Exercise, Shear Stress, and Flow-Mediated Dilation of Human Conduit Arteries

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Flow-mediated dilation (FMD) refers to the relaxation of vascular smooth muscle and the subsequent dilation of the vessel in response to increases in shear stress on the endothelial lining accompanying increases in blood flow. The phenomenon has been shown to be endothelium dependent and as such is used clinically and experimentally as an index of endothelial health. FMD can be assessed by imaging a conduit artery with ultrasound during a period of reactive hyperaemia, typically following a period of prior blood flow occlusion achieved by the inflation of a pneumatic cuff around the limb distal to the imaging site. Previous studies have shown that the health of the endothelium is predictive of the health of the cardiovascular system as a whole. This thesis set out to scrutinize the FMD test as a marker for endothelial health by testing the following five hypotheses:

1. A short burst of high shear is not adequate to elicit the FMD response.
2. Brachial artery dilation following 15 minutes of occlusion is a clearer indicator of endothelium dependent FMD than 5 minutes of occlusion with exercise.
3. Oscillating the post occlusion shear stress will decrease FMD compared to unidirectional shear).
4. Heavy dynamic hand grip exercise 6 minutes before an occlusion-only FMD protocol will result in an enhanced FMD response.
5. Long term bed-rest inactivity will attenuate the FMD response and an exercise program will preserve endothelial function.

The experiments documented in Chapter 2 found that a 20-s shear stress stimulus following 15 min of forearm circulatory occlusion was not adequate to induce an FMD response compared to longer durations of shear and there was a progressive

reduction in FMD when the magnitude of the initial peak shear was reduced by limiting the duration of prior occlusion. Also, the FMD response was correlated with the total shear to time of peak diameter for all shear durations and peaks that were studied while the same was not true of peak shear. In Chapter 3 it was revealed that an uncoupling of the shear-to-dilation ratio occurred when dynamic exercise was added to the FMD test as both 15 min of occlusion (15OC) and 5 min of occlusion with 1 min of exercise (1EXin5OC) yielded similar FMD responses, even though the shear stimulus was increased with the addition of exercise. Increased plasma nitrite during hyperaemia was observed only in the 15OC protocol, suggesting that the exercise in the 1EXin5OC protocol initiates dilatory mechanisms that are not as heavily reliant on the shear sensitive nitric oxide pathway . In Chapter 4 it was shown that 5 min of intense dynamic hand grip exercise (5EX) produced a greater dilation than either continuous (15OC) or intermittent (IO) shear following 15OC. Total shear to the time of peak diameter (AUC_{shear}) and peak shear were both correlated to %dilation following 15OC; however this relationship was lost during 5EX and IO. The results of this study echoed the suggestion in Chapter 3 that there was an uncoupling of the intensity of the shear stimulus and the magnitude of vasodilatation when exercise was introduced, and adds that it may be in part due to the oscillatory nature of the shear profile during exercise. The acute effects of local exercise on the FMD response following 15OC were examined in Chapter 5. FMD in the brachial artery was blunted following dynamic hand grip exercise, even though the shear stimulus was greater during PostEX. Nitrite was significantly elevated in CON at 15s while PostEX nitrite was significantly elevated at 30s post cuff release but not different from CON at 15s. The results of this study suggested that prior exercise had a

negative effect on FMD which may be related to exercise blunting post occlusion endothelial ṄO production. Chapter 6 examined the effect of 56 days of head-down tilt bed rest (HDBR) and an exercise countermeasure on conduit artery FMD following release of distal limb ischemia and NMD following sublingual administration of 0.3 mg of nitroglycerin. HDBR without EX decreased the resting diameter of the popliteal artery while EX increased the diameter. HDBR had no effect on the resting diameter of the brachial artery. FMD was elevated in all groups for the brachial but only in the non-exercisers for the popliteal. When change in resting diameter was taken into account the preserved FMD in EX was removed. NMD was not altered by HDBR in any group. There was enhanced endothelial function relative to intrinsic dilatory capacity in both the brachial and popliteal arteries post HDBR.

The results from Chapter 2 support hypothesis 1, showing that a 20 second burst of high shear stimulus was not adequate to elicit the FMD response during reactive hyperaemia. It is not clear whether hypothesis 2 was supported or not given that the results from Chapter 3 showed on the one hand that the %FMD did not change with the addition of exercise in the occlusion but on the other hand the shear to dilation ratio was altered. The finding, in Chapter 4, that FMD was not reduced when the hyperaemia was intermittent does not support hypothesis 3. In opposition to hypothesis 4, Chapter 5 showed that %FMD was reduced following bouts of heavy hand grip exercise; however the absolute magnitude of vessel diameter was similar in both post exercise and control tests. Finally, hypothesis 5 was also contradicted, with Chapter 6 showing that long term bed-rest enhanced rather than attenuated the FMD response in both arm and leg arteries, while an exercise countermeasure preserved pre-bed-rest FMD in the legs only. In addition

to the specific hypotheses tested, there was evidence that acute exercise evoked dilatory mechanisms in the conduit arteries that were not shear/endothelium dependent given that the shear to dilation relationship was uncoupled during, following, and in occlusion protocols that include exercise. The precise mechanisms by which this is achieved are still unknown, but it may be partially due to the oscillatory nature of the elevated blood flow during exercise. I conclude that inference of cardiovascular health from endothelial function by the evaluation of %FMD should be approached with caution, especially in the event that physical activity is involved.

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Chapter 3: Manuscript II Dilation and plasma nitrite reserve following different hyperaemia inducing protocols in the human brachial artery.

Chapter 4: Manuscript III Flow-mediated dilation of the human brachial artery in response to exercise and intermittent release of forearm occlusion.

Chapter 5: Manuscript IV Flow-mediated dilation of the human brachial artery is blunted following bouts of heavy forearm exercise.

Chapter 6: Manuscript V WISE 2005: Flow and nitroglycerin mediated dilation following 56 days of head down tilt bed rest with and without an exercise countermeasure.

Chapter 1 - **INTRODUCTION**

Statement of the Problem and Hypotheses

Cardiovascular disease (CVD) is a leading cause of morbidity and mortality across a wide range of populations. Early identification of those at high risk and intervention early in its development has been shown to reduce negative outcomes. For this reason design and development of simple and accurate methods of early detection of CVD has been the focus of much research. The vascular endothelium has been determined to be one of the first systems to be affected in the development of CVD (McLenachan, Williams, Fish, Ganz, & Selwyn, 1991), therefore assessment of endothelial function has been proposed as a valid method for early detection of CVD (Corretti, Plotnick, & Vogel, 1995). The endothelium responds to increases in blood flow generated shear stress by releasing vasodilatory factors leading to dilation of conduit arteries commonly referred to as endothelium dependent flow-mediated dilation (FMD). Measurement of conduit artery lumen diameter change, usually by echo Doppler ultrasound, in response to reactive hyperaemia following limb vascular occlusion, has been widely used as a non-invasive test of endothelial function. Although it has been reported that FMD in human conduit arteries is dependent on release of the vasodilator Ṅ0 from functional endothelial cells (Joannides et al., 1995), there is considerable controversy as to whether the $\dot{N}O$ dependence is preserved under different states of hyperemia (Green, 2005). Various methods of achieving the high shear stimulus have been employed in the literature. Different durations of occlusion, placements of the ultrasound probe relative to the occlusion site, and volumes of occluded tissue have been employed. A method used to increase the magnitude of the shear stimulus has been to add exercise of the occluded muscle mass. This has proven to increase the magnitude of the dilation, but may trigger dilatory mechanisms that

are not dependent on$\dot{N}0$ or the endothelium. It has been proposed that the increased FMD following occlusion with exercise is not due to the increase in peak shear but rather to the prolonged elevation of shear in contrast to the fast decaying response in occlusion alone, and that the response to prolonged shear is independent of the $\dot{N}0$ system (Mullen et al., 2001).

A regular schedule of moderate physical exercise is associated with a reduced risk of CVD and as such should be accompanied by preserved endothelial function. Indeed studies have shown that FMD is improved in diabetics (Flammer et al., 2007) and cardiac rehabilitation patients (Vona et al., 2009) following an exercise intervention. Padilla, Harris, & Wallace (2009) suggest that that the effects of an acute exercise bout on FMD could be able to predict the effects of chronic exercise, such as has been shown with blood pressure (Thompson et al., 2001). To date there are few studies examining the effect of acute exercise on FMD, and all, to the best of my knowledge, employed full body aerobic exercise. A 45 min bout of aerobic exercise improved brachial artery dilation following 5 min of occlusion in active, but attenuated in inactive overweight men (Harris, Padilla, Hanlon, Rink, & Wallace, 2008) , while Rundell, Hoffman, Caviston, Bulbulian, & Hollenbach (2007) found no changes in FMD following similar exercise. FMD from a post exercise occlusion protocol has been shown to be attenuated in kidney transplant patients (Cosio-Lima et al., 2006). Prior aerobic exercise has also shown to improve FMD in healthy subjects and counteract the endothelial dysfunction induce by the ingestion of a high fat meal (Padilla, Harris, Fly, Rink, & Wallace, 2006).

In order to further our understanding of the vasodilatory response to increases in shear stress and to come closer to a definitive test of $\dot{N}0$ dependent

FMD I have tested the following hypotheses:

1. **A short burst of high shear is not adequate to elicit the FMD response.** I developed protocols designed to compare bursts of reactive hyperaemia of identical peak shear but varying durations. Identical initial peak magnitude of shear was achieved by 15-min of circulatory occlusion distal to the measurement site in the brachial artery. Following the occlusion period different durations of shear stimulus were allowed, including a 3-min control condition and shorter durations achieved by re-inflating the occlusion cuff after periods of 20-, 40-, and 60-s. The second occlusion was maintained for an additional 2-min to allow sufficient time for the peak FMD response, at approximately 60 to 90 seconds post occlusion release, before a second cuff release which created conditions of reduced peak and cumulative shear. From these data I propose to examine the role of peak shear and total cumulative shear on FMD.
2. **Brachial artery dilation following 15min of occlusion is a clearer indicator of the function the endothelial $\dot{N}O$ vasodilatory pathway than 5min of occlusion with exercise.** Two protocols, 15 min of occlusion and 5 min of occlusion with 1 min of moderate hand grip exercise, were chosen to compare the FMD responses between occlusion only and occlusion plus exercise while maintaining comparable peak shear responses post cuff release. This study compared the dilation response as well as the

nitrite reserve as an index of ṄO production.

3. **Oscillating the post occlusion shear stress will decrease FMD compared to unidirectional shear.** I designed a study that compared the dilation of the human brachial artery in response to intermittent high shear following 15min of forearm occlusion (IO) to that following a sustained laminar shear stimulus during reactive hyperaemia following 15 min of occlusion (15OC). The IO was achieved by release/inflation of a blood pressure cuff in a two second cycle for 2 minutes, mimicking forearm muscular contraction, and was followed by another 2 minutes of occlusion.

4. **Acute exercise before an occlusion-only FMD protocol will result in an enhanced FMD response.** I designed a study of FMD following a 15 minute forearm occlusion protocol with and without prior heavy hand-grip exercise. FMD responses of the brachial artery were assessed on 2 occasions: 1) following 15 minutes of forearm occlusion (CON) and 2) following 15 minutes of forearm occlusion (PostEX) initiated 6 minutes after two bouts of heavy hand-grip exercise (EMD1 and EMD2). The dilation responses of EMD 1 and 2 were also contrasted.

5. **Long term bed-rest will attenuate the FMD response and an exercise program will preserve endothelial function.** I studied the dilation response of limb conduit arteries to increased blood flow (endothelium dependent) and nitroglycerin administration (endothelium independent) in the Women's International Space Simulation for Exploration (WISE) study, in which 24 women were

studied before and after 56 days of HDT, with and without an exercise countermeasure.

Literature Review

Flow-mediated dilation

Flow-mediated dilation (FMD) refers to the ability of a blood vessel to dilate in response to increased blood flow through its lumen. This phenomenon was first described by Schetzenmayer in 1933 in canine femoral arteries in situ (Rubanyi, 1995). FMD is mediated by increases in the shear stress of circulating blood acting on the endothelial cell lining of the interior lumen of blood vessels (Koller & Kaley, 1990; Silber et al., 2001). As blood travels parallel to the vessel wall (endothelial lining) the blood cells' velocities increase from zero at the wall to a maximal value some distance from the wall in accordance with the no-slip theorem of fluid dynamics. This theorem stems from the Navier-Stokes equation

$$\rho\left[\frac{\partial V}{\partial t} + (V \cdot \nabla)V\right] = -\nabla P + \mu\nabla^2 V + B$$

Equation 1 The Navier-Stokes equation of fluid dynamics

Where ρ is the density, μ is the viscosity of the fluid, B is the body force per unit volume acting on the fluid, ∇ is the del operator, and P and V are the pressure and velocity of the fluid, both functions of position and time. The no-slip model assumes an impermeable wall as the boundary condition, and allows for no relative motion between the wall and the fluid immediately in contact with the wall (Day, 1990). Shear stress at the wall is related to the slope of the velocity profile at the wall. Blood viscosity is dependent on the hematocrit, the aggregability, and the deformability of the red blood cells (Litwin, Chapman, & Stoliar, 1970). For the purposes of studies investigating FMD it is justified to assume viscosity of the

blood as a constant. As such, changes in shear rate are treated as analogous to changes in shear stress. To further simplify the calculations, it is assumed that the blood vessel is a uniform tube of circular cross section. Under these idealized "Newtonian" conditions the velocity profile in the vessel is parabolic and the shear rate can be expressed with the following equation:

$$8Vmean/Ø$$

Equation 2 Simplified shear rate in a blood vessel

where $Vmean$ is the mean blood velocity across the vessel and Ø is the vessel diameter (Lipowsky, 1995).

It has been demonstrated that FMD is indeed endothelium dependent (Holtz, Giesler, & Bassenge, 1983). In 1983 Holtz et al. showed, in chronically instrumented dogs, that increased flow to the coronary arteries induced dilation only in the presence of a normally functioning endothelium. This mechanism has since been confirmed in human conduit arteries as well as it has been demonstrated that blockage of the endothelial NOS by administration of L- NMMA abolished flow dependent dilation of the radial artery following reactive hyperaemia (Joannides et al., 1995).

The mechanisms by which increases in shear stress are translated to increased production of eNOS and other potential vasodilators are still poorly understood. It would appear that increases in shear stress are sensed by a mechano-sensitive mechanism on the endothelial cell wall. One possible candidate for the flow sensing structure is proteoheparan sulphate, a visoelastic, anionic polyelectrolyte (Siegel & Bevan, 1995). It has been described as a constituent of the

endothelial cell membrane (Bevan & Siegel, 1991) and seems to be localized in the vicinity of a Na^+ leakage system (Siegel et al., 1996). In this scenario an increase in flow would align the visoelastic coils in the direction of flow, producing a conformational change from random to filament structure, accompanied by an increased number of binding sites for Na^+. The consequence of this would be membrane hyperpolarization on the endothelial cell membrane, promoting the production and release of endothelium derived relaxing factors through increased Ca^{2+} influx (Qiu & Tarbell, 2000; Koller & Kaley, 1990; Paniagua, Bryant, & Panza, 2001). An endothelial cell environment with increased $[Ca^{2+}]$ sets the stage for the production of Ṅ0 via eNOS. This Ca^{2+}-dependent mechanism has been shown to act only transiently, though. Shear receptor stimulation is also associated with tyrosine kinase (TK) activity which up regulates the production of eNOS (Koller & Bagi, 2002). It appears as though this TK mediated phosphorylation-signalling cascade is responsible for the lasting phase of shear stress induced Ṅ0 release (Fleming, Bauersachs, Fisslthaler, & Busse, 1998). Figure 1.1 presents a summary of the mechanisms responsible for $\dot{N}0$ dependent FMD.

Reactive hyperaemia

In order to assess FMD, blood flow through a vessel must be increased. It has been observed that periods of circulatory arrest by application of external pressure, either by tourniquet or inflation of a pneumatic cuff, results in a large increase in blood flow following release of the occlusion pressure (Freeman, 1954; Gardiner & Salmoiraghi, 1956). This increase in blood flow is termed reactive hyperaemia and is almost certainly the result of a metabolic blood flow regulation mechanism (Hester et al., 1982). Blood flow is tightly linked to tissue metabolism. Any

alteration in metabolic activity or oxygen tension will result in compensatory alterations in blood flow. During periods of metabolic activity increased muscle blood flow is facilitated in part by action of metabolic bi-products to energy production, or metabolites, (CO_2, H^+, Pi, K^+, and adenosine) directly on the smooth muscle of resistance arterioles and/or indirectly by inhibiting norepinephrine release from nerve endings (Andersen & Saltin, 1985; Laughlin et al., 1996; Delp & Laughlin, 1998). The result is that these vessels are dilated in relation to vessels in other tissues thereby increasing the pressure difference (ΔP), especially in the event that the chemorecptors are stimulated thus initiating a pressor response (Freund, Hobbs, & Rowell, 1978), and simply by reducing vascular resistance in the direction of the active tissue. The result is increased vascular conductance which increases blood flow to the metabolically active region.

Metabolic activity is also associated with periods of reduced oxygen availability, as the tissue will use up the available oxygen for force production. The accumulation of vasodilator substances then ensues as metabolic processes are forced to produce energy with reduced oxygen supply. Alternatively some physiologists favour the “oxygen/nutrient demand theory” (Ebeigbe, Pickard, & Jennett, 1980; Rubanyi & Paul, 1985). The principles of this theory state that since oxygen is required to maintain vascular smooth muscle contraction, the absence of an adequate supply would result in relaxation and consequent dilation of the vessel. However, under some conditions vascular smooth muscle can maintain the contractile state for long periods in the presence of minute concentrations of O_2 (Duling & Pittman, 1975). Although it is controversial whether oxygen demand or vasodilator production is the cause of ischemia mediated vasodilation, the most likely scenario is that there is redundancy at work in this system (Wood, Litter, &

Wilkins, 1955; Crawford, Fairchild, & Guyton, 1959; Fairchild, Ross, & Guyton, 1966).

The degree of reactive hyperaemia is proportional to the amount of tissue occluded and to the duration of the limb occlusion. In experiments involving manipulation of pneumatic cuff placements the more distal on the limb the occlusion site (thereby occluding less muscle mass), the less reactive hyperaemia is observed (Berry, Skyrme-Jones, & Meredith, 2000; Betik, Luckham, & Hughson, 2004). Also, longer periods of circulatory arrest yield more prolonged elevations in blood flow following occlusion cuff release (Shakir, Gooden, & MacDonald, 1980; Carlsson, Sollevi, & Wennmalm, 1987; Imms, Lee, & Ludlow, 1988).

It is important to note the distinction between conduit and resistance artery dilation, due either to increased metabolic activity in the skeletal muscle or a reduced oxygen activity in the vascular smooth muscle. The reactive hyperaemia itself is due to the dilation of the resistance arteries controlling blood flow to the active muscle by either metabolic or oxygen/nutrient demand mechanisms. However the increased blood flow following the release of occlusion will, in turn, create a condition of increased shear stress in the resistance vessels thereby causing a further dilation that is likely mediated by the endothelial release of vasodilator substances. In this way reactive hyperaemia is capable of providing an index of FMD at the microcirculatory level (Strain, Chaturvedi, & Shore, 2005; Philpott et al., 2009), all be it limited due to the myriad other factors involved at the resistance vessel level (Ghiadoni, Versari, Giannarelli, Faita, & Taddei, 2008; Al-Qaisi, Kharbanda, Mittal, & Donald, 2008).

Flow-mediated dilation and activity level

It has long been known that prolonged inactivity results in significant deconditioning of many physiological systems, including the cardiovascular system. There is a body of evidence from animal and human experiments showing that the structure and function of the vascular system is subject to adaptations during long periods of inactivity or exposure to micro-gravity environments. Previous studies have shown that regular aerobic or resistive exercise is effective in preserving or improving endothelial and vascular function as a consequence of aging (Wray, Uberoi, Lawrenson, & Richardson, 2006) or in the presence of cardiovascular risk factors (Green et al., 2004; Olson, Dengel, Leon, & Schmitz, 2006), including inactivity (de Groot, Bleeker, & Hopman, 2006). Head-down-tilt bed rest (HDT) can be used to characterize the adaptations of vascular structure and endothelial function as a consequence of long term inactivity or micro-gravity exposure.

Regular physical exercise has been associated with beneficial changes in many cardiovascular risk factors. Exercise reduces the morbidity and mortality in the general public as well as those suffering from essential hypertension. Using reactive hyperaemia, measured by strain-gage plethysmography, as an index of flow- (Higashi et al., 1999a) and acetylcholine- (Higashi et al., 1999b) mediated dilation, Higashi et al. found that endothelial function was increased in hypertensive patients following a 12-week regimen of daily aerobic exercise. Using similar methods Taddei et al. (2000) showed that acetylcholine-mediated dilation was similar in sedentary and trained young men, blunted in sedentary elderly men compared to young, and somewhat maintained in elderly trained men compared to sedentary, concluding that regular physical activity prevented much of the age-

associated loss of endothelial function. Again, using acetylcholine-mediated vasodilation as an index of endothelial function, Kingwell et al. (1996) found that athletes showed an enhanced response, and in a subsequent study found that a four-week cycle training program increased basal Ṅ0 production in the forearm and decreased vasoconstriction in response to N(G)-monomethyl-L-arginine (L-NMMA) infusion (Kingwell, Berry, Cameron, Jennings, & Dart, 1997). The study of conduit artery FMD with regards to exercise training has been sparse. It has been demonstrated that 10 weeks of combined aerobic and anaerobic exercise training increased brachial artery FMD in healthy young men (Clarkson et al., 1999) and that habitual physical activity is correlated with the magnitude of brachial artery FMD in children (Abbott, Harkness, & Davies, 2002). The effect of exercise on conduit artery FMD seems to be more effective in those with clinically significant heart disease, in patients both over (Hambrecht et al., 2000) and under (Linke et al., 2001) 70 years of age. It is unknown what mechanisms might be responsible for restoring endothelial function in elderly and heart disease patients. A possible trigger for the change in status might be the repetitive increase in blood flow due to muscle demand which may facilitate arterial adaptations favouring a healthy endothelium. At this point there is little information available concerning the response of the endothelium to an acute bout of exercise (Moyna & Thompson, 2004).

Acute Exercise and FMD

As has been discussed above, exercise has been successfully used as an intervention to improve endothelial health, and as such would increase FMD in those expressing low FMD prior to intervention. However, the mechanisms of the

exercise response to FMD are not fully understood. The acute exercise model, with the FMD test being performed within the first 24h post exercise bout, can be useful in investigating the direct effects of exercise on FMD (Padilla, Harris, & Wallace, 2007). Recently, investigators have been interested in the FMD response following acute bouts of exercise. Treadmill walking has been reported to improve FMD evaluated 1h post exercise in active but not inactive overweight men, with the discrepancy left unexplained by the pro –and ant- inflammatory state as indicated by similar levels of IL-6 and TNF-α in both groups (Harris et al., 2008). However, Rundell et al. (2007) found no alteration in the FMD response measured 20-30 minutes after 30 min of aerobic cycling exercise in a young, healthy cohort.

A number of factors must be considered when interpreting the results of an FMD test performed after a bout of exercise. First, metabolically active muscle releases vasodilatory substances that cause vasodilation thereby increasing blood flow which can persist for over 1 h after cessation of exercise (Uehata et al., 1997). A difference in baseline diameter is known to be a limitation in inter-population comparison studies as large baseline diameters are associated with a low calculated FMD (Uehata et al., 1997). A post-exercise dilated artery may be associated with a relatively low FMD even when dilation is to the same absolute magnitude in pre and post tests. Second, a combination of exercise-induced and hyperaemia-induced shear stress may modify the overall stimulus for FMD. Although methods to control for the variability in reactive hyperaemic shear stress exist (Pyke & Tschakovsky, 2005), the impact of an altered pre-hyperaemic shear on the FMD measurement has not been clarified. Also, the impact of exercise-induced sympathetic activity on FMD should be addressed as it may alter the mechanism of the FMD response. Both acute lower body suction (Hijmering et al.,

2002) and mental stress and the cold pressor test (Lind, Johansson, & Hall, 2002) have been shown to result in a transient reduction in FMD ; although investigations in our lab indicate a dependency on the nature of the stimulus (Dyson, Shoemaker, & Hughson, 2006).

Patterns of Shear

In straight parts of the arterial tree, such as the brachial artery, laminar shear stress with a definite forward direction has anti-atherogenic effects (Lipowsky, 1995). Disturbed, as opposed to laminar, flow patterns have been demonstrated to cause sustained activations of atherogenic genes and enhancements of EC mitosis and apoptosis (Chien, 2008). Exercise results in a flow state that is disturbed by the mechanical compression on the arteries of the contracting muscle belly. However, the recovery blood flow after exercise results in a prolonged state of high laminar shear that may explain some of the anti-atherogenic effects of regular exercise (Niebauer & Cooke, 1996; Green et al., 2004). Endothelial cells align themselves in the direction of flow (Luscher & Corti, 2004) demonstrating that they have the ability to sense shear oscillations as well as shear magnitude. It has been found that cultured endothelial cells exposed to oscillatory shear stress for 12h expressed up to 5 fold greater extracellular fibronectin and laminin than cells in static culture, even though both exhibited a similar morphology (Thoumine, Nerem, & Girard, 1995). And indeed it has been found in humans that prior treatment with 30 min of enhanced retrograde shear, by incremental inflation of occlusion cuffs, resulted in impaired endothelial function as assessed by FMD (Thijssen, Dawson, Tinken, Cable, & Green, 2009). It is unlikely that 30 min of retrograde shear could have changed the morphology of the vascular endothelium, but it is worthy of note that direction as well as magnitude of shear has an effect on endothelial structure/function.

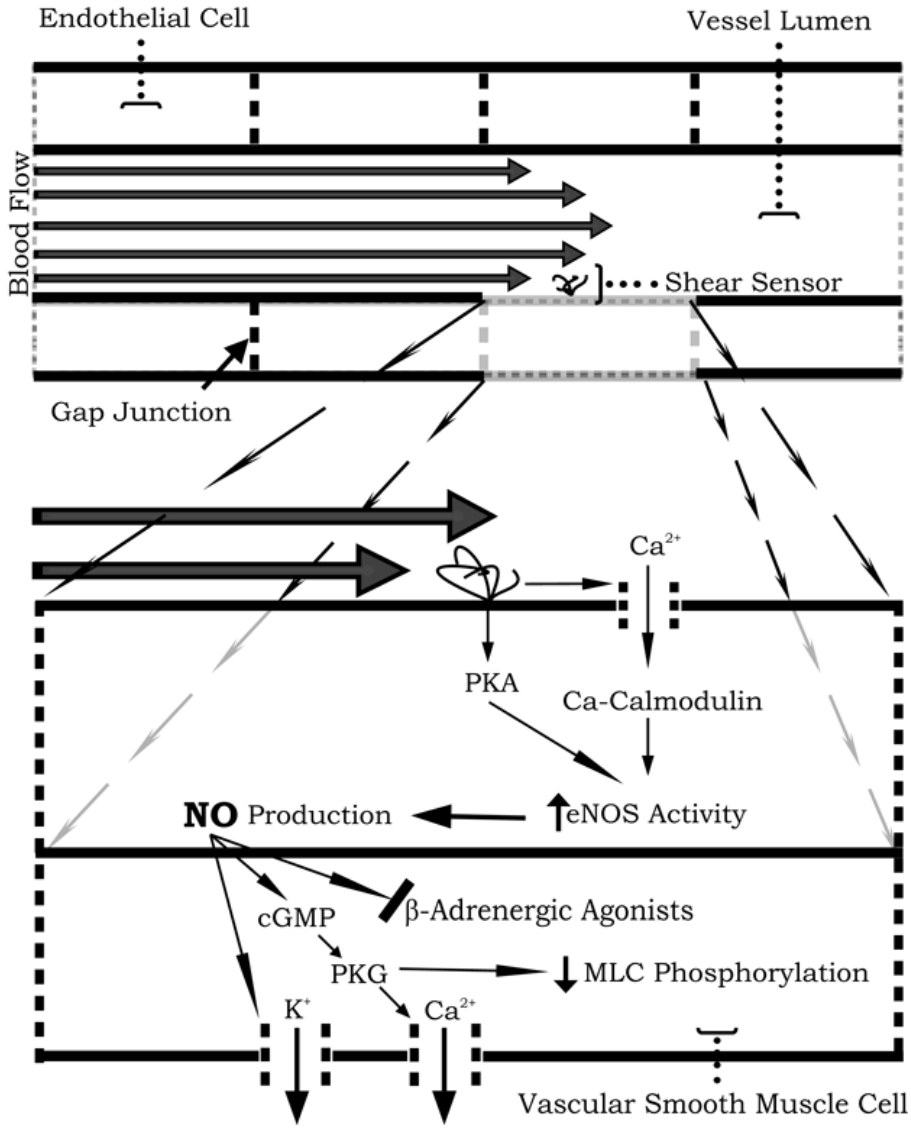

Figure 1.1 Pathways of shear stress initiated nitric oxide mediated vascular smooth muscle cell relaxation.

Elevated shear stress at the blood-endothelial cell interface causes a conformational change in the shear sensing glycoprotein on the luminal surface of the endothelial cell triggering Ca^{2+} influx and Ca-Calmodulin binding along with activation of Protein kinase A (PKA) which both contribute to elevated eNOS activity. eNOS activity results in formation of nitric oxide ($\dot{N}O$) which diffuses to the vascular smooth muscle cell (VSMC). In the VSMC $\dot{N}O$ promotes muscle relaxation, and therefore vessel dilation, by enhancing K^+ efflux, blockade of β-adrenergic agonists, and cGMP mediated increases in protein kinase G activation which directly and indirectly (through Ca^{2+} efflux) reduces myosin light chain phosphorylation.

Methodology

For the studies included in this thesis blood flow and shear rate were determined by combining the measurements of blood velocity, estimated by pulsed Doppler ultrasound, and arterial diameter, estimated by digital edge tracking measurements of echo Doppler ultrasound images, in the following equations:

$$A = \pi r^2$$

Equation 3 Cross-sectional area of an artery

$$Q = Vmean \times A\, min^{-1}$$

Equation 4 Blood flow through an artery

$$\gamma = 8 \times Vmean \times \emptyset^{-1}$$

Equation 5 Shear rate at the arterial wall

where $Vmean$ is mean blood velocity (cm/s) and Ø is the diameter of the artery (cm).

Area under the shear (AUC_{shear}) and diameter (AUC_{diam}) curves were calculated by approximating the definite integral by use of the trapezoidal rule on the interpolated values from the time of occlusion cuff release to the time of peak diameter (Figure 1.2).

Forearm Circulatory Occlusion

For the studies in Chapters 2 through 5 brachial artery blood flow was arrested by rapid inflation (E-20 Rapid Cuff Inflator, D. E. Hokanson, Issaquah, USA) of a standard blood pressure cuff to 250 mmHg. The occlusion cuff was placed just distal to the right elbow. Reactive hyperaemia was initiated by rapid deflation of the occlusion cuff.

For the study in Chapter 6 brachial and popliteal artery blood flow was arrested by manual inflation of a standard blood pressure cuff to supra-systolic pressure for four minutes. At the 1.5 minute mark of the occlusion period the subjects performed one minute of static exercise at 40% MVC. For the brachial tests this involved the squeezing of a pressurized rubber ball, while for the popliteal tests the subjects performed a toe-press in the prone position. Force was monitored on screen for verification of force maintenance.

Exercise Model

For the studies in Chapters 3 and 5 subjects performed rhythmic hand-grip exercise in the supine posture with the arm maintained at heart level. The subjects squeezed a hand-grip device set to either heavy (30% MVC, for exercise alone) or moderate (15% MVC, for exercise during occlusion) intensity at a cadence of one second contraction and one second relaxation. This model was chosen so as to affect a small muscle mass in the vicinity of the artery being assessed, and to limit the influence of central mechanisms on arterial tone, such as increased sympathetic activity.

Artery Diameter

Vessel diameter was monitored by M-Mode ultrasound imaging (MICROMAXX, Sonosite, WA) and stored on digital video tape. Video was collected continuously throughout the experimental protocols so as to minimize the risk of missing the peak diameter. Time of peak diameter has been reported to be in the range of 45 to 60 sec post cuff deflation (Betik et al., 2004; Berry et al., 2000), however there have been reports of large inter-individual differences (Black, Cable, Thijssen, & Green, 2008) and, to the best of my knowledge, the time course of dilation during the above described exercise models has not previously been characterized. M-mode imaging was chosen over B-Mode due to its ability to capture the image of the vessel wall at a higher resolution for continuous measurements (Stadler, Taylor, & Lees, 1997) and to be able to capture images over the course of the cardiac cycle, thus allowing for true mean diameter measures. However, some researchers favour B-Mode imaging over M-Mode, citing that there is a greater tendency for the image focus to drift from the centre of the artery when in M-Mode (Kelly, Kaiser, Dengel, & Bank, 2004). After the completion of the experiments, a new edge tracking software was made available to the lab which has the capability of calculating vessel diameter during real-time video play-back, which would have been able to generate mean values from B-mode images as well. Vessel diameters were measured by custom edge-detection software (Jorge Serrador and Brian Deegan) from still images extracted (KINO video editor) at every 5s of video. An average of the diameter across the M-mode image frame (2-3 cardiac cycles) was taken from the best image in each 15s window to represent mean arterial diameter. The measurements from the 15s windows were curve fit by cubic spline interpolation and Lenenberg-Marquardt peak fitting (Fityk,

www.unipress.waw.pl/fityk/) to output values second by second. Cubic spline interpolation is preferred over polynomial interpolation because the interpolation error can be made small even when using low degree polynomials for the spline to the inherent discretization (Reinsch, 1967). Given that image drifting over the entire protocol was not able to be avoided, thus limiting the frequency of "good" ultrasound images available for measurement, the fitting technique was employed to reduce the error that may have occurred due to missing the actual peak dilation or to making an overestimation of dilation due to random noise. Similar methods have been used previously in our lab, with the same rational (Dyson et al., 2006; Betik et al., 2004). For future studies the option of using the real-time edge detection software with B-Mode should be explored to avoid the need for mathematical modeling of the dilatation response. In addition to providing the baseline and peak diameters, the interpolated curve was used to determine the time to peak diameter and the second to second values were used in conjunction with second to second velocity measurements to generate estimates of blood flow (Equation 4) and arterial wall shear rate (Equation 5).

Blood Velocity

A 4-MHz Doppler probe was fastened above the brachial (proximal to the cubital fossa) or popliteal (in the popliteal fossa) artery with strips of surgical tape. The signal was directed 45° relative to the skin and the ultrasound gate was adjusted to encompass the total width of the artery. Mean blood velocity was determined from the mean output of the pulsed Doppler ultrasound signal (Multigon Industries, N.Y.). This method is non-invasive and is based on the physical principle that the high-frequency waves emitted by the probe will reflect back off of moving red blood

cells and other plasma particulate matter to the transducer at a frequency that is shifted in proportion to the velocity of the reflector. The Doppler shift is used to calculate the velocity of the circulating blood:

$$V = \frac{f_D \times C}{2f_t \times \cos\theta}$$

Equation 6 Blood velocity from Doppler shift

where V is velocity (m/s), f_t is the frequency of the ultrasound transmitted by the probe (MHz), f_D is the Doppler shift frequency (MHz), C is a constant (1570 MHz) representing the velocity of ultrasound through biological tissues, and θ is the angle of insonation of the Doppler probe (a constant of 45° relative to the probe/skin interface in the experiments in this thesis). Doppler ultrasound has long been used as a method for estimating blood velocity (Morris, Histand, & Miller, 1973). Numerous studies have tested its accuracy (Levenson, Peronneau, Simon, & Safar, 1981; Demolis, Asmar, Levy, & Safar, 1991; Tschakovsky, Shoemaker, & Hughson, 1995) and reproducibility (Shoemaker, Pozeg, & Hughson, 1996). Second by second values for MBV, used to match up with the interpolated diameter values, were derived by non-rounded Akima interpolation (QtiPlot, http://soft.proindependent.com/qtiplot.html). The Akima method is devised in such a way that the resultant curve will pass through the given points and will appear smooth and natural (Akima, 1970). Given the high sampling frequency of the MBV signal, the Akima interpolation values should be extremely close to the actual second to second values. Brachial artery MBV and cross-sectional area were used to calculate forearm blood flow (Equation 4) and arterial wall shear rate

(Equation 5).

Blood Pressure and Cardiac Output

Beat to beat mean arterial pressure was obtained non-invasively by finger photoplethysmograpy (Finometer PRO, Finepress Medical Systems, Netherlands). Cardiac output (CO) by Modelflow was calculated online by the proprietary software of the Finometer® device by analysis of the finger pressure waveform using a three element (aortic characteristic impedance, Z_0, Windkessel compliance, C_w, and peripheral resistance, R_p) non-linear equation dependent on the pressure-area relationship of the aorta. Age, sex, height, and weight for each subject were entered into the unit prior to testing and these parameters were used to determine the individual aorta pressure-area relationship. Pressure-area relationship allows for computation of Z_0 and C_w, while R_p is adapted by the model. This method has been shown to reliably follow changes in CO in cardiac surgery patients (Jansen et al., 2001).

$$Z0 = \sqrt{(\rho/AC)}$$

Equation 7 Aortic characteristic impedance.

$$\boldsymbol{C = dA/dp}$$

Equation 8 Vascular compliance per unit length of the aorta.

$$\boldsymbol{Cw = lC}$$

Equation 9 Windkessel compliance.

where ρ is the density of the blood, $\boldsymbol{A}$ is the aortic cross-sectional area, l is the length of the aorta.

Venous Blood Analysis

For the studies performed in Chapters 2, 4, and 5 venous blood samples were taken at close intervals (1 cc each, 15 to 30 seconds apart) before, during, and after exercise and occlusion. A catheter was placed in an antecubital vein in a retrograde fashion to maximize collection of blood from deep veins draining the finger flexor muscles. Whole blood samples were analyzed as soon as possible for lactate (Lactate Pro, Australia) and pH levels (PerpHect ROSS combination pH micro electrode, Thermo Scientific, MA). Heperinized samples were put on ice and analyzed for oxygen saturation and haemoglobin content by co-oximeter (Stat Profile pHOx, Nova Biomedical, MA). The remainder of the heparinized whole blood was centrifuged and the plasma separated and stored at -80 °C for subsequent analysis of nitrite concentration by colorimetric assay kit (Nitric Oxide Quantitation Kit, Active Motif, California). Nitrite was quantified by the addition of Griess reagent, which converts nitrite into a purple-colored azo compound. 100 µl of assay buffer was added to the blank wells and 100 µl of sample was added to the sample wells (each sample in duplicate). If the sample volume was less than 100 µl, the final volume was adjusted to 100 µl using the assay buffer. 50 µl of Griess reagent A was added to each well (blanks, standards and samples), immediately followed by the addition of 50 µl of Griess reagent B. Colour was then allowed to develop for 10 min at room temperature. Absorbance was read by spectrophotometer at 540 nm with a reference wavelength of 620 nm. The

absorbance values were averaged and the blank values subtracted from the mean values of the samples. To calculate the endogenous nitrite concentration of the samples the measured absorbance values were used with the standard curve of nitrite for that batch of samples.

There are a number of methodological considerations regarding the analysis and interpretation of plasma nitrite, especially during experiments involving exercise. First, it is difficult to determine whether the nitrite in the effluent venous blood is primarily derived from the micro-circulation or the conduit artery. Shear stress is increased throughout the circulation feeding the working muscle. It is likely that endothelial release in the micro-circulation will mirror that in the conduit arteries, however there was no way to test this in these studies. Also, in addition to being released from the vascular endothelium, $\dot{N}O$ is released in the exercising muscle (McConell & Wadley, 2008), primarily derived from neuronal NOS (nNOS). In addition to the concerns about the origin of the nitrite, there is the issue of $\dot{N}O$ metabolism in the vicinity of working muscle. Exercise creates an acute condition of oxidative stress, mediated by the production of the superoxide anion (O_2^-). It has been established that oxidative stress limits the bioavailability of endothelium derived $\dot{N}O$ (Thomas, Chen, & Keaney, Jr., 2003). Given these possible confounding factors, the relationship between the plasma nitrite results and endothelial $\dot{N}O$ synthesis at the conduit artery level in this study should be interpreted with caution.

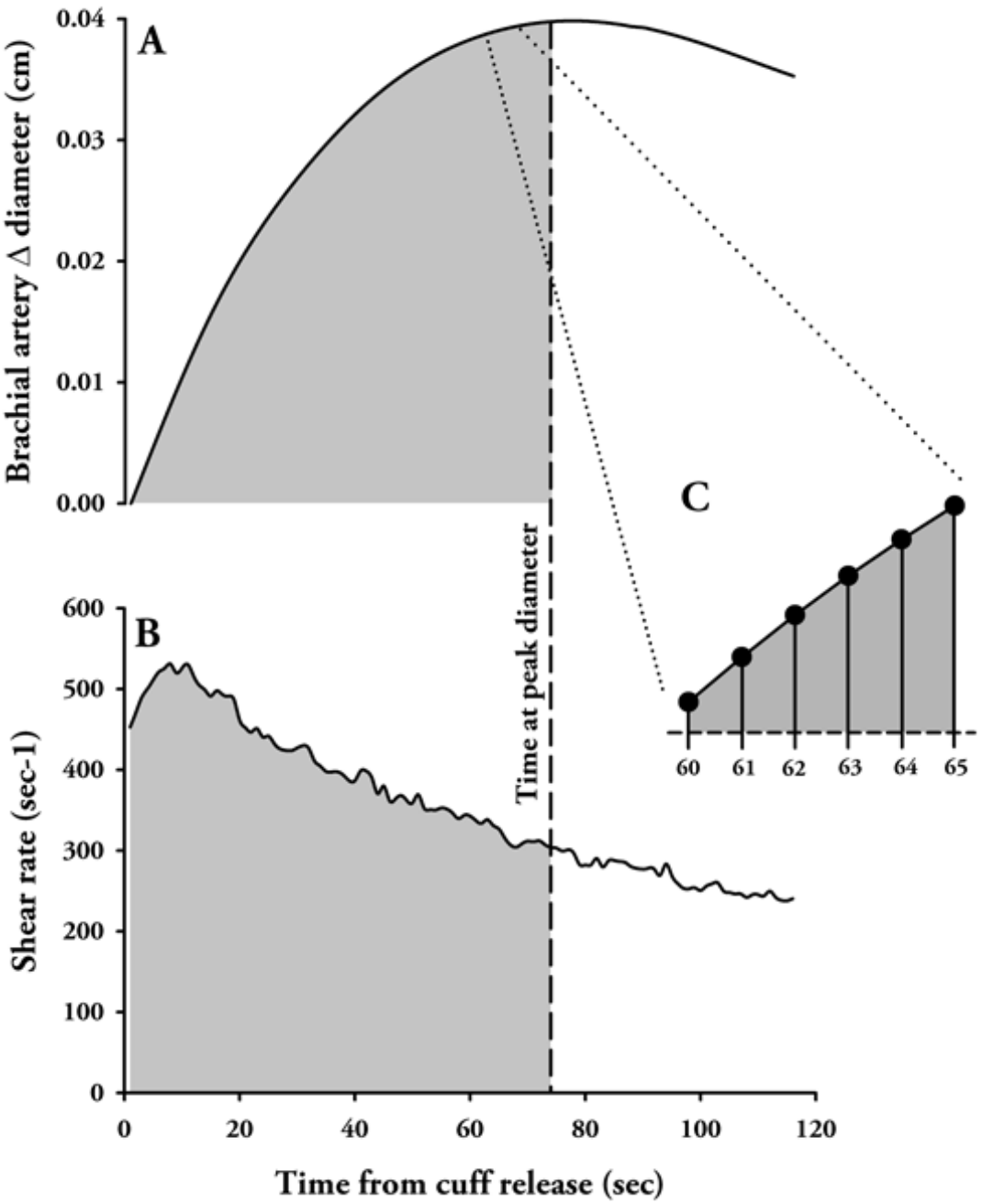

Figure 1.2 Area under the curve (AUC) integration

AUC to the time at peak diameter for change in diameter (A) and shear rate (B). The AUC was calculated by trapezoidal integration second by second (C).

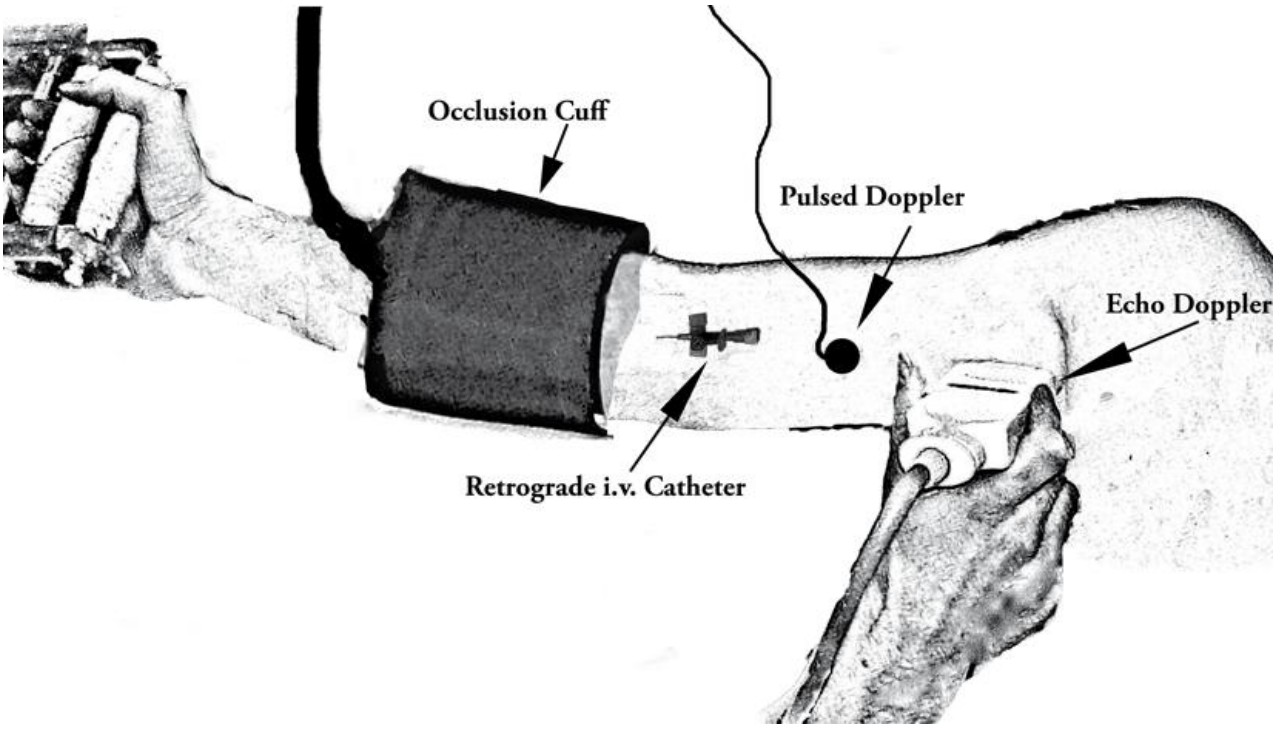

Figure 1.3 Experimental preparation for FMD.

Subject is in the supine position with the dominant arm extended at a right angle to the torso. The hand grip device is adjusted to fit comfortably in the hand. The occlusion cuff is placed just distal to the elbow. The venous catheter is inserted toward the hand in an antecubital vein. The pulsed (distal) and echo (proximal) Doppler probes are placed over the brachial artery at a distance that eliminates interference between the two.

Chapter 2 - **Sustained Hyperaemia Stimulus is Necessary to Induce Flow-Mediated Dilation of the Human Brachial Artery**

Summary

We studied the relative importance of the magnitude and duration of the shear stimulus to induce flow-mediated dilation (FMD) in the brachial artery of 10 healthy men by ultrasound imaging. The shear stress stimulus was induced by different durations of reactive hyperaemia following 15 min forearm occlusion. The control condition of continuous post-occlusion hyperaemia was compared to 20-, 40-, and 60- sec of reactive hyperaemia followed by reapplication of circulatory arrest for 2-min and a second cuff release. In response to the first cuff release, peak shear rate was not different between conditions, total shear during the first minute was reduced in the 40-s and further reduced in the 20 sec conditions. FMD in control (10.0±3.0%), 60 sec (10.5±3.2%) and 40 sec (7.8±3.6%) were greater than the 20 sec condition (2.9±2.8%). After the second cuff release, peak shear of the 20 sec condition was slightly reduced from the first release but 40 sec and 60 sec were progressively reduced. Total shear to peak dilation was reduced after the second cuff release for the 20 sec and 40 sec conditions and further after the 60 sec condition. FMD was maintained in the 20 sec condition (8.3±3.7%) but reduced in the 40 sec (3.7±1.7%) and 60 sec (1.5±2.6%). FMD was not related to peak shear rate after the first occlusion ($r=0.003$) but was after the second cuff release ($r=0.32$, $p=0.004$). The FMD response was correlated with the total shear to time of peak diameter after the first ($r=0.35$, $p<0.001$) and the second ($r=0.25$, $p=0.009$) cuff release. A sustained high shear stimulus was required to activate FMD.

Introduction

Endothelial dysfunction is associated with major risk factors for atherosclerosis and has been shown to be observed early in the course of atherogenesis, even before structural lesions can be found (Drexler & Hornig, 1999; Anderson, 1997; Verma, Buchanan, & Anderson, 2003). Invasive tests such as direct arterial infusion of vasoactive drugs (Drexler et al., 1992) and intracoronary catheterization (Antony, Lerebours, & Nitenberg, 1996) allow reproducible characterization of dysfunction, but the invasive nature of these techniques restricts their use in the general population. A non-invasive alternative is measurement of flow-mediated dilation (FMD) in response to reactive hyperaemia that follows release of circulatory occlusion (Green, 2005; Celermajer et al., 1992). FMD, like the invasive methods, is promoted as a sensitive biomarker of endothelial dysfunction associated with increased risk for cardiovascular disease (Gokce et al., 2003; Park, Charbonneau, & Schiffrin, 2001; Verma et al., 2003).

Although standardization of protocol to induce FMD has been advocated (Corretti et al., 2002) various sites and durations of occlusion are still employed. As a consequence of these different protocols, variable magnitude and duration of the shear stimulus will occur (Betik et al., 2004) and these could be responsible for achieving different FMD responses perhaps through activation of different dilatory mechanisms (Mullen et al., 2001; Tschakovsky & Pyke, 2005). Research on isolated rat cremaster 1A arteriole preparations suggested that the rate of onset of shear was the primary stimulus determining the magnitude of FMD (Butler, Weinbaum, Chien, & Lemons, 2000) and subsequent research with different occlusion protocols in humans suggested that peak and total shear stimuli were important determinants of FMD (Betik et al., 2004). Together these results suggested that a

short-duration, high shear rate stimulus should be capable of inducing a large FMD response but to our knowledge the role of high initial shear stimulus in isolation has not been investigated in humans. To test this hypothesis, we achieved the same initial magnitude of shear by performing 15-min circulatory occlusion distal to the measurement site in the brachial artery followed by different durations of shear stimulus including a 3 min control condition or interrupted shear stimulus by re-inflating the occlusion cuff after periods of 20-, 40-, and 60- sec. This second occlusion was maintained for an additional 2 min to allow sufficient time for the peak FMD response (Corretti et al., 2002) before a second cuff release which created conditions of reduced peak and cumulative shear. From these data we could independently examine the role of peak shear and total cumulative shear on FMD.

Methods

Ten healthy, physically active men with no family history of premature cardiovascular disease volunteered for the experiment. Subjects were instructed to refrain from consuming caffeine or high fat containing foods or beverages 24 hours, and to be fasted for three hours, preceding each experimental trial. Each subject gave written, informed consent for this study which was approved by the Office of Human Research Ethics at the University of Waterloo.

Physiological Data Collection

All measurements were performed while the participants were supine in a quiet, darkened room kept at a relatively constant temperature (21-23°C). Heart rate (Pilot 9200, Colin Medical Instruments, San Antonio, USA), mean arterial blood pressure (MAP) estimated by finger photoplethysmography (Finapres 2300, Ohmeda, Englewood, USA), and brachial artery mean blood velocity (MBV) measured by pulsed wave Doppler ultrasound (Model 500V, Multigon Industries, Mt. Vernon, USA) were collected using a data-acquisition system (PowerLab, ADInstruments, Colorado Springs, USA). The pulsed Doppler 4-MHz probe was fastened above the brachial artery proximal to the cubital fossa with strips of surgical tape. The signal was directed 45° relative to the skin and the ultrasound gate was adjusted to encompass the total width of the artery (Shoemaker et al., 1996). Continuous measurement of the velocity spectrum was obtained by directing the Doppler signal through a mean velocity processor enabling the determination of instantaneous MBV. The brachial artery was continuously imaged using a 7.5-MHz probe in B-mode (Model SSH-140A. Toshiba Inc., Tochigi-Ken, Japan) at a depth of 4 cm and recorded to s-VHS tape for later analysis.

Flow-Mediated Dilation Procedure

Brachial artery blood flow was arrested by rapid inflation (E-20 Rapid Cuff Inflator, D. E. Hokanson, Issaquah, USA) of a standard blood pressure cuff to 250 mmHg. The occlusion cuff was placed just distal to the right elbow. Reactive hyperaemia was initiated by rapid deflation of the occlusion cuff.

Experimental Design

Subjects reported to the laboratory for testing on four separate occasions separated by at least two days. All tests for a particular subject were performed at the same time of day in order to limit possible circadian rhythm effects. Each testing session consisted of one of the four FMD protocols administered in random order. After a supine rest period, the trials consisted of 1 min baseline data collection followed by the FMD procedure. Each of the 10 participants was familiarized to the FMD procedure one week prior to the commencement of testing.

FMD Protocols

The control trial consisted of 15-min forearm occlusion followed by 3-min monitoring during the period of reactive hyperaemia. The experimental trials consisted of identical 15-min forearm occlusion followed by one of 20-, 40-, or 60-sec reactive hyperaemia that was interrupted by a second cuff inflation. The second cuff inflation lasted 2 min to allow the expected time to see peak FMD response to the first hyperaemic period then monitoring continued for an additional 3 min after release of this second occlusion.

Data Analysis

Beat-to-beat brachial artery MBV was obtained by averaging MBV between consecutive R-waves from the ECG. Brachial artery diameter (D) was obtained from measurements of still images from the s-VHS tape using the electronic calliper function on the Toshiba ultrasound unit. Measurements were taken at specific time points during baseline, circulatory occlusion and the reactive hyperaemia period to permit detailed description of the time course of the FMD response. All measurements were taken during the diastolic phase of the cardiac cycle and were reported as the average of 4 measurements taken at each time point. The diameter values were then fit using an exponential curve fitting program in which best fit was defined by minimizing the residual sum of squares (Betik et al., 2004). The diameter curve was then used to determine circular cross sectional area ($A=\pi \times r^2$) of the artery for every point in time corresponding with MBV data. Brachial artery MBV and cross-sectional area were used to calculate forearm blood flow ($Q=v_{mean} \times A \times min^{-1}$) and shear rate ($\gamma = 8 \times v_{mean} \times D^{-1}$). Baseline forearm blood flow, expressed as ml/100ml/min, and shear rate, expressed as s^{-1}, were determined from averages over the last 30 sec of baseline. Peak shear rate was determined from 10-s averages beginning 5 sec after occlusion cuff release. Total shear rate ($s \cdot s^{-1}$) was calculated as the integration of the shear curve to the time of peak dilation (AUCshear).

Statistical Analysis

Statistical analysis software (SAS Institute, Cary, USA) was used to examine the shear rate, blood flow and FMD response for each of the high shear durations by

repeated measures analysis of variance by the glm procedure with one within subject factor. A probability of $p<0.05$ was accepted as statistically significant and any differences were subsequently analyzed with the Least Squares Difference *post hoc* test. Paired t-tests were run to identify significant differences in vessel diameter between baseline and peak FMD. Correlations between variables were calculated using the corr procedure, with $p<0.05$ indicating a significant correlation. Data are presented as means ±SD.

Variability of Diameter Measurements

In order to test for inter-rater variability, diameter values at baseline and peak FMD were compared between two technicians. Intra-rater variability was assessed by comparing baseline and peak values between two separate measures by the same technician.

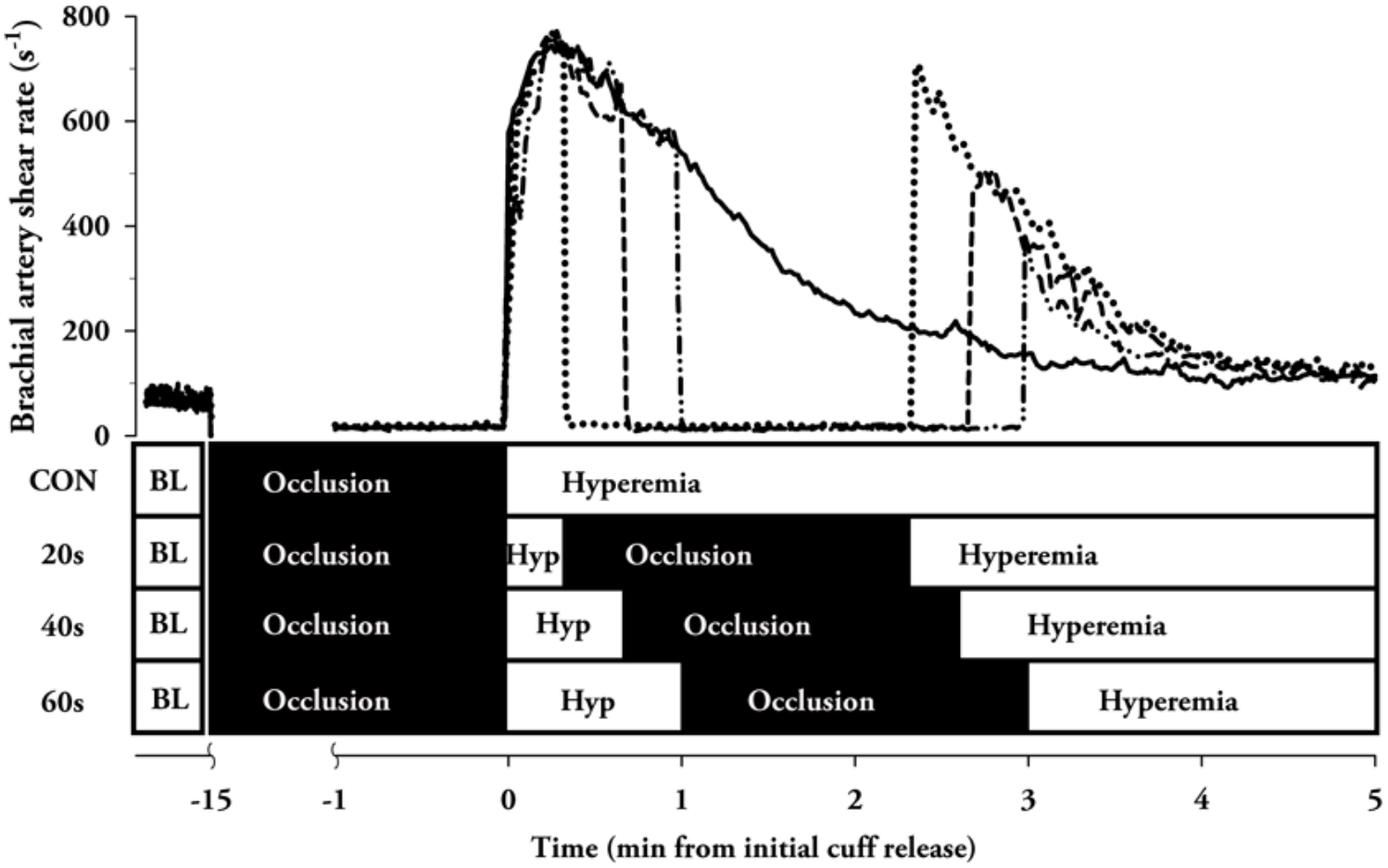

Figure 2.1 Illustration of the different shear duration protocols and representative tracings of the shear rate profiles for each protocol for one subject.

Results

Reactive Hyperaemia

On release of the cuff after 15-min circulatory occlusion, shear rate increased rapidly for all four conditions. The shear rate followed a similar time course for each condition until the rapid re-inflation of the occlusion cuff at 20-, 40- or 60-sec after the first cuff release (Figure 2.1). The cuff was then released 2 min later and the shear rate once again followed a pattern of rapid increase and slower decline (Figure 2.1). Peak shear rate was not different for the first cuff release across all protocols (Table 2.1). AUCshear after the first cuff release was not different for the control and 60-s condition but was significantly reduced for the 40 sec condition with a further significant reduction during the 20 sec condition (Table 2.1). After the second cuff release, peak shear for the 20 sec condition was not different from peaks observed with the first cuff release, but the peak shear values were reduced in the 40-s and 60-s conditions. AUCshear after the second cuff release was less than that observed after the first cuff releases for the 20 sec and 40 sec conditions and was further reduced for the 60 sec condition (Table2.1).

Flow Mediated Dilation

Brachial artery diameter increased significantly above baseline after the first cuff release in the control, 40 sec and 60 sec conditions (Figure 2.2). Vessel diameter decreased slightly during the two-minute re-occlusion period and diameter increased significantly above this value during the second cuff release for the 20 sec condition (Figure 2.2)

The dilation for the 20 sec (2.9±2.8%) and 40 sec (7.8±3.6%) conditions was less than for the control (10.0±3.0%) or 60 sec (10.5±3.2%) conditions. Following

the first cuff release, the FMD response in the control condition (10.0±3.0%) was not different from the 40-s (7.8±3.6%) and the 60 sec (10.5±3.2%) conditions, but FMD was significantly less in the 20 sec condition (2.9±2.8%) (Figure 2.2). The magnitude of the FMD response after this first cuff release was not related to the peak shear rate ($r=0.051$, $p=0.76$, but it did correlate with the total shear ($r=0.691$, $p<0.001$) (Figure 2.3).

The FMD response expressed as % increase above the diameter immediately before the second cuff release for the 20 sec (8.3±3.7%) condition was not different from the FMD response of the control, 40 sec and 60 sec conditions of the first cuff release, while it was greater than the FMD after the second cuff release of the 40 sec (3.7±1.7%) and 60 sec (1.5±2.6%) conditions (Figure 2.2). The FMD responses of the three second cuff release conditions were significantly correlated with both the peak shear rate ($r=0.60$, $p=0.007$, Figure 2.3) and AUCshear ($r=0.62$, $p<0.001$, Figure 2.3). For the second cuff release conditions, the AUCshear was highly correlated with peak shear ($r=0.96$, $p<0.001$).

When normalized for AUCshear there were no differences in FMD for any duration of hyperaemia during the first cuff release (Figure 2.4). During the second cuff release FMD normalized for AUCshear was significantly greater in the 20 sec protocol than the 40 sec protocol (20 sec: 0.0037±0.0025 vs 40 sec: 0.00019±0.00010, $P<0.05$).

To evaluate endogenous vasodilatory capability, independent of changes in baseline diameter a %FMD × BL diameter index was calculated. The response to the protocols showed the same significant differences as the %FMD alone (Figure 2.5).

Variability of Diameter Measurements

The intra-rater variability of diameter measurements was on average 3.5% (range 0 to 12.6%) with a Pearson Product Moment correlation Coefficient of 0.89 ($p<0.01$). The inter-rater variability rated on average 3.8% (range 0 to 16.2%) with a Pearson Product Moment correlation Coefficient of 0.87 ($p<0.01$).

Table 2.1 Mean responses during post-occlusion hyperaemic period for all subjects for the first and second cuff release expressed as peak shear rate and total shear to peak diameter.

	Peak shear, s^{-1}		Total shear to peak diameter, $s \cdot s^{-1}$	
	First release	Second release	First release	Second release
Control	853.4±188.6 [a]	--	50283.1±13877.8[a]	--
20 sec	857.1±219.6[a]	732.0±307.8[a]	15286.0±3971.3[d]	17650.5±17206.2[b,c]
40 sec	827.5±233.5[a]	505.7±131.9[b]	27414.4±9296.6[b]	20857.6±6285.6[c]
60 sec	807.6±117.8[a]	220.6±76.4[c]	39033.9±6720.0[a]	7425.4±5970.4[e]

Mean±SD. Means with the same letter are not significantly different (repeated measures ANOVA, $p<0.05$, LSD post hoc).

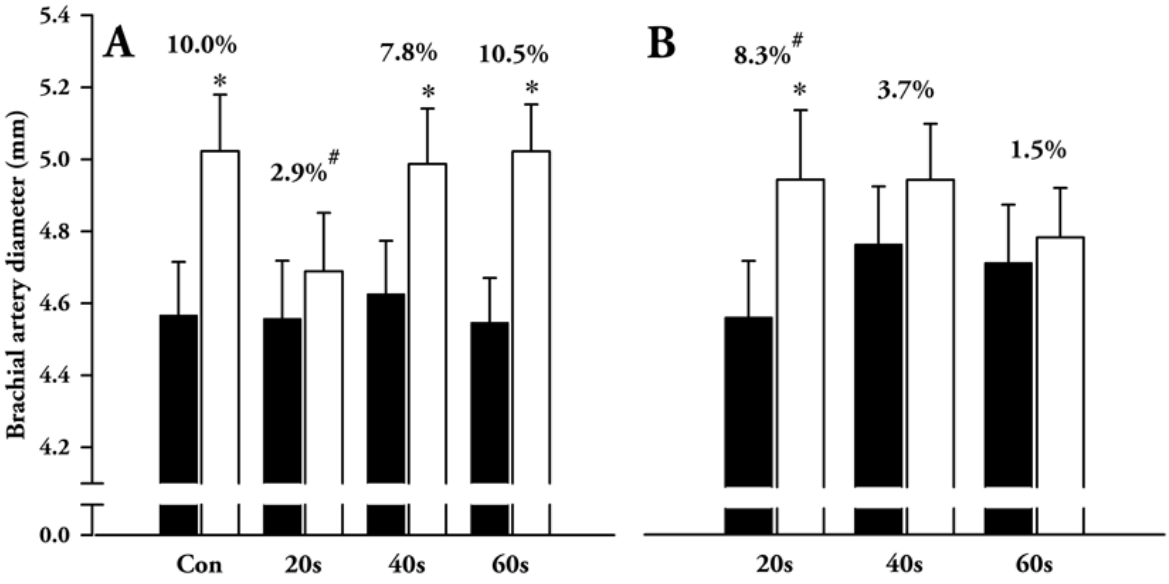

Figure 2.2 Mean baseline and peak diameter, as well as percent FMD for all durations of shear exposure following first and second cuff release.

Con, 40s, and 60s resulted in significant increases in diameter that were comparable. 20s was not sufficient to illicit a significant dilation (A). The only second cuff release that resulted in a significant increase in diameter was the 20s protocol. * significantly different from baseline # significantly different FMD, $p<0.05$.

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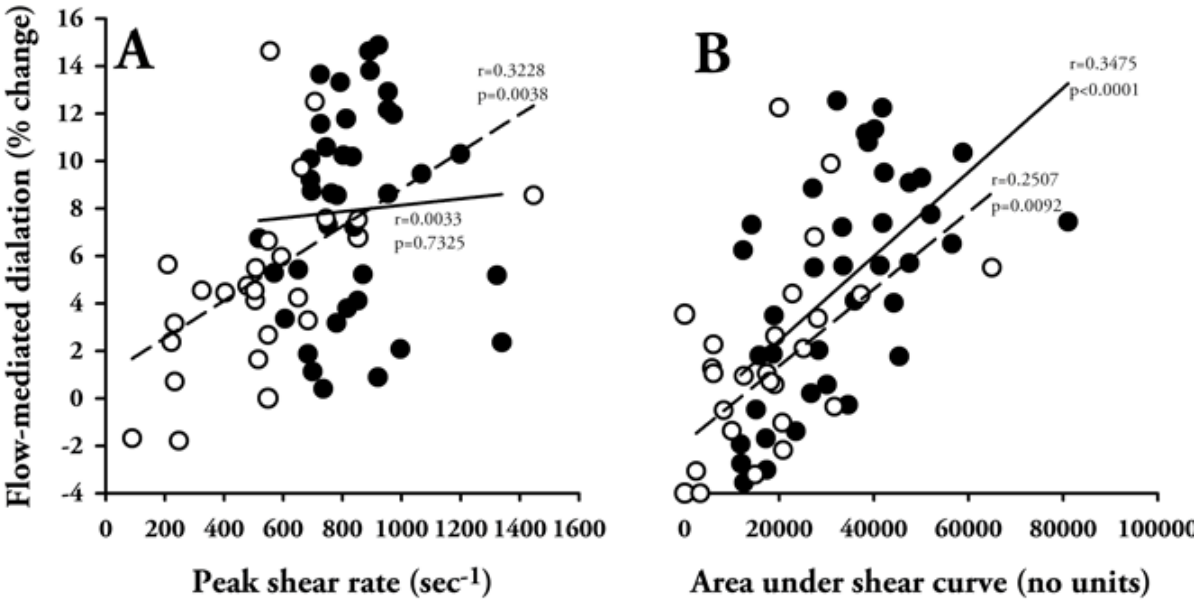


Figure 2.3 Correlation between peak shear rate and % FMD, and total shear and % FMD for both cuff releases.

•, solid line - first cuff release, ○, dashed line - second cuff release. %FMD was significantly correlated to peak shear only for the second cuff release (A). %FMD was significantly correlated to the area under the shear curve for both cuff releases (B).

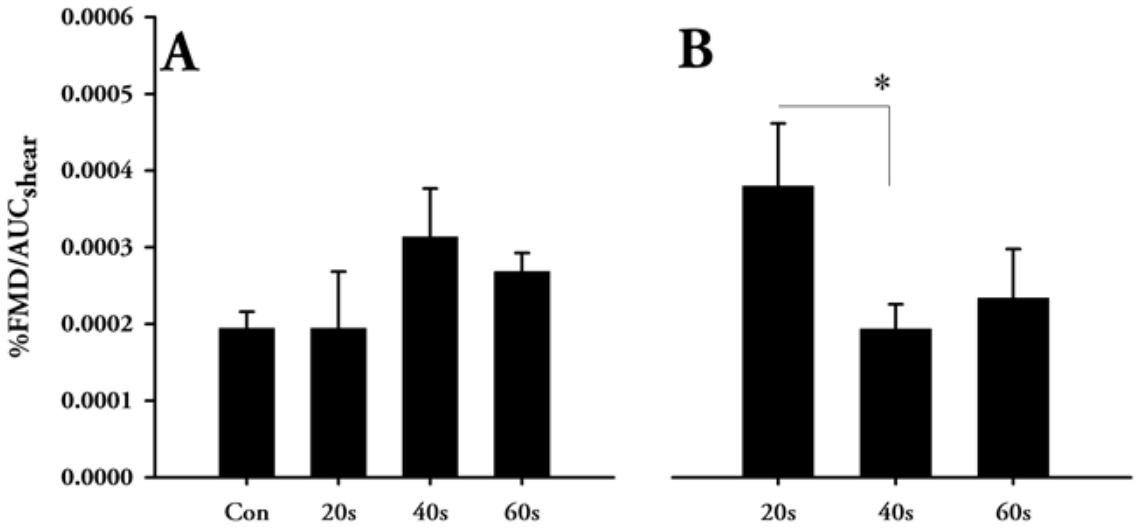


Figure 2.4 FMD normalized to the total shear to the peak diameter.

FMD/AUCshear was similar for Con, 20s, 40s, and 60s (A). During the second cuff the 20s protocol had a greater FMD/AUCshear than 40s (B). Means±SE * significantly different, $p<0.05$.

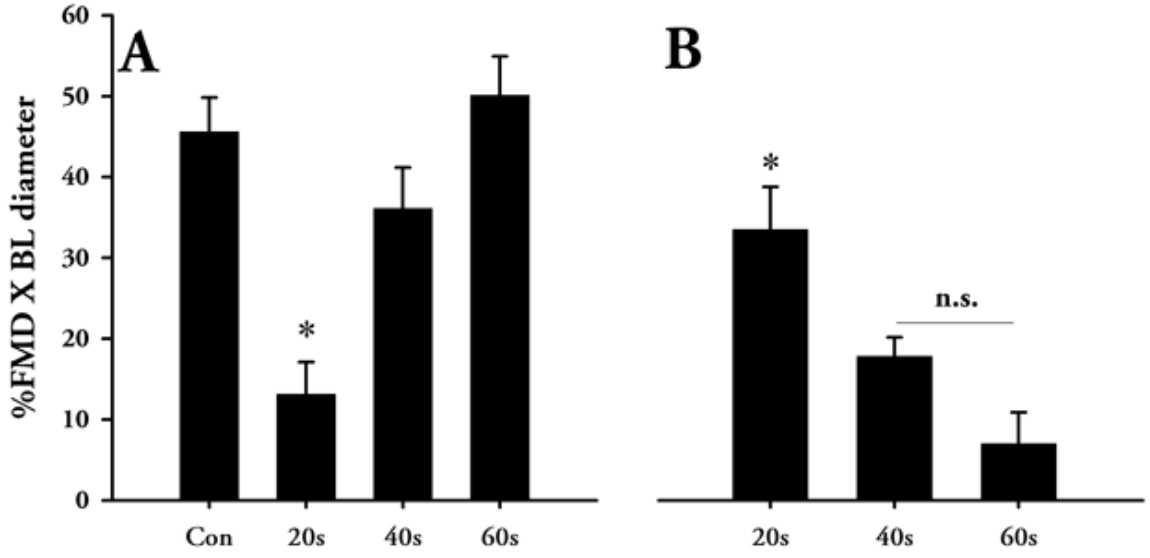


Figure 2.5 FMD normalized to the baseline diameter.

Using the %FMD X BL diameter index reveals that the change in baseline diameter had virtually no impact on the significance of the depressed FMD found in the first 20s cuff release (A), or the cuff releases following the 40s and 60s hyperaemia (B). Means±SE * significantly different from other contitions, $p<0.05$.

Discussion

The primary new finding of this research was that 20 sec of high shear rate stimulus following 15-min circulatory occlusion was insufficient to initiate more than a very small FMD response while after 40-s shear stimulus the magnitude of FMD was not significantly different from the response in either the 60 sec cuff release or the free flow, control conditions. Further we observed by these manipulations of blood flow through the brachial artery that the maximum FMD response, which was observed about 60 sec after cuff release, could be dissociated from the peak shear stimulus but was strongly linked to the total cumulative shear to the time of peak diameter. In this regard, when the %FMD was normalized for the cumulative shear to the time of peak diameter the differences in FMD were virtually abolished. The importance of the magnitude and duration of the shear stimulus in evoking FMD have been identified in previous research (Butler et al., 2000; Mullen et al., 2001; Tschakovsky & Pyke, 2005) but at the time of these investigations there were no studies examining the dilation response to a short duration of shear. In the interim period between the performance of these experiments and the writing of this manuscript Pyke & Tschakovsky (2007)) found similar results to these when controlling for shear duration by cuff re-inflation, and for shear magnitude by graded cuff release.

In previous research of FMD, the magnitude of the shear stimulus has been manipulated by changing the duration of occlusion, by adding ischemic exercise or by changing cuff position (Betik et al., 2004; Doshi et al., 2001; Mullen et al., 2001). The peak shear stimulus in the current study was identical in the control, 20 sec, 40 sec and 60 sec conditions and the shear stimulus remained identical until re-inflation of the occlusion cuff at these different times after the initial

release. The mechanisms responsible for FMD are probably initiated early in the hyperaemic period but if the stimulus is not maintained beyond 20 sec then the outcome is only minimal dilation. This result contrasts with research on isolated rat cremaster 1A arterioles that identified two fundamentally different responses to shear stress mediated by the endothelium: 1) an early rate sensitive response, and 2) a magnitude sensitive response contributing to both early and late responses (Butler et al., 2000). The observation in this study that exposure of the endothelium to a high shear rate for 20 seconds was not an adequate stimulus to achieve significant flow-mediated dilation suggests that, at least in human conduit arteries, the duration of the shear stimulus is important in the triggering of FMD. The apparent absence of an initial rate-sensitive response may be due to differences in the shear stress sensitivity between the micro-vessels studied by Butler et al. (2000) and the conduit vessel that we studied in this investigation. The time to peak dilation is much faster (<10 sec) in human skin micro-vessels as determined by laser Doppler indices (Shamim-Uzzaman et al., 2002) than the 60-90 sec for peak dilation in the brachial artery in the current research and in many other studies (Berry et al., 2000; Betik et al., 2004; Corretti et al., 2002; Jarvisalo et al., 2002).

In the time between the experiments in this study were performed and the manuscript was written, a study was published examining the dilation of the brachial artery after 10, 20, 30, 40, 50 sec, and full hyperaemia after 6 min of occlusion, as well as the dilation following occlusion+isometric exercise under 3 different levels of peak hyperaemia, as controlled by arterial compression (Pyke & Tschakovsky, 2007).They found a correlation between total shear to time of peak diameter and %FMD, in accord with our findings. However, they found no effect of

manipulating the peak shear rate following the occlusion+isometric exercise protocol, leading them to conclude that the sustained exposure to peak shear is responsible for FMD, independent of the peak stimulus. Our results, from the second cuff release, suggest that there is some interaction between peak and sustained shear to produce the FMD response. The discrepancies may be due to the exercise performed during the occlusion period in the Pyke & Tschakovsky (2007) study, or to the change in baseline diameter at the start of the 2nd protocol in the present study. Although, when baseline diameter was accounted for by the %FMD × BLdiameter index, as suggested by Mizia-Stec et al. (2007) we still found a significantly greater FMD following prolonged shear with a highest peak.

Early research into the mechanism underlying FMD in humans suggested that the entire response was mediated by shear stress-induced release of nitric oxide (Joannides et al., 1995). More recent research has suggested that FMD might be mediated by nitric oxide production in response to the relatively smaller shear stress stimulus during hyperaemia that follows 5 min circulatory occlusion; however, the FMD response to the stimulus after 15 min occlusion or sustained elevated blood flow by hand heating or pharmacological dilation of downstream resistance vessels might involve mechanisms largely independent of nitric oxide and prostaglandin production (Mullen et al., 2001). It is not clear from the study of Mullen et al. (Mullen et al., 2001) how the signal is differentiated between the 5 min and 15 min occlusion periods since the peak stimulus is slightly greater with the 15 min occlusion but the total stimulus is considerably increased. More recent evidence indicates that nitric oxide production is increased with sustained higher shear rates but there is also simultaneous activation of additional dilatory processes such as release of endothelium-derived hyperpolarizing factor and

activation of potassium channels to cause dilation (Bellien et al., 2006). Thus, in the current study where 20 sec of high shear stimulus evoked only a very small dilatory response, the stimulus did not appear large enough to sufficiently activate nitric oxide production and its second messenger systems or any of the other dilatory mechanisms.

Limitations

The model employed in this study achieved the flow stimulus by 15 min circulatory occlusion prior to cuff release for all time conditions. Re-inflation of the occlusion cuff after only 20 sec of hyperaemia would have trapped a large concentration of vasoactive metabolites that induced the first hyperaemic response and would have then contributed further to a dilatory stimulus with the additional 2 min of occlusion. On the second cuff release in the 20 sec condition the peak shear rate was only slightly reduced from that after the original 15 min occlusion but total shear to time of peak diameter was considerably reduced. Similarly for the 40 sec and 60 sec conditions, the peak and total shear after the second cuff release were progressively reduced as would be anticipated with the longer washout during the first hyperaemic period. A limitation of the method is that the brachial artery diameter was increased at the point of second cuff release in the 40 sec and 60 sec conditions due to the residual effects from the first cuff release. The FMD response above this elevated baseline was reduced and it is not known if the FMD would have been the same from the original resting baseline. However, we attempted to compensate for this discrepancy with the %FMD × BLdiameter index. The tight correlation between FMD and total shear from both the first and second cuff release experiments suggested that the same mechanisms were acting under all

conditions and that the magnitude of FMD was not affected, at least under the conditions of the current experiment, by the starting baseline diameter. The link between peak shear rate and FMD after the second cuff release was probably a function of the strong relationship (r=0.96) between peak shear and total shear to the time of peak diameter under these conditions. The specific mechanism cannot be identified from the current research as this would require simultaneous infusion of several drugs to block multiple pathways involved in response to short- and long-term shear stress stimuli (Bellien et al., 2006).

Conclusion

This research has shown that the human conduit artery FMD response was largely independent of peak shear rate and that it required shear stimulus for greater than 20 sec to activate the mechanisms responsible for FMD. On the other hand, the tight correlation between total shear to time of peak diameter and FMD suggested that human conduit artery FMD was dependent on the cumulative amplitude and duration of the shear stimulus. These data are consistent with the recent observation of involvement of nitric oxide, prostaglandins, endothelium-derived hyperpolarization factor, potassium c channels and other potential mechanisms in the FMD response to the sustained total shear stimulus (Bellien et al., 2006).

Chapter 3 - **DILATION AND PLASMA NITRITE RESERVE FOLLOWING DIFFERENT HYPERAEMIA INDUCING PROTOCOLS IN THE HUMAN BRACHIAL ARTERY**

Summary

This study examined the dilation response in the human brachial artery to 3 different hyperaemia inducing protocols. Ten healthy male varsity hockey players visited the lab on 2 separate occasions. Flow-mediated dilation (FMD) of the brachial artery was assessed with 2 protocols: 1) CON, following 15 minutes of forearm occlusion and 2) 1EXin5OC, 1 minute of moderate hand-grip exercise within 5 minutes of occlusion. Both protocols yielded FMD that were not significantly different 15OC: 9.8±4.6 and 1EXin5OC: 9.3±2.9 %change), even though the shear stimulus was increased with exercise (AUC_{shear}:15OC 27823.9±11170.9 vs. 1EXin5OC 31966.3±12437.6 no units, $p<0.05$; Peak shear: 15OC: 551.8±155.3 vs. 1EXin5OC: 576.3±164.4 sec^{-1}, $p<0.05$). 1EXin5OC had elevated mean arterial pressure by the end of occlusion (BL: 93.67±7.01, 15OC: 97.67±6.86 vs. 1EXin5OC: 109.74±14.54 bpm, $p<0.05$). CON had increased plasma nitrite during hyperaemia (0s: 1.05±0.37 vs. 15s: 1.81±0.90 μM, $p<0.05$) not seen in 1EXin5OC. Four of the participants also made a 3rd visit: maxEXin5OC, heavy hand-grip exercise to exhaustion in 5 minutes of occlusion. The FMD in this condition was not different from the other two (10.7±2.6 %change), while shear was significantly greater (AUC_{shear}: 63605.78±24618.01 no units; Peak shear: 995.8.3±177.4 sec^{-1}). The brachial artery was also constricted compared to BL at end occlusion in maxEXin5OC BL: 4.75±1.05, OC: 4.50±0.87 mm). The results of this study show that all of these conditions, while producing similar values for FMD, may not in fact be reflective of the same dilatory mechanisms.

Introduction

Flow-mediated dilation (FMD) describes an increase in blood vessel cross-sectional area in response to an increase in blood flow. The mechanism has been shown to be dependent on an intact endothelial layer (Pohl, Holtz, Busse, & Bassenge, 1986). A healthy endothelium will release nitric oxide (Ṅ0), a potent vasodilator, as a consequence of increased shear stress interacting with a shear sensitive receptor on the endothelial cell membrane (Joannides et al., 1995). As such FMD, usually in the brachial or radial artery, has been used as an indicator of endothelial cell function, and by extension, overall cardiovascular risk (Gokce et al., 2003).

In order to test FMD the blood flow through the artery of interest must be increased to a level that is sufficient to elicit a significant dilation. This is usually accomplished by temporarily occluding blood flow, either proximal or distal to the site of echo Doppler vascular imaging, thus causing a localized ischemic state which results in hyperaemia upon subsequent release of occlusion (Corretti et al., 2002). The standard duration of occlusion has been set at 5min (Corretti et al., 2002), however hyperaemia from this modest duration rarely elicits a dilation in excess of 5%, which makes it difficult to distinguish between normal and abnormal function due to the measurement error inherent in Doppler imaging (Gill, 1985). The addition of exercise within the occlusion period has been proposed to increase the demand for blood flow at end occlusion. While this method has been shown to increase hyperaemia and dilation up to double that of occlusion alone (Agewall, Whalley, Doughty, & Sharpe, 1999; Agewall, Hulthe, Fagerberg, Gottfridsson, & Wikstrand, 2002; Betik et al., 2004), there remains the possibility that exercise in and of itself has an effect on vascular tone that may mask any dysfunction of the endothelial Ṅ0 system. Both the prolonged duration of hyperaemia following

exercise in occlusion and the suggestion that longer periods of shear result in non-$\dot{N}O$ dependent dilation are suggestive of this hypothesis (Mullen et al., 2001; Bellien, Joannides, Iacob, Eltchaninoff, & Thuillez, 2003). Previous work in our laboratory has shown that a longer duration of occlusion (15min) can produce hyperaemia capable of eliciting a 10% dilation of the brachial artery without severely delaying the decay of the hyperaemia curve (Chapter 2). This study was designed to test the hypothesis that brachial artery dilation following an exercise in occlusion protocol would elicit a different FMD response compared to occlusion alone independent of differences shear stimulus. In addition, a third protocol employing exercise to exhaustion within 5 min of occlusion was tested to elicit a maximal hyperaemic response, thus testing the hypothesis that increasing shear will increase dilation above the ~10% FMD observed with the 15 min occlusion alone.

Methods

Ten healthy male varsity hockey players were recruited for inclusion in the study. The ten participants visited the lab on 2 separate occasions for brachial artery flow-mediated dilation (FMD) assessment: 1) control, following 15 minutes of forearm occlusion and 2) 1EXin5OC, 5 minutes of occlusion with 1 minute of moderate hand-grip exercise. The protocols were assigned in random order and the sessions were separated by at least 48 hours. Testing was performed at least 24 h following the participants' last exercise regimen and 72 h following an intense strength training session. The participants arrived to the laboratory in a rested state at least 2 h after eating and they were asked to abstain from caffeine 12 h and alcohol 24 h before testing. The participants were rested in supine position for 30 min before initiation of the protocols. The tests were completed in a quiet, air-conditioned laboratory at a temperature of ~ 22°C. Four of the participants made a 3rd visit after the other two: maxEXin5OC, heavy hand-grip exercise to exhaustion in 5 minutes of occlusion (Figure 3.1)

Flow-Mediated Dilation Procedure

Brachial artery blood flow was arrested by rapid inflation (E-20 Rapid Cuff Inflator, D. E. Hokanson, Issaquah, USA) of a standard blood pressure cuff to 250 mmHg. The occlusion cuff was placed just distal to the dominant elbow. Reactive hyperaemia was initiated by rapid deflation of the occlusion cuff.

Physiological Data Collection

Heart rate by 3 lead electrocardiogram (Pilot 9200, Colin Medical Instruments, San Antonio, TX), mean arterial blood pressure (MAP) estimated by finger

photoplethysmography (Finometer, FMS, The Netherlands), cardiac output (CO) estimated by ModelFlow (Finometer), and brachial artery mean blood velocity (MBV) measured by pulsed wave Doppler ultrasound (Model 500V, Multigon Industries, Mt. Vernon, USA) were collected and sampled at 1kHz using a data-acquisition (PowerLab, AD Instruments, Colorado Springs, CO) system running on a lap-top PC.

A pulsed Doppler 4-MHz probe was fastened above the brachial artery proximal to the cubital fossa with strips of surgical tape. The signal was directed 45° relative to the skin and the ultrasound gate was adjusted to encompass the total width of the artery.

Brachial artery diameter was monitored on the arm proximal to the inflation cuff by M-Mode ultrasound imaging (MICROMAXX, Sonosite, USA) and stored on digital video tape. The tapes were subsequently digitized to AVI video (KINO video editor). Vessel diameters were measured by custom edge-detection software (Jorges Serrador and Brian Deegan) from still images extracted (KINO video editor) at every 5s of video. An average of the diameter across the M-mode image frame (2-3 cardiac cycles) was taken from the best image in each 15s window to represent mean arterial diameter. The measurements from the 15s windows were curve fit by cubic spline interpolation and Lenenberg-Marquardt peak fitting (Fityk).

Brachial artery MBV and cross-sectional area ($A = \pi r^2$) were used to calculate forearm blood flow ($Q = vmean \bullet A\, min^{-1}$) and shear rate ($\gamma = 8 \times v_{mean} \times D^{-1}$). The MBV signal was filtered using 5 Hz low pass filter. Beat to beat MBV was interpolated to second to second by the non-rounded Akima method and matched with the interpolated diameter data. Baseline forearm blood flow, expressed as ml/100ml/min, and shear rate, expressed as s^{-1}, were determined from averages

over the last min before cuff inflation. Peak shear rate was determined from the peak value on the interpolated shear curve. Area under the shear (AUC_{shear}) and diameter (AUC_{diam}) curves were calculated by approximating the definite integral by use of the trapezoidal rule on the interpolated values from the time of occlusion cuff release to the time of peak diameter (QtiPlot).

Venous Blood Sampling

A catheter was inserted in an antecubital vein of the dominant arm in a retrograde fashion to maximize collection of blood from deep veins draining the finger flexor muscles. Blood samples (1 ml) were drawn in heparinized syringes at baseline and after cuff release (each 15 s from 0 to 90 s and at min 2 and 3 of hyperaemia). The heparinized whole blood was immediately put in an ice bath and centrifuged as soon as possible.The plasma was separated and stored at -80°C for subsequent analysis of nitrite concentration by colorimetric assay kit (Nitric Oxide Quantitation Kit, Active Motif, California). Plasma nitrite reserve was calculated the as relative nitrite increase during reactive hyperaemia in relation to the 0 sec values.

Statistical Analysis

Statistical analysis software was used to examine the differences in shear rate, blood flow, FMD, and blood factors for each protocol by repeated measures analysis of variance by the mixed procedure (SAS) with one within subject factor. A probability of $p<0.05$ was chosen to be accepted as statistically significant and any differences were determined by contrast adjustment.

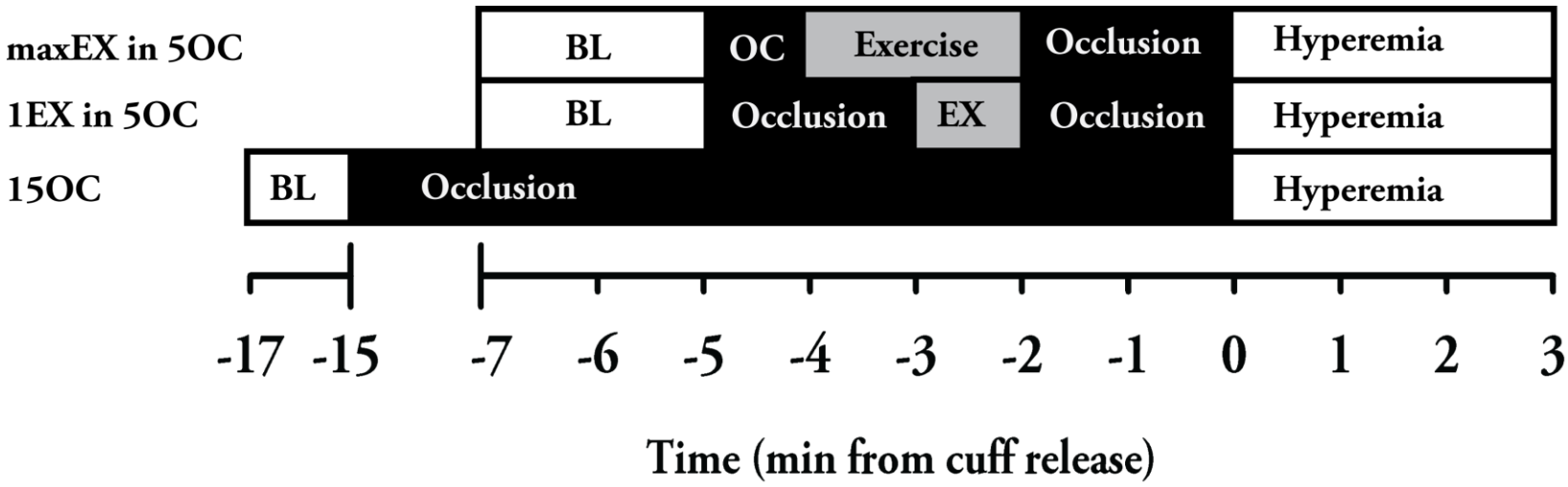


Figure 3.1 Three occlusion protocols.

maxEXin5OC, 3 min of baseline (BL) followed by exercise (EX) to exhaustion in 5 min of occlusion (OC) and 3 minutes of hyperaemia (HYP); 1EXin5OC, 3 min of BL followed by 5 min of OC containing 1 min of moderate EX then 3 min HYP; 15OC, 15 min OC followed by 3 min HYP.

Results

Maximal voluntary isometric contraction (MVC) strength was 54±6.2 kg during handgrip exercise as determined from the best of three attempts taken in supine position. The workloads during the moderate and heavy bouts were 7.55±1.01 W and 13.7± 0.7 W, respectively. The duration of the maxEX was 81.75±15.78s. There were no significant differences in any testing variable at BL between testing days.

Brachial Artery Diameter

Adding exercise to occlusion did not change the FMD response when peak diameter is compared to the diameter at end occlusion (15OC: 9.8±4.6 and 1EXin5OC: 9.3±2.9 %change, Figure 3.3). The 4 subjects who also did the maxEXin5OC protocol showed no differences in %FMD (10.7±2.6 %change, Figure 3.9 A) from end occlusion diameter compared to the other conditions, however there was a significant vasoconstriction during occlusion in maxEXin5OC (BL: 4.75±1.05 vs. OC: 4.50±0.87 mm, Figure 3.9 A).

Shear and Dilation

There were no significant differences between 15OC and 1EXin5OC for both the peak shear (15OC: 551.8±155.3 and1EXin5OC: 576.3±164.4, p=0.61) and AUC_{shear} to the time of peak diameter (15OC: 27823.9±11170.9, 1EXin5OC: 31966.3±12437.6 vs. maxEXin5OC: 63605.78±24618.01 no units, p=0.50, Figure 3.4). AUC_{shear} was correlated to AUC_{diam} only in 15OC (15OC: y=0.0001x-0.4164, R^2=0.7698, p=0.0009; 1EXin5OC: y=0.0005x+1.2457, R^2=0.1369, p=0.2927, Figure 3.8 A), as was the relationship between AUC_{shear} and percent dilation (15OC:

y=0.0003x+1.5522, R^2=0.5522, p=0.0178; 1EXin5OC: y0.0002+8.5883, R^2=0.0.0083, p=0.8018, Figure 3.8 B) and peak shear to % FMD(15OC: y=0.0238x-3.3449, R^2=0.6569, p=0.0045; 1EXin5OC: y=-0.0005+9.5610, R^2=0.0008, p=0.9373, Figure 3.8 C).

The 4 subjects who performed the maxEXin5OC protocol showed that both the peak shear (15OC: 551.8±155.3, 1EXin5OC: 576.3±164.4 vs. maxEXin5OC: 995.8.3±177.4 sec^{-1}, p<0.05) and AUC_{shear} to the time of peak diameter (15OC: 27823.9±11170.9, 1EXin5OC: 31966.3±12437.6 vs. maxEXin5OC: 63605.78±24618.01 no units, p<0.05, Figure 3.9 B) were higher in that protocol compared to the other 2 protocols. FMD was significantly smaller in maxEXin5OC when %FMD was normalized for the AUC_{shear} (15OC: 0.00035±0.00009, 1EXin5OC: 0.00032±0.00006 vs. maxEXin5OC: 0.00018±0.00004 no units, p<0.05, Figure 3.9 C).

Plasma nitrite

Figure 3.5 illustrates the venous plasma nitrite concentration following occlusion cuff release. Plasma nitrite was significantly elevated at 15s following cuff release in the 15OC protocol (0s: 1.05±0.37 vs. 15s: 1.81±0.90 μM, p<0.05), but not at any time up to 30s post cuff release in the 1EXin5OC protocol. By 30s post occlusion plasma nitrite in 15OC dropped below the stable level in 1EXin5OC (15OC: 0.83±0.22 vs. 1EXin5OC: 1.48±0.22 μM, p<0.05). When expressed as nitrite reserve from cuff release, nitrite in 15OC was elevated at 15s post cuff release compared to baseline and to any point during 1EXin5OC (15OC 15s:82.0±78.1 vs, 1EXin5OC 15s: 2.5±32.9 and 1EXin5OC 30s: 6.2±50.8 Δ%, p<0.05, Figure 3.5).

Heart rate, blood pressure, and cardiac output

Figure 3.7 illustrates the systemic cardiovascular responses at the end of the occlusion protocols. Although HR was elevated during the exercise bout (BL: 60.19±8.71, 15OC: 59.93±7.98 vs. 1EXin5OC: 74.71±12.25 bpm, $p<0.05$) it returned to BL and 15OC values by the time of cuff release (1EXin5OC: 60.54±8.85 bpm). MAP at end occlusion was significantly higher in the 1EXin5OC exercise protocol (BL: 93.67±7.01, 15OC: 97.67±6.86 vs. 1EXin5OC: 109.74±14 bpm, $p<0.05$).

The 4 subjects who also performed the maxEXin5OC protocol showed that CO was elevated in that protocol alone (BL: 6.9±1.9, 15OC: 7.2±1.8, 1EXin5OC: 7.0±1.4 vs. maxEXin5OC: 8.7±1.7 l/min, $p<0.05$). HR was elevated to a similar degree as was 1EXin5OC (74.73±12.32 bpm), while MAP was elevated significantly more than 1EXin5OC (123.27±9.65 bpm).

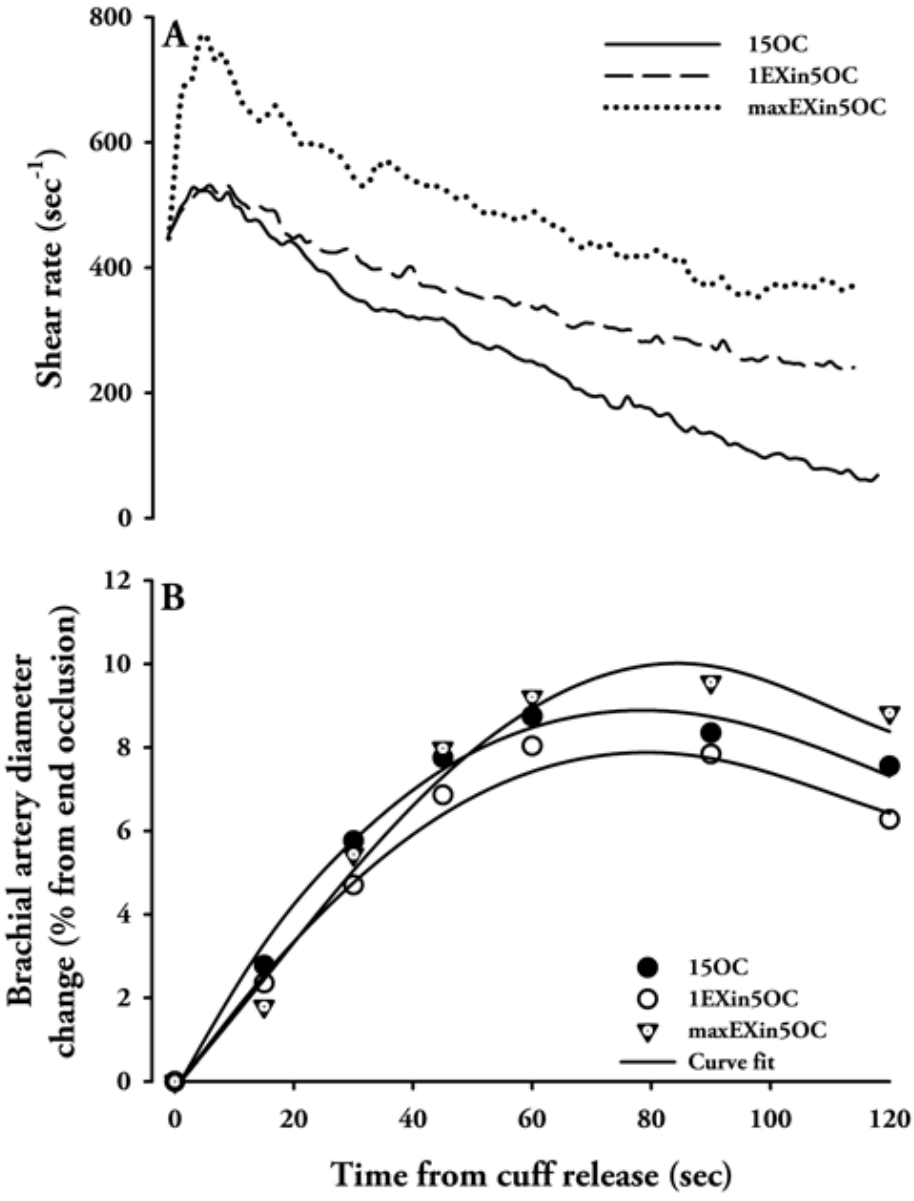


Figure 3.2 Shear and diameter profile post cuff release.

A. The shear profile for 15OC had a steeper decay than for the protocols involving exercise. Lines are means for 10 subjects (15OC and 1EXin5OC) and 4 subjects (maxEXin5OC).

B. The time course of diameter change was not different in any of the protocols. Symbols are measured means±SE and lines are curve fit means for 10 subjects (15OC and 1EXin5OC) and 4 subjects (maxEXin5OC).

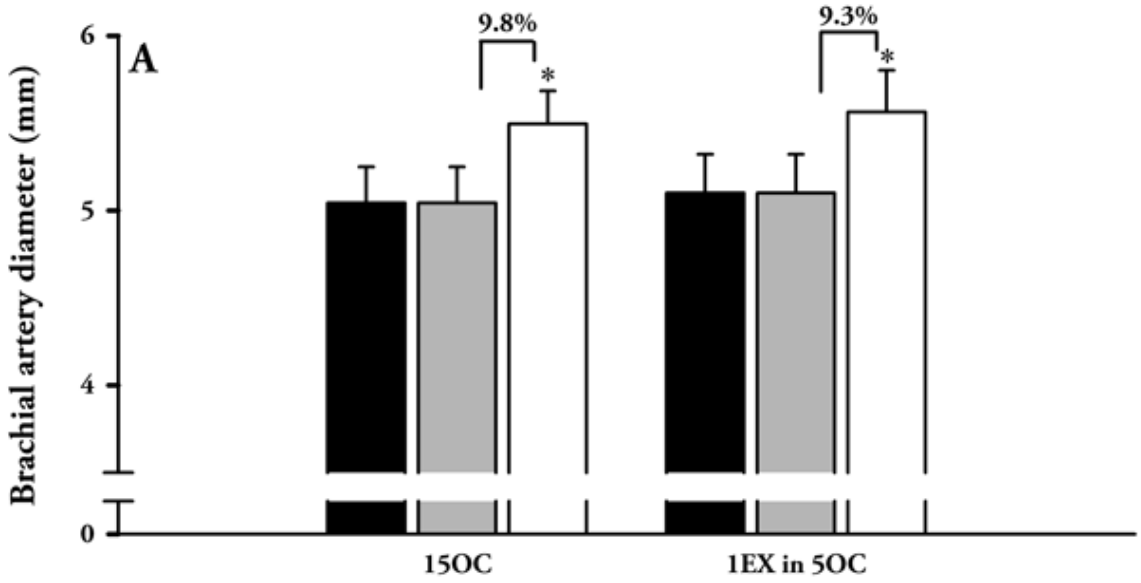


Figure 3.3 Brachial artery diameter increases following the 2 occlusion protocols.

Flow-mediated dilation was not different in either of the hyperaemia inducing protocols. Baseline (black bars), end of occlusion (grey bars), and peak after cuff release (white bars). Means ±SE, * significantly different from baseline. $P<0.05$.

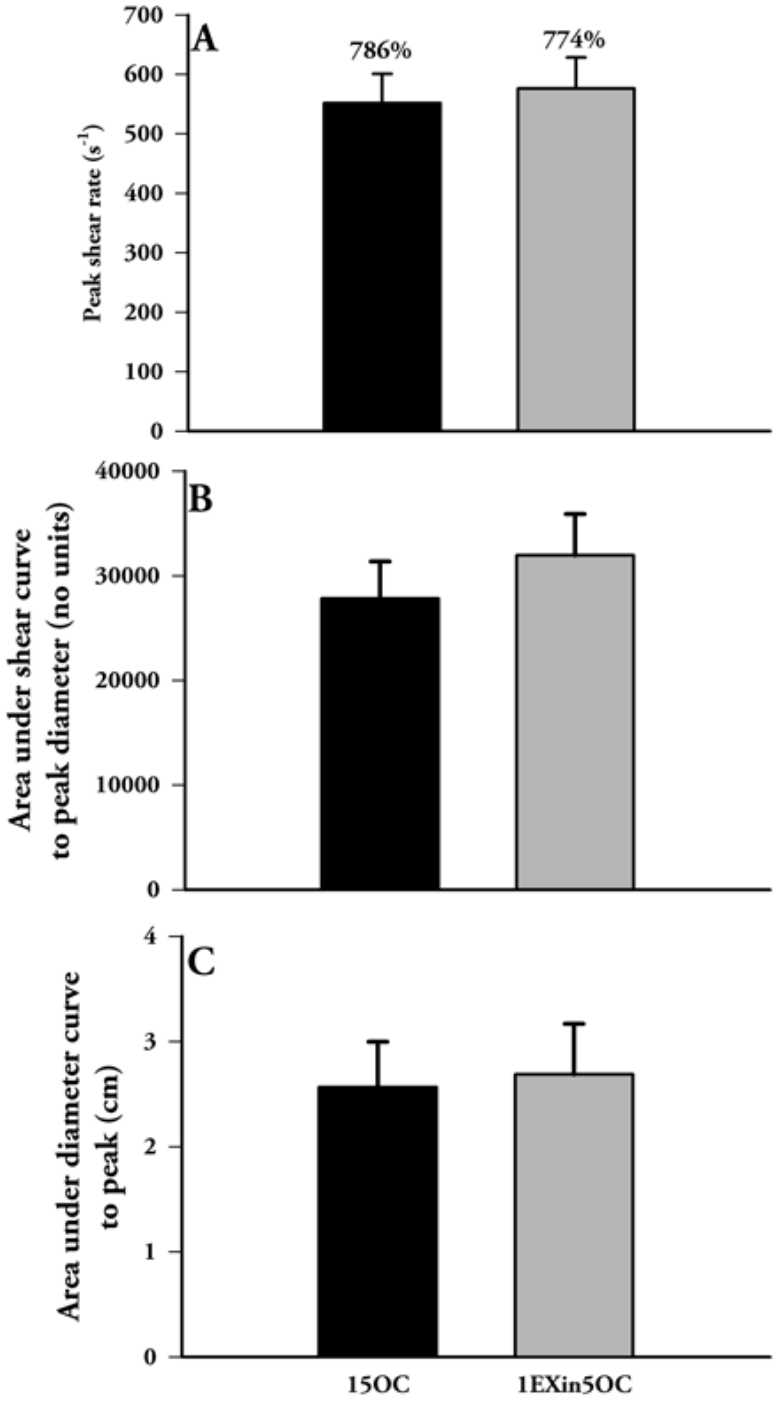


Figure 3.4 Peak shear and area under the shear rate and diameter curve for the 2 occlusion protocols.

There were no significant differences in peak (A) or AUC (B) shear or total dilation (C) between 15OC and 1EXin5OC. %'s are change in shear from baseline. Means ±SE.

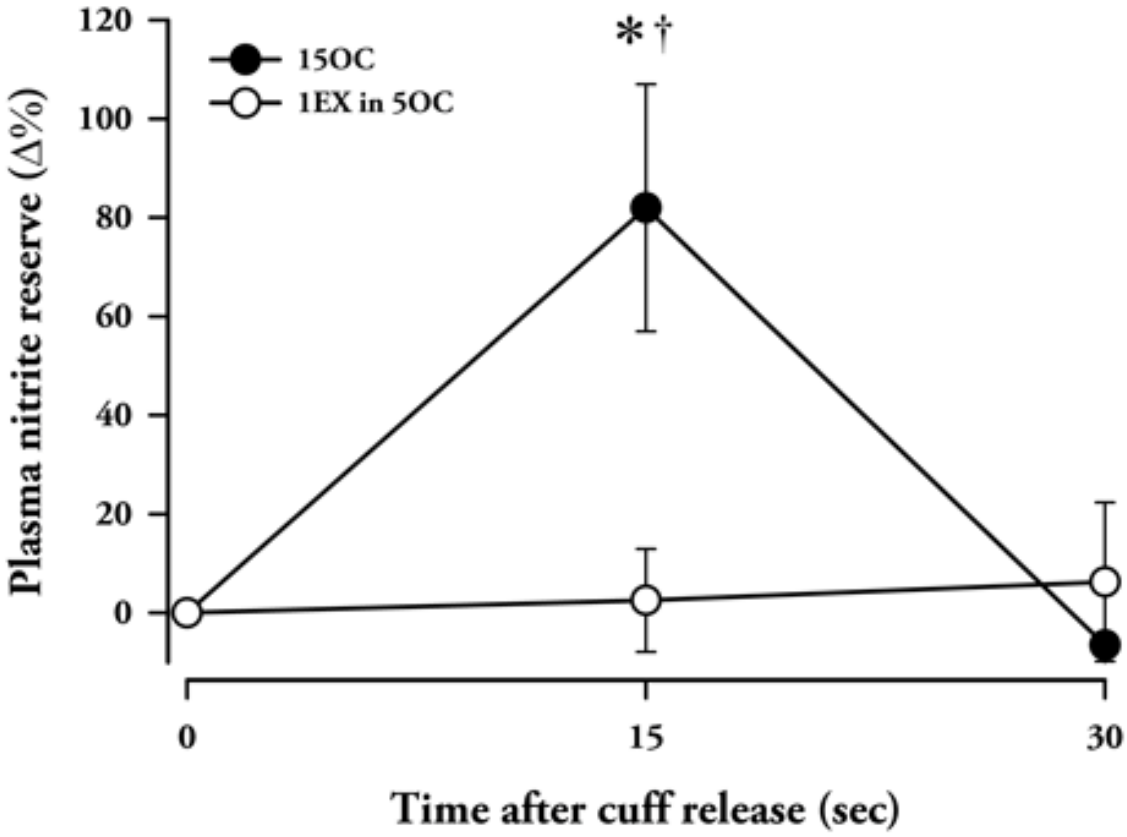


Figure 3.5 Plasma nitrite reserve following cuff release in the 15min and 5min with 1 min exercise occlusion protocols.

Plasma nitrite was elevated at 15 seconds of hyperaemia only in the 15 minute occlusion protocol. Means ±SE, * significantly different from baseline, † significantly different across conditions at the same time point. $P<0.05$.

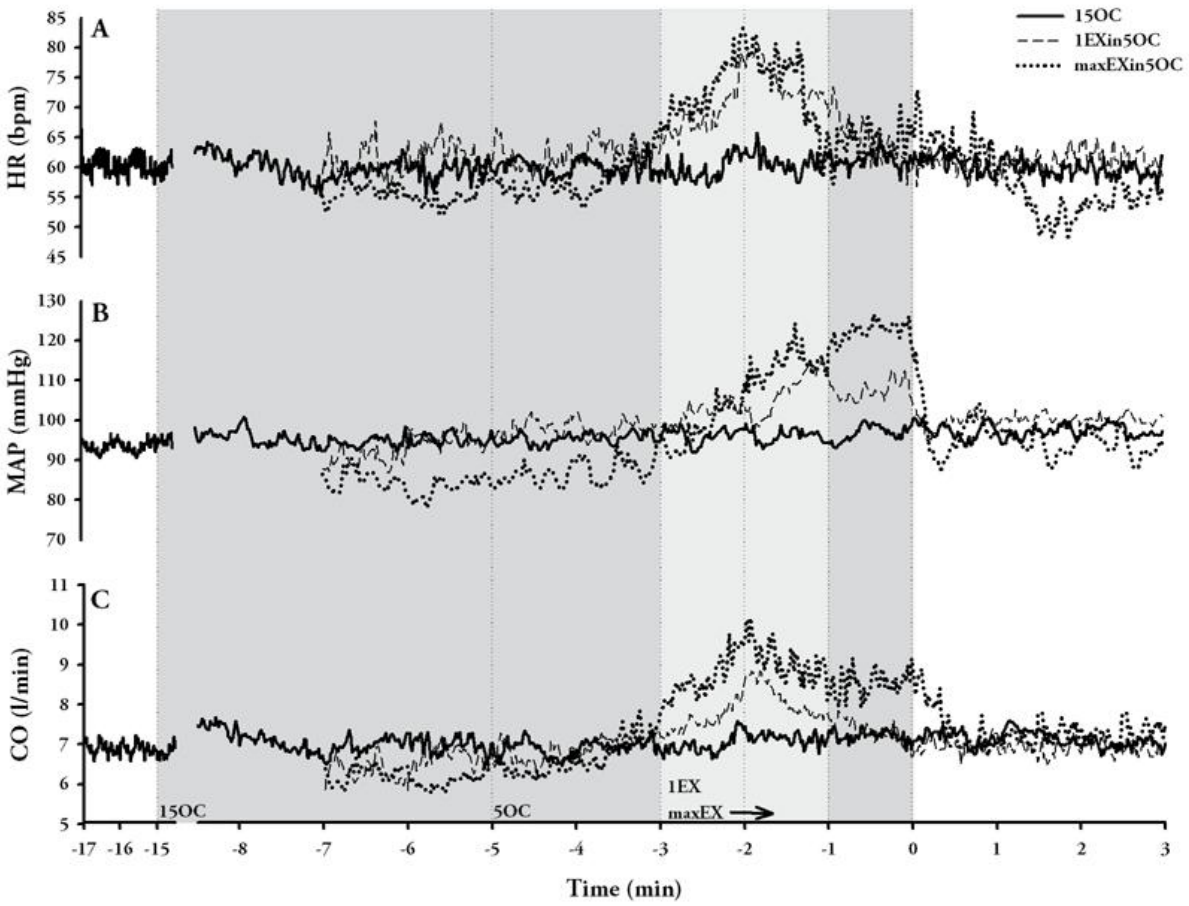


Figure 3.6 Systemic cardiovascular response throughout the 3 occlusion and exercise protocols.

Heart rate (HR) increased from start of exercise and fell steadily after 1EX, while maintaining the 1EX plateau to end EX in maxEXin5OC (A). Mean arterial pressure (MAP) increased from the start of exercise and continued to rise to the end of occlusion in both 1EXin5OC and maxEXin5OC (B). Cardiac output (CO) increased from the start of exercise, falling steadily after 1EX, but maintaining the 1EX plateau through to end occlusion in maxEXin5OC (C). Lines are means of 10 subjects for 15OC and 1EXin5OC, and of 4 subjects for maxEXin5OC.

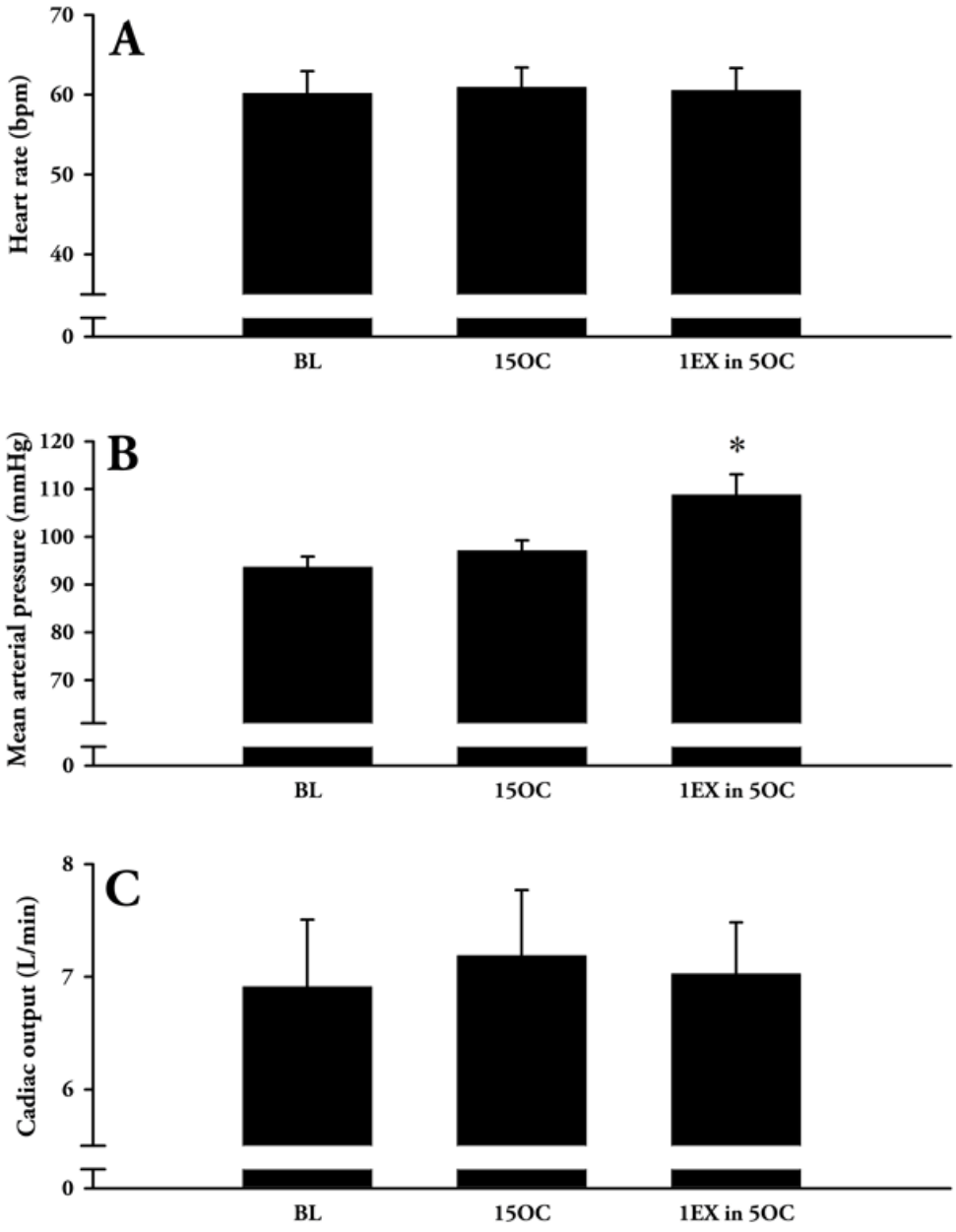


Figure 3.7 Mean arterial pressure at time of cuff release was altered in with EX in OC.

There was no affect of 15OC or 1EXin5OC on heart rate at the time of cuff release (A). Exercise in occlusion caused blood pressure to significantly increase at the end of occlusion (B). There was no affect of 15OC or 1EXin5OC on cardiac output (C). Means ±SE, * significantly different from baseline. $P<0.05$.

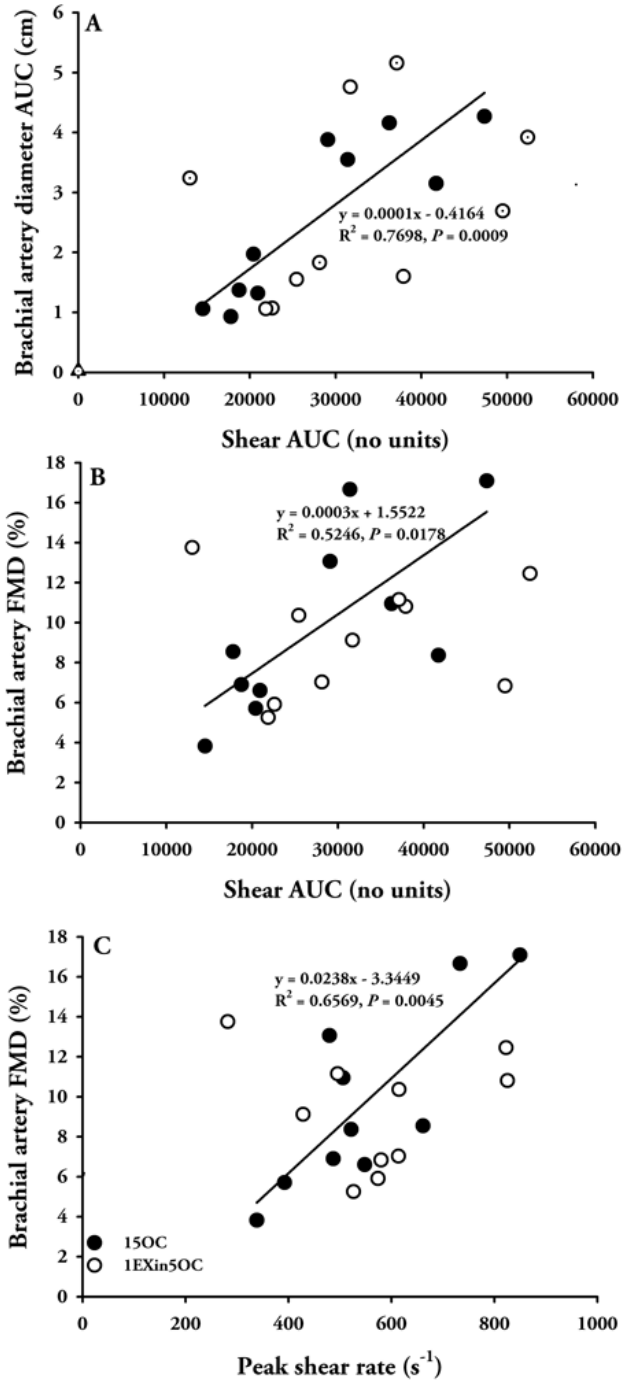


Figure 3.8 Relationships between shear and dilation.

A. AUCshear was correlated with AUCdiameter in 15OC, but not in 1EXin5OC (R2=0.1369, P=0.2927). B. AUCshear was correlated with %FMD in 15OC, but not in 1EXin5OC (R2=0.0083, P=0.8018). C. Peak shear was correlated with %FMD in 15OC, but not in 1EXin5OC (R2=0.0008, P=0.9373). Regression lines are for 15OC.

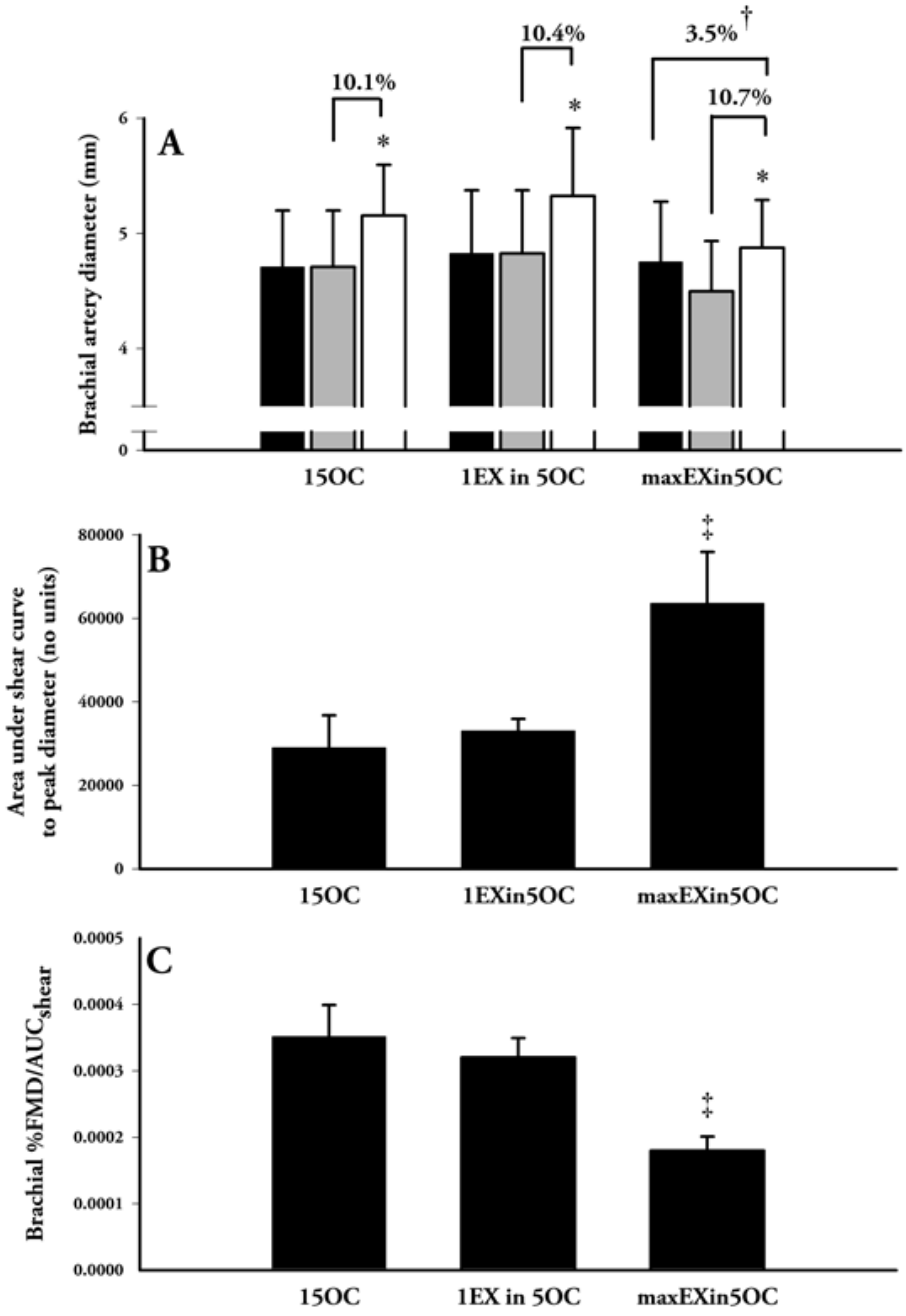


Figure 3.9 Diameter change, AUCshear, and FMD/AUCshear for the 4 subjects that performed all 3 occlusion protocols.

Percent FMD was not different in the three protocols (A), however baseline diameter at end occlusion was significantly smaller in maxEXin5OC. maxEXin5OC had a significantly greater AUCshear (B) and when %FMD was normalized for AUCshear it was significantly less than the other 2 protocols in maxEX5OC. n=4, Means ±SE, * significantly different from baseline, † significantly different FMD, ‡ significantly different from other conditions. $P<0.05$.

Discussion

We tested two protocols, 15 minute occlusion only reactive hyperaemia (15OC) and occlusion with exercise hyperaemia (1EXin5OC), in their ability to elicit FMD of the brachial artery under similar shear stimuli. Max exercise during occlusion (maxEXin5OC) was tested in 4 subjects to determine if increasing the shear response would result in greater FMD. The primary finding was that with the shear stimulus controlled for, occlusion and occlusion with exercise resulted in similar post cuff release FMD. That this was also true with the much higher shear following maxEXin5OC may indicate that the 15OC protocol reached a hypothetical %FMD ceiling that is independent of absolute peak or baseline diameter. Interestingly, the relationship between shear and FMD was lost when exercise was added to occlusion, suggesting that exercise may play a role in altering the mechanisms by which FMD is achieved. We also found that plasma nitrite in the forearm venous effluent was elevated in the occlusion only protocol, but not the 1EXin5OC (not measured in maxEXin5OC), supporting the $\dot{N}0$ dependence in the former.

Effects of Exercise on Baseline Diameter

We found that moderate exercise during the period of occlusion did not alter the baseline diameter of the brachial artery during the occlusion period. Others have reported constriction during occlusion following isometric exercise (Pyke & Tschakovsky, 2007) and no changes during a similar dynamic exercise to that employed in this study (Betik et al., 2004). Heavy exercise to exhaustion during occlusion caused a significant arterial constriction which could have been a result of local chemoreceptor activation in the vessels supplying the active muscle

(Rowell, 1997). This is supported by the observations that both blood pressure, and cardiac output were greatly affected by this protocol (Figure 3.6). A previous study in our laboratory has shown that increased chemoreceptor mediated sympathetic nerve activity is capable of causing vasoconstriction in a conduit artery (Dyson et al., 2006).

Shear and Dilation

As shear is the stimulus responsible for FMD it has been hypothesized that a greater shear stimulus will result in a greater FMD. In order to provide a greater hyperaemia, and therefore shear stimulus, following cuff release exercise during the occlusion period has been proposed. It has been reported that exercise during a 5 min occlusion period results in a greater magnitude of FMD than 5 min of occlusion alone (Betik et al., 2004; Agewall et al., 1999; Wendelhag, Fagerberg, & Wikstrand, 1999). Duration of occlusion can also be manipulated to change the hyperaemia at cuff release. Both 10 (Harris et al., 2009) and 15 (Mullen et al., 2001) min of occlusion have been shown to also increase post ischemic hyperaemia and FMD. There is a considerable amount of controversy however in the mechanisms for the increased FMD. Mullen et al. (2001) reported that the FMD following 15 min of occlusion was not $\dot{N}0$ mediated, from the finding that FMD was not reduced during infusion of L-NMMA while it was reduced after 5 min of occlusion. They attribute the discrepancy in mechanism to the sustained nature of the hyperaemia stimulus following 15 min of occlusion compared to relatively quicker decay of the shear curve in shorter durations. It has been shown that FMD following exercise in occlusion is $\dot{N}0$ mediated (Agewall et al., 2002). Shear following the maxEXin5OC protocol was significantly higher than the others, yet

absolute dilation was less, indicating perhaps that other, ṄO-independent, mechanisms are not responsive to shear above a given threshold, or that maximum flow-mediated dilation was achieved with all three protocols. When shear was the only factor, no exercise, there was a linear relationship between AUC_{shear} and AUC_{diam} and %FMD, and peak shear and %FMD. The finding that there was an uncoupling between shear and dilation in the exercise protocols suggests that an FMD test using exercise with occlusion does not investigate the same mechanism(s) as the pure reactive hyperaemia test.

Plasma Nitrite

The finding that plasma nitrite increased following 15OC, but not following 1EXin5OC, suggests that FMD protocols that include exercise involve dilatory mechanisms that are not dependent on endothelial release of ṄO. Although it was not possible to directly measure ṄO release in the conduit artery at the site of shear stimulation, it may be possible to make indirect measurements from the plasma nitrite concentrations in the effluent venous circulation. The plasma nitrite pool is under rather strict regulative control as is evidenced by the many pathways of ingestion (Lundberg & Govoni, 2004) and elimination (Doyle, Herman, & Dykstra, 1985). These processes are by far slower than the oxidation of ṄO to nitrite, and it has been shown that 70-90% of circulating plasma nitrite is derived by eNOS activity (Naghavi et al., 2003; Kleinbongard et al., 2006). Serum nitrite was found to reliably reflect changes in endothelial ṄO formation in human forearm after Ach infusion induced endothelium dependent dilation (Kelm, Preik-Steinhoff, Preik, & Strauer, 1999). As well, Rassaf et al. (2006), using a triiodide/ozone-based chemiluminescence assay, found that plasma nitrite reserve mirrored FMD

in healthy subjects, but not in those with cardiovascular disease risk factors. However, in that study nitrite concentration peaked at 50 s post occlusion while in the present study the peak was observed at 15 s. The cause for the discrepancy between the results of this study and those of Rassaf et al. (2006) are unknown, but could be related to differences in the nitrite detection techniques.

Conclusion

The results of this study show that all of these conditions, while producing similar values for FMD, may not in fact be reflective of the same dilatory mechanisms. It is possible, owing to the findings that shear was greater in 1EXin5OC and that nitrite reserve was increased only after 15OC, that the exercise present in 2 of the protocols called upon other mechanisms which could compensate for reduced endothelial release of $\dot{N}O$.

Chapter 4 - **DILATION OF THE HUMAN BRACHIAL ARTERY IN RESPONSE TO EXERCISE AND INTERMITTENT RELEASE OF FOREARM OCCLUSION**

Summary

This study examined the dilation of the human brachial artery in response to intermittent high shear following 15min of forearm occlusion (IO) and 5min of heavy hand-grip exercise (5EX), and a sustained laminar shear stimulus during reactive hyperaemia following 15 min of occlusion (15OC). Ten healthy young men visited the lab on three separate occasions: 1) 5EX, 5 minutes of hand-grip exercise 2) IO, 15 minutes of occlusion followed by 2 minutes of release/occlusion in a two second cycle, mimicking forearm muscular contraction, followed by another 2 minutes of occlusion and 3) 15OC, 15 minutes of occlusion followed by 3 minutes of reactive hyperaemia. The greatest brachial artery dilation was exhibited during EX, and there was no difference in the dilation following occlusion whether the hyperaemia was constant or intermittent (5EX: 13.4±6.7 vs. IO: 9.3±4.7 and 15OC: 9.8±1.4 %dilation). Total shear to the time of peak diameter (AUC_{shear}) and peak shear were both correlated to %dilation following 15OC, however this relationship was lost during 5EX and IO. The results of this study suggest that dilation in response to interrupted shear stimuli may not evoke the endothelial regulated $\dot{N}0$ FMD response that is typical of post occlusion hyperaemia.

Introduction

It is well established that increasing flow in a peripheral conduit artery results in a subsequent vasodilatation. This phenomenon, termed flow-mediated dilation (FMD), is mainly nitric oxide (Ṅ0) dependent (Joannides et al., 1995) and is initiated by the increase in shear stress at the blood-endothelium interface (Pohl et al., 1986). There are a number of ways to increase flow, and therefore shear stress, in a conduit artery. Methods include limb heating (Pyke, Hartnett, & Tschakovsky, 2008; Bellien et al., 2004), occlusion/reactive hyperaemia with or without exercise (Agewall et al., 1999; Corretti et al., 2002; Betik et al., 2004), and exercise alone (Padilla et al., 2007). Each of these tests have a distinct shear stress profile, with limb heating providing a gradual increase, reactive hyperaemia providing a rapid onset with gradual return to baseline (more gradual when exercise is added during the occlusion period), and exercise providing a rapid increase in shear which is sustained throughout the exercise period. However, the shear profile for exercise fluctuates during the course of exercise as muscle contraction compresses the artery, thus preventing forward flow periodically (Leyk, Essfeld, Hoffmann, Baum, & Stegemann, 1992). Another possible confounding factor for exercise is that it has a profound effect on metabolism and therefore may set in motion competing or parallel vasoactive mechanisms (Boushel, 2003; Saunders, Dinenno, Pyke, Rogers, & Tschakovsky, 2005; Tschakovsky et al., 2004). This effect would likely be present whether exercise alone or within a period of occlusion. It is known that strenuous exercise will engage the sympathetic nervous system, mainly through the chemosensitive α-adrenergic nerve fibres at the working muscle, thus increasing mean arterial pressure (MAP), heart rate (HR), and increasing vascular resistance. However, sympathetic nervous activity has been shown to have some

effects on $\dot{V}O_2$ metabolism (Engelke, Williams, Dietz, & Joyner, 1997) and FMD (Dyson et al., 2006). Of these methods heating has the least affect on metabolism, but the onset of hyperaemia is slow and therefore has been shown to produce only a small, ~2%, change in diameter (Bellien et al., 2004) which is not a large enough magnitude of FMD to be used as a clinical marker of endothelial function given the error inherent in Doppler imaging measurements.

This study was designed to test the hypothesis that oscillating the post occlusion shear stress will attenuate dilation compared to unidirectional, or continuous shear, and that dilation during exercise would be greater than the manually interrupted shear.

Methods

Ten healthy male varsity hockey players were recruited for inclusion in the study. The participants visited the lab on three separate occasions for brachial artery vasoactivity assessment: 1) EX, 5 minutes of hand-grip exercise 2) IO, 15 minutes of occlusion followed by 2 minutes of release/occlusion in a two second cycle, mimicking forearm muscular contraction, followed by another 2 minutes of occlusion and 3) 15OC, 15 minutes of occlusion followed by 3 minutes of reactive hyperaemia (Figure 4.1). The protocols were assigned in random order and the sessions were separated by at least 48 hours. Testing was performed at least 24 h following the participants' last exercise regimen and 72 h following an intense strength training session. The participants arrived to the laboratory in a rested state at least 2 h after eating and they were asked to abstain from caffeine 12 h and alcohol 24 h before testing. The participants were rested in supine position for 30 min before initiation of the protocols. The tests were completed in a quiet, air-conditioned laboratory at a temperature of ~ 22°C.

Circulatory Occlusion

Brachial artery blood flow was arrested by rapid inflation (E-20 Rapid Cuff Inflator, D. E. Hokanson, Issaquah, USA) of a standard blood pressure cuff to 250 mmHg. The occlusion cuff was placed just distal to the dominant elbow. Reactive hyperaemia was initiated by rapid deflation of the occlusion cuff. For the intermittent occlusion protocol the cuff was alternately inflated and deflated in a 2s cycle kept in time with a metronome.

Hand-Grip Exercise

Rhythmic hand-grip exercise was performed in the supine position using the dominant arm by lifting and lowering a weight a distance of 5 cm with a handgrip device. A metronome was used to ensure that the duty cycle was consistent at 0.5s contraction, 0.5s relaxation, and 1s pause.

Physiological Data Collection

Heart rate by 3 lead electrocardiogram (Pilot 9200, Colin Medical Instruments, San Antonio, TX), mean arterial blood pressure (MAP) estimated by finger photoplethysmography (Finometer, FMS, The Netherlands), cardiac output (CO) estimated by ModelFlow (Finometer), and brachial artery mean blood velocity (MBV) measured by pulsed wave Doppler ultrasound (MICROMAXX, Sonosite, USA) were collected and sampled at 1kHz using a data-acquisition (PowerLab, AD Instruments, Colorado Springs, CO) system running on a lap-top PC.

A pulsed Doppler 4-MHz probe was fastened above the brachial artery proximal to the cubital fossa with strips of surgical tape. The signal was directed 45° relative to the skin and the ultrasound gate was adjusted to encompass the total width of the artery.

Brachial artery diameter was monitored on the arm proximal to the inflation cuff by M-Mode ultrasound imaging and stored on digital video tape. The tapes were subsequently digitized to AVI video (KINO video editor). Vessel diameters were measured by custom edge-detection software (Jorges Serrador and Brian Deegan) from still images extracted (KINO video editor) at every 5s of video. An average of the diameter across the M-mode image frame (2-3 cardiac cycles) was taken from the best image in each 15s window to represent mean arterial diameter. The

measurements from the 15s windows were curve fit by cubic spline interpolation and Lenenberg-Marquardt peak fitting (Fityk). BL diameter was calculated as the average diameter from the interpolation for the 1 min before EX or OC; peak diameter was the maximal diameter reached by the interpolation for each stage of the protocol. Percent change in diameter (%EMD, for EX, %FMD for Post OC or IO) was calculated as the percent change from end occlusion to peak for %FMD, and from BL to peak for %EMD and %FMDbl.

Brachial artery MBV and cross-sectional area ($A = \pi r^2$) were used to calculate forearm blood flow ($Q = vmean \bullet A\, min^{-1}$) and shear rate ($\gamma = 8 \times v_{mean} \times D^{-1}$). The MBV signal was filtered using 5 Hz low pass filter. MBV was averaged beat to beat at rest and post occlusion, over the contraction to contraction cycle during exercise, and over the inflation to inflation cycle during intermittent occlusion (Figure 4.2). For exercise and intermittent occlusion MBV was also separated into its positive and negative components. The MBV values were then interpolated to second to second by the non-rounded Akima method and matched with the interpolated diameter data. Baseline forearm blood flow, expressed as ml/100ml/min, and shear rate, expressed as s^{-1}, were determined from averages over the last min before cuff inflation. Peak shear rate was determined from the peak value on the interpolated shear curve. Area under the shear (AUC_{shear}) and diameter (AUC_{diam}) curves were calculated by approximating the definite integral by use of the trapezoidal rule on the interpolated values from the time of occlusion cuff release to the time of peak diameter (QtiPlot).

Statistical analysis

Statistical analysis software was used to examine the differences in shear rate,

blood flow, FMD, and blood factors for each protocol by repeated measures analysis of variance by the mixed procedure (SAS) with one within subject factor. For differences within a protocol a repeated measures one way ANOVA was performed. A probability of $P<0.05$ was chosen to be accepted as statistically significant and any differences were determined by contrast adjustment.

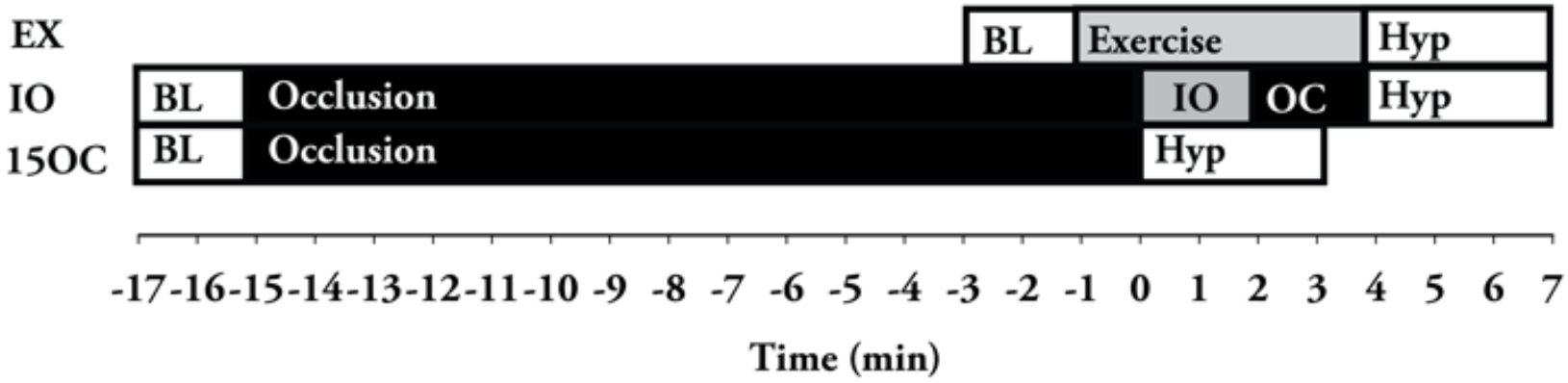


Figure 4.1 Exercise and occlusion protocols.

EX: 2 min of baseline (BL), 5 min of heavy hand grip exercise, and 3 min of post exercise hyperaemia (Hyp). IO: 3 minutes of BL, 15 minutes of forearm occlusion (OC), 2 minutes of intermittent occlusion (IO), 2 minutes of OC, and 3 minutes of reactive Hyp. 15OC: 2 min of BL, 15 min of OC, and 3 min of reactive Hyp.

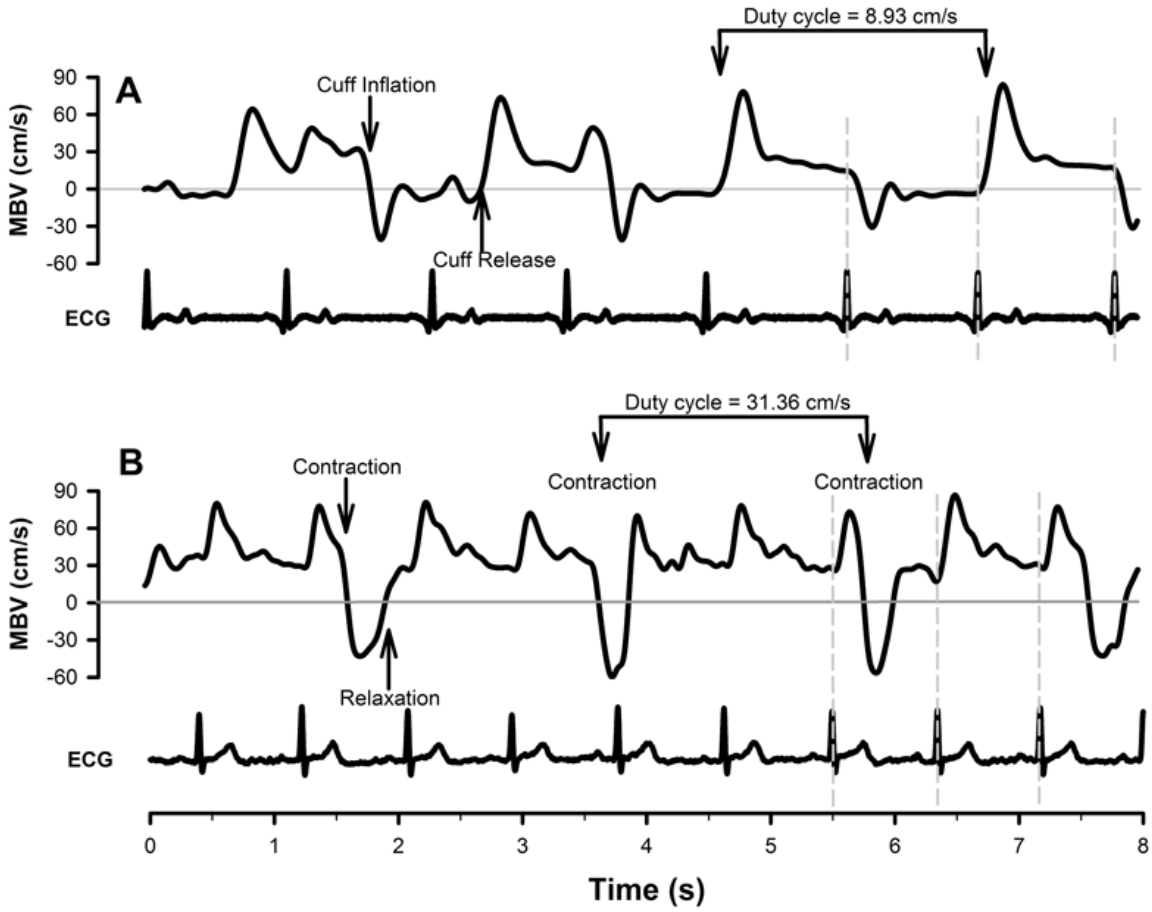


Figure 4.2 Representative tracings of MBV and ECG.

Representative velocity and ECG traces from one subject during IO (A) and 5EX (B) protocols. Both trials illustrate oscillations in the velocity response. IO had a 2 sec period of high (positive) shear, during cuff release, alternating with a 1 sec period of low (negative) shear, during cuff inflation. During 5EX there was 2 sec of high (positive) shear, during muscle relaxation, alternating with 0.5 sec periods of low (negative) shear, during muscle contraction. The MBV (cm/s) during oscillations were averaged over the duty cycle, while during rest and reactive hyperaemia it was averaged beat to beat on cue with the ECG signal.

Results

Maximal voluntary isometric contraction (MVC) strength was 54±6.2 kg during handgrip exercise as determined from the best of three attempts taken in supine position. The workload during the forearm exercise bouts was 13.7± 0.7 W, respectively. There were no significant differences in any testing variable at BL between testing days.

Brachial Artery Dilation

The greatest brachial artery dilation was seen during EX (EX: 13.4±6.7 vs. IO: 9.3±4.7 and 15OC: 9.8±4.6 %dilation, $P<0.05$, Figure 4.3 and Figure 4.5). Diameter did not increase following the release of 2 min occlusion after IO, even though the shear rate was significantly increased to a level similar to that following 15OC (Figure 4.3). The time to peak diameter was significantly different between all the protocols (Table 4.2, Figure 4.3). This longer time to peak dilation translated to a greater AUC_{shear} during EX (EX: 59541.38±24818.30 vs. IO: 23139.03±12491.45 and 15OC: 27823.86±11170.87, $P<0.05$, Figure 4.6).

Directional Shear

Figure 4.6 illustrates the peak shear for each of the stages of each protocol in both the positive and negative directions, and averaged over the cycle. Peak shear in the positive direction was greatest following during IO (IO: 799.2±230.7 vs. Post IO: 582.1±231.4, 15OC: 551.8±155.3, EX: 675.3±92.8, and PostEX: 570.6±125.8 s^{-1}, $P<0.05$). It must be noted that the peak shear for IO was achieved during only the first few cuff deflations, while in EX the shear increased throughout the exercise to about the 3rd min mark. Both the EX and the IO protocols resulted in shear in the

retrograde (negative) direction to go along with increases in positive shear. Peak negative shear was significantly greater during IO (EX: -140.3±95.7 vs. IO: -281.7±152.9 s^{-1}, P<0.05). EX having the significantly highest AUC_{shear} (EX: 59541.3±24818.3 vs. IO: 23139.0±12491.5 and15OC: 27823.86±11170.87, no units, *P*<0.05, Figure 4.6). This greater AUC_{shear} translate to similar differences in AUC_{diam} between the protocols (EX: 7.41±3.41 vs. IO: 3.16±2.15 and 15OC: 2.56±1.36 vs., cm, *P*<0.05, Figure 4.6)

Relationship Between Shear and Dilation

Figure 4.7 illustrates the relationships between shear and dilation for all the protocols. AUC_{shear} (R^2=0.5246) and peak shear (R^2=0.6569) were correlated to %FMD only in 15OC, over all or within any conditions. IO had significantly greater %dilation when normalized for the AUC_{shear} (IO: 0.00053±0.00046 vs. EX: 0.00026±0.00018 and Post 15OC: 0.00036±0.00010, p<0.05, Figure 4.9). The mean shear during exercise was not correlated to the percent dilation of the brachial artery (R^2=0.0005, Figure 4.8).

Heart Rate, Blood Pressure, and Cardiac Output

Table 4.1 outlines the systemic cardiovascular effects of the various stages of the 3 protocols. Heart rate, MAP, and CO all increased significantly from BL during 5EX. These indices all returned to BL values by 60s post EX.

Table 4.1 Heart rate, mean arterial pressure, cardiac output, and forearm blood flow during the different stages of the 3 experimental protocols.

		Heart rate (bpm)	Mean arterial pressure (mmHg)	Cardiac output (l/min)	Forearm blood flow (ml/min)
EX	BL	63±6	92.8±10.3	7.7±1.8	57.4±16.6
	End EX	75±9*	115±14.2*	9.4±1.6*	404.5±104.9*†
	PostEX	63±7	97.4±14.5	7.7±1.5	386.4±62.2*
15OC+IO	BL	58±11	87.0±5.4	6.5±1.0	58.9±15.4
	IO	60±9	92.8±5.7	7.0±1.1	326.5±81.3*
	OC	58±8	93.2±5.6	6.8±1.0	--
	PostOC	58±9	93.0±4.3	6.8±1.2	445.7±159.6*†
15OC	BL	60±9	93.7±7.0	6.9±1.9	59.1±14.5
	OC	61±8	97.1±6.9	7.2±1.8	--
	PostOC	59±8	96.8±5.3	7.2±1.7	447.6±114.0*†

BL: mean of values in the 60 seconds prior to protocol. End EX: mean of values during the last 60 seconds of exercise. PostEX: mean of values during the first 60 seconds after end exercise. OC: mean of values during the last minute of occlusion. IO: mean of values during the first minute of IO. Data are means ± standard deviation, n=10. *significantly different from BL †significantly highest within protocol, $P<0.05$.

Table 4.2 Change and time to peak diameter during exercise and IO, and after release of 15 min occlusion.

	Diameter change (mm)	Time to peak (sec)	$\frac{\Delta d}{\Delta t}$
EX	0.65± 0.33†	196.3±54.5*†	0.004±0.002†
IO	0.48±0.28	98.6±23.7	0.005±0.003
15OC	0.48±0.2	79.4±28.3*	0.007±0.003*

Values are means±standard deviation, *significantly different from IO †significantly different from 15OC, $P<0.05$

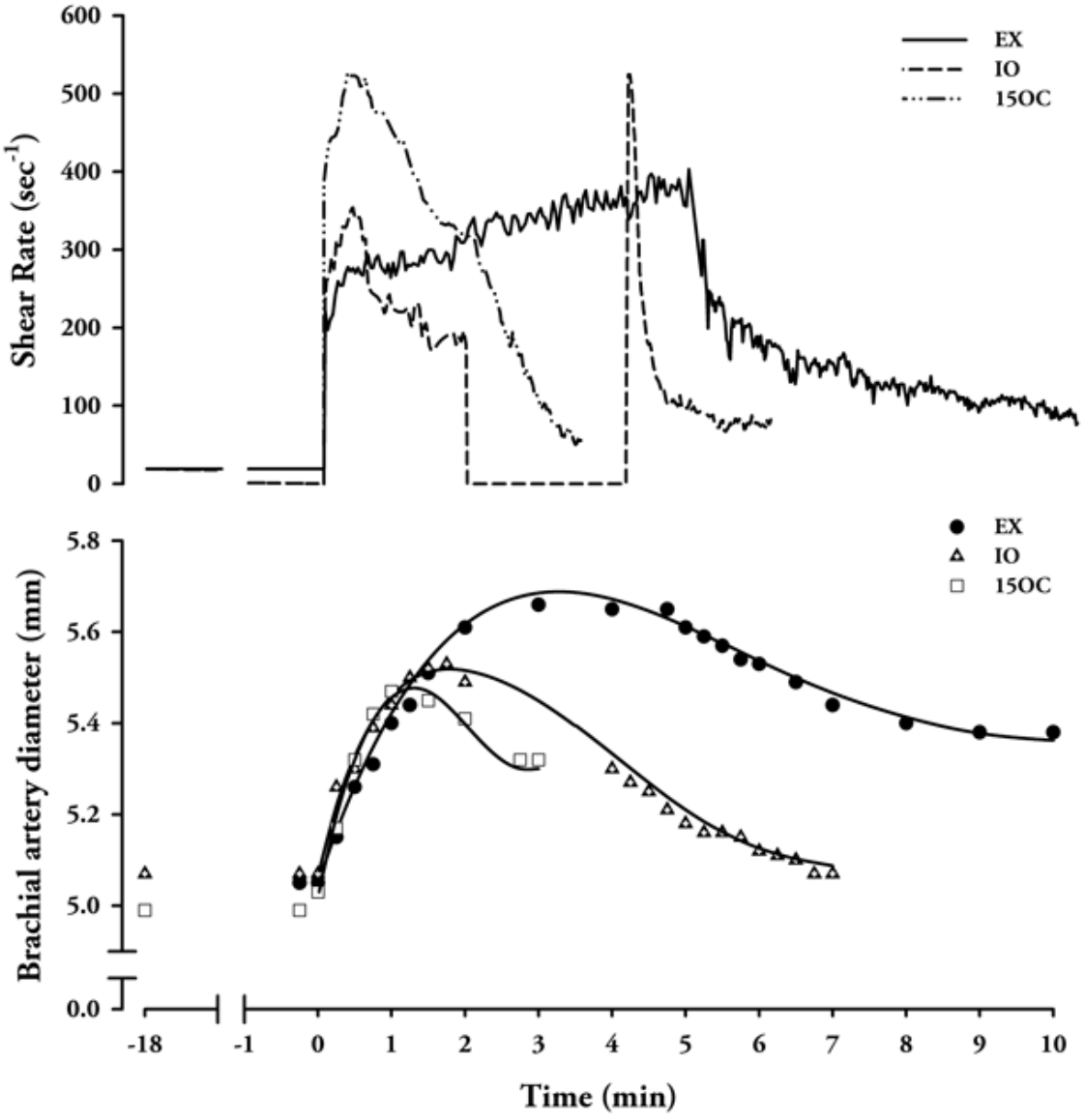
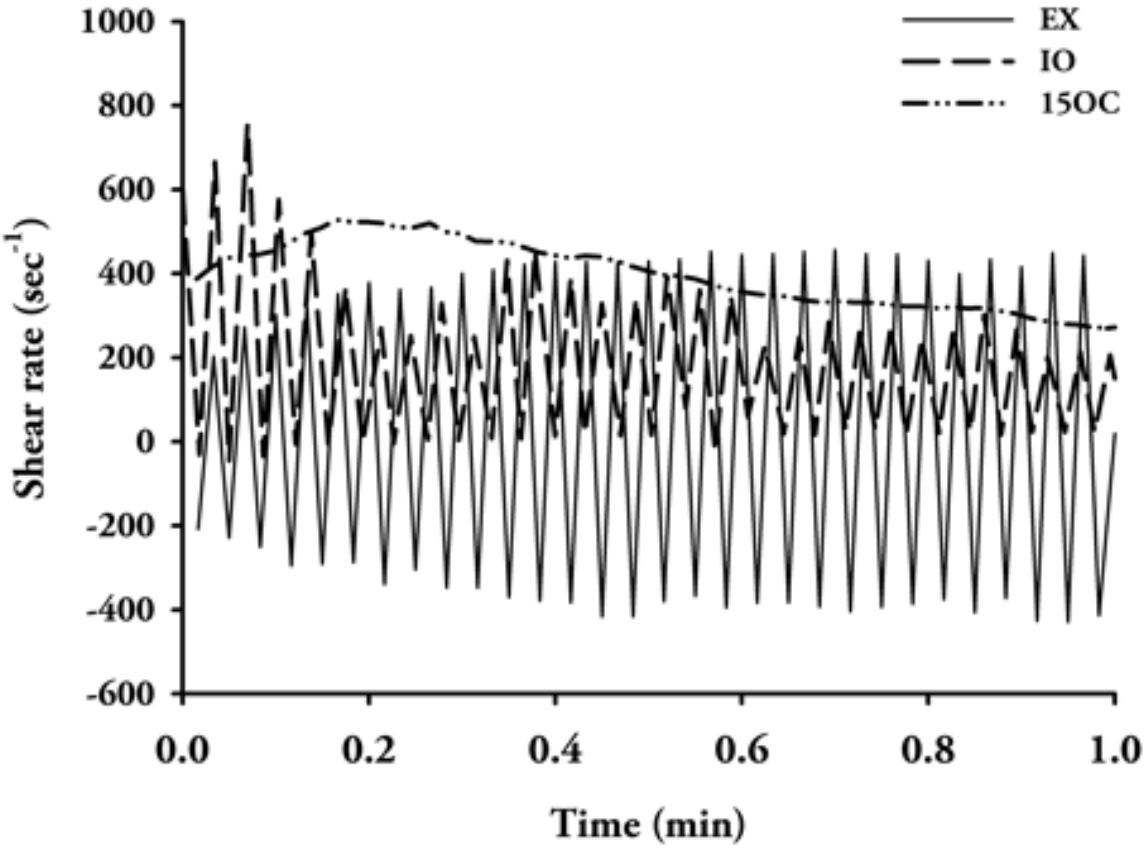


Figure 4.3 Brachial artery shear rate and dilation in response to the three different protocols.

A. Lines represent the mean shear rate of 10 subjects averaged over each contraction-relaxation cycle for EX, each inflation-deflation cycle for IO, and each cardiac cycle for 15OC. B. Symbols represent the measured diameter averaged over 10 subjects; lines represent the curve fit of each individual subject averaged over 10 subjects.



4.4 Brachial artery shear rate during the first minute of exercise, intermittent cuff release, and reactive hyperaemia.

Lines represent the mean shear rate of 10 subjects during contraction and relaxation phases for exercise (EX, solid line), inflation and deflation phases for intermittent occlusion (IO, dashed line), and over each cardiac cycle for reactive hyperaemia following 15 minutes of occlusion (15OC, dash-double dot line). IO achieved the highest shear rate, peaking at the 3rd cuff release. EX achieved consistent shear oscillations, with retrograde shear during the contraction phase. 15OC resulted in high shear upon cuff release, peaking at ~10 to 15 sec.

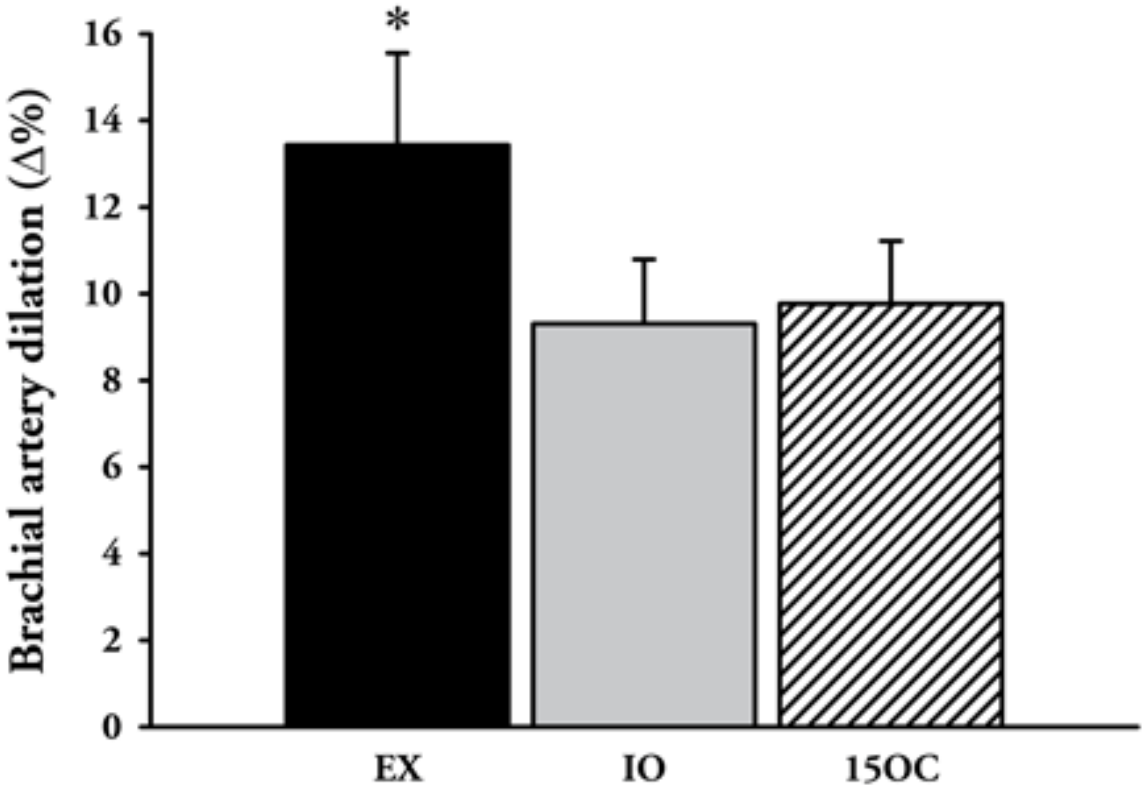


Figure 4.5 Percent dilation for the three dilation protocols.

Dilation during exercise (EX) was significantly greater than that during intermittent occlusion (IO) and post occlusion FMD (15OC). Means ±SE, *significantly different from IO and 15OC. $P<0.05$.

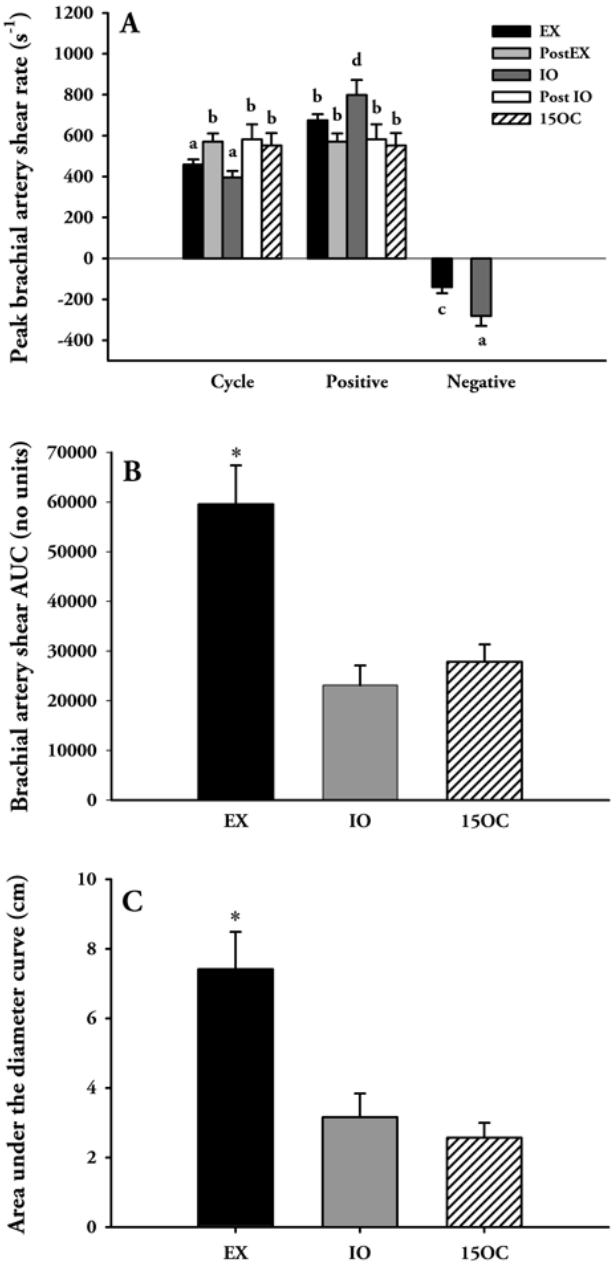


Figure 4.6 Peak and AUC_{shear} and $AUC_{diameter}$ during the three protocols.

The highest peak shear was achieved during intermittent occlusion (IO). Cuff oscillations caused a greater retrograde (negative) shear then that of muscle contraction (A). The highest total shear was achieved during exercise, average over the duty cycle (B). The greatest AUC diameter was seen during EX (C). Means ±SE, *significantly different from IO and 15OC , means with the same letter are not significantly different from each other. $P<0.05$.

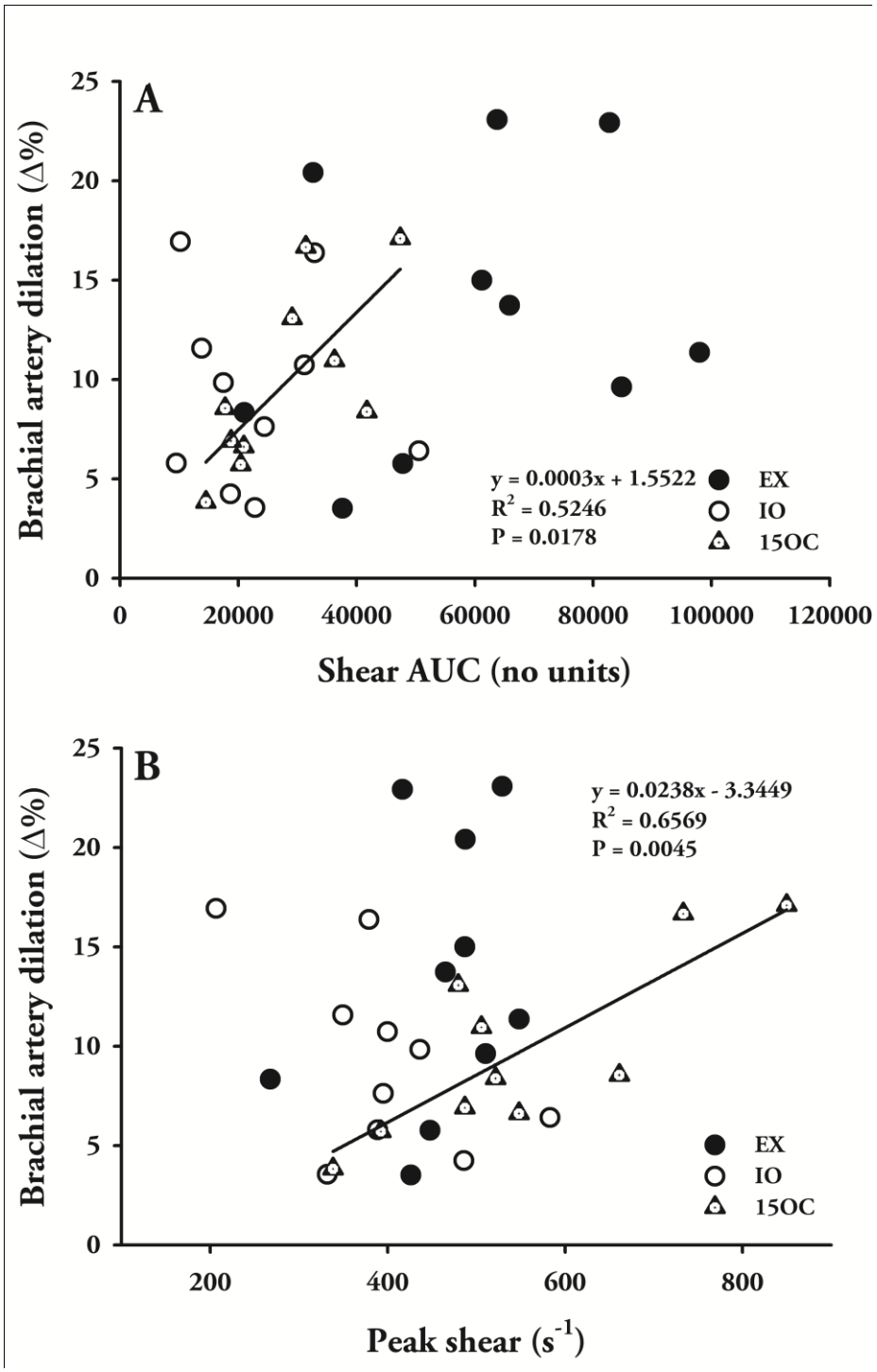


Figure 4.7 Relationships between shear and dilation.

Brachial artery dilation was correlated to shear AUC in 15OC but not during either EX or IO (A). Brachial artery dilation was correlated to peak shear in 15OC but not during either EX or IO (B). Regression line is for 15OC.

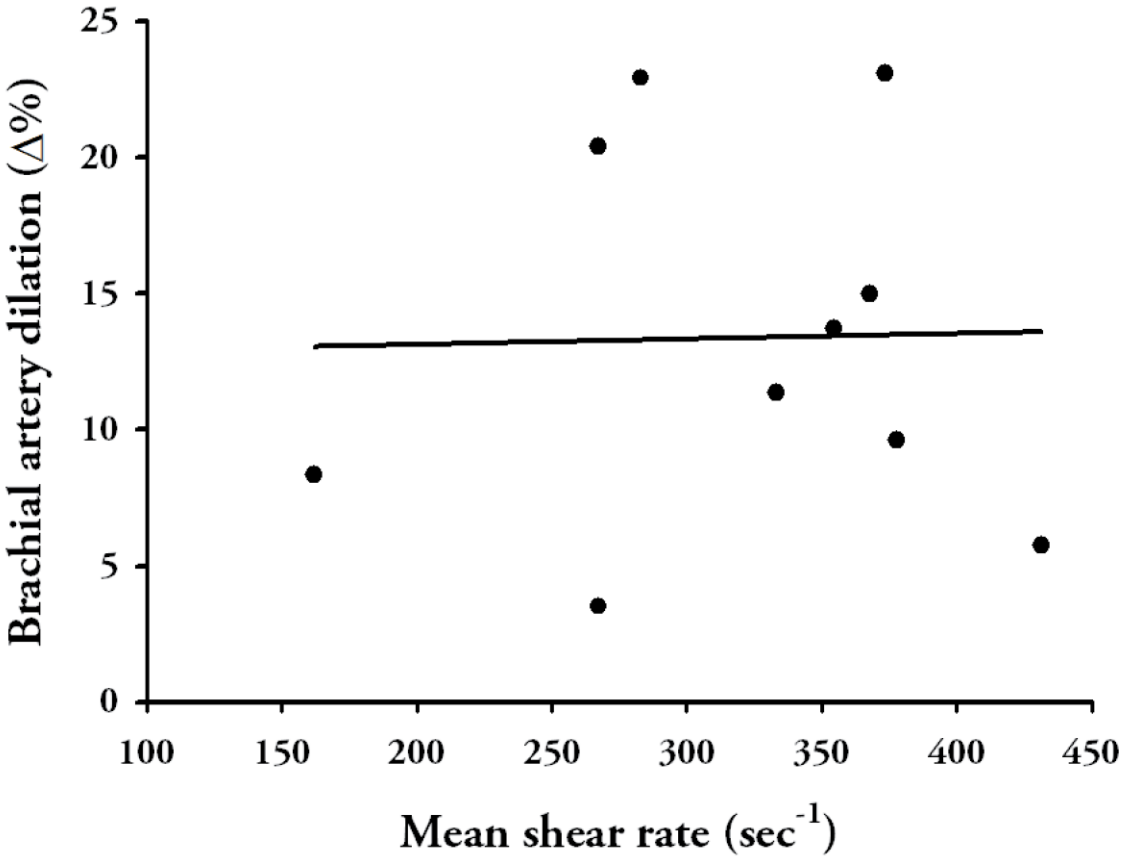


Figure 4.8 Relationship between mean shear during exercise and dilation of the brachial artery.

There was no correlation between the mean shear rate during exercise and the % dilation of the brachial artery.

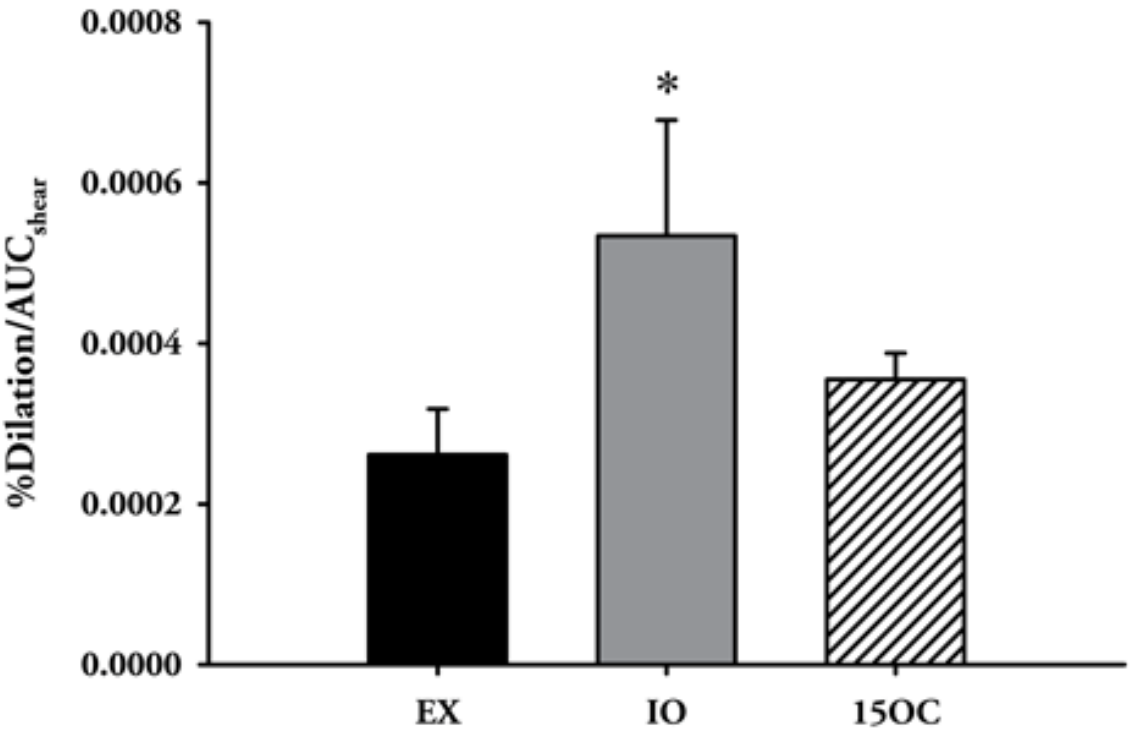


Figure 4.9 Brachial artery dilation normalized for total shear to peak diameter.

IO had a higher %FMD to AUCshear ratio than the dilation during EX or after 15OC. Means ±SE, * significantly different from EX and 15OC, $P<0.05$.

Discussion

The primary finding of this study is that, contrary to our hypothesis, oscillating the shear stimulus following cuff release (IO) did not attenuate FMD when compared to cuff release alone (15OC), and was actually shown to be enhanced when normalized to AUC_{shear}. In contrast, exercise (EX), another condition in which shear is oscillated, resulted in the greatest dilation, but when normalised to AUC_{shear} was comparable to the FMD from 15OC. Interestingly, shear rate, both peak and AUC_{shear}, were only correlated to % dilation in the 15OC protocol. These results suggest that, during exercise, and other conditions in which shear is oscillated, there is an uncoupling between the shear to dilation relationship.

We hypothesised that oscillating the shear during reactive hyperaemia would attenuate the dilation response based on reports that areas of turbulent blood flow are more susceptible to endothelial damage in the form of structural lesions (Prado, Ramos, Elias, Jr., & Rossi, 2008; Cunningham & Gotlieb, 2005). Also, it has been reported that a prior intervention of retrograde shear by inflation of a cuff at 25, 50, or 75 mmHg for 30 min resulted in attenuation of FMD in a pre – post test (Thijssen et al., 2009). We found that oscillating the shear response following15 min of occlusion did not have any effect on the acute endothelial response to a high shear stimulus.

The diameter increase was slowed during exercise, but not when the oscillations were passively applied. This would indicate that the slower dilation during exercise, in contrast to during reactive hyperaemia, was not due to the intermittent nature of the shear stimulus. It has been previously shown that longer time to peak dilation is associated with reduced endothelial function (Irace, Tschakovsky, Carallo, Cortese, & Gnasso, 2008; Black et al., 2008). However, it

may also be indicative of competing vaso-active mechanisms at the start of exercise, such as sympathetic vasoconstriction (Engelke et al., 1997).

In an attempt to assess endothelial function separately from the hyperaemic response there is a need to normalize the FMD to the shear stimulus. More and more investigators are advocating the use of AUC_{shear} to peak dilation as the normalizing factor (Harris et al., 2008; Pyke, Dwyer, & Tschakovsky, 2004; Thijssen et al., 2009). However, peak shear post cuff release has also been proposed as suitable normalization factor (Mitchell et al., 2004; Parker, Ridout, & Proctor, 2006; Wray et al., 2006). There is still considerable disagreement as to which factor contributes more to dilation with Pyke & Tschakovsky (2007) reporting that 56% of the FMD response was due to AUC_{shear} while Mitchell et al. (2004) report only 15% of the response as coming from AUC_{shear}. In the present study AUC_{shear} and peak shear followed similar trends over the conditions and thus we found it justifiable to normalize the dilation response to AUC_{shear}. Considering that the peak shear and AUC_{shear} may not be a proper representation of the dominating stimulus during exercise induced conduit artery dilation, the mean shear rate during exercise was also examined. Again, as was determined when using peak or AUC_{shear}, there was no correlation between the mean shear during exercise and the percent dilation.

The normalized results should, however, be considered with a note of caution. The results of the shear to dilation correlations indicate that the relationship between shear and dilation in the oscillating shear conditions are not as well defined as in the unidirectional reactive hyperaemia condition (Figure 4.7). A different shear stress to FMD ratio has been reported in subjects at moderate CVD risk compared to low risk (Padilla et al., 2009). That study was able to

distinguish differences in endothelial function between the groups only if FMD was normalised to AUC_{shear}. This may not be the case in the present study in which it appears that the relationship between shear and dilation is not present in oscillating shear conditions. If AUC_{shear} or peak shear are not determining factors for the peak dilation the question remains as to what is. Endothelial cells align themselves in the direction of flow (Luscher & Corti, 2004) demonstrating that they have the ability to sense oscillations in shear as well as magnitude of shear. There may be something intrinsic to oscillations, such as frequency, that triggers vasodilatory mechanisms, and these should be the focus of future studies.

Methodological Considerations

The results of this study are contrary to those of Pyke, Poitras, & Tschakovsky (2008), in which they found no differences in %FMD in experiments designed to compare passive forearm heating, heating with intermittent cuff pulses, and exercise under similar mean shear stimuli. Indeed, it has been previously reported that dilation during sustained periods of shear, with both gradual and stepwise increases in shear, by passive limb heating may not be primarily Ṅ0 mediated (Mullen et al., 2001). They demonstrated that the infusion of L-NMMA had no effect on the diameter increase in the radial artery during hand warming. However, the dilation was shown to be flow-dependent, as hand warming while the artery was occluded resulted in no dilation. Bellien et al. (2006) argues against the conclusions of Mullen et al. (2001), when they found that Ṅ0 blockade reduced the FMD from limb heating by 40%, while combined with endothelium derived hyperpolarizing factor (EDHF) blockade FMD was reduced by 70%. Pyke et al. (2008) suggest that EDHF may have compensated for the lack of available Ṅ0 in

the Mullen et al. (2001) study, similar to findings in skeletal muscle arterioles of eNOS knockout mice (Huang et al., 2001)and human coronary arterioles (Miura et al., 2001); however there are marked differences in the make-up of the endothelium in conduit arteries vs. arterioles and the mechanisms behind heat induced dilation still need to be determined. A draw back to the present study is that it did not employ methods to physically normalize the mean shear responses across all the protocols, and as such had to rely on post hoc mathematical normalization in the form of the AUC_{shear} to %FMD ratio. In this respect, the findings that IO had a greater %FMD relative to the shear stimulus than 15OC, and that EX had similar %dilation to 15OC relative to shear, may not be as significant as if the %FMD were determined at a controlled level of shear as was done by Pyke et al. (2008). The finding that the oscillating shear protocols did not show a clear relationship between shear and dilation is certainly troubling and could explain the discrepancies in the results from those of Pyke et al. (2008). The discrepancies between that study and the present one may also stem from differences in the methods used. The passive heating protocols used in the study by Pyke et al. (2008) achieved considerably lower peak shear rates than were shown in the present study. In particular, the cuff pulses in this study (IO) were preceded by a 15 min occlusion period which resulted in a rapid increase in shear (in the positive direction) for the first few cuff deflations, which were similar to the increases in peak shear derived from our heavy exercise model. It can be argued that an initial large burst of shear may trigger different dilatory mechanisms than those of a gradual onset. Although Pyke et al. (2008) employed an initial step increase in shear in order to bypass the slow rise in shear at the onset of the forearm heating protocol; the peak reached was still considerably lower than the

values derived from the protocol in this study. It should be noted however that it has been shown in Chapter 2 of this thesis that the first 20 sec burst of shear has a negligible impact on FMD, a finding that is supported by the lack of dilation in response to the burst of shear after the 2 min re-occlusion following IO. It should also be noted that the modes of exercise were also different, with isometric contractions being used in the Pyke et al. (2008) study while the present study used dynamic exercise. The isometric contractions achieved shear rates that were comparable to those achieved by the forearm heating protocol, in accordance with their goal of normalizing the mean shear rates across the protocols, and therefore were much lower than those achieved during the present study.

Conclusion

In conclusion, we found that oscillating the shear during reactive hyperaemia following 15 min of occlusion did not result in attenuated FMD compared to uninterrupted reactive hyperaemia. In contrast, the dilation was shown to be enhanced when normalized to AUC_{shear}. Also, active hyperaemia during a 5 min bout of heavy dynamic hand grip exercise resulted in a greater dilation response than that following 15 min of occlusion, whether the shear was oscillated or not. However, the exercise induced dilation was found to be similar to that following occlusion when normalized to AUC_{shear}. Finally, the relationship between shear and dilation seems to be lost during oscillating shear conditions.

Chapter 5 - **FLOW-MEDIATED DILATION OF THE HUMAN BRACHIAL ARTERY IS BLUNTED FOLLOWING BOUTS OF HEAVY FOREARM EXERCISE**

Summary

This study examined the dilation of the human brachial artery in response to reactive hyperaemia before and after 2 bouts of heavy hand-grip exercise. Nine healthy young men visited the lab on 2 separate occasions. Flow-mediated (FMD) and exercise-mediated (EMD) dilation of the brachial artery were assessed with 2 protocols: 1) following 15 minutes of forearm occlusion (CON) and 2) following 15 minutes of forearm occlusion (PostEX) initiated 6min after two bouts of heavy hand-grip exercise (EMD1 and EMD2). FMD was blunted following forearm exercise (CON: 10.0±4.8 vs. PostEX: 3.7±2.1 %FMD), even though the shear stimulus was greater during PostEX (CON, 29508.4±15146.4 vs. PostEX: 44992.6±15303.7, area under the curve to time of peak dilation (AUC_{shear}). Nitrite was significantly elevated in CON at 15s while PostEX nitrite was significantly elevated at 30s post cuff release but not different from CON at 15s. Oxygen uptake was higher throughout the first minute of hyperaemia period in CON compared to PostEX (0s; CON: 18.12±8.99 vs. PostEX: 28.74±18.40, 15s; CON: 42.52±23.4 vs. PostEX: 63.93±26.11, 60s; CON: 7.19±4.70 vs. PostEX: 19.45±10.70 ml/min). EMD was blunted during the second bout of heavy forearm exercise (EMD1: 14.5±6.4 vs. EMD2: 6.5±1.9 %EMD), even though AUC_{shear} was not different in that condition. There were significant correlations between peak shear and %FMD only in CON (CON: R^2=0.5259, P=0.097; PostEX: R^2=0.1072, P=0.396), but no significant correlations were found between shear and percent dilation in either of the exercise conditions. The results of this study suggest that prior exercise has a negative effect on %FMD which may be related to exercise blunting post occlusion endothelial $\dot{N}0$ production or to the effect of a change in baseline diameter at the onset of the FMD study.

Introduction

There is a preponderance of evidence suggesting that regular physical exercise is associated with lower risk of cardiovascular morbidity and mortality (Riopel et al., 1986; Henriksson, 1998; Petrella, Lattanzio, Demeray, Varallo, & Blore, 2005). Dysfunction of the vascular endothelium has been proposed as an early indicator of cardiovascular disease (CVD) (Willerson & Kereiakes, 2003), and has been shown to be a reliable predictor of associated risk factors of CVD such as smoking, hypercholesterolemia and type-1 diabetes in young subjects without evidence of established atherosclerotic disease (Thorne, Mullen, Clarkson, Donald, & Deanfield, 1998; Celermajer, Sorensen, Bull, Robinson, & Deanfield, 1994; Jarvisalo et al., 2004). Consequently it has been observed that regular physical exercise can improve endothelial function in those at risk for cardiovascular disease (Walther, Gielen, & Hambrecht, 2004; Gokce et al., 2002), and has been used as one of the strategies to repair a dysfunctional endothelium (Hambrecht et al., 2000; Higashi et al., 1999a; Hambrecht et al., 2000).

The primary factor leading to the restoration of endothelial function seems to be the chronic episodes of increased shear stress during exercise (Niebauer & Cooke, 1996; Green et al., 2004), which likely leads to upregulation of eNOS nRNA and protein resulting in greater Ṅ0 release in response to a subsequent high shear stimuli (Uematsu et al., 1995; Nishida et al., 1992). However, there appears to be no effect of chronic exercise training on the function of healthy endothelium (Moyna & Thompson, 2004).

Endothelial function is commonly evaluated by assessment of the vasodilator response of a conduit artery to a period of high shear stress during reactive hyperaemia, termed flow-mediated dilation (FMD). Echo Doppler

ultrasound is typically used to image the brachial artery, while reactive hyperaemia is usually initiated by rapid release of forearm occlusion (Corretti et al., 2002). It has been reported that adding exercise to the occlusion period increases the subsequent FMD with some reporting its dependence (Betik et al., 2004), and others independence (Agewall et al., 1999), on the magnitude of the shear stimulus. As well, exercise on its own has been reported to cause conduit artery dilation (Gaenzer et al., 2001), though, to the best of our knowledge, its dependence on endothelial Ṅ0 has not been studied.

Developing a greater understanding of the effects of acute exercise bouts on endothelial function may serve to increase the understanding of the effects of exercise during an FMD test as well as aid in predicting the effects of chronic exercise, similar to what has been shown with respect to blood pressure responses (Thompson et al., 2001). We assessed the effects of 2 prior bouts of heavy hand-grip exercise on a subsequent brachial artery FMD in response to reactive hyperaemia following 15 min of forearm occlusion. Also, the brachial artery dilation responses during the first and second bouts of exercise were contrasted.

Methods

Nine healthy young men were recruited for inclusion in the study. The participants visited the lab on two separate brachial artery FMD assessments: 1) CON, following 15 minutes of forearm occlusion and 2) EX, same as control but following two bouts of heavy forearm exercise. Figure 5.1 illustrates the experimental protocol. Reactive hyperaemia was initiated by rapid deflation of the occlusion cuff.

Flow-Mediated Dilation Procedure

Brachial artery blood flow was arrested by rapid inflation of a standard blood pressure cuff to 250 mmHg. The occlusion cuff was placed just distal to the right elbow.

Exercise Protocol

The exercise consisted of 5 minutes of dynamic hand-grip exercise at 30% MVC. The participant raised and lowered a weight attached to a pulley by squeezing a hand grip device. The duty cycle was set at 2 seconds and cadence was maintained by the participant paying attention to the beats of a metronome.

Physiological Data Collection

Heart rate, mean arterial blood pressure (MAP) estimated by finger photoplethysmography, and brachial artery mean blood velocity (MBV) measured by pulsed wave Doppler ultrasound were collected using a data-acquisition system running on a lap-top PC. The pulsed Doppler 4-MHz probe was fastened above the brachial artery proximal to the cubital fossa with strips of surgical tape. The signal

was directed 45° relative to the skin and the ultrasound gate was adjusted to encompass the total width of the artery.

Brachial artery diameter was monitored on the arm proximal to the inflation cuff by M-Mode ultrasound imaging and stored on digital video tape. The tapes were subsequently digitized to AVI video (KINO video editor). Vessel diameters were measured by custom edge-detection software (Jorges Serrador and Brian Deegan) from still images extracted (KINO video editor) at every 5s of video. An average of the diameter across the M-mode image frame (2-3 cardiac cycles) was taken from the best image in each 15s window to represent mean arterial diameter. The measurements from the 15s windows were curve fit by cubic spline interpolation and Lenenberg-Marquardt peak fitting (Fityk). BL diameter was calculated as the average diameter from the interpolation for the 1 min before EX or OC; peak diameter was the maximal diameter reached by the interpolation for each stage of the protocol. Percent change in diameter (%EMD, for EX, %FMD for Post OC or IO) was calculated as the percent change from end occlusion to peak for %FMD, and from BL to peak for %EMD and %FMDbl.

Brachial artery MBV and cross-sectional area ($A = \pi r^2$) were used to calculate forearm blood flow ($Q = vmean \bullet A\, min^{-1}$) and shear rate ($\gamma = 8 \times v_{mean} \times D^{-1}$). The MBV signal was filtered using 5 Hz low pass filter. MBV was averaged beat to beat at rest and post occlusion, over the contraction to contraction cycle during exercise, and over the inflation to inflation cycle during intermittent occlusion (Figure 4.2). For exercise and intermittent occlusion MBV was also separated into its positive and negative components. The MBV values were then interpolated to second to second by the non-rounded Akima method and matched with the interpolated diameter data. Baseline forearm blood flow, expressed as

ml/100ml/min, and shear rate, expressed as s^{-1}, were determined from averages over the last min before cuff inflation. Peak shear rate was determined from the peak value on the interpolated shear curve. Area under the shear (AUC_{shear}) and diameter (AUC_{diam}) curves were calculated by approximating the definite integral by use of the trapezoidal rule on the interpolated values from the time of occlusion cuff release to the time of peak diameter (QtiPlot).

Venous blood sampling

Venous blood samples were taken at close intervals (1 cc each, 15 to 30 seconds apart) before, during, and after exercise and occlusion. Whole blood samples were analyzed as soon as possible for lactate and pH levels. Heparinized samples were put on ice and analyzed for oxygen saturation and oxygen and haemoglobin content by co-oximeter (A. Faisal, personal communication). The remainder of the heparinized whole blood was centrifuged and the plasma separated and stored for subsequent analysis of nitrate and nitrite content by colorimetric assay kit (Nitric Oxide Quantitation Kit, Active Motif, California). Plasma nitrite reserve was calculated the as relative nitrite increase during reactive hyperaemia in relation to the 0 sec values.

Statistical analysis

Statistical analysis software was used to examine the differences in shear rate, blood flow, FMD, and blood factors for each protocol by repeated measures analysis of variance by the mixed procedure (SAS) with one within subject factor. For differences within a protocol a repeated measures one was ANOVA was performed. A probability of $p<0.05$ was be accepted as statistically significant and

any differences were determined by contrast adjustment.

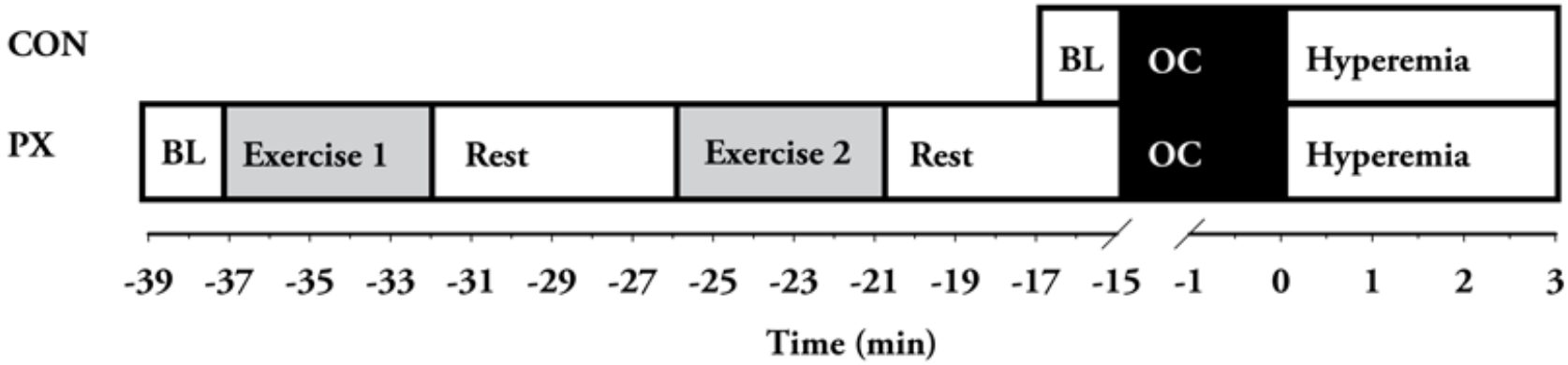


Figure 5.1 Post exercise FMD protocol.

Two bouts of heavy hand-grip exercise (Exercise1 and 2) were separated by 6 minutes of recovery (PX1 and PX2) followed by 15 minutes of forearm occlusion and 3 minutes of reactive hyperaemia (HYP). The control condition consisted of a single 15OC protocol followed by HYP.

Results

Brachial Artery Dilation

FMD was blunted following two bouts of heavy forearm exercise (CON: 10.0±4.8 vs. PostEX: 3.7±2.1 %FMD, $P<0.05$, Figure 5.2), even though shear AUC following cuff release was greater in that condition (CON: 29508.4±15146.4 vs. PostEX: 44992.6±15303.7, no units, Figure 5.3). The end occlusion diameter was significantly greater in the PostEX condition (CON: 5.0±0.7 vs. PostEX: 5.3±0.7 mm, $P<0.05$).

EMD was blunted during the second bout of heavy forearm exercise (EMD1: 14.5±6.4 vs. EMD2: 6.5±1.9 %EMD, Figure 5.5), even though shear AUC was not different in that condition (Figure 5.3). The starting diameter was significantly greater in the for the second exercise bout (EMD1: 5.0±0.6 vs. EMD2: 5.8±0.6 mm, $P<0.05$, Figure 5.5). Table 5.1 compares the change in diameter, time to reach peak diameter, and slope of the diameter curve (expressed as $\frac{\Delta d}{\Delta t}$) for all the conditions. Time to peak diameter did not change for FMD or EMD following an exercise bout. The smaller change in diameter during the post EX protocols (PostEX and EMD2) resulted in a more gradual slope of the diameter curve which reached statistical significance in the PostEX FMD protocol.

Plasma Nitrite

Figure 5.4 illustrates the blunting of plasma nitrite concentration during reactive hyperaemia following two bouts of heavy forearm exercise. Nitrite concentration in CON was significantly higher 15s after cuff release compared to that at the time of cuff release (0s: 0.96±0.50 vs. 15s: 1.77±0.97 μM, $P<0.05$). This was not the case

in PostEX. The nitrite value was significantly higher in CON than in PostEX at 15s post cuff release (CON: 1.77±0.97 vs. PostEX: 0.86±0.15 μM, $P<0.05$). When nitrite was expressed as the reserve from cuff release, PostEX nitrite was significantly elevated at 30s post cuff release and not different from CON at 15s (CON 15s: 82.0±79.1 and PostEX 30s: 49.3±121.2 Δ%, p=0.1335, Figure 5.4).

Post Ischemia $\dot{V}O_2$

Oxygen uptake was higher throughout the first minute of hyperaemia period in CON compared to PostEX (0s; CON: 18.12±8.99 vs. PostEX: 28.74±18.40, 15s; CON: 42.52±23.4 vs. PostEX: 63.93±26.11 , 30s; CON: 23.91±14.80 vs. PostEX: 41.48±20.48 , 45s; CON: 13.47±9.56 vs. PostEX: 24.31±15.05 , 60s; CON: 7.19±4.70 vs. PostEX: 19.45±10.70 ml/min, $P<0.05$, Figure 5.6).

Heart Rate, MAP, and CO

Figure 5.7 illustrates the systemic cardiovascular responses for each stage of the protocols. Heart rate, MAP, and CO were elevated during EX compared to BL and rest (HR; EX1: EX2: vs BL: R1: R2: bpm, MAP; EX1: EX2: vs BL: R1: R2: mmHg, CO: EX1: EX2: vs BL: R1: R2: ,$p<0.05$). There were no significant differences between EX1 and EX2.

Relationship Between Shear and Dilation

There were significant correlations between AUC_{shear} and %FMD in both CON and PostEX (CON: y=0.0002x+3.2149, R2=0.5139, P=0.097; PostEX: 0.0001x-0.6196, R^2=0.4916, P=0.035, Figure 5.8 B). Percent dilation was correlated to peak shear

only in CON (CON: y=0.0186x-3.1625, R^2=0.5259, P=0.097; PostEX: 0.0042x+0.0725, R^2=0.1072, P=0.396, Figure 5.8 D). There were no significant correlations between AUC_{shear} (Figure 5.8 A), peak shear (Figure 5.8 C), or mean shear (and percent dilation in any of the exercise conditions. The %FMD normalized for AUC_{shear} was significantly smaller in PostEX compared to control (CON: 0.000364±0.000148 vs. PostEX: 0.000083±0.000031, no units, $p<0.05$), but not different between the 2 exercise bouts (Figure 5.10).

Dilation and Baseline Diameter

To quantify vasodilatory capability independent of baseline diameter, %Dilation × BLdiameter was calculated. %Dilation × BLdiameter was attenuated for both the PostEX FMD (CON: 47.8±6.9 vs. PostEX: 19.4±2.8 %•mm, $P<0.05$, Figure 5.11 A) and the EMD during second of 2 exercise bouts (EMD1: 71.1±10.2 vs. EMD2: 35.2±3.9 %•mm, $P<0.05$, Figure 5.11).

Table 5.1 Change and time to peak diameter after cuff release before and after exercise, and during the first and second exercise bout.

		Diameter change (mm)	Time to peak (sec)	$\frac{\Delta d}{\Delta t}$
FMD	CON	0.48±0.20	74.7±27.0	0.007±0.003
	PostEX	0.19±0.08†	70.7±17.6	0.003±0.001†
EMD	EX1	0.71±0.31*	200.3±59.6*	0.004±0.002*
	EX2	0.36±0.12†	183.4±60.8*	0.002±0.001

Values are means±standard deviation, n=9 *significantly different from FMD †significantly different within subset, $P<0.05$

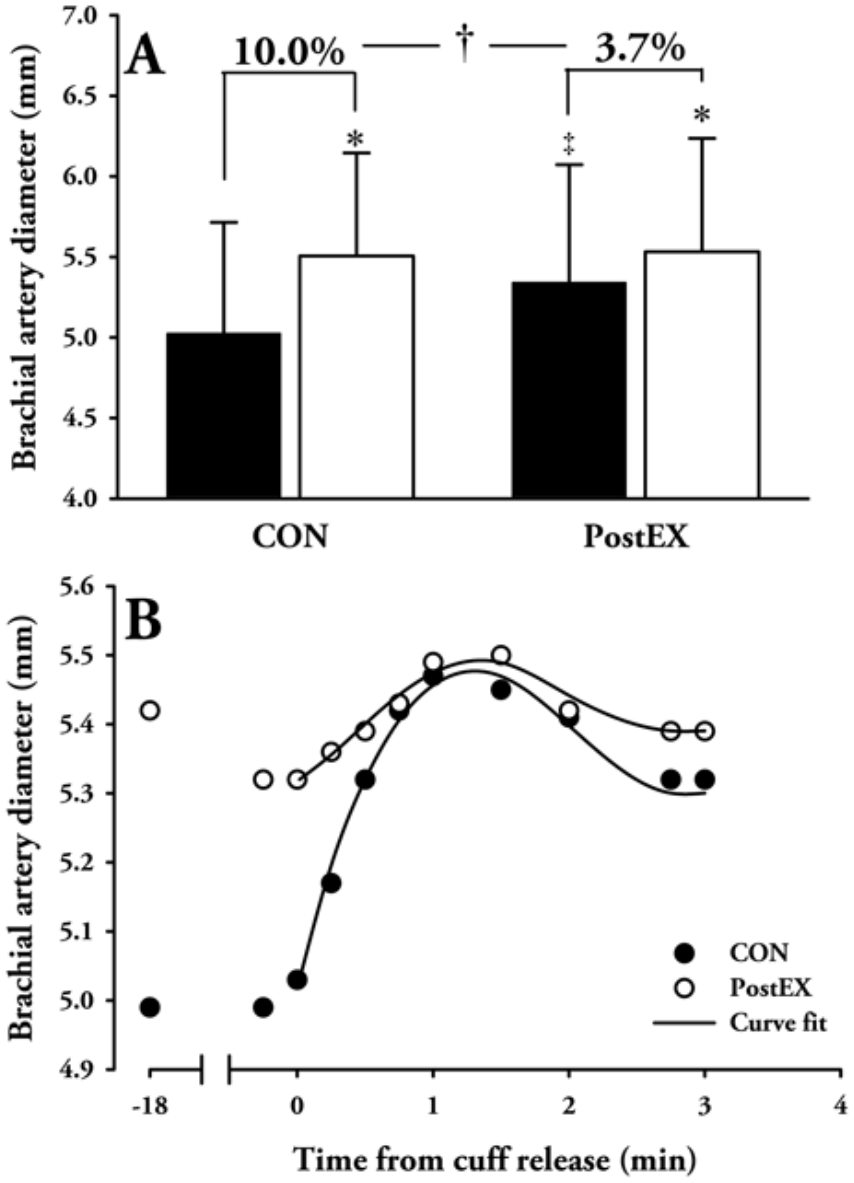


Figure 5.2 Flow-mediated dilation of the brachial artery in response to 15 minutes of forearm occlusion, with and without a prior exercise bout.

A. FMD was significantly smaller after a prior exercise bout. However, the diameter at end occlusion PostEX was significantly greater than in CON. Black bars indicate baseline, white bars peak diameter, mean over 9 subjects ± SE. *significantly different from baseline, †significantly different FMD, ‡significantly different across conditions, $p<0.05$. B. Points represent means for 9 subjects at the 15 second window diameter measurement. Lines represent the polynomial fit to peak diameter.

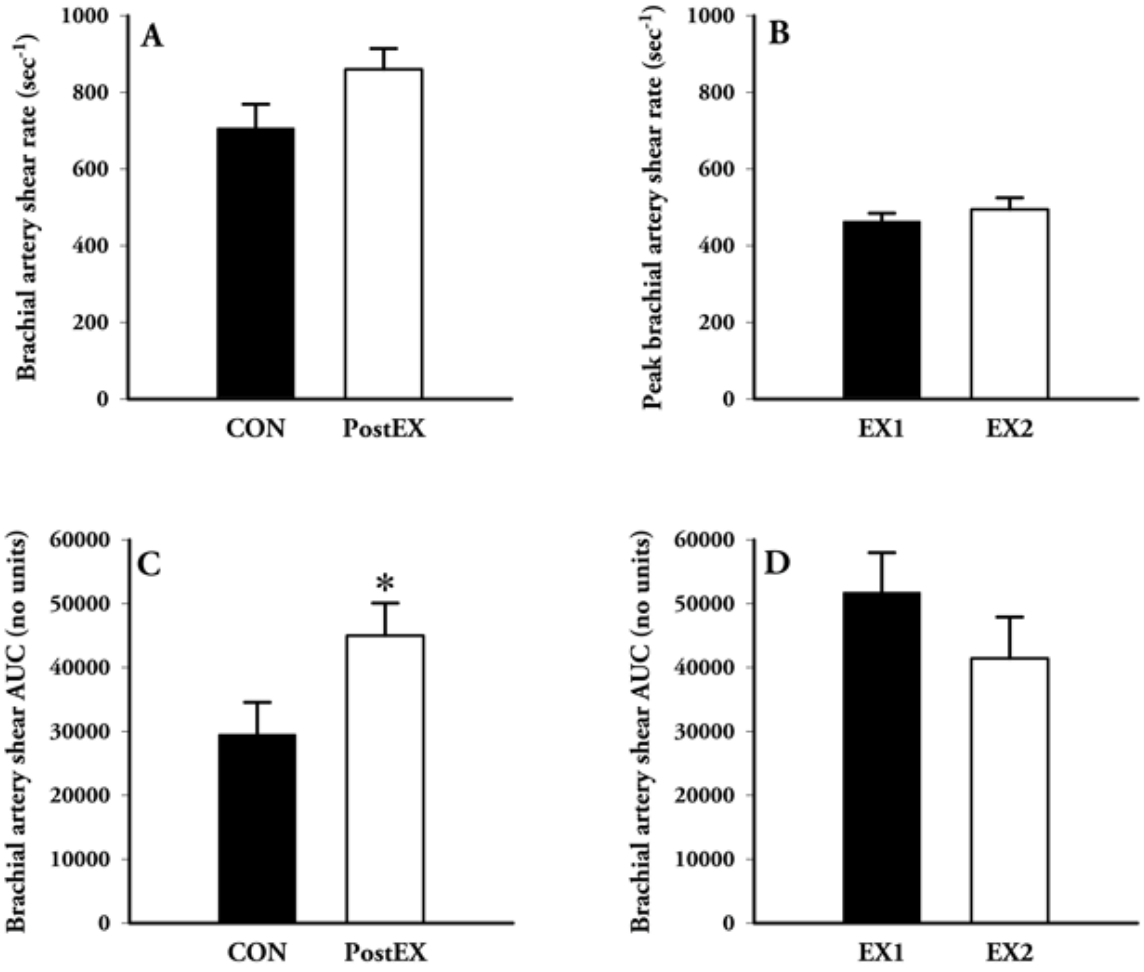


Figure 5.3 Peak and area under the shear curve for exercise and flow-mediated dilation, with and without a prior exercise bout.

The only significant difference found was that AUC was greater for the FMD occlusion protocol after an acute bout of forearm exercise (C) Bars represent means ±SE of 9 subjects, *significantly different from CON. $P<0.05$.

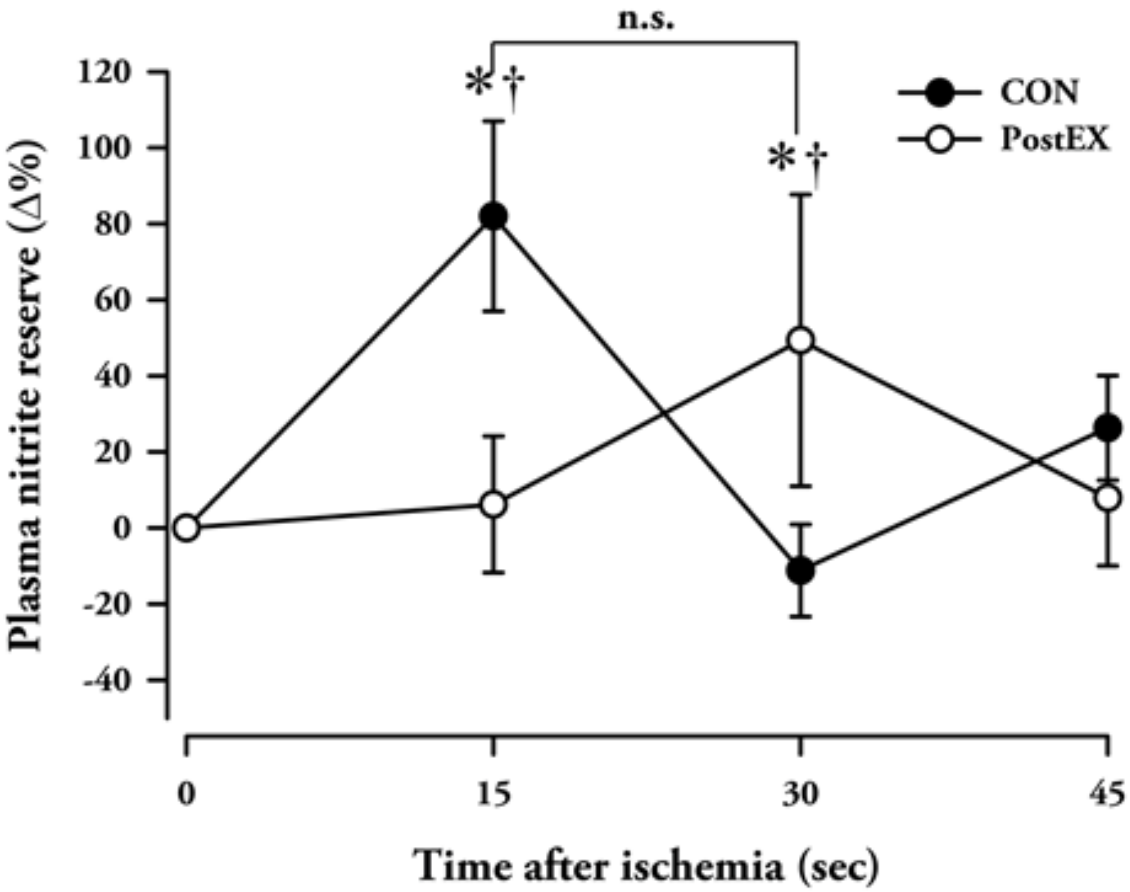


Figure 5.4 Plasma nitrite reserve following release of 15 minutes of forearm occlusion, with and without prior heavy exercise.

Plasma nitrite reserve was significantly elevated 15s after cuff release in the control condition while it was increased at 30s in the PostEX condition. The peaks in reserve were n o significantly different between conditions. Points represent means±SE for 9 subjects *significantly different from baseline, †significantly different across conditions at that time point, $p<0.05$.

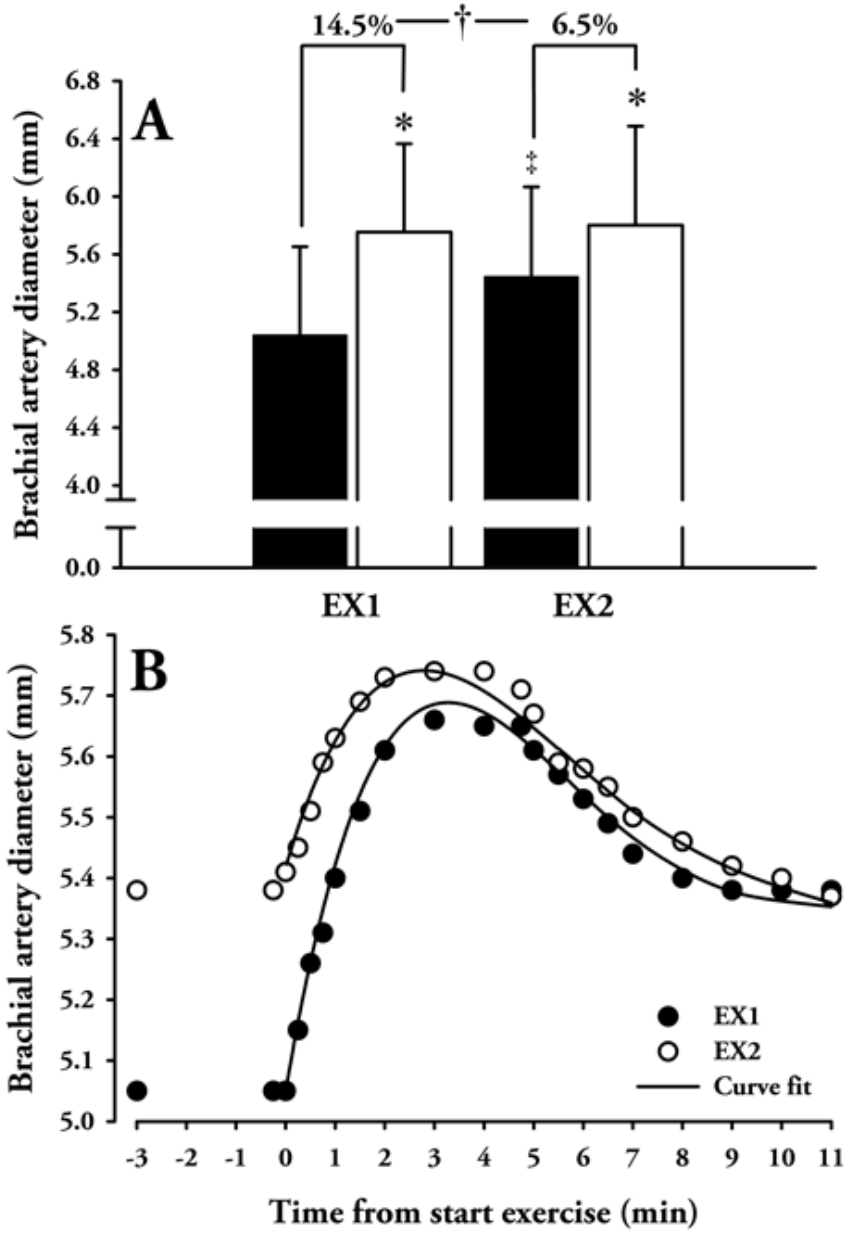


Figure 5.5 Exercise mediated dilation of the brachial artery during the first and second exercise bout.

A. Bout 2 had a significantly smaller dilation during exercise. However, the starting diameter was greater in EX2. Black bars indicate baseline, white bars peak diameter, mean over 10 subjects ± SE. *significantly different from baseline, †significantly different FMD, ‡significantly different across conditions, $p<0.05$. The diameter in the second exercise bout was significantly higher than the first up to the time of peak diameter. B. Points represent means for 9 subjects at the 15 second window diameter measurement. Lines represent the polynomial fit to peak diameter.

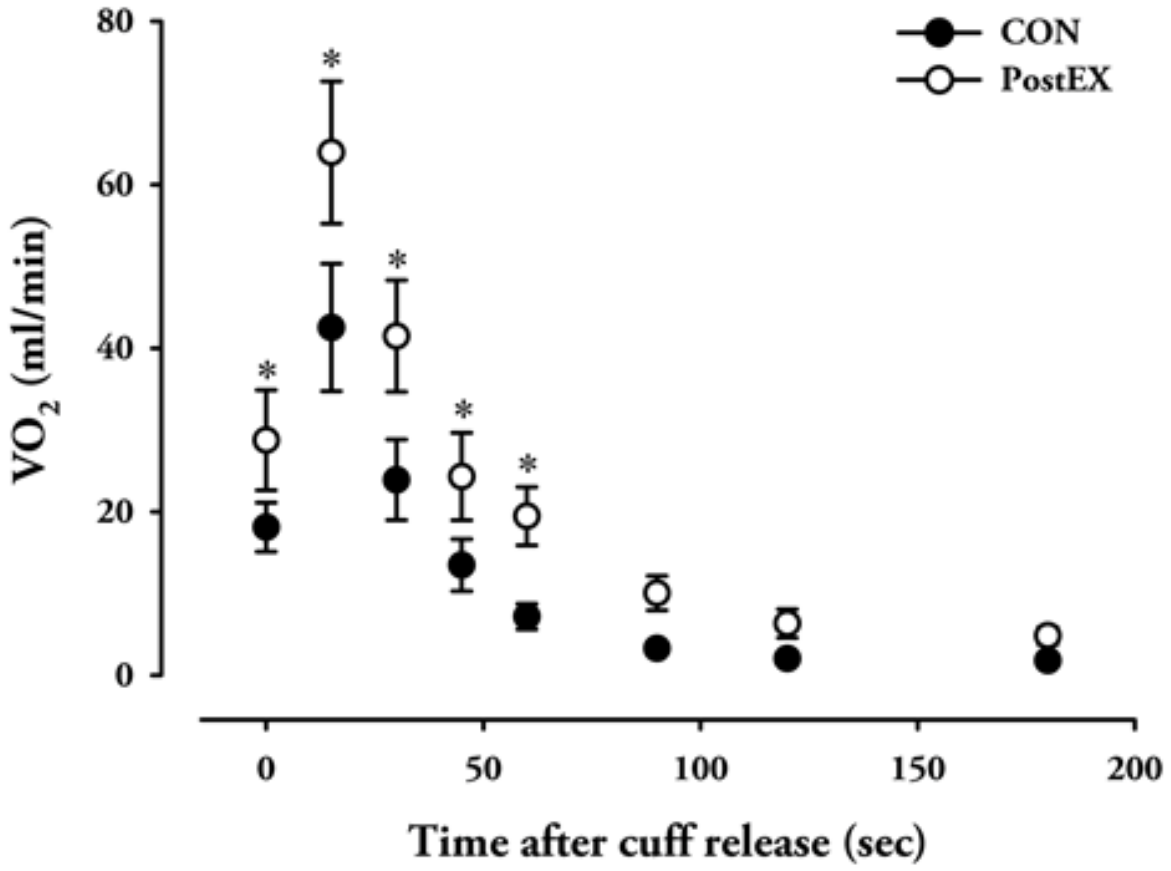


Figure 5.6 Oxygen consumption during reactive hyperaemia, before and after exercise.

Post occlusion $\dot{V}O_2$ was significantly greater after the bouts of heavy exercise as opposed to control from cuff release through 1 min. Points represent means±SE for 9 subjects $\dot{V}O_2$ calculated from (a-v)DO_2 and FBF. *significantly different between conditions, $P<0.05$.

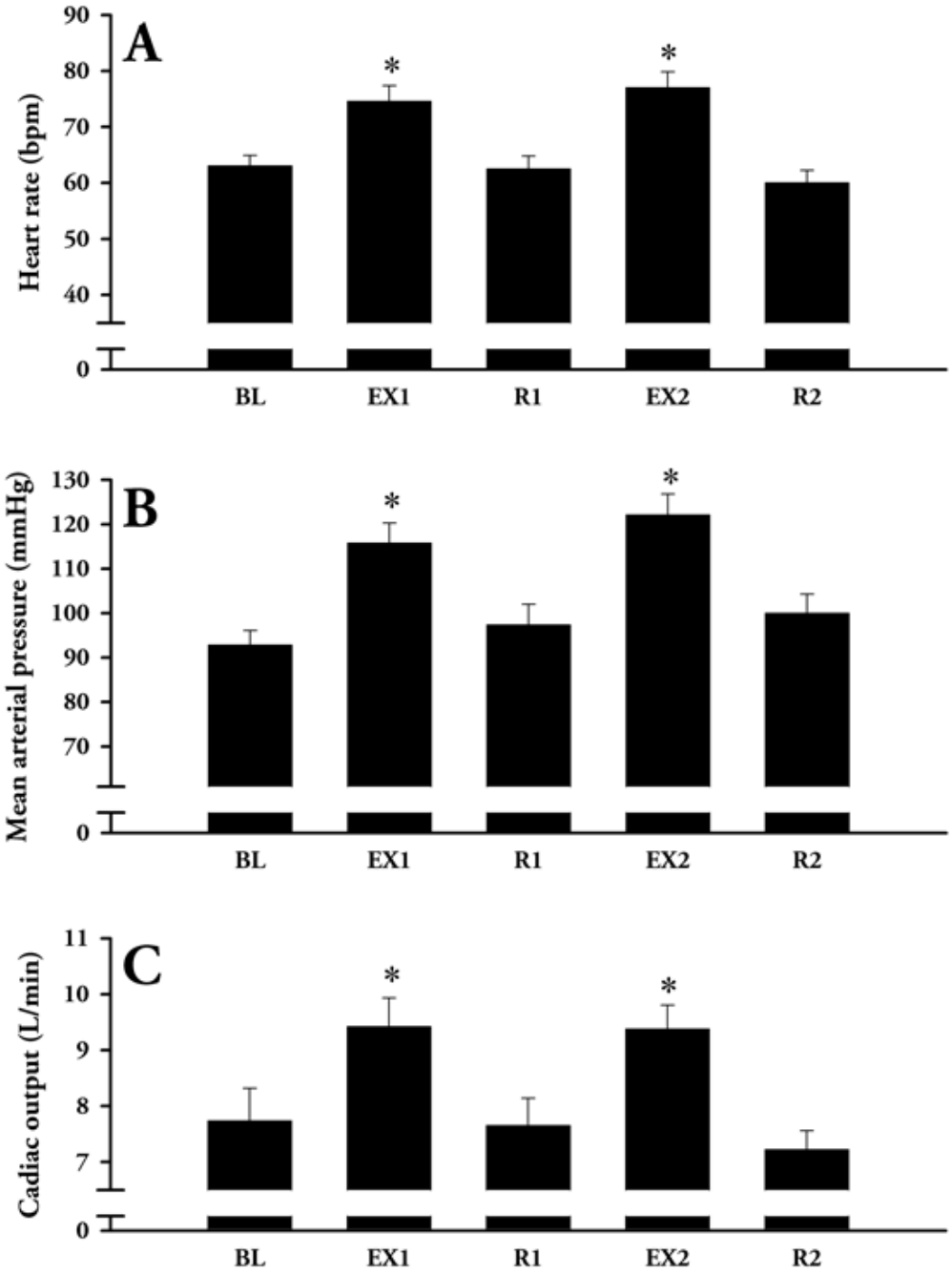


Figure 5.7 Heart rate, mean arterial pressure, and cardiac output increase during hand-grip exercise.

Heart rate, MAP, and CO were elevated during EX compared to BL and rest (R). There were no significant differences between EX1 and EX2. Bars represent means ±SE, *significantly different from BL, R1, and R2. $P<0.05$.

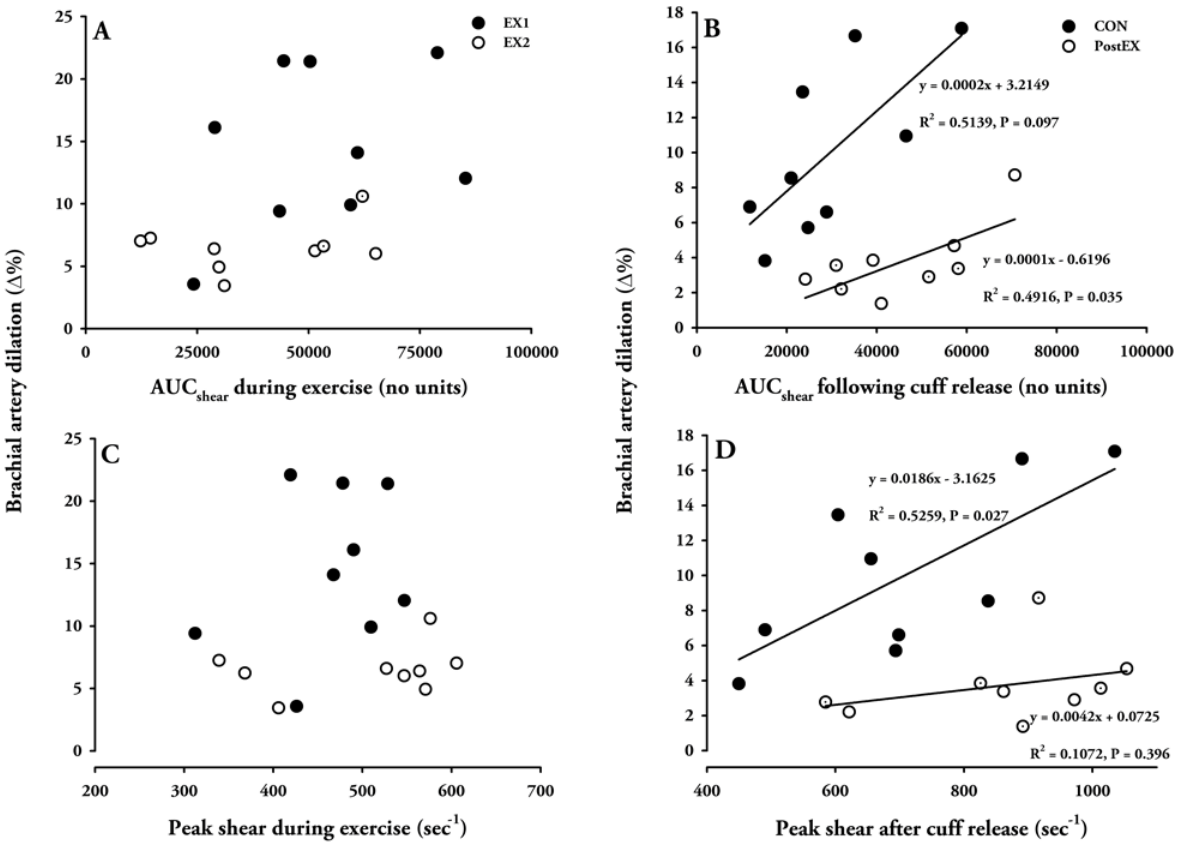


Figure 5.8 Relationship between peak shear and AUC_{shear} and percent dilation during exercise and reactive hyperaemia.

There was no correlation between either AUC_{shear} (A) or peak shear (C) and %EMD during the exercise protocols. AUC_{shear} and %FMD were both significantly correlated in CON and PostEX reactive hyperaemia (B). Percent dilation was correlated to peak shear only in CON (D).

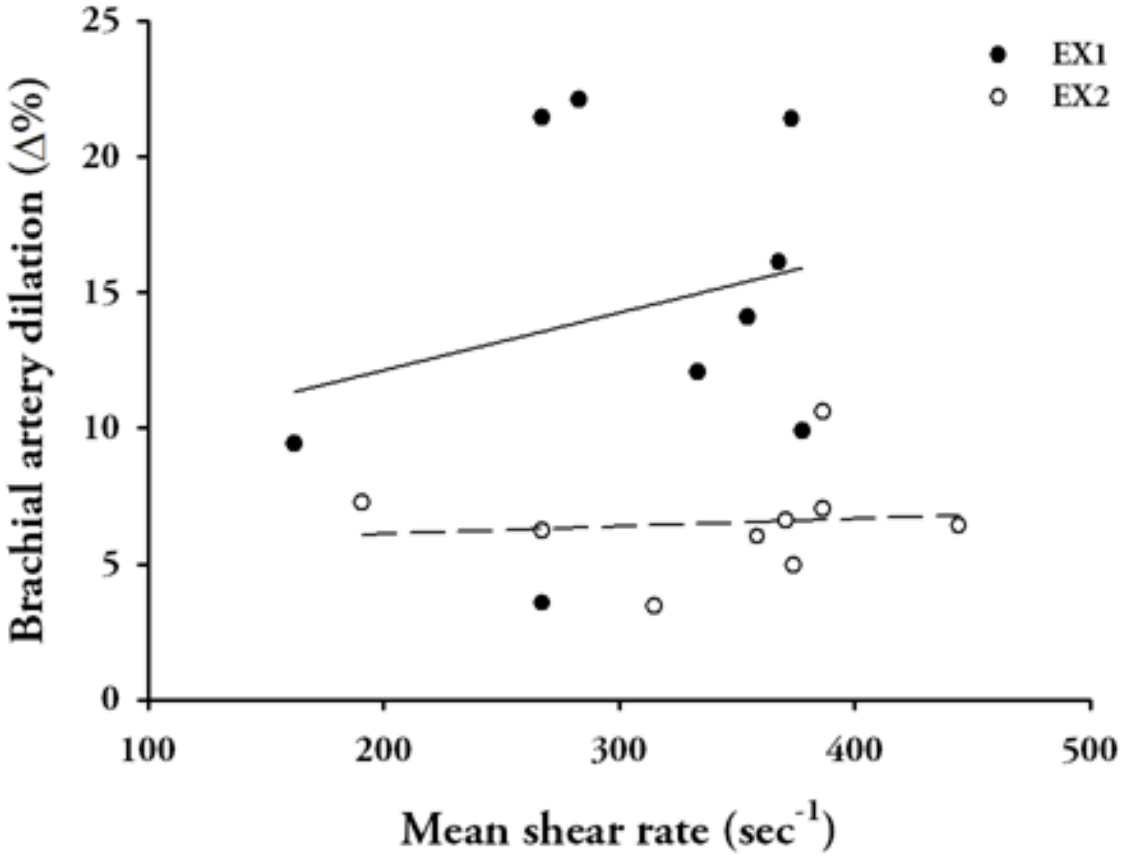


Figure 5.9 Relationship between mean shear rate during exercise and percent dilation.

There was no correlation between mean shear rate during exercise and percent exercise mediated dilation of the brachial artery.

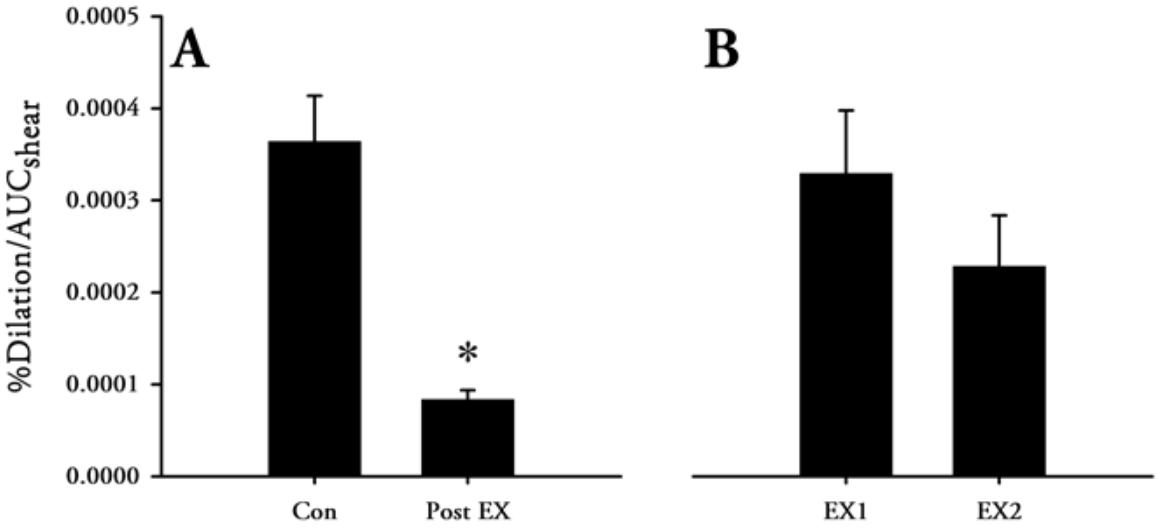


Figure 5.10 Dilation normalized for total shear to peak dilation following occlusion and during exercise.

When taking AUCshear into account, FMD following a bout of exercise is attenuated compared to control (A), while the dilation during exercise is similar in the first and second bouts (B). *significantly different from CON. P<0.05.

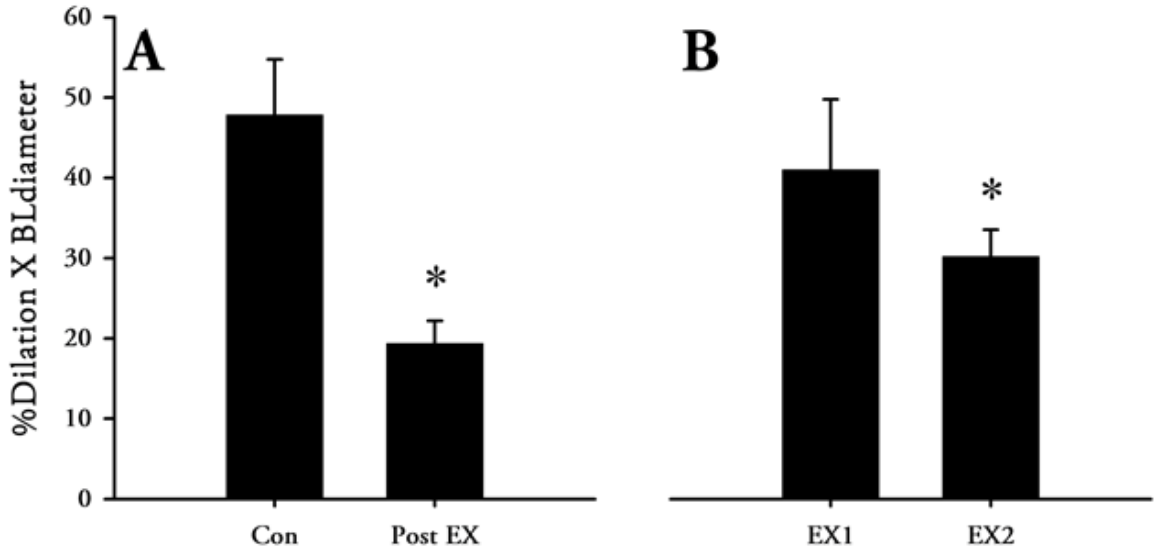


Figure 5.11 Dilation normalized for baseline diameter following occlusion and during exercise.

When using the %Dilation × BLdiameter index, FMD following a bout of exercise is attenuated compared to control (A), as is the dilation during exercise in the second of 2 bouts (B). *significantly different from Con or EX1. $P<0.05$.

Discussion

The major finding of the present study was that FMD was blunted when the test was preceded by 2 bouts of heavy hand grip exercise, and that the attenuation in FMD was still present when dilation was normalized for AUC_{shear} and the increased baseline diameter. Also, EMD was blunted in the second of the 2 exercise bouts, with this blunting still present when the increased baseline diameter was factored in.

Acute Exercise Models

Previous studies examining the effect of acute exercise on FMD have looked only at whole body aerobic exercise bouts (Padilla et al., 2007). Acute treadmill walking has been reported to improve FMD in active but not inactive overweight men, with the discrepancy left unexplained by the pro –and ant- inflammatory state as indicated by similar levels of IL-6 and TNF-α in both groups (Harris et al., 2008). The present study examined the effect of a prior exercise bout on FMD in the same limb as the exercising muscle. FMD in a young, healthy cohort was found not to be different after 30 min of aerobic cycling exercise (Rundell, Hoffman, Caviston, Bulbulian, & Hollenbach, 2007). It is a truly novel finding that FMD was blunted in healthy athletic males after acute hand-grip exercise.

Baseline Diameter

In the attempt to study the effects of acute exercise the time between the exercise and occlusion period may have been too short as evidenced by the finding that artery diameter was significantly greater at the time of cuff release following exercise compared to the control. However, it has been reported that conduit

artery dilation can persist for over an hour post exercise (Harris et al., 2008; Wallace, Bogle, King, Krasnoff, & Jastremski, 1999), thus limiting the time interval allowable for post exercise FMD testing, if one is concerned about having similar baseline diameters. Also shear is elevated after exercise for at least 2 hours (Padilla et al., 2007), and it is not clear what effect high baseline shear has on FMD. It is well established that consideration should be made for the size of the artery at baseline. Mizia-Stec, K., Gasior, Z., Mizia, M., Haberka, M., Holecki, M., Zwolinska, W., Katarzyna, K., and Skowerski, M. (2007) found that the larger baseline brachial artery diameter in men vs. Women was reflected in a lower FMD in men that is not seen with endothelium independent dilation (nitroglycerine), while both men and women had similar %dilation × BLdiamter index. There may be architectural factors that come into play that would limit or enhance the FMD response based on initial diameter (Daemen & De Mey, 1995). However, there may also be a role of the wall to lumen diameter ratio which has been shown to be linked to responsiveness to various vasoactive stimuli, at least in resistance vessels (Folkow, Grimby, & Thulesius, 1958; Folkow, 1978). Baseline artery diameter has been shown to be inversely correlated with the FMD response while this relationship was lost when normalized for AUCshear (Thijssen et al., 2008). Thus it may be interpreted that baseline diameter is more important than shear in determining %FMD. This is similar to what we found in the post exercise FMD, as shear was not correlated with %FMD as it was before exercise, indicating that the baseline diameter may have played a greater role in determining FMD post exercise. We did not test the FMD response of successive occlusion protocols, so the effects we have seen post exercise may not be fully due to the exercise per se but may have been related to the prior dilation. However, repetitive bouts of FMD

have previously been found not to affect the outcome of FMD tests (Harris, Padilla, Rink, & Wallace, 2006), and we attempted to address the situation of shifting baseline diameter by application of the %Dilation × BLdiameter index (Mizia-Stec et al., 2007).

Shear Oscillations

Endothelial cells align themselves in the direction of flow (Luscher & Corti, 2004) demonstrating that they have the ability to sense shear oscillations as are present during exercise. It may be that the oscillations in shear experienced by the endothelial cells during exercise cause then to be less sensitive to a subsequent high shear stimulus. It has been demonstrated that a prior intervention of accentuated retrograde shear by inflation of a cuff at 25, 50, or 75 mmHg for 30 min resulted in attenuation of FMD in a pre – post test (Thijssen et al., 2009). However, apart from manipulating the retrograde shear, these protocols also resulted in significantly reduced mean shear rate. Therefore the interpretation that it is the retrograde shear that is the main cause of reduced FMD may not be fully valid. It is possible that the reduced mean shear may have an effect similar to the effect of the prior exercise bout in the present study.

Systemic Effects of Exercise

The reports of enhanced FMD following acute exercise intervention have pointed to the reduction in sympathetic activity following exercise (Floras et al., 1989; Pober, Braun, & Freedson, 2004) as allowing for greater FMD in the post exercise state. Harvey, P J, Morris, B L, Kubo, T, Picton, P E, Su, W S, Notarius, C F, and Floras, J S (2005) found that a single bout of exercise increases FMD in older but not

younger women which could be explained by sympatholysis in the older women. Similar results have been found in men (Thijssen 2006). We were unable to test for sympathetic activity in this study, however it would appear that even if it was reduced in the post exercise state this had no effect on enhancing FMD as the final diameter reached was not different from control and percent FMD was lower in the post exercise state. The metabolic state of the muscle, characterized by oxygen consumption ($\dot{V}0_2$) as measured directly from oxygen extraction in the venous blood (A. Faisal, personal communication) and blood flow, was significantly higher following the post exercise occlusion protocol indicative of an altered metabolic state in the working muscle compared to that of control. Heart rate, mean arterial pressure, and cardiac output were all increased during the exercise bouts. Lingering effects of central cardiovascular control mechanisms or local muscle metabolic machinery may have interfered with endothelial release of $\dot{N}0$.

Plasma Nitrite

Although it was not possible to directly measure $\dot{N}0$ release in the conduit artery at the site of shear stimulation, Rassaf et al. (2006), using a triiodide/ozone-based chemiluminescence assay, found that the indirect measurement of plasma nitrite reserve in the venous effluent mirrored FMD in healthy subjects, but not in those with cardiovascular disease risk factors. Also, serum nitrite was found to reliably reflect changes in endothelial $\dot{N}0$ formation in human forearm after Ach infusion (Kelm et al., 1999). The nitrite reserve data in the present study suggest that there was no dysfunction of eNOS production post exercise as both CON and PostEX protocols had elevated nitrite post cuff release that was not significantly different from each other. However, it would seem that the response was slightly delayed in

the PostEX condition.

Conclusion

The brachial artery dilated to a similar absolute post occlusion diameter in an FMD test that followed acute exercise of the forearm muscles. However, baseline diameter was larger following exercise, resulting in a reduction in calculated post exercise FMD compared to control. This reduced FMD was evident even when the percent dilation was normalised to AUC_{shear} or to the increased baseline diameter. Similarly, percent dilation during the 2^{nd} of 2 exercise bouts was blunted, even when normalized to the increased baseline diameter. The relationship between AUC_{shear} and % dilation was not evident in the PostEX FMD test or in the 2^{nd} of 2 exercise bouts. Although the scientific rational for evaluating endothelial function in response to acute exercise is sound (Padilla et al., 2007), the results of the present study suggest that there needs to be further study into the influence of exercise mediated alterations in the vascular environment, particularly the exercise induced dilation, and their effects on subsequent dilation during reactive hyperaemia. The results of this investigation suggest that percent FMD results should be interpreted with caution in circumstances in which physical activity prior to the FMD test could have occurred.

Chapter 6 - **WISE 2005: Flow and nitro-glycerine mediated dilation following 56 days of head down tilt bed rest with and without an exercise countermeasure**

Summary

This investigation set out to determine the effect of 56 days of head-down tilt bed rest (HDBR) and an exercise countermeasure on endothelial dependent and independent vascular function. 24 women took part in this study. 8 subjects completed lower body resistance and aerobic exercise (EX, treadmill running 3-4 days per week for 40-min followed by 10-min of static LBNP, and resistive exercise on a flywheel device every 3rd day), 8 were given a protein supplement (NUT), and 8 were considered as the control group (CON). FMD was induced by release of distal limb ischemia and NMD by sublingual administration of 0.3 mg of nitro-glycerine before and after HDBR. HDBR without EX decreased the resting diameter of the popliteal artery while EX increased the diameter (CON: 4.6±0.4 to 3.7±0.7 and NUT: 4.5±0.5 to 3.8±0.5 vs. EX: 4.5±0.7 to 5.2±0.1 mm). HDBR had no effect on the resting diameter of the brachial artery. FMD was elevated in all groups for the brachial (EX: 6.9±1.6 to 11.3±2.3; CON: 5.2±3.5 to 13.6±8.5; NUT: 7.8±2.2 to 16.9±4.6%) but only in the non-exercisers for the popliteal (CON: 10.6±3.2 to 15.0±0.7; NUT: 6.4±4.9 to 13.1±5.7 %). When change in resting diameter is taken into account the preserved FMD in EX is removed (EX: 42.9±18.9 to HDBR 55.7±23.0; CON: 48.0±13.0 to 60.0±10.0; NUT: 27.5±19.8 to 51.4±31.3 %FMD × BLdiameter). NMD was not altered by HDBR in any group. There was enhanced endothelial function relative to intrinsic dilatory capacity in both the brachial and popliteal arteries post HDBR.

Introduction

The function of the vascular endothelium has repeatedly been shown to reflect the health and function of the cardiovascular system as a whole. Reduced function of the vascular endothelium has been linked to various cardiovascular disease states such as hypertension (Schmieder et al., 1997; Iiyama et al., 1996; Bolad & Delafontaine, 2005) and onset of atherosclerosis (Vapaatalo & Mervaala, 2001; Thorne et al., 1998; McLenachan et al., 1991; Mano et al., 1996), while enhanced function may be linked to orthostatic intolerance (Takase et al., 2000; Guazzi et al., 2004; Bonnin et al., 2001). Improved endothelial function, through interventions such as regular aerobic exercise (Clarkson et al., 1999; Brendle, Joseph, Corretti, Gardner, & Katzel, 2001) or Mediterranean diet (Sondergaard, Moller, & Egstrup, 2003; Cuevas & Germain, 2004), has been linked to reduced cardiovascular disease morbidity and mortality (Chan et al., 2006). A functional endothelium will react to increases in vessel wall shear stress by initiating a vasodilatory response mediated by the synthesis and release of endothelium-derived relaxant factor(s) (EDRF) (Pohl et al., 1986), primarily the free radical nitric oxide ($\dot{N}O$) (Palmer, Ferrige, & Moncada, 1987). Vessel wall shear stress is increased with increased blood flow and the resulting flow-mediated dilation (FMD) of a conduit artery, monitored by ultrasound imaging, used as a marker of vascular endothelial function (Kaiser & Sparks, 1986; Celermajer et al., 1994). Nitro-glycerine administration initiates a vasodilatory response (NMD) in conduit vessels by donating $\dot{N}O$ to the vascular smooth muscle independent of endothelial cell $\dot{N}O$ production (Gerson, Allen, Seltzer, Parker, & Markowitz, 1982), thus allowing for the study of the full dilatory capacity of the smooth muscle alone. (Allen, Cobb, Kraus, & Gow (2006) reported that individuals with CVD had a reduced nitro-

glycerine mediated dilation (NMD), to go along with reduced FMD, and also showed lower circulating nitric oxide metabolites ($\dot{N}0_X$) at rest, during exercise, and in recovery from exercise.

It has long been known that exposure to a micro-gravity environment results in significant deconditioning of many physiological systems, including the cardiovascular system. The most studied effect of micro-gravity induced cardiovascular deconditioning has been the post-flight orthostatic intolerance (OI). This condition manifests as the inability of astronauts to maintain an upright posture upon return from space. Although there has been extensive study into the possible mechanisms responsible for this phenomenon, including adaptations in the sympathetic nervous system, reductions in plasma volume, and decreased cardiac mass, there is still a considerable lack of understanding. There is a body of evidence from animal and human experiments, in simulated and real micro-gravity, showing that the structure and function of the vascular system is subject to adaptations (Delp, Colleran, Wilkerson, McCurdy, & Muller-Delp, 2000; Zhang, 2004).

Previous studies have shown that regular aerobic or resistive exercise is effective in preserving or improving endothelial and vascular function as a consequence of aging (Wray et al., 2006) or in the presence of cardiovascular risk factors (Olson et al., 2006; Green et al., 2004), including inactivity (de Groot et al., 2006). The purpose of this study was to characterize the adaptations of vascular structure and function to long term head-down-tilt bed rest (HDT) as a simulation of micro-gravity. To this effect we studied the dilation response of limb conduit arteries to increased blood flow (endothelium dependent) and nitro-glycerine administration (endothelium independent).

The Women's International Space Simulation for Exploration (WISE) was organized during 2005 at the MEDES facility in Toulouse, France jointly by ESA, NASA, CNES and CSA to study the benefits of potential countermeasures on a range of adaptations to long-duration head-down bed rest in women. Aerobic exercise was achieved with treadmill running in the supine position while inside a lower body negative pressure chamber with suction applied to achieve impact force equivalent to, or slightly greater than, the force of gravity during normal upright running (Cao et al., 2005). LBNP was applied for an additional 10-min to challenge the orthostatic response. The resistive exercise was accomplished with the flywheel device (Rittweger et al., 2005) that has been used in a previous long-term bed rest study of men. The nutritional intervention consisted of the addition of a daily protein supplement to the usual menu.

This study tested the hypothesis that long term bed-rest would attenuate the dilatory response to reactive hyperaemia in conduit arteries, revealing a dysfunctional endothelium indicative of cardiovascular disease risk. In addition it is reasoned that intervention with regular physical exercise would preserve endothelial function.

Methods

Twenty-four healthy women (age 25-40 years) took part in the study. Subjects were housed in the MEDES facility in Toulouse, France. Each subject completed 60 days of supervised, continuous 6° HDBR, with 20-day periods of additional testing and monitoring before and after bed rest in the Space Medicine Research Facility of the Centre National d'Etudes Spatiales in Toulouse, France. All experimental procedures were approved by the Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale, Midi-Pyrénées (France), Committee for the Protection of Human Subjects at Johnson Space Center, and local ethics committees, including the Office of Research Ethics, University of Waterloo. The entire protocol was in accordance with the declaration of Helsinki. Each subject was aware of her right to withdraw from the study for any reason without prejudice.

Subjects were randomly assigned to one of three groups: control, exercise, or nutrition. Throughout the study, hydration was maintained with 60 ml H_2O/kg/day, and caloric intake was set at 120% of the calories dictated by resting metabolic rate. This was modified for the exercise group to account for increased fluid requirements and energy expenditure. No nicotine, alcoholic beverages, or caffeine were permitted. Testing was completed on day 7 before the start of HDBR, and on day 56 of HDBR; for the exercise group day 56, testing took place 24 h after treadmill exercise.

Countermeasures

Subjects in the exercise group walked and ran 3 or 4 times per week in the supine position on a treadmill placed inside an LBNP chamber for 40 min at 40–80% $\dot{V}O_2$ peak in an interval fashion. The applied LBNP (-48 to -55 mmHg) provided 1–1.15 body weight of ground reaction force (Hargens et al., 2002). The treadmill exercise was followed by 10min of resting LBNP at the same level. Every third day, subjects performed flywheel resistance exercise while maintaining a head down position (1). The treadmill and flywheel exercises were normally not performed on the same day, and rest days were provided. The nutrition countermeasure group received a supplement of free branched-chain amino acids (3.6 g/day free leucine, 1.8 g/day free isoleucine, and 1.8 g/day free valine) that were added as a supplement (Friliver, Bracco, Italy) given at the main meals to attenuate nitrogen loss during bed rest (Stein, Schluter, Leskiw, & Boden, 1999).

Experimental Design

Nitro-glycerine (0.3 mg) was administered by sublingual spray. Once the effects of the drug had subsided the brachial and popliteal FMD protocols were performed. Brachial and popliteal artery blood flow was arrested by rapid inflation of a standard blood pressure cuff to supra-systolic pressure. The occlusion cuff was placed just distal to the right elbow for the brachial or the knee for the popliteal arteries. One minute isometric contraction was maintained before rapid cuff deflation to achieve the reactive hyperaemia.

Physiological Measurements

MAP was measured using finger-cuff plethysmography (Finometer, Finapres Medical, Amsterdam), and HR was determined from the electrocardiogram with all analog signals sampled in real time at 100Hz with an online acquisition and analysis system (PowerLab, ADInstruments; Castle Hill, New South Wales, Australia) and stored on a computer for subsequent analysis. Blood velocity (brachial and popliteal) was continuously monitored by 4Mz Doppler ultrasound probes and recorded to a digital data collection computer by data acquisition and control software (Notocord, Croissy-sur-Seine, France). Vessel diameter (brachial and popliteal for FMD, brachial, popliteal, and femoral for NMD) was monitored by M-Mode ultrasound imaging (Acuson 128XP, Paris, France) and stored on digital video tape. The tapes were subsequently digitized and vessel diameters were measured by edge-detection software from still images. Images at ~60 sec (FMD) and ~300 sec (NMD) were used to represent the peak response. Brachial artery MBV and cross-sectional area ($A = \pi r^2$) were used to calculate forearm blood flow ($Q = vmean \bullet A\, min^{-1}$) and shear rate ($\gamma = 8 \times v_{mean} \times D^{-1}$).

Results

Resting vessel diameter

Figure 6.1 shows the baseline and peak diameter (~60 sec post cuff release) during the FMD protocol. HDBR resulted in a decreased resting diameter of the popliteal artery in both the control and nutrition groups (CON, preHDBR 4.6±0.4 vs HDBR 3.7±0.7; NUT, preHDBR 4.5±0.5 vs HDBR 3.8±0.5 mm) while in the exercise group the diameter increased (EX, preHDBR 4.4±0.7 vs d56HDBR 5.2±1.1 mm). Resting diameter of the brachial artery did not change with HDBR in any group.

Flow-mediated dilation

Peak FMD was increased in all groups in the brachial (EX, preHDBR 6.9±1.6 vs HDBR 11.3±2.3; CON, preHDBR 5.2±3.5 vs HDBR 13.6±8.5; NUT, preHDBR 7.8±2.2 vs HDBR 16.9±4.6%), but only in the non-exercise groups in the popliteal (CON, preHDBR 10.6±3.2 vs HDBR 15.0±0.7; NUT, preHDBR 6.4±4.9 vs HDBR 13.1±5.7 %) arteries (Figure 6.2). FMD remained at preHDBR levels in the exercise subjects in the popliteal (EX, preHDBR 9.6±3.8 vs HDBR 10.6±3.2 %), but not the brachial (EX preHDBR 6.9±1.6 vs HDBR 11.3±2.2 %) artery (Figure 6.2). Peak shear rates in all arteries for all groups, resting and peak, remained the same following HDBR (Table 6.1). To evaluate endogenous vasodilatory capability of the popliteal artery, as baseline was changed by HDBR, %FMD × BLdiameter was assessed. %FMD × BLdiameter was greater for every group at HDBR (EX, preHDBR 42.9±18.9 vs HDBR 55.7±23.0; CON, preHDBR 48.0±13.0 vs HDBR 60.0±10.0; NUT, preHDBR 27.5±19.8 vs HDBR 51.4±31.3%)

Nitro-glycerine-mediated dilation

NMD was not changed by HDBR in either the brachial or popliteal artery for any group (Figure 6.4).

FMD/NMD

The ratio of FMD/NMD was increased following HDT for all groups in the brachial artery (EX, preHDBR 0.26±0.08 vs HDBR 0.38±0.05; CON, preHDBR 0.20±0.07 vs HDBR 0.48±0.12; NUT, preHDBR 0.26±0.05 vs HDBR 0.56±0.22, Figure 6.5 A). The ratio of FMD/NMD was increased following HDT only in the non-exercise groups in the popliteal arteries (CON, preHDBR 0.69±0.07 vs HDBR 0.91±0.13;

NUT, preHDBR 0.39±0.05 vs HDBR 0.79±0.22, Figure 6.5 B).

Table 6.1 Baseline and peak shear rate before and after 56 days of head down tilt bed rest.

		Brachial				Popliteal			
		Shear rate		Blood flow		Shear rate		Blood flow	
		Baseline	Peak	Baseline	Peak	Baseline	Peak	Baseline	Peak
Exercise	BDC	104.5±64.0	1750.9±936.8*	4.3±1.3	72.5±14.8*	50.0±15.5	856.2±238.5*	3.7±1.4	63.4±19.0*
	HDT	108.3±52.8	1595.4±871.4*	5.3±0.5	85.9±24.0*	41.6±15.0	834.7±290.0*	6.1±2.3	125.5±38.9*
Control	BDC	75.5±24.1	1534.3±647.4*	4.6±2.3	79.0±36.1*	45.3±12.3	741.4±265.4*	4.3±1.3	75.4±18.2*
	HDT	112.2±22.1	1695.7±422.9*	3.6±0.5	65.2±10.0*	52.2±17.7	864.6±236.6*	3.0±0.7	45.8±3.4*
Nutrition	BDC	115.0±28.1	1351.5±342.4*	5.8±2.4	75.8±44.4*	50.4±14.6	947.1±259.0*	3.6±1.3	65.9±14.7*
	HDT	110.4±43.2	1287.4±283.7*	4.4±1.3	54.7±12.5*	62.7±18.4	1047.9±281.2*	4.7±2.5	58.4±20.9*

*significantly different from baseline ($p<0.05$)

Table 6.2 Heart rate and mean arterial pressure response to the occlusion-with-exercise protocols before and after 56 days of head down tilt bed rest.

		Heart rate						Mean arterial pressure					
		Brachial protocol			Popliteal protocol			Brachial protocol			Popliteal protocol		
		Baseline	Exercise	Recovery	Baseline	Exercise	Recovery	Baseline	Exercise	Recovery	Baseline	Exercise	Recovery
Exercise	BDC	66.5±6.1	74.2±10.0*	62.8±7.0	70.3±4.1	81.3±8.7*	66.3±7.4	89.0±9.6	101.5±11.2*	90.9±10.1	90.3±9.6	98.5±11.6*	88.7±10.5
	HDT	65.6±9.2	74.3±9.1*	63.9±10.2	66.3±7.6	78.8±12.4*	69.7±6.5	85.5±8.2	97.4±9.2*	88.2±6.6	89.6±16.5	101.8±17.1*	94.5±13.7
Control	BDC	64.9±6.5	75.7±12.5*	65.5±6.7	69.5±8.2	76.3±11.9*	69.4±8.3	89.4±13.8	103.5±17.9*	89.8±9.8	94.0±11.3	104.3±16.5*	96.1±9.6
	HDT	75.2±9.7†	86.0±13.9*†	74.0±11.3†	76.9±13.7†	89.0±21.9*†	75.9±10.3†	88.8±12.9	100.4±19.2*	88..3±11.2	90.9±13.7	102.4±20.5*	94.5±13.2
Nutrition	BDC	60.3±10.0	73.2±14.8*	59.7±8.8	64.5±7.2	78.9±13.9*	63.0±6.3	86.6±10.0	97.5±10.3*	87.8±9.2	97.2±8.0	105.7±6.4*	96.8±6.2
	HDT	73.5±12.6†	87.2±13.3*†	72.2±7.3†	76.5±11.1†	89.1±15.5*†	75.5±9.4†	81.4±8.6	94.6±9.7*	82.9±7.4	85.4±5.6	92.9±7.0*	85.9±6.4

*significantly different from baseline, †significantly different from BDC ($p<0.05$)

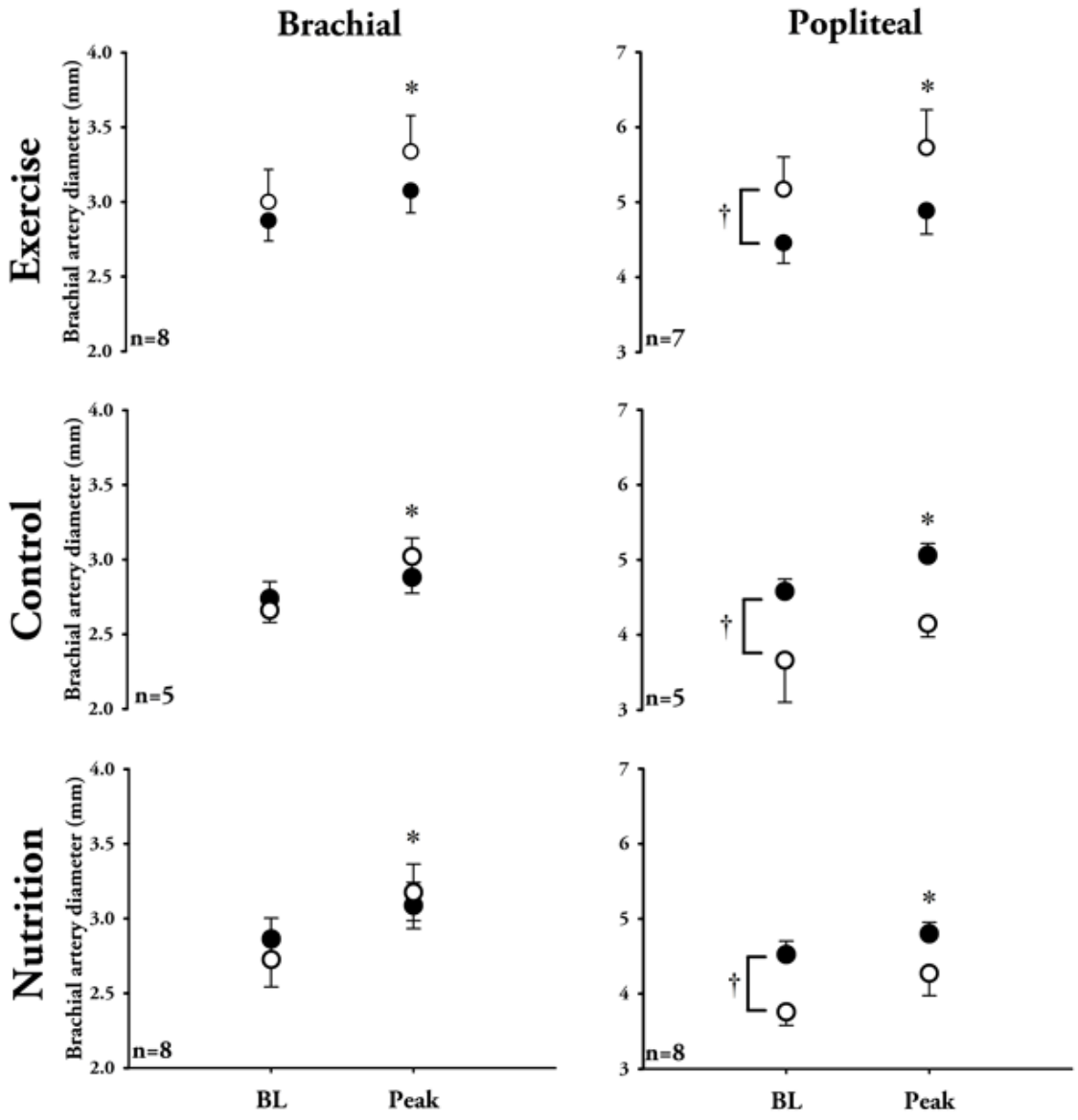


Figure 6.1 Diameter of the brachial and popliteal arteries following release of an occlusion cuff before and after 56 days of head down tilt bed rest for exercise, control, and nutrition.

Symbols represent Means±SE diameter (mm) at baseline (BL) and 60 sec after occlusion cuff release (Peak). Closed circles: Pre-HDBR, Empty circles: 56 days-HDBR. *significantly different from baseline, †significantly different at baseline, $p<0.05$

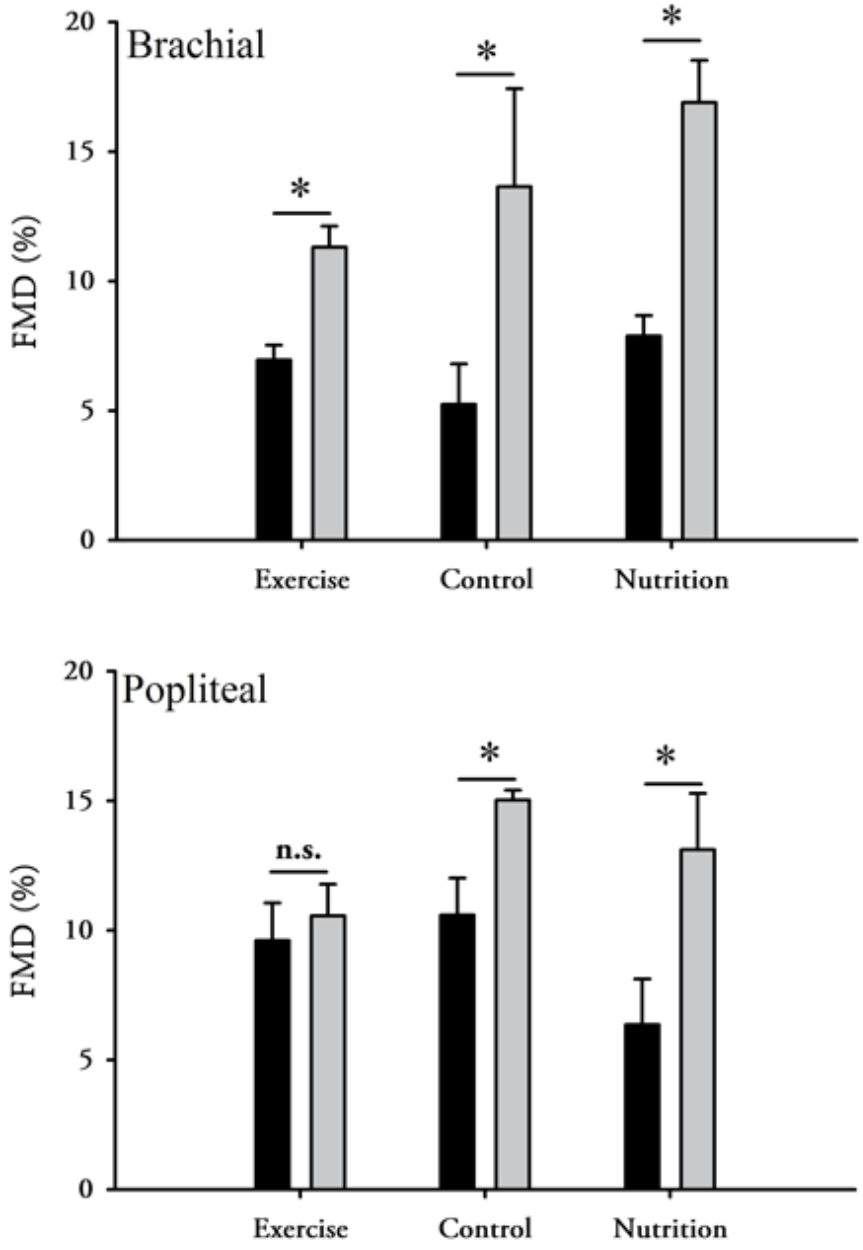


Figure 6.2 FMD before and after 56 days of head down tilt bed rest for exercise countermeasure, control, and nutrition groups.

FMD of the brachial artery was greater in every group at 56 days of head down tilt. For the popliteal artery the increased FMD at 56 days head down tilt is attenuated in the exercise group. Black bars: pre-HDBR, Grey bars: 56 days-HDBR (60 sec post cuff release or 5 min post NG spray), Means±SE, *significantly different dilation, $p<0.05$.

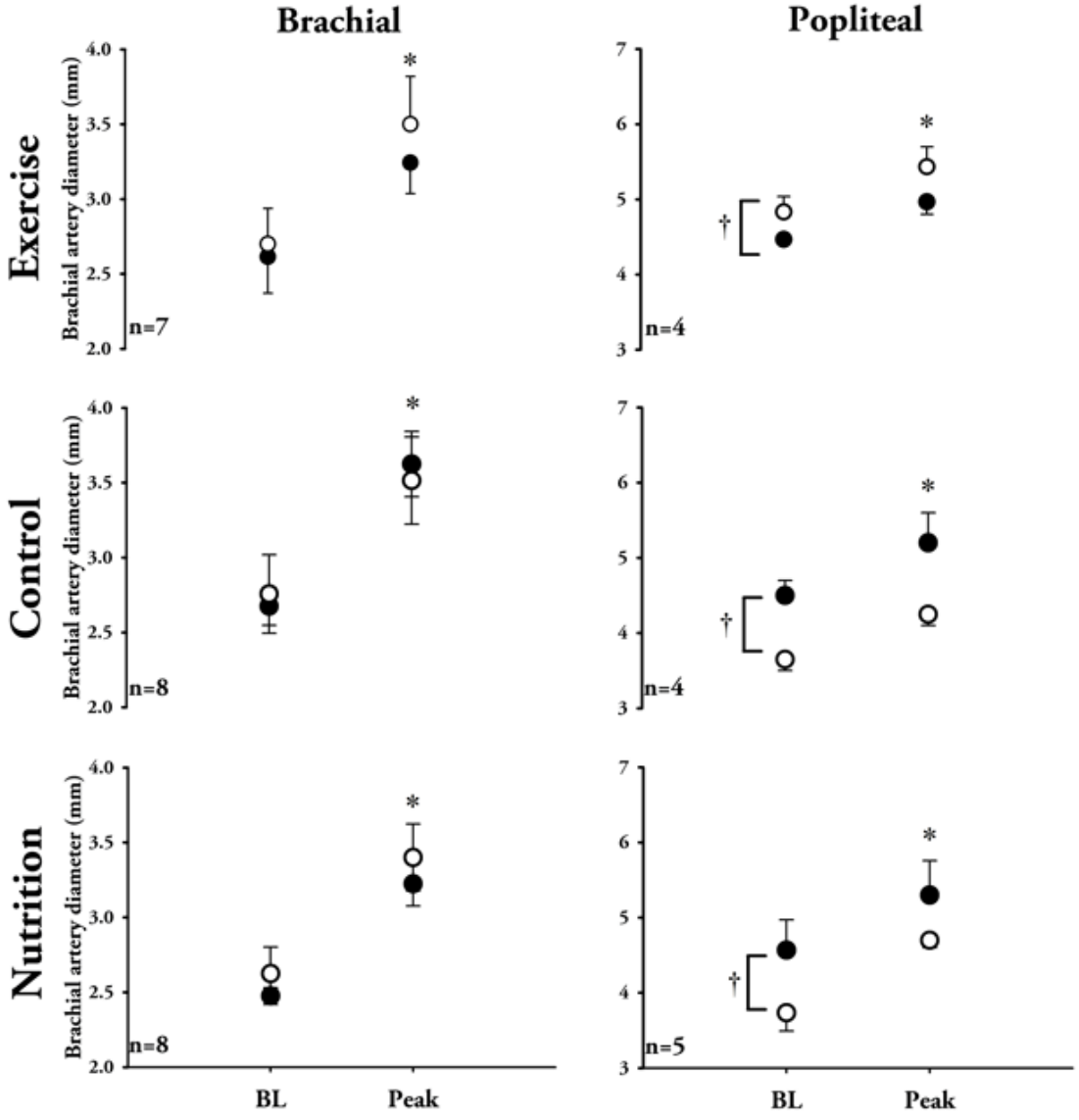


Figure 6.3 Diameter of the brachial and popliteal arteries following sublingual nitro-glycerine before and after 56 days of head down tilt bed rest for exercise, control, and nutrition.

Symbols represent Means±SE diameter (mm) at baseline (BL) and 5 min after nitroglycerine spray (Peak). Closed circles: Pre-HDBR, Empty circles: 56 days-HDBR. *significantly different from baseline, †significantly different at baseline, $p<0.05$

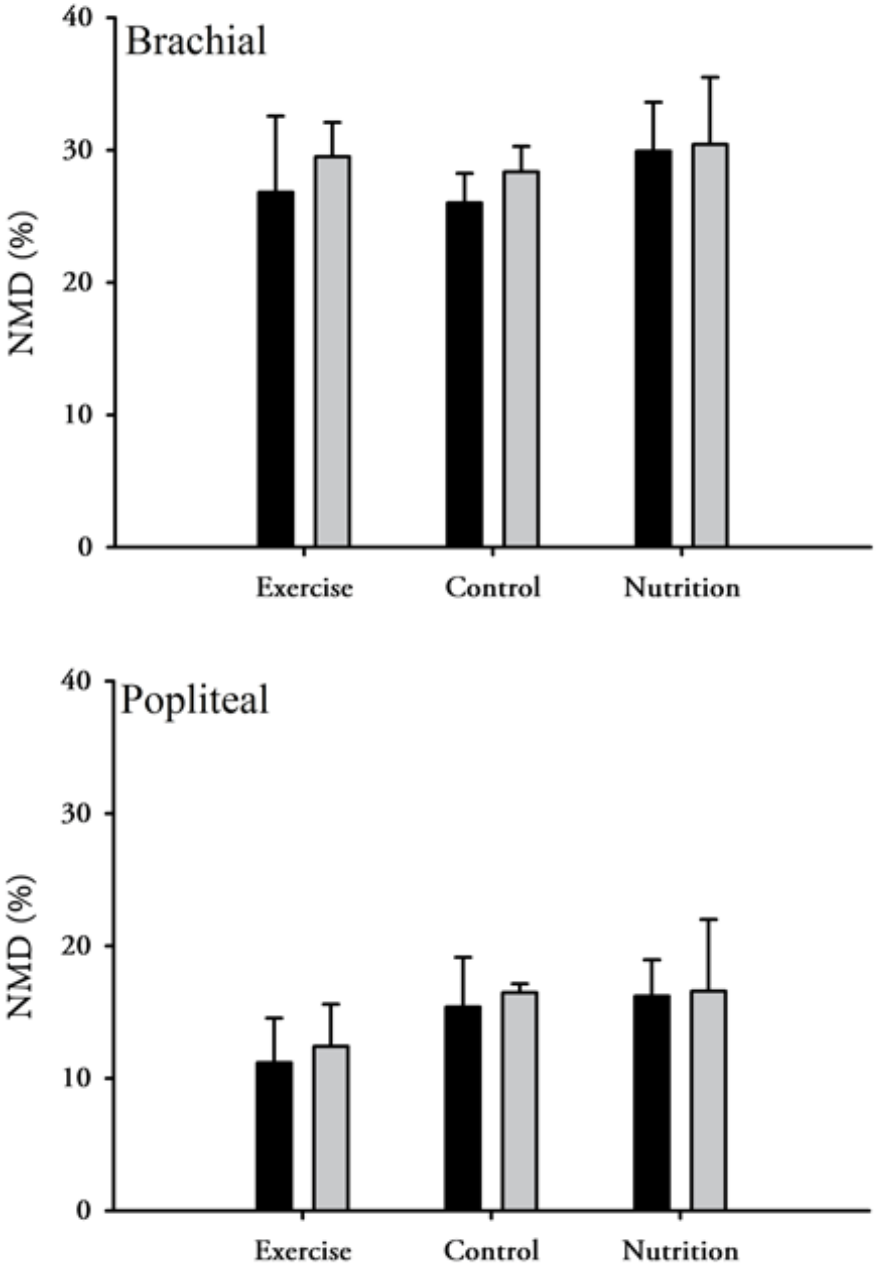


Figure 6.4 NMD before and after 56 days of head down tilt bed rest for exercise countermeasure, control, and nutrition groups.

There was no effect of 56 days of head down tilt on brachial (A) or popliteal (B) NMD. Black bars: pre-HDBR, Grey bars: 56 days-HDBR (60 sec post cuff release or 5 min post NG spray), Means±SE.

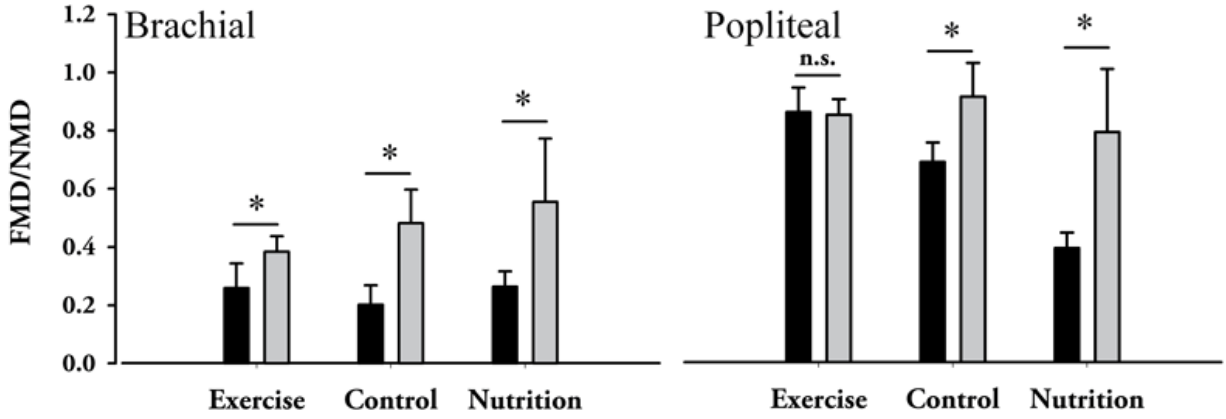


Figure 6.5 The ratio between nitro-glycerine and flow-mediated dilation before and after 56 days of head down tilt bed rest.

The FMD to NMD ratio was greater post 56 days of head down tilt in the brachial of all groups, while the ratio was preserved in the popliteal of the exercise group. Black bars: pre-HDBR, Grey bars: 56 days-HDBR, Means±SE, *significantly different, $p<0.05$.

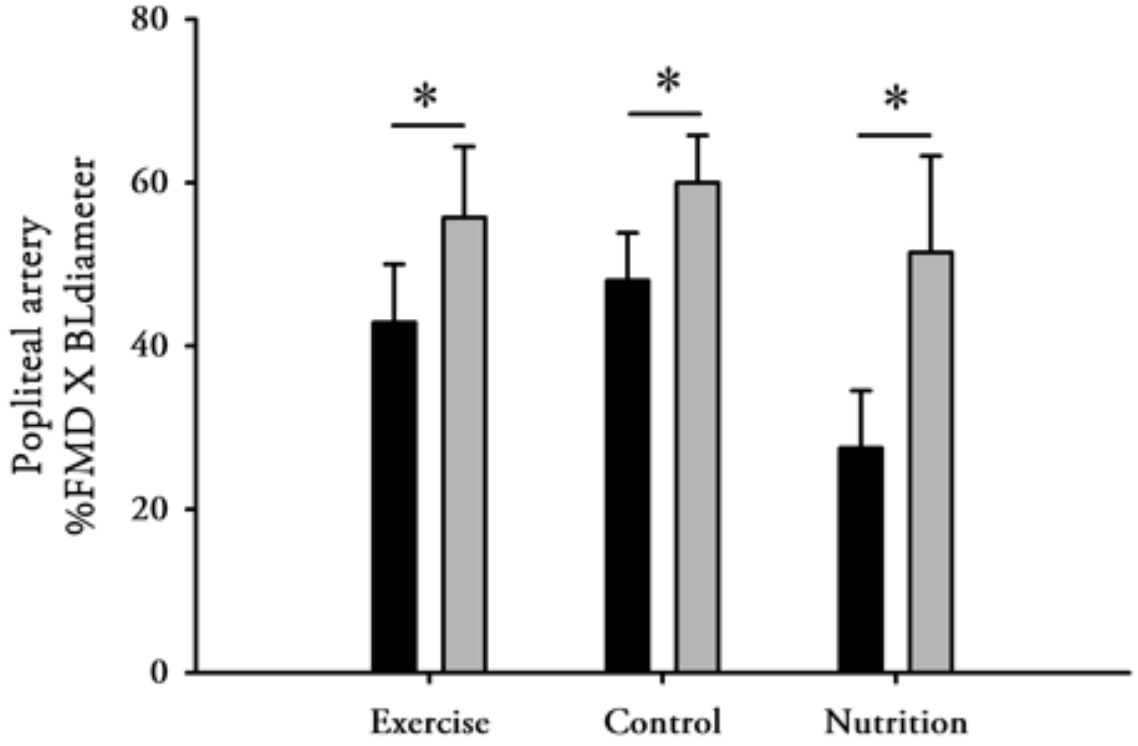


Figure 6.6 Popliteal FMD, independent of baseline diameter, before and after 56 days of head down tilt bed rest for exercise countermeasure, control, and nutrition groups.

%FMD × BLdiameter of the popliteal artery is enhanced in all groups after 56 days of head down tilt bed rest. Black bars: pre-HDBR, Grey bars: 56 days-HDBR. Means±SE, *significantly different, $p<0.05$.

Discussion

The main findings of this investigation were that FMD was augmented by HDBR in the brachial artery regardless of activity level, while pre-HDBR popliteal FMD was preserved post-HDBR in the group of subjects that performed regular exercise. The changes in FMD were still evident when %FMD × BLdiameter was used to account for the reduction in the luminal cross-sectional area of the popliteal artery. The baseline cross-sectional area of the brachial artery was not affected by HDBR. The increased dilatory response to shear in the conduit vessels may create an increased risk of orthostatic intolerance (OI) upon resumption of an upright posture in terrestrial gravity. The exercise countermeasure used in this study appears to counteract the enhancement of endothelial function in the lower limbs and may help to reduce the risk of OI.

Shear Rate

Peak shear rates following cuff release were not affected by HDBR or by either of the countermeasures. This would seem to rule out the change in shear stimulus being the cause of altered FMD. However, we were unable to calculate total shear to peak diameter due to a lack of frequent measurable ultrasound images. Shear rate is determined by three factors: 1) blood viscosity, 2) blood flow, and 3) vessel cross sectional area. We have no data for blood viscosity in this study; however hematocrit, a suitable marker, was shown to be similar in all groups before and following bed rest (unpublished data). Blood flow was reduced in the legs of the non-exercisers and increased in the exercisers, while the opposite effect was seen with respect to cross-sectional area. It is difficult to speculate as to which of reduced area or reduced flow is the cause or effect.

Exposure to periodic bouts of high shear, as are evident during physical exercise, has been proposed as being a factor in vasculogenisis. Demiot et al. (2007) found, in these same subjects, that there was an increase in circulating endothelial cells in the non-exercising groups. One interpretation is that these cells could have originated from atrophic capillaries. A reduction in capillary density could explain the decreased flow during hyperaemia in the non-exercisers. In any event the shear rate at rest and during the reactive hyperaemia stimulus of the FMD protocol were unchanged following bed rest in all groups, thus strengthening the argument that the endothelium had undergone an adaptation.

Vascular Deconditioning

Previously it has been found that 52 days of HDBR resulted in a reduction in the diameter and augmented FMD and NMD of the of the superficial femoral artery, with only the baseline diameter effect being attenuated by vibration exercise (Bleeker et al., 2005). They found that deconditioning did not affect the FMD/NMD relationship, concluding that deconditioning had a greater effect on general smooth muscle cell $\dot{N}0$ responsiveness than on endothelial function. This was not the case in the present study where we found an augmented FMD/NMD ratio due to no change in NMD post HDBR. Our results are more in line with those of Bonnin et al. (2001) who found that FMD, but not NMD, measured at the end of 7 days of HDBR was increased compared to before HDBR, and previous animal data showing a reduction in endothelium-dependent dilatation with 14 days of hind-limb unloading in rats (Schrage, Woodman, & Laughlin, 2000; Woodman et al., 2001). Bonnin et al. (2001) showed that FMD was negatively correlated with the duration of orthostatic tolerance, suggesting that post HDBR orthostatic

intolerance is at least partially related to enhanced endothelial function. Hindlimb unloaded rats showed a significant increase in tissue NOx content and an upregulation of iNOS protein compared to controls and was associated with marked attenuation of hypertensive response to norepinephrine and a significant increase in hypertensive response to aminoguanidine, suggesting enhanced iNOS-derived NO generation (Vaziri, Ding, Sangha, & Purdy, 2000). The authors speculate that if true in humans, administration of an iNOS inhibitor may reduce the instances of orthostatic intolerance after long term HDBR. Assessment of orthostatic tolerance was not within the scope of this paper. Further, over-all responsiveness to vasoconstrictor stimuli also seem to be blunted after HDBR as the ability of the cold pressor test to lower forearm blood flow was shown to be less in a post- vs. in a pre-HDBR test (Shoemaker et al., 1998).

FMD and Orthostatic Stress

It has been demonstrated that FMD is greater in the head up tilt (HUT) position than in the supine position, and that the magnitude of FMD is dependent on angle of tilt (Guazzi et al., 2004). However, the results of that study are in direct conflict with the results of studies examining FMD under lower body negative pressure (LBNP) which showed no change in (Dyson et al., 2006) or attenuation of (Hijmering et al., 2002) the FMD response to reactive hyperaemia. LBNP is used to unload the baroreceptors and simulates the fluid shifts associated with HUT without physical movement of the body. The findings that HUT, but not LBNP, may increase the degree of FMD suggest the possibility that endothelial function is partially influenced by mechanisms that are linked to spatial orientation. It may be speculated that these mechanisms also play a role in the adaptations to

prolonged bed rest.

Exercise and the Endothelium

It has been reasoned that repeated episodes of increased shear stress during regular exercise lead to the restoration of endothelial function (Niebauer & Cooke, 1996; Green et al., 2004), likely due to up-regulating eNOS nRNA and protein resulting in greater Ṅ0 release in response to a high shear stimulus (Uematsu et al., 1995; Nishida et al., 1992). The finding that FMD was preserved only in the lower limb is suggestive that the effects of exercise are local, at least in the conditions of the present investigation. However, previously it has been demonstrated that run trained subjects have a higher FMD than sedentary, however this could be due to the higher hyperaemia following occlusion in the trained subjects (Libonati, 2007). In general it has been found that predominantly lower body focused aerobic exercise is beneficial in restoring endothelial function in a systemic fashion in those suffering from (Walther et al., 2004) or exhibiting risk factors for (Higashi et al., 1999a) CVD. To the best of our knowledge there is no precedent research examining the role of exercise in normalising previously enhanced endothelial function, such as we found with HDBR.

Conduit vs. Micro-circulation

The results of this study contrast with the findings of impaired endothelial function following HDBR in the microvasculature of these same subjects. Demiot, C, Dignat-George, F, Fortrat, J O, Sabatier, F, Gharib, C, Larina, I, Gauquelin-Koch, G, Hughson, R, and Custaud, M A, (2007) found that endothelium-dependent vasodilatation in the cutaneous micro-circulation at the level of the calf, as

assessed by laser Doppler after iontophoresis of acetylcholine, was significantly reduced by HDBR only in those of the non-exercising groups. The discrepancies in results of these investigations may be explained by intrinsic differences in endothelial function between the conduit arteries and the micro-circulation. It has been shown that there is no linear relationship between microvascular FMD, assessed by laser Doppler following iontophoretic administration of acetylcholine, and brachial FMD (Hansell, Henareh, Agewall, & Norman, 2004). It is possible that HDBR promotes the pathogenesis of CVD in the micro-circulation, while increasing risk for orthostatic intolerance due to hypersensitivity of the endothelium in the conduit arteries to flow/shear stimuli.

Conclusion

The multifaceted effects of this countermeasure demand further investigation to determine its overall usefulness. In particular, there should be an assessment made on increasing the number of muscle groups activated during the countermeasure as this study found that predominantly lower body focused exercise targeted endothelial function in the lower limbs. These data demonstrated that EX was effective in countering the bed rest-induced changes in endothelium-dependent vascular responses in the popliteal artery.

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Chapter 7 - **General Discussion**

The results of the six manuscripts in this thesis have demonstrated the following:

I. A short burst of high shear stimulus was not adequate to cause a discernable degree of FMD. (Chapter 2)

II. Intermittent post occlusion hyperaemia increased the %FMD compared to continuous hyperaemia when normalised to the AUC_{shear} stimulus. (Chapter 4)

III. Adding exercise to the occlusion period enhanced post occlusion hyperaemia, but not FMD. Also, the linear relationship between AUC_{shear} and FMD was lost when exercise was added to the occlusion period. (Chapter 3)

IV. Exercise itself was not able to provide as robust a dilation response as mechanically initiated shear oscillations when both were normalised to the AUC_{shear} stimulus. (Chapter 4)

V. While the absolute diameter reached in the post occlusion period was the same in the control condition or when the FMD test was preceded by bouts of heavy hand grip exercise, calculated percent FMD following reactive hyperaemia was less in the post exercise condition due to a shift in baseline diameter. The reduced %FMD in the post exercise condition stresses the caution that should be used when interpreting values of %FMD in the evaluation of endothelial function, especially in instances where pre test physical activity may be involved. Similar results were found during the 2^{nd} of 2 exercise bouts. (Chapter 5)

VI. 56 days of HDBR was associated with enhanced FMD, but not NMD, of the brachial and popliteal arteries, with a predominately lower body focused exercise countermeasure preserving pre-HDBR FMD in the popliteal.

(Chapter 6)

From these results some insight can be gained, and several questions have been raised, concerning the effects of exercise on the vascular endothelium as well as in the FMD test for endothelial function. These will be the foci of the final discussions.

The FMD test

The purpose of the FMD test is to quantify vascular dilation in response to a shear stimulus. It has been reported, in this thesis (Chapter 2) as well as in other studies (Betik et al., 2004; Silber et al., 2001), that a larger stimulus results in a greater dilation. Results from this thesis suggest that this is not the case when comparing a similar shear stimulus derived from long period of occlusion to one from a shorter period of occlusion with added exercise. In the studies in this thesis shear was linearly correlated to dilation in the non-exercise conditions, while the linearity was lost when exercise was added, either during (Chapter 3) or before (Chapter 5) the period of occlusion. This suggests the possibility that the dilation that accompanies exercise, both exercise alone and when embedded in occlusion, is mediated by more than just the shear stimulus. Indeed, it was found that there was no significant increase in plasma nitrite concentration following the protocol that employed 1 min of moderate exercise in 5 min of occlusion, even though this protocol did elicit a shear stimulus and dilation that was not different from 15 min of occlusion alone (Chapter 3). It should also be noted that the relationship between AUC_{shear} and FMD following occlusion alone may be limited to healthy adults (Thijssen et al., 2009). The subjects in our studies were all highly trained athletes, so it is safe to assume that the linearity should hold with them; however caution should be used when interpreting shear to dilation response in a clinical population.

Limb occlusion alone, with the cuff distal to the site of ultrasound monitoring, does not seem to have any effect on the baseline state of the artery (Corretti et al., 2002). However, it has been reported that 5 min of distal arm occlusion resulted in an increase in brachial artery diameter of 0.1mm that

reached statistical significance in young adults and children, but not in elderly, subjects (Thijssen et al., 2008). This vasodilatation was perhaps due to local transmural pressure changes (Laughlin, Newcomer, & Bender, 2008) or local tissue displacement influencing image acquisition. However, this was not the case in the studies in this thesis, nor in any other study to the best of my knowledge (Betik et al., 2004; Parker et al., 2006). In the occlusion protocols employed in this thesis the brachial artery was measured just distal to the axilla, which was perhaps far enough away from the cuff placement to not be subject to image distortion. Therefore, it is more than likely the case that the dilation observed in these studies, and with others using the distal cuff placement with ample space between cuff and probe, is truly a change from baseline diameter.

There is some controversy as to whether the 15 min of occlusion used as the control condition for endothelium dependent FMD in the experiments for this thesis produces an FMD which is $\dot{N}0$ dependent. It has been reported that L-NMMA blocks the FMD response after 5 min but not 15 min of occlusion indicating that only the former is $\dot{N}0$ dependent (Mullen et al., 2001). However, in contrast to the studies in this thesis Mullen et al. (2001) showed that 5 and 15 min of occlusion had similar peak shear stimuli. This may be due to the nature of the arteries examined. Mullen et al. (2001) examined the radial artery, which has a much smaller diameter than the brachial, used in this thesis. The peak shear to hand occlusion would be much less than for forearm occlusion and as such could have been maximized at the 5 min mark of occlusion. The fact that there was no attenuation in FMD response following 15 min occlusion can be explained thusly: increased AUC_{shear} could increase the production of $\dot{N}0$ while attaining the maximal dilation of ~10%. The specific dose of L-NMMA could therefore have been blocking

redundant production of eNOS. Perhaps a larger dose would have resulted in an attenuation, or perhaps the dilation is mediated by Ṅ0 in physiological conditions but other compensatory mechanisms take over when Ṅ0 is blocked. It has been previously reported that EDHF can play a major part in flow-mediated dilation, at least during a sustained high shear stimulus during limb heating (Bellien et al., 2006).

The results of the studies in this thesis suggest that exercise-mediated dilation may not be a reliable indicator of endothelial function. The controversy arises from the fact that the shear stimulus during exercise is oscillatory in nature and that muscular work produces a number of metabolic by-products that leak into the surrounding circulation. In any event, either of those factors, or a combination of the two, appears to alter the shear to dilation relationship that is established in tests of FMD that employ strict reactive hyperaemia via distal limb occlusion and even those studies using sustained a high shear stimulus during limb heating.

Currently the only method that is proven effective to measure Ṅ0 dependent FMD is the 5 min occlusion protocol (distal to the elbow) without exercise. However, the relatively small FMD (~5%) requires great precision in ultrasound technique (should be performed by a skilled technician) in B-mode with real time edge tracking to capture the full dilation curve. However, caution must also be used when accepting the declaration of Ṅ0 dependence, as administration of L-NMMA may reduce post ischemia hyperaemia (Meredith et al., 1996). Meredith et al. (1996) found that L-NMMA infusion significantly reduced the peak and AUC of flow by 15% when normalized to the reduction in baseline flow. This flow attenuation, and perhaps not Ṅ0 blockade, may contribute to the reduced FMD

seen with L-NMMA. It is vital that the shear to dilation relationship for pure endothelial dependent FMD be characterized in order for the test to be clinically or experimentally viable.

Another concern when testing for endothelial function is the baseline architecture of the artery. For example, the baseline brachial artery diameter is larger in men than in women. This is reflected in a lower FMD in men than women that is not seen in NMD. If FMD is multiplied by the baseline diameter (%FMD × BLdiamter index), the differences between male and female FMD disappear (Mizia-Stec et al., 2007). There may be architectural factors that come into play that would limit or enhance the FMD response based on initial diameter (Daemen & De Mey, 1995). However, there may also be a role of the wall to lumen diameter ratio which has been shown to be linked to responsiveness to various vasoactive stimuli, at least in resistance vessels (Folkow et al., 1958; Folkow, 1978). In the studies in this thesis we confront two distinct modes of changes in arterial baseline diameter. The first mode is an acute change in the wall to lumen ratio brought on by a previous dilation inducing protocol, such as in the second cuff release in Chapter 2 and the previous exercise bouts in Chapter 5. The second mode was one in which it is likely that there were changes in architectural factors beyond a simple change in initial diameter. It is unknown what effects these would have on subsequent endothelial function and if the %FMD × BLdiamter index would negate any baseline size bias in either or both of these cases. It is for this reason that all aspects of diameter change were reported in the manuscripts. It is hoped that continued research in this field, combined with the finding in these and other similar investigations, will develop a consensus wherein a clearer picture of the structure-function relationship in the vascular endothelium can be established.

The increased hyperaemia resulting in a greater % dilation following 15 min over 5 min of occlusion makes it an attractive method for determining FMD. It seems rather clear that adding exercise to shorter periods of occlusion is not appropriate, given the dissociation from the AUC_{shear} stimulus, even in cases where the baseline diameter was not affected. AUC_{shear} should be reported, but its use as a normalising factor is still under consideration. Baseline diameter changes should also be considered. It is important to note that the use of the %FMD × BLdiamter index, while apparently effective in normalizing %FMD between subjects having different baseline diameters, particularly between males and females (Mizia-Stec et al., 2007), has not been validated in the cases of acute changes of baseline diameter within the same vessel. The differences between individuals are most certainly architectural in nature, while acute changes in baseline diameter (in these cases following exercise) are likely the result of multiple vaso-regulatory mechanisms which can interact with the flow-mediated response. The results of this thesis would suggest that the use of the 5 min occlusion protocol should be recommended for determination of FMD, until such time that the 15 min occlusion protocol is determined to be $\dot{N}0$ dependent, or the exercise mediated changes in diameter are fully characterized.

Exercise and Endothelial Function

The results of the studies in this thesis have shown that exercise does indeed affect the physiologic milieu in which a particular blood vessel will dilate in response to an increase in flow. Shear following 15 min occlusion, both total and peak, was directly correlated to dilation (Chapter 3, Chapter 4, and Chapter 5), while when the test was preceded by forearm exercise (Chapter 5), or included forearm exercise during occlusion (Chapter 3), this relationship was uncoupled. In contrast, a long period of HDBR resulted in an increase in FMD in both upper and lower limbs with no change in NMD (Chapter 6). This was in agreement with the findings of Bleeker et al. (2005) in the superficial femoral artery, but not with the findings of Demiot et al. (2007) who found impaired endothelial function in the microcirculation. The discrepancies with Demiot et al. (2007) may be related to differences between adaptations in the micro- vs. conduit circulation. Indeed, there is doubt as to the linearity of the relationship between micro-vascular FMD, assessed by laser Doppler following iontophoretic administration of acetylcholine, and brachial FMD (Hansell et al., 2004).

Regular exercise is among the strategies used to repair a dysfunctional endothelium (Hambrecht et al., 2000; Higashi et al., 1999a; Hambrecht et al., 2000) and it is reasoned that it is the increased shear stress during the exercise bouts that is primarily responsible for the restoration of endothelial function (Niebauer & Cooke, 1996; Green et al., 2004). Upregulation of both eNOS nRNA and protein have been shown to result from exercise and initiate greater Ṅ0 release in response to a given high shear stimulus, such as from a reactive hyperaemia test (Uematsu et al., 1995; Nishida et al., 1992). Exercise training may also enhance endothelial function in healthy adults as well, run trained subjects have a

higher FMD than matched sedentary control subjects (Libonati, 2007).

Acute bouts of exercise have also been shown to be beneficial to endothelial function. Harris et al. (2008) found that acute treadmill walking improved FMD in active but not inactive overweight men. Reduction in sympathetic activity following exercise (Floras et al., 1989; Pober et al., 2004) may account for the greater FMD in the post exercise state. Harvey et al. (2005) explained increased FMD in older but not younger women after a single bout of exercise by sympatholysis in the older women. Similar results have been found in men (Thijssen 2006). Previous studies examining the effect of acute exercise on FMD have looked only at whole body aerobic exercise bouts (Padilla et al., 2007). In this thesis the effect of a prior exercise bout on FMD was studied in the same limb as the exercising muscle (Chapter 5). In contrast to the studies using whole body exercise, the acute forearm exercise utilised in this thesis resulted in a smaller %FMD response to reactive hyperaemia. As was discussed above, it is important to note the discrepancies in the baseline diameters and as such the greater diameters in the post exercise baseline state in Chapter 5 were taken into consideration using the %FMD × BLdiameter index. It would not be possible to test FMD with similar diameters pre and post acute exercise as it has been reported that artery dilation can persist for over an hour post exercise (Harris et al., 2008; Wallace et al., 1999). The %FMD × BLdiameter index showed that FMD was attenuated independent of the baseline diameter shift, however this index has not been proven to be valid in an acutely changed vessel.

The results of this thesis suggest that acute exercise has the effect of masking a functional endothelium by producing a lower value for %FMD while the peak diameter in response to the hyperaemia stimulus is similar to control. Of

particular concern is the uncoupling of the relationship between shear and dilation when exercise is introduced to the FMD protocol. The experiments in this thesis were unable to discern which aspect(s) of exercise are responsible for these effects. It is possible that it could be related to metabolic by-products produced during the bout or to the oscillatory nature of the shear stimulus during the bout. An alternative explanation may be that the change in arterial diameter during exercise, which persisted to the baseline of the post-EX FMD test, masked the fully functioning dilatory mechanisms. Indeed, the experimental results in Chapter5 showing that absolute brachial artery diameter was similar in both pre and post exercise tests suggest maintained vascular function. There is no indication that these effects should last long term, and in fact the acute negative effects may translate to positive effects if the exercise is performed periodically. That long duration inactivity enhanced FMD in both upper and lower limbs was surprising, if not novel. The mechanisms by which exercise preserved pre bed rest FMD, and its implications on orthostatic tolerance need to be further examined.

Conclusions

This thesis attempted to

1. Evaluate different methods of testing flow-mediated endothelium dependent dilation in human conduit arteries.
2. Characterize the effects of exercise, acute and regular, on the results of FMD tests.

The results of the first study presented above provide evidence that a sustained high shear stimulus is required to generate an FMD response, and that the magnitude of FMD is dependent on the total shear to the time of peak dilation, not the peak shear post cuff release. The next two studies showed that the addition of exercise to the prior occlusion or adding oscillations to the post occlusion shear produce similar FMD, however the dependence on total shear to peak dilation is lost. If endothelial function is to be characterized by a an increase in lumen diameter to a given shear stress then protocols involving exercise or shear oscillations would not make good candidates for tests. It is possible that the exercise during occlusion may confound the results of the FMD evaluation by the initiation of sympathetic nerve activity, evidenced by elevated blood pressure, or the use of non-$\dot{N}0$ dilators that may or may not be shear rate sensitive, evidenced by the lack of increase in plasma nitrite following occlusion with exercise.

The last two studies looked at the effects of acute and regular exercise on subsequent FMD tests. It was found that brachial artery endothelial function was blunted following an acute heavy hand grip exercise, as revealed by a lower FMD following 15 minutes of forearm occlusion. The reasons for this blunting are as yet unknown, but may be related to a lag in the shear to $\dot{N}0$ release machinery, given that plasma nitrite elevation was delayed compared to the control FMD test and the

shear to dilation relationship was uncoupled as with the previous studies involving exercise. The long term effects of exercise were shown to be quite the opposite. Inactivity resulted in an increase in shear to dilation sensitivity, which was effectively attenuated by the addition of an exercise countermeasure in the exercising limbs.

Future Considerations

This collection of studies examined the outcomes of various methods employed to elicit a flow-mediated dilation response in conduit arteries, an index of the function of the vascular endothelial Ṅ0 system, and to determine the possible influence of physical activity, both acute and regular, on this response. The protocols and measurements in these experiments were by and large non-invasive in nature. While this affords some advantages, such as the ability to observe physiological phenomena in a predominantly undisturbed state, the ability to determine the mechanistic processes underlying the measured results is compromised. The following list outlines my recommendations for further study of the issues presented in each chapter.

- **Chapter 2:** The FMD protocol in this experiment used a 15 min period of forearm occlusion as the priming condition to produce the reactive hyperaemia stimulus. The assumptions were made that dilation after this duration of occlusion was i) flow-dependent ii) mediated by the vascular endothelium and iii) initiated by Ṅ0. There is the possibility that ischemia of the conduit vessel itself during this long a period of circulatory occlusion might initiate a propagated dilation upstream of the occlusion. This type of cell to cell communication has been reported in feed arteries after arterioles were treated with the endothelium dependent vasodilator acetylcholine (Uhrenholt, Domeier, & Segal, 2007). Although the likelihood of this being the case with our subjects is low, it would be beneficial to have confirmation of the flow-dependence of post 15 min occlusion dilation. The endothelium and Ṅ0 dependence of the dilation to this protocol could be confirmed with the blockade of various endothelial cell mediated dilator substances by

infusion of such drugs as L-NMMA to block eNOS (Rees, Palmer, Hodson, & Moncada, 1989), ketorolac tromethamine to block prostaglandins (Momen, Cui, McQuillan, & Sinoway, 2008), and brefeldin A to block endothelium-derived hyperpolarizing factors (Bauersachs, Fleming, Scholz, Popp, & Busse, 1997).

- **Chapter 3:** This study examined the effect of the addition of dynamic forearm exercise to an occlusion protocol on the subsequent dilation of the brachial artery following cuff release. The results of the loss of the shear-to-dilation relationship and the lack of observable increase in plasma nitrite in the forearm venous effluent when exercise was added to the occlusion, let me to the speculative conclusion that the mechanisms for dilation in this protocol were different from those following 15 min of occlusion alone, and that the dilation was not mediated by $\dot{N}0$. As was stated above for the experiment in Chapter 2, this type of study would benefit from the addition of specific dilator blockade to provide a more robust indication of the mechanistic differences, if any, between the protocols. In addition, the exercise was of a severity that elicited changes in central and peripheral cardiovascular indices indicative of sympathetic nervous system activation. Involvement of the autonomic nervous system in either enhancing or attenuating the FMD response cannot be ruled out without employing sympathetic blockade in similar experiments. Isolation of sympatheticly mediated responses could be achieved using doxazosin for α1-receptor blockade (Vincent, Elliott, Meredith, & Reid, 1983) and propranolol for β-receptor blockade (Prichard & Gilliam, 1964), or alternatively full sympathetic blockade could be applied in an animal model (Brown &

Maycock, 1942).

- **Chapter 4:** The main concern with the dilation results in this study was that they were not achieved under the same shear stress conditions. The exercise bout had by far the greatest total shear to the time of peak dilation and this corresponded to a higher % dilation than that reached post 15 min of occlusion, both when the shear was continuous and intermittent. However, when mathematically normalized to the shear stimulus exercise dilation was equivalent to that following 15 min of occlusion and the intermittent achieved the greatest dilation response. It wou*l*d be beneficial to attempt to physically normalize the shear stimuli during these conditions, perhaps using arterial compression (Pyke, Poitras, & Tschakovsky, 2008). Again I must stress the usefulness of local vasodilator blocking agents in the future investigation of the FMD response. These studies could shed more light as to the possible mechanistic differences between exercise and reactive hyperaemia induced vasodilatation, and serve to differentiate the effects of local metabolic activity and the intermittent shear stimulus.
- **Chapter 5:** The finding that %FMD following 15 min of occlusion was lower after acute forearm exercise compared to control may have been confounded by the fact that baseline diameter at the end of occlusion was greater in the post exercise condition. The reduction was still evident when the diameter change was mathematically normalised to baseline diameter, but that would not compensate for a possible absolute FMD ceiling. It would be beneficial to more fully characterize FMD in response to steadily increasing shear to determine if there is a ceiling to FMD that is independent of the full vasorelaxation capacity of the smooth muscle. This could be achieved in an

animal model using isolated cannulated vessels. There are a number of factors that are affected by exercise, including the release of vasoactive metabolites into the circulation, influence on the sympathetic nervous system, and, as observed in this investigation, a delay in the endothelial ṄO machinery during a subsequent reactive hyperaemia. Once again, local vasodilator and sympathetic nervous blockade would help to determine mechanistic differences pre and post acute exercise, if they exist.

- **Chapter 6:** The final study in this thesis found that FMD was enhanced following 56 days of head down tilt bed rest in both upper and lower limb conduit arteries; while a mainly lower body focused exercise regimen attenuated the response in the lower, but not upper, limb. It also found that endothelium independent NMD was not affected by HDBR, suggesting that the endothelium, and not the smooth muscle reactivity to ṄO, was the target of the adaptation. Previous studies using the rat hind limb unloading model also found that the endothelium was affected (Schrage et al., 2000; Woodman et al., 2001) but also note that iNOS was up-regulated (Vaziri et al., 2000), perhaps increasing ṄO bioavailability via that pathway. Future studies should investigate the effects of long term unloading on endothelial function after selective blockade of iNOS by aminoguanidine, N(6)-(l-iminoethyl)-L-lysine or S-methylisothiourea (Chan, Wang, Wang, & Chan, 2001). Additionally, a thorough analysis of the orthostatic tolerance of these subjects should be performed in order to determine the relative contribution, if any, augmented FMD had on the incidences of orthostatic intolerance following HDBR.

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