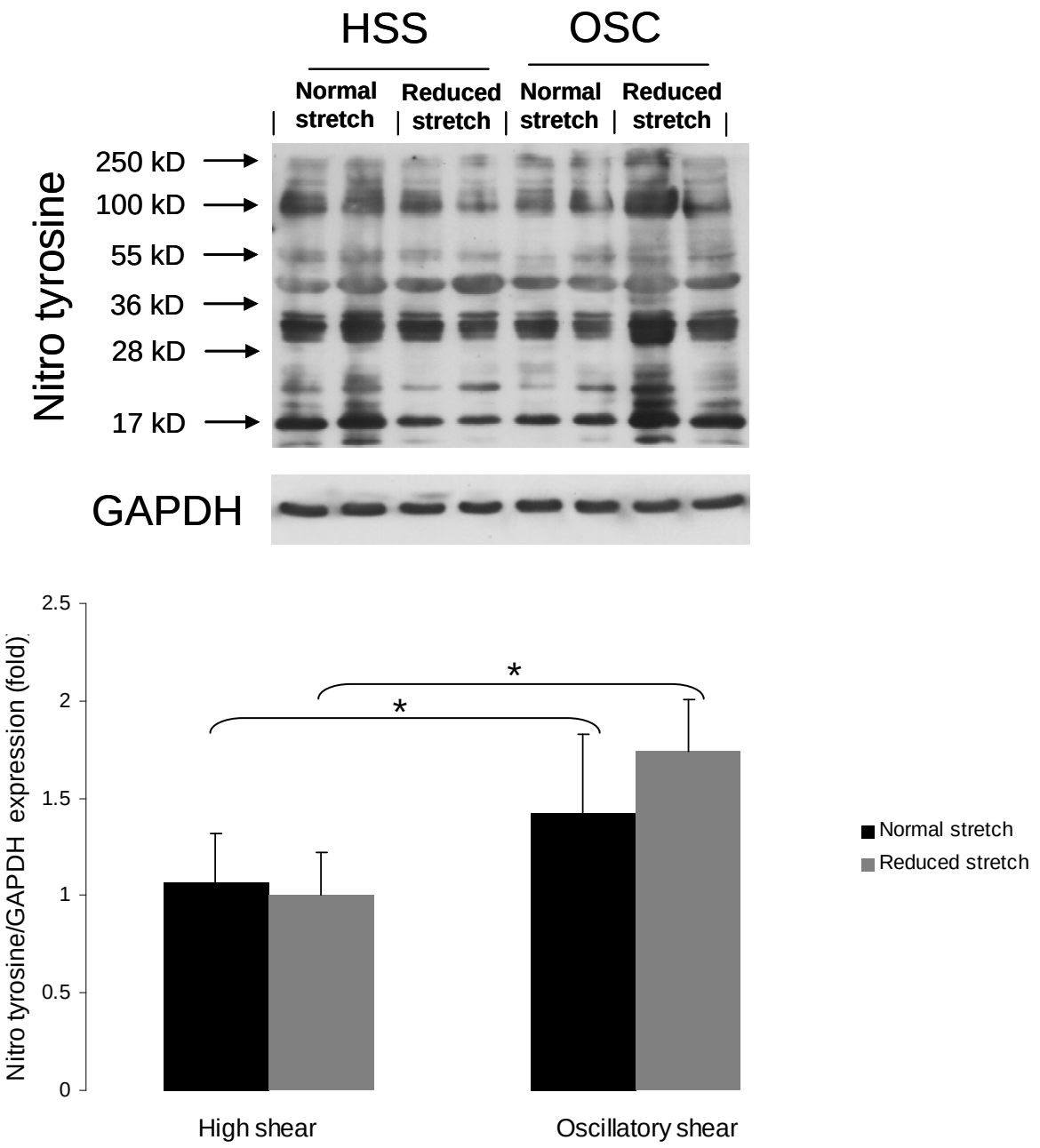

Figure 5a: *The effect of reduced cyclic stretch and oscillatory flow on nitro tyrosine expression, as evaluated by immunoblot. Data are expressed as mean ± SD, n=6, *P<0.001.*

Immunostaining analysis revealed that nitro tyrosine was predominantly located in the intimal and medial layers (below).

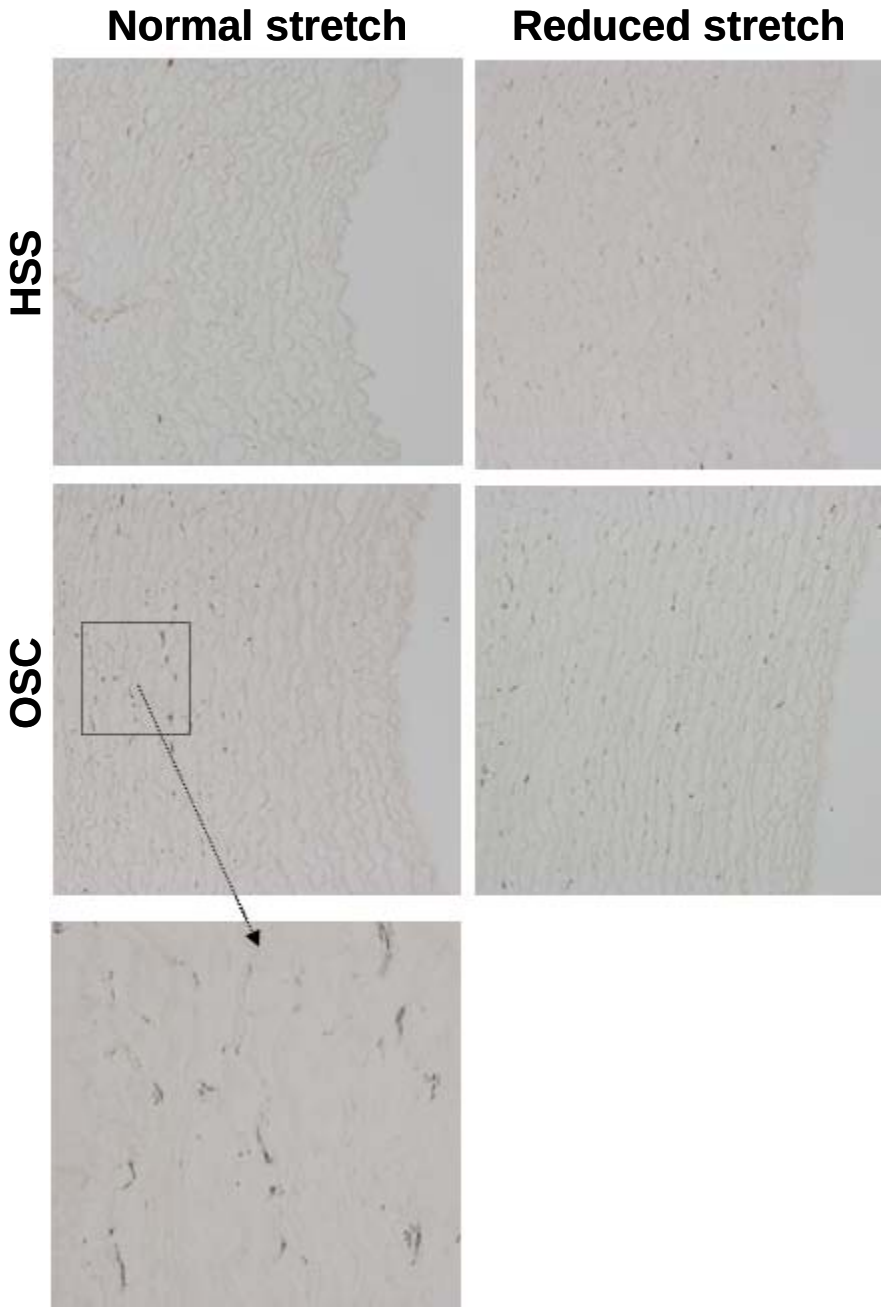

Figure 5b: *Nitro tyrosine was visualized in brown by immunostaining on arterial cross sections using a DAB substrate kit. All images were taken at the same contrast and luminescence levels at 10 times magnification.*

Discussion

The current study demonstrates that shear stress and cyclic stretch play significant roles in the progression of endothelial dysfunction, and points out their individual and spatial contribution in triggering vascular disease. Oscillatory flow and reduced stretch both proved detrimental to endothelial function, whereas oscillatory flow alone, dominated total endogenous vascular wall superoxide anion production. Yet, analysis of oxidative stress in just the endothelial region showed a more pronounced formation of superoxide anion by reduced cyclic stretch than by oscillatory shear. These results emphasize an important distinction between shear and stretch mediated effects to the vascular wall. Similarly, western blot analysis revealed that eNOS and nitro tyrosine expression were also more modulated by oscillatory flow, than by reduced stretch. These data suggest that shear and stretch stimulate regions of the vascular wall differently, yet it is their combined effects which contribute to vascular disease.

Previous works have related reduced compliance [16] and oscillatory shear stress [17] to modulation of eNOS expression and endothelial cell homeostasis [18-20]. However, when shear stress and cyclic stretch have been studied in the past, often these two very important stimuli have been coupled, or studied under such a wide variety of experimental conditions that it has been difficult to determine their individual contributions to vascular disease. In our study we have performed experiments coupling high and oscillatory shear stress with normal and reduced stretch, for 72 hours, and studied how shear stress and cyclic stretch, two very different mechanical forces, can work independently or in concert to evoke athero-prone endothelial responses.

To study if the effects of shear stress and cyclic stretch do indeed have an effect on vascular endothelial functionality we performed vascular reactivity studies on tissue segments from four experimental groups: HSS/normal stretch, HSS/reduced stretch, OSC/normal stretch and OSC/reduced stretch. Using the HSS/normal stretch group as reference, we noticed that introducing an oscillatory flow or reducing the stretch both caused decreases in endothelial functionality upon BK stimulation, in the order of 85%. Moreover the effect of an oscillatory flow combined with a reduced stretch nearly completely abolished any sign of endothelial functionality. These results indicate that on a functional level, both shear stress and circumferential stretch are imperative for maintaining endothelial function. Moreover, no significant differences in KCl contraction or SNP relaxation among the four groups were observed, confirming that changes in bradykinin relaxation were due to oscillatory flow and reduced stretch and not to loss of vascular smooth muscle function.

To investigate the origins of endothelial dysfunction in regards to shear stress and stretch we first investigated whether eNOS protein expression is modulated differently by reduced cyclic stretch and oscillatory shear. The most obvious result shows that oscillatory flow significantly down-regulates eNOS production, which is in agreement with previous findings [21]. Interestingly, there was no statistical difference between the high shear/reduced stretch and the oscillatory shear/normal stretch groups, indicating that in spite of the knowledge that shear stress is a more significant regulator of eNOS, normal stretch conditions are fundamental to retain the protective effects of wall shear stress.
Fluctuations in oxidative stress, namely in the direction of increased levels of ROS, are known to have dramatic effects on endothelial health and vascular function. Basal levels of ROS are essential to vascular cell physiologic function and contribute to the signalling required for vascular remodelling [22, 23]. Yet increases in their production have been shown

to decrease vascular levels of NO through reactions forming peroxynitrite [24], causing inflammation and potentially even cell death [25]. Thus, we found it interesting to investigate how shear stress and cyclic stretch modulate ROS production in the vascular wall. To visualize ROS production arterial cryosections were stained with DHE, a cell permeable compound which forms ethidium upon reacting with $O^{\cdot}{}_2$-. Quantification was performed by multiplying the percent area for the region of interest of the arterial cross section expressing ethidium by the average intensity of ethidium expression. It was found that oscillatory flow more significantly up regulated total DHE intensity when compared to reduced stretch. Yet, at the endothelial layer, reduced stretch is a more significant modulator of DHE than oscillatory flow. These results suggest that reduced stretch and oscillatory flow both induce ROS production in the vascular wall, but most importantly they explain that increases in ROS due to reduced arterial compliance pose a much higher risk to endothelial dysfunction than oscillatory flow.

As mentioned above, arterial ROS production is mediated at many levels. The present study focus on the NOX cascade, known to be a major source of ROS in vascular cells [26]. To measure the activation of the NOX we investigated the regulation of two crucial components, p22-phox and p47-phox. P22-phox was increased by both oscillatory shear and reduced shear stress, but more significantly by oscillatory shear. These results were further supported by immunostaining which showed that p22-phox expression was highly expressed in the endothelial and intimal region. The expression of p47-phox component was significantly up-regulated in the combination of oscillatory shear and normal stretch. These results suggest that p22-phox and p47-phox could play significant roles in the development of vascular ROS production, but do not necessarily act in concert. At physiologic pH, it is known that high levels of vascular ROS can react with NO to form peroxynitrite, a potent oxidant, thus lowering NO bioavailability. Due to the very short half-life of peroxynitrite, we can measured nitro-tyrosine formation, the by-product of the reaction between peroxynitrite and tyrosine protein residues[9]. The measurement of nitro-tyrosine indirectly reflects the amount of NO which is being removed from the vasculature due to ROS scavenging. Our results showed that oscillatory shear significantly up-regulates nitro-tyrosine expression, indicating that the increases in ROS due to oscillatory flow are causing more reactions with vascular NO, decreasing its bioavailability and creating a more athero-prone endothelial environment.

To conclude, this study demonstrates for the first time in an arterial tissue model the individual contributions of shear stress and cyclic stretch to endothelial injury due to increased superoxide anion production. Our results have shown that oscillatory flow is a more dominant stimulus than reduced cyclic stretch with regards to down-regulation of eNOS, increased levels of total vascular ROS and nitro-tyrosine regulation, the combination of which can be linked to a decreased in NO bioavailability. This study also demonstrated the involvement of two NOX components, namely p22-phox and p47-phox, in the modulation of ROS expression. Finally, the most interesting finding from this study was that ROS production in the endothelial region is significantly higher and more significantly modulated by reduced stretch than oscillatory shear. This point suggests that although oscillatory shear was shown to more significantly modulate total ROS production, reduced stretch is the more dangerous risk for endothelial injury. These findings agree with results from tissue reactivity studies where we were able to see significant decreases in endothelial functionality due to both oscillatory flow and reduced stretch. Because perturbed shear stress and reduced arterial compliance have both been implicated in the initiation and attenuation of arterial disease, this work gives insight to how oscillatory shear stress and reduced compliance provoke endothelial dysfunction and increased risk of atherosclerosis over time.

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Paper IV

Differential effects of reduced cyclic stretch and perturbed shear stress within the arterial wall and on smooth muscle function

T. Thacher[1], Rafaela F. da Silva[1,2,3], Nikos Stergiopulos[1]

[1] Laboratory of Hemodynamics and Cardiovascular Technology (LHTC), Swiss Federal Institute of Technology Lausanne (EPFL)
[2] Department of Neurosurgery, Geneva University Hospital
[3] Department of Neurosciences, University Medical Center, University of Geneva Medical School

Abstract

Cyclic circumferential stretch and shear stress act in concert yet are capable of independently mediating arterial smooth muscle function, modulating the production of superoxide and stimulating arterial remodeling.

Porcine carotid arteries were perfused *ex-vivo* for 72 hours. Groups combining normal (5%) and reduced (1%) stretch with high shear (6±3 dynes/cm^2) and oscillatory shear (0.3±3 dynes/cm^2) stress were created, while maintaining a pulse pressure of 80±10 mmHg.

Total superoxide production, fibronectin expression and gelatinase activation were mediated by shear stress, but expression in the endothelial region was mediated by reduced cyclic stretch. By plotting intensity versus radius we saw that superoxide and gelatinase activity were in part mediated by stress distributions through out the vascular wall, while fibronectin and p22-phox were much less or not at all. These findings, when coupled with our results from tissue reactive studies, suggest that the arterial remodeling process triggered in the endothelial region due to reduced stretch causes the most significant changes in arterial smooth muscle function.

We have found that the remodeling process triggered by reduced compliance in the endothelial region of large conduit arteries has a more profound detrimental effect to smooth muscle function than that brought on by perturbed shear stress. This work provides new insight by suggesting that although mechanical stimuli such as cyclic stretch and shear stress are known to augment similar markers of vascular remodeling, the location of their expression throughout the vascular wall differs greatly and this can have dramatic effects on vascular function.

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Introduction

Arteries are constantly exposed to dynamic mechanical forces comprising of shear stress and cyclic circumferential stretch. These stresses determine arterial structure and morphology; modulation of which leads to chronic adaptive remodeling within the arterial wall, a situation which has been linked to the progression of vascular diseases such as hypertension [1] and atherosclerosis [2, 3].

It has been documented that areas of the vasculature exposed to low mean shear stress and cyclic reversal of flow direction (also called oscillatory flow, OSC) are at higher risk of aggressive inward vascular remodeling, marked by matrix reorganization and cellular proliferation [4], than those exposed to a high unidirectional shear stress (HSS). Similarly, it has been suggested that reduced arterial compliance may also promote vascular remodeling [5], in which the mechanisms also involve matrix reorganization and intimal cellular proliferation. In most previous works these two stimuli have been simultaneously coupled *in-vivo*, making it very difficult to understand the individual contributions of cyclic stretch and shear stress to vascular remodeling. A recent *in vivo* study has addressed how altered circumferential wall tension and shear stress affects aortic remodeling in rats [6], yet how these mechanical forces are perceived and transformed into cellular signals through out the vascular wall is still unclear. Some attempts have also been made to study the individualistic effects of these stimuli *in-vitro*, however the use of different cell lines in a wide variety of stretch systems and flow chambers has made it very difficult to draw definitive conclusions. In the present study we found a compromise between the *in-vivo* and *in-vitro* works of the past by studying the autonomous effects of shear stress and cyclic stretch and their relation to remodeling throughout the arterial wall.

Mechanisms implicated in the initiation of chronic adaptive arterial remodeling are numerous, but recent evidence has implicated the production of reactive oxygen species (ROS) to be an important mediator of hypertensive vascular remodeling [7]. All vascular cells are capable of producing ROS, chronic increases of which have been shown to be mediated by the membrane bound NADPH oxidase subunit p22-phox [8]. Vascular remodeling is a process which requires the breakdown and reorganization of extracellular matrix (ECM), allowing enhanced cellular migration and proliferation in the intima region. Matrix metalloproteinases (MMPs) are a family of enzymes capable of degrading the ECM, of which MMP-2 has been shown to play a principal role [9]. Another important mediator of remodeling is fibronectin, which is deposited by a cellular mediated process and helps rebuild the ECM. Fibronectin has also been shown to be an important regulator of cell proliferation and migration in vascular tissue [10].

The first goal of this study is to understand how shear stress and cyclic stretch individually modulate crucial components of vascular remodeling, such as superoxide production, fibronectin expression, MMP expression/activation, and relate this to arterial smooth muscle cell functional changes. The second goal of the study is to see how the expression of these factors is modulated regionally, throughout the vascular wall.

Materials and Methods

Arterial preparation

Left internal carotid arteries of 6 month old pigs weighing between 120 – 150 kg were taken from a local slaughter house (Bell SA, Cheseaux-sur-Lausanne, Switzerland). Loosely

attached adventitial tissue was removed and a 3.5 cm segment, 1 cm distal to the primary bifurcation was obtained. Arterial segments were then mounted on to the *ex-vivo* arterial support system (EVASS, see details below). The longitudinal stretch ratio was determined to be 1.3 times the un-pressurized un-stretched length. To reduce arterial compliance, a silicon cuff (Statice Sante, Besançon, France) of 6 – 8mm (depending on the outer diameter) and of 0.2 ± 0.05 mm thick was placed around the arterial segment. Reduction of cyclic circumferential stretch obtained with the cuff was roughly 80%, when compared to the un-cuffed segments.

Ex-vivo arterial support system: EVASS

The EVASS system used in this study offers a nice compromise between *in-vitro* and *in-vivo* setups, allowing the perfusion of arterial tissue segments while allowing precise control of perfusion pressure and flow. More precise details on EVASS have been given previously [11]. The arterial perfusions were maintained for 72 hours with a medium cocktail, as previously described [5]. Perfusion flow was adapted to create either a pulsatile unidirectional high shear stress (HSS) with a mean value of 6 dyne/cm^2 and amplitude of 3 dyne/cm^2, or an oscillatory shear stress (OSC) with a mean value of 0.3 dyne/cm^2 and amplitude of 3 dyne/cm^2, both with a frequency of 1 second. Perfusion pressure was fixed at 80 mmHg with a pulse pressure amplitude of ±10 mmHg. Resulting strains were 4 – 5% for the un-cuffed segment, which is in the physiologic range of pulsatile stretch for the porcine carotid, and less than 1% for the cuffed segment, mimicking a less compliant arterial segment.

Arterial function analysis

Arterial rings were tested before and after the perfusion to determine their capacity to achieve smooth muscle mediated contraction. Arterial rings were mounted in an organ bath (EMKA Technology), equilibrated in a Krebs buffer solution at 37^{o}C, infused with 5% CO_2 and 95% O_2. During the equilibration period resting tension was repeatedly adjusted to 2 grams. Arterial rings were pre-contracted with 80 mmol/L KCl until a constant maximum contraction was achieved. Next tissues were rinsed with krebs solution until baseline tension was achieved; doses from 10^{-8} to 10^{-4} mol/L of Norepinephrine (NE) were introduced and dose response curves were obtained. Endothelial functionality was a criteria for inclusion in our study and was assessed using Bradykinin (Bk) (data not shown).

Immunofluorescence, dihydroethidium and gelatinase

After the perfusion, a 5 mm long segment of the artery was rinsed with 0.9% NaCl, frozen in OCT compound (Tissue-Tek) and stored at -80^{o}C for further analysis. For p22-phox and fibronectin staining, sections of 5 μm were cut, air dried and fixed in 100% acetone for 5 minutes at -20^{o}C. Sections were permeablized with 0.1% Triton X-100 in PBS for 10 minutes, then incubated for 60 minutes with rabbit anti-p22^{phox} (p22-phox)(1:500, Santa Cruz Biotechnology) or rabbit anti-fibronectin (fibronectin)(1:500, Sigma-Aldrich) in 10% normal goat serum in PBS. The p22-phox sections were then incubated with Alexa Fluor 568 goat anti-rabbit IgG (Invitrogen) as a secondary antibody for 45 minutes. Sections were examined on a Leica DMI 4000 microscope at 10 times magnification. All steps were performed at room temperature.

For gelatinase activity detection, 5 μm thick arterial cryosections (as described above) were incubated for 4 hours at 37^{o}C with 25 μg/ml of DQ gelatin (EnzCheck Gelatinase assay kit, Molecular Probes) in 10% agarose (Sigma) in PBS. Sections were examined on a Leica DMI 4000 microscope at 10 times magnification. All steps were performed in the dark.

Dihydroethidium (DHE) is a cell permeable compound which forms ethidium upon reacting with superoxide anion. For detection 5 µm arterial cryosections (as described above) were incubated for 30 minutes at 37 ^{o}C with 5 µmol/L dihydroethidium (DHE) (FluoProbes) in PBS, then rinsed for 1 minute in PBS. Sections were examined on a Leica DM5500 at 20 times. All steps we performed in the dark.

Protein extraction and Western blot analysis

Protein expression was assessed using standard Western blot techniques. Protein was extracted from the samples with a Brij-35 lyses buffer (50 mmol/L Tris pH 7.5, 1mol/L NaCl, 2M Urea, 0.1% Brij-35, and 1 protease inhibitor cocktail (Roche)). 20 µg of protein was electrophoresed, after which the proteins were transferred to a nitrocellulose filter (Amersham). Filters were incubated with mouse anti-MMP-2 (MMP-2) (1:500, Milipore) and mouse anti-Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) (1:1000, Chemicon International) followed by ECL-peroxidase labelled anti-mouse secondary antibodies (1:5000, Amersham). MMP-2 protein expression was normalized to GAPDH.

Image analysis

Images were analyzed using Metamorph (Meta imaging series 7.0). Auto-fluorescence of elastin was subtracted from each image as to not interfere with staining quantifications. Quantifications were performed by multiplying the percent area of the arterial region of interest expressing the stain, by the average intensity of the stain. Endothelial regions were defined by hand using a WACOM graphic tablet.

Statistics

The data are reported as mean values ± standard deviation. Two-way analysis of variance (ANOVA) statistics were performed to assess significant differences between shear stress and cyclic stretch groups. A value of $p < 0.05$ was considered significant.

Results

Reduced stretch modulates smooth muscle contractile capacity

Smooth muscle contractile function, as assessed at the 10^{-4} M dosage of Norepinephrine, was significantly modulated for both HSS and OSC groups by reduced stretch with a 20% and 24% decrease respectively.

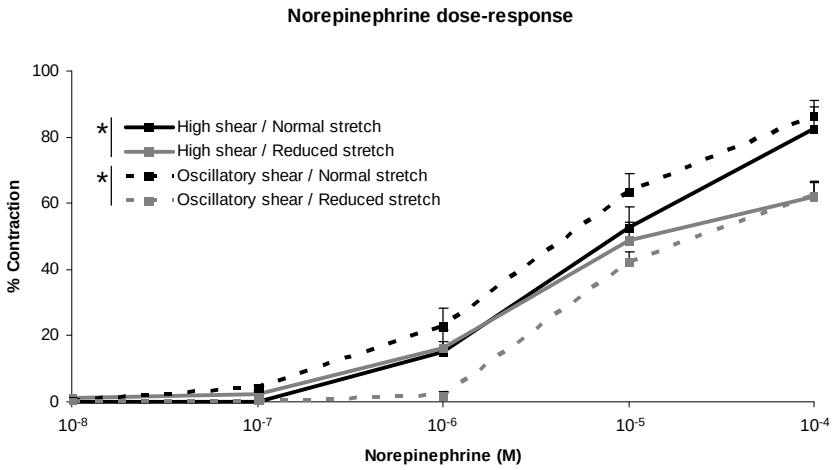

Figure 1: *Norepinephrine (NE) dose-dependent contraction capacity of porcine carotid arterial segments after 72 hours of ex-vivo perfusion. Data are represented as mean ± SEM, n = 6, *p<0.05.*

Radial expression of superoxide anion is modulated by reduced cyclic stretch and oscillatory shear

Radial vascular ROS magnitude and distribution is modulated almost identically by both reduced stretch and oscillatory flow, which maintain a sustained level of ROS through out the measured region. The combination of the two stimuli are additive, nearly doubling the amount of superoxide anion present in the vascular wall, which is sustained through out the region of interest.

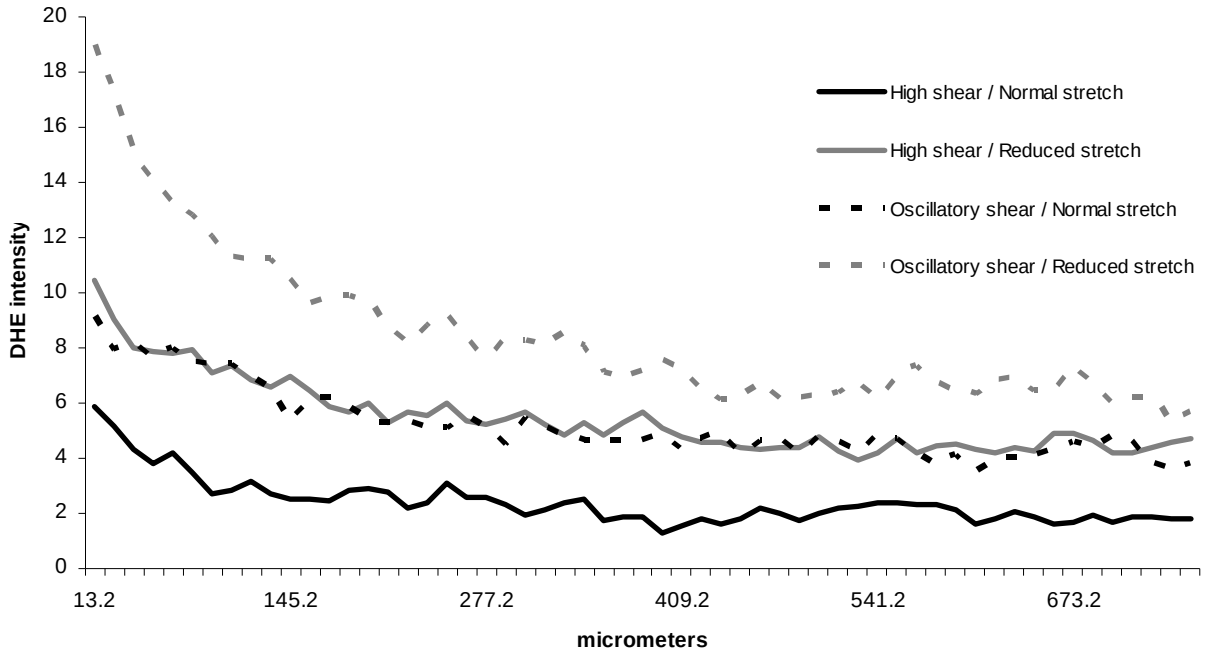

Figure 2a: *The effect of reduced cyclic stretch and oscillatory shear stress on dihydroethidium staining versus arterial wall radius. Curves represent the average of four images from each artery, n = 6.*

Representative dihydroethidium stained images used for analysis have been included (below).

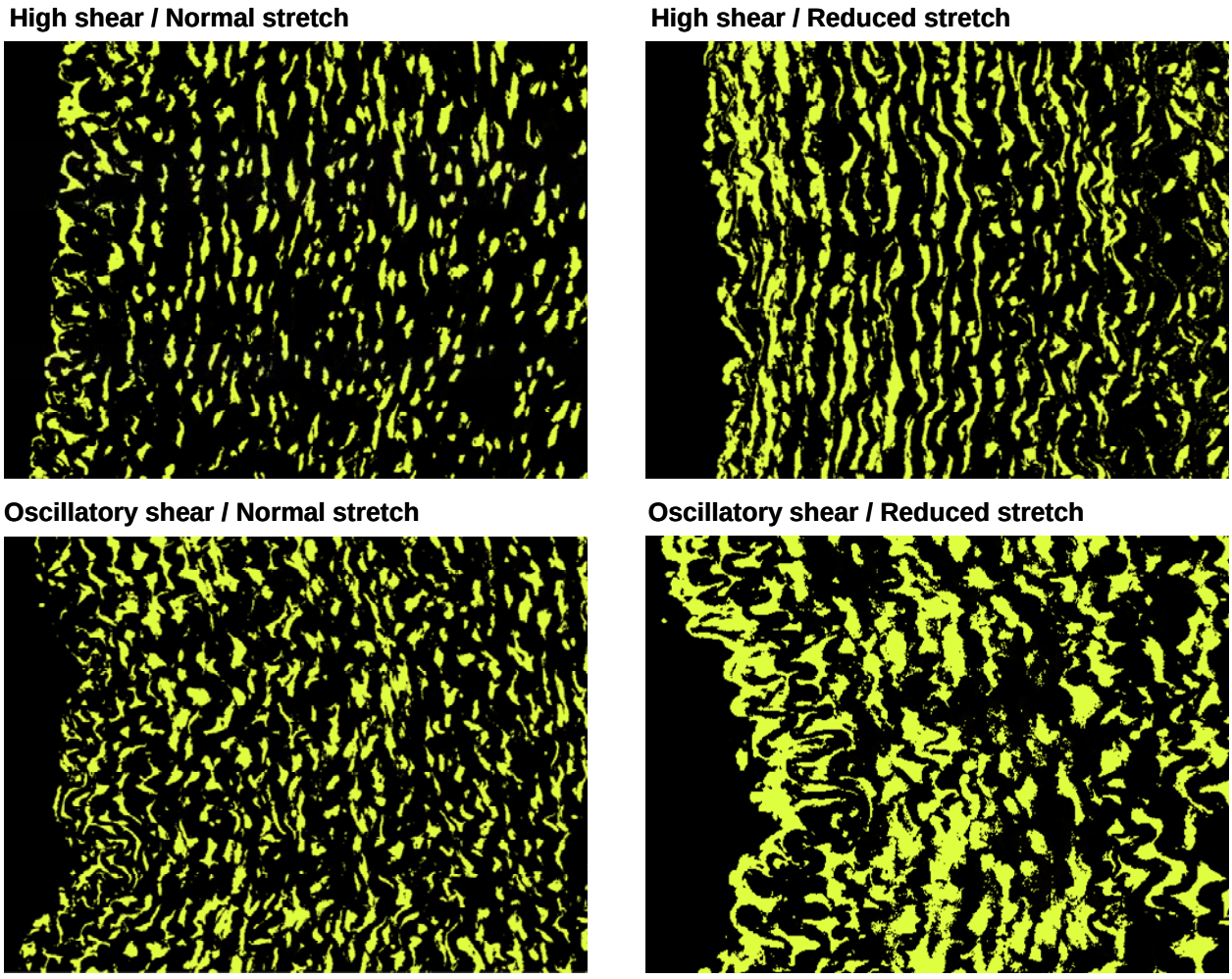

Figure 2b: *DHE is represented in yellow; all images were taken at the same contrast and luminescence levels at 10x magnification. Arterial lumen orientation is facing left.*

Radial expression of p22-phox correlates with superoxide ion production, but suggests it is not the only factor responsible for its measured increases

The radial magnitude of p22-phox expression is modulated by reduced stretch and oscillatory shear stress, the two effects being additive and correlating well with the magnitude of DHE expression. In the first 130μm reduced stretch and oscillatory shear stimulate nearly identical amounts of p22-phox, at which point they diverge and oscillatory shear is shown to be the more prominent stimulus of p22-phox. The combination of both reduced stretch and oscillatory shear creates a sustained amount of vascular p22-phox.

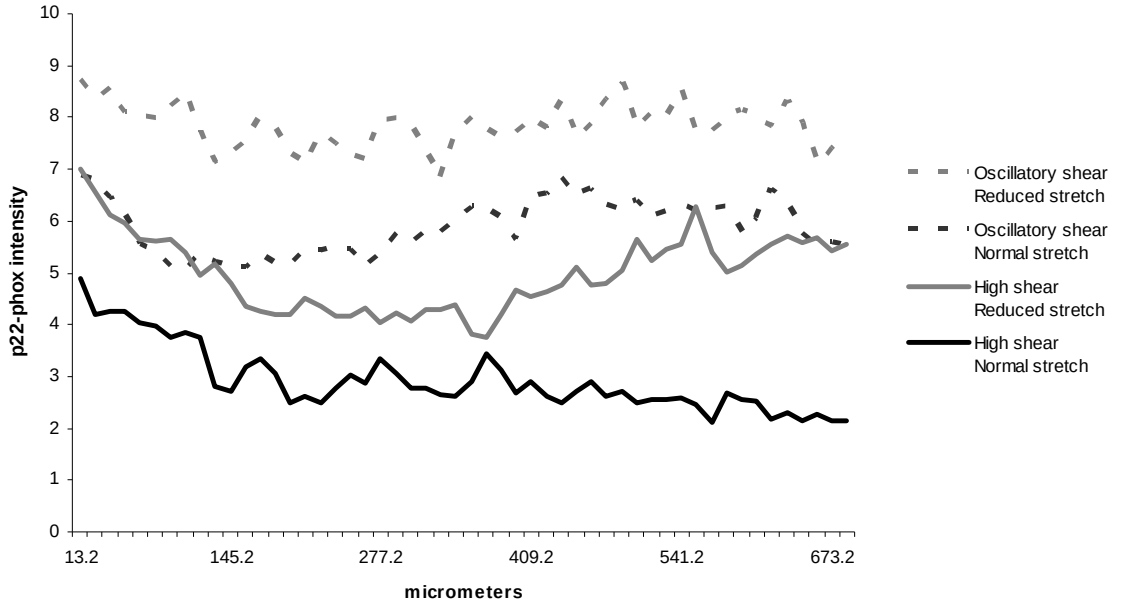

Figure 3a: *The effect of reduced cyclic stretch and oscillatory shear stress on p22-phox expression versus arterial wall radius. Curves represent the average of four images from each artery, n = 6.*

Representative p22-phox images used for analysis have been included (below).

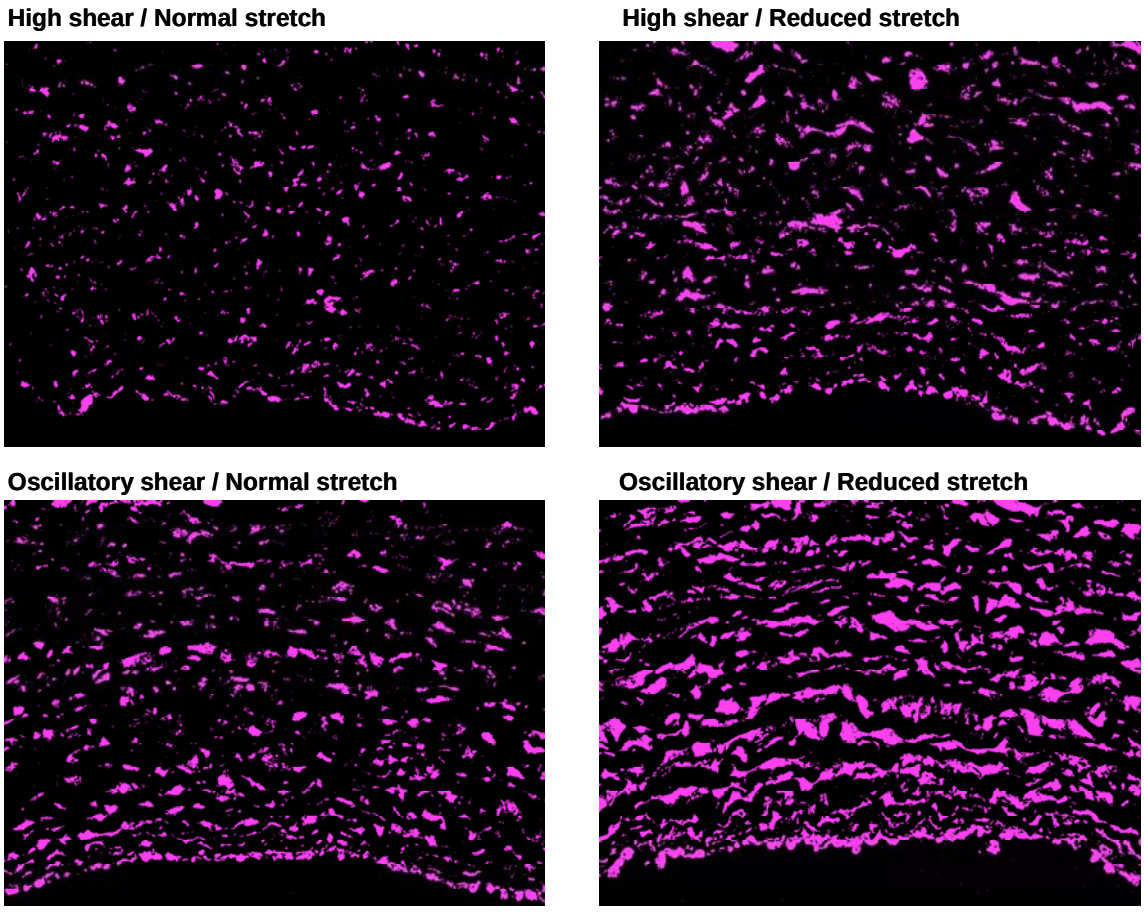

Figure 3b: *p22-hox is represented in purple; all images were taken at the same contrast and luminescence levels at 10x magnification. Arterial lumen orientation is facing down.*

MMP-2 is significantly modulated by shear stress, but not stretch

In arteries exposed to OSC shear stress MMP-2 protein expression was significantly up-regulated in both normal and reduced stretch groups (21% and 18% respectively).

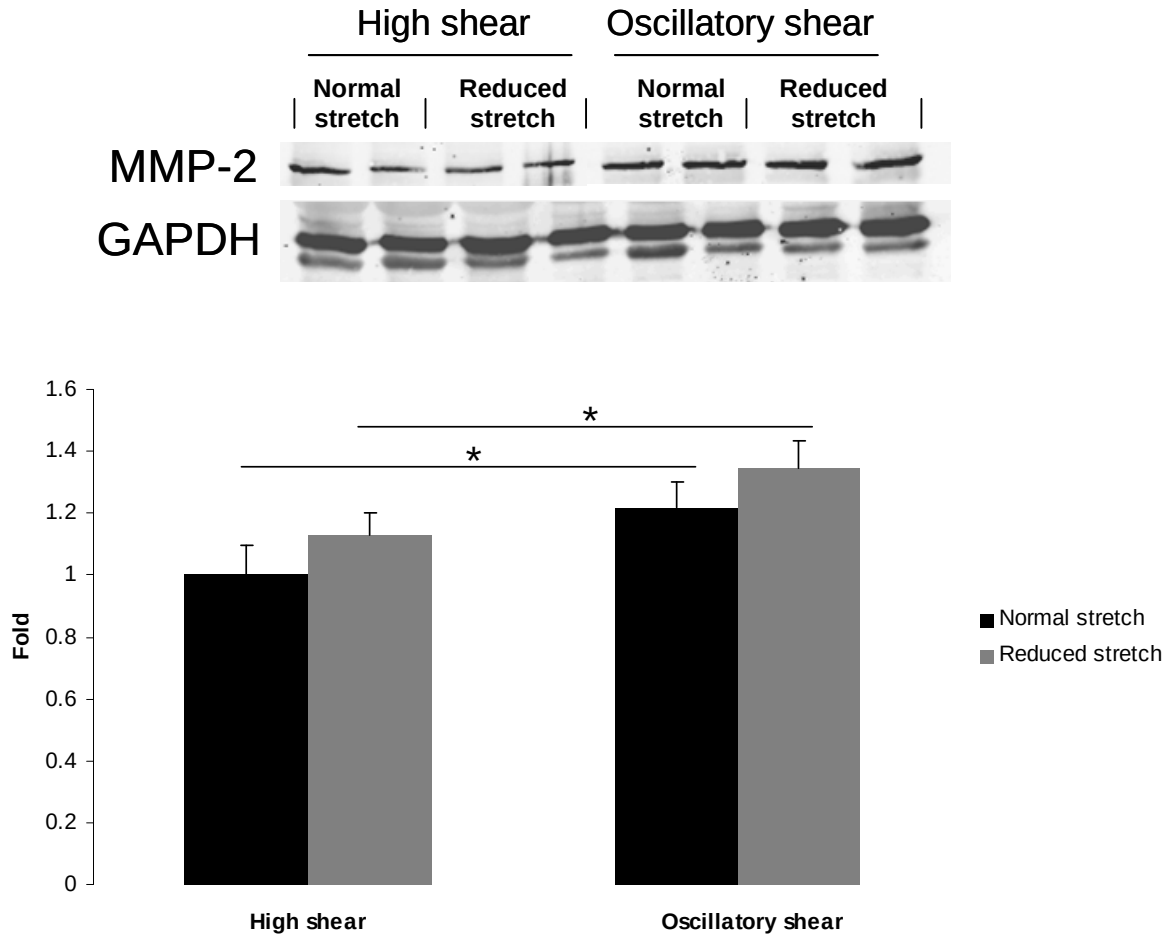

Figure 4: *The effect of reduced cyclic stretch and oscillatory shear stress on MMP-2 expression as evaluated by immunoblot. Data are expressed as mean ± SEM, n = 6, *p<0.05.*

Reduced stretch activates gelatinase in the endothelial region, while oscillatory shear increases gelatinase activity through out the vascular wall.

When focusing on the endothelial region, arteries exposed to a reduced stretch show significant increases in gelatinase activity for both HSS and OSC groups (66% and 50% respectively).

Figure 5a: *The effect of reduced cyclic stretch and oscillatory shear stress on endothelial gelatinase activity as evaluated by image analysis. Data are expressed as mean ± SEM, n = 6, *p<0.05.*

Representative gelatinase images used for analysis have been included (below).

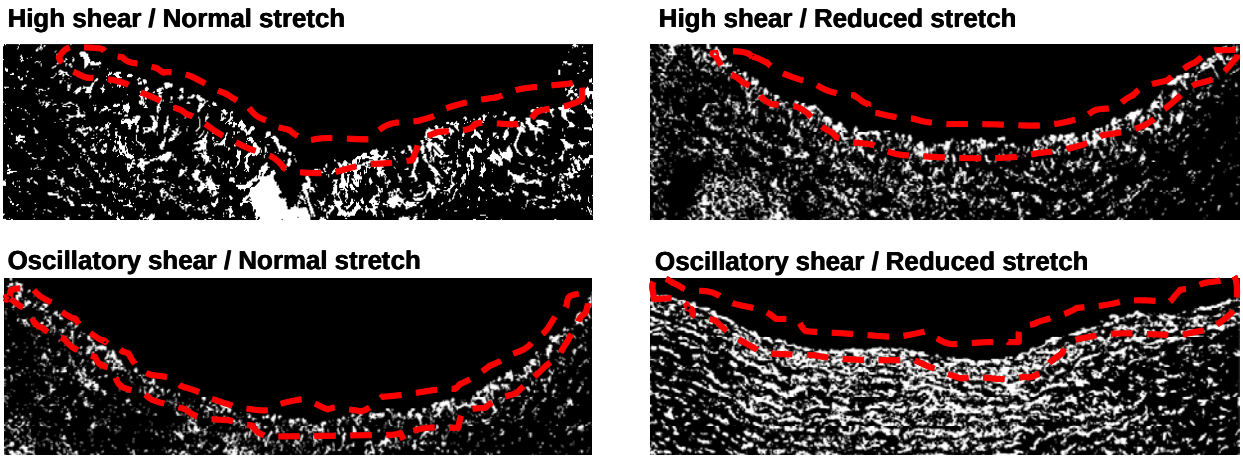

Figure 5b: *Gelatinase activity is represented in white and was evaluated at the endothelium. All images were taken at the same contrast and luminescence levels at 10x magnification. Arterial lumen orientation is facing up.*

Analysis of total vascular gelatinase activation was significantly increased by OSC shear for both the normal and reduced stretch groups (67% and 32% respectively).

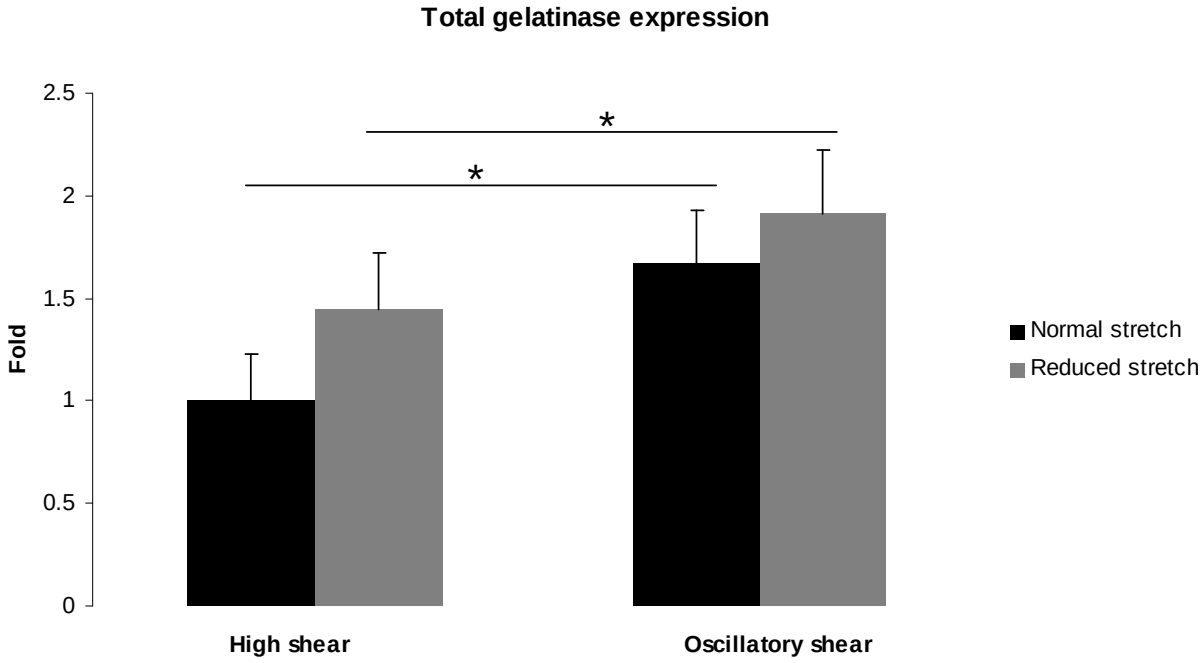

Figure 5c: *The effect of reduced cyclic stretch and oscillatory shear stress on total gelatinase activity as evaluated by image analysis. Data are expressed as mean ± SEM, n = 6, *p<0.05.*

Representative gelatinase images used for analysis have been included (below).

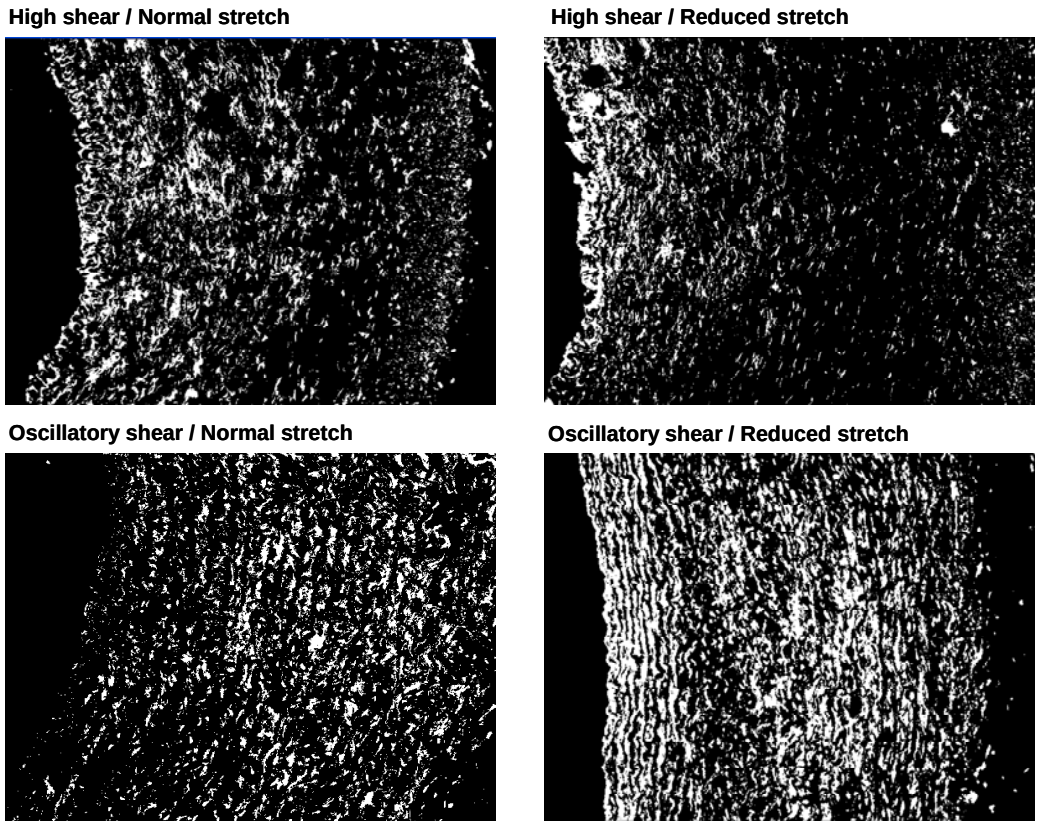

Figure 5d: *Gelatinase is represented in white and was quantified over the entire region. All images were taken at the same contrast and luminescence levels at 10x magnification. Arterial lumen orientation is facing left.*

Reduced cyclic stretch and oscillatory shear stress modulate gelatinase activity differently in the arterial wall

In the first 100 μm of the arterial wall we see that the magnitude of gelatinase is modulated by reduced stretch and OSC shear and the shapes of the curves are similar. From 100 μm to 400 μm gelatinase activity for the HSS 5% group steadily declines and introduction of either reduced stretch or OSC shear reduces this decline. The combination of reduced stretch and OSC shear causes the activation of gelatinase to peak in this region. From 400 μm to 600 μm we see all four groups come together and begin to behave in a similar manner.

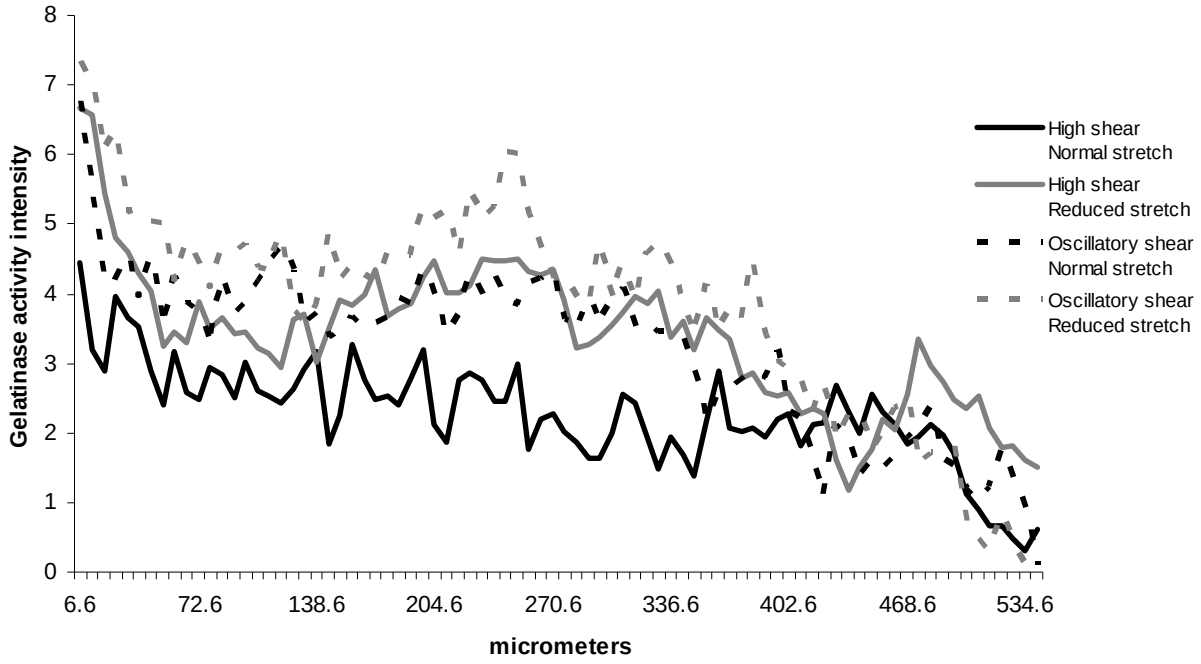

Figure 5e: *The effect of reduced cyclic stretch and oscillatory shear stress on gelatinase activity versus arterial wall radius. Curves represent the average of four images from each artery, n = 6.*

Reduced stretch modulates the expression of fibronectin in the endothelial region, while oscillatory shear increases gelatinase activity through out the vascular wall

When focusing on the endothelial region, arteries exposed to reduced stretch show significant increases in fibronectin expression for both HSS and OSC groups (40% and 41% respectively).

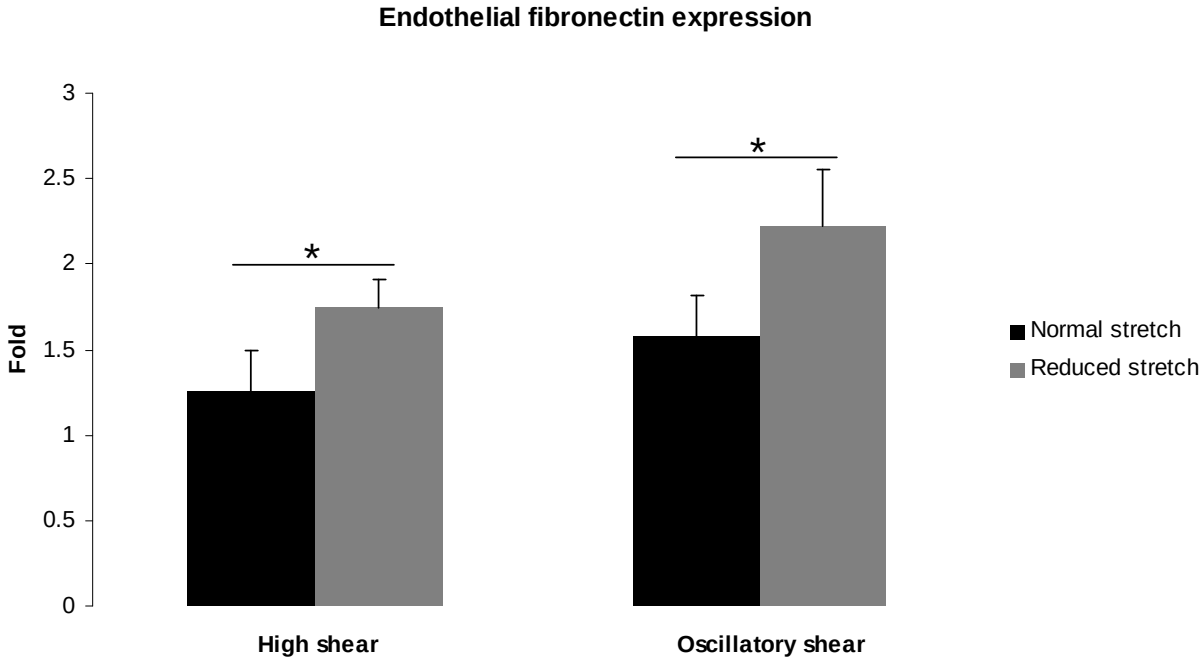

Figure 6a: *The effect of reduced cyclic stretch and oscillatory shear stress on endothelial fibronectin expression as evaluated by image analysis. Data are expressed as mean ± SEM, n = 6, *p<0.05.*

Representative fibronectin images used for analysis have been included (below).

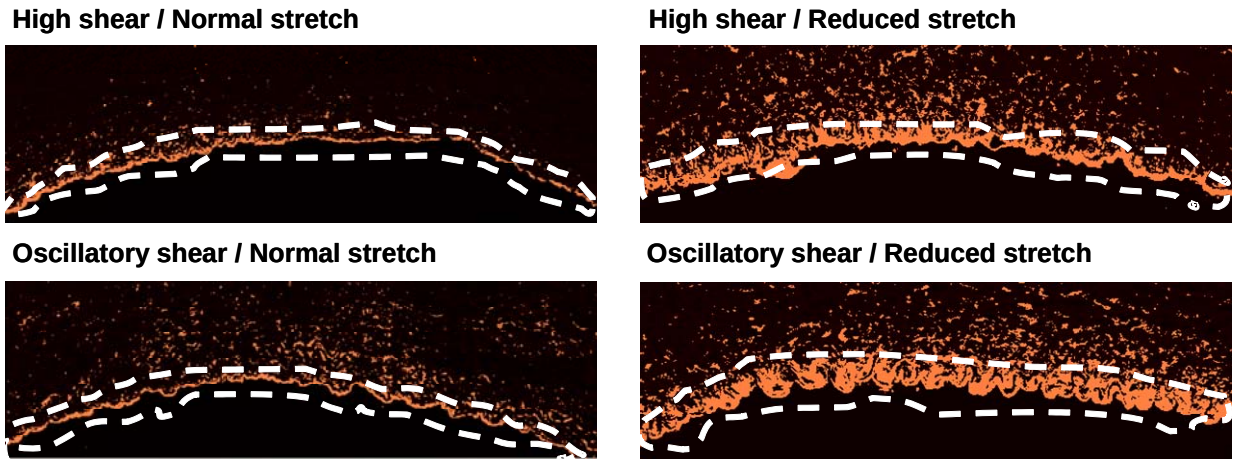

Figure 6b: *Fibronectin is represented in orange and was evaluated at the endothelium. All images were taken at the same contrast and luminescence levels at 10x magnification. Arterial lumen orientation is facing down.*

Total vascular fibronectin expression was significantly increased by OSC shear for both the normal and reduced stretch groups (68% and 74% respectively).

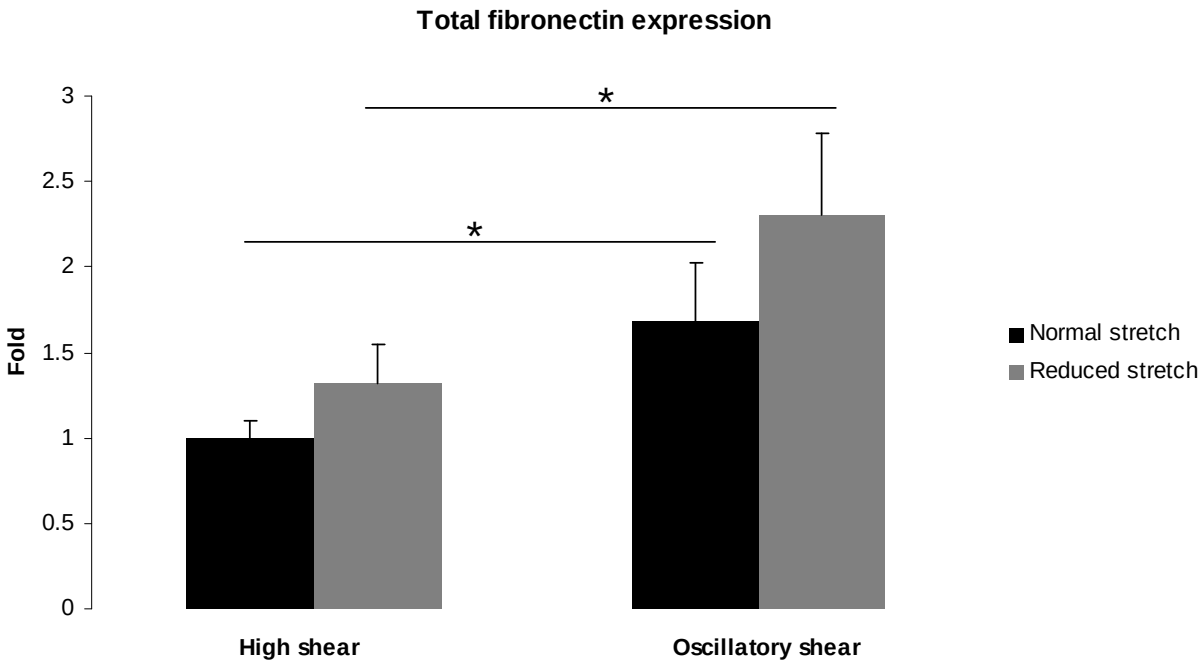

Figure 6c: *The effect of reduced cyclic stretch and oscillatory shear stress on total fibronectin expression as evaluated by image analysis. Data are expressed as mean ± SEM, n = 6, *p<0.05.*

Representative fibronectin images used for analysis have been included (below).

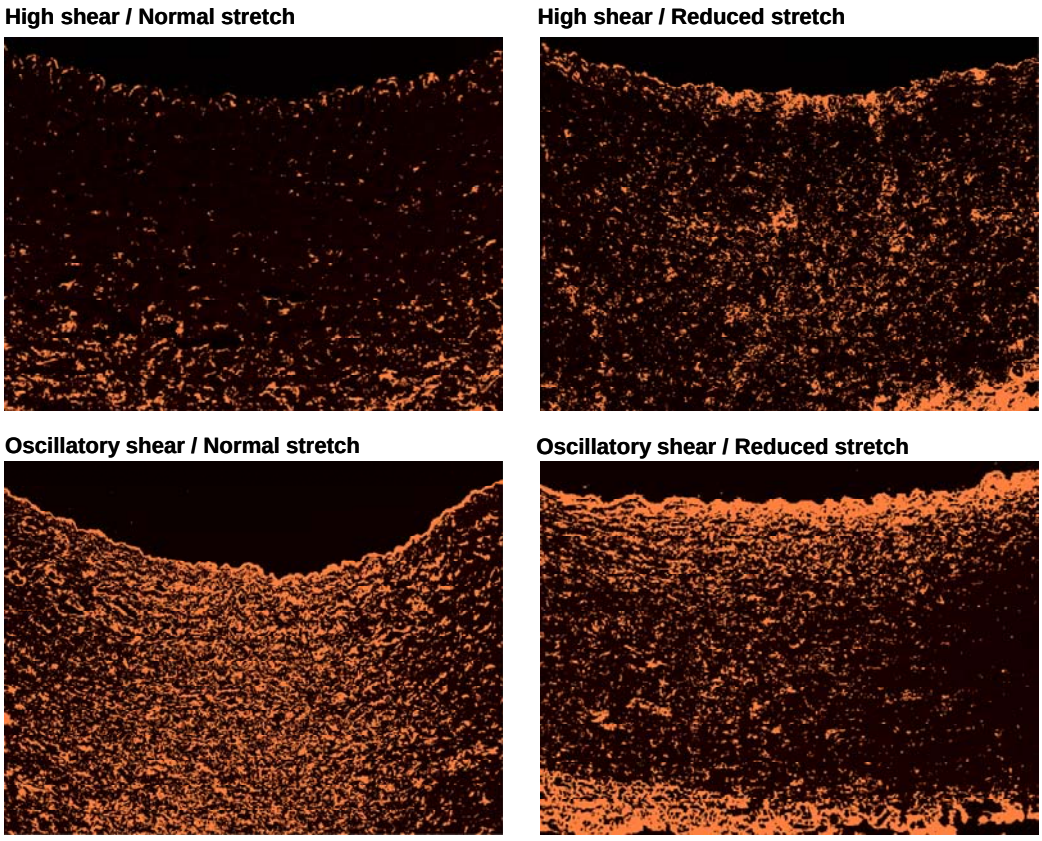

Figure 6d: *Fibronectin is represented in orange and was quantified over the entire region. All images were taken at the same contrast and luminescence levels at 10x magnification. Arterial lumen orientation is facing up.*

Reduced cyclic stretch and oscillatory shear stress modulate fibronectin expression differently in the arterial wall

From the endothelium penetrating 0.05mm into the arterial wall, we see that the magnitude of fibronectin expression is modulated by both reduced stretch and OSC shear, but the shapes of the curves fall into two distinct groups, modulated by shear. The expression of fibronectin in the HSS groups decreases exponentially with radius, where as the OSC groups have a more sustained expression before diminishing. As the radius increases we see that reduced stretch, OSC shear and the combination of the two still modulate the magnitude of fibronectin expression. The shapes of the curves for the four groups follow a similar trend, steadily increasing with radius.

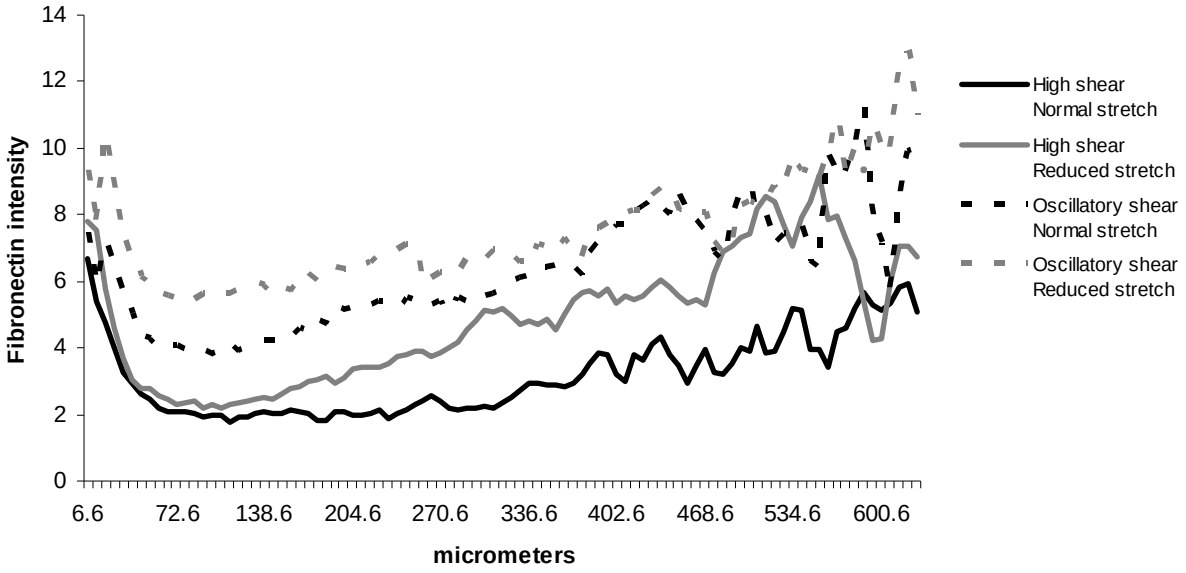

Figure 6e: *The effect of reduced cyclic stretch and oscillatory shear stress on fibronectin expression versus radius in the arterial wall. Curves represent the average of four images from each artery, n = 6.*

Discussion

Previous works have shown that reduced arterial compliance [12] and oscillatory shear stress [13] stimulate the production of vascular superoxide [14], which in turn contributed to the processes of arterial remodeling [15]. However, the experimental conditions traditionally used to study shear stress and cyclic stretch have often left these two very important stimuli coupled, or exploited in such a way that is less physiologic, making it difficult to determine their individual contributions to vascular disease. In our study we have performed experiments coupling high and oscillatory shear stress with normal and reduced stretch and studied how shear stress and cyclic stretch, two different mechanical stimuli, modulate remodeling throughout the arterial wall.

Arterial stiffness is strongly affected by endothelial cell signaling [16] and smooth muscle contractile capacity [17]. By limiting the range of arterial adaptability in response to hemodynamic stimuli the artery becomes more susceptible to vascular disease, thus provoking vascular remodeling [18]. To understand the effects of shear stress and cyclic stretch on arterial smooth muscle contractile capacity we performed vascular reactivity studies on tissue segments from our four experimental groups: HSS/5% stretch, HSS/1% stretch, OSC/5% stretch and OSC/1% stretch. We noticed introducing an oscillatory shear had little effect,

while reducing stretch caused significant decreases in smooth muscle contractile capacity for both the HSS and OSC groups. Indicating that on a functional level, reduction of cyclic stretch has a more dramatic effect on smooth muscle contractile capacity than oscillatory flow.

Fluctuations in superoxide ion production increasing vascular levels of ROS are understood to have dramatic effects on vascular health, especially those occurring in the endothelial region [19]. Basal levels of ROS are necessary for vascular cell physiologic function, increases of which have been shown to accelerate and contribute to the signaling required for vascular remodeling [20, 21]. Dramatic increases of ROS have been shown to scavenge vaso-protective molecules such as NO, thus modulating vascular tone [22]. Although ROS production is mediated at many levels, the predominant system producing ROS in vascular cells is recognized as being the NADPH oxidase cascade [23], which is mediated, in part, by the expression of p22-phox [24]. Thus we found it interesting to see how shear stress and cyclic stretch modulate ROS production and the expression of p22-phox in the vascular wall. Upon staining with DHE we found that oscillatory flow up regulated the total formation of superoxide anion as compared to reduced stretch [result accepted in Journal of Vascular Research]. Yet when we focused on just the endothelial region it was found that reduced stretch is a more significant modulator of superoxide than oscillatory flow [result accepted in Journal of Vascular Research]. Upon measuring the intensity of DHE staining versus radius, both reduced stretch and oscillatory shear have similar effects in the expression of DHE and combined they have a significant additive effect. This distribution suggests that increased superoxide is a process that is at least partly mediated by the variation of stress throughout the vascular wall due to change in radius. When looking at the expression of p22-phox vs. arterial wall radius, we see that its expression follows a similar trend, but the shape of the curves indicate that expression is nearly independent of radius. This feature could help explain the sustained increases of superoxide production as radius increases, throughout the arterial wall.

Arterial wall remodeling in response to mechanical stimuli is a process that involves increased cellular proliferation and reorganization of the ECM [25]. MMP-2 is a very important ECM protein which is suspected to play a significant role in arterial matrix reorganization, a process governed by both its expression and activation. Also known to be actively involved in ECM remodeling, as well as cellular migration and proliferation, is fibronectin [26]. Our results showed that total MMP-2 expression was significantly up regulated by oscillatory shear stress for both the normal and reduced stretch groups. Comparing this to total MMP activation, as assessed by gelatinase activity, we observed a similar trend. Yet when we observed gelatinase activation at just the endothelial region, we saw it was significantly modulated by reduced stretch. To further investigate this regional distribution of gelatinase activity we plotted gelatinase activity versus arterial radius and found a trend suggesting the distribution of gelatinase activation is strongly mediated by stress variations due to radius. When the same analysis was performed for fibronectin expression, we found that again, total arterial fibronectin expression was mediated by oscillatory shear stress for both normal and reduced stretch groups. Analysis of just the endothelial region showed that reduced stretch was the significant up-regulator. When plotting fibronectin expression versus radius we observed that in the endothelial region expression is strongly related to the stress distribution in this area, yet as radius increases this relation is slightly inversed.

To conclude, this study demonstrates for the first time in an arterial tissue model the individual contributions of shear stress and cyclic stretch to arterial remodeling while emphasizing how these processes are mediated and distributed through out the vascular wall.

Our results showed that total superoxide production, fibronectin expression and gelatinase activation are predominantly mediated by oscillatory shear stress, while their expression in the endothelial region are mediated by reduced cyclic stretch, correlating well with results from total MMP-2 expression. By plotting intensity versus radius for these markers of vascular remodeling we were able to see that superoxide production and gelatinase activity followed trends indicating their expression was in part mediated by stress distributions through out the vascular wall due to changing radius, while fibronectin and p22-phox were much less or not at all. Most importantly these findings, when coupled with our results from tissue reactive studies, suggest that the arterial remodeling process triggered in the endothelial region due to reduced stretch causes the most significant changes in arterial smooth muscle function. Because perturbed shear stress and reduced arterial compliance have both been implicated in the initiation and progression of vascular disease, this work provides a new perspective into how these stimulus are perceived through out the vascular wall.

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Conclusion

Conclusion

Cardiovascular diseases remain the leading cause of morbidity and mortality in the western world, the most prevalent of these being atherosclerosis. The regular occurrence of atherosclerotic plaques in regions of curvature and branch points, and magnified by ailments such as high blood pressure, suggests hemodynamics, arterial wall mechanics and geometry play significant roles in plaque formation.

The first goal of this thesis was to establish the importance of cyclic arterial stretching for maintaining healthy arterial function. Thus, we performed ex vivo experiments where we reduced the cyclic stretch of the arterial wall and searched for evidence of arterial wall remodeling and endothelial dysfunction. We have shown that a decrease in cyclic stretch leads to changes in SMC phenotypes and reduces their contraction capacity. Furthermore, we found the increases in MMP-2 which associate with decreases of PAI-1 and increases in Ki67, suggesting that the decrease in cyclic stretch may promote wall remodeling, SMC proliferation and migration. We next demonstrated that decreasing cyclic stretch leads to a significant decrease in the functionality of the endothelium which could be caused in part by interrupting eNOS Ser1177 phosphorylation, thus reducing the production of endothelial derived nitric oxide. We also measured significant increases in superoxide anion production and coupled this data with marked increases in p22-phox and p47-phox, both key players in the membrane bound superoxide producing NOX system. These results indicated that reducing an artery's ability to stretch physiologically will significantly contribute to creating a dysfunctional endothelium.

The second goal of this thesis is to develop an experimental model which allows the effects of shear stress and cyclic stretch to be studied individually and see how these two different forces modulate markers of vascular disease differently throughout the vascular wall. We once again performed ex vivo experiments, but this time modulated both shear stress and cyclic stretch, creating four different experimental conditions. Our results indicated that oscillatory flow is a more dominant stimulus than reduced cyclic stretch with regards to down-regulation of eNOS, increased levels of total vascular superoxide anion and nitro-tyrosine regulation, the combination of which can be linked to a decreased in nitric oxide bioavailability. This study also demonstrates the involvement of two NOX components, namely p22-phox and p47-phox, in the production of superoxide anion. But perhaps the most interesting finding from this study was that superoxide anion production in the endothelial region is significantly higher and more significantly modulated by reduced stretch than oscillatory shear. This point suggests that although oscillatory shear was shown to more significantly modulate total ROS production, reduced stretch is the more dangerous risk for endothelial injury. These findings agree with results from tissue reactivity studies where we were able to see significant decreases in endothelial functionality due to both oscillatory flow and reduced stretch. We next demonstrate that total superoxide anion production, fibronectin expression and gelatinase activation are predominantly mediated by oscillatory shear stress, while their expression in the endothelial region is mediated by reduced cyclic stretch, correlating well with results from total MMP-2 expression. By plotting intensity versus radius for these markers of vascular remodeling we are able to see that superoxide production and gelatinase activity follows trends indicating their expression is in part mediated by stress distributions through out the vascular wall due to changing radius, while fibronectin and p22-phox are much less or not at all. Most importantly these findings, when coupled with our results from tissue reactive studies, suggest that the arterial remodeling

process triggered in the endothelial region due to reduced stretch causes the most significant changes in arterial smooth muscle function.

To conclude, we have shown that cyclic stretching due to the pulsatile nature of blood flow is an essential stimulus regulating arterial remodeling and endothelial viability. We have performed experiments allowing for the autonomous effects of cyclic stretch and shear stress to be studied. Most importantly we have shown how cyclic stretch and shear stress stimulate the vascular wall in different regions and compared this data to arterial functionality studies. These results indicate that although reduced cyclic stretch may not stimulate the total expression of certain markers of vascular disease as much as an OSC shear stress, it does so in specific regions of the vascular wall which can, in fact, have a more detrimental effect on arterial function. As reduced arterial cyclic stretching is associated with the aging process, our work gives insight into the progression of vascular disease over the course of a person's life time.

Future perspectives

The experimental design described in chapters 3 and 4 of this thesis offers the unique capability of separating the effects of reduced cyclic circumferential stretch and perturbed shear stress with regards to the initiation and progression and development of arterial disease. Future work developing the experimental model could also incorporate modulation of pressure, therefore completely describing the individual components of the primary dynamic mechanical forces stimulating the vascular wall. By understanding how the individual effects of different mechanical forces affect the expression of various markers of vascular disease we will better understand which areas to focus on with regards to the development of therapeutic treatments.

Further work regarding this model should be focused on linking the expression of more specific markers of vascular disease to endothelial and smooth muscle cell dysfunction, with the hope of determining which have the most profound effects and under which conditions. Elements which are known to upset NO bioavailability, such as eNOS coupling, phosphorylation (of residues other than Ser1177), and the arginase pathway combined with the expression of different NOX components (other than p22 and p47-phox) would be interesting to study in relation to our model. Determining the link between inflammatory elements, such as NF-κB and the production of vascular NOX derived ROS is another interesting avenue. Perhaps by further understanding how the individual components of NF-κB and NOX interact it would be possible to regulate their expression within non threatening levels.

Future applications of our perfusion model could be to further understand how arterial hemodynamics, affect venous remodeling and progression of disease. Currently, treatment for renal failure involves implementing a hemodialysis access point where a vein and an artery are connected using a synthetic graft. Most often, the cause of failure of such a graft is due to occlusion at the vein graft junction. If a study similar to the one I have just undertaken was to be performed using venous segments, and we were able to learn more about how to control vascular remodeling and plaque formation of veins exposed to arterial hemodynamic conditions, we might be able to formulate a therapeutic strategy, effectively convincing the vein to become more arterial and better able to accommodate the induction of arterial blood flow. Presently, solutions using degradable hydro gel technology

pioneered by leaders in the biomaterials domain, such as Prof. J. Hubbell, have been proposed. Imagine surrounding the affected vein site with a hydro gel, thus providing mechanical support to the venous wall, while imbedding the hydro gel with co-factors simultaneously stimulating the remodeling, proliferative and phenotypic changes necessary for the venous wall to become more arterial.

But perhaps we find that we are far away from controlling pathological development of the vascular wall, venous or arterial, and that eliminating the initiation of certain aspects of vascular disease and plaque development is something which is currently therapeutically unrealistic. If this is the case I think the most promising area of development in our field involves plaque rupture and the distinction between vulnerable vs. invulnerable plaque. We know that vascular tissue is a wonderfully adaptive medium, and perhaps if we could find a way to stabilize plaques, while allowing the vascular wall to remodel and accommodate sufficient blood flow we could have a therapeutic strategy able to be implemented with the tools and knowledge currently available.

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Acknowledgments

When I made the decision to begin a PhD at the EPFL I had no idea how profoundly that would shape my life. It was a big deal for me at that time; leaving family, friends and a life which I knew so well to begin something unknown to me in a foreign place. But the best part about it is that if I were able to go back and make that decision again, I wouldn't change a thing. Coming out to Switzerland was one of the best decisions I've ever made. Life is good here in Lausanne, and regarding places one could be a PhD student it doesn't get much better than the EPFL. But the point is, without the help and support of key individuals not only would my academic experience here been less fruitful, it also wouldn't have been any fun. For this I need to thank all of you.

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Curriculum Vitae

Tyler Thacher
Nationality: American
Marital status: Single
Born Dec. 8th 1980

Chemin des Cedres 9
1004 Lausanne, CH
tyler@tntarch.com
tel. +41.78.865.63.90

Objective To obtain a position in industry managing clinical research, product engineering and development

Work history

Mar 2005 – present **École Polytechnique Fédéral de Lausanne (EPFL)**
Laboratory of Hemodynamics and Cardiovascular Technology
1015 Lausanne, Switzerland

I am near completion of a PhD in the Biotechnology and Bioengineering program under the supervision of Prof. Nikos Stergiopulos. My work is focused on how circumferential stretch in major arteries influences the initiation and development of atherosclerosis.

Jun 2003 – Jan 2005 **Laboratory Technician**
Lab of Dean Matt Tirrell
Materials Research Laboratory, UCSB
Santa Barbara, CA, USA

I designed peptide amphiphiles for research group members. My work allowed our lab greater freedom in peptide design, while lowering costs and increasing productivity.

Sep 2002 – Jun 2003 **RISE Internship (funded by the National Science Foundation)**
Lab of Dean Matt Tirrell
Materials Research Laboratory, UCSB
Santa Barbara, CA, USA

My work yielded information regarding the most efficient method to produce mono-disperse polystyrene colloids, used to then functionalize with peptide amphiphiles for bioactivity studies.

Education

Sep 1998 – Jun 2003 **University of California at Santa Barbara (UCSB)**
Santa Barbara 93106, CA, USA

Bachelor of Chemical Engineering

Post-grad courses (Science and Engineering, EPFL)
Biomaterials, Biophysics of the Cytoskeleton, Biomicroscopy, Hemo/Biomechanics of the Cardiovascular System, Vascular Biology, Cardiovascular Drug Delivery, Rodent cardiovascular phenotyping

Post Grad courses (Management of Technology Doctoral school, EPFL)
Topics in Economics of Innovation in Life Science and Technology
The Organization Design for Innovation, Technology and Product Development

Post Grad courses (Swiss School of Public Health, UNIL/CHUV)
Formation de base pour investigateurs cliniques: introduction aux méthodes de recherche clinique (Basic training for clinical investigators: introduction to clinical studies/trials)

Teaching
Directed two students: one from TUE (Holland) and one from EPFL in projects for their master's theses EPFL.
I am also an assistant for the 3rd year level BioFluid mechanics course at the EPFL

Guest lecturer in "Engineering as a profession" at Cabrillo College, Aptos CA. A course meant to inspire College and High school students to pursue engineering

Skills
- General laboratory apparatus' and procedures, Solid Phase Peptide Synthesis
- Computer skills: Photoshop, Matlab, Adobe Premiere, Metamorph, Hysis, Distil, Microsoft Office

• Microscope training: Leica TCS-SP2 Confocal invert/upright, Leica DM5500 moving stage, Leica DMI 4000
• Scientific writing: English corrections for 9 manuscripts produced in the l'Hôpital ophtalmique Jules-Gonin

Awards
1st place, Young investigator award at the Biomechanics in Vascular Biology and Cardiovascular Disease meeting 2009

Oral/Podium presentations
World Congress of Biomechanics (WCBM): Munich, Germany, July 29th - August 4th, 2006.
Biomedical Engineering Society (BMES): Chicago, Illinois, USA, Oct 11th - 14th, 2006.
The 2nd Symposium on Biomechanics in Cardiovascular Disease: Shear Stress in Vascular Biology: Rotterdam, Netherlands, April 19th – 20th, 2007.
4th European Meeting on Vascular Biology and Medicine: University of Bristol, United Kingdom, September 17th-20th, 2007.
13th Cardiovascular Biology and Clinical Implications Meeting: Muntelier, Switzerland, October 4th -5th, 2007.
European Society of Biomechanics: Lucerne, Switzerland, July 6th – 9th, 2008
Bioengineering 08: Imperial College, London, United Kingdom, September 18th – 19th, 2008
Lunchtime seminar series: EPFL, Lausanne, Switzerland, December 18th, 2008
3rd Switzerland-Japan Workshop on Biomechanics 2009: Engelberg, Switzerland, Sep 1st-4th, 2009

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Languages English (mother-tongue) and French (conversational)

Hobbies Surfing, snowboarding, swimming, volleyball, ceramic art

References

Matthew Tirrell
Professor and Chair, Department of Bioengineering
University of California, Berkeley
mvtirrell@berkeley.edu
ph: 1-510-642-5833

Nikos Stergiopulos
Professor of Bioengineering and Biotechnology
Swiss Federal Institute of Technology Lausanne (EPFL)
nikolaos.stergiopulos@epfl.ch
ph: 41-21-693-8658