THE EFFECT OF HANDGRIP EXERCISE DUTY CYCLE ON BRACHIAL ARTERY FLOW MEDIATED DILATION

by

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Lay Abstract

The endothelium is the inner layer of cells that lines all arteries. A healthy endothelium protects the arteries against atherosclerosis (the buildup of fatty plaque in the arteries), and other cardiovascular diseases, by releasing several vasoprotective and vasodilatory (artery widening) substances. Shear stress, which is the frictional force of blood flow on endothelium, is the stimulus for the release of vasodilators. Dilation in response to increased shear stress is termed flow mediated dilation (FMD). FMD can be exploited to assess endothelial health non-invasively; with greater health corresponding to a greater magnitude of FMD in response to a given shear stress stimulus. Shear stress in the large artery of the upper arm (brachial artery) can be increased by rhythmic handgrip exercise, and the resultant FMD can be observed via echo ultrasound imaging. Handgrip exercise is a relatively new method of eliciting increases in shear stress for FMD assessment, and as such there is no standard pattern of relaxation and contraction durations (duty cycle) in use. Duty cycle may be important because it influences the shear stress pattern via blood flow inhibition during contraction and enhancement during relaxation. Previous work has shown that the pattern of shear stress may impact the FMD magnitude. The purpose of this thesis was to determine whether different duty cycles, which create the same mean shear stress, elicit a similar magnitude of FMD. The primary finding of the thesis was that there was no impact of duty cycle on FMD magnitude, and experimenters are free to use whichever duty cycle they are comfortable with. This will help to expand our understanding of FMD and will be used to refine the methods of testing cardiovascular health. These methods may be used in the future to contribute to the development of interventions to slow the development and progression of cardiovascular disease.

Technical Abstract

Shear stress is the frictional force exerted on the vascular wall by blood flowing through an artery. It is a major regulator of endothelial cell function, which is essential for vasoprotection and local regulation of vascular tone. Using handgrip exercise (HGEX) to increase shear stress is an increasingly popular method for assessing brachial artery (BA) endothelial cell function via flow-mediated dilation (FMD, dilation which increases with improved endothelial function). However, different exercise duty-cycles (ratio of handgrip relaxation to contraction in seconds) produce different patterns of BA shear stress with different anterograde and retrograde flow magnitudes. PURPOSE: To determine the impact of HGEX duty-cycle on BA %FMD while maintaining a constant mean shear stress. METHODS: N=16 healthy males. BA diameter (BAD) and blood velocity (BV) were assessed via echo and Doppler ultrasound. Shear stress was estimated as shear rate (SR=BV/BAD) and reported as mean SR during the last minute of baseline (target 10 s^{-1}) and each minute of HGEX (75 s^{-1}). Subjects performed 3 six minute HGEX trials on each of 2 separate days (like trials averaged). Each trial was one of 3 randomly ordered HGEX duty-cycles (1:1, 3:1, 5:1). %FMD was calculated as the increase in BAD from baseline to the end of HGEX and at each minute (subset N=10) during HGEX. RESULTS: Data are means ± SD. As intended, mean SR was similar between duty-cycles (main effect, p=0.835), despite significant differences in anterograde and retrograde SR (P<0.001). There was no impact of duty cycle on blood pressure (p=0.188) or heart rate (p=0.131) responses. End exercise %FMD (4.0 ± 1.3%, 4.1 ± 2.2%, 4.2 ± 1.4%, p=0.860) and minute by minute %FMD (main effect p=0.939; interaction, p=0.545) were also not different between duty-cycles. CONCLUSION: Distinct HGEX duty-cycles create markedly different shear stress patterns in the BA. However, duty cycle had no impact on %FMD magnitude suggesting that mean shear stress is the most important stimulus for FMD in the BA. Using a 5:1 duty cycle may yield the best vessel image and diameter measurement quality due to the long period of arm stability between contractions.

Co-Authorship

Trevor King was responsible for reviewing the relevant literature related to the study, determining the knowledge gap, and designing the study with feedback from Dr. Kyra Pyke. All data collection analysis was conducted by Trevor King with feedback from Dr. Pyke. The introduction, literature review, manuscript, and general discussion were written by Trevor King with feedback from Dr. Pyke. Dr. Pyke was the principal investigator on the grant that funded this research, and was therefore involved in the study design and organization of the project. Dr. Pyke also provided Trevor with training on data collection and analysis.

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List of Abbreviations

AC - Arterial compression

Ach - Acetylcholine

AUC - Area under the curve

BA – Brachial artery

BAD – Brachial artery diameter

BMI – Body mass index

BV – Blood velocity

Ca^{2+} - Calcium ions

cAMP - cyclic adenosine monophosphate

cGMP - Guanosine-3',5'-cyclic monophosphate

cGMP –PK - cGMP protein kinase

Cl^- - chloride

CVD - Cardiovascular disease

CVSRL - Cardiovascular Stress Response Lab

EDHF – Endothelial derived hyperpolarizing factor

EDRF - Endothelium derived relaxing factor

eNOS - Endothelial nitric oxide synthase

FMD - Flow mediated dilation

$G_{\alpha q/11}$ - Heterotrimeric G protein subunits alpha q and 11

HGEX – Handgrip exercise

HR – heart rate

K^+ - Potassium

K_{Ca} - Calcium dependent potassium channels

MAP – mean arterial pressure

MVC – Maximal voluntary contraction

NO – Nitric oxide

PG – prostaglandins

RH – Reactive hyperemia

ROS - Reactive oxygen species

SNA - Sympathetic nervous activity

SR – Shear rate

T1D - Type 1 diabetes

TC – total cholesterol

TD - Time delay

TG - Triglycerides

Chapter 1

Introduction

Although the mortality rate has declined in recent years, cardiovascular disease remains the leading cause of death in North America (126). A risk factor which is gaining usage and utility as a clinical research tool and an early warning sign is the assessment of endothelial dysfunction(153; 154). The endothelium is a single layer of cells located on the inside of vessel walls and is in direct contact with the blood. A properly functioning endothelium is essential for vasoprotection (43) and local regulation of vascular tone (37), which is due at least in part to the actions of nitric oxide (NO) (43; 108). Endothelial dysfunction is associated with the loss of that vasoprotection (55), and may play a role in the initiation of atherosclerosis (19; 37).

In response to an increase in blood flow, the endothelium acts by releasing several vasoactive substances, including NO, which results in vasodilation. An increase in blood flow leads to an increase in shear stress, which is the tangential force exerted on a vessel wall by blood flowing through an artery. It is a major regulatory force in the vasculature both acutely and chronically and is known to influence both vascular structure (30) (artery wall thickness, lumen area and vessel size) and endothelial cell function (22; 23; 98; 123; 142; 143). The endothelial dependent vasodilation stimulated by an increase in shear stress is termed flow mediated dilation (FMD). Endothelial function is usually assessed by measuring FMD in response to an experimenter imposed increase in shear stress. Endothelial dysfunction, in addition to resulting in a reduction in its vasoprotective actions, is associated with a reduced FMD response (less dilation), allowing us to use FMD in the assessment of endothelial dysfunction. Several acute and chronic lifestyle factors influence endothelial function (eg. cigarette smoking (16), caffeine

(29), alcohol (2), high fat meals (39), mental stress (44), exercise training (21)). FMD is often impaired in populations with risk factors for cardiovascular disease (15), is a predictor of all cause mortality in those with existing cardiovascular disease (72), and is a predictor of cardiovascular events in healthy subjects (153; 154).

In order to assess FMD, a shear stress stimulus must be created in the conduit artery of interest. Reactive hyperemia (RH) has been used extensively in past research as the standard method for transiently increasing shear stress in order to assess FMD. This is accomplished by temporary limb occlusion (primarily 5 minutes) and release which elicits an immediate, transient increase in conduit artery (usually the brachial artery) blood flow and stimulates an FMD response. However, interpretation of the significance of the magnitude of FMD may be complicated by the uncontrolled nature of the RH stimulus. For example, smaller vessels tend to experience a larger shear stress stimulus and thus produce a larger FMD response (116). The larger FMD response could therefore be falsely interpreted as indicative of superior endothelial function/artery health vs. that of larger arteries. This has led some researchers to look for alternative methods of assessing FMD.

Utilizing handgrip exercise (HGEX) or arm/ hand skin heating to induce prolonged increases in conduit artery shear stress has become an increasingly popular method for assessing FMD (104; 119; 134). Exercise is how shear stress is increased in daily life, making it a physiologically relevant method for FMD assessment. However, FMD resulting from RH and HGEX may arise from distinct endothelial dilator mechanisms. Some evidence suggests that HGEX produces FMD through NO independent mechanisms (96), whereas other research suggests that this dilation is NO dependent (151). Early FMD research focused on NO as the primary mechanism for the endothelium's vasoprotective properties (28; 43; 68) This has lead to

doubts regarding the clinical utility of HGEX FMD responses. In support of HGEX, evidence exists that exercise induced FMD is impaired in older healthy subjects, and subjects with type 1 diabetes (51). Similar impairments in FMD assessed via skin heating, which also creates a prolonged increase in shear stress, have been observed in subjects with type 1 diabetes as compared to controls (10). Therefore, although the bulk of the evidence for the clinical relevance of FMD stems from research using RH, this research suggests that FMD resulting from prolonged increases in shear stress may also be clinically relevant. Due to this, researchers will likely continue to investigate the utility of HGEX FMD results for the assessment of vascular health in a research setting.

An additional benefit of HGEX is its ability to create a sustained shear stress stimulus. This sustained stimulus allows for the assessment of the resultant time course of brachial artery vasodilation. The time course of dilation refers to the time to onset, speed, and number of phases of dilation resulting from the shear stress stimulus. Dilation that follows a similar time course is likely to have been initiated by similar vasodilatory mechanisms. Previous research suggests that the human brachial artery experiences 2 or 3 phases of dilation when exposed to prolonged bouts of shear stress (117). The mechanisms behind each phase of dilation likely differ (36; 96; 133), and animal studies suggest that these mechanisms are also vessel and bed specific (7; 36; 133).

The development of the HGEX technique for FMD assessment has involved contributions from several groups (104; 118; 134; 151). As such, there are no set norms for performing HGEX in order to elevate shear stress and quantify the resultant FMD, and several protocols have been reported to date (67; 104; 119; 150; 151). Handgrip exercise is characterized by periods of relaxation, during which shear stress in the conduit artery that feeds the active muscle (where FMD is measured) is relatively high, followed by periods of contraction, during

which shear stress is reduced to close to zero. Another feature of the shear stress pattern is that at the onset of each contraction, a brief period of retrograde (reverse) shear stress is created. Thus, when different studies use different duty cycles (ratio of contraction : relaxation) to elevate shear stress for FMD assessment (52; 67; 118; 150; 151), different patterns of shear stress are created. The endothelium's response (ie. Vasodilatory mechanisms, magnitude of dilation, and rate of dilation) may be specific to the nature of the shear stress stimulus (ie. magnitude, rate of increase, pattern, or duration of the stimulus). Different patterns of shear stress have previously been shown to alter the magnitude of FMD (140; 142), and retrograde shear stress has been associated with proatherogenic effects in vivo (140) and in vitro (156). In contrast, other data (119) has indicated that when the brachial artery is exposed to handgrip exercise induced fluctuations in shear stress, it is the mean shear stress stimulus that determines the FMD response magnitude. This study (119) more accurately represents the magnitude of shear stress fluctuations that occur during HGEX, however it did not assess the effects of different patterns of HGEX or any differences in retrograde shear stress that are associated with different patterns.

It therefore remains unclear whether different patterns of shear stress which result from different HGEX ratios of relaxation and contraction (duty cycles) have an important impact on the FMD response. This is important for researchers who intend to continue studying HGEX induced FMD in order to develop repeatable and clinically relevant protocols. Further analyses on different duty cycles and patterns of shear stress and their effect on FMD are required in order to compare the results of previous research studies using HGEX FMD.

With this as a background, the specific objectives and hypotheses of this thesis were as follows:

Specific Objectives

To determine whether different duty cycles of forearm contraction to relaxation, while maintaining mean shear stress, elicit different FMD responses.

Specific Hypothesis

When the same mean shear stress is achieved in all duty cycles there will be no significant differences in the FMD response that they elicit.

The following chapter is a literature review which provides background information about the endothelium, the acute effect of shear stress on endothelial function, and HGEX FMD assessment. This is followed by chapter 3 which details the experiment performed to address the influence of different duty cycles on FMD. The last chapter provides a general discussion of the utility of FMD, endothelial function and the future direction of FMD assessment.

Chapter 2

Literature Review

Background

Shear stress is the tangential force exerted on a vessel wall by blood flowing through an artery. An increase in luminal blood flow and thus internal wall shear stress results in the vasodilation of an artery, a phenomenon which is termed flow mediated dilation (FMD). In a groundbreaking study, Furchgott and Zawadzki (37) established that the single layer of endothelial cells (cumulatively called the endothelium) which lines the inside of the vessel walls produce a vasodilator, termed endothelium derived relaxing factor (EDRF). Later animal studies determined that FMD was dependent upon the presence of the endothelium (114; 136). Further studies deduced that the EDRF released in response to blood flow was nitric oxide (NO) (95; 129). NO is a bioactive substance which has many vasoprotective (combats atherosclerosis) properties (77; 94). The release of NO and other vasodilators from the endothelium also results in FMD. Impaired FMD has been implicated as a predictor of cardiovascular events in those with cardiovascular disease, independent of traditional risk factors (19; 74; 112). In asymptomatic individuals there is a modest association of FMD with cardiovascular disease risk which is at least as predictive as traditional risk factors (62).

This review will focus on 1) the mechanistic pathway by which increases in shear stress are converted to arterial dilation, 2) currently utilized flow mediated dilation (FMD) tests and the utility of handgrip exercise for the creation of shear stress and assessment of FMD, 3) different

patterns of shear stress and their influence on endothelial function, and 4) the assessment of FMD onset dynamics.

Conversion of shear stress to chemical signals

There are likely multiple pathways by which endothelial cells sense shear stress and convert it to a chemical signal (shear stress transduction), resulting in the release of vasodilators. The type of shear stress impacts the signal transduction pathway; for example, laminar and oscillatory shear stress stimulate different pathways to determine which vasoactive substances are acutely released. The following are involved in pathways of shear stress transduction.

Ion channels

Flow responsive ion channels participate in sensing shear stress. With an increase in shear stress, there is an activation of inward rectifying potassium (K^+) channels (figure 1). This flux leads to the activation of outward rectifying chloride (Cl^-) channels. This flux initiates transmembrane hyperpolarization and drives calcium ions (Ca^{2+}) into the cell (86), the magnitude of this flux is regulated by non-selective cation channels (145). Ca^{2+} enters through two ion channels: P2X purinoreceptors and transient receptor potential channels (75; 152). The influx of Ca^{2+} into the endothelial cells leads to the activation of signaling pathways that end in the release of vasodilators. NO, which is a regulator of vascular tone, FMD, and vascular remodeling, is generated due to Ca^{2+} induced release of endothelial nitric oxide synthase (eNOS) (99) from the calveolae (small invaginations in the membrane) (124). When phosphorylated, eNOS is activated, and produces NO (99). Levels of intracellular Ca^{2+} are also important to the synthesis

of vasodilating EDHF (61). Both extracellular (entering from outside) and intracellular (released from sarcoplasmic reticulum) Ca^{2+} are important in liberating arachidonic acid in shear induced prostacyclin PGI_2 production (12).

There are 3 theories as to how shear stress activates these flow sensitive ion channels. First, the physical interaction between blood flow and the ion channels may push open the channels due to the drag force. However, mathematical calculations have shown that the energy required to achieve this is greater than that experienced in vivo (9). A second theory involves ion channel interaction with the cyctoskeleton. It is suggested that shear stress changes the mechanical tension and ionic current of the cytoskeleton, allowing ion channel activation (100). The prevailing theory is that of cell membrane fluidity. As blood moves across the cell surface, it alters the viscosity of the lipid bilayer, leading to the activation of G-proteins in the cell membrane (54), which transduce shear stress to ion channels. In summary, the mechanisms underlying ion channels sensing shear stress are incompletely understood however flow responsive ion channels likely play a major role in the release of vasodilators.

Cell membrane components

The plasma membrane is internally lined with small invaginations called caveolae. Caveolae are composed of scaffolding proteins and participate in receptor mediated endocytosis. They work in conjunction with Ca^{2+} signaling (65) by providing compartmentalization of eNOS. In response to an increase in Ca^{2+}, caveolae-bound eNOS is released into the cytoplasm allowing eNOS to become phosphorylated and subsequently produce NO (64). The interrelationship between calveolae and shear stress induced Ca^{2+} concentration is highlighted by the fact that flow

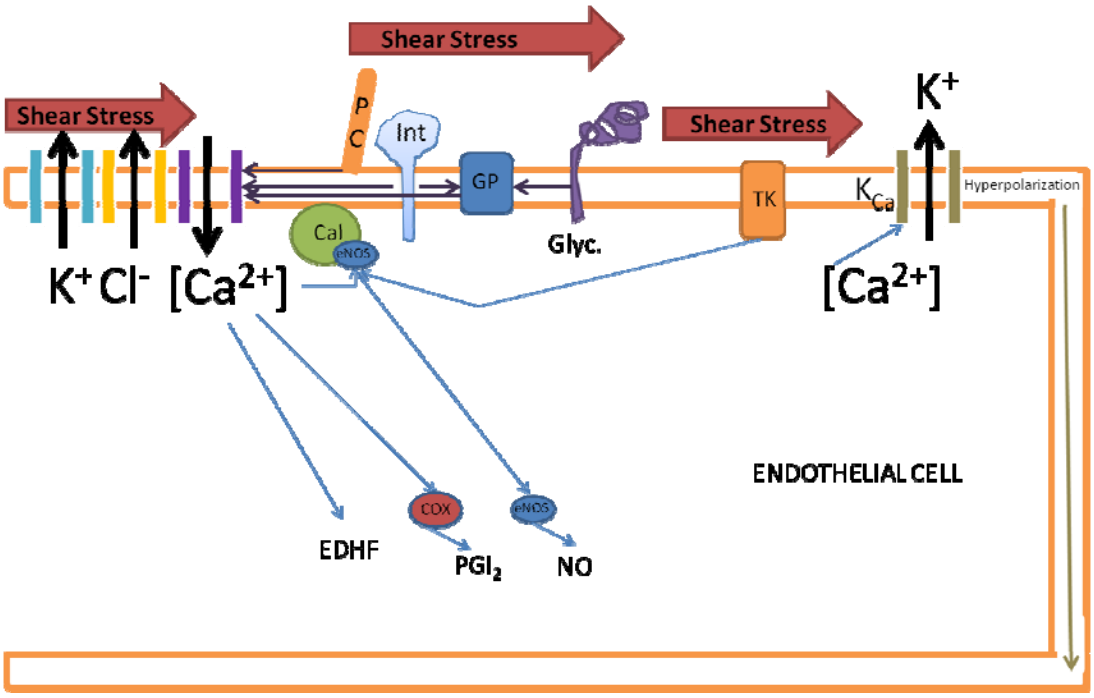


Figure 2-1. Schematic of mechanotransduction of shear stress using ion channels on an endothelial cell. Shear stress activates shear sensitive ion channels (K^+ outflow, Cl^- outflow and activation of Ca^{2+} channels). PC, Int, and GP also sense shear stress, and transmit it to the cytoskeleton, leading to the activation of Ca^{2+} channels. Glycocalyx sense shear stress and activate GP. These steps lead to an increase in Ca^{2+} concentration inside the endothelial cell. Ca^{2+} directs the release of eNOS from the calveolae and the synthesis of EDHF and activation of COX. Ca^{2+} also activates Ca^{2+} dependent K^+ channels, leading to membrane hyperpolarization which is transmitted through gap junctions to the smooth muscle. COX and eNOS synthesize PGI_2 and NO, which along with EDHF move to the smooth muscle and participate in smooth muscle relaxation. NO, Nitric Oxide; EDHF, Endodthelium-derived hyperpolarizing factors; PGI_2, prostacyclin; Ca^{2+}, Calcium ions; K^+, Potassium ions; Cl^-, chloride ions; PC, primary cilia; Int, integrans; GP, G-proteins; TK, Tyrosine kinase; Cal, calveolae; eNOS, endothelial nitric oxide synthase; Glyc, glycocalyx; K_{Ca}, calcium dependent potassium channels; COX, cyclooxygenase.

induced Ca^{2+} responses begin at the calveolae (64), and also that prolonged (1 hour) exposure to shear stress increased calveolae concentration threefold (109).

G-proteins are small GTPases located in the cell membrane that act downstream of Integrins (cell membrane receptors involved in cell adhesion), and are involved in cytoskeletal organization and changes in gene expression due to shear stress (144). G-proteins are also involved in the immediate increase in NO production that occurs following an increase in shear stress (78), and G-protein activation begins one second following flow onset (53). G-protein activation results in an increase in intracellular Ca^{2+} (88), leading to the release of eNOS from the calveolae and synthesis of EDHF (61) and PGI_2 (via cyclooxygenase) (12). G-protein coupled receptors may also independently mechanotransduce shear stress. Shear stress activates bradykinin B2 G-protein coupled receptors via cell membrane fluidity changes, leading to the mechanotransduction of shear stress (18). Ras GTPase is activated through G-proteins, and this leads to the transcription of eNOS (144). Li *et al* (84) observed that cultured endothelial cells increased G protein (GTPase Ras) activation during only the first few minutes of shear stress exposure, indicating that it may exert its transcriptional influence during the first few minutes of the signaling pathway, and vasodilational influence within seconds.

Tyrosine kinase receptors are involved in the control of many protein cascades. In endothelial cells, tyrosine kinase receptors are activated by shear stress through phosphorylation (82). These cascades involve Activation of PI3-kinase and Akt signal transduction pathways resulting in eNOS phosphorylation and subsequent NO production (26).

Extracellular components

Cell adhesion molecules participate in the sensing of shear stress. Integrins (a cell adhesion molecule) and platelet cell adhesion molecules are activated by shear stress and lead to the initiation of the Ras ERK transduction pathway (102) which begins after 1 minute (101) and lasts for about 4 minutes of shear stress (83). The Ras ERK pathway is involved in immediate early gene expression via transcription of eNOS (24). Integrans may also transmit shear stress to the cytoskeleton (83) which activates ion channels.

The Glycocalyx is a network of glycoslated transmembrane proteins which protrude outward, and are coiled when experiencing no flow (135). In response to an increase in shear stress, they uncoil and stretch in the direction of flow (135). The conformational change creates forces which are propagated across the membrane, stimulating the cytoskeleton within the endothelial cell. This leads to an increase in Na^+ binding sites (135), and activation of ion channels and G-proteins (113). These changes lead to the initiation of several signaling pathways (113; 135).

Primary cilia, which also protrude out from the endothelial cell membrane, bend in response to shear stress, allowing for the transmission of shear stress to dilation (58). This bending allows for an influx of Ca^{2+} (1; 58), leading to Ca^{2+} signaling pathways (1). Primary cilia are thought to be involved in the sensing of shear stress during low flow states, due to their high density in areas of oscillatory flow (97). Also, it has been observed that during periods of high shear stress primary cilia disassemble (63).

Irregular patterns of shear stress

Retrograde shear stress is a period of reverse blood flow that moves towards the heart, and oscillatory shear stress is the alternation of retrograde and anterograde (forward) shear stress. Although much is known about the consequences of sustained retrograde and oscillatory shear stress (increase in expression of atherogenic genes (137; 156), increase in oxidative stress (148) (see '*Shear pattern and Endothelial function' section*), the specific mechanisms by which distinct shear stress patterns are differentiated and transduced by the endothelium to produce distinct responses is a newer area of research. In vitro, flow adapted endothelial cells display sudden transient increases in intracellular Ca^{2+} concentration which peak after about 25 seconds of flow reversal (90). Melchior and Frangos (90) investigated a G-protein pathway (heterotrimeric G protein subunits alpha q and 11 ($G\alpha_{q/11}$) in retrograde flow induced Ca^{2+} responses. Inhibition of $G\alpha_{q/11}$ delayed the Ca^{2+} response by 10 seconds, but did not alter the magnitude. Inhibition of IP3 (a downstream receptor of $G\alpha_{q/11}$ which causes the release of intracellular Ca^{2+} stores) completely attenuated the Ca^{2+} response, indicating that the Ca^{2+} increase in response to retrograde flow occurs through release of intracellular calcium. This suggests an adaptive pathway in which the endothelial cells respond to the acute presence of retrograde flow by releasing Ca^{2+} which may result in the release of various vasodilators.

Oscillatory shear stress may differentially affect endothelial cell function. A study by Helmlinger *et al* (56) observed that while steady anterograde and retrograde shear caused an increase in intracellular Ca^{2+} concentration, oscillatory shear did not. The frequency of oscillatory shear stress may also be important for endothelial cell function and gene expression. In vitro oscillatory shear stress administered at the frequency of 0.2 and 1 Hz results in fully activated K^+ channels, but only minimally activated Cl^- channels and a fully hyperpolarized, then

minimally depolarized membrane (85). In addition, a greater frequency of 5 Hz does not activate either K^+ of Cl^- channels, suggesting that high frequencies with little net directional flow may be too fast to endothelial cell ion channels to detect (9) (resulting in no influx on Ca^{2+}(56)). Using a similar method, Gautam *et al* (41) exposed bovine endothelial cells to 1 Hz of oscillatory shear stress, and Cl^- channels were not activated. However, the Cl^- channels were partially activated by oscillatory shear stress at lower frequencies, and fully activated (to the level achieved by steady flow) by non-reversing pulsatile shear stress. These results together suggest that flow sensitive ion channels (which provide a rapid response to changes in shear stress) contribute to the differentiation of different types of shear stress by the endothelium.

Summary

Several mechanisms are suggested to be involved in the sensing of shear stress and its conversion into a chemical signal. Shear sensitive ion channels which result in membrane hyperpolarization and the influx of Ca^{2+} may be the first response to increases in shear stress and the presence of irregular flow. When stimulated by shear stress, other cell membrane and extracellular components also contribute to the production and release of vasodilators (NO, prostaglandins, EDHF). Much of the eNOS present in the cells is in bound in caveolae, and its release results in the production of NO. In addition to the acute impacts that were discussed, shear stress alters the expression of approximately 3000 endothelial genes (60), indicating that the complexities of all pathways initiated by shear exposure remain incompletely elucidated.

From vasodilators to vasodilation

As described in the previous section, the conversion of shear stress into a chemical signal results in the production of several vasodilators, among which are NO, EDHF, and prostoglandins (PGI_2, PGH_2). NO is a small, neutral, relatively hydrophobic nonelectrolyte in an aqueous solution (95). EDHF is not a single molecule but a group of molecules that includes epoxyeicosatrienoic acids, hydrogen peroxide, carbon monoxide, hydrogen sulfide, and C-natriuretic peptide (45). Prostaglandins are lipid compounds derived from fatty acids. The vasodilators which are produced may depend on the nature of the shear stress stimulus (ie. duration (96), magnitude (96; 151) or direction (56; 90) of shear stress), and the artery in which vasodilation occurs (115). Although the literature is scarce in human arteries, the prevailing theory that the initiation of a step increase in shear stress is NO dependent; whereas once the stimulus is prolonged, the contribution of NO declines (96; 120). However, these results may not carry over to all conduit arteries (115; 151) or subjects(110). Once vasodilators are produced, they must diffuse from the endothelial cell to the smooth muscle cell. The properties of NO and Prostaglandins allow their diffusion from the endothelial cells to the smooth muscle cells through the membrane (45; 95). EDHF may hyperpolarize the smooth muscle through gap junctions which bridge the endothelial cells and smooth muscle cells (27).

The distance that NO and other vasodilators can diffuse is a function of the vessel diameter, the hemoglobin present, and the amount of oxidative stress experienced (146). Oxidative stress is a term used to describe excess exposure to reactive oxygen species (ROS). Local degradation of NO occurs when ROS interact with NO to form peroxynitrite. In vitro, it has been shown that exposure to endogenous or exogenous O_2 reduces endothelium-dependent

dilation to Ach (50; 71). There is an exhaustive amount of in vivo human evidence suggesting that oxidative stress induced destruction of NO contributes to endothelial dysfunction (33; 103; 130). In addition to ROS, NO also reacts with deoxy- and oxyhemoglobin to form nitrosylhemoglobin, methohemoglobin and nitrate (146), reducing the concentration of the vasodilator that reaches the smooth muscle.

As vasodilators reach and diffuse into the smooth muscle, they cause vasorelaxation through decreasing Ca^{2+} concentration. NO stimulates guanylate cyclase, which leads to the creation of Guanosine-3',5'-cyclic monophosphate (cGMP) (125). cGMP protein kinase (cGMP-PK) has been show in vitro to upregulate calcium dependent potassium channels (K_{Ca}) 2 fold, and in the presence of ATP, cGMP directly upregulates K_{Ca} channels 8 fold (125). In addition to this, there evidence that both NO and EDHF directly activate K_{Ca} channels in aortic smooth muscle (13; 27). EDHF exert this action by hyperpolarizing the smooth muscle cell membrane (32). Hyperpolarization and the activation of K_{Ca} channels inhibit voltage gated Ca^{2+} channels, reducing the concentration of Ca^{2+} in the smooth muscle cell (32), leading to relaxation of the muscle and vasodilation of the artery (13). Studies on rat aortas indicate that PGI_2 asserts its vasodilatory action through a PGI_2-PGE_1 receptor (149), leading to the generation of cyclic adenosine monophosphate (cAMP) (5; 79), activation of K_{Ca} channels and decreasing intracellular Ca^{2+} (3; 8).

The change in artery diameter in response to the increase in shear stress is termed flow mediated dilation (FMD). FMD is usually measured as the percent change in diameter from a low shear stress baseline to the maximal dilation (138), or dilation at the end of the shear stress stimulus (119). In humans, Echo ultrasound is used to non-invasively measure the diameter changes that occur by obtaining an image of a conduit artery. While we use change in diameter

as an index of endothelial function, structural factors (and levels of oxidative stress) can influence the degree to which the production of vasodilators and level of smooth muscle relaxation translate into the actual change in diameter (138). Lind (77) examined arterial stiffness and FMD in young and old patients. It was determined that in elderly patients, a stiffer artery (more collagen, less elastin, larger wall thickness) was indicative of a lower FMD. This suggests that the natural occurrence of stiffer arteries among the elderly leads to a reduction in FMD

In summary, the endothelium releases several vasodilators, including NO, EDHF (which is not a single molecule, but a group of vasodilators), and prostaglandins (PGI_2, PGH_2, etc.). These vasodilators diffuse from the endothelium into the smooth muscle, where they initiate pathways which lead to a reduction in Ca^{2+} concentration and relaxation of the muscle. Vessel wall structural factors and oxidative stress interact with the functioning of the endothelium and smooth muscle cells to influence the magnitude of dilation that occurs.

Shear pattern and endothelial function

The functioning of the endothelium is essential for preventing cardiovascular disease. Oscillatory shear stress is a potential mechanism for the onset of endothelial dysfunction. Bifurcations in the arterial tree modify laminar blood flow, creating areas of low shear stress and oscillatory shear stress. Both of these areas are prone to the development of atherosclerotic lesions (20). There is a natural increase in resting levels of retrograde and oscillatory shear stress that occurs with age in the femoral (155) and brachial arteries (106). High levels of oscillatory shear stress have been shown to have pro-atherogenic effects in vitro (90; 111; 156). Ziegler *et al* (156) found that 24 hours of oscillatory flow in vitro upregulated endothelin-1 (pro-atherogenic)

and downregulated eNOS (anti-atherogenic) expression. Another study (111) found that 6 hours of low level retrograde shear stress evoked significant transcriptional changes in multiple antherogenic genes, including elevated endothelin-1, platelet-derived growth factor A, platelet-derived growth factor B, and transforming growth factor-beta. In humans, retrograde flow can be artificially elevated using different levels of occlusion (140). Thijssen *et al* (140) introduced 3 degrees of limb occlusion (25, 50 and 75 mmHg) to subjects on 3 separate days which elicited progressing degrees of retrograde shear stress for 30 minutes, while anterograde shear stress remained constant. An FMD test on the brachial artery via RH was done before and after each acute intervention. It was determined that the artificial elevation of retrograde shear without altering anterograde shear resulted in the acute reduction of FMD which is dose-dependent in nature (140). In contrast to the findings that oscillatory shear stress produced endothelial dysfunction, Padilla *et al* (106) showed that the age related increase in the brachial artery oscillatory shear was likely due in part to a reduction of NO availability. There is likely a synergistic effect of reduced NO availability and proatherogenic gene upregulation via oscillatory shear stress that contribute to endothelial dysfunction and the development of cardiovascular disease with age.

Methods of FMD assessment

Reactive hyperemia

In 1992, Celermajer *et al* (17) published a paper introducing their technique of assessing FMD non-invasively in healthy subjects. Their method involved peripheral conduit artery diameter assessments before during and after distal limb occlusion (for 4.5 minutes, now

primarily 5 minutes). Following release of occlusion a large transient increase in blood flow is produced (reactive hyperemia (RH)), and the resulting increase in shear stress stimulates an FMD response. This dilation had been previously shown to be endothelium dependent (37). Shear stress can be estimated in areas of unidirectional flow by the equation: Shear Stress = viscosity x velocity/diameter (46). It can also be assumed that blood viscosity will be relatively consistent (47; 105) within subjects and shear rate (SR) can be used as an adequate surrogate (67; 68; 116). In this case we can substitute shear stress for SR which is calculated as: SR = velocity/diameter.

A significant limitation of the RH technique is the uncontrolled nature of the stimulus. This presents a major obstacle to interpretation if the stimulus magnitude changes pre vs. post an intervention or is significantly different between groups. For example, smaller vessels experience a larger shear stress stimulus (116) (even while flow is not different in these vessels (116)), and thus there is a larger FMD response (4; 17). This is a problem because older populations (which exhibit a blunted FMD response) tend to have larger vessel diameters (57), suggesting that their shear stress stimulus is reduced with age. Perhaps the strongest evidence that RH provides differential shear stress stimuli included 2045 subjects in the Framingham heart study (92), which assessed diastolic shear stress in response to a reactive hyperemia stimulus. Increased age, pulse pressure, mean arterial pressure, body mass index, prevalent cardiovascular disease, fasting blood glucose and prescribed antihypertensive treatment were associated with blunted hyperemic shear stress. Normalization of the % FMD response with the shear stress area under the curve (AUC) until the time of peak dilation (%FMD/shear stress AUC) has been suggested as a way to account for differences in the uncontrolled RH stimulus magnitude (120). However, normalization of the FMD response is not recommended by all researchers (6; 139). The Y intercept of within and between subject shear stress stimulus- FMD response relationships is variable and a non-zero y-

intercept biases normalized responses at the high and low stimulus range (6). In addition, if a response ceiling is reached this could result in underestimating response magnitude with normalized responses. The difficulty in accounting for stimulus differences mathematically has lead some researchers to look for other methods to try to control SR and shear stress (116; 119).

Using non RH shear stress stimuli has been avoided in the past due to fear of evoking non NO dependent responses, thus considerable attention has been given to the importance of adhering to the 'standard' RH test technique (5 min occlusion, occlusion cuff distal to the site of FMD measurement) in order to elicit a NO dependent FMD response (120). The quest to isolate an exclusively NO mediated FMD response is driven by the belief that NO bioavailability, and therefore NO mediated vasoprotection, was the sole reason for FMD's link to disease. This would mean that only NO dependent FMD responses would provide clinically relevant information regarding endothelial function. However, many vasodilators can be released from the endothelium in response to shear stress (NO, prostaglandins, endothelial derived hyperpolarizing factor), and while the shear stress profile may influence the predominant vasodilator involved, there is currently conflicting evidence in the literature regarding a clear pairing of shear profiles and vasodilatory mechanisms. FMD stimulated by a brief RH stimulus (created via release of a 3-5 min occlusion) has been shown by Joannides *et al* (68) and others (96) to be largely NO dependent, however long duration shear profiles and studies in the coronary arteries have been found to variably create NO independent FMD (96; 132), and NO dependent FMD (11; 151). The notion that NO dependence is a critical condition of FMD's clinical relevance has recently been called into question and it is likely that regardless of the vasodilatory mechanism, FMD confers important information about vascular health (48; 115). For example when the occlusion cuff is placed proximal to the site of FMD measurement, the response is minimally dependent on

NO (28), however, RH mediated FMD with proximal or distal occlusion cuff positions (48) have both been shown to be independent predictors of future events (70; 93) and survival (35; 91) in subjects with cardiovascular disease (CVD) or CVD risk. These results suggest that researchers have the flexibility to use non-standard shear stress patterns for FMD assessment.

Heating and small muscle mass exercise

Utilizing handgrip exercise or arm/ hand skin heating induced increases in shear stress has become an increasingly popular method for assessing FMD (104; 119; 134). Exercise is arguably the most physiologically relevant cause of increases in shear stress because exercise is how shear stress is elevated in daily life. Exercise results in an increase in shear stress due to rapid vasodilation of the resistance vessels feeding the exercising muscle, causing an increase in flow in the upstream conduit arteries (25). Skin heating also lowers vascular resistance thus increasing blood flow and shear stress in the upstream arteries, including the brachial artery during forearm heating (116; 117). Both of these methods afford greater stimulus control than reactive hyperemia. Increasing and decreasing the intensity of handgrip exercise or the temperature of a water bath during skin heating will alter downstream vascular resistance, and therefore shear stress in the feeding conduit artery. A data charting program can be used to display live measurements of blood velocity during handgrip exercise or heating. This allows even greater precision in stimulus control because by rearranging the SR equation, experimenters can use a subject's artery diameter and determine the target brachial artery blood flow velocity required to reach a desired SR (67; 119). The equation is rearranged to become:

Target Velocity = desired SR x Artery Diameter

When performing handgrip exercise this display will allow the subject to target the blood velocity by increasing or decreasing their contraction intensity. When utilizing skin heating, temperature changes can be used to create increases or decreases in blood velocity as required to achieve the target. Not only do these methods create a more uniform shear profile than RH (between subjects and visits), but they also allow the shear stress stimulus to be maintained over a longer period of time. The magnitude of this stable maintained stimulus is easier to characterize than the constantly changing reactive hyperemia profile, and it also allows a quantification of response dynamics (see dynamics section).

The putative mechanisms underlying vasodilation resulting from heating/exercise are up for debate. Mullen *et al* (96) assessed radial artery FMD during infusion of an eNOS inhibitor. They found that the normal 5 minute cuff occlusion RH stimulus resulted in an attenuated FMD following eNOS inhibition; however this same inhibition did not attenuate FMD as a result of RH following 15 minutes of cuff occlusion or progressive hand heating. This suggested that the FMD response to a sustained stimulus might not be NO dependent. In opposition to this, Bellien *et al* (11) assessed the shear stress to FMD slope of the radial artery obtained during progressive hand heating with various vasodilator blockades. The slope was decreased (vasodilation attenuated) in response to a blockade of NO and also to EDHF. In addition, the blockade of both NO and EDHF synergistically reduced to FMD response suggesting a functional interaction between the 2 mechanisms of vasodilation. Wray *et al* (151) used progressive handgrip exercise to create a shear stress stimulus in the brachial artery. In agreement with the findings of Bellien *et al* (11), the inhibition of eNOS reduced FMD at the highest intensity of exercise by 70%. These results together suggest that NO is important in the FMD resulting from sustained shear stress stimuli, however, the method of shear stress administration, or the presence of non-responders (there is

heterogeneity in the vasodilator pathways which underlie FMD (110)) may affect the extent to which NO confers vasodilation with these stimuli.

Dilation dynamics

The dynamic characteristics of a response refer to the time delay (TD), which is the time between the onset of the stimulus and the onset of the response, and the tau which is an estimation of the rate of response (time to 63 % of peak response development). Kinetics of oxygen uptake (87) and blood flow responses (131) have been measured for many years. The dynamics of human conduit artery FMD is a relatively new area of research. Assessing the dynamic response rather than only the peak response magnitude is desirable because different populations may exhibit distinct dynamic responses that are not captured by peak response measurements only. Pyke *et al* (117) determined the dynamic characteristics of brachial artery FMD in response to 2 step increases in heating induced SR (20 min stimulus duration): A large step increase (baseline to maximal blood velocity elicited with forearm heating) and a small step increase (50 % of maximal increase). All subjects displayed a rapid phase 1 dilation and a slower phase 2 dilation. Parameters for dilation dynamics (TD, Tau) and percent contribution of phase 1 to end trial dilation were not sensitive to SR magnitude for either phase. In addition, the above mentioned study by Pyke *et al* (117) supported the results that in the brachial artery, neither TD or Tau of phase 1 dilation in response to handgrip exercise induced increases in shear stress were sensitive to stimulus magnitude. This is in line with the concept of dynamic linearity (81) wherein the same rate of response development occurs, regardless of the magnitude of stimulus increase. This suggests that the same control mechanisms are responsible for the adaptation to

different stimulus magnitudes. The brachial and radial arteries' FMD dilation dynamics were compared by King *et al* (73) using forearm and hand exercise. They observed that the radial artery exhibited a shorter Phase 1 time delay and a larger percent contribution of phase 1 to maximal dilation than the brachial artery. In summary, current research suggests that the conduit arteries may exhibit a biphasic response which is influenced by 2 distinct mechanisms that are proportionally sensitive to shear stress magnitude, but dynamics that are not sensitive to shear stress magnitude.

The particular mechanisms behind phases 1 and 2 (36; 96; 133) are likely vessel and bed specific (7; 36; 133), and further study in humans is required. Frangos *et al* (36) studied cultured endothelial cells and proposed that nitric oxide production occurred via 2 separate mechanisms, which were elicited by different patterns of shear stress. Cells exposed to rapid changes of shear stress produced NO via a G-protein dependent burst, and those exposed to smooth or transitioned shear stress expressed a sustained production of NO via G-protein independent methods (36). This suggests that the first phase of dilation observed in cultured human umbilical vein endothelial cells (36) may have been mediated by G-proteins during rapid adaptation, whereas during slow adaptation it was not. A study by Azzawi *et al* (7) used isolated rat coronary arteries. They found that in these arteries, a step increase in shear stress created a transient dilatory response (7). This response was endothelium dependent, and inhibition by charybdotoxin and apamin (EDHF precursor) completely abolished the response. In addition, inhibition of eNOS (NO synthesis) and cyclooxygenase (PG synthesis) altered the response and made it more transient in nature (7). This suggests the importance of several endothelial vasodilator pathways during the first phase in rat coronary arteries. In contrast, when rat soleus and gastrocnemius muscles were exposed to a step increase in flow which was sustained for 20 minutes, phase 1

dilation was not inhibited by the blockade of either eNOS or cyclooxygenase (133). However, the sustained vasodilation (minutes 2 to 20) was eliminated by the blockade of eNOS, suggesting a crucial role of NO in phase 2 dilation in these vessel beds. In the same study (133) blockade of K^+ channels inhibited phase 1 dilation in both beds, but the effect was greater in the gastrocnemius. Also, phase 2 constriction in the absence of NO was found to be mediated by Endothelin-1. As in animal data indicating stimulus duration dependency of mechanisms, in human radial arteries, the dilatory response to transient shear stress (via RH) was found to be NO mediated, but in contrast to other studies (133) the response to sustained shear stress (prolonged episodes of RH, hand warming, and distal infusion of acetylcholine (Ach)) was not NO mediated (96).

Summary

There is clearly a large variation in FMD response dynamics depending on the blood vessels and bed. There is no clear consensus on the mechanisms underlying each phase of dilation. More human studies are required in order to determine the mechanisms behind the dilatory response to sustained shear stress stimuli. The best estimate of the human conduit artery dilation response includes an NO mediated phase 1, followed by phases 2 and 3 which may or may not be NO dependent (96; 151). Multiple phases of dilation suggest a need for the analysis of the magnitude of each phase, and not just peak dilation. Also, it has been suggested that alteration in the dynamics (TD, Tau) of a particular phase may indicate an alteration of the mechanism involved in FMD (117).

Potential limitations of using handgrip exercise to elevate shear stress for FMD assessment

Until recently, there were several concerns regarding the use of exercise induced increases in shear stress to assess FMD. Firstly, exercise may result in increases in sympathetic nervous activity (SNA) (14). A study by Hijmering *et al* (59) showed that concomitant sympathetic nervous activation via baroreceptor unloading (using lower body negative pressure) significantly attenuated FMD obtained using the RH technique. This attenuation was fully removed by loco-regional alpha-adrenergic blockade by intra-arterial infusion of phentolamine (59). Other studies have shown mixed results (31; 141). Dyson *et al* (31) found that FMD was only blunted by one of four different methods for raising SNA (cold pressor test), and Thijssen *et al* (141) found that the cold pressor test blunted FMD in young but not older subjects. However, in a study by Pyke *et al* (119), when similar SR profiles were created with forearm heating (no increase in SNA) and handgrip exercise, both conditions elicited the same %FMD, suggesting that the small increases in SNA that may occur during handgrip exercise do not effect FMD magnitude (119).

A second possible concern is that, exercise influences the metabolic environment of the muscle. Metabolites that build up in the active muscles may initiate conducted vasodilation (80). The concern was that this conducted vasodilation could reach the level of the conduit artery. In this scenario changes in conduit artery diameter would not solely reflect shear stress mediated dilation (FMD) (147). However, Pyke *et al* (119) addressed this concern. When forearm exercise was performed while keeping flow at baseline (a scenario in which metabolite accumulation is present but an increase in conduit artery shear stress is not) it was observed that there were no changes in brachial artery diameter (these data have since been confirmed in the radial artery

(67)). This indicates that conducted vasodilation does not reach the conduit arteries in the forearm circulation (119).

Finally, contraction induced impedance to flow results in a fluctuating pattern of shear stress with brief periods of retrograde flow. As described earlier, large amounts of retrograde flow have been shown to acutely inhibit FMD (140). However, as stated above, Pyke *et al* (119), observed that passive heating induced increases in shear stress (no shear fluctuation) and exercise induced increases in shear stress to the same mean shear magnitude resulted in a very similar FMD response. In addition, oscillatory flow, often associated with endothelial dysfunction, contains a net flow near zero, whereas exercise consists of predominantly anterograde flow with brief periods of retrograde flow. This suggests that the mean shear stimulus is transduced by the endothelium (119), and that exercise induced fluctuations are not a concern. However, the study by Pyke *et al* (119), investigated only a 3:2 duty cycle (ratio of relaxation to contraction) and there is a need for the evaluation of different duty cycles, which provide differing patterns and magnitudes of retrograde and anterograde shear stress.

Summary

RH is the most widely used method of assessing FMD; however the stimulus is brief and uncontrolled, which is a limitation. Heating and exercise both provide a viable means by which to increase shear stress for FMD assessment with greater stimulus control.

Does FMD resulting from sustained shears tress stimuli convey clinically important information?

The vast majority of previous research linking FMD to cardiovascular risk/disease utilized the reactive hyperemia technique. However, while there are some negative reports (96; 104), emerging evidence indicates that FMD resulting from more prolonged stimuli also conveys important information regarding vascular health. Grzelak *et al* (51) demonstrated that FMD induced by handgrip exercise is impaired in older healthy subjects, and subjects with type 1 diabetes vs. young healthy controls (51). These differences between groups were also significantly exaggerated with handgrip exercise vs. RH mediated FMD. In addition, Gaenzer *et al* (38) observed that cycling exercise induced femoral artery FMD was impaired in smokers vs. non smoking controls. Bellien *et al* (10) also reported that skin heating induced radial FMD was impaired in subjects with type 1 diabetes as compared to controls. This evidence supports the notion that handgrip and heating FMD tests may confer clinically relevant information regarding endothelial function. There is much interest in using FMD tests as clinical research tools and this evidence of clinical utility may stimulate greater interest in handgrip exercise approaches. This highlights the need to improve our understanding of the impact of factors such as duty cycle in order to ensure that FMD data is collected, interpreted and compared appropriately.

Handgrip exercise duty cycles in FMD assessment protocols

Currently, there is no standard handgrip exercise duty cycle, therefore different research teams often use different patterns of relaxation and contraction in order to elevate shear stress and elicit an FMD response. In a study by Wray *et al* (150), subjects exercised at incremental intensities from 5 to 60 % of their max work rate at a frequency of 0.5 Hz (1 contraction every 2 seconds, however the experimenters had subjects perform dynamic exercise where they

transitioned directly from concentric to eccentric contraction such that the relaxation period was eliminated, producing a '0 : 2' duty cycle). Another progressive handgrip exercise study by Wray *et al* (151) had subjects try to limit contraction duration to <25% of the duty cycle for 3 minutes at each stage (0.75 : 0.25 duty cycle). Grzelak *et al* (51) used dynamic handgrip exercise with a spring loaded hand exerciser (8 J of work), and the subjects squeezed 30 times in approximately 30 seconds (0.5 :0.5). A study by Padilla *et al* (104) used a 1 second relaxation to 1 second contraction duty cycle at 10 percent of the subject's maximal voluntary contraction intensity for 5 minutes. Pyke *et al* used a 3 s relaxation, 2s contraction duty cycle for 10 (119) and a 6 (118) minute exercise bouts. The same research group also implemented a 5 to 3 duty cycle in the brachial and radial arteries in order to increase relaxation time (67). This clearly demonstrates that a wide range of protocols are in use.

In two previous studies by Pyke and colleagues that examine the same population (118; 119), a duty cycle of 3:2 and a mean SR of 65 s^{-1} resulted in a very similar magnitude of FMD (~6.5 – 7%). However, when that same group assessed FMD in the same population (young healthy males) but used a duty cycle of 5:3, a SR of 60 s^{-1} resulted in an FMD of 2.5% and SR of 80 s^{-1} resulted in an FMD of only 5.4% (67). While hardly conclusive, this data could indicate that the duty cycle plays a role in determining the FMD magnitude even when mean shear is matched.

As stated above, rhythmic handgrip exercise is characterized by a fluctuating pattern of shear stress in the brachial artery (119). This is created by a period of contraction induced impedance of flow, which may include a brief period of retrograde flow, followed by a period of relatively enhanced hyperemia during relaxation (119) (Fig. 2-2). The variability in shear stress exposure with varying duty cycles lays in the frequency of contraction, and ratio of relaxation to

contraction time (Fig. 3-3). If mean SR is equal in both conditions, during a duty cycle where the ratio of relaxation to contraction are equal, the peak shear stress during relaxation will be higher than that of a duty cycle which has a longer period of relaxation in relation to contraction. This is because the former duty cycle's SR will have to be higher during relaxation to compensate for the more frequent contraction induced impedances to flow. This results in one duty cycle that has a higher peak shear stress than the other, even though the mean shear stress is the same (figure 3).

The second source of variability which arises with different duty cycles is that more frequent contraction results in more frequent periods of retrograde shear stress (Figure 3). High levels of oscillatory flow are found to be pro-atherogenic in vitro (90; 111; 156) and in vivo (20) (see '*Shear pattern and Endothelial function')*. Acute bouts of retrograde shear stress (30 min) also acutely impair FMD (140). However, there is evidence that suggests that the endothelium transduces the mean stimulus, and that fluctuations about that mean do not alter the FMD magnitude. In the study by Pyke *et al* (119) described above, in which the same mean shear stress was created in fluctuating, steady state, and handgrip exercise, all three conditions resulted in the same relative FMD after 10 minutes of sustained stimulus. Padilla *et al* (107) matched brachial artery mean SR in subjects using 2 conditions which elicited different patterns of shear stress in the brachial artery: lower body cycling exercise (high peak shear stress with periods of retrograde shear stress) and forearm heating (lower peak shear stress, no retrograde shear stress). They found that brachial artery FMD was not different between conditions. In summary, the strongest evidence in the literature suggests that mean shear stress is the important factor in determining % FMD, not peak shear stress or retrograde shear stress. However, studies that involve direct comparisons of different shear patterns at the same mean shear are limited.

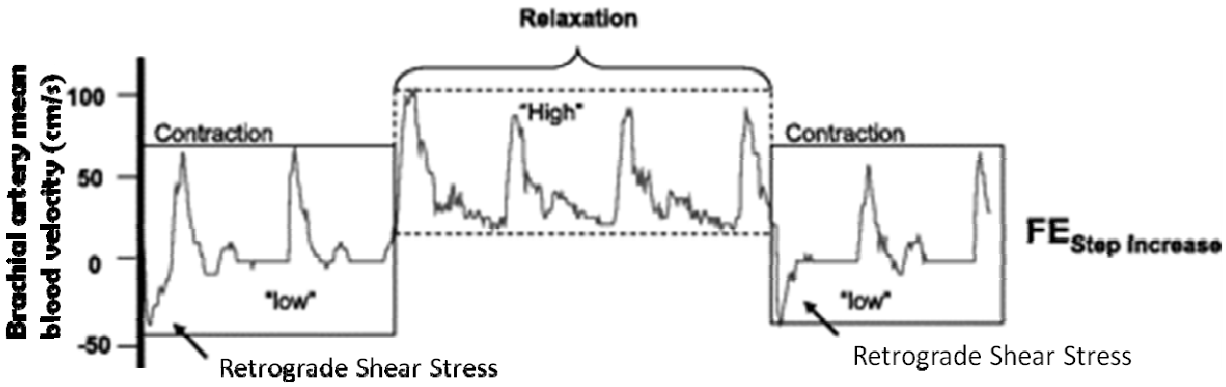


Figure 2-2. Diagram of blood velocity during rhythmic handgrip exercise. Blood velocity is lower during times of contraction than times of relaxation. A brief period of retrograde flow occurs at the onset of each contraction. Adapted from Pyke et al (95).

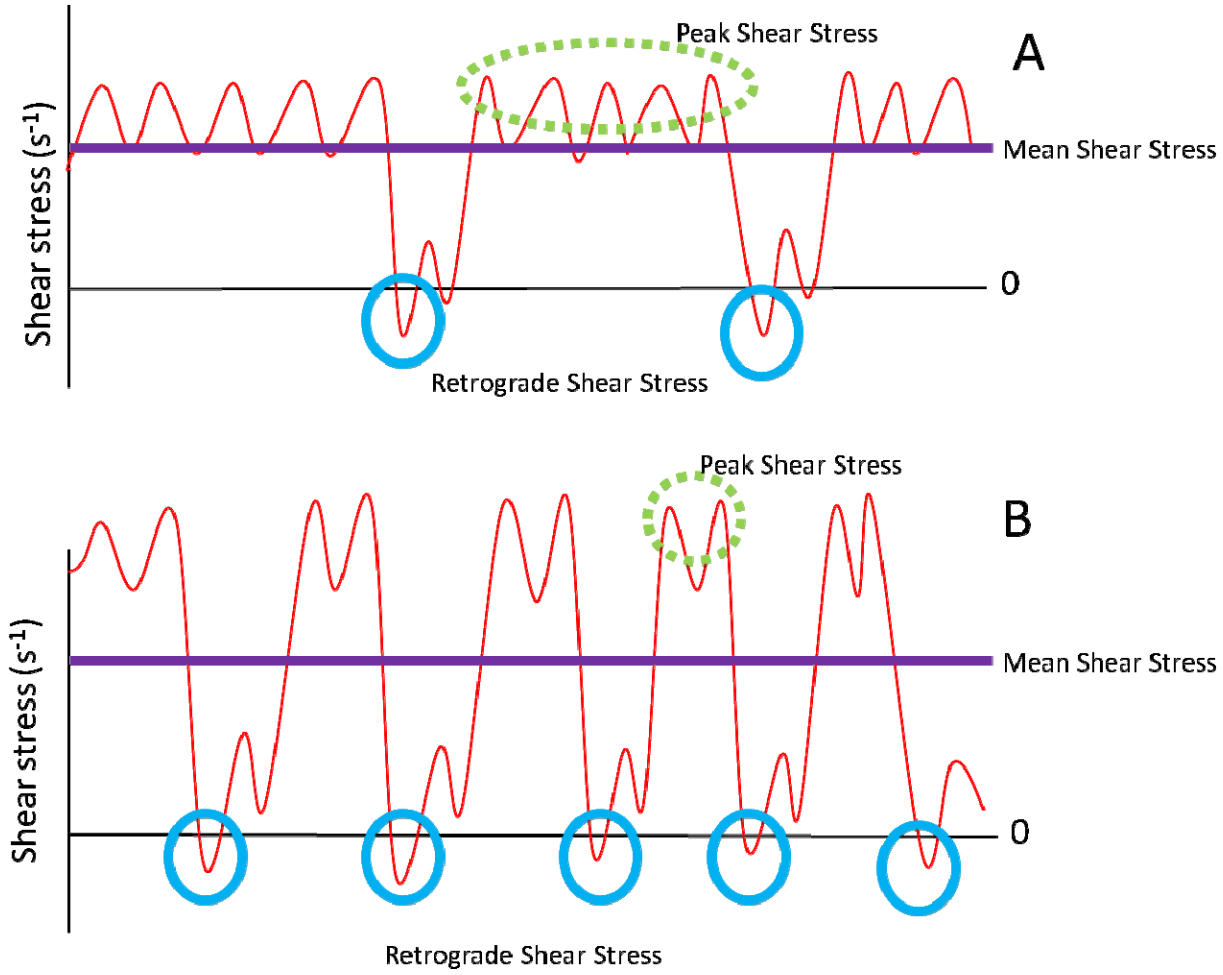


Figure 2-3. Schematic of shear stress during 2 different duty cycles: A = Duty cycle with long period of relaxation and short period of contraction. B = Duty cycle with short period of relaxation and short period of contraction. Both duty cycles have the same mean shear stress, however, duty cycle B has a higher mean shear stress during relaxation and more frequent periods of retrograde shear stress. Dashed circles highlight the peak shear stress, and solid circles highlight periods of retrograde shear stress.

Summary

Due to the lack of stimulus standardization in past studies of sustained shear stress stimuli for FMD, it is difficult to compare the results of different studies. Studies have used different stimulus durations, methods for increasing shear stress (ie. heating, exercise) and different shear stress patterns. There is a need for a greater understanding of the impact of duty cycle (and therefore shear pattern) on FMD magnitude. While there is some suggestion that duty cycle may impact FMD magnitude, the most direct investigation of the impact of shear pattern to date suggests that the mean SR stimulus is the most important factor contributing to conduit artery FMD (107; 119).

Conclusion

The mechanisms underlying the conversion of a shear stress stimulus to FMD may vary with the nature of the stimulus and of the phenotype of the vessel of interest. Recent concerns regarding the process of stimulating flow mediated dilation via RH have led some researchers to utilize a sustained shear stress stimulus in order to assess FMD. Forearm exercise has arisen as a viable alternative method for elevating shear stress; however there are many inconsistencies in methodology (ie. duty cycle) between research groups. Separate duty cycles are prone to the creation of different patterns of shear stress, and more research must be done in order to improve our understanding of the effects of these methodological differences.

Chapter 3

Manuscript

Introduction

The vascular endothelium is essential for vasoprotection (43) and local regulation of vascular tone (37). In response to an increase in blood flow associated shear stress, the endothelium acts by releasing several vasoactive substances (11; 108), resulting in an endothelium dependent vasodilation (flow mediated vasodilation (FMD)). Endothelial function is often assessed by measuring FMD in response to an experimenter imposed increase in shear stress. FMD is typically impaired in populations with risk factors for cardiovascular disease (15) and is a predictor of cardiovascular events in healthy subjects (153; 154).

Reactive hyperemia (RH), achieved via the release of temporary limb occlusion, has been used extensively in past research as the standard method for increasing shear stress in order to assess FMD (17; 138). It elicits an immediate, transient, uncontrolled increase in conduit artery blood flow that stimulates an FMD response. However, the RH stimulus may vary between groups or conditions (128; 143), complicating the interpretation of FMD response differences, and the transient profile is not ideal for all FMD investigations (117; 119).

Handgrip exercise (HGEX) or arm/ hand skin heating induce prolonged increases in conduit artery shear stress, and these methods are now appearing more frequently in FMD assessment studies (104; 119; 134). Notably, shear stress is typically increased by exercise (not RH) in daily life, making it a physiologically relevant method for FMD assessment. In addition,

the rapid and sustained elevation of shear stress permitted with exercise allows for the assessment of the time course of brachial artery vasodilation (dynamics) (117; 119) which may provide important information independent from the response magnitude. Although the bulk of the evidence supporting the clinical relevance of FMD stems from research using RH, it has recently been reported that exercise induced FMD is impaired in older healthy subjects, and subjects with type 1 diabetes (51). This suggests that FMD in response to HGEX induced increases in shear stress may be useful in detecting clinically relevant endothelial dysfunction.

There are no set norms as to how HGEX should be performed in order to elevate shear stress and quantify the resultant FMD, and several protocols have been reported to date (51; 67; 104; 117; 119; 150; 151). HGEX is characterized by periods of relaxation, during which shear stress is relatively high, followed by periods of contraction, during which shear stress is reduced to close to zero. Another feature of the shear stress pattern is that at the onset of each contraction, a brief period of retrograde (reverse) shear stress is created. Thus, when different studies use different duty cycles (ratio of relaxation : contraction) to elevate shear stress for FMD assessment, different patterns of shear stress are created. Different patterns of shear stress have been shown to alter the magnitude of FMD (140; 142), and retrograde shear stress has been associated with proatherogenic effects in vivo (140) and in vitro (156). While it is clear that the mean shear stress stimulus influences the magnitude of FMD (107; 119), the impact of duty cycle induced alterations in shear stress pattern remains unknown.

The purpose of the present study was therefore to determine the impact of HGEX duty cycle on FMD magnitude. A secondary purpose was to determine the impact of duty cycle on FMD dynamics. This was achieved by creating the same mean brachial artery shear stress

stimulus via three distinct duty cycles that created three distinct shear stress patterns. The hypothesis was that duty cycle does not influence HGEX FMD.

Methods

Subjects

16 healthy, recreationally active male volunteers aged 18-28 years who were recruited by advertisements across the Queen's University campus (Kingston, Ontario) participated in the study (Consent form Appendix F). Health status was confirmed via a medical screening questionnaire for risk factors associated with endothelial dysfunction or the presence of any cardiovascular disease. The study procedures were approved by the Health Sciences Human Research Ethics board at Queen's University, which operates under the terms of Helsinki, and all subjects completed a consent form approved by the same board.

On an initial visit subjects were introduced to the HGEX protocol and it was ensured that a clear image of the brachial artery, and a clear blood velocity signal, with no interference from adjacent veins, could be obtained. Subjects also completed a seven day physical activity questionnaire on this initial visit, which has been shown to correlate well with daily physical activity values measured by accelerometer (66). Following the initial visit subjects participated in two experimental visits separated by at least 2 days and at most 2 weeks. In the experimental visits, subjects were instructed to abstain from food for 6 hours before the study and to avoid alcohol, caffeine, and exercise for 12 h before the study. The experimental visits took place in the morning, beginning between 8 and 10 am, in a quiet, temperature-controlled room (20-23 °C). Subjects completed all trials on each visit, resulting in two replicates of each trial. The order of

trials on each day was randomized, and like trials on separate days were averaged to result in a single response to each duty cycle in each subject.

Subject monitoring

Heart rate (HR) was measured with three ECG electrodes placed on the chest and abdomen. Blood pressure was monitored throughout the protocol using a Finometer device (Finometer PRO, Finapres Medical Systems). A pneumatic cuff was fitted around the right middle finger with the hand placed at heart level.

Brachial artery blood flow velocity and diameter measurements

Brachial blood flow velocity was obtained using Doppler ultrasound operating at 4 MHz (Vivid i2 GE Medical Systems). The Doppler shift frequency spectrum was analyzed via a Multigon 500P TCD spectral analyzer where mean velocity was determined as a weighted mean of the spectrum of Doppler shift frequencies. The corresponding voltage output was sampled continuously (Powerlab; AD Instruments and stored (LabChart; AD Instruments) for analysis at a later time.

Brachial artery diameter was obtained using ultrasound imaging technology operating at 12-MHz in B-mode (Vivid i2 GE Medical Systems). Ultrasound parameters were set to optimize longitudinal B-mode images of the lumen/arterial wall interface. For reasons previously described (117) data were collected using an insonation angle of 68°, which did not vary from trial to trial. Image recording from the Vivid i was achieved with a VGA to USB frame grabber (Epiphan systems Inc.) and recorded as .avi files on an independent PC using commercially available software (Camtasia Studio, TechSmith).

Experimental procedures

Subjects lay supine with their left arm extended out to the side and the handgrip device was placed in their left hand. While the subject was resting, the ultrasound probe was placed over their left brachial artery. The probe was positioned to achieve an optimal image and blood flow velocity signal. The real time blood flow velocity was displayed on a computer screen as a moving average (moving average of the last 4 seconds for the 1:1 and 3:1 duty cycles, and a moving average of the last 6 seconds for the 5:1 duty cycle).

During the initial visit subjects were asked to perform two isometric maximal voluntary contractions (MVC) using a handgrip dynamometer. Subjects then performed a series of contractions at each of the 3 duty cycles to identify the contraction intensity (% MVC) that elicited the blood flow velocity required to achieve an exercise target shear rate (SR) of 75 s^{-1}. SR (an estimate of shear stress without blood viscosity) was calculated as: *mean blood flow velocity/vessel diameter*. The blood flow velocity required to achieve the target SR was calculated for each subject as: *required velocity = 75s^{-1} x brachial artery diameter*. To perform the calculation, the brachial artery diameter for each subject was estimated by manual caliper placement on the ultrasound image before the trials commenced.

During each of the two experimental visits (Fig. 3-1), all parameters were measured for one minute (pre-compression baseline) and then, to ensure a uniform baseline SR in all subjects, arterial compression (AC) was performed for four minutes (AC baseline). To achieve this, a researcher placed two fingers on the subject's arm distal to the ultrasound probe and applied

Figure 3-1. Timeline for one experimental visit. Subjects each performed 2 sessions on separate days and the order of duty cycle was randomized between subjects and within subjects. AC, arterial compression; BA, brachial artery; FMD, Flow mediated Dilation; SR, Shear Rate.

pressure to the brachial pulse. The compression force was adjusted based on continuous blood velocity output (an online moving average) in order to maintain the SR at 10 s^{-1}. To achieve a rapid increase in SR upon compression release, during the last 10 seconds of the AC, subjects performed a number (ranging from one to three) of contractions at the designated %MVC (as determined during the initial visit). Upon compression release the subjects performed 6min of HGEX. During the HGEX, force feedback was displayed continuously for the subjects on a computer data acquisition system (Powerlab, AD Instruments). Subjects achieved the necessary % MVC and duration for each contraction by displacing the force readout line to the desired level in time with the prescribed duty cycle (1:1, 3:1, or 5:1; chosen because these duty cycles spanned all previously used ratios of relaxation to contraction). Brachial artery blood velocity was monitored continuously throughout the trial, and experimenters coached subjects through minor increases and decreases in force production in order to maintain the required blood velocity. Three HGEX trials were completed for each participant on each study visit, and a minimum of ten minutes or the time until the diameter returned to baseline separated the trials. Data were collected on 19 subjects; however 3 subjects were excluded from the analysis due to inadequate image quality during baseline and/or recovery.

Blood testing

At the end of one of the two experimental visits, a fingerprick blood sample was taken and blood cholesterol and glucose levels were measured using a Cholestech LDX system. For 4 subjects, LDL values resulted in a reading of 'NA' due to their values being outside of the detectable range. These subjects' LDL values were excluded from the group mean. For 3

subjects, triglyceride values read <45 mg/dL. For these subjects, a value of 45 mg/dL was included in the group mean.

Data analysis

Blood velocity was analyzed offline to characterize the contraction vs. relaxation velocities and the magnitude and duration of retrograde shear. Blood velocity was also analyzed offline in 3-s average time bins for mean shear stress determination.

Vessel diameter was analyzed using automated edge-detection software (Encoder FMD & Bloodflow v3.0.3, Reed Electronics). This program allows the user to identify a region of interest on the portion of the image where the walls are most clear. It then identifies and tracks the walls of the artery via the intensity of the brightness of the walls vs. the lumen of the vessel. The program collects one diameter measurement for every pixel column in the region of interest. It uses the median diameter as the diameter for that frame. The program allows for the removal of erroneous data points due to vessel wall tracking errors. The diameter data was then compiled as 3-s time bins. Repeat trials were averaged and treated as a single response for each duty cycle. Missing data due to erroneous wall tracking was interpolated to facilitate calculation of the average.

Shear stress was estimated as SR. SR was combined into one minute time bins during baseline, AC baseline and exercise. The first 21 seconds of recovery were used to characterize SR immediately post-exercise (recovery). The AUC of the retrograde SR during each contraction was calculated using an automated analysis function that detected all blood velocity below 0 cm/s. Cumulative AUC was calculated by summing all retrograde AUC data from the 6 min exercise bout.

Percent FMD was calculated as the % change in artery diameter from baseline to the new dilated diameter using the equation:

$$\%FMD = \left[\frac{(\text{Recovery Diameter} - \text{AC baseline diameter})}{\text{AC baseline diameter}}\right] X\ 100\ \%$$

where 'Recovery Diameter' is the average diameter during the 21s recovery period. Recovery diameter was used to calculate the %FMD outcome due to the superior image stability during that interval, when the arm is still vs. during exercise when movement is occurring (particularly in the 1 to 1 duty cycle, Appendix C). In a subset of 10 subjects with stable images and wall tracking during exercise in all trials (manually determined via observation of images and wall tracking during analysis), FMD was also calculated for each minute of exercise and the diameter measured during exercise was used for FMD dynamics analysis

Custom designed software was used to determine the phase 1 dilation dynamics using exponential curve fitting on 3 second average diameters. The software determines the time from onset of stimulus to onset of response (time delay; TD1), and the time to 63 percent of response magnitude (Tau1). The model allows for the separate curve fitting of each phase of response. The dynamics of the second or third phase were not quantified because of the variability in onset and presence of these phases.

Statistical analysis

One way (Appendix D) (factor duty cycle : 1:1, 3:1 and 5:1) and two way (Appendix E) repeated measures ANOVAs (factors duty cycle and time) were used to analyze stimulus and

response parameters as appropriate. Level of significance was set at $P<0.05$ and significant differences for RM-ANOVA were further assessed using Tukey post hoc tests. All statistics were calculated using SigmaPlot 11.0 (Systat Software Inc.) (San Jose, CA). All data are expressed as means ± SD.

Results

Subject characteristics

Subject characteristics are shown in Table 1. Mean values are within the normal healthy range (42), and subjects were not highly trained, indicating that these factors would not be likely to negatively or positively impact FMD.

	Calories/ Day	Height (cm)	Weight (kg)	BMI (kg/m^2)	TC (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	TG (mg/dL)	Glucose (mg/dL)
Mean	2832.4	180.4	78.6	24.1	156.4	51.3	91.7	84.1	87.9
SD	357.4	6.7	8.4	1.7	26.0	21.6	14.9	54.7	6.4

Table 1. Anthropometric data for subjects. n=16, LDL n = 12, TG n = 13. Calories/Day = average calories expended per day over a one week period. BMI = body mass index. TC = total cholesterol. HDL = high density lipoproteins. LDL = low density lipoproteins. TG = Triglycerides. Glucose = fasting blood glucose.

Heart rate and mean arterial pressure

There was no impact of duty cycle on mean arterial pressure (main effect of duty cycle, $p = 0.102$). There was a small but significant effect of duty cycle on heart rate (1 to 1, 60.8 ± 6.5 bpm; 3 to 1, 59.6 ± 6.5; 5 to 1, 59.5 ± 6.5; main effect of duty cycle $p=0.009$). HGEX caused a significant increase in these variables in all trials (MAP: 98.1 ± 7.7 mmHg to 102.1 ± 7.9 mmHg, main effect of time $p<0.001$, HR: 57.6 ± 6.9 bpm to 61.0 ± 6.3 bpm, main effect of time $p<0.001$). (Graphs depicting HR and MAP over time may be found in Appendix A)

Shear rate

Full shear rate profile

SR profiles are shown in Fig 3-2. Neither Pre-compression baseline SR (main effect, $p=0.92$) nor AC baseline SR ($p = 0.95$) were significantly different between duty cycles. SR was successfully matched during HGEX between duty cycles (no significant differences in mean SR between duty cycles, $p = 0.84$), and reached steady state by minute 2. As expected, given the requirement of the same mean shear stress with a different ratio of contraction to relaxation time, the mean handgrip force applied (% MVC) for each duty cycle was significantly different (1 to 1, 15.0 ± 4.6 %; 3 to 1, 21.1 ± 6.1 %; 5 to 1, 25.0 ± 7.3%, $p<0.001$). SR during the first 21 seconds of recovery was significantly different between duty cycles (1 to 1 = 82.67 ± 8.6 s^{-1}; 3 to 1 = 69.11 ± 6.4 s^{-1}; 5 to 1 = 64.61 ± 5.21 s^{-1}, $p < 0.001$).

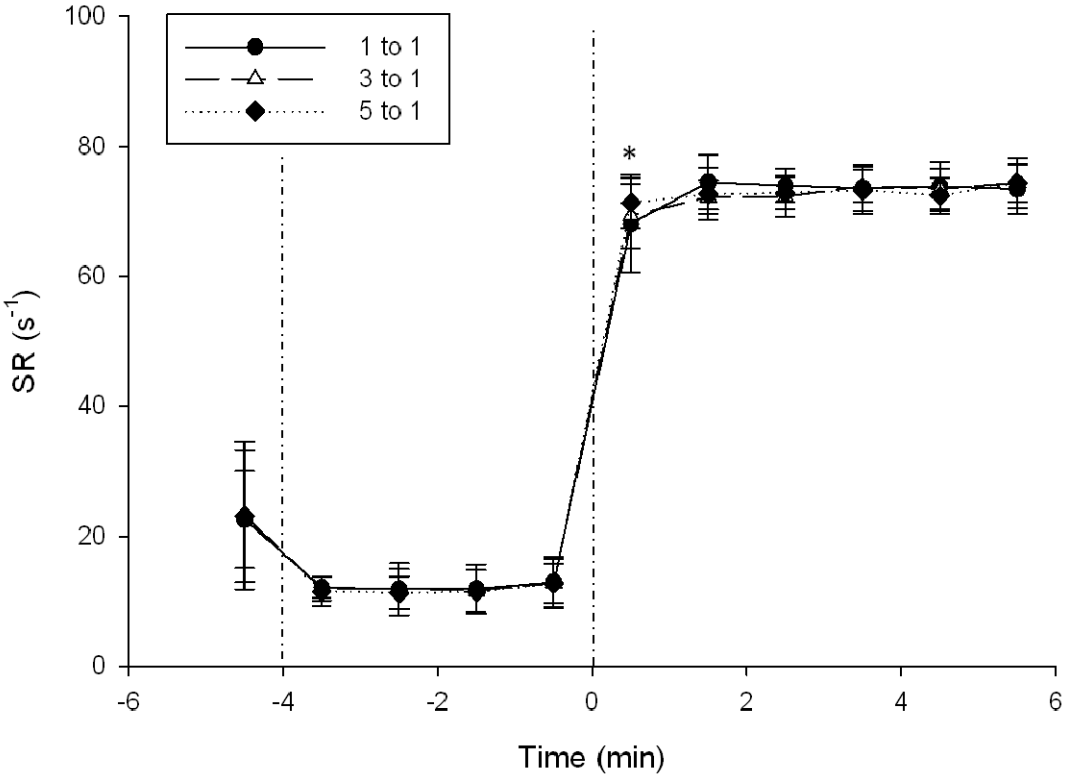


Figure 3-2. 1 minute average shear rate (SR) for duty cycles during 1 minute baseline, 4 minutes of arterial compression (AC) baseline, and 6 minutes of exercise. There was no difference in mean SR between duty cycles during pre- compression baseline, AC baseline, 6 minutes of exercise. * denotes that minute 1 of handgrip exercise was significantly lower than minutes 2 through 6. Data are mean ± SD.

Shear rate pattern.

The differences in SR pattern are depicted in Fig 3- 3. During the contraction period, 5 to 1 had the greatest retrograde AUC (Fig. 3-3A, $p < 0.001$), mean Nadir SR (Fig. 3-3 B $p = < 0.001$), and lowest mean SR (Fig. 3-3 C, $p < 0.001$). However, when subjects performed the 1 to 1 duty cycle, due to the larger total number of contractions, they experienced a significantly greater cumulative retrograde AUC over the 6 min exercise bout vs. the other duty cycles (Fig. 3-3 D $p < 0.001$). During the relaxation phase, all duty cycles had a significantly different mean SR (Fig. 3-3 C, $p < 0.001$).

Brachial artery diameter and FMD

There was no effect of duty cycle ($p = 0.114$) or AC on baseline diameter (Pre compression diameter: 0.393 ± 0.028 cm; AC diameter: 0.390 ± 0.028 cm, $p=0.096$). There was no impact of duty cycle on %FMD at the end of the 6min exercise bout ($p = 0.860$) (measured from AC baseline to recovery) (Fig. 3-4). In a subset of 10 subjects in which all 6 HGEX trials had acceptable wall tracking during exercise, there was no effect of duty cycle on FMD during exercise (Fig. 3-5) (Duty cycle main effect $p = 0.939$).

FMD dynamics

The time course parameters of Phase 1 dilation in the subset ($n = 10$) with acceptable wall tracking during HGEX are displayed in Fig 3-6. There was no difference between duty cycles in Phase 1 Gain ($p = 0.192$), Time Delay ($p = 0.889$), or Tau ($p = 0.085$). (Examples of Curve fitting in Appendix B)

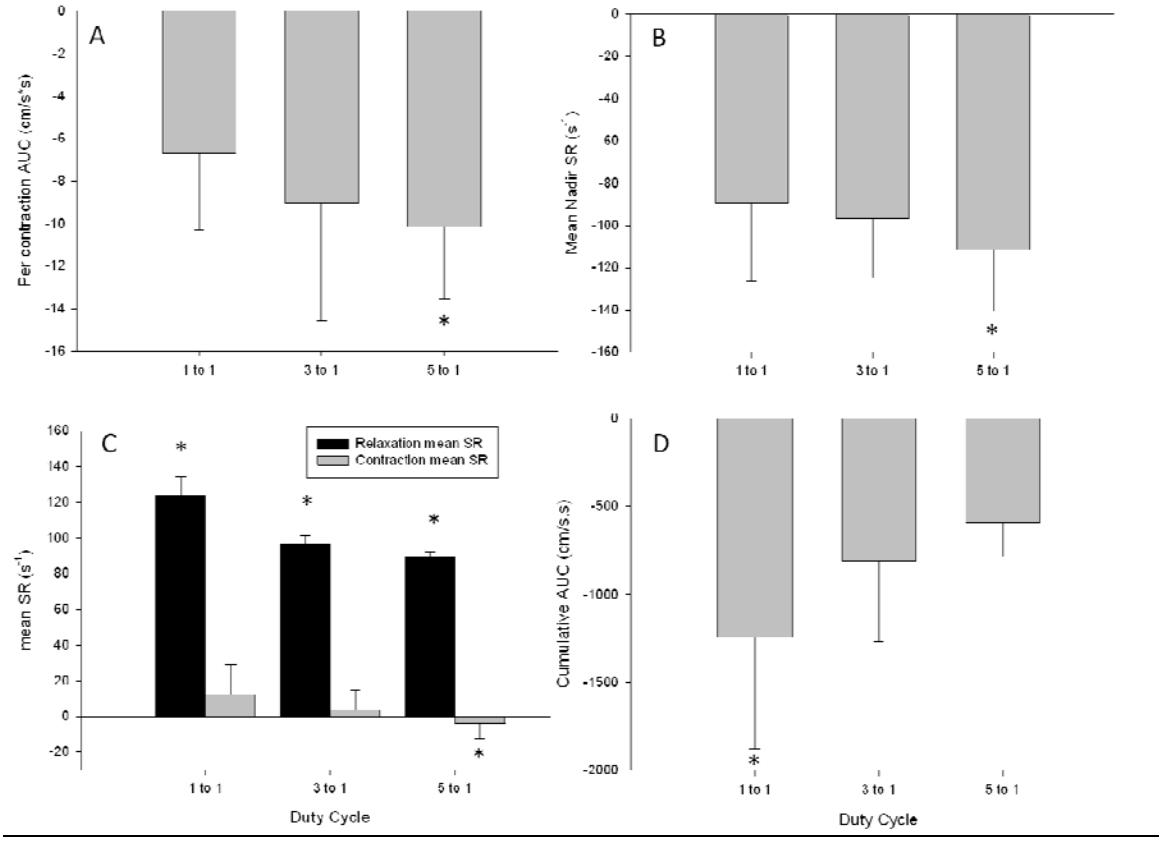


Figure 3-3. Characterization of shear rate (SR) pattern. A: mean area under the curve (AUC) of retrograde SR during contraction. B: mean SR nadir (minimum SR value) during contraction. C: Mean SR during relaxation (black bars) and contraction (grey bars). D: Cumulative area under the curve (AUC) of retrograde SR for 6 minutes of exercise. * represents significantly different from all other duty cycles. Data are mean ± SD.

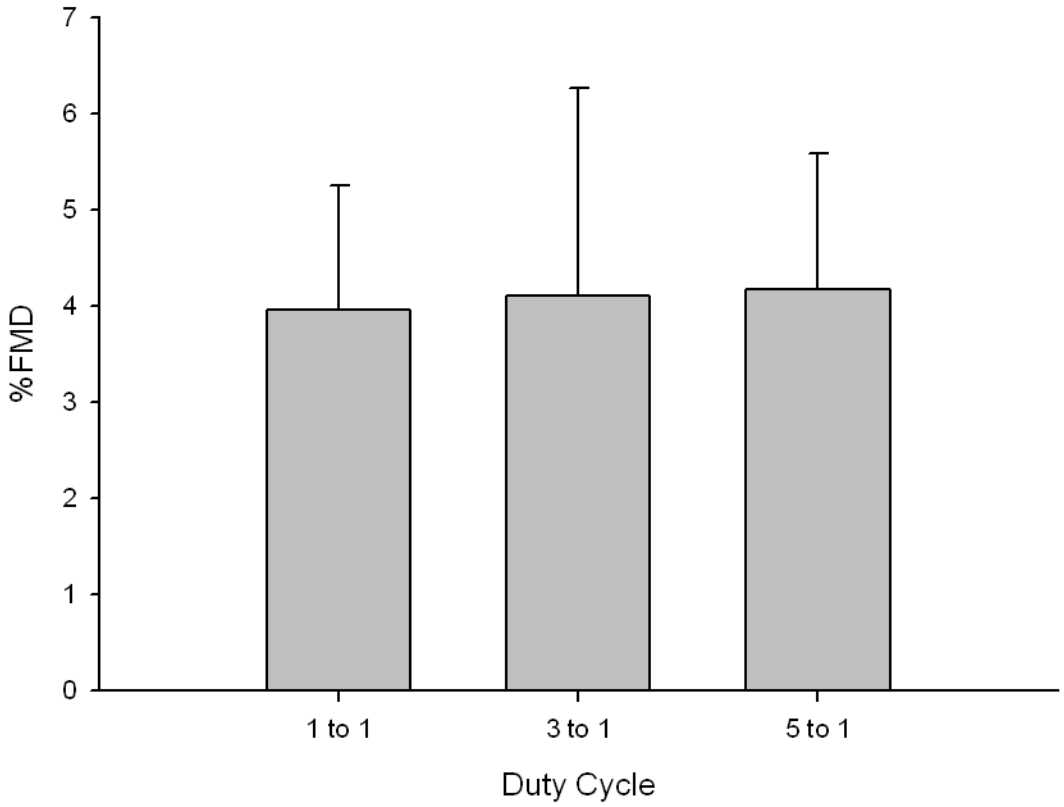


Figure 3-4. Percent flow mediated dilation (% FMD) following three different duty cycles. There was no significant difference between any duty cycles (p = 0.860). Diameter measurements for FMD calculation were collected from the first 21 seconds after exercise. Data are mean ± SD.

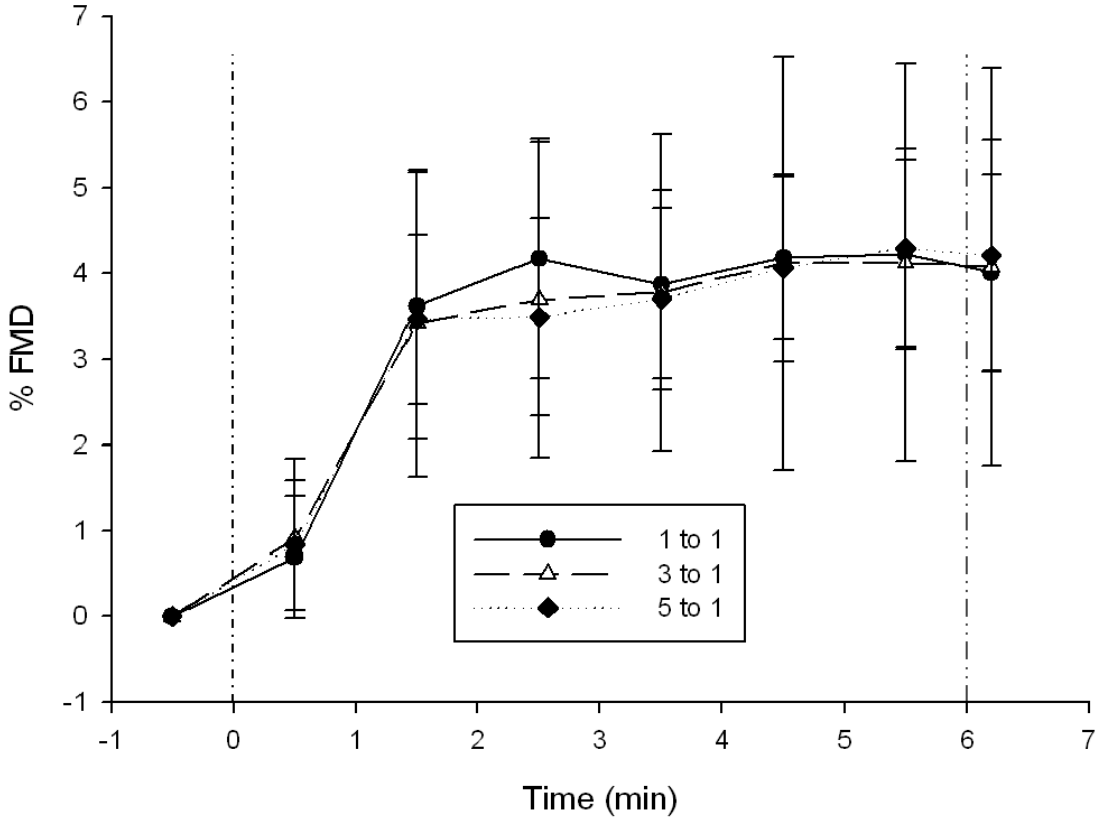


Figure 3-5. Time course of percent flow mediated dilation (% FMD). Subset N = 10. Data are 1 minute averages from last minute of arterial compression (AC) baseline through 6 minutes of handgrip exercise and 21 seconds of recovery. There was no difference in FMD between duty cycles (main effect p = 0.939, duty cycle x time interaction p = 0.545). All data mean ± SD.

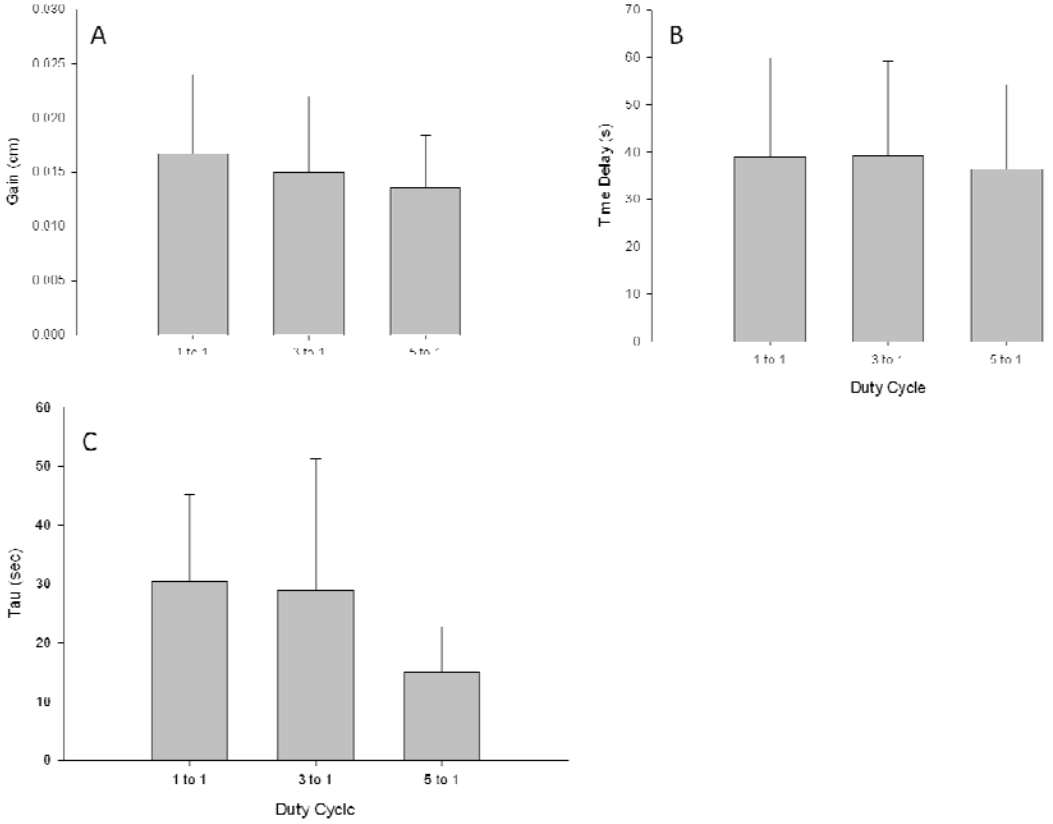


Figure 3-6. Phase 1 dilation dynamics. A: Gain of phase 1 dilation. B: Time delay from onset of stimulus to onset of dilation. C: Tau (time required to reach 63 % of peak dilation) of phase 1. No significant differences between duty cycles were determined Phase 1 Gain (p=0.192), Time Delay (p=0.889), or Tau (p=0.085). Data are mean ± SD.

Discussion

Increasingly, HGEX is being employed to elevate conduit artery shear stress for FMD assessment (51; 104; 119; 151). Duty cycle has a significant impact on the pattern of the shear stress stimulus; however, there has been little examination of the impact of duty cycle on FMD. The present study performed the first direct comparison of FMD elicited by the same mean shear stress but distinct duty cycle associated shear stress patterns. The primary finding of this study was that there was no significant impact of duty cycle on %FMD. There was also no definitive impact of duty cycle on the dynamics of the Phase I dilation response. In agreement with previous findings (107; 119), this suggests that the mean shear stress stimulus is the most important determinant of HGEX FMD.

Handgrip exercise for FMD assessment

Utilizing HGEX to elevate shear stress for FMD assessment has historically been avoided due to concerns of conducted vasodilation (134) and a potential lack of NO dependence that would limit its utility for assessing NO bioavailability and the associated level of vasoprotection (96). However, it has recently been shown that conduit artery vasodilation during exercise is shear mediated (not conducted upwards from the resistance vasculature) (119), and at least partially NO dependent (151).

Although there have been some observations to the contrary (96), emerging evidence suggests that sustained shear stress stimulated FMD is impaired in high risk subjects as compared to controls. Grzelak *et al* (51) assessed the FMD of patients with type 1 diabetes (T1D) and found that HGEX elicited significantly lower FMD in diabetic subjects vs. controls, and was more effective at illustrating the age related decline in endothelial function than RH mediated

FMD. Similarly, Findlay *et al* (34), observed that HGEX mediated brachial artery FMD was impaired in young smokers vs. controls while RH mediated FMD was not . Femoral artery FMD in response to cycling has also been reported as impaired in smokers (38). Forearm heating, which produces a sustained shear stress stimulus comparable to HGEX (although without the contraction induced fluctuations), identified a distinction between subjects with T1D and controls (10). Taken together these studies indicate that HGEX mediated FMD may be a powerful research tool for characterizing endothelial function in clinical and healthy populations.

The current body of research lacks a standard duty cycle for HGEX induced FMD assessment. Grzelak *et al* (51) used a duty cycle in which the subject squeezed once per second (0.5 to 0.5 duty cycle), and FMD was measured post-exercise. Wray *et al* (151) used a similar protocol in which subjects were instructed to squeeze at 1 Hz, limiting contraction time to 25% of a second (0.75 to 0.25). In another study by the same group, a 0.5 Hz duty cycle was used, but subjects alternated between concentric and eccentric contraction rather than contraction and relaxation (0 to 2 duty cycle) (150). Other studies have implemented duty cycles of 1 to 1(104), 3 to 2 (118; 119), and 5 to 3 (67). In combination with the findings of the present study, the observation that a variety of studies with distinct exercise duty cycles/shear stress patterns have found impaired FMD in at risk populations (10; 34; 38; 51) suggests that the ability of exercise induced FMD to detect endothelial dysfunction is not duty cycle specific.

Sympathetic nervous activity (SNA) has variably been shown to blunt FMD when substantially elevated (31; 59). In the present study, as in others (67; 119), there was a modest elevation in blood pressure and heart rate during HGEX, suggesting that there may have been an increase in SNA. However, previous work has shown that the FMD response to the same mean shear stress stimulus was not different during HGEX (with any associated SNA elevation), vs.

during forearm heating (no elevation in SNA). This suggests that a HGEX induced elevation in SNA is unlikely to influence FMD. Importantly, in the present study, hemodynamic variables were similarly elevated from baseline in all duty cycles (Delta MAP, $p = 0.118$; Delta HR, $p = 0.096$, indicating a similar SNA activation), and any blunting of FMD due to SNA would be unlikely to influence our conclusions regarding the impact of duty cycle. If other duty cycles or exercise intensities used in future research result in greater HR, MAP, or SNA increases than those discussed, concerns regarding an SNA mediated blunting of FMD would need to be revisited.

Mean shear rate profile

AC was performed for 4 minutes to ensure a uniform baseline SR in all subjects (AC baseline) and to allow a rapid increase in SR from baseline to the target. The later is required for a meaningful assessment of response dynamics. The SR of 10 s^{-1} was selected because it was lower than the baseline SR for all subjects. This strategy of AC was first done by Pyke *et al* (119), who also implemented a natural increase in shear stress (no AC) to the same mean magnitude as achieved in the AC condition and found no difference in FMD between the trials. Therefore there is no reason to suspect that the AC employed in the current study influenced the FMD magnitude.

The mean SR during steady state exercise (minutes 2-6) was 73.4 ± 2.2 s^{-1}, which was very close to the 75 s^{-1} target and was not significantly different between duty cycles (Fig. 2). Practically, the 5 to 1 duty cycle offered some advantages. During HGEX, the 5 to 1 duty cycle had less frequent impedance to blood flow, making it easier to target blood velocity. Additionally (see following section) the less frequent contraction improved arm and image stability.

SR during recovery was significantly different between all duty cycles, The 1 to 1 duty cycle had the largest recovery SR likely due to the removal of more significant contraction induced impedance to flow in that condition. Refer to '*SR pattern and the impact of HGEX duty cycle on FMD'* section for a discussion of the potential influence of recovery SR on the calculation of FMD from the diameter measured during recovery.

Shear rate pattern and the impact of handgrip exercise duty cycle on FMD

Each duty cycle produced a different pattern of shear stress. A major difference between the 3 duty cycles was that there were varying amounts of retrograde shear stress being experienced by the brachial artery (Fig. 3). Retrograde shear stress has been shown to be acutely detrimental to endothelial function in vivo (140), ex vivo (156), and in vitro (90). The different patterns in the present study arose from variation in the frequency and intensity of contraction. The magnitude of FMD was not different between duty cycles (Fig. 4), suggesting that mean shear stress (which was matched across duty cycles) is the most important stimulus characteristic influencing FMD. Therefore, although previous research suggests that retrograde shear stress may be detrimental to FMD (140), the range of retrograde shear exposure with HGEX in the present study and others (119) appears to be insufficient to impact FMD. Thijssen *et al* (140) elicited 3 degrees of retrograde SR using forearm cuff inflation for a 30 minute time period. Their results showed that prior exposure to retrograde flow acutely impaired subsequent RH mediated brachial artery FMD; however the experimenters did not see a change in FMD following their retrograde intervention that created the smallest increase in retrograde SR. This suggests that there may be a threshold of retrograde SR that is required to alter endothelial function, or that the impact of retrograde shear in this condition was too small to be acutely

relevant. The % FMD created by different duty cycles in this study was 4.0 %, 4.1 %, and 4.2 % respectively, which were not significantly different and were within the normal range of %FMD found in previous studies on similar populations (67; 118). The observation that there was no significant impact of duty cycle suggests that the range of retrograde SR experienced in the present study may not have crossed this threshold. This is in agreement with previous research identifying that the SR pattern created through HGEX produces an FMD which is not different from that induced during passive limb heating (no retrograde shear) when the same mean shear stress stimulus is experienced in both conditions (119). However, the current study extends these findings by investigating varying patterns of shear stress which included more frequent contractions (and therefore bursts of retrograde shear). The results suggest that duty cycle does not influence FMD magnitude, and thus experimenters have flexibility in choosing the duty cycle with which they are most comfortable. Also, this supports the notion that the results of studies using different duty cycles can be compared.

The current study assessed minute by minute FMD in a subset of 10 subjects. This is an important inclusion because it allows one to identify whether HGEX bouts shorter than 6 minutes would be likely to elicit FMD that is influenced by duty cycle. Similar to the findings at the end of exercise, there was no difference in minute by minute HGEX FMD between duty cycles over the whole 6 minute bout (main effect $p = 0.939$, duty cycle x time interaction $p = 0.545$) (Fig. 5). Subset analysis was required because during HGEX, the arm moves with each contraction. This movement during contraction can cause difficulty in maintaining the clarity of the image, resulting in a failure of the wall tracking program to identify the wall consistently, thus compromising the artery diameter measurement. Erroneous measurements are removed during image analysis; however frequent contraction may inhibit the reappearance of wall clarity

between contractions ('movement artifact'). Movement artifact occurred most frequently in the 1 to 1 duty cycle, suggesting that its use may be impractical for FMD measures made during exercise

Although the image is stable during the recovery period, which is desirable, SR during this period was different between duty cycles (elevated vs. the target in 1 to 1 and lower than the target in 3 to 1 and 5 to 1). This raises the possibility that the 21s recovery diameter was influenced by a distinct shear stress stimulus in each duty cycle; however this in unlikely for several reasons. First, with large changes in shear stress, the mean time delay for dilation and reconstriction has been shown to be 38 and 20 seconds respectively (89), suggesting that the diameter would be unlikely to respond significantly to a modest change in SR within the 20 second recovery period. Second, as stated above, SR increased or decreased during recovery (1 to 1 and 3 to 1/5 to 1 respectively), yet in the subset analysis, the %FMD during the last minute of exercise and %FMD during recovery were not different, suggesting no response to the altered shear stress. This indicates that that diameter measurements made during recovery are an accurate portrayal of the artery diameter during the immediately preceding bout of exercise. Therefore when movement artifact is an issue, diameter measurements made immediately upon recovery provide an additional opportunity to record a clear image. This is in line with other researchers who have also elected to calculate HGEX induced FMD from recovery measurements (51; 104).

The impact of handgrip exercise duty cycle on FMD phase 1 dynamics

Response dynamics provide additional insight into the mechanisms at play during FMD. Current research suggests that the mechanisms which cause the initiation of dilation (Phase 1)

may be different from those which maintain it (Phase 2 and 3) (36; 96; 133). The specific mechanisms responsible for these phases are likely vessel and bed specific (7; 36; 133) and further study is required to tease out the mechanisms involved in each phase in humans.

In the present study, a subset of subjects (n = 10, described previously) were used to analyze dilation onset dynamics and no significant difference between duty cycles was identified (Gain p=0.192, Tau p=0.085, and Time Delay p = 0.889). However, the study was only powered to detect large differences in these variables due to the considerable amount of variation in dynamic parameters. A source of variability may have stemmed from the first minute of HGEX, where the mean shear stress was moderately lower (~4 s^{-1}) than the remaining 5 minutes. This indicates that in some subjects, SR did not immediately rise to the target as anticipated and this stimulus variability may have influenced the dynamics. There was a trend for a faster tau in the 5 to 1 duty cycle. This trend may have been due to the stimulus in this condition. In the first 30 seconds of exercise in the 5 to 1 duty cycle SR was modestly but significantly greater than in the 1 to 1 duty cycle (P = 0.001, data not shown), indicating that the SR rose more quickly during the 5 to 1 condition. However, high sensitivity of tau to the rate of increase of the SR stimulus was not reported by Pyke *et al* (119). In a comparison of HGEX with AC (immediate SR increase) and HGEX without preceding AC (slow increase in SR towards target), there were no observed differences in tau. It is possible that the longer intervals (5s vs. 3s or 1s) of uninterrupted relaxation shear stress influenced the mechanisms at the onset of dilation. However, 1 to 1 and 3 to 1 relaxation duration (and relaxation and contraction shear rates) differed substantially and their mean tau was quite similar. The explanation for and relevance of the trend in tau are unclear, and further research regarding the impact of shear stimulus pattern on dilation dynamics is required.

Limitations

The subjects used in this study were young healthy males, and the observations regarding the impact of duty cycle cannot be generalized to other populations. An older or diseased population may use different mechanisms of dilation, or respond differently to different peak shear rates and amounts of retrograde shear stress. However, this study group was chosen in order to observe a relatively large FMD response which would improve the ability to detect any duty cycle dependent differences in FMD. Future research should investigate the impact of duty cycle in other populations.

All possible duty cycles were not assessed in this study and our results may not be all encompassing. Other studies have used longer and shorter contraction periods; however our focus was on the ratio of contraction to relaxation. In addition, a smaller ratio would likely deteriorate vessel image quality, and a larger ratio may not enable shear stress to elevate to an adequate magnitude. The duty cycles assessed in this study covered most of the ratios of contraction to relaxation used in other studies.

Our study did not include viscosity measurements, a variable which is necessary for the calculation of shear stress. However, in most studies SR is calculated as a surrogate for shear stress (138). Padilla *et al* (105) assessed several magnitudes of FMD normalized to the shear stress stimulus when calculated with either assumed or measured viscosity and found no difference in their results. This suggests that measuring viscosity doesn't meaningfully influence to quantification of the shear stimulus. Supporting this, within this population there is evidence that variability in viscosity is small (47). In addition, in the present study design, all three duty

cycles were measured on both testing days and the presented data is the average across days, therefore any day to day variability in viscosity would not impact our results.

Conclusion

The primary objective of this study was to determine the impact of HGEX duty cycle on FMD magnitude. The major finding was that there was no difference in % FMD when the same mean shear stress magnitude was created with three different duty cycles. The three duty cycles created significantly different patterns of shear stress including distinct anterograde and retrograde shear stress magnitudes. These findings are in agreement with previous research (107; 119) suggesting that the endothelium transduces the mean shear stress stimulus. While the data suggests that there is minimal impact of duty cycle, therefore affording flexibility in experimenter choice, the selection of a 5 to 1 duty cycle may be advantageous due to greater ease in targeting a blood flow velocity, and greater arm and therefore vessel image, stability during exercise.

Chapter 4

General Discussion

Cardiovascular disease is the leading cause of death in North America (126), and considerable attention has been given to the early detection of this disease. Atherosclerosis, which is the buildup of plaque in the arteries, leads to more severe cardiovascular diseases such as angina, peripheral artery disease, stroke, and myocardial infarction. The body's resistance to the progression of atherosclerosis involves the endothelium. The health of this single layer of cells that lines the inside of every artery may determine a large proportion of cardiovascular risk. The onset of a dysfunctional endothelium can be thought of as the first step in the progression of atherosclerosis (127). Historically, the release of the vasodilator NO has been implicated as the reason for the endothelium's vasoprotective nature. A healthy endothelium, which releases NO and other vasodilators, will inhibit platelet aggregation (121; 122) and suppress vascular smooth muscle cell proliferation (40), both of which are steps in the progression of atherosclerosis.

It is the vasodilatory action of the endothelium that allows us to assess its health non-invasively, with greater health corresponding to a greater magnitude of dilation in response to a vasodilatory stimulus. The magnitude of dilation can be observed using echo ultrasound images. Shear stress (the frictional force of blood on the endothelium) is the stimulus used in the Cardiovascular Stress Response Lab (CVSRL- research facility where Chapter 3 study was completed) to induce vasodilation, via a phenomenon known as flow mediated dilation (FMD). The FMD of the brachial artery (highly accessible) correlates with FMD in the coronary arteries (4) (clinical relevance but less accessible), therefore brachial artery FMD can be used to assess cardiovascular risk. Handgrip exercise (HGEX) creates an oxygen demand in the forearm (and

resultant microvascular vasodilation), eliciting an increase in shear stress in the upstream brachial artery, which results in FMD. An advantage of handgrip exercise over other methods of increasing shear stress is that the variation in contraction intensity can be used to control the magnitude of the shear stress stimulus. The use of HGEX for FMD assessment is relatively new, and therefore there are no guidelines for its use. The study described in chapter 3 set out to determine whether different patterns of relaxation to contraction ('duty cycle'), while maintaining the same mean shear stress, elicited a similar FMD response. The primary finding of this study was that there was no significant impact of duty cycle on FMD. There was also no clear impact of duty cycle on the dynamics of the Phase I dilation response. In agreement with previous findings (107; 119), this suggests that the mean shear stress stimulus is the most important determinant of HGEX FMD.

Reconciling differences in pattern data

There are frequently discrepancies between in vitro data, in vivo animal data, and in vivo human data, and this has been the case in the endothelium's response to shear stress (7; 36; 117; 133). There are several reasons why this may occur: 1) Differences may be a reflection of the different vascular beds that are being used. Different vessel beds dilate differently in response to shear stress (7; 133). This may be due to different patterns of shear stress that naturally occur in separate vascular beds. 2) Animals experience shear stress differentially. Animal vessel sizes are often drastically different from human vessel sizes and within humans vessel size is a major factor influencing FMD (92). Smaller animals have different heart rates than humans, which varies the frequency of shear stress peaks. Animal limbs are also used differently from human

limbs; for example a limb that is frequently experiencing static contraction may be exposed to different levels of perfusion than one which is frequently relaxed. 3) In vitro studies don't perfectly replicate the milieu of stimuli or internal environment present in human and animal studies. Therefore, one must be cautious when extrapolating the results of in vitro and animal studies to humans.

As described in chapter 3, a major difference observed between duty cycles was the amount of retrograde (reverse) shear stress and the magnitude of shear stress experienced between contractions. Research indicates that retrograde shear stress acutely impairs endothelial function (140). In addition to acute affects, prolonged periods of retrograde shear stress are detrimental to arterial function. In areas where there are high levels of oscillatory shear stress in the arteries, there is a higher prevalence of atherosclerosis (76). This prolonged exposure to oscillatory shear stress likely activates different pathways in the endothelial cells (63; 97). However, in the study described in chapter 3, despite significant differences in shear pattern, the magnitude of FMD was not different. A reasonable conclusion is that the magnitude or duration of retrograde shear stress experienced in the current study was not great enough to influence FMD. Although the chapter 3 study did not include a zero retrograde control group (thus in theory its possible that FMD was affected similarly by retrograde shear in all duty-cycles), a study by Pyke *et al* (119) found that HGEX induced FMD, with retrograde shear stress with the range that we tested, was no different than hand heating induced FMD (no retrograde shear).

Future directions of exercise induced FMD research

Whole body aerobic exercise is protective against cardiovascular disease by about 40 % more than can be attributed to improvements in traditional risk factors alone (49). Some researchers have suggested that the 'risk factor gap' is at least partially closed by an improvement in endothelial function (69). Endothelial function is also an independent risk factor for cardiovascular disease (35; 153; 154) indicating that it covers at least part of this risk factor gap. The utilization of HGEX to test FMD eliminates concerns regarding reactive hyperemia (RH) shear stress stimulus adaptations that can occur with training, and impairments in the stimulus that occur with disease (i.e. with HGEX induced increases in shear stress it can be ensured that FMD is tested with the same stimulus throughout a training program and/or between groups) . Further studies with HGEX FMD may help us more clearly study extent to which conduit artery endothelial function covers this risk factor gap.

Although there are studies demonstrating the clinical relevance of HGEX FMD (34; 51), and studies supporting the relevance of other sustained shear stress stimuli (10; 38), HGEX lags far behind RH in research quantity and quality. Studies in subjects who have type 1 diabetes have shown a reduction in FMD with sustained shear stress stimuli which is more pronounced than RH in magnitude (51) and more sensitive than RH mediated FMD at detecting dysfunction (10). However, in contrast to RH mediated FMD, the use of HGEX to increase shear stress was not successful in detecting a change in FMD in response to an acute high fat meal (104). The mechanisms behind HGEX induced dilation are shown to be at least in part due to NO (151), suggesting that HGEX likely does convey clinically relevant information. The ability of HGEX FMD to detect endothelial dysfunction must be investigated in a range of populations and the

ability of HGEX FMD to predict the development of cardiovascular disease and the incidence of cardiovascular events must be established.

Dilation dynamics may provide additional clinically relevant information that is independent of FMD magnitude. Separate phases of dilation likely indicate separate mechanisms of dilation being activated (36; 96; 133). If this is true, then a delayed or reduction in magnitude of a particular phase could help to isolate a problem with a particular mechanism. However, the measurement of dilation dynamics is complicated due to a large variability in responses making it difficult to detect differences (demonstrated in chapter 3) and there is variability in the number of phases, even in a healthy population (117). In order to counteract this variability, multiple trials may be averaged in the same subject. In addition, it appears that frequently one phase of dilation is not completed before the next phase begins, and in order to measure the completion of the second or final stage, a very long bout of exercise would be required. Most handgrip exercise protocols are relatively short (51; 67; 118), and phase 1 likely accounts for majority of the FMD (73; 119). While not without its challenges, the quantification of phase 1 dynamics may have the most potential for investigation because it can be fully captured in a 5 minute bout of HGEX. Future studies are needed to determine whether phase 1 dynamics provide any important information about endothelial function or vascular health that is independent from FMD.

Barriers to widespread use of handgrip exercise FMD

Handgrip exercise induced FMD may not be for everyone. The HGEX FMD performed by our group requires a handgrip dynamometer, echo and Doppler ultrasound, various computer programs, and the skills to operate each of these. Many research groups do not have the

ultrasound technology that allows for the simultaneous measurement of Doppler and echo ultrasound signals, which is important for quantifying or targeting shear stress (138). Also, some populations may not be able to contract hard enough to elevate blood flow, or the act of handgrip exercise may be painful. In the chapter 3 study, subjects contracted for 6 minutes, which may not be sustainable in some populations. As described above, the clinical relevance of HGEX is yet to be fully explored and differing durations of exercise or duty cycles may provide different clinically relevant information. While emerging evidence indicates that HGEX may be a powerful research tool, the sophisticated equipment and operator requirements, and the barriers to application in some groups will limit its universal adoption.

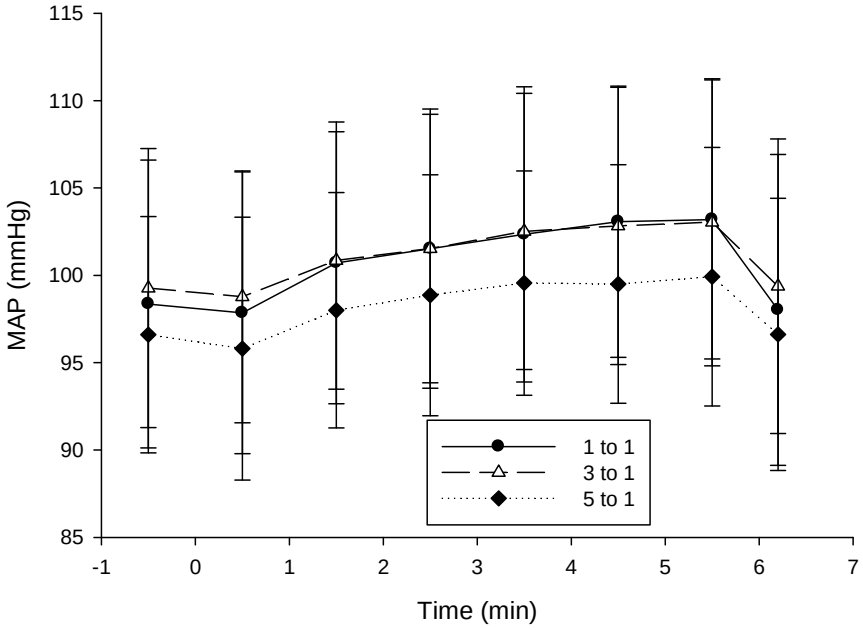
Conclusion

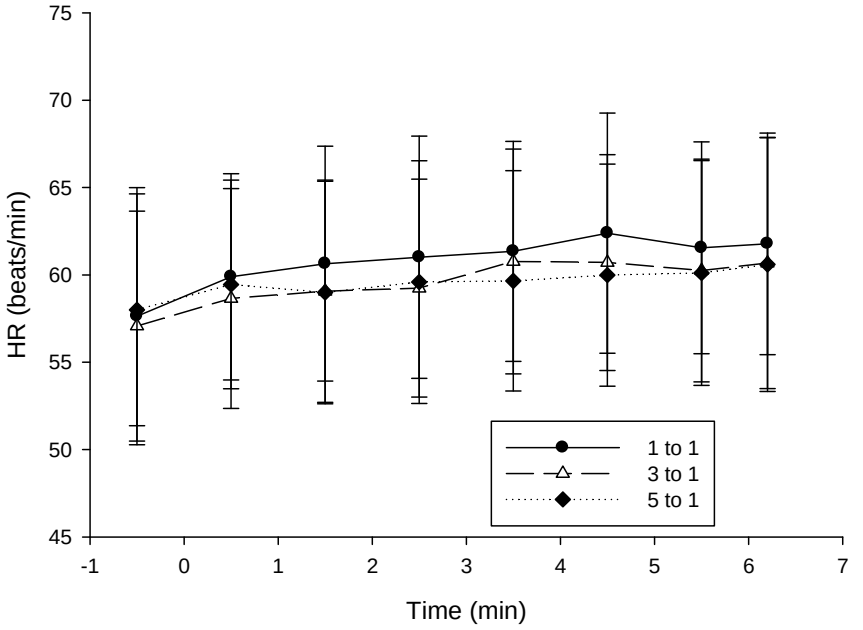
The study described in this thesis was the first to investigate the impact of different duty cycles of relaxation to contraction on the resultant HGEX FMD. We determined that there is no impact of duty cycle on FMD, supporting the position that the endothelium responds to the mean shear stress stimulus. Based on the results of this study our group has adopted the use of a 5 to 1 duty cycle for continuing research. This duty cycle allows for an accurate measure of artery diameter during HGEX because the longer periods of relaxation allow an experimenter to obtain a movement-free image for a longer period of time. The observations made while conducting this study indicate that future research should avoid the use of a duty cycle with frequent contraction (less than 3 seconds of relaxation) in order to assess diameter during exercise. However, if more frequent contraction is required, diameter measurements made immediately upon recovery can provide an accurate measurement of FMD stimulated by the

shear stress stimulus during exercise. Endothelial function is an independent risk factor which can be assessed through the use of HGEX FMD. This is a relatively new technique which requires more research in order to gain widespread acceptance. However, due to the advantage of stimulus control that accompanies HGEX FMD, it is likely that broader adoption of this technique will facilitate research which improves our understanding of the endothelium's response to shear stress.

Appendix A

Heart Rate and Mean Arterial Pressure



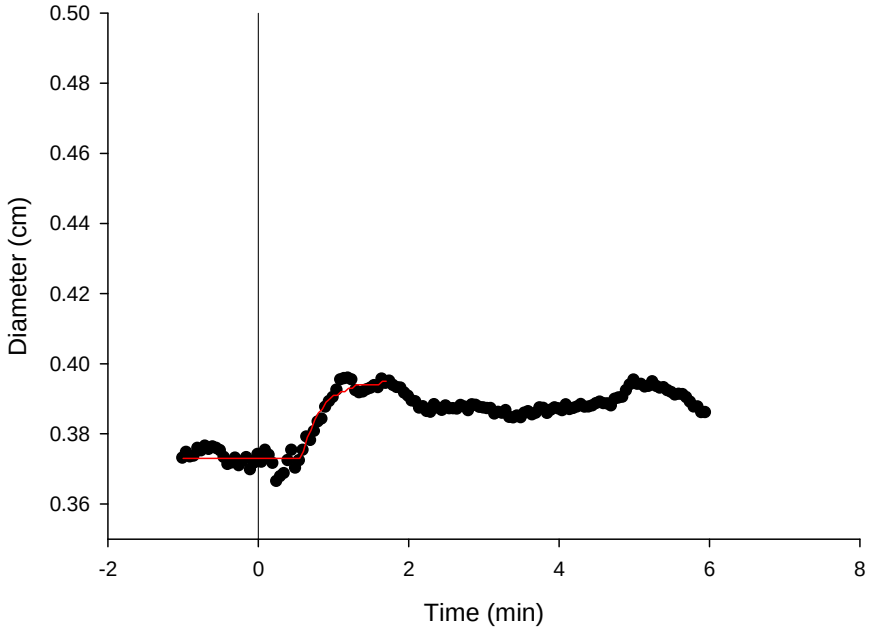


One minute average heart rate and mean arterial pressure in all subjects from baseline to recovery. There was no significant difference between duty cycles at any time point. There was a main effect of time. HR = heart rate, MAP = mean arterial pressure.

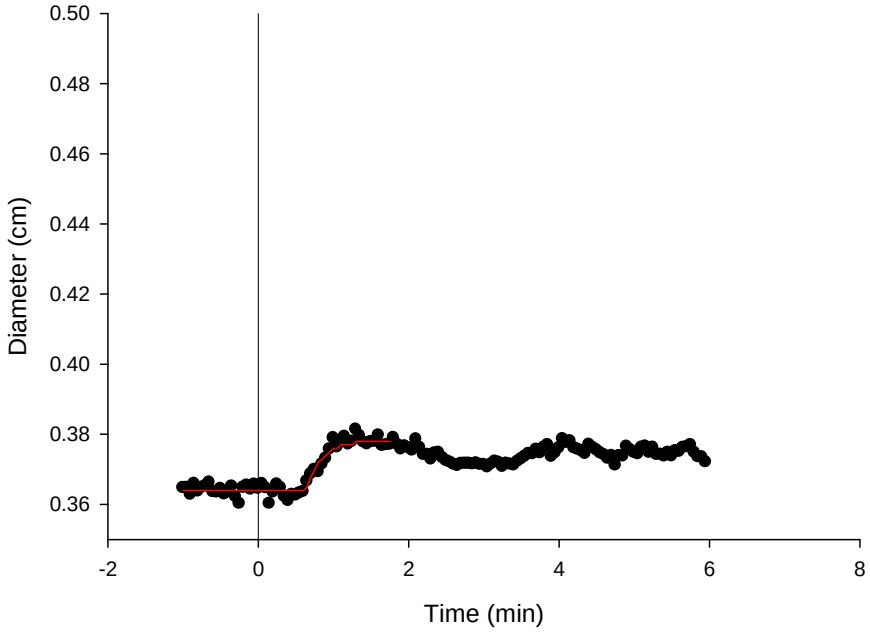
Appendix B

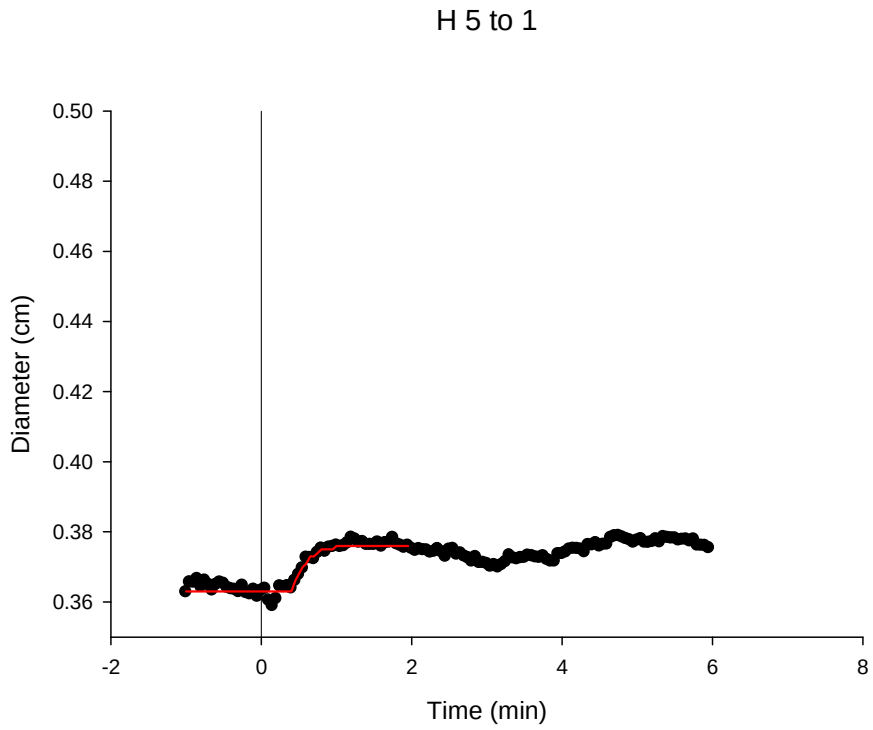
Representative Curve Fits

H 1 to 1


0.50
0.48
0.46
0.44
0.42
0.40
0.38
0.36
Diameter (cm)
-2
0
2
4
6
8
Time (min)

H 3 to 1

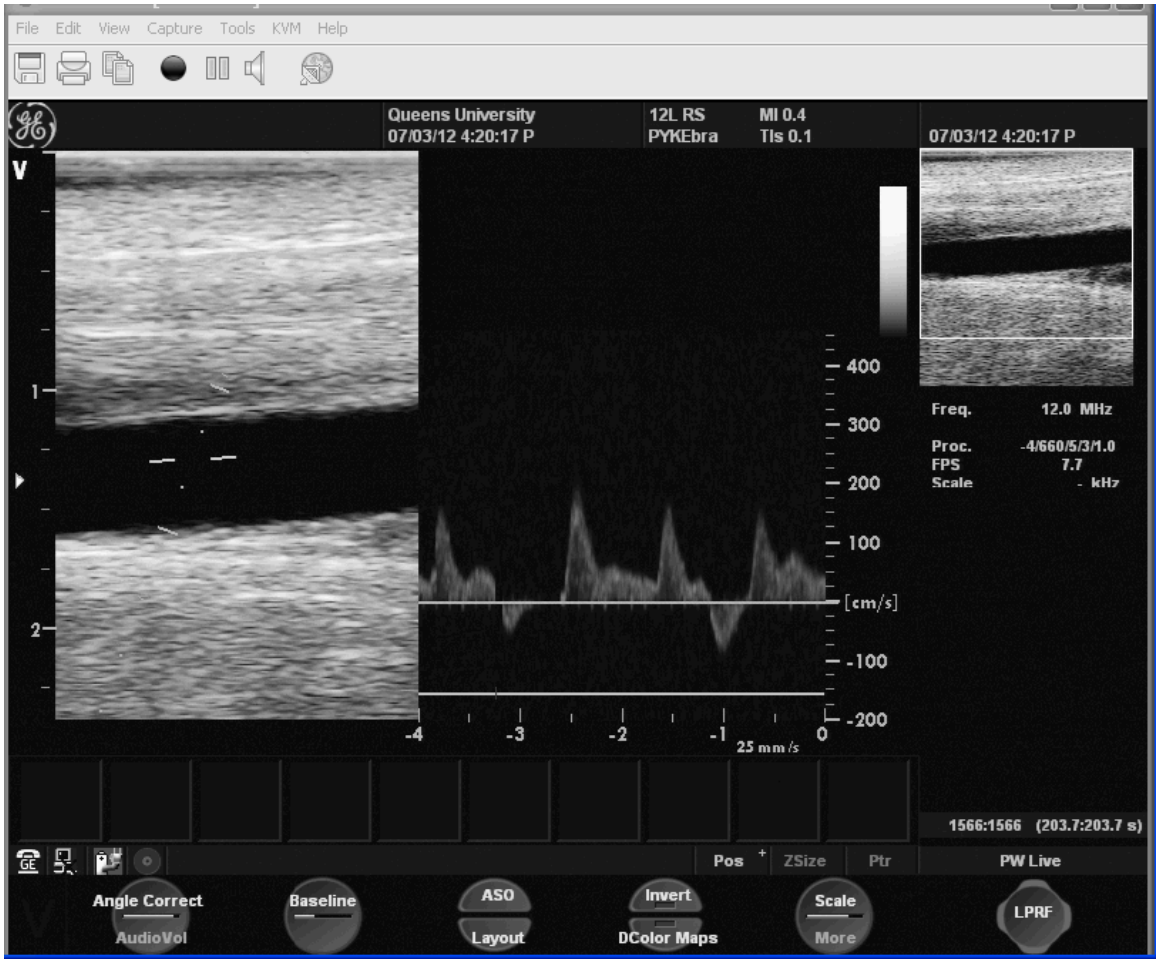

0.50
0.48
0.46
0.44
0.42
0.40
0.38
0.36
Diameter (cm)
-2
0
2
4
6
8
Time (min)



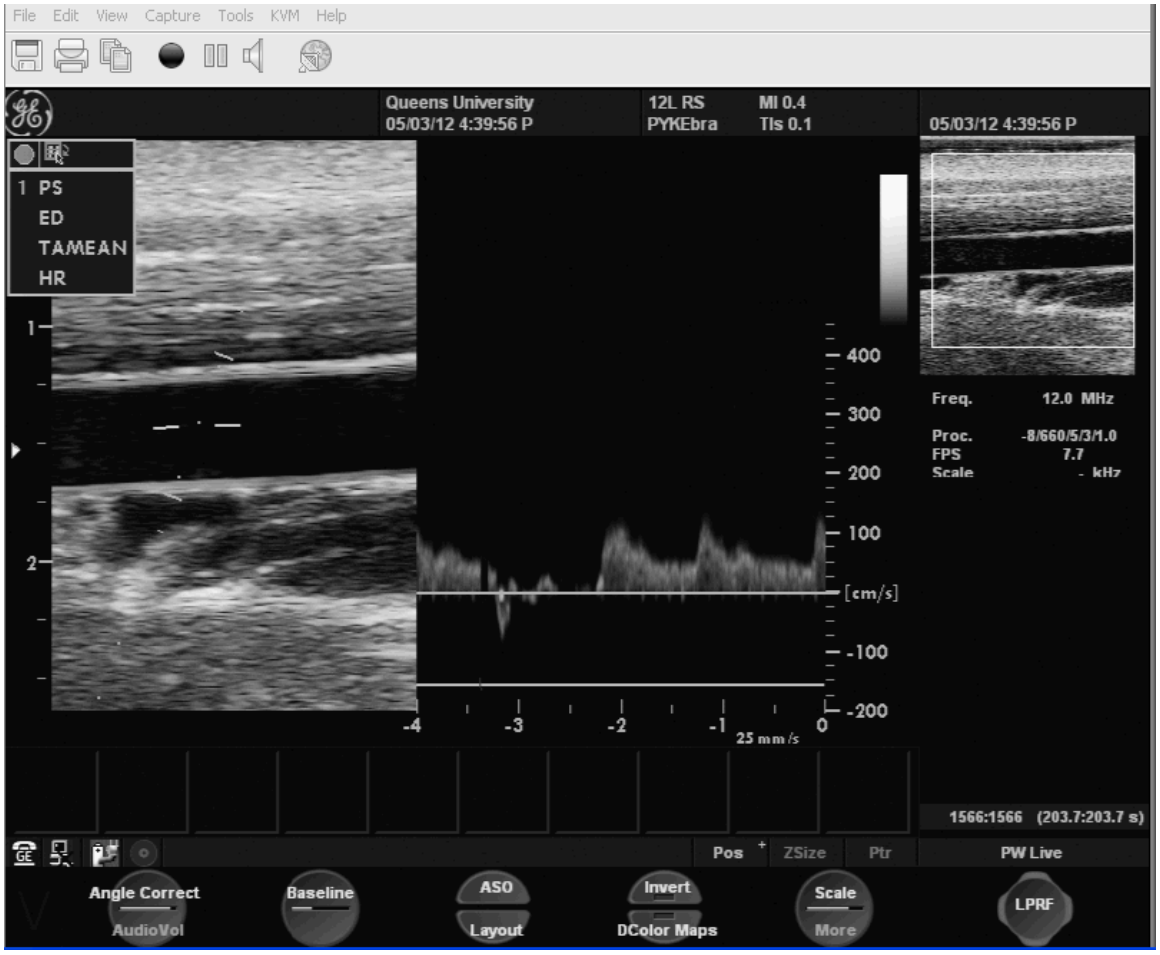
Representative subject (H) diameter measurements (3 second averages) are graphed over time during baseline and 6 minutes of exercise. Red line represents curve fit created by curve fitting program. Black line indicates exercise onset.

Appendix C

Representative Echo Ultrasound Images



Example of an echo ultrasound image with movement artifact (left side of screen). There are no clear inner walls. Right side of the screen displays blood velocity (Doppler ultrasound).



Example of an echo ultrasound image without movement artifact (left side of screen). Inner walls are clearly present. Right side of the screen displays blood velocity (Doppler ultrasound).

Appendix D

Example 1 way RM ANOVA Statistical Output

One Way Repeated Measures Analysis of Variance Friday, August 10, 2012, 1:23:42 PM

Data source: Nadir Stats in Retrograde SR

Normality Test (Shapiro-Wilk) Passed (P = 0.413)

Equal Variance Test: Passed (P = 0.421)

Treatment Name	N	Missing	Mean	Std Dev	SEM
SR 1 to 1	16	0	-89.614	36.875	9.219
SR 3 to 1	16	0	-96.637	27.839	6.960
SR 5 to 1	16	0	-111.524	28.958	7.239

Source of Variation	DF	SS	MS	F	P
Between Subjects	15	41223.408	2748.227		
Between Treatments	2	4005.385	2002.692	17.793	<0.001
Residual	30	3376.603	112.553		
Total	47	48605.396			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001). To isolate the group or groups that differ from the others use a multiple comparison procedure.

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
SR 1 to 1 vs. SR 5 to 1	21.910	3	8.261	<0.001	Yes
SR 1 to 1 vs. SR 3 to 1	7.023	3	2.648	0.164	No
SR 3 to 1 vs. SR 5 to 1	14.887	3	5.613	0.001	Yes

Appendix E

Example 2 way RM ANOVA Output

Two Way Repeated Measures ANOVA (Two Factor Repetition) Tuesday, August 14, 2012, 4:10:06 PM

Data source: only perf and good stats in time course of dilation graphs

Balanced Design

Dependent Variable: FMD

Normality Test (Shapiro-Wilk) Passed (P = 0.955)

Equal Variance Test: Passed (P = 0.873)

Source of Variation	DF	SS	MS	F	P
Subject	9	163.812	18.201		
duty cycle	2	0.775	0.388	0.0636	0.939
duty cycle x Subject	18	109.715	6.095		
time	5	246.255	49.251	67.812	<0.001
time x Subject	45	32.683	0.726		
duty cycle x time	10	2.523	0.252	0.890	0.545
Residual	90	25.507	0.283		
Total	179	581.271	3.247		

The difference in the mean values among the different levels of duty cycle is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in time. There is not a statistically significant difference (P = 0.939).

The difference in the mean values among the different levels of time is greater than would be expected by chance after allowing for effects of differences in duty cycle. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of duty cycle does not depend on what level of time is present. There is not a statistically significant interaction between duty cycle and time. (P = 0.545)

Power of performed test with alpha = 0.0500: for duty cycle : 0.0500
Power of performed test with alpha = 0.0500: for time : 1.000
Power of performed test with alpha = 0.0500: for duty cycle x time : 0.0500

Least square means for duty cycle :

Group	Mean
1 to 1	3.462
3 to 1	3.342
5 to 1	3.310

Std Err of LS Mean = 0.319

Least square means for time :

Group	Mean
0.500	0.810
1.500	3.501
2.500	3.786
3.500	3.786

4.500 4.125
5.500 4.220
Std Err of LS Mean = 0.156

Least square means for duty cycle x time :

Group	**Mean**
1 to 1 x 0.500	0.693
1 to 1 x 1.500	3.619
1 to 1 x 2.500	4.176
1 to 1 x 3.500	3.873
1 to 1 x 4.500	4.185
1 to 1 x 5.500	4.228
3 to 1 x 0.500	0.907
3 to 1 x 1.500	3.418
3 to 1 x 2.500	3.695
3 to 1 x 3.500	3.784
3 to 1 x 4.500	4.119
3 to 1 x 5.500	4.132
5 to 1 x 0.500	0.831
5 to 1 x 1.500	3.465
5 to 1 x 2.500	3.488
5 to 1 x 3.500	3.703
5 to 1 x 4.500	4.071
5 to 1 x 5.500	4.301

Std Err of LS Mean = 0.168

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **time**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
5.500 vs. 0.500	3.410	6	21.915	<0.001	Yes
5.500 vs. 1.500	0.720	6	4.624	0.024	Yes
5.500 vs. 2.500	0.434	6	2.788	0.374	No
5.500 vs. 3.500	0.434	6	2.787	0.375	Do Not Test
5.500 vs. 4.500	0.0953	6	0.613	0.998	Do Not Test
4.500 vs. 0.500	3.315	6	21.302	<0.001	Yes
4.500 vs. 1.500	0.624	6	4.012	0.070	No
4.500 vs. 2.500	0.339	6	2.176	0.642	Do Not Test
4.500 vs. 3.500	0.338	6	2.175	0.642	Do Not Test
3.500 vs. 0.500	2.976	6	19.128	<0.001	Yes
3.500 vs. 1.500	0.286	6	1.837	0.784	Do Not Test
3.500 vs. 2.500	0.000172	6	0.00111	1.000	Do Not Test
2.500 vs. 0.500	2.976	6	19.127	<0.001	Yes
2.500 vs. 1.500	0.286	6	1.836	0.784	Do Not Test
1.500 vs. 0.500	2.690	6	17.291	<0.001	Yes

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs.

1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

Appendix F

Consent Form

School of Kinesiology and Health Studies

Queen's University

Kyra E. Pyke, Ph.D., Principle Investigator

Study performed in Room 400D, School of Kinesiology and Health Studies

CONSENT FORM

FOR RESEARCH PROJECT ENTITLED:

The effect of Handgrip exercise duty cycle on brachial artery flow mediated dilation

This is an important form. Please read it carefully. It tells you what you need to know about this study. If you agree to take part in this research study, you need to sign this form. Your signature means that you have been told about the study and what the risks are. Your signature on this form also means that you want to take part in this study.

Purpose of the Study:

You are being invited to participate in a research study directed by Dr. Kyra Pyke to evaluate the variability of the arterial response to different patterns of increased blood flow. Dr. Pyke or a graduate student investigator will read through this consent form with you and describe the procedures in detail and answer any questions you may have. This study has been reviewed for ethical compliance by the Queen's University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board.

The purpose of the study is to 1) examine the variability in the magnitude of brachial artery (large artery in the upper arm) widening (dilation) following three different patterns of increased blood flow

Benefits For You:

There are no direct benefits to you by participating in this study.

Description of Experiment and Risks:

What will happen? During this study, you will take part in the specific experimental procedures outlined below.

HEART RATE MEASUREMENTS:

Heart rate is continuously monitored by an electrocardiogram (EKG) through 6 spot electrodes on the skin surface. The electrodes are placed on the chest and abdomen and they can detect the electrical activity that makes your heart beat.

RISKS: This procedure is entirely safe. In a very small group of individuals, a skin rash might occur from the adhesive on the electrodes. There is no way of knowing this ahead of time. The rash, if it develops, will resolve itself within a day or so. Avoid scratching the rash and keep clean.

BLOOD PRESSURE MEASUREMENTS:

A small cuff is fit around your finger. This cuff inflates to pressures that match the blood pressure in your finger, so you feel the cuff pulsing with your heart beat. It shines infrared light through your finger to measure changes in the size of your finger with each heart beat.

RISKS: This technique is non-invasive and poses no risk.

LIMB BLOOD FLOW AND BLOOD VESSEL DIAMETER MEASUREMENTS: The blood flowing through your brachial (above the elbow) artery can be detected, and your artery size measured using Doppler and imaging ultrasound. A probe will be placed on the skin over your artery and adjustments in its position will be controlled by hand by the investigator. High frequency sound (ultrasound) will penetrate your skin. The returning sound provides information on blood vessel size and blood flow.

RISKS: This technique is non-invasive and poses no risk.

HANDGRIP EXERCISE: You will be asked to perform handgrip squeezing exercise. You will be asked to perform maximal contractions (duration ~2s) and up to 4 6 min of rhythmic contractions at an intensity between 10-70% of your maximal force.

RISKS You may experience muscle soreness in the muscles of your forearm for 24-72 hours after performing the handgrip exercise, much as you would if you had been lifting weights.

ARTERIAL PULSE COMPRESSION- To ensure that blood flow starts at the same level in every subject, an experimenter will apply pressure with their finger on your brachial pulse at your elbow to control blood flow into your forearm for 4-8 min prior to the initiation of exercise. The arterial pulse compression should cause no discomfort. If there is pain, immediately notify the investigator and the compression will be adjusted or removed. At the end of the ~8 min the investigator will remove the compression and there will be an increase in blood flow through your brachial artery as you begin exercising.

RISKS: This technique is non-invasive and poses no risk.

FINGER PRICK BLOOD TEST: To measure your fasting blood glucose and cholesterol levels small lancet (sharp edge) will be placed over your finger tip and depressed to draw a drop of blood. This blood will be collected with a thin tube and used to measure the amount of sugar and cholesterol in your blood.

RISKS: Brief discomfort will be associated with the finger prick itself and an a modest amount of soreness may persist at the site for 1-2 days. As with any break in the skin there is risk of infection although it is very small in this case. This skin will be cleaned prior to the finger prick and a bandaid will be applied immediately following collection of blood.

[redacted]

7 DAY PHYSICAL ACTIVITY RECAL: This is a questionnaire that will ask you to report your physical activity levels over the past 7 days.

RISKS- This poses no risk

How long will it take?

On an initial screening visit you will be asked to lie down while we will use ultrasound to get an image of the artery in your upper arm to make sure that we can get clear pictures. You will also be asked to perform 3 maximal voluntary contractions (separated by 1 min rest) and some repeated handgrip contractions to allow you to practice and to allow us to determine the right exercise intensity for the subsequent visits. This visit will take approximately 20-30 min.

 Visits 2 and 3 will each take a maximum of 3 h. While lying down and resting, you will be instrumented for heart rate, blood pressure and blood flow (ultrasound) measurements. After a 30 min rest period you will undergo 3 trials of handgrip exercise with different contraction to relaxation cycles (e.g. 1s of contraction followed by 1-5s of relaxation). Your blood flow and vessel diameter will be measured with ultrasound throughout. A minimum of 10 min of rest will occur between each trial. After the completion of the exercise trials the finger prick blood test will be performed.

Talking and Movements:

Talking or moving during the times that we are taking measurements will cause variations in the measurements we are making If you have any discomfort, please let us know immediately and we can temporarily break from data collection. However, if everything is comfortable, please maintain a very quiet posture. Even very slight movements interfere with our experiments.

Special Instructions:

Participants are asked to not exercise, eat, drink alcohol or caffeine during the 12 hours prior to the study. You should empty your bladder immediately prior to starting the test. When the study is finished, we will have you sit in the laboratory for a short time to allow you to readjust to the upright posture. These precautions should be enough to prevent any sensations of dizziness. Please be aware that sensations of dizziness are not normal and you should let us know if you experience any discomfort before you leave the laboratory.

Attached Medical Screening Form:

This questionnaire asks some simple questions about your health. This information is used to guide us with your entry into the study. Current health problems indicated on this form which are related to cardiovascular diseases exclude you from the study.

Safety Precautions:

Safety precautions for the study will include the following:

- Before entering the study you will be screened using a medical screening form. You will not be able to enter the study if anything is found which indicates that it is dangerous for you to participate.

- We will continuously monitor your heart rate and blood pressure, and you will be laying on your back . These precautions allow us to quickly identify if you are experiencing an unusual response and simply stopping the experimental manipulation will allow you to quickly recover.

Confidentiality:

All information obtained during the course of the study is strictly confidential and will not be released in a form traceable to you, except to you and your personal physician. Your data and any personal health information reported on the health questionnaire, will be kept in locked files which are available only to the investigators and research assistants who will perform statistical analysis of the data. There is a possibility that your data file, including identifying information, may be inspected by officials from the Health Protection Branch in Canada in the course of carrying out regular government functions. The study results will be used as anonymous data for scientific publications and presentations, or for the education of students in the School of Kinesiology and Health Studies at Queen's University.

Study Compensation

You will receive $10 per hour of your time in the laboratory for expenses and imposition on your time incurred by your participation in this study.

Freedom to Withdraw from the Study

Your participation in this study is voluntary. You may refuse to participate or you may discontinue participation at any time during the duration of the study without penalty and without affecting your future medical care.

Subject Statement and Signature Section

I have read and understand the consent form for this study. I have had the purposes, procedures and technical language of this study explained to me. I have been given sufficient time to consider the above information and to seek advice if I choose to do so. I have had the opportunity to ask questions which have been answered to my satisfaction. I am voluntarily signing this form. I will receive a copy of this consent form for my information.

If at any time I have further questions, problems or adverse events, I will contact:

Kyra E. Pyke, Ph.D.

pykek@queensu.ca

(Principal Investigator)

Room 301C, School of Kinesiology and Health Studies 28 Division st.

Queen's University, Kingston, ON, K7L 3N6

Tel: (613) 533-6000, ext, 79631

Jean Cote

Department head

Undergraduate/Graduate office, School of Kinesiology and Health Studies

Queen's University, Kingston, ON, K7L 3N6

Tel: (613) 533-6601

If I have any questions concerning research subject's rights, I will contact:

Dr. Albert F. Clark, Chair of the Queen's University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board

Office of Research Services

Fleming Hall, Jemmett Wing 301

Queen's University, Kingston, ON, K7L 3N6

Tel: (613) 533-6081

By signing this consent form, I am indicating that I agree to participate in this study.

_____	_____
Subject Signature	Person obtaining consent Signature
_____	_____
Subject Name (please print)	Person obtaining consent Name (please print)
_____	_____
Date (day/month/year)	Date (day/month/year)

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