THE IMPACT OF BASELINE ARTERY DIAMETER ON HUMAN FLOW-MEDIATED VASODILATION: A COMPARISON OF BRACHIAL AND RADIAL ARTERY RESPONSES TO MATCHED LEVELS OF SHEAR STRESS

by

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A thesis submitted to the School of Kinesiology and Health Studies

In conformity with the requirements for

the degree of Master's of Science

Queen's University

Kingston, Ontario, Canada

(September, 2010)

Abstract

Flow-mediated dilation (FMD) can be used to assess the risk of atherosclerosis; however, an inverse relationship between vessel size and FMD has been identified using reactive hyperemia (RH) to create a shear stress (SS) stimulus in human conduit arteries. RH creates a transient and uncontrolled SS stimulus that is inversely related to baseline arterial diameter. It is therefore unclear whether differences in FMD between groups with non-uniform artery sizes are indicative of differences in vascular health or due to the creation of a greater SS stimulus in smaller vessels. Unlike RH, exercise can effectively create sustained and controlled increases in conduit artery SS. The purpose of this study was to compare the FMD responses of two differently sized upper limb arteries (brachial (BA) and radial artery (RA)) to matched graded levels of SS. Using exercise, three distinct sustained shear rate stimuli were created ((SR)=blood flow velocity/vessel diameter; estimate of SS) in the RA and BA. Artery diameter and mean blood flow velocity were assessed with echo and Doppler ultrasound respectively in 15 healthy male subjects (19-25yrs). Data are means ±SE. Subjects performed 6-min each of adductor pollicis and handgrip exercise to increase SR in the RA and BA respectively. Exercise intensity was modulated to achieve uniformity of SR between the RA and BA at three SR targets ($40s^{-1}$, $60s^{-1}$, $80s^{-1}$). Three distinct SR levels were successfully created (steady state exercise: $39.8\pm0.6s^{-1}$, $57.3\pm0.7s^{-1}$, $72.4\pm1.2s^{-1}$; $p<0.001$ between SR levels). The %FMD at the end of exercise was greater in the RA vs. BA (SR40 RA: 5.4±0.8%, BA: 1.0±0.2%; SR60 RA: 9.8±1.0%, BA: 2.5±0.5%; SR80 RA: 15.7±1.5%, BA: 5.4±0.7%; $p<0.001$). The mean slope of the within-subject FMD-SR dose-response regression lines was significantly greater in the RA (RA: 0.33±0.04, BA: 0.13±0.02; $p<0.001$) and a strong within-subjects relationship between FMD and SR was observed in both arteries (RA r^2: 0.92±0.02; BA r^2: 0.90±0.03). These findings suggest that the response to SS is

not uniform across differently sized vessels, which is in agreement with previous RH studies. Future research is required to investigate the potential mechanisms that mediate the functional differences observed between differently sized vessels.

Acknowledgements

First, I would like to thank my supervisor Dr. Kyra Pyke for her guidance and support. Dr. Pyke was always encouraging, understanding and happy to answer my sometimes very silly questions. Her enthusiasm for research and her faith in my abilities has helped me develop as a researcher and has made my time as a graduate student both pleasurable and unforgettable. I had the honour of being Dr. Pyke's first graduate student and I honestly could not have asked for a better supervisor.

I would also like to thank my colleague and good friend, Jen. Without Jen, this project would not have been possible. I could always count on Jen to be there for data collection and her incredible sense of humour helped me get through the discouraging times I faced during this project. I would also like to thank all the Cardiovascular Stress Response Lab volunteers, Keith, Ingrid, Trevor, and the graduate students of the Vascular Control Lab for enthusiastically assisting with data collection and for bringing laughter into the lab when it was most needed. A special mention goes out to Morgan and Kaitlyn who bravely became cardiovascular graduate students for one day each to help me with data collection when no one else could.

Additionally, I would like to thank all my friends, especially Marta who I could always rely on for a pep talk, and my Dad for always believing in me. Finally, I'd like to thank Mark for putting up with me when this project took over my life and mostly, for his beautiful arteries, which he was always willing to let me use for my research.

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Chapter 1

Introduction

Initially believed to be simply a physical barrier between blood and the vessel lumen, the endothelial cells that line the arteries are now known to be critically involved in the maintenance of vascular homeostasis. The endothelium senses changes in hemodynamic forces acting against the vessel wall and in response, releases vasoactive substances including nitric oxide (NO) and prostacyclin (PGI_2) (Buga *et al.*, 1991;Okahara *et al.*, 1998). The endothelium also has important antiatherogenic properties including acting as an antithrombotic vascular lining, inhibiting smooth muscle cell proliferation, and deterring platelet adherence and aggregation to the vessel wall (Celermajer, 1997;Cooke & Dzau, 1997;Rubanyi, 1993). Therefore, the endothelium plays an important role in both modulating vascular smooth muscle tone and maintaining vascular health. In 1986, the link between the endothelium and vascular health was identified and since then, a great deal of research has been carried out to further understand endothelial cell function (Ludmer *et al.*, 1986).

Shear stress is the tangential force acting against the vessel wall as a result of blood flow and is an important mechanical stimulus for the release of vasoactive substances from the endothelium (Davies & Tripathi, 1993). Shear stress is directly proportional to the blood flow velocity and blood viscosity and is inversely related to artery diameter (Gnasso *et al.*, 2001). Increases in shear stress trigger a cascade of events

that lead to the release of vasodilators (e.g. NO, endothelial-derived hyperpolarizing factor (EDHF) and PGI_2) by the endothelium, which subsequently causes vasodilation (Celermajer, 1997;Davies, 2009). This mechanism is termed endothelial-dependent flow-mediated dilation or FMD. By detecting changes in shear stress levels and modulating vascular tone accordingly, the endothelium may function to maintain vessel wall shear stress within physiological limits (Koller *et al.*, 1993).

Endothelial dysfunction is characterized by a change in the availability and/or activity of endothelial vasoactive factors including NO and PGI_2. Functionally, this results in an impaired FMD response (Celermajer *et al.*, 1992;Corretti *et al.*, 1995;Drexler *et al.*, 1989). NO and PGI_2 have important antiatherogenic properties such as inhibiting platelet aggregation and adherence to the vessel wall (Celermajer, 1997;Cooke & Dzau, 1997;Oemar *et al.*, 1998;Rubanyi, 1993). Thus, endothelial dysfunction also leads to a reduction in vasoprotection, which may accelerate the formation of atherosclerotic lesions. Supporting its role in the pathogenesis of atherosclerosis, endothelial dysfunction precedes the development of cardiovascular disease and is frequently identified in asymptomatic individuals with risk factors for atherosclerosis, such as cigarette smoking and hypercholesterolaemia (Celermajer *et al.*, 1992;Celermajer *et al.*, 1993). Endothelial function is further impaired in individuals with diagnosed coronary artery disease (CAD) (Celermajer *et al.*, 1992). Identification of endothelial dysfunction, which manifests itself as impaired FMD, therefore has the

potential to be used as a predictive tool for assessing risk of developing cardiovascular disease (Yeboah *et al.*, 2009) .

The reactive hyperemia (RH) test is currently the most common method of assessing FMD in humans and uses a temporary forearm occlusion via a sphygmomanometric cuff (Corretti *et al.*, 1995;Pyke & Tschakovsky, 2005). Occlusion of the forearm vessels creates an ischemic environment, evoking vasodilation and thus, a reduction in forearm vascular resistance. When the cuff is deflated, brachial artery blood flow velocity is immediately and briefly increased (due to the downstream dilation of the forearm vessels) and then decays over a period of ~1 minute. This leads to an increase in shear stress in the brachial artery and as a result, the vessel dilates (Berry *et al.*, 2000;Corretti *et al.*, 2002;Pyke & Tschakovsky, 2005;Korkmaz & Onalan, 2008). The shear stress stimulus created with RH is transient and uncontrolled. As such, the magnitude of the shear stress imparted on the vessel cannot be maintained or easily manipulated via this methodology. This is a serious limitation of RH (Pyke *et al.*, 2004).

Exercise has recently been explored as an alternative method of increasing shear stress in order to assess FMD (Pyke *et al.*, 2008b). With exercise, a rapid reduction in the vascular resistance in the muscle bed is created, which leads to a sustained increase in upstream conduit artery blood flow and shear stress (Delp & Laughlin, 1998;Shoemaker *et al.*, 1998). The magnitude of the shear stress stimulus is dependent on the exercise intensity with higher exercise intensities eliciting a greater stimulus (Pyke *et al.*, 2008b). Exercise is the most common cause of significant shear stress increases in conduit

arteries in vivo and therefore, exercise induced increases in shear stress are a highly physiologically relevant stimulus for FMD (Duffy *et al.*, 1999;Gielen & Hambrecht, 2001;Gielen *et al.*, 2001). Handgrip exercise induced increases in shear stress have been used to examine brachial artery FMD in a limited number of studies (Grzelak *et al.*, 2010;Padilla *et al.*, 2006;Pyke *et al.*, 2008b) and this methodology presents several distinct advantages over the traditional RH technique: 1) by manipulating the exercise intensity, shear stress can be controlled at specific magnitudes, and a uniform shear stress stimulus can be achieved over multiple trials both within and between subjects (Jazuli *et al.*, 2010). This facilitates comparison of FMD responses and eliminates the need for post hoc efforts to account for the stimulus magnitude; 2) the capacity for stimulus control permits measurement of FMD responses over a specific range of shear stress stimuli for an investigation of the shear stress stimulus-FMD response relationship. This is important because although several studies have reported a positive relationship between shear and FMD (Leeson *et al.*, 1997;Padilla *et al.*, 2009;Pyke & Tschakovsky, 2007;Pyke *et al.*, 2008b), the characteristics of the relationship (e.g. the presence of a threshold stimulus and the slope) and the factors that affect those characteristics (e.g. disease or vessel size) remain incompletely understood; and 3) while it can be difficult to precisely identify the portion of the transient RH stimulus that is relevant to FMD, the sustained shear stress stimulus created with exercise is easily quantified.

One important unresolved issue in endothelial research is the impact of baseline diameter on FMD responses. Multiple studies have reported a strong inverse relationship

between baseline vessel diameter and the FMD response both within an artery and between vessels with different diameters (Herrington *et al.*, 2001;Silber *et al.*, 2001;Thijssen *et al.*, 2008). Specifically, small arteries tend to show a larger FMD response magnitude relative to larger vessels. As a result, it can currently be difficult to compare the health of endothelial cells in groups with varying baseline artery sizes (for example, the young vs. the elderly and men vs. women) because artery size remains a confounding factor. For example, if a smaller FMD response magnitude is found in a group with a larger average baseline artery diameter, it is unclear if this observation is indicative of endothelial dysfunction in this group or if it is related to benign differences related to baseline artery size. It is possible that there are intrinsic differences in the endothelial cell response to shear stress in differently sized vessels; however, previous studies have typically used RH protocols to induce increases in shear stress. Importantly, with RH, the shear stress stimulus generated is inversely related to vessel diameter such that smaller arteries tend to experience a greater shear stress stimulus relative to larger arteries (Herrington *et al.*, 2001;Pyke *et al.*, 2004). **It is therefore possible that in previous studies, smaller vessels experienced a greater shear stress stimulus relative to larger vessels, potentially accounting for the observed inverse relationship between baseline artery size and the FMD response.** Moreover, given the distinct hemodynamic environments in the arm and the leg, in between limb comparisons it is difficult to isolate the impact of baseline diameter per se.

With this as a background, the aim of the study described in chapter three was to determine whether vessel size dependent differences in FMD remain under conditions in which shear stress is matched across vessel size.

1.1 Specific Objectives

1) To investigate the impact of baseline artery diameter on the magnitude of the FMD response using exercise to create an equal and sustained shear stress stimulus in two differently sized upper limb arteries (brachial and radial). Investigating two distinctly sized conduit arteries in the upper limb allows the impact of baseline diameter to be investigated with a within subjects design, and avoids the potential for upper vs. lower limb heterogeneity (Proctor & Newcomer, 2006).

2) To examine the impact of vessel size on the shear stimulus-FMD response relationship.

1.2 Hypotheses

1) Creating the same shear stress stimulus in two differently sized arteries (brachial and radial arteries) will elicit a similar FMD response in both arteries.
2) There will be a similar and significant positive relationship between the shear stress stimulus and the FMD response in both the radial and brachial arteries.

Chapter 2

Literature Review

2.1 Vascular Biology of the Endothelium

2.1.1 Shear Stress

Shear stress is the tangential force acting against the vessel wall as a result of blood flow. It is directly proportional to the blood flow velocity (V) and blood viscosity (η) but is inversely related to the vessel diameter (D) (Gnasso *et al.*, 2001):

$$\text{Shear stress} = \frac{V \times \eta}{D}$$

It is the elevation in wall shear stress that is the stimulus for endothelial dependent flow mediated dilated (FMD) (Gnasso *et al.*, 2001;Melkumyants *et al.*, 1989). Increases in blood velocity elicit increases in shear stress, which in turn leads to dilation. According to the above equation, at a given blood velocity, dilation of the vessel (increased denominator) leads to a reduction in shear stress. FMD may therefore act as a negative feedback mechanism that maintains vessel wall shear stress within physiological limits (Koller *et al.*, 1993).

It is difficult to measure shear stress accurately *in vivo* due to the pulsatile nature of blood flow and the fact that it is necessary to measure blood velocity and viscosity in very close proximity to the vessel walls (Wootton & Ku, 1999). In conduit arteries however, blood flow is generally unidirectional and laminar making it possible to

estimate shear stress. A surrogate measure of shear stress is shear rate (shear rate = V/D) (Pyke & Tschakovsky, 2005;Widlansky, 2009). Shear rate measurements are however limited since they do not account for the effect of blood viscosity on shear stress.

Increases in blood viscosity have been found to lead to vasodilation while reductions in blood viscosity result in vasoconstriction (Melkumyants *et al.*, 1989). Therefore, variations in blood viscosity impact shear stress levels and can affect endothelial mediated changes in vascular smooth muscle tone. However, there is evidence to suggest that in young healthy individuals, measurement of blood viscosity is not critical for estimation of the shear stress stimulus (Padilla *et al.*, 2008). Padilla et al. (2008) compared the calculated shear stress with and without use of viscosity measurements in a young healthy population and found that the inclusion of viscosity measurements did not significantly alter shear stress measurements. Thus, in this particular study population, the addition of viscosity measurements may not be required to obtain an adequate estimate of shear stress.

2.1.2 Shear Stress Transduction

When endothelial cells detect an increase in shear stress, an array of responses is triggered that ultimately lead to the conversion of the mechanical signal of shear stress into a biochemical response (mechanotransduction) (Davies & Tripathi, 1993). Mechanotransduction can be broken down into three main events: 1) physical deformation of the cell surface; 2) intracellular stress transmission; and 3) conversion of the physical signal of shear stress into a biochemical response (Figure 2-1). If the shear

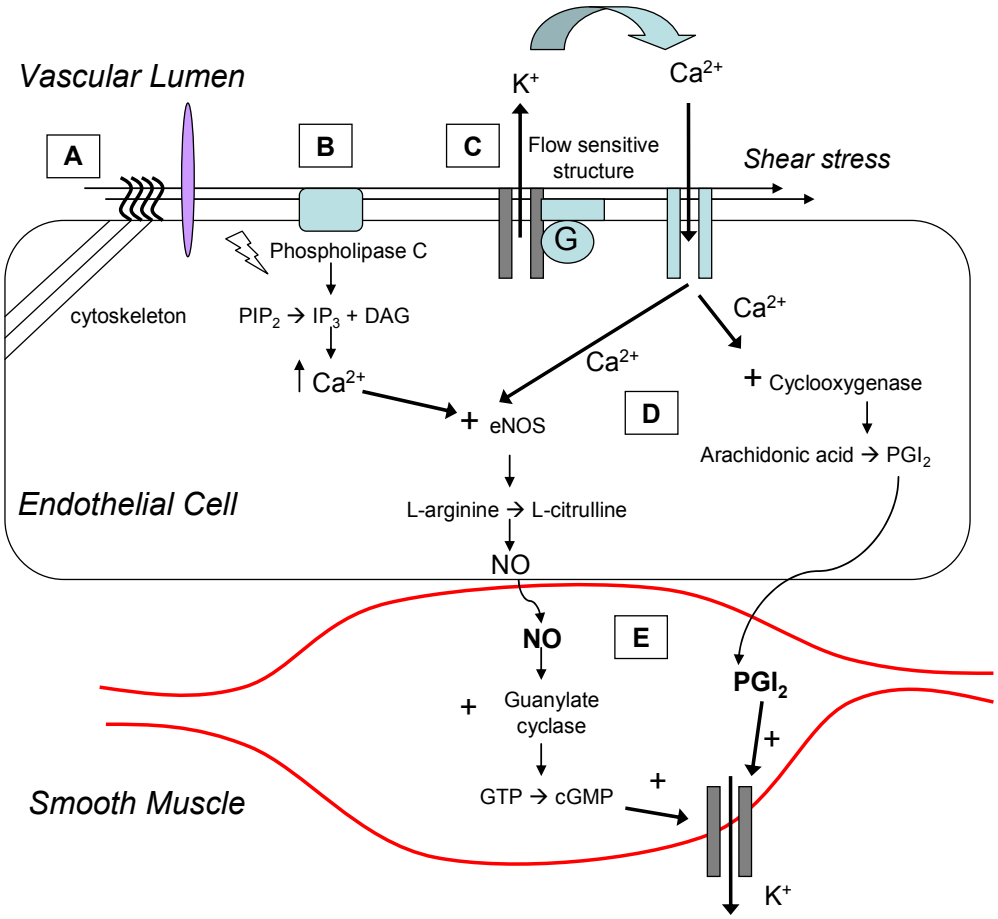

Figure 2-1: A model for shear stress mechanotransduction by the endothelial cell. A) Extended cellular structures including the glycocalyx and cilia transmit the mechanical signal of shear stress to the cytoskeleton. B) Deformation of endothelial plasma membrane proteins leads to phospholipase C activation, increasing intracellular Ca^{2+} via the IP_3 pathway. C) Increases in shear stress deform the flow-sensitive membrane structure associated with a G-protein modulated K^+ channel, producing K^+ efflux. This triggers Ca^{2+} influx into the cell. D) The increased intracellular [Ca^{2+}] activates endothelial nitric oxide synthase (eNOS) and cyclooxygenase, stimulating nitric oxide (NO) and prostacyclin (PGI_2) production respectively. E) NO and PGI_2 then diffuse into the smooth muscle cell, where NO activates guanylate cyclase, stimulating conversion of GTP into cyclic GMP (cGMP). cGMP and PGI_2 stimulate K^+ channel activity, resulting in K^+ efflux and ultimately smooth cell muscle relaxation. + = positive activation. Figure adapted from Davies *et al.* 2009 and Ohno *et al.*, 1993.

stress increase is sustained for several hours, acute biochemical responses to changes in shear stress are followed by changes in gene expression and cytoskeleton redistribution (Davies & Tripathi, 1993). Prolonged changes in local hemodynamics lead to adaptive endothelium-dependent artery wall remodelling (Davies, 2009) in that chronic increases in flow promote vessel restructuring to form a larger diameter while the opposite effect results with long-term reductions in flow (Cooke & Dzau, 1997). A brief overview of the acute biological responses to changes in shear stress will be provided as these are the responses most relevant to the short-term increase in shear stress that is elicited via exercise-based FMD assessments.

Physical Deformation

An important role for the extended cellular structures in detecting changes in shear stress has recently emerged. Shear stress acts as an external mechanical stressor causing displacement of external cellular structures such as the glycocalyx and primary cilia. Both of these structures are connected to the cytoskeleton at their base and project into the flow region of the luminal cell surface (Davies, 2009). As the outermost interface between the endothelial cell and blood flow, the glycocalyx is exposed to and detects changes in shear stress. It is a glycoprotein-rich extension of the cell surface that projects up to 0.5 μm from the endothelial plasma membrane (Davies, 2009). Increases in blood-flow associated shear stress displace the glycocalyx and it is postulated that the glycocalyx transmits this increase in shear to the cytoskeleton (Tarbell *et al.*, 2005).

Thus, the deformation of the glycocalyx by shear stress plays an important role in contributing to force transmission. The importance of the glycocalyx in shear stress mechanotransduction was demonstrated in a study that found that flow-mediated production of nitric oxide was hindered when glycocalyx components such as the glycosaminoglycan heparin sulphate were selectively cleaved (Florian *et al.*, 2003).

The primary cilia are another extended cellular structure potentially involved in detecting and transmitting the mechanical signal of shear stress. Changes in shear stress induce physical alterations in the primary cilia, potentially triggering mechanisms of shear stress transduction (Davies, 2009). Both cells with and without cilia are flow-responsive (Davies, 2009); thus, further research is required to decipher to specific role of the cilia in detecting change in shear stress.

Force Transmission

Increases in shear stress cause physical deformation of the endothelial cell surface (Davies, 2009). The cytoskeleton and integrins (membrane proteins that are the site of cytoskeletal anchorage to the plasma membrane) play an imperative role in transmitting this surface deformation throughout the body of the endothelial cell (Davies & Tripathi, 1993;Davies, 1995). The cytoskeleton generates a force equilibrium within the cell. An increase in blood flow-associated shear stress causes external loading onto the cell, disturbing this force equilibrium (Davies & Tripathi, 1993). The external forces acting on

the cell surface are subsequently transmitted throughout the cell via the cytoskeleton as solid mechanical stresses (Davies & Tripathi, 1993).

The Biochemical Response

One of the first endothelial responses to the mechanical stimulus of shear stress is the activation of flow-sensitive potassium (K^+) ion channels found in the endothelial cell membrane (Olesen *et al.*, 1988). K^+ channels are associated with flow-sensitive membrane structures via cytoskeletal elements and activation of these channels is modulated by pertussis toxin-sensitive G proteins (Ohno *et al.*, 1993). Increases in shear stress deform the flow-sensitive membrane structure, increasing the probability of the opening of the adjacent K^+ channel (Ohno *et al.*, 1993). The opening of shear-sensitive potassium channels leads to K^+ efflux, which subsequently leads to hyperpolarization of the endothelial cell. As a result, there is an increase in the driving force for Ca^{2+} entry into the cell. Endothelial nitric oxide synthase (NOS), an enzyme that is critical to the generation of NO, can be activated by Ca^{2+} (Ohno *et al.*, 1993). Increases in intracellular Ca^{2+} levels is also important in the synthesis of endothelial derived hyperpolarizing factor (EDHF) (Huang *et al.*, 2001;Mombouli & Vanhoutte, 1997). Additionally, production of the vasodilator prostacyclin (PGI_2) can be induced via Ca^{2+} mediated increases in release of arachidonic acid (the precursor for PGI_2) (Bhagyalakshmi & Frangos, 1989). Thus, influx of Ca^{2+} resulting from activation of flow-sensitive K^+ channels can lead to the production of vasodilators.

Another mechanism leading to an increase in calcium concentration is the inositol triphosphate (IP_3) pathway. Physical deformation of endothelial plasma membrane proteins leads to phospholipase activation and subsequent cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) to form IP_3 and diacylglycerol (DAG). IP_3 and DAG release triggers release of intracellular Ca^{2+} and may also initiate immediate influx of extracellular Ca^{2+} (Davies & Tripathi, 1993;Davies, 1995).

NO and PGI_2 diffuse from the endothelium into the smooth muscle cell. NO activates guanylate cyclase, leading to the conversion of GTP into cyclic GMP (cGMP) (Archer *et al.*, 1994). cGMP and PGI_2 stimulate K+ channel activity leading to in K+ efflux. This eventually results in hyperpolarization of the smooth muscle cell, inhibition of voltage-gated Ca^{2+} channels, and ultimately smooth muscle relaxation (Archer *et al,* 1994; Feletou *et al.*, 2000).

2.1.3 Vasodilators involved in the FMD Response

The primary result of shear stress transduction is the release of the endothelial factors prostacyclin (PGI_2), nitric oxide (NO), and endothelial derived hyperpolarizing factor (EDHF) (Busse *et al.*, 2002;Joannides *et al.*, 1995;Okahara *et al.*, 1998). PGI_2 is a prostanoid that is produced from arachidonic acid via the enzymatic actions of cyclooxygenase and PGI_2 synthase (Okahara *et al.*, 1998). NO is generated via the conversion of L-arginine into L-citrulline by the enzyme endothelial nitric oxide synthase (eNOS) (Celermajer, 1997;Kawashima & Yokoyama, 2004). PGI_2 and NO have been

extensively studied and are both known to be released by the endothelium in response to transient increases in shear stress (Koller *et al.*, 1994;Huang *et al.*, 2001). The inability of NO and PGI_2 to fully account for the dilatory response observed with increases in shear stress has led to the proposal of the existence of an EDHF. However, the chemical identity and mechanism of action of EDHF remain unclear (Luksha *et al.*, 2009). The putative role of EDHF in mediating the FMD response will be discussed in a later section.

In human peripheral conduit arteries, NO appears to play a more significant role than PGI_2 in FMD. Joannides et al. (1995) found that inhibition of NO synthesis with N^Gmonomethyl-L-arginine (L-NMMA) completely eliminated flow-dependent dilation in the radial artery following the release of a 3 min wrist occlusion. Inhibiting PGI_2 on the other hand, had no effect on FMD. A similar pattern was observed in study by Mullen et al. (2001) who found that radial artery FMD in response to a brief shear stimulus created with the release of a 5 min wrist occlusion depended solely on NO and was unaffected by inhibition of PGI_2. Findings from a study by Pyke et al. (2010) however, challenge the view that NO is obligatory for human FMD. In this study, it was found that L-NMMA administration did not affect radial artery FMD in response to different shear stimulus profiles (created with the release of 5 or 10 minutes of cuff occlusion) in healthy individuals. It is therefore possible that there are multiple endothelial phenotypes such that in some individuals another vasodilator (such as EDHF) may compensate for an

absence of NO, which would account for the unaffected FMD observed with NO blockade (Bellien *et al.*, 2006;Huang *et al.*, 2001;Pyke *et al.*, 2010).

Brief vs. Sustained Increases in Shear Stress

Studies suggest that the mechanisms that mediate the FMD response are dependent on the duration of the hyperaemic stimulus. Mullen et al. (2001) examined the role of NO and PGI_2 in radial artery dilation in response to both a transient and more sustained hyperemia. As mentioned previously, it was found that radial artery FMD in response to a brief shear stimulus (achieved using reactive hyperemia with a 5-minute cuff occlusion) was abolished when NO activity was inhibited via administration of the NO synthesis inhibitor L-NMMA. To examine the mechanisms involved in the FMD response to prolonged periods of hyperemia, reactive hyperemia with 15-minutes cuff occlusion and hand heating were used with blockade of NO and PGI_2. Both L-NMMA and cyclooxygenase had no significant effect on radial artery dilation, indicating that the mechanisms that mediate the FMD response to prolonged hyperemia are independent of both NO and PGI_2 (Mullen *et al.*, 2001).

Similarly, Kooijman (2008) demonstrated that FMD in the superficial femoral artery is NO dependent only after brief increases in shear stress. L-NMMA administration almost completely abolished FMD in the superficial femoral artery following reactive hyperemia with a 5-minute occlusion period. In contrast, the FMD response was unaffected by NO inhibition following prolonged increases in shear stress

achieved via reactive hyperemia with a 10-minute occlusion. Thus, these studies suggest that in the radial and superficial femoral arteries, the FMD response to brief increases in shear may be NO-mediated, whereas FMD after sustained increases in shear stress is NO-independent. The results of a study by Bellien et al. (2006) however, indicate the involvement of NO in the FMD response to sustained increases in shear stress cannot be ruled out and that it is possible that an insufficient dose of L-NMMA was given in previous studies. Bellien et al. (2006) used heating to achieve a sustained shear stress stimulus in the radial artery with the temperature being increased in gradual steps. L-NMMA was administered throughout the trial with the dose being increased at the end of each temperature stage. Interestingly, with this increasing dose of L-NMMA, maximal radial artery FMD in response to hand skin heating was attenuated by ~40%. This suggests that the FMD response to sustained increases in shear stress may in fact involve NO and that the duration and doses of L-NMMA infusion in previous studies may have been insufficient to achieve effective blockade of NO synthesis.

Similar to hand skin heating, handgrip exercise creates sustained increases in shear stress. Wray et al. (2010) examined the mechanisms that mediate exercise-induced FMD and found that administration of L-NMMA blunted brachial artery FMD by 30-60% during high intensity dynamic handgrip exercise (20 to 24 kg). This suggests that NO plays a major role mediating exercise-induced FMD, which is in contrast to the NO-independent FMD response observed with heating (Mullen *et al.*, 2001). Thus, there is

conflict in the literature regarding the mechanisms involved in the FMD response to sustained increases in shear stress.

The Role of EDHF

It has been suggested that another important vasodilator, EDHF, exists based on the observation that PGI_2 and NO fail to completely account for the FMD response (Feletou & Vanhoutte, 2000;Luksha *et al.*, 2009). EDHF is postulated to promote the opening of calcium-activated potassium channels, thereby leading to membrane hyperpolarization and smooth muscle relaxation (Rubanyi, 1993). Research has shown that the relative contribution of EDHF in mediating vessel dilation increases as vessel size decreases (Tomioka *et al.*, 1999). It has also been found that EDHF responses in mice are more prominent after inhibition of eNOS, suggesting that EDHF may act as a 'backup' vasodilator in cases where NO synthesis is compromised (Huang *et al.*, 2001). The main candidates for EDHF are epoxyeicosatrienoic acids, which are products of endothelial cytrochrome P450 epoxygenases (Huang *et al.*, 2005). Evidence for the role of cytrochrome P450 metabolites as EDHF comes from the observation that EDHF-mediated responses are increased by factors that enhance the expression of cytochrome P450 in the endothelium (Fisslthaler *et al.*, 1999). Most research regarding EDHF has been carried out in animal models; thus, future research aimed at understanding the role of EDHF in the FMD response in human arteries is necessary.

Bellien et al. 2006 is one of the few studies that provide insight into the role of EDHF in human conduit arteries. They investigated the role of EDHF and its interaction with NO in radial artery FMD. As mentioned above, in this study, hand skin heating was used to create sustained increases in shear stress in the radial artery. Blockade of calcium-activated potassium channels via tetraethylammonium chloride (TEA) in combination with inhibition of NO with L-NMMA was found to reduce radial artery FMD significantly more (64% reduction) than administration of L-NMMA (39% reduction) or TEA alone (14% reduction). Similarly, infusion of fluconazole (an inhibitor of cytochrome P450 (CYP) epoxygenase 2C9) with L-NMMA reduced radial artery FMD significantly more (71% reduction) than infusion of L-NMMA or fluconazole alone (18% reduction). Thus, there is a functional interaction between EDHF and NO pathways in the FMD response (Bellien *et al.*, 2006). Moreover, this study suggests a loss of NO synthesis may trigger a compensatory release of CYP-related EDHF to maintain the FMD response to sustained shear stimuli (Bellien *et al.*, 2006). These findings therefore suggest a role for EDHF in peripheral conduit artery FMD.

2.2 Endothelial Dysfunction and Disease

Endothelial dysfunction results from physical or cellular damage to the endothelium and is regarded as an important early event in the development of atherosclerosis (Celermajer, 1997). Endothelial dysfunction is characterized by a disruption in the normal bioavailability of the relaxing factors such as NO and PGI_2

(Celermajer, 1997;Rubanyi, 1993;Kawashima & Yokoyama, 2004). These substances have important antiatherogenic properties such as inhibiting platelet aggregation and adherence to the vessel wall, inhibiting leukocyte-endothelial interactions, and deterring smooth muscle proliferation (Celermajer, 1997;Rubanyi, 1993;Schafer & Bauersachs, 2008). Thus, endothelial dysfunction leads to a reduction in vasoprotection and functionally results in an impaired FMD response. Endothelial dysfunction has been observed in asymptomatic individuals with risk factors for atherosclerosis, such as cigarette smoking and hypercholesterolaemia (Celermajer *et al.*, 1992;Celermajer *et al.*, 1993). Identification of endothelial dysfunction, which manifests itself as impaired FMD, therefore has the potential to be used as a predictive tool for assessing risk of developing CAD (Yeboah *et al.*, 2009).

2.2.1 The Mechanisms of Endothelial Dysfunction

As previously mentioned, endothelial dysfunction manifests itself as a reduction in NO bioavailability (Celermajer, 1997;Kawashima & Yokoyama, 2004;Oemar *et al.*, 1998;Cooke & Dzau, 1997). Proposed mechanisms of endothelial dysfunction that work via a decrease in NO bioavailability include: reduced L-arginine availability, alterations in normal eNOS transcriptional activity, and augmented destruction of NO by reactive oxygen species (ROS).

L-arginine is the key precursor molecule for the formation of NO (Figure 2-2). Thus, decreased availability of L-arginine may contribute to a reduction in NO

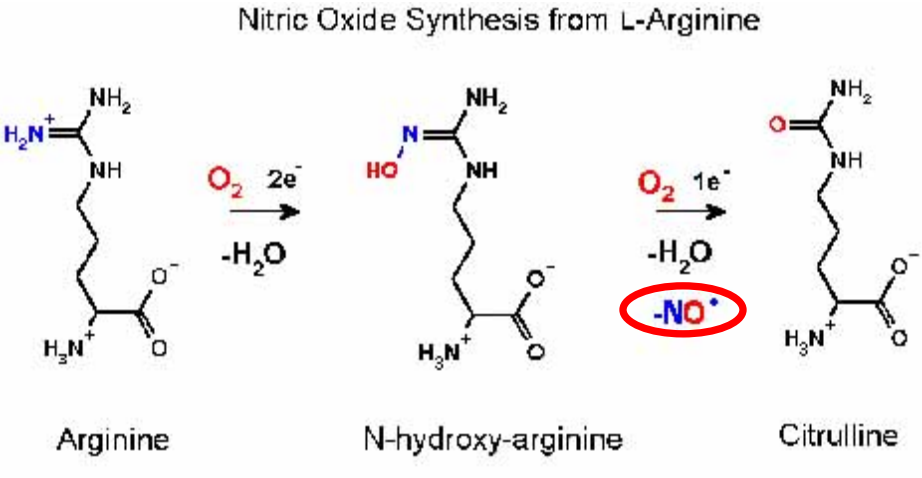

Figure 2-2: Chemical pathway in the formation of nitric oxide via eNOS. L-arginine is oxidized to L-citrulline and NO via eNOS. This reaction is oxygen and $NADPH^-$ dependent.

production and availability. Accumulation of oxidized low-density lipoprotein (LDL) in the vascular wall (as seen in patients with hypercholesterolemia) can interfere with the synthesis and release of L-arginine from intracellular stores (Drexler, 1999). Oxidized LDL can also impair transport of arginine into endothelial cells (Drexler, 1999). Acute administration of L-arginine has been shown to improve endothelial function in hypercholesterolemic individuals, supporting the notion that endothelial dysfunction maybe associated with reduced L-arginine availability (Creager *et al.*, 1992).

A decrease in the activity and expression of eNOS at the vessel wall may also account for the reduced NO levels observed in atherosclerosis. Reduced transcription and increased instability eNOS mRNA may be responsible for this deficiency in eNOS levels (Kawashima & Yokoyama, 2004). In addition, dysfunctional eNOS has been identified to play a role in reducing NO levels and promoting the development of atherosclerosis. eNOS can become dysfunctional when there are reduced levels of tetrahydrobiopterin (BH4) (Kawashima & Yokoyama, 2004). BH_4 is a cofactor of eNOS that is essential for the proper transfer of electrons to oxidize L-arginine (Kawashima & Yokoyama, 2004). When vascular tissue levels of BH4 are deficient, eNOS oxidizes molecular oxygen rather than L-arginine, generating superoxide (O_2^-) and hydrogen peroxide (H_2O_2) instead of NO (Kawashima & Yokoyama, 2004;Anderson, 2003). Superoxide anions degrade NO, reducing NO availability (Celermajer, 1997;Kawashima & Yokoyama, 2004). Notably, endothelial dysfunction has been reversed in patients with coronary artery disease after long-term administration of the natural antioxidant ascorbic acid (vitamin C)

(Gokce *et al.*, 1999). This lends support to the contention that NO breakdown via ROS plays an important role in the development of endothelial dysfunction.

2.3 Measuring FMD

As previously described, the loss of normal endothelial function plays a pivotal role in the development of atherosclerosis and cardiovascular disease. Normally, vasodilation of the coronary arteries is observed in response to increased blood flow. However, when endothelial dysfunction is present at sites of coronary plaques, an opposite response (vasoconstriction) occurs (Ludmer *et al.*, 1986;Gordon *et al.*, 1989). In coronary arteries, FMD is critical to ensuring adequate myocardial perfusion during periods of increased demand (Duffy *et al.*, 1999;Gielen & Hambrecht, 2001;Gielen *et al.*, 2001). Thus, endothelial dysfunction is clinically significant as it may contribute to the pathogenesis of myocardial ischemia (Zeiher *et al.*, 1991;Zeiher *et al.*, 1995). As previously mentioned, endothelial dysfunction has been detected in the coronary arteries of humans with advanced atherosclerosis (Ludmer *et al.*, 1986). However, the methods used to measure coronary endothelial function are highly invasive. Therefore, development and implementation of non-invasive methods of assessing endothelial function is required.

Endothelial function in the brachial artery has been experimentally shown to be reflective of coronary endothelial function (Anderson *et al.*, 1995;Takase *et al.*, 1998). Takase et al. (1998) found that the FMD response to increases in shear stress in the coronary and brachial arteries are highly correlated ($r = 0.78$) (Takase *et al.*, 1998). Since

assessment of brachial artery endothelial function *can* be done non-invasively, it has the potential to be used as a surrogate measure of coronary endothelial function. Thus, endothelial dysfunction in the coronary arteries can be predicted by non-invasively investigating the response of peripheral arteries.

2.3.1 Reactive Hyperemia Test

The reactive hyperemia (RH) test is the most frequently used method of assessing FMD in humans. With RH, the forearm is temporarily occluded via a sphygmomanometric cuff (Corretti *et al.*, 1995;Pyke & Tschakovsky, 2005). Occlusion of the forearm vessels creates an ischemic environment, evoking vasodilation and thus a reduction in forearm vascular resistance. When the cuff is deflated, brachial artery blood flow velocity is immediately and briefly increased (due to the downstream dilation of the forearm vessels) and then decays over a period of ~1 minute. This leads to an increase in shear stress in the brachial artery and as a result, the vessel dilates (Berry *et al.*, 2000;Corretti *et al.*, 2002;Pyke & Tschakovsky, 2005;Korkmaz & Onalan, 2008). Brachial artery diameter and blood velocity are measured by imaging the brachial artery via ultrasound technology. FMD is then calculated by taking the percent change in artery diameter from baseline diameter to the maximum diameter after cuff release (Berry *et al.*, 2000;Corretti *et al.*, 2002;Pyke & Tschakovsky, 2005).

Limitations of Reactive Hyperemia

A large, transient, and uncontrolled shear stress stimulus is created with RH. As such, the magnitude of the shear stress imparted on the vessel cannot be maintained or controlled via this methodology, which is a serious limitation (Pyke *et al.*, 2004). With the use of RH, maximum blood flow occurs immediately upon cuff release and then gradually declines. However, peak vessel diameter is observed approximately 60 seconds after cuff release (Figure 2-3) (Corretti *et al.*, 1995). Therefore, there is no point at which the level of shear stress is constant making it impossible to link vessel diameter changes to a specific shear stress stimulus (Pyke *et al.*, 2008b). Furthermore, there is great inter-subject variability in the magnitude of the shear stress stimulus created via RH (Pyke *et al.*, 2004). This makes it difficult to compare vascular health between subjects or groups using FMD when the shear stress stimulus experienced with RH varies substantially (Pyke *et al.*, 2004). For example, de Groot *et al.* (2004) found no significant difference in the brachial artery FMD responses of spinal-cord injured individuals relative to controls. However, the RH-induced increase in shear was significantly higher in the brachial artery of spinal-cord injured individuals. Thus, differences in the magnitude of the stimulus created with RH made it difficult to carry out reliable comparisons regarding the vascular health of these two groups because it was unclear in this instance whether differences in the FMD were indicative of differences in endothelial function or the shear stress stimulus.

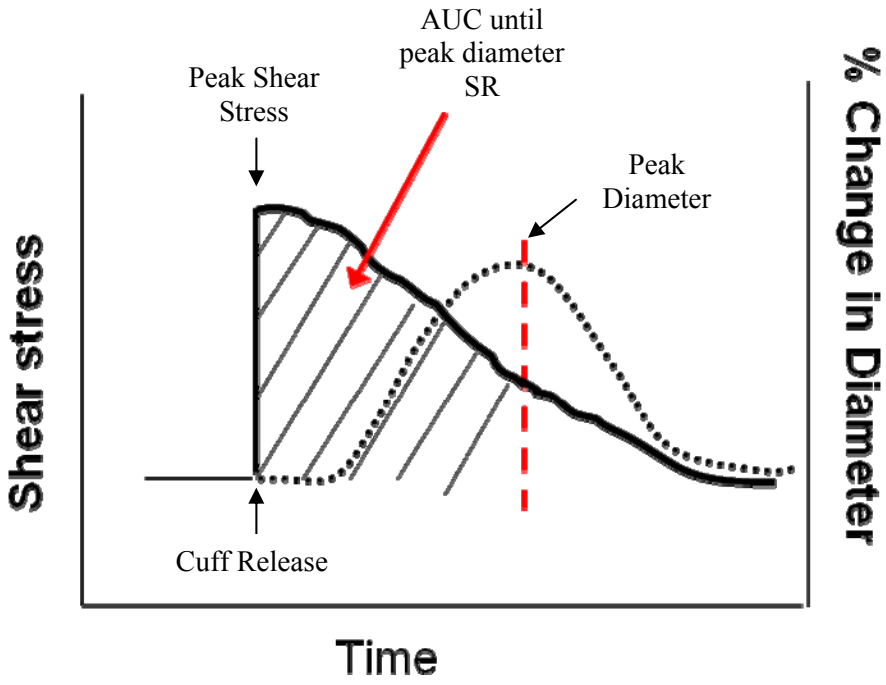

Figure 2-3: The relationship between peak vessel diameter and % change in diameter over time with RH. The peak shear stress occurs immediately after cuff release and then gradually decays. Maximum FMD however, is observed approximately 60 seconds after cuff release. The area under the curve (AUC) until the time to peak dilation (shaded region) is measured and taken as the relevant shear stress stimulus. The %FMD is then divided by AUC shear stimulus to provide a normalized measure of FMD. Figure adapted from (Pyke & Tschakovsky, 2005).

To circumvent these limitations, it has recently been proposed that the FMD response should be normalized to the imposed shear stimulus, providing a measure of FMD that is relative to the shear stress stimulus. This is achieved by dividing the %FMD by the area under the curve (AUC) of the shear rate stimulus (shear rate is surrogate measure of shear stress) until the time of peak dilation. This time interval is considered to be the 'relevant' shear stress stimulus since it represents the entire portion of the stimulus profile that may have contributed to FMD response (Pyke & Tschakovsky, 2005) (Figure 2-3). The normalization of the FMD response is supported by the findings of Pyke & Tschakovsky (2007) who examined the independent contributions of the peak and the duration of the reactive hyperemia and found that the shear rate duration (AUC) rather than the peak post cuff release shear rate was critical in determining the peak FMD response (Pyke & Tschakovsky, 2007). In this study, a main effect of duration of the hyperemic stimulus on %FMD response was identified; however, this effect was abolished upon normalization of the FMD response to the shear rate AUC. In designs that manipulate the shear stimulus magnitude between trials, Padilla et al. (2008) and Padilla et al. (2009) have also found that normalization of FMD eliminates between trial differences in the response. These data indicate that the influence of variable shear rate stimuli on the FMD response is abolished with normalization.

However, normalization of the FMD response not yet been widely adopted by the scientific community and has been criticized for several reasons. First, it is unclear what portion of the shear rate AUC can always be deemed the 'relevant stimuli'. For example,

there are substantial differences in the time to peak diameter between different arteries (brachial artery: ~85 s vs. superficial femoral artery: ~172 s) (Thijssen *et al.*, 2008). Therefore, uncertainty regarding the appropriate shear characterization is a limitation to using normalization. Additionally, recent studies have criticized the statistical validity of ratio normalization in FMD research (Atkinson *et al.*, 2009). Finally, the FMD-shear stress dose-response relationship is incompletely characterized; it is unclear if the relationship is linear and the presence of a threshold stimulus or ceiling response has not been identified. Thus, we cannot be confident that we are not introducing more error with normalization and that ratio normalization of FMD is an appropriate way of expressing FMD.

2.3.2 Alternative Methods of Assessing FMD

The limitations of RH highlight the importance of exploring alternative methods that allow the experimenter to create a sustained shear stress stimulus. Limb heating, exercise, and distal vasodilator infusion are examples of alternative methods of increasing shear stress in a conduit artery. The shear stress profiles associated with these alternative methods differ from that observed with RH in terms of the rate of onset and the peak magnitude of the shear stress stimulus (Figure 2-4). Unlike the stimulus created with RH, these methods result in more gradual increases in shear stress that eventually reach a sustained plateau (Pyke & Tschakovsky, 2005). The creation of sustained and controlled increases in shear stress means that the FMD response can be better characterized since it can be associated with a specific stimulus. Arterial compression can be used in

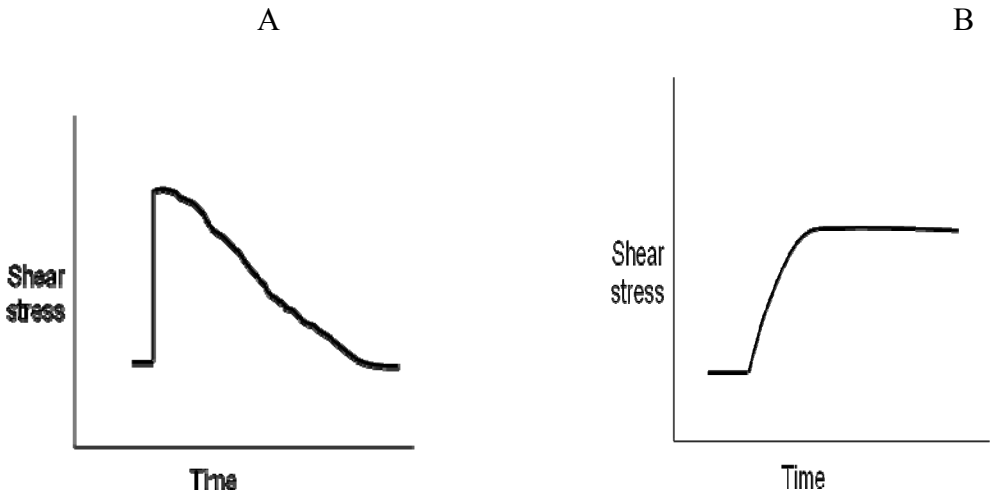

Figure 2-4: Hypothetical shear rate profiles for A) reative hyperemia and B) exercise, limb heating or distal vasodilator infusion. A) Upon release of the cuff, there is a large peak in shear stress that decays over time. B) In contrast, a gradual increase in shear that reaches a steady state plateau is created with exercise, limb heatiing or distal vasodilator infusion.

combination with heating or exercise to create step increases in shear stress. Via this design, quantification of FMD response dynamics is possible (Pyke *et al.*, 2008a). Therefore, the use of alternative methods of increasing shear can be advantageous as the FMD response can be better characterized with these techniques.

Exercise as a Method of Assessing FMD

Exercise has recently been explored as a method of assessing FMD. With the use of exercise, sustained increases in artery wall shear stress can be created at specific magnitudes. Therefore, unlike RH where the stimulus is uncontrolled, mathematical normalization of the FMD response to the shear stimulus created with exercise is not required. Upon commencing exercise, the oxygen demand of the tissue increases. A rapid reduction in vascular resistance in the exercising muscle bed is induced to facilitate increased oxygen delivery (Delp & Laughlin, 1998;Shoemaker *et al.*, 1998). This leads to sustained increases in upstream conduit artery blood flow, leading to an increase in shear stress. The magnitude of the reduction in vascular resistance is dependent on the exercise intensity; higher intensity = greater reduction in resistance. Thus, the magnitude of the conduit artery shear stress stimulus is also dependent on the exercise intensity in that higher exercise intensities elicit a greater shear stress stimulus (Pyke *et al.*, 2008b). By manipulating the exercise intensity, a uniform shear stress stimulus can be achieved over multiple trials within subjects and also between subjects. Moreover, exercise is the most common cause of significant shear stress increases in conduit arteries in daily living.

Therefore, studying the FMD responses to exercise-induced increases in shear stress is beneficial as it represents a more relevant physiological stimulus for FMD. This is highlighted by observations that in coronary arteries, FMD is critical to ensuring adequate myocardial perfusion during exercise, making FMD an important part of the exercise response (Duffy *et al.*, 1999;Gielen & Hambrecht, 2001;Gielen *et al.*, 2001).

As previously mentioned, there is evidence to suggest that mechanisms that mediate exercise-induced FMD are similar to those found with RH in that NO plays a significant role in mediating the FMD response (Wray *et al*., 2010). These findings suggest that the impaired FMD responses detected with RH may also be detectable via exercise since the FMD response to these different stimuli may be mediated via similar mechanisms. Although, it has been established that RH induced FMD in peripheral conduit arteries is impaired in individuals with disease or risk factors for disease, the relationship between exercise-induced FMD and vascular health is unclear. A study by Gaenzer et al. (2001) provided insight into the value of exercise as a method of detecting endothelial dysfunction. An impaired femoral artery FMD response was detected with cycling in cigarette smokers. Furthermore, a strong correlation between FMD induced in the brachial artery via RH and exercise-induced FMD in the femoral artery ($r = 0.88$, $p<0.001$) was identified. Similarly, Grzelak et al. (2010) found that brachial artery FMD in response to handgrip exercise-induced increases in shear stress is attenuated in individuals with type I diabetes. The findings of these studies suggest that FMD in response to exercise has potential as an index of clinically relevant endothelial function.

Although the findings of Gaenzer et al. (2001) and Grzelak et al. (2010) are promising, a study by Padilla et al. (2006) found that the use of exercise-induced FMD does not detect acute high fat meal induced endothelial dysfunction. The FMD responses to RH and handgrip exercise following ingestion of a high fat meal (a known perturbation to endothelial function (Vogel *et al.*, 1997)) were compared and it was found that the FMD response to RH was attenuated while FMD in response to handgrip exercise was unchanged. More research is therefore required to verify the predictive value of exercise-induced FMD in assessing the risk of developing cardiovascular disease.

Potential Confounding Factors Associated with the Use of Exercise to Assess FMD

During exercise, a fluctuating pattern of blood flow and therefore shear stress is induced. The fluctuating pattern is the result of contraction-induced impedance to flow, which can include a short period of reverse flow during a contraction and hyperemia during relaxation. It has been shown that exposure to repetitive forward and reverse cycles of flow (oscillatory flow) can negatively affect the endothelial response to shear stress (Gambillara *et al.*, 2006). In addition, in bovine aortic endothelial cells, oscillatory flow has been found to transiently increase endothelin-1 expression and downregulate eNOS levels, which can negatively impact endothelial function (Ziegler *et al.*, 1998). Therefore, there is the potential that the fluctuating pattern of blood flow associated with exercise may have a negative influence on the FMD response. Recently, Pyke et al. (2008b) investigated the FMD response associated with handgrip exercise in the brachial

artery. The FMD responses to the same mean step increase in shear rate were compared in three conditions: 1) stable stimulus via forearm heating, 2) fluctuating shear via forearm heating and rhythmic forearm cuff inflation and deflation, and 3) exercise induced fluctuating shear with handgrip exercises. It was found that the magnitude of the FMD response was the same in all three cases indicating that with the same mean shear stimulus, the FMD response was independent of the shear stress pattern. Therefore, when exposed to shear fluctuations generated by muscle activation, the endothelium transduces the mean shear stimulus (Pyke *et al.*, 2008b).

Studies have shown that vasodilation in active skeletal muscle with elevated metabolites can be transmitted to upstream vessels through conducted vasodilation (Neild & Crane, 2002). This is achieved via gap junctions, which allow electrical signals to be rapidly conducted through endothelial cells (Emerson *et al.*, 2002;Segal & Jacobs, 2001). Hyperpolarization can therefore move upstream into the arterioles that feed the muscle, contributing to the vasodilation observed in vessels remote from those in direct contact with the exercising muscle and its metabolites (Segal & Jacobs, 2001). It is possible that conducted vasodilation associated with exercise may be a non-shear related contributor to conduit artery vasodilation (Shoemaker *et al.*, 1997). To examine the effect of muscle activation and the influence of conducted vasodilation on the FMD response, Pyke et al. (2008b) implemented a condition that included handgrip exercise with arterial compression to prevent an increase in blood flow associated shear stress. Via this design, brachial artery shear rate was maintained at near baseline, while producing the metabolic

stimulus required for the initiation of conducted vasodilation. However, no vasodilation was observed in this condition, indicating that conducted vasodilation did not reach the level of the brachial artery. Therefore, this evidence suggests that the dilatory response in conduit arteries observed during exercise is not a result of conducted vasodilation, and rather solely reflects the response to the elevation in shear stress.

Exercise causes an increase in sympathetic nervous system activity (SNA). Increases in SNA can affect vascular tone (for example, sympathetic activation leads to vasoconstriction in active muscle resistance vessels (Buckwalter *et al.*, 1997)) and has been shown to blunt FMD responses (Hijmering *et al.*, 2002). Thus, the influence of sympathetic activation on endothelial function is another factor to consider when using exercise to assess FMD. Hijmering et al. (2002) found that elevating SNA (via lower body negative pressure), significantly attenuated the FMD in response to reactive hyperemia. In contrast, Dyson et al. (2006) implemented various conditions to increase SNA including: lower body negative pressure, a mental arithmetic test, a cold pressor test, and the muscle chemoreflex. They found that FMD was generally unaffected by increases in SNA. Shoemaker et al. (2007) investigated the mechanisms that mediate the increase in blood pressure observed during isometric handgrip exercise and found that an elevation in cardiac output rather than an increase in sympathetically mediated peripheral vasoconstriction is primarily responsible for the increase in blood pressure. In addition, the handgrip exercise and heating with rhythmic cuff inflation conditions of the Pyke et al. (2008b) experiment (same shear magnitude and pattern) did not differ in their FMD

response, while only exercise would be expected to increase SNA (blood pressure did increase modestly in the handgrip exercise condition). This, in combination with the findings of Shoemaker et al. (2007) and Dyson et al. (2006), make it unlikely that sympathetic activation affects brachial artery FMD during handgrip exercise.

2.4 The Relationship between Shear Stress and the FMD Response

A consensus regarding the nature of the relationship between vascular wall shear stress and FMD has yet to be reached. Applying increasing magnitudes of RH occlusion duration times (which thereby increases the shear rate AUC) within subjects has been found to lead to a progressive increase in %FMD (Leeson *et al.*, 1997;Padilla *et al.*, 2008;Padilla *et al.*, 2009) demonstrating a positive relationship between shear stress and FMD. Pyke & Tschakovsky (2007) manipulated the reactive hyperemia duration within a subject and also found that FMD increased linearly with an increasing shear stimulus ($r^2 = 0.56$; $p < 0.001$). Betik (2004) created a range of RH stimuli via changing cuff positions and adding ischemic handgrip exercise and found a significant correlation between brachial artery diameter and peak shear rate ($r = 0.49$, $P < 0.002$). Additionally, studies that have employed phase-contrast magnetic resonance angiography have reported that FMD in both the brachial and femoral arteries is proportional to the hyperemic shear created via RH (Silber *et al.*, 2001;Silber *et al.*, 2005). This linear FMD-shear relationship has also been observed when increases in shear are created via handgrip exercise or hand skin heating demonstrating that this relationship exists with different

shear profiles (Joannides *et al.*, 2002;Pyke *et al.*, 2008b). There is evidence to suggest that the relationship between FMD and shear stress changes with disease; specifically, the slope of the FMD-shear stress relationship has been shown to be attenuated in individuals with moderate risk for cardiovascular disease relative to individuals who are at low risk (Padilla *et al.*, 2009). Additionally, data from Kelly & Snow (2007) and Shoemaker et al. (1997) indicate that there may be a threshold shear stimulus at which point the vessel begins to dilate.

The above studies employed a within subjects design in that each subject was exposed to multiple shear stress magnitudes. In contrast, Thijssen et al. (2009) used a between subjects design and found no significant correlation between FMD and the shear rate AUC in older adults and children and only a modest relationship in young adults ($r^2 = 0.14$, $p = 0.005$). Similarly, Thijssen et al. (2008) examined the correlations between %FMD and shear rate AUC until peak diameter in the radial, brachial, popliteal and femoral arteries and reported no relationship between these variables in any artery. The inconsistent findings regarding the relationship between shear stress and FMD magnitude is largely due to experimental design differences. The effect of between-subject variation on the FMD-shear relationship was removed in studies that performed repeated measurements within the same subjects. Thus, the lack of association between FMD and the shear stress stimulus observed in the studies by Thijssen et al. (2009) and (2008) (which both employed between-subject designs) may be attributable to a greater degree of intersubject variability of the FMD-shear relationship (Widlansky, 2009). It is clear

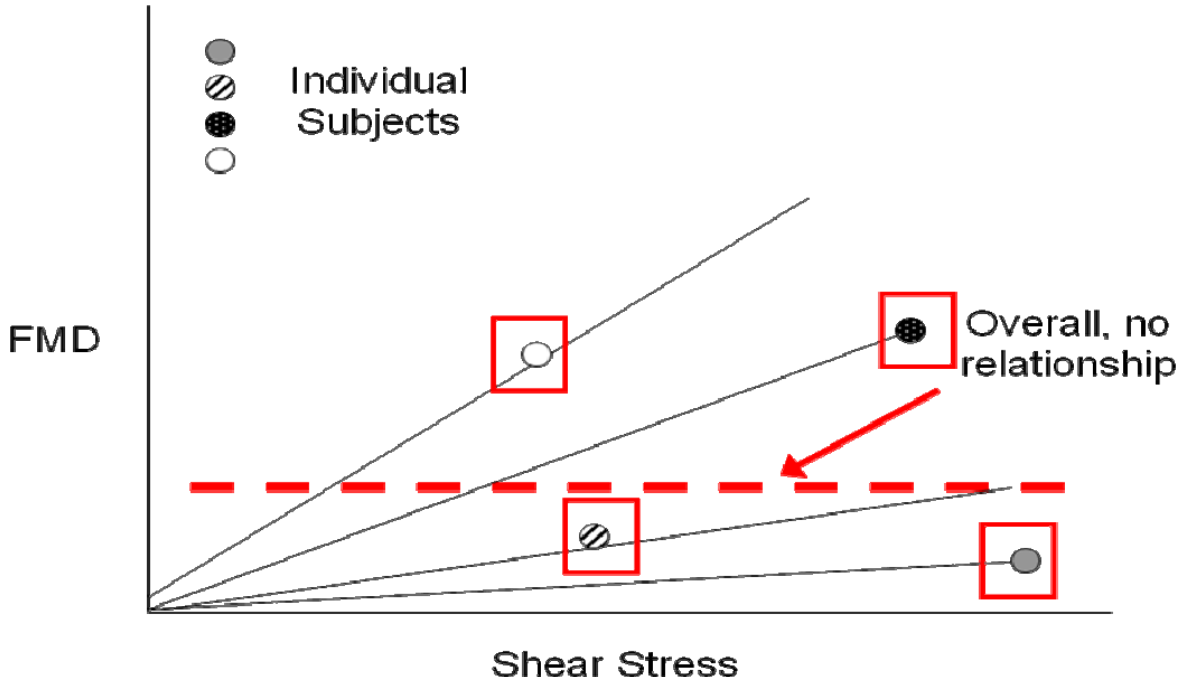

Figure 2-5: Hypothetical data illustrating how the relationship between shear stress and FMD can be confounded by a between-subjects analysis. The thin black lines each represent the positive relationship between shear stress and FMD in each individual. Each distinctly shaded circle represents a single individual and their response to a 5-min RH test. Due to the random nature of RH, the stimulus created with a 5-min RH is variable between individuals (Pyke & Tschakovsky, 2007). Moreover, the response of each individual to a given shear stimulus varies due to between individual differences in vascular function (illustrated here as the distinct slopes of the individual shear-FMD dose response relationship) (Padilla *et al.*, 2009). Each red square represents the sampling of a single subject response to a 5-min RH test, as would be done in a between subjects design. Each individual exhibits a positive linear relationship between FMD and shear stress; however this relationship is lost when conducting a between-subject analysis because of differences in endothelial function across subjects. This may lead to the erroneous conclusion that there is no relationship between shear stress and FMD (adapted from Thijssen et al., 2010, under review).

that there is a need to further examine the association between shear stress and the magnitude of the FMD using improved study designs. Between-subject differences in endothelial function can confound the relationship between FMD and shear stress (Figure 2-5); thus, a heavier emphasis on *within*-subject designs is required in order to clarify the relationship.

2.5 The Relationship between Baseline Artery Diameter and the FMD Response

Multiple studies have reported a strong inverse relationship between baseline brachial artery diameter and FMD response magnitude (Herrington *et al.*, 2001;Silber *et al.*, 2001;Thijssen *et al.*, 2008). It is possible that there are intrinsic differences in the endothelial cell response to shear stress in differently sized vessels; however, previous studies have typically used RH protocols. With RH, the shear stress stimulus generated is inversely related to vessel diameter: smaller arteries tend to experience a greater shear stress relative to larger arteries (Herrington *et al.*, 2001;Pyke *et al.*, 2004). Thus, it remains unclear whether the inverse relationship between baseline artery size and FMD is due to limitations in experimental procedures or whether there truly are intrinsic differences between differently sized arteries. Multiple studies have compared the FMD responses of various differently sized arteries located in both the upper and lower limbs.

The importance of shear stimulus magnitude on the baseline diameter-FMD relationship was demonstrated by Pyke et al. (2004) who found that there is a significant inverse relationship between baseline diameter and the shear rate 1-minute AUC using

RH ($r^2 = 0.42$, $p < 0.001$) and a significant inverse relationship between baseline diameter and FMD. This FMD-baseline diameter relationship was significantly attenuated with normalization of the brachial artery RH FMD to the shear rate-AUC (unnormalized $r^2 = 0.65$, normalized $r^2 = 0.19$). In addition, there was virtually no relationship between baseline diameter and FMD when the shear stress stimulus was uniform across diameters (created with heating and arterial compression).

Joannides et al. (2002) characterized the radial artery FMD responses of women (artery size: 2.32 mm) and men (artery size: 2.76 mm) using both RH and hand skin heating. When shear stress was increased via RH, women had a significantly greater FMD response relative to men (13% vs. 9%). However, when gradual and sustained increases in shear stress were created with hand skin heating, the increase in diameter for a given increase in shear stress was the same in men and women. In other words, in response to the same increase in shear stress, large and small radial arteries dilated the same absolute amount (and small arteries by a larger percent amount). Hormonal differences between the sexes, primarily the higher estrogen levels in females, may contribute to the different FMD responses observed in males in females. However, Joannides et al. (2002) stipulate that gender differences in radial artery FMD are due to the fact that baseline diameter is smaller in females, thus leading to the creation of a larger shear stress stimulus with RH (Joannides *et al.*, 2002). This reinforces the need to further examine the relationship between artery size and the FMD response with methodologies that allow for creation of a controlled shear stress stimulus.

Thijssen et al. (2008) examined the FMD responses of the radial (~3 mm), brachial (~4 mm), femoral (~10 mm), superficial femoral (~8 mm), and popliteal (~6 mm) arteries using RH. A correlation including data from all arteries revealed a significant inverse relationship between artery size and FMD response magnitude ($r = -0.57$, $r^2 = 0.33$, $p < 0.001$) (Thijssen *et al.*, 2008). Artery size and FMD remained significantly correlated when the FMD responses were normalized to the shear rate AUC, although the relationship was weakened ($r = -0.48$, $r^2 = 0.23$, $p < 0.001$). Within-artery correlations between baseline vessel diameter and FMD for each artery were also examined and a significant correlation between FMD and baseline diameter was identified in the radial, brachial and popliteal arteries. Within an artery, when baseline diameter was correlated with normalized FMD responses, the relationship between baseline diameter and FMD was eliminated for all examined arteries suggesting that differences in the shear rate experienced in differently sized vessels account for the inverse relationship between FMD and shear stress. However, as previously described, there are limitations to normalization; therefore, it is difficult to make definitive conclusions based on these data. Nonetheless, based on the findings from their study, Thijssen et al. (2008) proposed that intrinsic differences in vessel wall structure in differently sized arteries may affect the magnitude of the FMD response.

Walther et al. (2008) compared upper and lower limb vasculature in swimmers, cyclists, and sedentary individuals using RH. It was observed that when FMD was normalized for the shear rate AUC, the FMD response of the brachial artery (~4 mm) was

greater than the response observed in the larger superficial femoral artery (~6.5 mm) in all three groups. Nishiyama et al. (2007) investigated differences in FMD in the popliteal (~7 mm) and brachial (~4.7 mm) arteries. They first compared the FMD responses of these two arteries after a 5 minute cuff occlusion in each limb and found the brachial artery tended to have a larger FMD than the popliteal artery, although this difference did not reach statistical significance. Using this protocol lead to a significantly higher shear rate in the smaller brachial artery (Nishiyama *et al.*, 2007). Thus, to experimentally match shear rate in both arteries, the cuff duration in the arm was reduced from 5 minutes to ~60s while maintaining the 5 minute cuff occlusion in the leg. Interestingly, when shear rate was experimentally matched between these two arteries, Nishiyama et al. (2007) found that the larger popliteal artery had a significantly greater FMD response than the brachial artery. The authors also computed the normalized FMD response following a 5-min occlusion period for both arteries and again found that the larger popliteal artery had a significantly greater dilation for a given shear rate than the brachial artery. A similar study by Parker *et al.* (2006) compared the FMD response of the brachial artery (~3 mm) to the larger popliteal artery (~6 mm) in young and old women after 5 minutes of forearm and calf cuff occlusion respectively. Similar to the findings of Nishiyama et al. (2007), the unnormalized FMD response of the smaller brachial artery was found to be significantly greater than that of the popliteal artery. However, after normalizing the FMD responses, no significant difference was found between the brachial and popliteal arteries in either age group, which is in contrast to the findings of Nishiyama et al.

(2007). It is important to note that these studies compared vessels located in different limbs, which is a major design limitation if a primary goal of a study is to isolate the impact of baseline diameter on FMD. The legs experience greater hydrostatic pressures relative to the arms; during standing, blood pressure measured at the ankle level is 65 mmHg greater than it is in the arm (Procter and Newcomer, 2006). Moreover, the legs are involved in the frequent work of locomotion and therefore contain a muscle mass that is approximately threefold greater than the arm (Walter and Richardson, 2006). Limb differences are highlighted by the observation that vascular dysfunction can occur in the lower extremities without significant changes in arteries of the upper limbs (Sanada *et al.*, 2005). Thus, comparing functional responses between arteries located in the upper and lower limbs may introduce error as differences in the responses may not be attributable to heterogeneity in vessel size but rather, due to upper vs. lower limb differences.

It is certainly possible that there are intrinsic differences in the endothelial cell response to shear stress in differently sized vessels. Additionally, there may be differences in the mechanisms that mediate FMD. For example, research has shown that the relative contribution of EDHF in mediating FMD increases as vessel size decreases (Tomioka *et al.*, 1999), which demonstrates that there may be size dependent differences in the endothelial response to shear. Other possible non-shear related reasons that may account for heterogeneity in the FMD responses of differently sized arteries include: variations in the amount of vasodilator reaching the smooth muscle of the vessel (Mulvany & Aalkjaer, 1990), non-uniform smooth muscle responses (Daemen & De

Mey, 1995), or an effect of potential structural differences between arteries of varying baseline diameter (Thijssen *et al.*, 2008).

The FMD response is dependent on the shear stress stimulus, with a greater shear stimulus evoking a greater FMD response. The use of RH makes it extremely difficult to create similar shear stress profiles in differently sized arteries or in groups with heterogeneous vessel diameters since this test inherently generates a larger stimulus in smaller vessels. Investigation into the reasons behind the inverse relationship between vessel size and FMD response magnitude is required in order to further our understanding of endothelial function and how to interpret FMD responses. The next chapter covers the specific study of this thesis which investigates how the FMD responses of two differently sized vessels compare in response to graded, matched levels of shear stress created via handgrip exercise.

Chapter 3

Manuscript

3.1 Introduction

The endothelial cells that line the arteries are critically involved in the maintenance of vascular homeostasis. In healthy arteries, increases in blood-flow associated shear stress lead to the release of vasodilators (e.g. NO, EDHF and PGI_2) by the endothelium, which subsequently causes an endothelium-dependent, flow-mediated dilation (FMD) (Buga *et al.*, 1991;Celermajer, 1997;Luksha *et al.*, 2009;Okahara *et al.*, 1998). An inverse relationship between baseline vessel diameter and the magnitude of the conduit artery FMD response in the arm has been reported by several studies (Herrington *et al.*, 2001;Silber *et al.*, 2001;Thijssen *et al.*, 2008). Specifically, small arteries tend to show a larger FMD response magnitude relative to larger vessels. As a result, it can currently be difficult to use FMD to compare endothelial function in groups with varying baseline artery sizes (for example, the young vs. the elderly and men vs. women) because it may be unclear whether a smaller FMD response in a large artery reflects endothelial dysfunction or a benign effect of artery size. It is possible that there are intrinsic differences in the endothelial cell response to shear stress in differently sized vessels; however, methodological limitations of previous studies have prevented a definitive conclusion.

In human FMD studies, an increase in artery wall shear stress is typically achieved by releasing forearm occlusion (reactive hyperemia (RH)). Large, transient shear stress increases are created with RH; thus, the shear stress imparted on the vessel cannot be maintained or controlled via this methodology, which is a serious limitation (Pyke *et al.*, 2004). With RH, the shear stress stimulus generated is inversely related to vessel diameter such that smaller arteries tend to experience a greater shear stress relative to larger arteries (Herrington *et al.*, 2001;Pyke *et al.*, 2004). It is therefore possible that in previous studies, smaller vessels experienced a greater shear stress stimulus relative to larger vessels, potentially accounting for the inverse relationship between baseline artery size and the FMD response. In support of this, the relationship between baseline diameter and FMD within a given vessel has been eliminated in studies that have accounted for the stimulus magnitude with ratio normalization (FMD response/shear stimulus magnitude) (Pyke *et al.*, 2004;Thijssen *et al.*, 2008) or have controlled the stimulus magnitude independent of artery size (Pyke *et al.*, 2004).

Between vessel comparisons have also been conducted, most often comparing smaller arm arteries with larger leg arteries (Nishiyama *et al.*, 2007;Parker *et al.*, 2006;Walther *et al.*, 2008). Some of these studies have also attempted to account for the RH stimulus magnitude (Nishiyama *et al.*, 2007;Parker *et al.*, 2006) with conflicting results. However, in these studies conclusions regarding the impact vessel size per se are complicated by the potential for a limb specific hemodynamic effect (Proctor & Newcomer, 2006) on endothelial function.

Handgrip exercise has recently been explored as an alternative method of increasing shear stress in order to assess FMD (Pyke *et al.*, 2008b). Exercise creates a rapid reduction in the vascular resistance in the muscle bed, which leads to a sustained increase in upstream conduit artery blood flow (Delp & Laughlin, 1998;Pyke *et al.*, 2008b;Shoemaker *et al.*, 1998). The magnitude of the shear stress stimulus is dependent on the exercise intensity, such that higher exercise intensities elicit a greater shear stress stimulus (Pyke *et al.*, 2008b). Exercise is arguably the most common cause of significant shear stress increases in conduit arteries. Therefore, using exercise to increase shear stress is of interest because it creates a physiologically relevant stimulus for studying FMD (Duffy *et al.*, 1999;Gielen & Hambrecht, 2001;Gielen *et al.*, 2001). Furthermore, by manipulating the exercise intensity, a uniform shear stress stimulus can be achieved over multiple trials both within and between subjects (Jazuli *et al.*, 2010;Pyke *et al.*, 2008b).

Endothelial dysfunction plays a pivotal role in pathogenesis of atherosclerosis and is characterized by a blunted FMD response (Celermajer *et al.*, 1992). Given the crucial role of the endothelium in maintaining vascular health, it is important to understand the response characteristics of the endothelium and the factors that impact the endothelial response to shear stress. Several studies have attempted to characterize the shear stress stimulus-FMD relationship by manipulating the reactive hyperemia technique to create a range of stimuli (Betik *et al.*, 2004;Padilla *et al.*, 2008;Padilla *et al.*, 2009;Pyke & Tschakovsky, 2007). Emerging evidence indicates that risk factors for atherosclerosis alter the slope of the relationship (Padilla *et al.*, 2009). As mentioned

above, the impact of baseline artery size on the FMD response is currently unclear and to date, there has been no clear examination of the impact of vessel size on the shear stress stimulus-FMD response relationship.

With this as a background, the main objectives of the present study were 1) to investigate the impact of baseline artery diameter on the FMD response magnitude using handgrip exercise to create matched, sustained increases in shear stress in two differently sized arteries; and 2) to examine the impact of vessel size on the dose-response relationship between shear stress and FMD. To achieve this, we created three distinct, matched levels of shear stress both within and between subjects via handgrip exercise in the radial (RA) and brachial (BA) arteries (~2 and ~4 mm in diameter, respectively). This design allows for within subject, within limb comparisons, providing a superior isolation of the impact of vessel size by eliminating the influence of between limb and between subject differences in endothelial function (Pyke *et al.*, 2004). Our results suggest that 1) when exposed to matched levels of shear stress, the FMD response of the smaller RA is greater than that of the BA; and 2) there is a strong positive relationship between handgrip exercise induced increases in shear stress and FMD, however, the characteristics of that relationship differ somewhat between the RA and BA.

3.2 Methods

3.2.1 Subjects

Fifteen healthy, non-smoking males (19 to 25 years old) from the Queen's University community participated in this study. Health status was confirmed via a

medical screening questionnaire for risk factors associated with endothelial dysfunction. 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.

Subjects were instructed to abstain from food for 4 hours prior to the study and to avoid alcohol, caffeine and exercise for 12 hrs before the study. Each subject participated in testing over 3 days. These 3 visits occurred within a 14 day period and experiments were performed at the same time of day (± 2 hrs) with the exception of one subject who completed two morning visits and one late afternoon visit. Experiments were performed in a quiet, temperature controlled room (20-25 °C). Subjects completed all trials on each visit, resulting in three replicates of each trial; order of arterial examination was counterbalanced between subjects (brachial first vs. radial first) and the trials were randomized and performed in a way that balanced the order that each SR level was tested over three visits.

3.2.2 Subject Monitoring

Heart rate was continuously monitored using a 3-lead electrocardiogram (ECG). Blood pressure was monitored throughout each trial using a Finometer device (Finometer PRO, Finapres Medical Systems).

3.2.3 Brachial Artery Blood Velocity and Diameter Measurements

Brachial (BA) and radial artery (RA) blood flow velocities were obtained using Doppler ultrasound operating at 4 MHz (Vivid i2 GE Medical Systems) and were

measured continuously during each trial. The Doppler shift frequency spectrum was analyzed via a Multigon 500P TCD (Multigon Industries). The corresponding voltage output was continuously sampled at 1000 Hz and stored (Powerlab, ADInstruments) for analysis at a later time.

For reasons previously described (Pyke *et al.*, 2008a) scans were performed at an insonation angle of 68º for all trials. The ultrasound probe was placed over the RA or BA to acquire an optimal image of the vessel and a clear arterial blood velocity signal with no interference from adjacent veins. Once in position, a guide was adhered to the skin so that the same region of the vessel would be imaged across trials. This region was marked for repeatability in subsequent visits. BA and RA diameters were obtained using two-dimensional gray-scale ultrasound imaging technology operating at 12 MHz in B-mode (Vivid i2 GE Medical Systems). The images were recorded in AVI format on an external PC with commercially available software (Camtasia Studio, TechSmith) for later analysis.

3.2.4 Experimental Procedures

Subjects lay supine with both arms extended out to their sides. Ultrasound measurements were conducted on the left arm while blood pressure was measured in the right arm. There were a total of six trials per visit; three trials in the RA and three in the BA, all of which had the following in common. Baseline BA or RA images and blood velocity before exercise commenced were recorded for 1 min (Figure 3-1). Subjects then performed handgrip exercise for 6 min. Blood flow velocity was continuously monitored

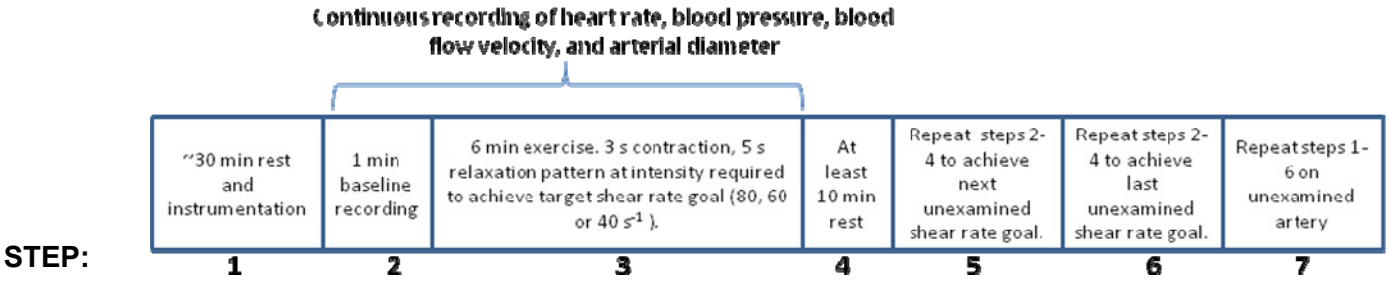

Figure 3-1: Timeline of experimental procedure. This procedure was repeated for all three experimental visits.

throughout each trial and a minimum of 10 min were allotted between trials to permit BA or RA diameter and blood flow to return to baseline values. It has previously been shown that there is no systematic change in FMD over four closely spaced exposures to the same magnitude of handgrip exercise induced increases in shear stress (Jazuli *et al.*, 2010).

Radial Artery

To create a sustained increase in shear stress in the RA, a rhythmic dynamic handgrip exercise that focuses on the contraction of the adductor pollicis muscle was performed by subjects to minimize contraction of forearm muscles and general arm movement (Figure 3-2A). Subjects squeezed an air bladder with a pressure sensor. Subjects first performed two maximum voluntary contraction (MVC) attempts so that exercise intensity in the exercise trials could be quantified relative to their maximal contraction intensity. Subjects then performed this adductor pollicis exercise (3 s contraction; 5 s relaxation duty cycle) at the required intensity to achieve and maintain the target blood velocity necessary to achieve the target SR ($40s^{-1}$, $60s^{-1}$, or $80\ s^{-1}$). Using the measured artery diameter (D), the blood flow velocity (V) required to achieve the target SR was calculated ($V = SR \times D$). Mean contraction intensities at SR40, SR60, and SR80 were 27 ± 3% MVC, 45 ± 3% MVC, and 65 ± 4% MVC, respectively. Blood flow velocity was continuously recorded and displayed (8 s moving average) on a computer screen so that the subject had visual feedback on how close they were to the

A

B

Figure 3-2: A) Adductor pollicis exercise used to increased shear in the RA. B) Handgrip exercise used to increase shear in the BA. Subjects squeezed an air bladder equipped with a pressure transducer. 3 s contraction, 5 s relaxation duty cycle was used for both types of exercise.

target blood flow velocity. The subject was coached through minor increases and decreases in exercise intensity in order to maintain the target blood flow velocity.

Brachial Artery

Subjects performed a rhythmic, dynamic handgrip exercise to create sustained increases in shear stress in the BA (Figure 3-2B). The same exercise protocol as described in the RA was followed for the BA. Mean contraction intensities at SR40, SR60, and SR80 were 11 ± 1% MVC, 25 ± 2% MVC, and 39 ± 4%MVC, respectively. In a subset of subjects, a fourth BA trial was performed. In this trial, subjects exercise at ~60% of their MVC, following the timeline outlined in Figure 3-1.

Arterial Compression

To determine whether mechanisms of vasodilation other than shear-induced FMD occur in the RA, arterial compression was used to prevent increases in shear stress during exercise. The subject's RA pulse was located just proximal to the wrist. Blood flow velocity was controlled via manual experimenter finger pressure applied to the radial pulse, with increased pressure leading to reductions in blood flow velocity. The subject performed the adductor pollicis exercise (duty cycle: 3-s contraction; 5-s relaxation) at an MVC of 50 ± 7%, (which corresponded to the creation of a SR of $60s^{-1}$) for 3-min (Figure 3-3). Blood velocity was continuously displayed as an 8-s moving average, which allowed the experimenter to adjust compression to maintain blood flow velocity at

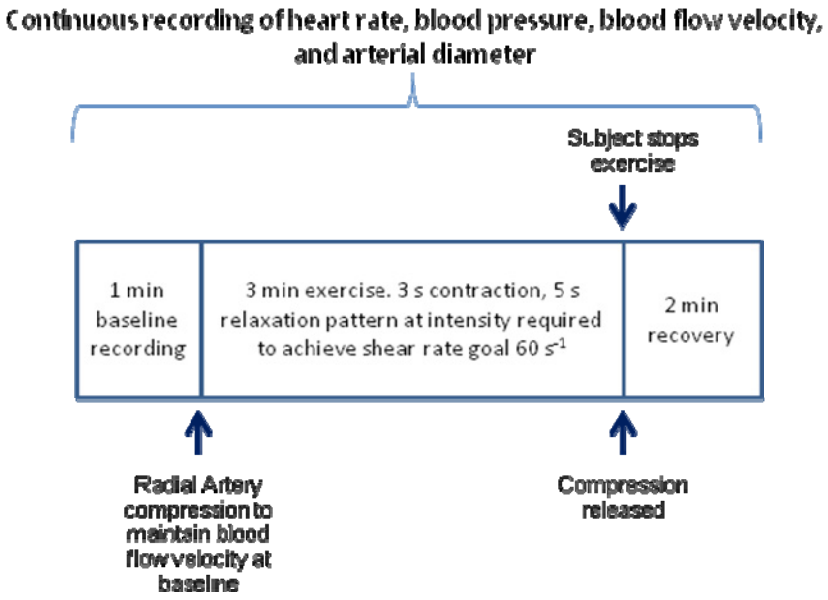

Figure 3-3: Timeline for arterial compression exercise trial performed in the radial artery.

baseline values. At the end of the 3-min exercise period, the subject stopped exercise and compression was released. A 2-min recovery period was recorded.

3.2.5 Data Analysis

Artery blood velocity

RA and BA blood velocities were analyzed offline in 3 s average time bins. A mean blood velocity profile for each subject at each SR condition (SR40, SR60 and SR80) was obtained from the average of the three trials per condition (with the exception of two subjects who had one trial for RA SR80 and two subjects who had two trials for RA SR80 due to difficulties in achieving this SR target).

Artery Diameter

RA and BA diameter were 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 vessel walls are clearest (Woodman *et al.*, 2001). It then identifies and tracks the artery walls via the intensity of the brightness of the walls relative to the vessel lumen. 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. A continuous diameter measurement is provided (30 frames per second). Once the diameter measurements have been collected by the program, erroneous data points (due to vessel wall tracking errors) can be removed.

The diameter data were compiled as 3 s time bins. This allows for the time alignment and averaging of multiple trials. Missing data due to software diameter tracking errors in individual trials were interpolated to facilitate calculation of the average. Thus, a diameter measurement and a corresponding blood velocity measurement, time aligned for every 3 s, were obtained. FMD was reported as the percent change in diameter (%FMD) or the absolute change in diameter from the baseline measurement before exercise commenced to the average diameter during the last minute of exercise.

Shear Rate

Shear rate (SR; an estimate of shear stress without viscosity) was calculated as the mean blood flow velocity/vessel diameter. The 3 s average SR was averaged into 21 s time bins. SR is reported as the mean SR during the last 5 min of exercise or the mean increase in SR from baseline to the average SR during the last 5 min of exercise.

Statistical Analysis

Data are reported as means ±SE, unless otherwise stated. Repeated-measures ANOVAs were used to compare the FMD responses and the SR stimuli created across artery conditions (baseline, SR40, SR60, SR80). To test the hypothesis that the RA and BA would have similar FMD responses to similar SR stimuli with FMD increasing as the

SR increased, the FMD responses at SR targets 40, 60, and 80 s^{-1} were compared. The factors were artery (RA and BA), and shear condition (SR40, SR60 and SR80).

To test the hypothesis that a significant positive relationship exists between SR and FMD, linear regression analyses examining the relationship between FMD (%FMD or absolute change diameter) and SR (mean shear rate or change in shear rate from baseline) were examined using 1) mean within-subject data; 2) across-subject mean data; and 3) between subject data. To test the hypothesis that there is no relationship between baseline artery size and FMD when SR is controlled, regression analyses between %FMD and baseline artery diameter were conducted for the RA and BA.

To test the hypothesis that increases in radial artery diameter where due to exercise-induced shear stress increases during exercise trials and not other vasodilatory mechanisms, within the arterial compression condition, arterial diameter during exercise and release was compared to that during baseline using one-way repeated measures ANOVA. The level of significance was set at $P < 0.05$ for all tests. Significant results for repeated-measures ANOVA tests were further assessed using Tukey's post hoc tests. Statistics were calculated using Sigmaplot version 11. Slope and intercept data resulting from individually plotted stimulus response relationships were compared via paired t-tests. Regression results resulting from between subject comparisons were performed with GraphPad Prism 5. Where slopes were identified as significantly different, regression lines were assumed to differ; as such, y-intercepts were not tested.

3.3 Results

3.3.1 Subject Characteristics

Refer to Table 3-1.

3.3.2 Heart Rate

Refer to Figure 3-4A. There was no significant difference between RA and BA baseline HR ($p = 0.979$); however, HR was consistently higher in the RA vs. BA during exercise ($p < 0.001$). There was a significant effect of exercise on HR in both the RA and BA, with HR increasing as the SR target increased ($p < 0.001$). In the SR 80 condition, HR increased by ~5 beats per minute (bpm) in the BA ($p < 0.001$ vs. baseline) and by ~8 bpm in RA ($p < 0.001$ vs. baseline). A significant increase in HR was also observed during exercise in the radial compression trials ($p < 0.001$ vs. baseline). Subject's average HR significantly increased from baseline during the BA 60%MVC exercise trial (52.7 ± 2.0 vs. 63.5 ± 1.9 beats/minute, $p < 0.001$).

3.3.3 Blood Pressure

Refer to Figure 3-4B. Mean arterial pressure (MAP) did not differ between the RA and BA across all artery conditions (baseline, SR 40, SR 60, SR 80; $p = 0.716$). There was a main effect of exercise on MAP in both the RA and BA ($p < 0.001$). In the BA exercise, MAP increased modestly at SR 80 (~5 mmHg vs. baseline). This elevation in MAP during RA exercise was more pronounced at SR 80 (~10mmHg vs. baseline). MAP significantly increased from baseline during the BA 60%MVC exercise trial (93.5 ± 3.6 vs. 104.1 ± 3.8 mmHg, $p < 0.001$). A significant increase in blood pressure was also

Table 3-1: Subject Characteristics

Age (years)	22 ± 2.2
Weight (kg)	78.7 ± 6.1
Height (cm)	181.7 ± 5.6
Average Resting HR (beats/min)	60.8 ± 9.7
Averaging Resting MAP (mmHg)	97.3 ± 8.6

Values are means ± SD. HR: Heart Rate, MAP: Mean arterial pressure.

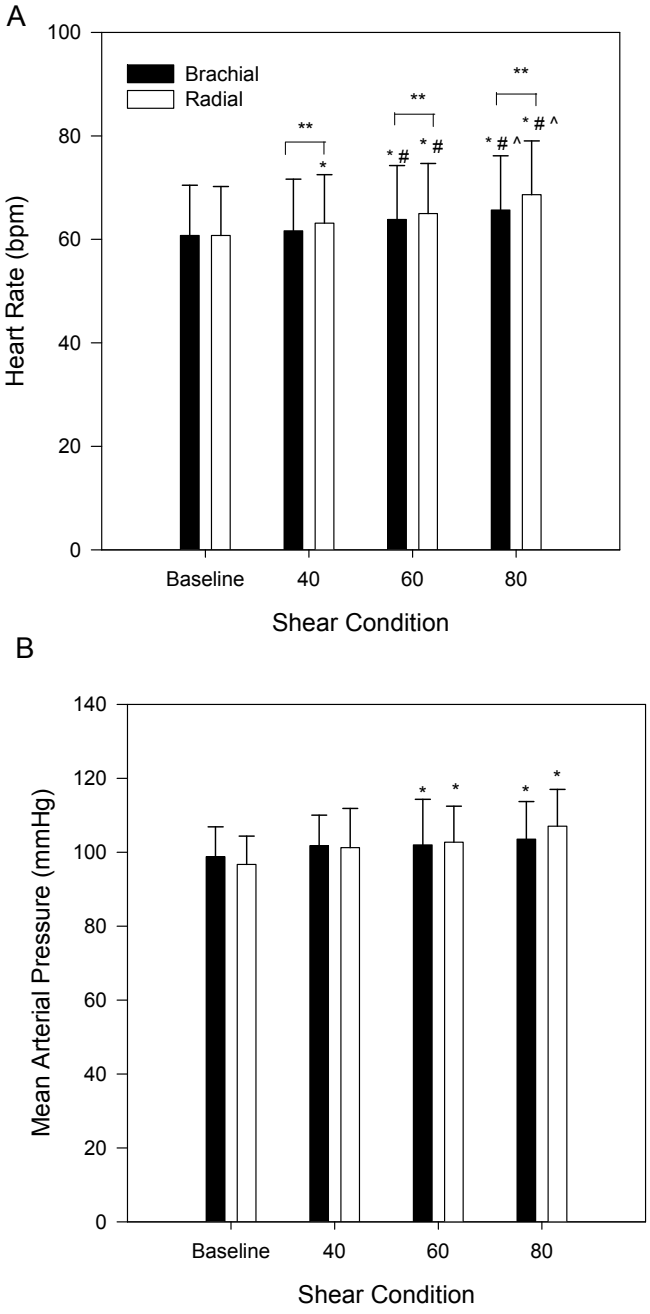

Figure 3-4: A) Heart rate (HR) and B) mean arterial pressure (MAP) for each artery at each shear condition. HR was consistently higher in the RA vs. BA during exercise; MAP did not differ between the RA and BA across all artery conditions. * Significantly different from baseline within artery. ^ Significantly different from SR 60 within artery. #Significantly different from SR 40 within artery. ** Significant difference between arteries.

observed in the radial compression trial with a ~10 mmHg elevation in MAP during exercise ($p = 0.005$ vs. baseline).

3.3.4 Quantification of Shear Rate Stimuli

Baseline SR was significantly lower in the RA at all SR levels (Target SR 40: RA: $11.8 \pm 0.7 s^{-1}$ vs. BA: $17.4 \pm 1.2\ s^{-1}$; Target SR 60: RA: $11.6 \pm 0.6 s^{-1}$ vs. BA: $17.8 \pm 1.0 s^{-1}$; Target SR 80: RA: $11.0 \pm 0.5 s^{-1}$ vs. BA: $18.01 \pm 1.3\ s^{-1}$; $p < 0.001$). The target SR level of the trial did not have an effect on the baseline SR in either artery ($p = 0.894$). BA and RA SR profiles for each target SR (40, 60 and 80 s^{-1}) are shown in Figure 3-5. Three distinct SR levels were successfully created (steady state SR taken during last 5-min of exercise: $39.8 \pm 0.6 s^{-1}$, $57.3 \pm 0.7 s^{-1}$, $72.4 \pm 1.2 s^{-1}$; $p < 0.001$ between SR levels). SR was well matched in the RA and BA at SR40 and SR60; however, SR was slightly greater in BA at SR80 (RA: $70.9 \pm 1.5 s^{-1}$, BA: $73.8 \pm 0.8 s^{-1}$; $p = 0.017$). As a result of the lower baseline shear rate in the RA, Delta SR was significantly greater in the RA at all SR levels ($p < 0.001$).

3.3.5 Baseline Artery Diameter

The baseline diameter was smaller in the RA vs. the BA (2.06 ± 0.05 mm vs. 3.78 ± 0.12 mm; $p < 0.001$). The target SR level of the trial did not have an effect on the baseline diameter in either artery ($p = 0.066$).

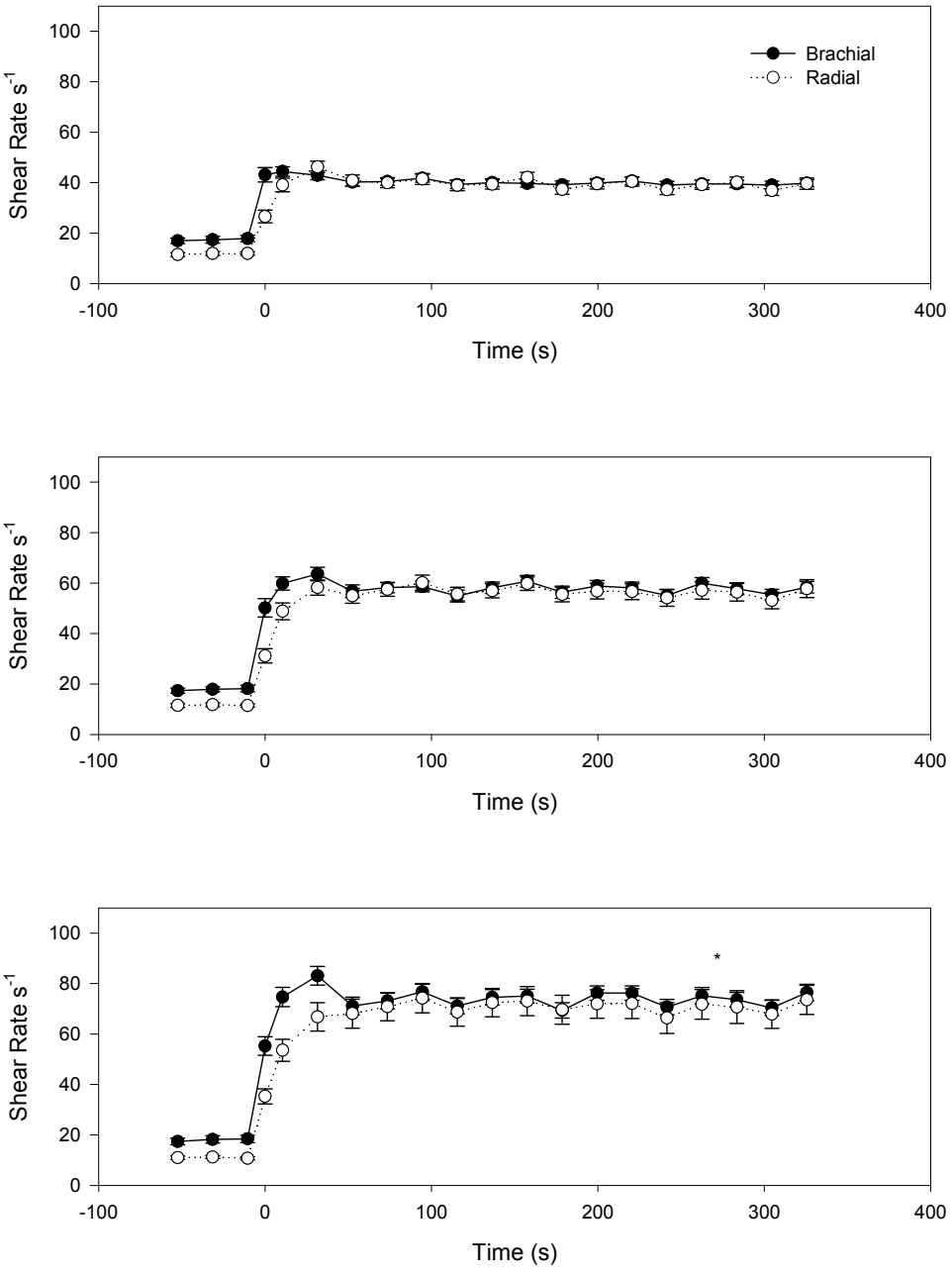

Figure 3-5: BA and RA SR profiles over time for each SR level. A) SR profiles for target SR 40; B) SR profile for target SR 60; c) SR profiles for target SR 80. SR was well matched at SR targets 40 and 60 but was slightly higher in the BA at SR 80 (p = 0.017). *Significantly greater than RA.

3.3.6 %FMD and Absolute Diameter Changes

The average %FMD response profiles of the BA and RA at each SR level are shown in Figure 3-6. The %FMD at the end of exercise (last minute of exercise) was greater in the RA vs. BA at every SR target (SR40: RA: 5.4 ± 0.8%, BA: 1.0 ± 0.2%; SR60 RA: 9.8 ± 1.0%, BA: 2.5 ± 0.5%; SR80 RA: 15.7 ± 1.5%, BA: 5.4 ± 0.7%; $p < 0.001$). The %FMD was different across SR levels in both the RA and BA ($p < 0.001$). The %FMD in response to each shear target represents a significant increase from baseline diameter in all conditions with the exception of BA SR40 ($p = 0.063$ vs. baseline).

FMD was also quantified using absolute change in diameter. The RA had a greater absolute change in diameter at each SR level relative to the BA (SR40: RA: 0.011 ± 0.001 cm, BA: 0.004 ± 0.001 cm; SR 60: RA: 0.020 ± 0.002 mm, BA: 0.009 ± 0.002 cm; SR80: RA: 0.032 ± 0.003 cm, BA: 0.020 ± 0.002 cm; $p < 0.001$). As SR level increased (SR40 to SR60 to SR80), the absolute diameter change significantly increased both in the RA and BA ($p < 0.001$).

3.3.7 FMD-SR Dose-Response Relationship

Within Subject %FMD-SR Relationship

SR was quantified as the mean SR experienced in the last five minutes of exercise because this represented the steady state period. The mean slope of the individual within-

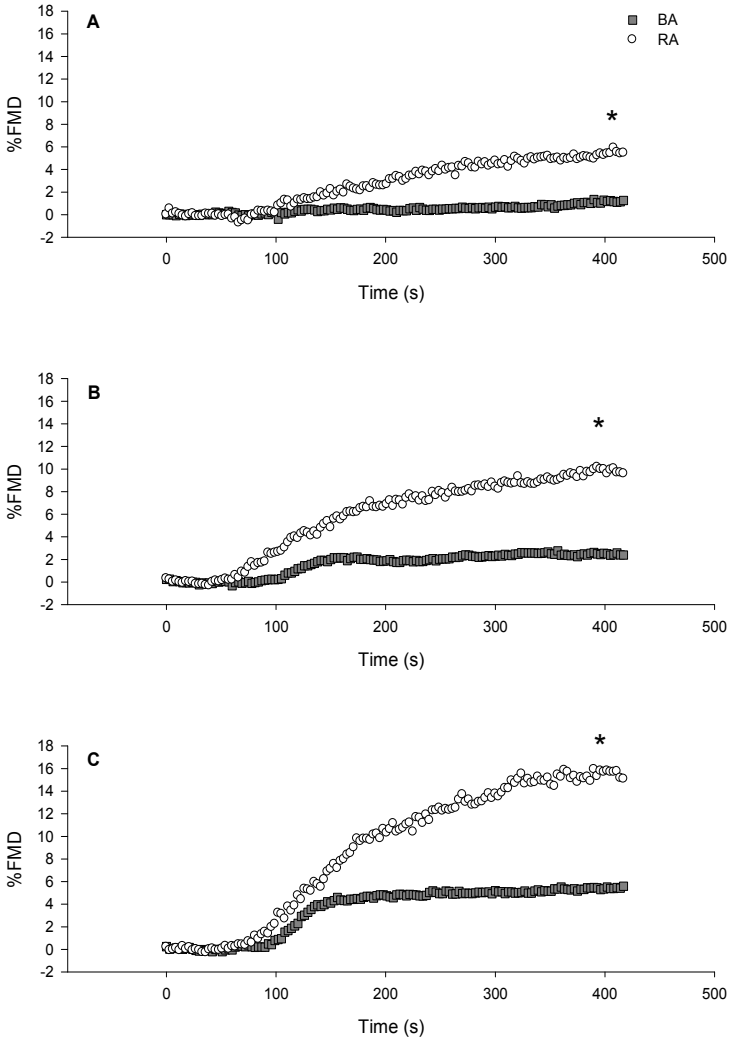

Figure 3-6: %FMD response profiles for the RA and BA at A) Target SR 40; B) Target SR 60; and C) Target SR 80. At all three target SRs, the RA dilated significantly more than the BA. * Significantly different from BA %FMD.

subject %FMD-SR dose response lines was significantly greater in the RA (RA: 0.33 ± 0.04, BA: 0.13 ± 0.02; $p < 0.001$) and a strong relationship between %FMD and SR was observed in both arteries (RA r^2: 0.92 ± 0.02; BA: 0.90 ± 0.03). The Y- intercepts were borderline but found to be not significantly different (RA: -7.96 ± 1.76; BA: -4.44 ± 0.63; $p = 0.08$). The x-intercepts were significantly different between arteries (BA: 33.02 ± 1.96 s^{-1}; RA: 18.36 ± 5.21 s^{-1}; $p = 0.019$). Figure 3-7 presents an individual sample data set.

Across-Subject %FMD-SR Relationship

When the data was expressed as the mean FMD and mean SR of all subjects at each shear target, linear regression revealed a strong positive relationship between %FMD and SR in both the RA and BA (RA r^2: 0.98; BA r^2: 0.95) (Figure 3-8). The difference between the RA and BA slopes approached significance (RA: 0.33, BA: 0.13; $p = 0.06$); the y-intercepts were found to significantly differ (RA: -7.91, BA: -4.38; $p = 0.018$).

The slopes and y-intercepts of the %FMD-SR regression lines of the RA and BA were also compared when the regression was calculated using all subject data from every SR target (Figure 3-9A). A significant positive relationship between %FMD and SR was identified in both RA and BA (RA r^2: 0.31, $p < 0.001$; BA r^2: 0.45, $p < 0.001$) and the

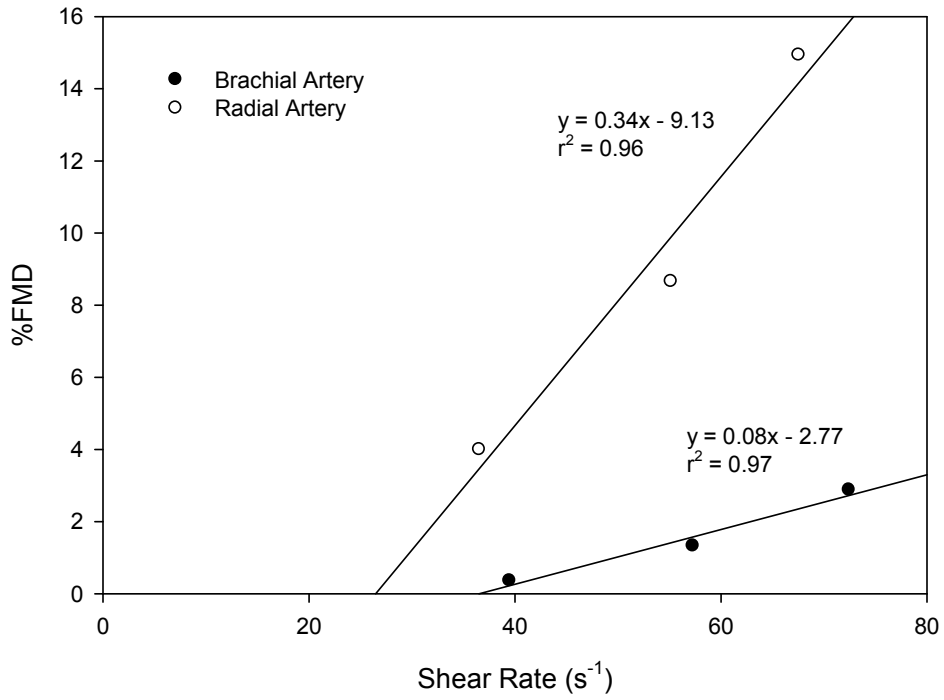

Figure 3-7: Sample of an individual %FMD-SR dose-response relationship in the RA and BA.

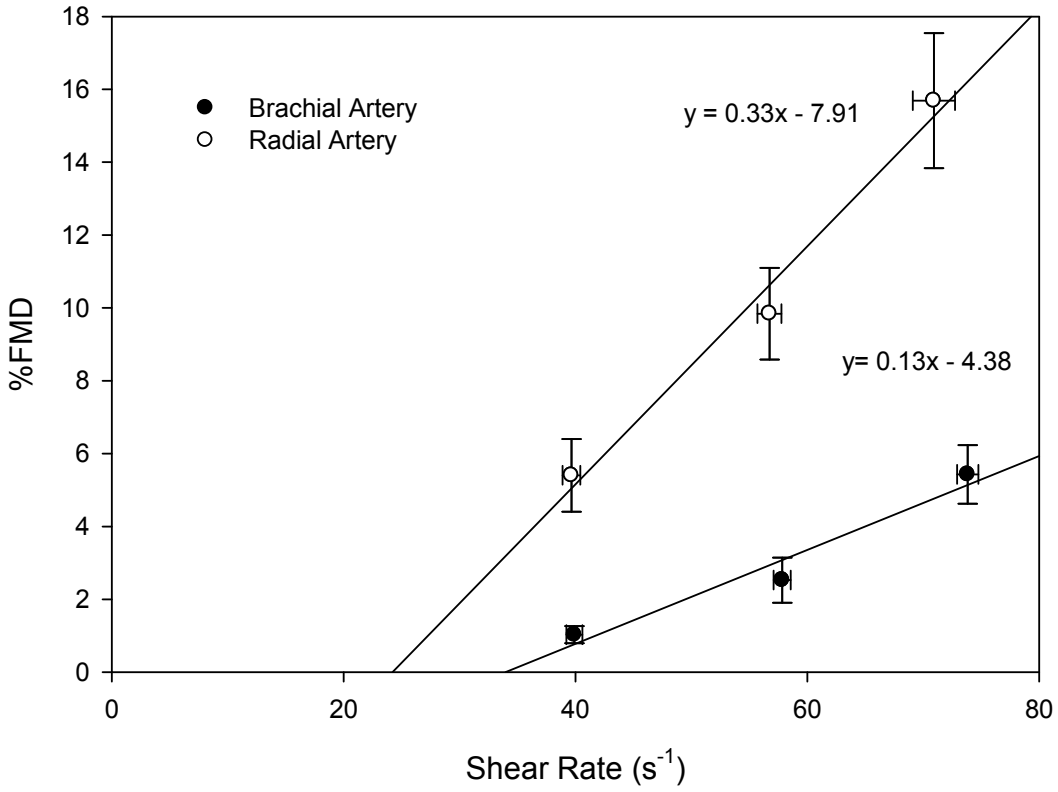

Figure 3-8: Across-subject mean %FMD-SR dose response relationships for RA and BA. A strong positive relationship between %FMD and SR was identified in both the RA and BA (RA r^2 = 0.98; BA r^2: 0.95). The RA slope was found to be greater and this approached significance (p = 0.06). The y-intercepts were found to significantly differ (p = 0.018).

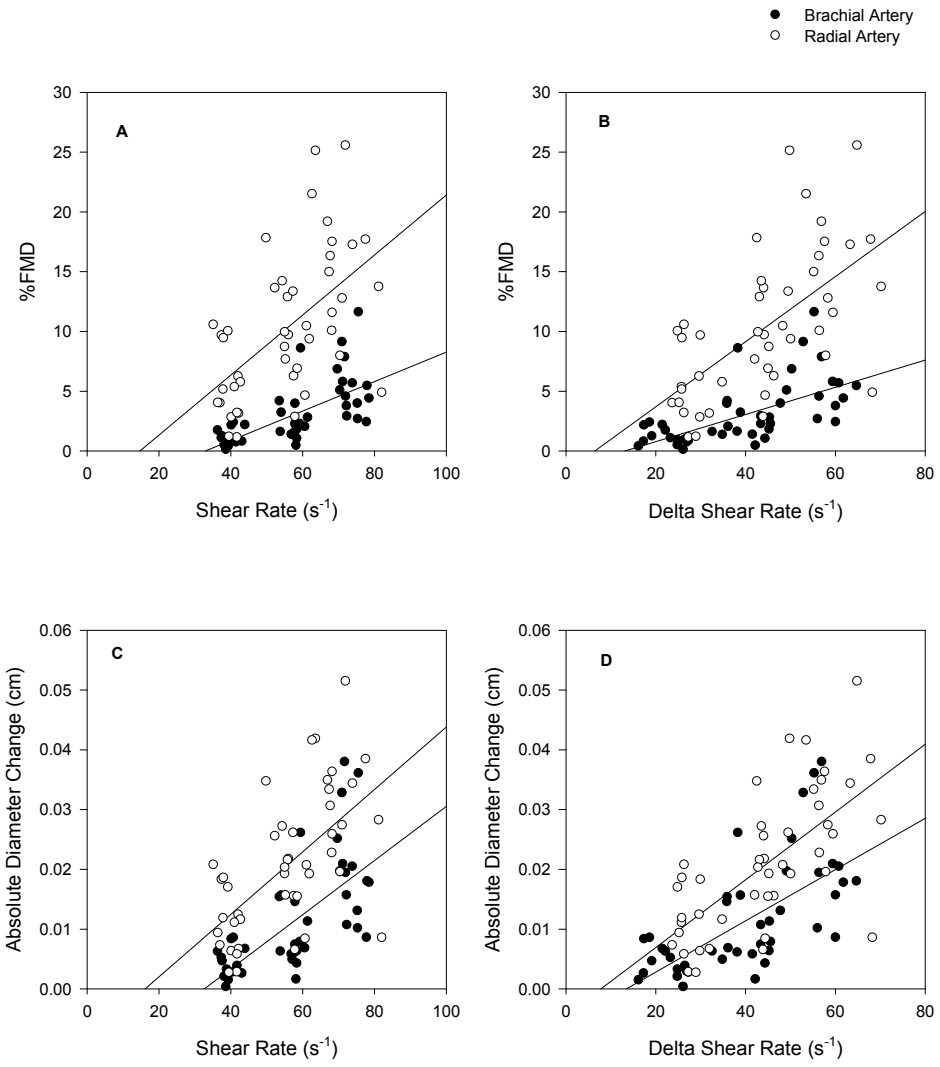

Figure 3-9: The relationship between FMD and shear using all subject data. A) Relationship between SR and %FMD in the RA and BA (RA r^2: 0.31, $p < 0.001$; BA r^2: 0.45, $p < 0.001$; RA y-intercept: -3.7, BA y-intercept: -4.1, did not test). The slope of the RA was larger than that of the BA ($p = 0.036$). B) Relationship between delta SR and %FMD in the RA and BA (RA r^2: 0.38, $p < 0.001$; BA r^2: 0.40, $p < 0.001$; RA y-intercept: -1.8, BA y-intercept: -1.5, did not test). The RA had a significantly greater slope than the BA ($p = 0.006$). C) Relationship between SR and absolute diameter change in the RA and BA (RA r^2: 0.37, $p < 0.001$; BA r^2: 0.47, $p < 0.001$). The slopes of the two lines were found not to differ but there was a significant difference in the y-intercepts ($p < 0.0001$). D) Relationship between delta SR and absolute diameter change in the RA and BA (RA $r^2 = 0.45$, $p < 0.0001$; BA $r^2 = 0.43$, $p < 0.0001$). The slopes of the two lines were found not to differ but there was a significant difference in the y-intercepts ($p < 0.0001$).

slope of the RA was greater than that of the BA (RA: 0.25 vs. BA: 0.12, $p = 0.036$). Similarly, a significant positive relationship between %FMD and the increase in SR from baseline (delta SR) was identified in both RA and BA (RA r^2: 0.38, $p < 0.001$; BA r^2: 0.40, $p < 0.001$) (Figure 3-9B). The RA had a significantly greater slope than the BA (RA: 0.12, BA: 0.27; $p = 0.006$).

The data were also characterized using absolute diameter change. A significant positive relationship between absolute diameter change and SR was identified in both the RA and BA (RA r^2: 0.37, $p < 0.001$; BA r^2: 0.47, $p < 0.001$) with a non-significant difference between the slopes of the two lines (RA: 0.00052 vs. BA: 0.00045, $p = 0.588$) (Figure 3-9C). The y-intercepts significantly differed (RA: -0.0084, BA: -0.015; $p < 0.0001$). This indicates that the regression lines are parallel but distinct.

A similar trend was observed when the absolute diameter change and delta SR dose-response relationship was examined (RA $r^2 = 0.45$, $p < 0.0001$; BA $r^2 = 0.43$, $p < 0.0001$) (Figure 3-9D). The slopes of the two lines were found not to differ (RA: 0.00057, BA: 0.00043; $p = 0.255$) but there was a significant difference in the y-intercepts (RA: -0.0058 vs. BA: -0.0044; $p < 0.0001$), which indicates that the regression lines are distinct.

60%MVC Brachial Artery Trials (Within-Subject Comparisons)

In a subset of 6 subjects, a fourth BA trial consisting of 6-min of exercise performed at an intensity of 60% of each individual's maximum voluntary contraction (MVC) was completed. This was done to create a larger range of %FMD values and to

verify that the relatively low BA dilation observed across subjects was due to a low SR stimuli. The stimulus-response regression lines of the RA and BA (with the 60%MVC trial) were then re-examined (see Figure 3-10 for an individual data set). The %FMD was 13.0 ± 1.5% in this trial. The strong within-subject relationship between shear and FMD was maintained with the addition of this fourth shear rate (BA r^2: 0.92 ± 0.03).

3.3.8 Relationship Between Baseline Artery Size and %FMD within each artery

Linear regression analysis of RA baseline diameter vs. %FMD across all subjects revealed a modest but significant, negative relationship (slope = -122.70, $r^2 = 0.18$, $p = 0.004$). The relationship between baseline RA diameter and absolute change in diameter approached significance ($r^2 = 0.08$, $p = 0.055$). In contrast, no significant relationship between baseline artery size and %FMD was identified in the BA (slope = -9.19, $r^2 = 0.02$, $p = 0.312$) (Figure 3-11). Similarly, there was no relationship between baseline brachial artery diameter and absolute change in diameter ($r^2 = 0.003$, $p = 0.907$).

3.3.9 Correlations in FMD between the RA and BA

There was no correlation between the %FMD responses (at target SR 80: $r^2 = 0.05$, $p = 0.413$) or the absolute diameter change (at target SR 80: $r^2 = 0.02$, $p = 0.624$) of the RA and BA. The %FMD-SR slopes of these arteries were also uncorrelated ($r^2 = 0.003$, $p = 0.841$).

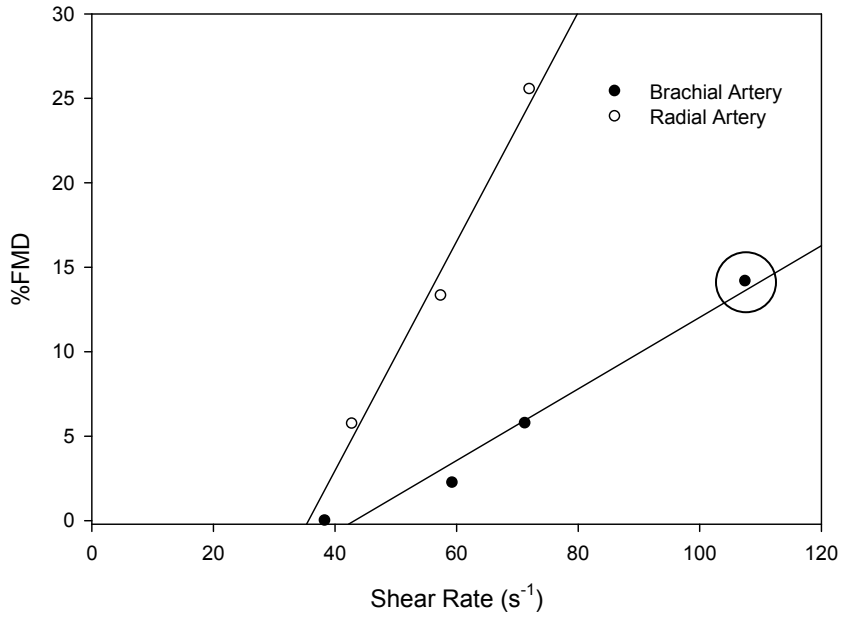

Figure 3-10: Sample of an individual %FMD-SR dose-response relationship in the RA and BA, including the fourth BA trial. The subject performed one trial of handgrip exercise at 60% MVC for 6 min, creating the highest SR stimulus and correspondingly, the greatest FMD response. This fourth BA trial is circled for clarity.

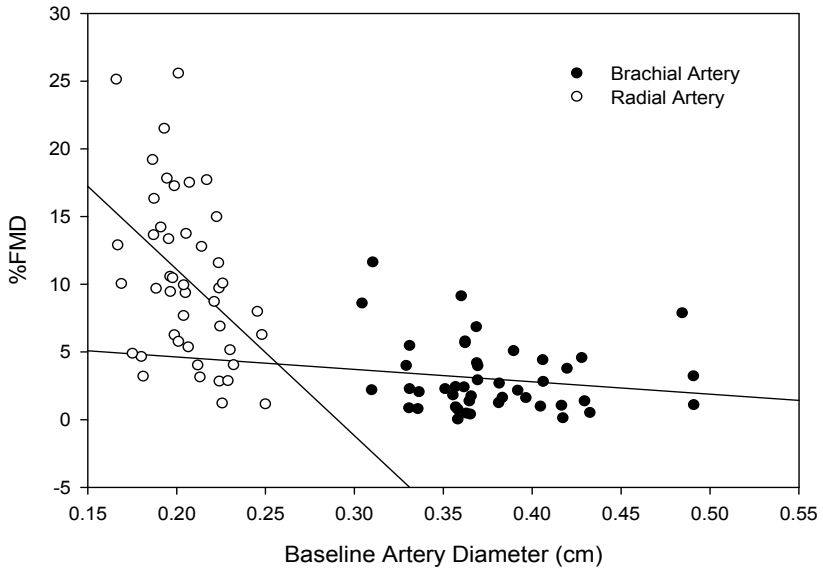

Figure 3-11: %FMD-baseline artery diameter relationship (data from all subjects and SR trials included). A significant negative relationship between baseline artery diameter and %FMD was identified in the RA ($r^2 = 0.18$, $p = 0.004$) but not in the BA ($r^2 = 0.02$, $p = 0.312$). This same trend (significant relationship in the RA, but not in the BA) was also observed when each shear condition was plotted separately.

3.3.10 Radial Arterial Compression

In a subset of 11 subjects, the shear stimulus elicited via exercise was prevented via arterial compression to assess whether mechanisms of vasodilation other than RA shear-induced FMD contributed to the observed dilation during exercise. The average diameter during the last minute of exercise (exercise trial was 3 min total) was not significantly different from baseline diameter (2.22 ± 0.08 mm vs. 2.18 ± 0.09 mm, respectively; $p = 0.386$). Peak release diameter (2.36 ± 0.08 mm) was significantly greater than baseline diameter ($p < 0.001$) and the average diameter during the last min exercise ($p < 0.001$).

3.4 Discussion

This study was designed to compare the FMD responses of two differently sized vessels to graded, matched handgrip exercise induced levels of shear stress (SS). The experimental approach has two distinct advantages over previous explorations: 1) the ability to target a sustained shear rate stimulus independent of vessel size, and 2) it allows within subject, within limb comparisons providing a superior isolation of the impact of vessel size by eliminating the influence of between limb and between subject differences in endothelial function. The primary findings were that 1) the smaller radial artery (RA) dilated more than the larger brachial artery (BA) in response to matched SS stimuli; 2) the RA and BA FMD responses increased as the SS stimulus increased; 3) the slope of the %FMD-shear stimulus regression line was greater in the RA relative to the BA; and

4) within the BA, there was no relationship between baseline diameter and %FMD; however, a modest inverse relationship was detected between these variables in the RA. These data indicate that 1) there are intrinsic differences in the arterial response to graded increases in SS in differently sized vessels, specifically the RA and BA; 2) there is a linear dose-response relationship between FMD and handgrip exercise induced increases in SS in the RA and BA across the range of stimuli that were tested; and 3) within the BA, but not the RA there appears to be no influence of baseline diameter per se on %FMD.

3.4.1 The Impact of Baseline Artery Diameter on FMD

Between Artery Differences

It has been previously shown that when comparing the FMD responses of different vessels using between limb models, smaller arteries generally dilate more in response to RH-induced increases in SS (Nishiyama *et al.*, 2007;Parker *et al.*, 2006;Thijssen *et al.*, 2008;Walther *et al.*, 2008). To our knowledge, this is the first study to create matched increases in SS via handgrip exercise in two differently sized arteries located within the same limb. In the present experiment, three graded and matched levels of shear rate (SR) were created in the RA and BA via handgrip exercise. It was expected that creating matched levels of SR in these two vessels would eliminate the differences in FMD observed in previous studies, which used RH where smaller arteries tend to experience a greater shear stimulus relative to larger ones (Nishiyama *et al.*, 2007;Parker

et al., 2006). However, our study found that at all three matched SR levels, the RA dilated significantly more than the BA, indicating that smaller arteries do in fact have a greater FMD response relative to larger arteries. A larger FMD response in a smaller artery has also been observed by Nishiyama et al. (2007) and Parker et al. (2006) who compared the FMD responses of the popliteal (~5.5-7 mm) and brachial (~3-4.7 mm) arteries after a 5 minute cuff occlusion in each limb and found that the smaller brachial artery tended to have a larger unnormalized FMD (a normalized measure of FMD is expressed by dividing the %FMD by the area under the curve shear stimulus). However, after attempting to account for the stimulus magnitude by normalization (Nishiyama *et al.*, 2007;Parker *et al.*, 2006) or decreasing the brachial cuff occlusion duration to match the RH to that in the popliteal artery (Nishiyama *et al.*, 2007), Parker et al. (2006) found no differences in the FMD in the brachial and popliteal arteries and Nishyama et al. (2007) found that the popliteal artery had a larger response than the brachial artery. These 'stimulus corrected' results are in contrast with that of the present study and may be due to the between limb comparison. Comparing vessels located in different limbs is a major design limitation when trying to identify an effect of baseline artery diameter because the upper and lower limbs are exposed to different hemodynamic environments. During standing, blood pressure measured at the ankle level is 65 mmHg greater than it is in the arm (Proctor & Newcomer, 2006). Moreover, the legs are involved in the frequent work of locomotion and therefore contain a muscle mass that is approximately threefold greater than in the arm (Wray & Richardson, 2006). Consequently, differences in the

responses may be partially attributable to hemodynamically mediated upper vs. lower limb differences in endothelial function. In addition, due to the uncontrolled nature of the stimulus created with RH, normalization of the FMD response has been proposed to be a more appropriate method of expressing FMD. However, studies have criticized the validity of ratio normalization in FMD research (Atkinson *et al.*, 2009;Widlansky, 2009). Thus, it is unclear how well normalization 'accounted for' the differing stimulus between vessels in the work of Parker et al. (2006) and Nishiyama et al. (2007).

The results of the current study are in partial agreement with findings of Thijssen et al. (2008), who examined the FMD responses of the radial (~3 mm), brachial (~4 mm), femoral (~10 mm), superficial femoral (~8 mm), and popliteal (~6 mm) arteries using RH and reported a significant inverse relationship between artery size and FMD response magnitude ($r^2 = 0.33$, $p < 0.001$). Artery size and FMD remained significantly correlated when the FMD responses were normalized to the shear rate AUC, although the relationship was weakened ($r^2 = 0.23$, $p<0.001$) (Thijssen *et al.*, 2008).

The current findings are generally in agreement with the unnormalized results of previous studies, which report an inverse relationship between vessel size and the FMD response. Thus, the FMD responses of differently sized vessels cannot necessarily be expected to be uniform, even when vessels experience the same shear stress stimulus.

Within Artery Differences

Multiple studies using RH protocols have reported an inverse relationship between artery size and FMD in the brachial artery (Herrington *et al.*, 2001;Pyke *et al.*, 2004;Silber *et al.*, 2001). In contrast with previous studies, no relationship between baseline artery diameter and %FMD ($r^2 = 0.02$, $p = 0.312$) was observed in the BA (range in BA baseline diameter: 3.1 mm to 4.9 mm). Similarly, there was no relationship between baseline BA diameter and absolute change in diameter ($r^2 = 0.003$, $p = 0.907$). This may be attributable to the fact that in the present study, the shear stimulus was controlled so that every subject received a similar increase in shear. This is in agreement with the findings of Pyke et al. (2004) who found that creating uniform increases in shear stress with forearm heating and arterial compression virtually eliminated the relationship between baseline brachial artery diameter and FMD that was observed in the same subjects when RH was used to create the shear stress stimulus. Normalization of the BA RH FMD to the shear rate-AUC resulted in a similar dramatic attenuation of the relationship between FMD and baseline diameter (unnormalized $r^2 = 0.65$, normalized $r^2 = 0.19$). In combination with the present data, this indicates that in the BA, the frequently reported FMD-diameter relationship is mediated by vessel size dependent differences in the shear stimulus rather than intrinsic size dependent differences in vascular function. Importantly, this indicates that controlled increases in SR may be used to effectively compare BA FMD in groups with distinct baseline diameter. However, in the present study a modest but significant relationship between baseline artery diameter and %FMD

was observed in the RA ($r^2 = 0.18$, $p = 0.004$). The relationship between baseline RA diameter and absolute change in diameter approached significance ($r^2 = 0.08$, $p = 0.055$). This indicates that the effect of artery size on endothelial responsiveness is inherently different between the RA and BA. It is possible that a 'threshold' artery size exists, whereby artery size differences within a vessel impacts the FMD response when the vessel in question is smaller than the 'threshold diameter'. Differences in artery structure and endothelial sensitivity (which will be discussed in the next section) may play a role in mediating the variations in responsiveness across vessel diameters.

3.4.2 Possible Mechanistic Differences across Differently Sized Arteries

The mechanisms that could account for the functional differences observed between differently sized vessels fall into three main categories and will be discussed in turn, with the understanding that any combination of factors could contribute.

The Response of the Endothelial Cells

There may be intrinsic differences in the endothelial cell response to shear stress, such that the endothelial cells of smaller vessels are more sensitive to increases to shear stress. For example, the endothelial cells of smaller vessels may have a lower shear stress detection threshold. The existence of a threshold stimulus, at which point the vessel begins to dilate, was demonstrated in the pig iliac artery by Kelly & Snow (2007). In this study, shear stress was increased in a stepwise fashion. When shear stress exceeded a threshold value, the artery dilated (Kelly & Snow, 2007). Although the current study was

not specifically designed to identify a threshold stimulus, the FMD-SR regression line predicts that RA dilation begins at a lower SR relative to the BA, which is demonstrated by the fact that the regression line of the RA has a smaller X-intercept value (refer to Figure 3-8 and Figure 3-9). Therefore, the endothelial cells of smaller vessels may have a lower threshold, such that they begin to dilate at a lower shear stress relative to larger arteries.

Additionally, artery size may impact the relative proportion of vasodilator released by the endothelium. For example, it is possible that the endothelial cells of smaller vessels release more endothelial derived hyperpolarizing factor (EDHF) relative to larger arteries. Research has shown that in both human and animal models, the relative contribution of EDHF in mediating FMD increases as vessel size decreases (Luksha *et al.*, 2009;Tomioka *et al.*, 1999;Urakami-Harasawa *et al.*, 1997). It has been reported that the relative contribution of EDHF in mediating vasodilation increases only in arteries with a diameter smaller than 300µm (Luksha & Kublickiene, 2005). Although the RA is greater than 300µm in diameter, there still remains a precedent for size-dependent differences in vasodilatory mechanisms. Therefore, artery size may impact the nature of the response of the endothelial cell to increases in shear stress leading to downstream differences in the mechanisms that mediate FMD.

Structural Differences

Structural differences between differently sized vessels may also account for heterogeneity in the FMD responses. In porcine coronary arteries, the ratio of smooth muscle to endothelial cells decreases as vessel size decreases (Laughlin *et al.*, 2003). Thus, in smaller vessels, there may be fewer smooth muscle cells for a given number of endothelial cells. This means that the smooth muscle of smaller arteries may be exposed to proportionally more vasodilator (as there is a greater number of endothelial cells per smooth muscle cell), potentially contributing to a greater FMD response. Therefore, it is possible that with a similar production of vasodilator per endothelial cell, the amount of vasodilator reaching the smooth muscle varies with artery size.

Moreover, the diffusion distance from the endothelium to the outermost layers of the smooth muscle decreases in smaller vessels (Mulvany & Aalkjaer, 1990); thus, the dilatory response of smaller arteries may be greater because vasodilator diffusive conductance increases with a smaller diffusion distance. Additionally, it been postulated that EDHF diffuses more effectively into the smooth muscle of smaller vessels possibly because the half life of EDHF may be too short to diffuse through the smooth muscle layers of larger arteries (Urakami-Harasawa *et al.*, 1997).

Differences in artery morphology and wall structure have also been postulated to contribute to heterogeneity in the FMD response (Thijssen et al. 2008). Thijssen et al. (2008) have hypothesized that differences in the wall-to-lumen ratio across differently sized vessels play a role in determining differences in artery responsiveness. This theory

stems from the observation that in hypertension, there is an increase in the wall-to-lumen ratio of resistance vessels and an associated hyper-reactivity to vasoconstrictors (Folkow *et al.*, 1958;Folkow, 1978). It is unclear however, how this translates to the current model. Thijssen et al. (2008) have extrapolated that this structural attribute of smaller arteries may contribute to the larger FMD responses observed in smaller vessels and report emerging evidence to suggest that in both men and women, smaller vessels possess relatively more smooth muscle cells, therefore leading to a greater wall-to-lumen ratio (Thijssen, personal communications). This theory is not yet fully developed; however, further research is being conducted to better understand how size-associated structural differences can impact vessel function (Thijssen, personal communications).

Differences in artery wall composition across differently sized vessels may also impact FMD responses. For example, an increased elastin relative to collagen content in smaller arteries could contribute to a greater distensibility of the artery (HARKNESS *et al.*, 1957). Although it has been shown in dogs that the relative proportions of elastin and collagen are similar in vessels that are ~2 and ~4 mm in diameter (HARKNESS *et al.*, 1957) (comparable in diameter to the RA and BA, respectively), it remains possible that in humans, artery size may have an impact on artery wall composition. For example, Segers et al. (2009) found that in humans, arterial compliance differs between the RA and BA. Thus, differences in artery wall composition may influence the responses of the BA and RA.

Smooth Muscle Sensitivity

Responsiveness of the smooth muscle to vasoactive substances may also vary across vessels (Daemen & De Mey, 1995), which may account for functional differences between differently sized arteries. This diversity in smooth muscle cell function may be attributable to the presence of different subpopulations of smooth muscle cells and/or non-uniform smooth muscle cell phenotypic properties (for example, differential expression of contractile and cytoskeletal proteins) across different vascular beds (Daemen et al 1995). Thijssen et al. (2008) found that conduit artery responsiveness to glyceryl trinitrate (GTN), an exogenous NO-donor, was non-uniform across different vessels (including the femoral, superficial femoral, popliteal, brachial and radial arteries). Since GTN is an endothelium-independent stimulus, this observation indicates that heterogeneity at the level of smooth muscle responsiveness may exist across different vessels.

3.4.3 Correlations in FMD between Different Vessels

Brachial and coronary artery FMD responses have been shown to be well correlated, suggesting that endothelial function is systemic in nature (Anderson *et al.*, 1995;Takase *et al.*, 1998). However, correlations in FMD responses between peripheral arteries have not yet been reported. In the present study, the relationship between RA and BA FMD responses was examined. There was no correlation between the %FMD responses (at target SR 80: $p = 0.413$) or the %FMD-SR slopes of these arteries ($p = 0.841$). This is in contrast to the reported correlation in the FMD responses of the brachial

and coronary arteries (Anderson *et al.*, 1995;Takase *et al.*, 1998). However, the brachial and coronary arteries studied to establish this connection were much more similar in diameter than the RA and BA. In the study by Takase et al. (1998), the diameters of the brachial and coronary arteries were 3.84 mm and 3.21 mm, respectively. The difference in diameter is much more pronounced between the RA and BA (RA: 2.06 mm; BA: 3.78 mm). Therefore, the previously discussed intrinsic differences associated with vessel size between the RA and BA may contribute to functional heterogeneity between these arteries. This may hinder our ability to detect a correlation in the FMD responses of the RA and BA. Moreover, there may be some conducted vasodilation occurring with exercise in the RA, as will be discussed in a later section, which may also be a contributing factor to the lack of correlation observed in the responses of the RA and BA. The lack of correlation between the BA and RA FMD responses suggests that further studies are needed to determine whether the radial FMD responses can also predict coronary function

3.4.4 %FMD vs. Absolute Diameter Change

A consensus regarding the most appropriate method of characterizing the FMD response has yet to be reached. Typically, FMD is expressed as the percentage change in diameter from baseline. However, the FMD response can also be characterized as the absolute change in diameter (expressed in centimetres; FMD_{cm}) (Corretti *et al.*, 2002). Calculation of percent change in FMD is influenced by baseline diameter; for a given absolute change in diameter, smaller arteries will have a greater %FMD. As a result of

this mathematical relationship, it has been proposed that expressing absolute change in diameter is a more sound method of reporting FMD since it is not influenced by baseline diameter (Corretti *et al.*, 2002).

However, expressing the change in diameter in response to increases in shear stress as %FMD vs. FMD_{cm} may provide a more physiologically relevant measure of endothelial function. Shear stress is directly proportional to the blood flow velocity and blood viscosity but is inversely related to the vessel diameter (Gnasso *et al.*, 2001). When blood velocity increases, shear stress also increases, leading to FMD. Dilation increases vessel diameter, thereby reducing shear stress. FMD therefore acts as a negative feedback mechanism that maintains vessel wall shear stress within physiological limits (Figure 3-12). When two differently sized vessels dilate by the same %FMD, they both normalize shear stress by the same amount. However, if they dilate by the same absolute amount, the smaller artery is bringing shear back down to a greater degree than the larger artery (Figure 3-13). If the physiological role of FMD is to normalize shear to attenuate fluctuations from baseline values, then it is more appropriate to compare the %FMD responses of two differently sized arteries to determine if there is a difference in responsiveness to increases in shear stress. By using %FMD, we can directly compare the degree to which each artery normalized shear, which therefore allows us to determine if there is a difference in the endothelial function.

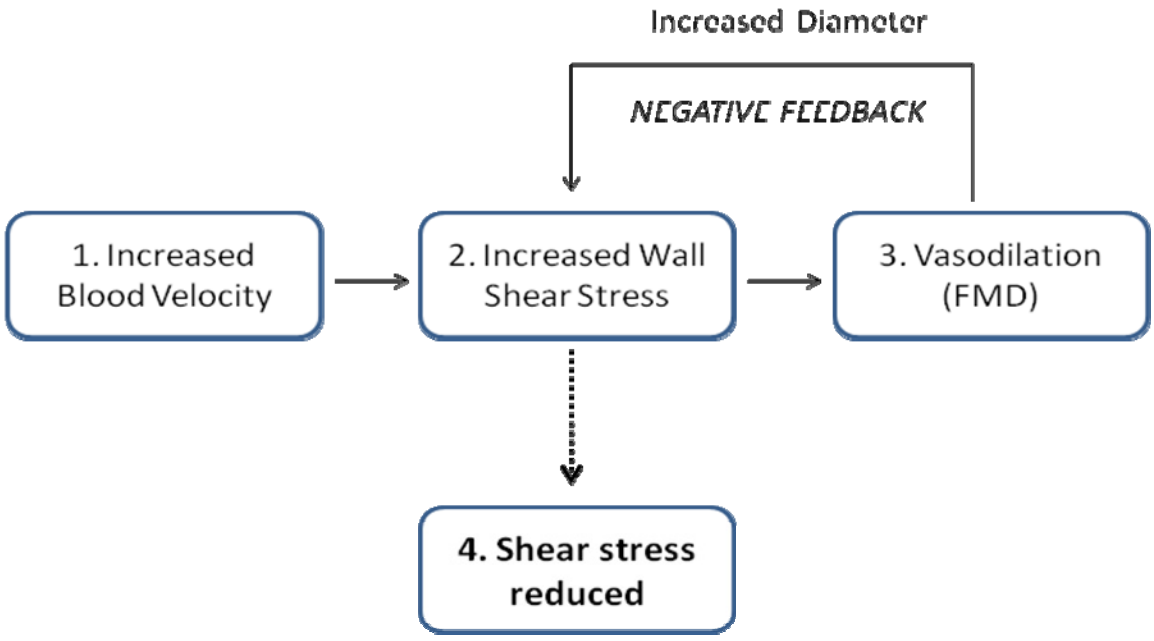

Figure 3-12: FMD acts as a negative feedback mechanism to maintain shear rate within physiological limits. The vessel dilates in response to increases in shear stress, thereby reducing shear stress. Adapted from Pyke M.Sc. Thesis, 2003.

Baseline Diameter	0.2 cm	0.4cm
Shear Rate	75 s^{-1}	75 s^{-1}
%FMD	15%	15%
New Shear Rate:	65.2 s^{-1}	65.2 s^{-1}
FMD_{cm}	0.02 cm	0.02 cm
New Shear Rate:	68.2 s^{-1}	71.4 s^{-1}

Figure 3-13: An example demonstrating that when two differently sized vessels dilate by the same %FMD (15%), they both normalize in shear stress to the same degree. If they dilate by the same absolute amount (0.02 cm) however, the 'new' shear rate level is lower in the smaller artery relative to the larger one, indicating a difference in the degree of shear stress normalization.

3.4.5 FMD-Shear Stress Relationship

The within subject relationship between %FMD and shear rate was found to be strong and highly significant in both the RA and BA (RA r^2: 0.92 ± 0.02; BA: 0.90 ± 0.03). Similar results were found when the data was expressed as three points that describe the mean stimulus and response at each shear target (Figure 3-7). This is consistent with the findings of Padilla et al. (2009) who applied five increasing magnitudes of RH-induced shear stimuli within a subject and found that FMD increased linearly with an increasing shear stimulus. In a reported sample subject, they found an r^2 of 0.978, indicating a strong within-subject dose-response relationship between shear stress and FMD.

In accordance with previous studies (Padilla *et al.*, 2009;Pyke & Tschakovsky, 2007;Pyke *et al.*, 2008a), the FMD-SR regression lines of the RA and BA were also compared when the regression was calculated using all subject data from every SR target (Figure 3-9). A significant positive relationship between %FMD and SR was identified in both RA and BA (RA r^2: 0.31, $p < 0.001$; BA r^2: 0.45, $p < 0.001$) (Figure 3-9A). Similarly, a significant positive relationship between %FMD and the increase in shear rate from baseline (delta SR) was identified in both RA and BA (RA r^2: 0.38, $p < 0.001$; BA r^2: 0.40, $p < 0.001$) (Figure 3-9B). Although no previous study has compared the stimulus-response relationship in the BA and RA, studies manipulating RH to create multiple stimulus magnitudes have demonstrated a strong positive relationship between

shear and FMD in the BA, which is in agreement with the findings of the current study (Betik *et al.*, 2004;Padilla *et al.*, 2009;Pyke & Tschakovsky, 2007). In addition, a strong positive relationship between shear and FMD has been shown in the radial (Joannides *et al.*, 2002;Mullen *et al.*, 2001) and brachial arteries (Pyke *et al.*, 2008a) with more sustained increases in shear stress with distal acetylcholine infusion or distal limb heating.

3.4.6 The Impact of Baseline Artery Diameter on the FMD-Shear Stress Relationship

Although the %FMD responses of both the RA and BA demonstrated a linear relationship with SR, the slope of the regression line was significantly steeper in the RA. Thus, for a given increase in shear rate, there is a larger %FMD response in the RA relative to the BA. However, within the BA, FMD at SR 40 only reached marginal significance ($p = 0.063$); thus, it is possible that inclusion of this potentially 'below threshold' point may have reduced the slope of the %FMD-SR relationship in the BA. As such, a comparison of the slopes of the RA and BA %FMD-SR relationship without inclusion of the BA SR40 trial was conducted and the slope of the relationship was still found to be significantly greater in the RA. In addition, the mean x-intercept of the individual %FMD-SR stimulus regression lines was significantly different in the BA and the RA. This predicts that a distinct absolute shear stress threshold for the initiation of FMD exists in these arteries. Although no previous studies have compared the responses on the BA and RA, the existence of a threshold stimulus for conduit artery FMD is supported by both animal (Kelly & Snow, 2007) and human data (Shoemaker *et al.*,

1997). In contrast to the %FMD-SR regression data, the slopes of the FMD_{cm}-SR regression lines of the RA and BA were not significantly different from each other; however, the x- and y-intercepts of the RA and BA regression lines were found to be significantly different (Figure 3-9C).

By comparing responses of men and women, two studies have examined the SR-FMD relationship in distinctly sized radial (Joannides *et al.*, 2002) and brachial (Levenson *et al.*, 2001) arteries. Women were found to have significantly smaller baseline diameters and a steeper %FMD-shear stress relationship (but a similar absolute FMD- shear stress relationship (Joannides *et al.*, 2002)). While this observation is consistent with the current paper's finding that the smaller RA had a significantly steeper %FMD–SR slope, it is in contrast with the present study's finding that within the BA FMD was unaffected by baseline diameter. In addition, these studies were deriving distinct diameters via gender comparison (Joannides *et al.*, 2002;Levenson *et al.*, 2001). This makes conclusions regarding baseline diameter effects difficult because there may be differences in endothelial function as a result of gender per se.

3.4.7 Brachial 60%MVC Trial

The FMD response of the BA at the SR80 target (actual SR: 73.8 ± 0.8 s^{-1}) was 5.4 ± 0.7%. This is slightly lower than the ~6% brachial artery dilation reported by Pyke et al. (2008b) in response to a shear rate of ~65 s^{-1} created via handgrip exercise. Thus, to ensure that a large range of FMD was tested in this sample population, a larger shear stimulus was created via the brachial 60% MVC trial. A considerably greater FMD

response (13.0 ± 1.5%) was observed at this higher SR level (102.3 ± 7.6 s^{-1}). The creation of a greater shear stimulus allowed for the verification that a good relationship between FMD and shear rate still exists in the BA with a stimulus that causes a greater FMD response. The strong within-subject relationship between shear and FMD was maintained with the addition of this fourth shear rate (n = 6; BA r^2: 0.92 ± 0.03).

3.4.8 Considerations of Heart Rate and Blood Pressure

An exercise-associated increase in SNA could lead to an underestimation of FMD as increases in SNA mediate vasoconstriction (Buckwalter *et al.*, 1997). Hijmering et al. (2002) found that elevating SNA (via lower body negative pressure) significantly attenuated the FMD in response to RH. However, this is not a universal finding. Dyson et al. (2006) found that increases in SNA (achieved via various stimuli, including lower body negative pressure, a mental arithmetic test, a cold pressor test, and the muscle chemoreflex) generally had no effect on FMD.

The mild increase in heart rate and blood pressure observed in the current study with both handgrip and adductor pollicis exercise may indicate a potential increase in sympathetic activation. However, Shoemaker et al. (2007) investigated the mechanisms that mediate the increase in blood pressure observed during isometric handgrip exercise and found that an elevation in cardiac output rather than an increase in sympathetically mediated peripheral vasoconstriction is primarily responsible for the increase in blood pressure. In addition, Pyke et al. (2008b) compared FMD responses of handgrip exercise and heating with rhythmic cuff inflation (same shear stress magnitude and pattern).

These conditions did not differ in their FMD response, while only exercise would be expected to increase SNA and indeed HR and MAP were only increased in the exercise condition. Taken together, these data indicate that sympathetic activation is unlikely to impair the FMD response during handgrip exercise. Heart rate (HR) and mean arterial pressure (MAP) tended to be greater during adductor pollicis exercise (although MAP did not reach statistical significance); therefore, in the unlikely event that sympathetic activation did blunt FMD, this would have lead to a relative underestimation RA FMD and our conclusions would not be affected.

3.4.9 Consideration of Baseline Shear Rate

Previous studies have variably characterized the shear stress stimulus as the absolute shear stress (Joannides *et al.*, 2002) or the increase in shear from baseline (Pyke *et al.*, 2008b). Therefore, it is currently unclear which characterization of shear stress is most appropriate. Baseline SR was significantly lower in the RA; thus, the increase in SR from baseline (delta SR) was significantly greater in the RA relative to the BA. To determine the effect of this non-uniform baseline SR, the %FMD responses of the RA and BA were compared in a subset of 7 subjects where delta SR was not significantly different between the two arteries for the SR80 target trial. The FMD response of the RA was significantly greater than that of the BA (16.5 ± 2.0 % vs. 5.5 ± 1.3%, $p = 0.002$) in this subset, indicating that differences in the FMD response between these two arteries is not attributable to differences in baseline SR. Moreover, the slope of the %FMD-delta SR regression line was significantly greater in the RA relative to the BA (RA: 0.27, BA:

0.12; p = 0.006), which is consistent with the relationship observed between the %FMD-SR regression lines (RA: 0.25 vs. BA: 0.12, p = 0.036) (Figure 3-9A and Figure 3-9B). Additionally, when shear stress is expressed as delta-SR, the x-intercepts (suggestive of threshold stimulus) of the RA and BA %FMD-delta SR regression lines become more similar. This suggests that while the %FMD-SR regression lines predict that the absolute shear stress required to initiate vasodilation is distinct, perhaps the *increase* in shear from baseline required for the initiation of a vasodilatory response is similar between the RA and BA.

3.4.10 Potential Non-FMD Contributions to Changes in Radial Artery Diameter

Vasodilation can be transmitted upstream to sites remote from those in direct contact with the exercising muscle and its metabolites (conducted vasodilation) (Neild & Crane, 2002). This is achieved via gap junctions, which allow electrical signals to be rapidly conducted through endothelial cells (Emerson *et al.*, 2002;Segal & Jacobs, 2001). Hyperpolarization can therefore travel to vessels upstream from the site of contracting muscle, potentially mediating a vasodilatory response (Segal & Jacobs, 2001). As such, it has been proposed that conducted vasodilation associated with exercise may be a non-shear related contributor to conduit artery vasodilation (Shoemaker *et al.*, 1997). Pyke et al. (2008b) examined the effect of handgrip exercise and the influence of conducted vasodilation on the BA FMD response vasodilation and found that conducted vasodilation did not reach the level of the BA. However, no study had investigated whether conducted vasodilation reached the RA during intrinsic hand muscle exercise.

In the RA compression trial, RA SR was maintained at near baseline during adductor pollicis exercise. Via this design, the effects of conducted vasodilation could be isolated as the metabolic stimulus required for conducted vasodilation was present without an increase in blood flow (preventing FMD). The observation that there was no significant vasodilation during the RA compression trial indicates that the dilation observed in the exercise trials was flow-mediated and not associated with conducted vasodilation. Although the average change in diameter from baseline to the end of compression was 0.00 cm, upon examination of individual data, three subjects had a 0.010-0.015 cm increase in diameter during compression. This suggests that in some subjects, a potential for RA conducted vasodilation may exist. However, in the remaining subset of 8 subjects without conducted vasodilation, the slopes %FMD-SR regression lines were re-examined and the RA slope was still found to be significantly greater than the BA ($p = 0.023$). Therefore, the FMD response in the RA was greater and thus a potential for RA conducted vasodilation is unlikely to fully account for our results. Only one trial of RA compression was conducted in the current study; therefore, future studies with repeat trials are required to confirm these findings. If conducted vasodilation occurs in RA of some subjects, then this presents a significant limitation to the use of exercise-induced increases in shear stress to investigate FMD.

3.4.11 Potential Limitations

No measurements of viscosity

The shear stress stimulus was estimated as shear rate. Thus, viscosity measurements were not taken into account when quantifying the shear stimulus, which is a limitation of this study as variations in blood viscosity impact shear stress levels and can therefore affect the FMD response (Melkumyants *et al.*, 1989). However, within a period of 6-8 weeks, it has been shown that there is minimal variation in blood viscosity within a subject (Gnasso *et al.*, 1996). The primary goal of this study was to create three distinct shear rate levels *within* a subject over repeated visits (which were completed within a two week period); therefore, it is unlikely that estimating shear stress by calculating shear rate had a significant impact on our between artery comparisons. Moreover, there is evidence to suggest that in young healthy individuals, measurement of blood viscosity is not critical to acceptable quantification of the shear stress stimulus (Padilla *et al.*, 2008). Padilla et al. (2008) compared the calculated shear stress with and without use of viscosity measurements in a young healthy population and found that the inclusion of viscosity measurements does not significantly impact shear stress measurements. Therefore, it is unlikely that our ability to create three distinct equidistant stimuli was compromised by expressing shear stress as shear rate.

Rate of increase in shear rate

Butler et al. (2000) have demonstrated that rate of the shear stress increase can affect the vasodilatory response, with more rapid increases in shear stress resulting in a

greater response. In rat cremaster arterioles, more dramatic step increases in shear stress stimulated a greater short term vasodilation (4-5 min into onset of stimulus) relative to slower, more gradual increases in shear stress (Butler *et al.*, 2000). Upon visual inspection, the speed of the shear increase appears to be faster in the BA relative to the RA. As such, if this magnitude of difference is important, it could be expected that the BA would dilate more in response to the faster rate of shear increase. Therefore, had the speed of shear increase been uniform between the RA and BA, RA dilation would be expected to be in further excess of BA. As such, the slightly faster rate of shear increase in the BA is not expected to compromise the study findings.

Smooth muscle sensitivity

Finally, differences in smooth muscle sensitivity were not tested in the current study. Additional trials with GTN administration may have provided better insight into whether differences in responsiveness between the RA and BA are attributable to heterogeneity in the smooth muscle response. Thissijen et al. (2008) found that sublingual GTN resulted in a greater percent dilation in the radial artery vs. the brachial artery suggesting that there may be differences in the smooth muscle sensitivity to NO in these vessels.

3.5 Conclusions

We found that when the RA and BA were exposed to matched, graded shear rate levels, the smaller RA had a greater FMD response. In addition, while both the RA and BA demonstrated a strong, positive %FMD-shear rate relationship, the slope of this relationship was steeper in the RA. Furthermore, the differing x-intercepts suggest that the RA has a lower threshold shear rate for initiation of vasodilation. Therefore, we conclude that there are intrinsic differences in the FMD response in the BA and RA. In within artery comparisons, we found that with a uniform handgrip exercise induced increase in shear rate, there was no relationship between baseline diameter and FMD within the BA; however, a modest inverse relationship between these variables was present in the RA. As a result, we conclude that in the BA, but not in the RA, vessel size per se does not have an impact on the %FMD response. Further research is required to investigate the physiological differences between differently sized vessels that account for the non-uniform responses to similar shear stimuli observed in this study.

Chapter 4

General Discussion

The main objective of the current study was to investigate the impact of artery size on the FMD response. To achieve this, matched levels of shear stress were created in two differently sized vessels (RA and BA) via handgrip exercise. Previous experiments have typically used RH to explore the relationship between baseline artery diameter and FMD. Thus, the use of handgrip exercise is unique to this study. Handgrip exercise provides a simple method of creating increases in conduit artery SS. Unlike RH, the stimulus created with exercise is controlled and sustained and can be matched between subjects; therefore, post hoc efforts to account for differing stimulus magnitudes are not required. Arterial compression in combination with forearm heating can also be used to create controlled, sustained increases in shear stress; however, forearm heating is a time consuming technique and arterial compression can be quite uncomfortable for the subject. In contrast, exercise can create rapid, controlled increases in shear stress and is relatively comfortable. Moreover, it has been shown that repeated exposure to handgrip-induced increases in shear stress (as was done in the current study design) does not alter endothelial reactivity (Jazuli *et al.*, 2010). Thus, handgrip exercise may provide a superior method of creating increases in conduit artery shear stress in future FMD studies. A caveat to using handgrip exercise to create increases in shear stress is that the relationship between exercise-induced FMD and vascular health is currently unclear

(Gaenzer *et al.*, 2001;Grzelak *et al.*, 2010;Padilla *et al.*, 2006). Thus, further research is required to verify the predictive value of exercise-induced FMD in assessing the risk of developing cardiovascular disease.

It was hypothesized that creating matched levels of SR in the RA and BA via handgrip exercise would eliminate the vessel size dependent differences in FMD observed in previous studies. However, at all three matched SR levels, the RA dilated significantly more than the BA, indicating that on a between vessel basis, smaller arteries do in fact have a greater FMD response relative to larger arteries. Furthermore, the slope of the %FMD-shear stimulus regression line was greater in the RA relative to the BA, which is a novel finding of this study. The results of this study indicate that there are intrinsic differences in the arterial sensitivity to SS in differently sized vessels, specifically the RA and BA. The mechanisms that could account for the functional differences observed between differently sized arteries include: 1) differences in the response of the endothelial cells; 2) an effect of potential structural differences between arteries of varying baseline diameter; and 3) non-uniform smooth muscle responses. *Within* the BA however, no relationship between baseline artery diameter and FMD was observed. This is consistent with the findings of Pyke et al. (2004) who demonstrated that creating uniform increases in shear stress with forearm heating and arterial compression virtually eliminated the relationship between baseline BA diameter and FMD. In contrast, an inverse relationship between FMD and baseline artery diameter was still observed *within* the RA, even when the shear stimulus was controlled. This again indicates that

intrinsic differences in the arterial response to SS exist across differently sized vessels. The results of the present study in combination with the results of Pyke et al. (2004) suggest that within the brachial artery, FMD responses are not influenced by baseline diameter per se. However, caution should be exercised when comparing the FMD responses of groups with differing baseline RA diameters as heterogeneous responses may be due to an effect of artery size rather than differences in endothelial function that reflect differences in vascular health.

Future Directions

To extend the findings of the current study, future experiments could utilize exercise to compare the responses of differently sized lower limb arteries to matched, graded increases in shear stress. Examination of the larger lower limb arteries may be prove to be methodologically easier than the use of the smaller upper limb vessels. For example, large increases in blood velocity were difficult to create in the RA. This is partially attributable to the fact that the adductor pollicis exercise used in this study engages a small muscle mass; however, the use of adductor pollicis exercise was necessary as it minimized forearm contraction (which makes imaging of the RA very difficult). The limited range of blood velocities attainable in the RA and its small size therefore made it difficult to create multiple distinct SR levels while maintaining optimal ultrasound images. Thus, it may be desirable to compare the responses of larger, differently sized arteries located within the same limb (for example, the popliteal and the

femoral artery) to determine if an effect of baseline artery diameter on functional responses exists not only in the upper limb vessels, but also across various conduit arteries.

Chapter 5

Summary and Conclusions

Using handgrip exercise, it was possible to create matched, graded levels of SR in two differently sized vessels. This was expected to elicit similar FMD responses in the RA and BA; however, it was found that the smaller RA dilated significantly more in response to matched SS stimuli. Furthermore, the slope, x-, and y-intercepts of the SR stimulus-%FMD relationship differed in the BA vs. the RA. This study therefore demonstrated that there are intrinsic differences in the arterial response to SS in these differently sized vessels. Finally, it appears that within the BA, there is no effect of baseline diameter per se on the FMD response; however, baseline diameter does appear to have an intrinsic influence on FMD responses within the RA. The differences between the BA and RA, two vessels located very close together in the upper limb, demonstrate that the FMD responses of different vessels cannot necessarily be expected to be uniform or correlated. The lack of correlation between the BA and RA FMD responses suggests that further studies are needed to determine whether the radial (or other conduit artery) FMD responses can also predict coronary function as the relationship to coronary function has only been investigated in the brachial artery (Anderson *et al.*, 1995;Takase *et al.*, 1998). Future research is also required to investigate the potential mechanisms that mediate the functional differences observed between differently sized vessels.

In conclusion, the findings of this study indicate that two differently sized blood vessels in the arm (the radial and brachial arteries) respond differently to the frictional force (known as shear stress) associated with increases in blood flow. Thus, differences in artery size between vessels can influence functional responses. However, within the larger brachial artery, baseline artery size does not affect the FMD response. Thus, artery size is not a confounding variable when comparing the brachial artery FMD responses between groups with varying brachial artery sizes, provided uniform levels of shear stress are created across all brachial artery diameters. We can therefore use brachial artery FMD to compare vascular health between groups with differently sized brachial arteries (for example the young vs. the elderly), provided that blood flow is controlled to create similar increases in shear stress in all vessels. In contrast, even when all radial arteries experienced a similar stimulus, a relationship between baseline radial artery size and FMD was observed, such that smaller radial arteries tended to have a larger FMD response. This indicates that caution is warranted when comparing FMD responses between groups with differently sized radial arteries because there may be an inherent effect of radial artery size on the FMD response. As such, heterogeneous radial artery FMD responses may not necessarily provide information regarding differences in vascular health, but rather may be an effect of artery size.

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Chapter 6

Appendices

6.1 Example Statistical Output

To determine if there is a main effect of artery on shear rate level and if shear rate significantly differed across shear rate levels:

Two Way Repeated Measures ANOVA (Two Factor Repetition) Tuesday, June 29, 2010, 2:41:09 PM

Data source: last 5 min in 2 way anova for SR.JNB

Balanced Design

Dependent Variable: Col 4

Normality Test (Shapiro-Wilk) Passed (P = 0.643)

Equal Variance Test: Passed (P = 0.544)

Source of Variation	DF	SS	MS	F	P
Subject	14	382.337	27.310		
Artery	1	45.697	45.697	2.043	0.175
Artery x Subject	14	313.189	22.371		
SR level	2	15961.849	7980.925	1323.319	<0.001
SR level x Subject	28	168.868	6.031		
Artery x SR level	2	27.775	13.887	4.260	0.024
Residual	28	91.280	3.260		
Total	89	16990.994	190.910		

Main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor.

The effect of different levels of Artery depends on what level of SR level is present. There is a statistically significant interaction between Artery and SR level. (P = 0.024)

Power of performed test with alpha = 0.0500: for Artery : 0.150
Power of performed test with alpha = 0.0500: for SR level : 1.000
Power of performed test with alpha = 0.0500: for Artery x SR level : 0.568

Least square means for Artery :

Group	Mean
Brachial	57.196

Radial 55.771
Std Err of LS Mean = 0.705

Least square means for SR level :

Group	**Mean**
40.000	39.786
60.000	57.287
80.000	72.378

Std Err of LS Mean = 0.448

Least square means for Artery x SR level :

Group	**Mean**
Brachial x 40.000	39.917
Brachial x 60.000	57.833
Brachial x 80.000	73.838
Radial x 40.000	39.655
Radial x 60.000	56.741
Radial x 80.000	70.917

Std Err of LS Mean = 0.466

6.2 Data from Jazuli et al. (2010) Variability Study demonstrating no systematic change in FMD over four closely spaced exposures to the same magnitude of handgrip exercise induced increase in shear stress

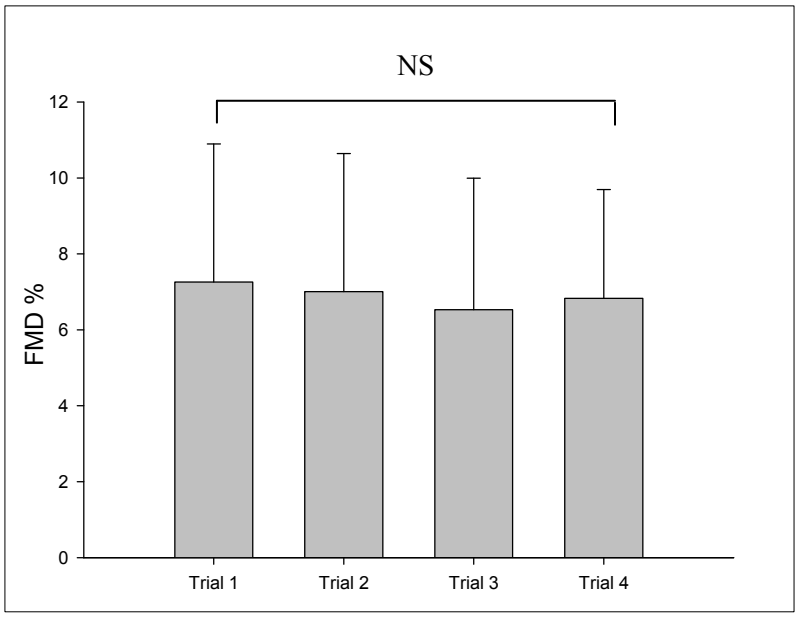

Figure 6-1: Brachial artery %FMD across 4 trials of handgrip exercise. The %FMD was similar across the 4 trials with a mean of 6.9 ± 3.4%