Impact of repeated increases in shear stress via reactive hyperemia and handgrip exercise: no evidence of systematic changes in brachial artery FMD

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Submitted 26 July 2010; accepted in final form 17 December 2010

Pyke KE, Jazuli F. Impact of repeated increases in shear stress via reactive hyperemia and handgrip exercise: no evidence of systematic changes in brachial artery FMD. Am J Physiol Heart Circ Physiol 300: H1078–H1089, 2011. First published December 24, 2010; doi:10.1152/ajpheart.00736.2010.—Reactive hyperemia (RH) creates an uncontrolled, transient increase in brachial artery (BA) shear stress (SS) for flow-mediated dilation (FMD) assessment. In contrast, handgrip exercise (HGEX) can create similar, sustained SS increases over repeated trials. The purpose of this study was to examine the impact of repeated SS elevation via RH or HGEX and the relationship between RH and HGEX %FMD. BA diameter and blood velocity were assessed with echo and Doppler ultrasound in 20 healthy subjects. Visit A consisted of four 6-min HGEX trials (HGEX trials 1–4) at the intensity required to achieve a shear rate (SR = mean blood velocity/BA diameter; an estimate of SS) of 65 s⁻¹. Visit B consisted of four RH trials (RH trials 1–4). The RH SR area under the curve (AUC) was higher in trial 1 versus trial 3 and trial 4 (P = 0.019 and 0.047). The HGEX mean SR was similar across trials (mean SR = 66.1 ± 5.8 s⁻¹, P = 0.152). There were no differences in %FMD across trials or tests (RH trial 1: 6.9 ± 3.5%, trial 2: 6.9 ± 2.3%, trial 3: 7.1 ± 3.5%, and trial 4: 7.0 ± 2.8%; HGEX trial 1: 7.3 ± 3.6%, trial 2: 7.0 ± 3.6%, trial 3: 6.5 ± 3.5%, and trial 4: 6.8 ± 2.9%, P = 0.913). No relationship between subject’s RH %FMD and HGEX %FMD was detected (r² = 0.12, P = 0.137). However, with response normalization, a relationship emerged (RH %FMD/SR AUC vs. HGEX %FMD/mean SR, r² = 0.44, P = 0.002). In conclusion, with repeat trials, there were no systematic changes in RH or HGEX %FMD. The relationship between normalized RH and HGEX %FMD suggests that endothelial responses to different SS profiles provide related information regarding endothelial function.

endothelial function; Doppler ultrasound; shear stress profile; flow-mediated dilation

WHEN BLOOD FLOW through a vessel increases, the resultant increase in shear stress on the vascular endothelium causes an endothelial-dependent vasodilation [flow-mediated dilation (FMD)]. The magnitude of this vasodilatory response can be taken as an index of endothelial function. The most common endothelial function test performed in humans assesses conduit artery FMD after a transient increase in blood flow-associated shear stress generated by the release of a temporary limb occlusion [reactive hyperemia (RH)] (7). FMD tested in this fashion is impaired in a myriad of disease states, and early endothelial dysfunction is thought to play an important role in the pathogenesis of atherosclerosis (5, 51). FMD (and therefore endothelial function) can also be impaired in a transient fashion with insults such as a high-fat meal (13, 47, 56) or an experience of mental stress (6, 8, 14, 42).

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RH after the release of limb occlusion results in a transient and uncontrolled increase in shear stress. This can complicate interpretation because it may be unclear whether differences in FMD response magnitude are a result of differences in endothelial function or differences in the magnitude of the shear stress to which the vessels were exposed. Accounting for the magnitude of the stimulus has been attempted with both an analysis of covariance procedure (25) and by performing a ratio normalization in which the magnitude of the FMD response is divided by the magnitude of the stimulus (25, 31, 38). Both of these procedures have received criticism (18, 46, 50), and the problem of accounting for the stimulus magnitude remains an unresolved issue in human endothelial function research.

In contrast to RH, exercise results in a sustained, contraction intensity-dependent (55) increase in shear stress that can be targeted at a specific magnitude (36). This eliminates the need for post hoc normalization procedures. While the mechanisms responsible for FMD in response to a sustained stimulus versus a transient RH stimulus may vary (29), there is growing evidence that FMD resulting from non-RH increases in shear stress is impaired in individuals with cardiovascular disease risk factors (2, 12, 17). Indeed, in studies by Bellian et al. (2) and Grzelak et al. (17), heating and handgrip exercise (HGEX)-induced increases in shear stress, respectively, resulted in larger FMD differences between control and interest groups (type I diabetic and healthy older subjects) than did FMD after RH-induced increases in shear stress. Therefore, testing FMD in response to exercise-induced increases in shear stress may provide clinically useful information. In light of its physiological relevance and opportunity for stimulus control, using exercise-induced increases in shear stress to investigate FMD may be an important avenue for future endothelial function research.

Protocols that endeavor to assess the acute impact of a specific manipulation on FMD (e.g., mental stress or changes in shear stress magnitude) or that simply wish to do repeat trials for averaging purposes (14, 38) must perform multiple FMD tests over a short period of time. It would therefore be a concern if repeated exposure to shear stress systematically blunted or potentiated endothelial responses. Harris et al. (19) performed five RH trials with a 30-min intertrial interval and did not find any systematic changes in FMD magnitude. To our knowledge, no study to date has examined FMD after repeated HGEX-induced increases in shear stress. This is essential to begin to incorporate exercise-induced shear stress stimuli into human endothelial function studies. As mentioned above, there is some evidence that distinct vasodilatory mechanisms are initiated by different shear stress stimulus profiles (29). However, the degree to which the function of these mechanisms is associated is unclear. The relationship between FMD responses
elicited by different shear stress profiles has been minimally studied and could provide insights regarding whether endothelial sensitivity to shear stress is profile specific.

With this as a background, the primary objectives of the present study were to 1) identify if there is a systematic change in the FMD response to repeated increases in shear stress created with HGEX and 2) examine the relationship between FMD created with HGEX- versus RH-induced increases in shear stress. In the study by Harris et al. (19), the stimulus was reported in only a subset of subjects and was characterized as the peak hyperemic flow rather than the recommended shear rate area under the curve (AUC) until the time of peak diameter measurement (18, 37, 38). Thus, it remains possible that changes in the stimulus magnitude across the five trials masked a change in endothelial responses. Therefore, a secondary objective was to identify whether there is a systematic change in the shear stress stimulus after the release of repeated forearm occlusion and to confirm the lack of a systematic change in the resultant FMD response. We hypothesized that there would be no systematic change in FMD over four trials of HGEX and that there would be a relationship between FMD after RH- and HGEX-induced increases in shear stress. Finally, given the previously reported stability of FMD across multiple trials of RH (19), we hypothesized that there would be no systematic change in the magnitude of the postocclusion shear rate stimulus.

METHODS

Subjects

Twenty healthy nonsmoking male (n = 10) and female (n = 10) subjects between 19 and 37 yr from the Queen’s University community in Kingston, Ontario, Canada, volunteered to participate. Menstrual phase was not controlled; however, the phase at the time of testing was estimated via the reported cycle day. Nine of the ten female subjects were tested during the follicular phase of their menstrual cycle and one in the luteal phase. For all females, both visits testing was estimated via the reported cycle day. Nine of the ten female subjects was confirmed with a medical screening questionnaire for risk factors associated with endothelial dysfunction. The study procedures were approved by the Health Sciences Human Research Ethics Board at Queen’s University, which operates under the terms of Helsinki, and all subjects completed a consent form that was approved by the same board. Subjects were instructed to abstain from alcohol, caffeine, and exercise for 12 h before the study (43). Each subject participated in the study via a three-lead ECG. Blood pressure was measured throughout the exercise protocol. Exercise was mainly aerobic and consisted of a 6-min cycle ergometer at approximately 60% max heart rate on a cycle ergometer (Cybex, Medelec Systems, Amsterdam, The Netherlands). HR and mean arterial pressure (MAP) are reported as the mean of the 1-min baseline period and the mean of the 2-min cuff release period in the RH trials and the mean of the last minute of exercise in the HGEX trials.

Brachial artery blood flow velocity and diameter. Brachial artery blood flow velocity was measured continuously in all experiments with Doppler ultrasound operating at 4 MHz (GE Vingmed System 5, GE Medical Systems, London, ON, Canada). All scans were performed at an insonation angle of 68° for reasons that have been previously discussed (35). The Doppler shift frequency spectrum was analyzed via a Multigon 500M TCD (Multigon Industries, Yonkers, NY) spectral analyzer, from which mean velocity was determined as a weighted mean of the spectrum Doppler shift frequencies. The corresponding voltage output was, in turn, continuously sampled and stored (Powerlab, AD Instruments, Colorado Springs, CO) for later analysis.

The ultrasound probe was positioned over the left brachial artery to obtain a clear arterial blood flow velocity signal, with no interference from adjacent vein blood flow. Once in position, the probe was secured with a guide adhered to the skin. The brachial artery was imaged by two-dimensional grayscale ultrasound imaging in B-mode with the same probe operating at 10 MHz. The probe operator was able to make corrections to probe placement to maintain an optimal blood flow velocity signal and vessel image for the duration of the experiment. The images were recorded in Digital Imaging and Communications in Medicine format for future analysis with automated edge detection software (53).

Experimental Procedures

Subjects lay supine with both arms out to their sides. Blood pressure was measured on the right arm, whereas ultrasound measurements were performed on the left arm. Subjects rested supine for a minimum of 20 min before the initiation of the first trial. HGEX trials. The HGEX involved squeezing an air-filled bladder (PyHaH, Flemington NJ) equipped with a pressure transducer (Utah Medical Products, Midvale, UT). Before the initiation of forearm exercise trials, subjects performed three maximum voluntary contractions (MVCs). Subjects then performed a series of contractions at different intensities to identify the contraction intensity (%MVC) that elicited the blood flow velocity required to achieve a shear rate of 65 s−1. This target shear rate was selected because we have previously determined that it results in a significant FMD response (36) and pilot work indicated that the typical exercise intensity required to achieve it could be performed comfortably in four closely spaced trials. The target shear rate was achieved in each trial for each subject as follows. Shear rate is an estimate of shear stress without viscosity and was calculated as shear rate = mean blood flow velocity/vessel diameter. The blood flow velocity required to achieve the target shear rate was calculated for each subject as velocity = 65 s−1 × brachial artery diameter. To perform this calculation for each subject, the brachial artery diameter was estimated by a manual caliper before the trials started. During the forearm exercise, isometric handgrip force feedback was displayed continuously for the subjects on a computer data-acquisition system (LabChart, AD Instruments). Subjects achieved the target force and duration for each contraction by displacing the force readout line to the desired level in time with a 2-s contraction/3-s relaxation duty cycle metronome. Brachial artery blood flow velocity was continually monitored throughout the trial, and experimenters coached the subjects through minor increases and decreases in force production to maintain the desired blood flow velocity. One minute of all variables was recorded as baseline, and the subject was then instructed to begin exercising. Subjects were instructed to only contract their forearm muscle and to limit arm movement. This allows the maintenance of upper arm ultrasound measurements throughout the exercise protocol. Exercise was maintained for 6 min, and recovery was recorded for a further 2 min. After 10 min of rest after completion of a trial, diameter was measured manually via electronic callipers, and the next trial was commenced if
the artery diameter was no longer elevated compared with the initial baseline. If the artery was still vasodilated, diameter was assessed every ~30 s, and the next trial was initiated upon the return to baseline diameter. Four HGEX trials (HGEX trials 1–4) were performed in succession (mean intertrial time: 13.5 ± 2 min).

RH trials. An occlusion cuff was placed on the forearm just below the antecubital fossa (distal to site of brachial artery ultrasound measurement). The cuff was inflated for 5 min at a pressure of 250 mmHg. One minute of all variables was recorded as baseline, and the cuff was then inflated. Forearm occlusion was maintained for 5 min, and recording of all variables resumed 1 min before occlusion cuff release. After cuff release, all variables were recorded for a further 2 min. After 10 min following completion of a trial, diameter was measured manually via electronic callipers, and the next trial was commenced if the artery diameter was no longer elevated compared with the initial baseline. If the artery was still vasodilated, diameter was assessed every ~30 s, and the next trial was initiated upon the return to baseline diameter. Four RH trials (RH trials 1–4) were performed in succession (mean intertrial time: 13.5 ± 3 min, \( P = 0.984 \) vs. the HGEX intertrial time).

Data Analysis

Brachial artery blood flow velocity. As previously described (35, 36, 38), blood flow velocity was analysed offline using the data-acquisition software program LabChart (AD Instruments) in 3-s average time bins.

Brachial artery diameter. Vessel diameter was analyzed using an updated version of the automated edge detection software package (FMD/Blood Flow Acquisition and Analysis, Reed Electronics, Perth, WA, Australia) described by Woodman et al. (53). This program allows the user to identify a region of interest on the portion of the image where the walls are most clear. It then identifies and tracks the walls of the artery via the intensity of the brightness of the walls versus the lumen of the vessel. The program collects one diameter measurement for every pixel column in the region of interest. It uses the median diameter as the diameter for that frame. The program is triggered to the ECG signal and provides a diameter measurement for every R-wave (corresponding to end diastole). The program allows for the removal of erroneous data points due to vessel wall tracking errors. The diameter data was compiled as 3-s time bins allowing alignment with the 3-s average velocity data. In the HGEX trials, FMD is reported as the percent change in diameter from baseline to the average diameter in the last minute of exercise. In the RH trials, FMD is reported as the percent change in diameter from baseline (before cuff occlusion) to the peak 3-s average diameter time bin.

Shear rate. Shear rate (an estimate of shear stress without viscosity) was calculated as mean blood flow velocity/vessel diameter and was used to quantify the shear stimulus for FMD. This was calculated as a 3-s average from the 3-s average diameter and blood flow velocity data. The shear stimulus during the HGEX trials is reported as the mean shear rate during the last 5 min of exercise (steady state). The shear stimulus during the RH trials is reported as the peak 3-s average shear rate and the AUC of the stimulus from cuff release until the time of peak diameter measurement (SR AUC).

Statistical analysis. One- and two-way repeated-measures ANOVA were used to compare the baseline, shear rate stimulus, and FMD response parameters across the four repeat RH and HGEX trials. Trial agreement was further assessed with an intraclass correlation coefficient (ICC) and examination of the within-subject variability via the root mean square error (RMSE) from the repeated-measures ANOVA (9, 49). The coefficient of variation (within-subject SD/within-subject mean \( \times 100 \)) for the baseline diameter and RH peak diameter and FMD were 1.95%, 2.21%, and 28.71%, respectively, and are comparable with previous reports (9, 10, 38, 39). The level of significance was set at \( P < 0.05 \), and significant differences from ANOVAs were further assessed using Tukey’s post hoc tests. The relationship between %FMD in the RH trials and %FMD in the HGEX trials was assessed with linear regression. Cook’s distance was calculated to detect potential outliers (21). A sex comparison was not the objective of this investigation. However, for completeness, sex comparisons of FMD magnitude and shear stimulus magnitude were compared via \( t \)-tests using subject data pooled across the four trials of each test. With the exception of the ICC (SPSS 17.0), all statistics were calculated using SigmaPlot 11 (Systat Software, Chicago, IL). Data are reported as means ± SD.

RESULTS

HR and MAP

Baseline variables are shown in Fig. 1. There were no differences in baseline MAP or baseline HR between the two tests (RH vs. HGEX; \( P = 0.642 \) and 0.548, respectively). There was, however, a main effect of trial on hemodynamic variables. Baseline MAP was slightly lower in trials 1 and 2 versus trial 4 (\( P < 0.01 \)). Baseline HR was slightly higher in trial 1 versus trials 3 and 4 (\( P < 0.05 \)). There were no changes in HR or blood pressure from baseline in the RH test; however, HGEX modestly increased these variables [change in HR and MAP from baseline to cuff release (RH) vs. change from baseline to the last minute of exercise (HGEX): RH −0.2 ± 3.5 beats/min and HGEX 4.3 ± 7.5 beats/min for HR, \( P < 0.001 \); and RH 0.62 ± 4.9 mmHg and HGEX 7.4 ± 7.8 mmHg for MAP, \( P < 0.001 \)].

Baseline Shear Rate, Vascular Conductance, and Brachial Artery Diameter

There was a main effect of both test (RH vs. HGEX) and trial for baseline shear rate in that it was slightly but significantly higher in the HGEX trials versus the RH trials (\( P < 0.001 \)) and trial 1 had a slightly but significantly higher baseline shear rate than all other trials (\( P < 0.001 \); Fig. 1). These differences in calculated shear rate (−2 s\(^{-1}\)) represent differences in blood flow velocity of ~1 cm/s on a 3.2-mm baseline diameter. Baseline forearm vascular conductance was slightly higher in RH trial 1 versus subsequent trials (\( P < 0.003 \)). Forearm vascular conductance was slightly lower in trials 2 and 4 of the RH condition versus the HGEX condition (\( P < 0.02 \)). There was a significant interaction between trial and test for baseline diameter (\( P = 0.024 \)). Post hoc analysis revealed that in trial 1 only, the baseline diameter in the RH test was modestly but significantly larger than the baseline diameter in the HGEX test (\( P = 0.031 \)). Within the RH test, trial 1 and 2 diameters were slightly larger than the trial 4 diameter (\( P = 0.033 \) and 0.043, respectively). There were no significant differences in baseline diameter between trials within the HGEX test.

Shear Rate Stimulus Magnitude

The AUC of the shear rate until the time of peak diameter measurement (RH trials) was significantly higher in trial 1 versus trials 3 and 4 (\( P = 0.019 \) and 0.047, respectively; Fig. 2A); however, there were no significant differences in the peak postcuff release shear rate across the four trials (\( P = 0.116 \); Fig. 2B). As intended by targeting the shear rate at 65 s\(^{-1}\) in each trial, in the HGEX test there were no significant differences in the steady-state shear rate between trials (\( P =
There were also no significant trial differences in the %MVC of the exercise required to create the target shear rate (trial 1: 27.2 ± 13.5%, trial 2: 27.4 ± 14.2%, trial 3: 26.4 ± 14.5%, and trial 4: 26.9 ± 13.7%). The individual and mean differences between trials 1 and 4 are shown in Fig. 2D. For the mean shear in the HGEX trials, the mean difference between trial 1 and trial 4 was near zero (1.9 ± 5.9 s⁻¹). For the peak shear rate and shear rate AUC in the RH test, the trial 1–trial 4 differences were above zero, reflecting the somewhat larger trial 1 stimulus magnitude, although this was not statistically significant for the peak shear. Figure 3 shows the shear rate across all four trials in each individual subject. The RH shear rate AUC ICC was 0.580 and the RMSE was 702.59 s⁻¹. The RH peak shear rate ICC was 0.828 and the RMSE was 17.97 s⁻¹. The HGEX steady-state shear rate ICC was 0.603 and the RMSE was 3.3 s⁻¹. The average shear rate profile for the RH and HGEX trials is shown in Fig. 4. Sex did not have a significant impact on the magnitude of the shear stress stimulus in either test (P = 0.255 and 0.796 for the RH and HGEX tests, respectively).

**FMD Response Magnitude**

There were no significant differences in %FMD between trials (trials 1–4, P = 0.913) or between tests (RH vs. HGEX, P = 0.913; Fig. 5). There were also no significant differences in the absolute change (in mm) in diameter across trials (trials 1–4, P = 0.910) or tests (RH vs. HGEX, P = 0.957). In the RH test, when the %FMD was normalized to the magnitude of the shear rate stimulus (%FMD/SR AUC), there were still no
significant differences between trials ($P = 0.197$). Figure 5C shows a plot of the individual subject and mean differences between trials 1 and 4 (trial 1 value – trial 4 value). The mean values for both tests were very close to zero (RH: $0.1 \pm 3.5\%$ and HGEX: $0.4 \pm 3.1\%$). Figure 6 shows %FMD across all four trials in each individual subject. The RH %FMD ICC was 0.446 and the RMSE was 2.3%. The HGEX %FMD ICC was 0.716 and the RMSE was 1.8%. In the RH trials, there were no differences in the time to peak diameter measurement between trials (overall mean: $52.6 \pm 19.8\ s$, $P = 0.954$). The average %FMD profile for the RH and HGEX trials is shown in Fig. 4. Sex did not have a significant impact on %FMD in either test (RH or HGEX, $P = 0.212$ and 0.819 for the RH and HGEX tests, respectively).

**Relationship Between %FMD in the RH Trials Versus %FMD in the HGEX Trials**

There was no significant relationship between %FMD in the RH and HGEX trials when the RH %FMD was expressed without accounting for the stimulus magnitude ($r^2 = 0.12$, $P = 0.137$; Fig. 7A). No outliers were detected in this analysis. However, when the %FMD was normalized to the magnitude of the SR stimulus (RH %FMD/SR AUC = RH %FMDnorm; HGEX %FMD/mean SR in the last 5 min of exercise = HGEX %FMDnorm) ($31, 32, 38$), the relationship between these variables was improved ($r^2 = 0.19$, $P = 0.056$). Cook’s distance assessment revealed one outlier (circled point in Fig. 7B), and, when this point was removed from the regression analysis, a reasonably strong relationship between RH %FMDnorm and HGEX %FMDnorm emerged ($r^2 = 0.44$, $P = 0.002$). Due to the very small variability in the HGEX stimulus as a result of the target, a similar relationship emerged between RH %FMDnorm and unnormalized HGEX %FMD ($r^2 = 0.40$, $P = 0.003$).

**DISCUSSION**

This study was designed to investigate brachial artery FMD responses over repeated exposures to two distinct shear stress stimulus profiles. The primary novel findings were as follows. First, there were no systematic changes in FMD over four closely spaced exposures to HGEX-induced increases in shear stress. Second, there were also no systematic changes in FMD with four repeat exposures to RH-induced increases in shear stress.
stress, and this occurred despite a decrease in the RH stimulus magnitude after trial 1. Finally, accounting for the shear rate stimulus magnitude permitted detection of a relationship between RH- and HGEX-induced FMD, such that individuals who had a large normalized RH %FMD response also tended to have a large normalized HGEX %FMD response. These data indicate that 1) there is no systematic blunting or potentiating of endothelial responses to shear stress with repeated exposure.
to RH or a typical (36, 41, 48) HGEX protocol, 2) it is appropriate to use HGEX to investigate FMD in studies that require multiple trial designs, and 3) there is a degree of uniformity in endothelial sensitivity to shear stress across distinct stimulus profiles.

Impact of Multiple, Closely Spaced Exposures to an Increase in Shear Stress

Many research designs that use FMD require that multiple trials be repeated over a short time interval (4, 32, 38). This raises the issue of whether repeated exposure to elevated shear stress might potentiate or blunt the endothelium’s ability to respond. Stability of the shear rate stimulus and FMD responses over the four trials was assessed by repeated-measures ANOVA and examining the ICC and RMSE (9, 19, 49). In agreement with Harris and colleagues (%FMD ICC range: 0.450 – 0.811), the FMD response indicated “fair to good agreements” in both the RH and HGEX trials. The RMSE for the RH condition was slightly lower than that reported by De Roos et al. (9) (2.8%). The shear rate stimulus data ICCs were in a similar range (RH shear rate AUC: 0.580, RH peak: 0.828, and HGEX steady-state shear: 0.603). The ICC is influenced by the between-subject’s variability, such that if there is little between subject’s variability, the ICC will be depressed even if the trial-to-trial variability is small (49) (e.g., shear rate in the HGEX trials, where shear rate was targeted to the same level in all subjects). The RMSE is independent from between-subject variability, and overall the analysis indicated greater trial-to-trial stability of the shear rate stimulus in the HGEX trials (Fig. 3A and low HGEX RMSE), which was as expected given the targeting of the shear rate. The trial-to-trial %FMD stability was similar between the two conditions but may be modestly greater in the HGEX trials. This is consistent with previous findings comparing FMD after RH and more controlled stimuli (shear rate elevated with heating and controlled by arterial compression) (34) and suggests that factors other than stimulus variability play a significant role in determining %FMD variability.

In agreement with the present study findings, we (34) have previously reported that two RH trials in succession do not result in changes in the FMD response or the shear rate stimulus magnitude (34). Harris et al. (19) performed two to five RH trials over a 2-h period. The shortest intertest interval was 30 min, and, in agreement with the present study, they (19)
found no systematic changes in FMD across the five trials. The present study extends these findings in two important ways. First, Harris et al. (19) measured brachial artery diameter at discrete postcuff release time points rather than continuously. We are thus able to contribute the novel finding that the time to reach peak diameter was not significantly different between trials. This further supports the stability of the endothelial response. Second, in the RH trials, the present study quantified the shear rate AUC until the time of peak diameter measurement (SR AUC) in all participants. This has been identified as our best estimate of the relevant stimulus for FMD (38, 44). In the study by Harris et al. (19), the stimulus was characterized as the peak blood flow after cuff release in a subset of six subjects and was not found to be different between trials. The present study identified that there was a significantly larger SR AUC in trial 1 versus trials 3 and 4, with no difference in the time to peak diameter measurement (Fig. 2). This suggests that there was a greater dilation of the forearm vessels in trial 1, and, indeed, analysis of forearm vascular conductance (in ml·min⁻¹·mmHg⁻¹) revealed that it was higher during the trial 1 release period than that of the other trials. This suggests that the forearm resistance vessel response to occlusion decreases after an initial cuff inflation. However, as discussed below (Consideration of Baseline Variables), baseline forearm vascular conductance was larger in trial 1 of the RH test versus the other RH trials (Fig. 1), reflecting a lower tone in the resistance vessels, and this may have potentiated the occlusion-induced vasodilation. This association between an increased baseline conductance and an increased postocclusion shear stimulus has been previously reported (28).

Despite the AUC stimulus variability, %FMD did not differ between RH trials. A smaller stimulus in later trials with the same FMD might suggest an increase in sensitivity to shear stress; however, %FMD normalized to the shear stimulus magnitude also did not differ between trials (P = 0.197). Taken together, this suggests that the between-trial stimulus differences were not of sufficient magnitude to impact FMD response magnitude and that endothelial reactivity to shear remained stable over repeat exposures to RH.

In addition to RH, the present study provides the first report of FMD created with repeated HGEX-induced increases in shear stress. HGEX creates a sustained stimulus that is highly physiologically relevant as this is the primary way in which shear stress is elevated in vivo. It has the added advantage that the shear stress stimulus magnitude can be controlled and thus matched across trials (36), eliminating the need for post hoc normalization. HGEX results in forearm muscle metabolite production and a distinct fluctuating pattern of shear stress, such that shear is higher during muscle relaxation and lower during muscle contraction (36). However, when the same mean increase in brachial artery shear stress is achieved with HGEX and passive forearm heating (no muscle activation, no fluctuation in shear pattern), it has been shown to result in the same pattern and magnitude of FMD (36). In addition, Pyke et al. (36) found that when exercise was performed and brachial artery shear stress was not allowed to increase, no brachial artery vasodilation occurred. This indicates a lack of conducted vasodilatory signals in the conduit artery, confirming that conduit artery vasodilation with HGEX is mediated only by shear stress (36). As a whole, this indicates that neither the exercise shear pattern nor the muscle activation alter the FMD response and that conduit artery diameter changes in response to exercise-induced increases in shear stress reliably reflect a FMD response.

The present study demonstrated, for the first time, that, similar to FMD resulting from RH, repeated increases in shear stress via HGEX do not result in a systematic change in FMD magnitude. One of the concerns that motivates investigations of FMD with repeat shear stress exposure (19) is that acute elevations in shear could alter FMD responses. To gain insights into this concern, previous studies have evaluated RH-induced FMD before and after an intervention that acutely elevates shear stress. In young healthy subjects, Tinken et al. (45) found that RH-induced FMD was enhanced immediately after an exposure to light dynamic HGEX and two other interventions designed to acutely increase brachial artery shear stress (forearm heating and recumbent cycling). This is in contrast to the present study in which repeated exposure to elevated shear

Fig. 6. Individual %FMD values in each of the four trials for each subject. A: RH trials. B: HGEX trials.
Mechanisms Responsible for FMD in Response to RH- and Exercise-Induced Increases in Shear Stress

Recent evidence suggests that the mechanisms responsible for FMD in response to brief versus sustained increases in shear stress may be distinct. Mullen et al. (29) found that while radial artery FMD after the release of 5 min of occlusion (brief stimulus) could be virtually abolished with NO synthase inhibition, FMD resulting from sustained stimuli elicited with a 15-min occlusion or hand warming was unaffected by a N-monomethyl-L-arginine infusion, suggesting an NO-independent FMD response. Subsequent studies have found some role for NO in FMD resulting from sustained increases in shear stress created with both hand warming (3) and HGEX (54). Furthermore, and also in contrast to the findings of Mullen et al. (29) and others (24), it has recently been shown that even FMD after a brief stimulus created with 5 min of occlusion may not always be NO dependent (33). While it is clear that substantial confusion remains regarding the identification of predominant FMD mechanisms, it remains probable that the stimulus profile has an impact on which mechanisms are engaged. Thus, recognizing the distinct shear rate profiles created with RH and exercise (Fig. 4), the mechanisms underlying the respective FMD responses may be at least partially distinct. We sought to determine whether the FMD response to these two distinct profiles was correlated and found that when RH %FMD was left in its “raw” form (i.e., not normalized to the stimulus magnitude) no relationship could be detected. However, when %FMD was normalized to the magnitude of the shear rate stimulus, a significant relationship emerged.

The RH stimulus was uncontrolled and highly variable between subjects (SR AUC between-subject range: ~5,400–2,100), whereas the exercise stimulus was controlled near 65 s⁻¹ in all subjects (between-subject range: ~70–50 s⁻¹). The emergence of a relationship between RH %FMD and HGEX %FMD with response normalization suggests that the influence of intrinsic differences in the RH stimulus magnitude relative to the enforced stimulus in the HGEX trials in each subject masked the relationship between HGEX %FMD and RH %FMD. Ratio normalization is an imperfect method of accounting for the shear stimulus magnitude in RH protocols and has received considerable criticism (1, 50). However, no more appropriate alternative has been identified, and ratio normalization has been shown to effectively control for manipulated differences in the shear stimulus magnitude in RH protocols and to enable the detection of FMD differences between risk factor groups (32). However, the latter has not always been shown (44).

The relationship between HGEX %FMDnorm and RH %FMD (r = 0.66, r² = 0.4, P = 0.002) is in agreement with the findings of Gaenzer et al. (12). They found a significant relationship between the FMD in the femoral artery in response to 150-W cycling exercise and the brachial artery response to RH (r = 0.88, P < 0.001). That they found a relationship between exercise FMD and unnormalized FMD in response to RH may be due to the exercise being performed at 150 W (creating variable increases in shear rate) rather than at an

stress did not result in an enhancement of FMD magnitude. The duration of shear stress elevation may be important because in the study by Tinken and colleagues (45), the shear stress was elevated for a period of 30 min (vs. 6-min bouts of exercise in the present study) between the RH FMD tests. It is also possible that exposure to sustained increases in shear stress (several minutes of shear above baseline) only enhances the magnitude of FMD when it is elicited with a brief RH shear stress profile. In contrast to the findings of Tinken and colleagues (45), in medicated hypertensive subjects, McGowan and colleagues (28) found that four sets of a 2-min continuous contraction held at 30% MVC significantly reduced RH-mediated FMD from 3% down to 2%. Although levels of oxidative stress were not assessed, the authors hypothesized that the reduction in FMD was likely due to a decrease in nitric oxide (NO) bioavailability brought on by an increase in ROS production during the exercise protocol (40). It is likely that the rhythmic exercise in the present study created less ischemia and ROS production than a continuous isometric contraction and that this may contribute to the conflicting results. Further work is required to fully elucidate the impact of repeat acute exposure to elevated shear stress on FMD in all situations and populations.
intensity chosen to achieve a predetermined shear rate. In the design of Gaenzer and colleagues (12), intrinsic resistance vessel function was involved in determining both exercise and RH stimuli, potentially improving their correlation (i.e., an individual with a large exercise blood flow response might also have been more likely to have a large RH). A similarity in relative stimulus magnitude with exercise and RH would allow a relationship in exercise- and RH-induced FMD to be detected with unnormalized %FMD measurements more easily. Taken together, the results of the present study and those of Gaenzer et al. (12) suggest that despite the potential for mechanistic differences, FMD elicited by the distinct stimulus profiles of RH- and exercise-induced increases in shear stress provide related information regarding endothelial function. Stated differently, it seems that endothelial sensitivity to shear stress may not be entirely stimulus profile specific (i.e., those with a large ability to respond to one stimulus profile may be more likely to have a large ability to respond to another stimulus profile).

Important from a clinical perspective, Gaenzer and colleagues (12) reported that both exercise- and RH-induced FMD were impaired in smokers. This suggests that FMD resulting from exercise-induced increases in shear stress may provide clinically relevant information (30). In addition, Grzelak et al. (17) reported that FMD after HGEX-induced increases in shear stress was reduced in elderly subjects and subjects with type I diabetes. NO serves many vasoprotective functions (26), and it is generally thought that an impairment in FMD after a standard 5-min RH test reflects a decrease in NO bioavailability (16). The recent report by Wray et al. (54) showing that brachial artery HGEX-induced FMD may be reduced by up to 60% with NO synthase blockade suggests that the decrements in exercise-induced FMD could also occur via a reduced NO bioavailability mechanism. However, there is some conflicting evidence on the ability of exercise-induced FMD to detect changes in endothelial function. Padilla et al. (30) found that unlike RH, exercise-induced FMD was unable to detect high-fat meal-induced decrements in endothelial function. The degree to which FMD mechanisms and the endothelial sensitivity to shear stress are specific to the characteristics of the stimulus profile continues to be an important avenue in endothelial function research.

Consideration of Baseline Variables

Subjects’ HR, brachial artery shear rate, and forearm vascular conductance were slightly higher, and blood pressure was slightly lower, in trial 1 versus trial 4 (Fig. 1). Subjects entered the laboratory from a warm summer environment, and the minimum 20-min rest period before trial 1 may have been insufficient for subjects to reach stable levels in adjustment to lying still in the cool (18°C) room temperature. Whole body exposure to cool temperatures can result in a sympathetically mediated increase in blood pressure and a decrease in skin blood flow, with no change or a slight decrease in HR (22, 52). Although there is some concern that increases in sympathetic nervous activity (SNA) can blunt FMD (23), dramatic interventions to increase SNA have also been shown to have no effect (11). Furthermore, we (36) have previously performed FMD tests at 22 and 18°C in the same subjects and found no differences in FMD responses to the same shear stimuli. Thus, it is unlikely that in the present study changes in sympathetic activity influenced the FMD responses. However, a longer “lead-in” rest period may be advisable to obtain greater stability in baseline parameters. Finally, modest differences in baseline diameter were noted between tests and within the RH trials. To address this, FMD was calculated as both a percent change (%FMD) and an absolute change (in mm) from baseline, with similar results.

Limitations

This study examined FMD in a mixed population of men and women. Use of oral contraceptives and variations in hormone levels over the menstrual cycle can influence the magnitude of the FMD response and were not controlled in the present study (20, 27). However, comparisons were performed on a within-subject basis, decreasing the importance of between-subject differences in menstrual phase and use of oral contraceptives. Nine of the ten female subjects were tested within 3 days (5 subjects on consecutive days) to limit the degree of hormonal variations between their RH trials and their HGEX trials. However, it is possible that some women had significant differences in hormone levels between the two test days. While this might have influenced the absolute magnitude of their FMD, it is unlikely to have altered the potential for systematic changes in endothelial sensitivity to shear over repeat trials, which was the major issue addressed by this study. In support of this, within women specifically, there were no significant differences in RH %FMD or HGEX %FMD between trials (P = 0.54 and 0.71, respectively). If there were differences in the menstrual cycle phase between days in female subjects, this may have altered day-to-day endothelial function and resulted in an underestimation of the relationship between normalized RH and HGEX FMD responses, as these tests were performed in two separate visits. This would not, however, alter the study conclusions.

We did not measure blood viscosity and instead used shear rate as an estimate of shear stress. However, in a recent study examining the effect of the shear stimulus on FMD, Padilla et al. (31) found that using measured viscosity or an assumed constant viscosity had no effect on their results. Although differences between visits might exist, it is unlikely that viscosity changed significantly within a given subject over the brief testing period in each visit (15); thus, the lack of viscosity measurements should have a minimal impact on our primary finding that there is no systematic change in FMD across repeat, closely spaced shear exposures. However, error in our estimate of shear stress due to differences in viscosity might have made us less effective in accounting for the stimulus magnitude and, again, caused us to underestimate the relationship between normalized RH %FMD and HGEX %FMD.

We did not perform any blockade studies and can therefore only speculate regarding the impact of repeat exposure to shear stress on the underlying FMD mechanisms and on mechanistic differences between the two tests. Furthermore, we did not measure endothelium-independent vasodilation. Therefore, it remains a possibility that endothelial reactivity to shear stress was in fact changing with repeat exposure to shear stress but that changes in smooth muscle responsiveness masked this effect. In support of the stability of smooth muscle responses, Tinken et al. (45) investigated the brachial artery dilatory
response to glyceryl trinitrate administration before and after exposure to 30 min of mild dynamic HGEX, forearm heating, and recumbent cycling and found that these stimuli had no effect. However, it remains possible that repeat exposure to acute bouts of increased shear stress, as in the present study, impact smooth muscle responses.

Finally, in HGEX trial 1, subjects had already been exposed to an increase in brachial artery shear stress during the previous MVCs and submaximal contractions used to determine the %MVC that would be required to elicit the blood flow velocity target required to achieve a shear rate of 65 s⁻¹. Thus, it is possible that the response in HGEX trial 1 differs from what would have been observed if the brachial artery had not experienced these increases in shear rate. However, if HGEX is going to be used to test FMD as described in this study, MVCs and submaximal contractions before the first official measurement will be required. Thus, the data reported represent what can be expected in a typical experimental design.

Summary and Conclusions

Four closely spaced exposures to RH- or HGEX-induced increases in shear stress did not alter the magnitude of brachial artery FMD responses. In the RH protocol, the SR AUC decreased after trial 1, indicating a reduction in the resistance vessel response to ischemia; however, this did not impact the FMD response magnitude. This supports the position that it is appropriate to design experimental protocols that require multiple FMD tests in a short time period. The relationship between normalized %FMD in response to RH and HGEX suggests that endothelial sensitivity to shear stress is not entirely profile specific and that endothelial responses to these different shear rate profiles provide related information regarding endothelial function. As a whole, these data support the value of exploring HGEX protocols in the assessment of human endothelial function.

ACKNOWLEDGMENTS

The authors thank Dr. Michael Tschakovsky for the use of equipment.

GRANTS

This work was funded by a Natural Sciences and Engineering Research Council of Canada Discovery Grant and by Canada Foundation for Innovation and Ontario Ministry of Research and Innovation Leaders Opportunities Funding (to K. E. Pyke). F. Jazuli was supported by a Natural Sciences and Engineering Council of Canada Discovery Grant and by Canada Foundation for Innovation

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