Peak vs. total reactive hyperemia: which determines the magnitude of flow-mediated dilation?

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Pyke KE, Tschakovsky ME. Peak vs. total reactive hyperemia: which determines the magnitude of flow-mediated dilation? J Appl Physiol 102: 1510–1519, 2007. First published December 14, 2006; doi:10.1152/japplphysiol.01024.2006.—We investigated the independent contributions of the peak and continued reactive hyperemia on flow-mediated dilation (FMD). J) For the duration manipulation experiment (DME), 10 healthy males experienced reactive hyperemia durations of 10 s, 20 s, 30 s, 40 s, 50 s, or full reactive hyperemia (RH). 2) For the peak manipulation experiment (PME), eight healthy males experienced reactive hyperemia trials with three peak shear rate magnitudes (large, medium, and small). Data are means ± SD. For the DME, peak shear rate was not different between trials (P = 0.326). Shear rate area under the curve (AUC) was P < 0.001. Peak %FMD was dependent on shear rate AUC: 10 s, 2.7 ± 1.3; 20 s, 6.2 ± 1.9; 30 s, 7.9 ± 2.9; 40 s, 8.3 ± 3.2; 50 s, 7.9 ± 3.2; full RH, 9.3 ± 4.1, with 10 and 20 s less than full RH (P < 0.001). For the PME, peak shear rate was different between trials (large, 1.049 ± 285.8; medium, 726.4 ± 228.8; small, 512.8 ± 161.8; P < 0.001). AUC of the continued shear rate was not (P = 0.412). Peak %FMD was unaffected by peak shear rate (large, 7.0 ± 2.7%; medium, 7.4 ± 2.6%; small, 6.6 ± 1.8%; P = 0.542). Peak and AUC shear stimulus were not significantly related in full RH (r² = 0.35, P = 0.07). We conclude that the shear stimulus AUC, not the peak itself, is the critical determinant of the peak FMD response. This indicates AUC as the best method of quantifying reactive hyperemia shear stimulus for %FMD normalization.

The magnitude of FMD is related not only to the health of the vascular endothelium, but also to the magnitude of the imposed stimulus (25, 27). Importantly, the magnitude of the shear stimulus created with reactive hyperemia is influenced by several factors and may be quite variable between subjects or potentially between groups (11, 16, 25, 27). Thus, if a small %FMD response is observed, it is important to know if it is the result of a small imposed shear stimulus or if it is in fact revealing an underlying endothelial dysfunction. One way to address this is to normalize the %FMD response to the magnitude of the shear stimulus imposed (peak FMD response/shear stimulus).

The peak of the shear stress stimulus profile created with reactive hyperemia occurs ~4–7 s post-cuff release and then rapidly decays, returning to baseline within 2 min. In contrast, the peak diameter adaptation is typically not observed until 45–75 s post-cuff release (5). Thus the vessel is exposed to the peak stimulus and then an additional 35–65 s of continued elevated shear stress prior to the development of the peak response. Despite the relatively prolonged exposure to elevated shear stress with reactive hyperemia, often only the peak shear stress or blood flow post-cuff release is used to quantify the stimulus for FMD (1, 7, 17).

Some studies have more closely scrutinized the role of peak vs. continuation characteristics of the reactive hyperemia stimulus in determining FMD (3, 15, 20) and suggest the peak is not solely responsible. However, these studies used alterations in occlusion time and addition of ischemic handgrip exercise to establish different peaks and durations of hyperemia in combination, and thus their findings do not partition the role of each. Furthermore, it remains unknown what effect the duration of zero shear along the brachial artery endothelium during occlusion has on the subsequent FMD response. Thus differences in occlusion time may be a confounding influence in these studies.

The current study was therefore designed to systematically isolate and investigate the relative contribution of the peak vs. the continuation of a reactive hyperemia shear stimulus following a standard 5 min of occlusion in determining the FMD response. This was achieved by holding the peak shear stimulus constant while manipulating the shear stimulus duration [duration manipulation experiment (DME)] or by holding the continuation of the shear stimulus constant while manipulating the magnitude of the peak shear stimulus [peak manipulation experiment (PME)]. For the DME we tested the hypothesis that J) the peak stimulus by itself (10 s of hyperemia) does not determine FMD and 2) that longer stimulus continuation would result in progressively larger FMD responses. For the PME we tested the hypothesis that the peak FMD would increase with the peak stimulus magnitude.
METHODS

Subjects

Ten (DME) and eight (PME) healthy, nonsmoking male subjects between 19 and 32 years of age from the Queen’s University student population volunteered to participate. Health status of the subjects was confirmed with a medical screening questionnaire for risk factors associated with endothelial dysfunction. Each subject served as his own control. This study was approved by the Health Sciences Human Research Ethics Board at Queen’s University. All subjects completed a consent form that was approved by the Health Sciences Human Research Ethics Board at Queen’s University. Subjects were instructed to abstain from alcohol, caffeine, and exercise for 12 h prior to the study and to abstain from food for 4 h prior to the study. Each subject performed all trials (DME, 6 trials; PME, 3 trials) at the same time of day in a temperature-controlled room (22°C).

Subject monitoring. Heart rate was monitored throughout each study via three-lead ECG. Blood pressure was measured continuously via arterial tonometry (Colin 7000, Trudell Medical, London, ON, Canada).

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). All scans were performed at an insonation angle of 68 degrees, which still provides valid estimates of flow velocity as long as insonation angle is maintained accurately (19). This angle was selected as it allows the vessel to be perpendicular to the ultrasound beam, which yields superior image quality. Image quality is critical to the determination of FMD.

The ultrasound probe was oriented over the brachial artery to achieve a clear arterial blood velocity signal, with no interference from adjacent vein blood flow. Once in position, the probe was secured with a clamp stand and 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 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 minor corrections to probe placement to maintain an optimal velocity signal and vessel image throughout the experiment while maintaining consistent insonation angle within subject trials. The images were recorded in Digital Imaging and Communications in Medicine (DICOM) format for future analysis with a custom automated edge-detection software (32).

Experimental Protocols

For all protocols, subjects lay supine with both arms out to their sides. Blood pressure was measured on the right arm, while ultrasound measurements were performed on the left arm. An occlusion cuff was placed below the area of brachial artery blood flow velocity and diameter measurement, just proximal to the antecubital fossa. Baseline brachial artery images and blood flow velocity prior to occlusion cuff inflation were recorded for 1 min. The occlusion cuff was then inflated to 300 mmHg for 5 min. The brachial artery blood flow velocity and vessel images were recorded for the final 1 min during occlusion immediately prior to the initial cuff release and for 2 min thereafter.

Shear stimulus DME experiments. To manipulate the duration (continuation) of the reactive hyperemia, the occlusion cuff was reinflated after allowing a defined period of hyperemia. This reinfation was a progressive increase in cuff pressure from 0 to 300 mmHg over 2–3 s to avoid a sudden retrograde pressure and velocity burst. This allowed for a constant peak shear stimulus and a range of shear stimulus durations within each subject. Given the nature of reactive hyperemia, there were obviously differences in the peak shear stimulus between subjects. Subjects underwent six trials with different hyperemic durations; 10 s, 20 s, 30 s, 40 s, 50 s and “full reactive hyperemia” (full RH; see Fig. 1A). The order of these trials was randomized. Baseline conditions were re-established between trials. Subjects performed the same six trials on 3 different days. All 3 test days took place within a 7-day period. Each subject’s responses were averaged across test days for each hyperemic duration condition to provide an individual mean response.

Shear stimulus PME. Isometric handgrip exercise at 30% maximal voluntary contraction (MVC; subjects received oscilloscope feedback on force output relative to the target level of 30%) was performed with the occluded forearm for 30 s to 1 min starting at the 2nd min of occlusion (exercise was terminated when an increase in mean arterial pressure was noted; see Fig. 1B).

On release of the 5-min forearm occlusion, brachial artery blood flow velocity was controlled via downstream arterial compression (finger pressure over brachial pulse downstream of ultrasound probe site). Briefly, mean blood flow velocity was continuously displayed on the data acquisition computer as a 3-s moving average, allowing the experimenter to use the velocity feedback to target specific velocities on cuff release by varying downstream arterial compression. To apply appropriate downstream arterial compression on occlusion release, the

<table>
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<th>A</th>
<th>Cuff inflation</th>
<th>Cuff Release</th>
<th>Pre-occlusion Baseline</th>
<th>Occlusion Baseline</th>
<th>Post-peak stimulus magnitude controlled with arterial compression</th>
<th>Release Period</th>
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<th>Cuff Release</th>
<th>Pre-occlusion Baseline</th>
<th>Occlusion Baseline</th>
<th>Peak stimulus magnitude controlled with arterial compression</th>
<th>Post-peak stimulus magnitude controlled at 50% peak stimulus magnitude with arterial compression</th>
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Fig. 1. A: duration manipulation experiment (DME) protocol. One minute of baseline was recorded before occlusion cuff inflation. An occlusion baseline was recorded 1 min before occlusion cuff release. After 5 min of occlusion, the cuff was released for a duration that depended on the trial [full reactive hyperemia (RH), 50 s, 40 s, 30 s, 20 s, or 10 s period]. B: peak manipulation experiment (PME) protocol. One minute of baseline was recorded before occlusion cuff inflation. After 2 min of occlusion, subjects performed 30 s–1 min of ischemic forearm contraction at 30% maximal voluntary contraction (MVC). One minute prior to occlusion cuff release, a second baseline was recorded (occlusion baseline). After 5 min of occlusion, depending on the trial, the cuff was released completely (large peak), released to 50 mmHg with arterial compression to target 75% of the normal peak hyperemia (medium peak), or released to 50 mmHg with arterial compression to target 50% of the normal peak hyperemia (small peak).
brachial pulse was located and its position was marked on the skin prior to starting the experiment. Initially a trial was performed with full release of cuff occlusion and no downstream arterial compression. This trial was used to establish the “normal” peak shear stimulus magnitude. For targeting purposes (see below) the peak stimulus was defined as the velocity in the first 9-s post-cuff release. In subsequent trials, subjects were exposed to three peak magnitudes; large (L; full release of cuff occlusion, no downstream arterial compression upon occlusion release), medium (M; occlusion release to 50 mmHg cuff pressure and downstream arterial compression targeting 75% of the normal peak brachial artery blood flow velocity magnitude for a given subject), and small (S; occlusion release to 50 mmHg cuff pressure and downstream arterial compression targeting 50% of the normal peak brachial artery blood flow velocity magnitude for a given subject).

Peak targets were maintained for the first 9-s post-occlusion release. After 9 s, arterial compression was used to keep brachial artery blood flow velocity from exceeding the “small peak” magnitude in all trials (see Fig. 1B). This control was maintained for 2 min post-occlusion release. Thus the first 9-s post-occlusion release (the peak stimulus) was different between trials (S, M, or L), but the post-peak stimulus magnitude was the same. Subjects underwent three trials of each peak magnitude. Baseline conditions were reestablished between trials. For each subject, the three trials were averaged to provide a mean response per subject for each peak stimulus manipulation condition.

Data Analysis

**Brachial artery blood flow velocity.** Blood flow velocity was analyzed off-line in 3-s average time bins. Velocity bins from multiple trials were averaged to provide an average blood flow velocity profile for each subject in every condition.

**Brachial artery diameter.** Vessel diameter was analyzed using an automated edge-detection software package (FMD/blood flow acquisition and analysis) described in Woodman et al. (32). This program allows the user to identify a region of interest (ROI) on the portion of the image where the walls are most clear. It then identifies and tracks the walls of the artery via the intensity of the brightness of the walls vs. the lumen of the vessel. The program collects one diameter measurement for every pixel column in the ROI. 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 diameter data were averaged into 3-s time bins, allowing multiple trials to be time aligned and averaged. These averaged data were then plotted over time, and a line of best fit was applied using custom software that allows the user to select the type of exponential function and manipulate the function parameters [time delay and time constant (τ)] to form a curve that is the best fit for the data (see Fig. 2B). With the use of the function parameters, another custom program was then applied to provide a corresponding diameter estimate for every 3 s. Thus a diameter measurement and a velocity measurement, time aligned for every 3 s, was obtained.

FMD is reported as the peak % change in diameter (%FMD) from the occlusion baseline measurement prior to release of forearm occlusion. This baseline measurement was used as adjustments to the image were often necessary during occlusion and thus the probe position and image during the occlusion baseline were the same as those during the release period. The occlusion baseline measurements tended to be slightly smaller than the preocclusion baseline measurements, but the pattern of FMD was the same regardless of which baseline was used in the calculation (data not shown).

**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 stimulus for FMD. The peak shear stimulus was calculated as the AUC of the shear rate in the first 9 s post-cuff release. This characterization was selected because it 1) provides a more robust quantification than the use of a single time point, 2) does not exceed the time frame of the 10-s trial in the DME, and 3) facilitates comparison with the post-peak stimulus, which was also characterized as an AUC.

**Statistical Analysis**

One- and two-way repeated measures ANOVA were used to compare both the stimulus (shear rate) and response (% change in diameter) parameters. The level for significance was set at $P < 0.05$, and significant differences for ANOVA were further assessed using Tukey’s post hoc tests. All statistics were calculated using Sigmapstat 2.03 (SPSS, Chicago, IL). Values are expressed as mean ± SD. AUC is shear rate × time. This is s$^{-1} \times$ s, and therefore does not have a unit symbol.

**RESULTS**

**Heart Rate and Blood Pressure**

Average blood pressures and heart rates at baseline, during last minute of occlusion, and following occlusion cuff release are shown in Table 1.

**DME.** There was no main effect of duration of the shear stimulus on the heart rate or the blood pressure ($P = 0.893$ and $P = 0.602$, respectively).

**PME.** There was a main effect of baseline period, as heart rate and blood pressure were slightly elevated in the occlusion baseline period compared with the initial baseline ($P = 0.018$ and $P \leq 0.001$, respectively; Table 1). Average blood pressure and heart rate post-occlusion cuff release returned to levels that were not significantly different from the initial baseline ($P = 0.181$ and $P = 0.517$, respectively; Table 1). There was no main effect of peak shear stimulus magnitude on heart rate or blood pressure ($P = 0.970$ and $P = 0.530$, respectively).
Brachial Artery Baseline Diameter

Brachial artery diameter in the DME subjects was slightly but significantly less in occlusion baseline vs. initial baseline (3.63 ± 0.27 vs. 3.67 ± 0.28 mm, P = 0.002). Brachial artery diameter for the PME subjects was also slightly but significantly less in occlusion baseline vs. initial baseline (3.82 ± 0.47 vs. 3.88 ± 0.48 mm, P = 0.009). There was no main effect of trial on baseline diameter for either the DME or PME in either the preocclusion baseline or occlusion baseline conditions (P = 0.843 and P = 0.509 for the preocclusion baseline and P = 0.553 and P = 0.247, respectively, for occlusion baseline). The absence of an increased occlusion baseline in the PME indicates that no conducted vasodilation took place as a result of ischemic exercise in the PME. The within-subjects coefficient of variation for baseline diameter was 0.95 ± 0.41% for the DME and 1.01 ± 0.74% for the PME. This demonstrates good repeatability of baseline measures and is similar to other studies using automated diameter analysis (13).

Shear Rate Profiles

DME. There was no main effect of shear stimulus duration condition on initial baseline shear rate (P = 0.663; average shear rate: 20.5 ± 9.0 s⁻¹). There was also no main effect of shear stimulus duration condition on occlusion baseline shear rate (P = 0.668; average shear rate: 3.1 ± 1.1 s⁻¹). The mean shear stimulus profile created is shown in Fig. 3A. As anticipated, there was no main effect of shear stimulus duration condition on the peak shear rate (9 s AUC not different across DME trials; range 1.173.7 ± 245.2 to 1.122.4 ± 222.4, P = 0.326; see Fig. 4A). As intended, the range of cuff reinflation times resulted in a post-peak shear stimulus (defined as the AUC from 9 to 120 s) that was significantly different between trials (main effect of cuff reinflation time P < 0.001; see Fig. 4B). However, the shear stimulus that could contribute to the development of the peak %FMD response could only be the shear stimulus occurring prior to the peak %FMD. We term this the “relevant” shear stimulus. When the shear stimulus was quantified in this fashion, the 40 s, 50 s, and full RH trials were not significantly different (see Fig. 4C).

PME. There was no main effect of peak shear stimulus condition on the initial baseline shear rate (P = 0.618; average shear rate: 21.5 ± 7.4 s⁻¹). There was also no main effect of peak shear stimulus condition on occlusion baseline shear rate (P = 0.367; average shear rate: 6.1 ± 2.2 s⁻¹). The mean shear stimulus profile created is shown in Fig. 3B. As intended, there was a main effect of peak shear stimulus condition on the peak shear stimulus (AUC first 9 s: L, 1,049.0 ± 285.8; M, 726.4 ± 228.8; S, 512.8 ± 161.8; P < 0.001; see Fig. 4D). Post-peak arterial compression was successful in creating a uniform post-peak shear stimulus magnitude in all trials [defined as the AUC from 9 to 120 s: L, 7,741.2 ± 2,026.6; M, 7,615.2 ± 1,930.4; S, 7,820.7 ± 2,141.4; P = 0.412; (see Fig. 4E)]. The relevant post-peak shear stimulus was also not significantly different between trials (P = 0.342; see Fig. 4F).

Stimulus Manipulation Effect on Peak %FMD

DME. There was a main effect of altered peak duration of the shear stimulus on the peak %FMD response under conditions of equal peak shear stimulus magnitude (see Fig. 5A). Post hoc analysis revealed specifically that 10 or 20 s of reactive hyperemia resulted in a significantly smaller peak %FMD than did the full RH exposure. Thus at least 30 s of hyperemia was required to achieve a %FMD response that was not significantly different from that achieved with a full RH period. The peak %FMD also increased linearly with the AUC of the shear stimulus (r² = 0.56; P < 0.001; see Fig. 5C). There was a main effect of shear stimulus duration on the time to peak %FMD (time from shear stimulus initiation to peak response measurement in seconds; see Fig. 6A; 10 s, 21.9 ± 5.1; 20 s, 31.2 ± 5.9; 30 s, 37.5 ± 6.4; 40 s, 44.4 ± 11.9; 50 s, 51.9 ± 13.4; full RH, 43.5 ± 15.7; P < 0.001).

PME. There was no main effect of altered peak shear stimulus magnitude on the peak %FMD response when post-peak shear stimulus magnitude was kept constant (L, 7.0 ± 2.7%; M, 7.4 ± 2.6%; S, 6.7 ± 1.84%; P = 0.542; see Fig. 5B). There was also no main effect of peak shear stimulus magnitude on the time to peak %FMD (Fig. 6B; L, 89.3 ± 25.5; M, 101.3 ± 19.9; S, 98.0 ± 19.9; P = 0.361). The peak diameter is typically reported at ~60 s, and there was also no

Table 1. Heart rate and blood pressure

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<td><strong>PME</strong></td>
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<tr>
<td>HR, beats/min</td>
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<td>82.1 ± 6.8*</td>
<td>79.8 ± 7.3</td>
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</tr>
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</table>

Values are means ± SD. DME, duration manipulation experiment; PME, peak manipulation experiment; HR, heart rate; MAP, mean arterial pressure.

*Significantly different from initial baseline; NS, not significant.
main effect at this earlier time point (L, 6.3 ± 2.3%; M, 5.7 ± 1.9%; S, 5.1 ± 1.9%; P = 0.194). There was also a poor correlation between the peak shear stimulus and the peak %FMD (r² = 0.11; P = 0.12; see Fig. 5D).

Relationship of the Peak and the AUC of the Reactive Hyperemia Shear Stimulus

In the full RH trial of the DME experiment (the only trial with no stimulus manipulation), there was no statistically significant relationship between the peak shear stimulus and the relevant shear stimulus AUC (r² = 0.35, P = 0.07; see Fig. 7).

Impact of Response Normalization

DME. When the peak %FMD response was normalized to the relevant shear stimulus AUC (peak %change/relevant shear stimulus AUC), there was no longer a main effect of shear stimulus duration conditions on peak %FMD (P = 0.06; see Fig. 8A). In contrast, the main effect of shear stimulus duration on peak %FMD response remained when %FMD was normalized to the peak stimulus (see Fig. 8B). No response normalization was performed for the PME as there were no differences in the %FMD response or the relevant shear stimulus AUC between trials.

DISCUSSION

To date it has not been clearly determined which portion of the shear stimulus profile is relevant to the development of the peak %FMD response in a reactive hyperemia test (27). The %FMD response magnitude is taken as an index of endothelial function and it is critical to discern whether small %FMD responses are the result of a small imposed stimulus or whether they are a true indicator of endothelial dysfunction. The main novel finding of this study was that the independent contribution of the peak shear stimulus is minimal, and it is the shear rate AUC, and not the peak itself, that is the critical determinant of the peak FMD response.

This conclusion is supported by four key observations. First, despite a uniform peak shear stimulus magnitude in all trials, exposure to only the peak hyperemia (10-s trial DME) resulted in minimal %FMD, and at least 30 s of reactive hyperemia was required to observe a “maximal” %FMD response (Fig. 5A). Second, exposure to the same post-peak shear stimulus resulted in a uniform %FMD response regardless of the magnitude of the peak shear stimulus (Fig. 5B). Third, when the peak %FMD in the DME was normalized to the AUC of the shear stimulus until the time of peak diameter measurement, the differences between trials were eliminated (Fig. 8A). Finally, there is a
Shear Stimulus Profiles: Peak vs. Continuation as the Determinant of Peak %FMD

In this experiment we precisely and independently manipulated the peak shear stimulus and the shear stimulus duration created with reactive hyperemia (Fig. 3). Previous studies have used techniques including varied occlusion periods, occlusion cuff positions, and ischemic handgrip exercise to create a range of stimulus magnitudes (3, 15, 20). However, none of the experimental approaches used were able to systematically manipulate one variable and hold the other constant.

Isolated manipulation of peak vs. duration of shear stimulus. Joannides et al. (15; radial artery) and Leeson et al. (20; brachial artery) both manipulated the peak and the duration of the shear stimulus created with reactive hyperemia by employing a range of occlusion periods (30 s–10 min). Both groups observed that even with no increase in the peak RH, longer hyperemic durations elicited larger peak %FMD responses. These results agree with ours in that they demonstrate that increased shear stimulus duration in the absence of an increase in the peak shear stimulus results in a larger %FMD response. However, our study extends the findings of these previous studies in several important ways.

1) It applies specifically to the current standard 5-min occlusion reactive hyperemia test.
2) We have quantified shear rate and isolated the impact of the peak vs. the continued AUC of the reactive hyperemia shear stimulus on the peak %FMD. 3) Our design allows for conclusive, specific recommendations regarding reactive hyperemia peak vs. AUC in %FMD response normalization.

With the DME we found that 10 s of reactive hyperemia resulted in minimal %FMD. The peak shear stimulus was the same in all trials so this provides strong evidence that with a 5-min occlusion reactive hyperemia test, the peak stimulus by itself is not responsible for determining the %FMD response. In fact, at least 30 s of hyperemia was required to see a “maximal” %FMD response, which is consistent with the duration of the hyperemia being an important determinant of the %FMD response. In this study the shear stimulus decayed rapidly and the majority of the relevant shear stimulus magnitude had already elapsed by 30 s post-occlusion release. It is quite possible that if the elevation in shear stimulus was of longer duration, the 40, 50, and full release conditions would have yielded progressively larger %FMD responses.

In the PME, the post-peak shear stimulus (continuation of shear stimulus) was constant between trials. The peak magnitudes were substantially and significantly different with the medium and small peak magnitudes at 69% and 48% of the large peak magnitude, respectively. It was previously observed in isolated vessels that an impulse increase in shear stress results in a G protein-dependent burst of nitric oxide release (9). This previous observation would suggest that the sudden increase in shear stress at the onset of reactive hyperemia is responsible for initiating the endothelium-dependent vasodilation. In the present study, we observed that the magnitude of the peak shear stimulus had no impact on the magnitude of the %FMD response under conditions of equal continued reactive hyperemia. Taken together with the observation in the DME that the initial 10 s of reactive hyperemia in isolation result in minimal dilation, it appears that the independent contribution of this short burst of vasodilator release creates minimal dilation and is “overwhelmed” by the effect of the continued elevated shear stimulus. Thus peak shear stimulus magnitude plays little role by itself in determining the %FMD response. Rather, this study supports the hypothesis that the magnitude of the shear stimulus continuation is the key determinant of the %FMD response magnitude.
%FMD: correlation with peak and duration of shear stimulus. Figure 5, C and D, respectively, plot 1) %FMD against the relevant shear stimulus AUC across the reactive hyperemia duration conditions in the DME experiment and 2) %FMD against the peak shear stimulus AUC in the PME experiment. These correlations reveal that as the duration of shear stimulus following release of occlusion is allowed to increase from the 10 s to full RH conditions, %FMD increases proportionally (Fig. 5C). This relationship is a strong one ($r^2 = 0.56, P < 0.001$). In contrast, when the peak shear stimulus is manipulated independently, there is virtually no change in %FMD magnitude as indicated by a minimal slope and non-significant $r^2 = 0.11$ (Fig. 5D; $P = 0.120$). The much steeper slope in Fig. 5, C vs. D, translates into a much greater sensitivity of %FMD to the duration of shear stimulus than to the peak shear stimulus when each is manipulated independently.

Consideration of Blood Viscosity

Because we were unable to measure blood viscosity in this experiment, we could not quantify shear stress. However, shear stress is simply the product of shear rate and viscosity. Therefore we used shear rate as an estimate of the shear stimulus. Within a subject during a given experimental session, viscosity will be stable so changes in shear rate represent proportional

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**Figure 7.** DME full RH trial. Relationship between the peak shear stimulus (9-s AUC) and the continued (post peak relevant) shear stimulus AUC ($r^2 = 0.35, P = 0.07$).
Changes in shear stress. It is possible that there were day-to-day fluctuations in blood viscosity (DME only, PME had only 1 test day) that might have influenced the magnitude of the stimulus (shear stress). However, viscosity fluctuations are unlikely to affect our conclusions for a number of reasons. First, the 3 test days in the DME were performed within a 7-day period, at the same time of day under the same fasting conditions, and this should have minimized variability (30). Second, all trials were performed each day and averaged together to form a single mean response for each subject. Thus, even if viscosity did vary between days, it was accounted for equally in all DME conditions and therefore would not affect our conclusions. Third, blood viscosity measured three to five times per subject over a period of 6–8 wk demonstrates a very low intrasubject variability (mean coefficient of variation 2.72% range 0.96–4.9%; Ref. 10).

**Consideration of Cuff Reinfalation Effect**

In the DME experiment, the duration of the reactive hyperemia was controlled by reinfalation of the occlusion cuff at specific time points. It could be suggested that this reinfalation affects the vessel mechanical environment in a way that initiates a vasoconstrictor response and therefore the blunting of FMD in the 10- and 20-s reactive hyperemia trials could be confounded by an activation of vasoconstriction. The basis of this concern originates in the observations that there is an apparent reduction in vessel diameter during the downstream occlusion period (21).

There are a number of lines of evidence within the present study, however, that argue against a confounding effect of downstream cuff reinfalation-induced vasoconstriction. With continuous tracking of vessel diameter from baseline through the first 2 min of occlusion and from the last minute of occlusion until 2 min after occlusion release, we consistently observe the following as indicated in a representative brachial artery diameter response in Fig. 2, A and B: 1) there is no change in vessel diameter during the first 2 min of occlusion; 2) the average change in diameter from preocclusion baseline to the last minute of occlusion in the present study was only 1% (3.63 ± 0.27 vs. 3.67 ± 0.28 mm); 3) the FMD response initiates ~14–20 s and reaches a peak value within 60–90 s, which is during a time period where there is no cuff inflation-induced vasoconstriction; and 4) the %FMD in the 10 s vs. full RH was 2.7 ± 1.3 vs. 9.3 ± 4.1, which, if explained by a counteracting vasoconstrictor influence, would require that influence to be substantial. Taken together, these observations argue strongly against cuff reinfalation-induced vasoconstriction as a confounder in this study.

**Consideration of Ischemic Handgrip Exercise**

The post-peak shear stimulus in the PME was more prolonged than that in the DME due to the addition of a brief period (30 s–1 min) of ischemic handgrip exercise. Ischemic handgrip exercise has previously been shown to increase the duration of reactive hyperemia (3, 31). The prolonged hyperemia was necessary to control the post-peak shear stimulus magnitude and ensure that it was uniform between trials. The prolonged stimulus resulted in a delayed attainment of peak diameter [average 96 s vs. the roughly 60 s that is typically reported in the literature (5)].

The prolonged stimulus and its effect on response development warrants consideration for several reasons. First, it could be suggested that a long period of post-peak elevated shear stimulus would potentially “wash out” the impact of the brief peak shear stimulus. However, if this were the case in the current PME study, we would expect a peak shear stimulus effect at the typical peak %FMD time of 60 s. Instead, the %FMD was virtually identical between PME conditions at this time. Thus we do not believe that a wash out effect can explain our findings.

Second, the prolonged stimulus in our study may have affected the %FMD response mechanisms responsible for the observed peak %FMD. Mullen et al. (Ref. 26; using a 15-min period of occlusion) and Agewall et al. (Ref. 2; using ischemic handgrip exercise) have shown that a prolonged reactive hyperemia results in a peak %FMD response that is not (primarily) nitric oxide mediated. This may be due to the engagement of a second, delayed-onset mechanism. This is in contrast to the response that is created with a 5-min occlusion without ischemic exercise, which is thought to be nitric oxide dependent (7, 17, 22). However, it must be noted that the timing of the peak %FMD in these previous prolonged hyperemia studies would have been delayed relative to the ~60 s time of 5-min occlusion studies (5). As stated above, in the current PME study, the peak %FMD at 60 s was virtually identical between conditions. Thus we believe our findings are relevant for both early and later acting %FMD mechanisms.

A final consideration regarding ischemic handgrip in this study is the potential effect on sympathetic activation via the muscle chemoreflex. In the PME, heart rate and blood pressure were transiently elevated by performing ischemic handgrip exercise. This has been observed by others in a similar setting (3). The magnitude of the elevation in these variables (2.1 beats/min and 3.2 mmHg) suggests mild sympathetic activation. Sympathetic activation created via lower body negative pressure has been reported to blunt the %FMD response (12).
In contrast to these findings, a recent study by Dyson et al. (8) found no impact of either lower body negative pressure or peak metaboreflex-induced sympathetic activation on the %FMD in healthy young males. In our study, the increases in heart rate and blood pressure were minor, not different between trials, and they returned to baseline within seconds of cuff release. Thus, in the unlikely event that there remained a mild level of sympathetic activation during the FMD response, it should not have affected the response magnitudes and therefore we do not believe sympathetic activation confounds our findings.

%FMD Response Normalization Recommendations

We recently stressed the importance of accounting for the magnitude of the stimulus when interpreting %FMD responses (28), and this approach is beginning to come into practice (6). However, the practice of stimulus normalization has been hindered because there has been no clear evidence as to which shear stimulus quantification should be used to normalize responses. In the current study, %FMD response normalization with the AUC of the shear stimulus until the time of peak diameter measurement eliminated the differences in the response seen in the DME (Fig. 7A). This time interval was selected because it represents the entire shear stimulus that could be contributing to the response. In contrast, when the %FMD response was normalized to the magnitude of the peak shear stimulus, the differences in %FMD between trials were maintained.

One final consideration with regard to normalization recommendations needs to be addressed. In the DME study, the duration of the stimulus was artificially manipulated by reinflating the occlusion cuff mid-hyperemia. Perhaps without this intervention the magnitude of the peak shear stimulus and the continuation of the shear stimulus (post-peak AUC) would be very closely related, making the peak shear stimulus an acceptable “normalization factor” as is suggested by a previous publication from our laboratory (27). However, in the full RH trial where the duration of the shear stimulus was not manipulated, there was a poor relationship between the peak shear stimulus and the post-peak shear stimulus ($r^2 = 0.35; P = 0.07$; see Fig. 6). This lack of correlation between the peak and the continuation of the stimulus has also been reported by others (15). In addition Ishibashi et al. (14) observed that while healthy subjects and subjects with multiple risk factors for cardiovascular disease had the same peak reactive hyperemia, the duration of the hyperemia was significantly attenuated in risk factor subjects. This observation is critical because it has ramifications for the interpretations of diagnostic/predictive FMD studies performed in high-risk groups. Combined, these data confirm that the peak and the duration of the reactive hyperemia can vary independently (peak does not always represent duration). Therefore, because the duration of the stimulus is important [demonstrated by the current study and shown by others (3, 13, 16)] it should be measured and used in response normalization.

In conclusion, independent manipulation of the continuation of reactive hyperemia had an impact on the peak %FMD response, whereas independent manipulation of peak reactive hyperemia did not. We conclude that the continued shear rate AUC is the critical determinant of the peak %FMD response and needs to be accounted for in quantifying the stimulus for FMD. Therefore, normalization of the peak %FMD response to the total AUC of the shear rate stimulus until the time of peak diameter measurement is the appropriate strategy to account for shear stimulus contributions to %FMD.

REFERENCES


