Interruption exercise abolishes the diurnal variation in endothelial-dependent flow-mediated dilation in humans

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THE FLOW-MEDIATED DILATION (FMD) technique provides information about endothelial function of conduit arteries and, when the appropriate methodology is used, it has been reported that the FMD-response is nitric oxide (NO)-mediated vasodilation. Therefore, we measured brachial artery flow-mediated dilation (FMD) in 10 males (mean age = 28 yr, SD = 7), before and after a bout of intermittent cycling at 70% peak oxygen uptake on separate days beginning either at 0800 or 1600. Edge-detection and wall-tracking software was used to measure changes in arterial diameter, while shear rate (SR) was assessed using simultaneously derived blood flow velocity and B-mode diameter data. The FMD data were analyzed before and after normalization for SR with repeated-measures models. Before exercise, mean ± SD FMD was 7 ± 3% in the morning compared with 11 ± 6% in the afternoon (P = 0.01). This diurnal variation persisted after data were normalized for SR, which was found to be unaffected by time of day (P = 0.33). Postexercise SR was higher than at baseline (pre-exercise) (P = 0.01) to a similar extent at both times of day. FMD was unaffected by exercise in the morning (P = 0.96) but decreased by 4 ± 3% following exercise in the afternoon (P = 0.01) so that postexercise measurements did not differ between times of day. These data indicate that endothelium-dependent FMD is lower in the morning, compared with other times of day, to determine whether the magnitude of the proposed FMD stimulus accounts for the observed effects (37). Therefore, it is currently unclear whether the diurnal variation in FMD is due to diurnal differences in intrinsic endothelial NO-vasodilator system function or in SR related to blood flow changes (i.e., differences in the stimulus per se).

Everyday life for most individuals consists of intermittent bouts of physical activity, which vary in intensity and duration. Interruption exercise also characterizes many leisure and sports activities. Therefore, it is important to examine the effects of such “real-world” physical activity in the studies on diurnal variation to understand the changes in circulatory control with time of day. Interestingly, recent studies on diurnal variation in postexercise circulatory responses have indicated that postexercise blood pressure and brachial SR are higher in the morning than in the afternoon (25, 27). The purpose of this study was 1) to investigate the contribution of diurnal variation in SR to that in endothelial-dependent FMD, and 2) to explore whether diurnal variation in FMD is present following a preceding bout of intermittent exercise. Our primary hypothesis was that FMD will be lower in the morning compared with the afternoon and that this diurnal variation will not be explained by differences in SR.

METHODS

Participants. Ten normotensive males aged 28 ± 7 years, with mean ± SD body mass of 75.6 ± 6.3 kg, height of 1.79 ± 0.04 m and peak oxygen uptake (V̇O₂ peak) of 45.7 ± 6.7 ml·kg⁻¹·min⁻¹ participated in the study. All participants were nonsmokers, had no history of cardiovascular disease, were not taking any medication, and all reported that they were engaged in regular physical activity >2 h/wk on a self-report questionnaire. The study conformed to the Declaration of Helsinki and was approved by the Institutional Ethics Committee. All participants were informed of the methods before providing written informed consent.

Research design. Participants attended the laboratory on four separate occasions, with the first visit for familiarization purposes, the second visit for measurement of peak oxygen uptake, and then two visits for completion of the main experimental trials involving FMD measurements prior to and following an intermittent exercise protocol in the morning (AM) and the afternoon (PM). The two trials were administered in a counterbalanced order and were separated by 7–10 days. The light intensity in the laboratory was controlled at ~200 lux, and temperature was maintained at 21°C. At both times of day, the protocol began after a 12-h abstinence from caffeine, 24-h abstinence from alcohol, and strenuous exercise, and at least a 4-h fast (participants ate a standard carbohydrate breakfast only between 0800 and 0900 on the day of the afternoon test).

Familiarization. During their first visit to the laboratory, participants were familiarized with the equipment and exercise protocol, and anthropometric measurements were recorded. Height (m), body mass...
measurement (kg), and resting blood pressure (three serial measurements with a mercury manual sphygmomanometer) were determined.

Measurement of peak oxygen uptake. On the second visit to the laboratory, \( \text{VO}_2\peak \) was determined using a progressive continuous protocol (7). As a standard warm-up, participants performed 10 min of submaximal cycling on an ergometer (KettleTec Sport, Worcestershire, UK). Power output was set initially at 50 W and was increased in 25-W increments every 2 min until volitional exhaustion or the point at which the subject could no longer maintain the required pedal cadence (≥60 rev/min). Expired gases were collected using an on-line collection system that sampled every 10 s (MetaMax 1; Cortex Biophysic, Leipzig, Germany). Oxygen uptake was then plotted against work rate, and the exercise work rate (i.e., watts) corresponding to 70% \( \text{VO}_2\peak \) was interpolated using a linear regression equation.

Experimental protocol. Participants reported to the laboratory at 0700 ready to begin exercise at 0800 in the morning exercise condition, and at 1500 ready to begin exercise at 1600 in the afternoon exercise condition. Baseline measurement of conduit artery FMD was obtained prior to any exercise. The exercise protocol consisted of three 10-min bouts of semisupine cycling at 70% \( \text{VO}_2\peak \) with each bout separated by 10-min rest periods (seated on cycle ergometer). The exercise protocol was intermittent in nature, as previous research by our group has indicated that this type of exercise protocol at this intensity mediated greater postexercise cardiovascular responses compared with a continuous exercise protocol (24, 28). Following the exercise protocol, participants remained seated on the ergometer for a period of 20 min. Postexercise conduit artery FMD measurements were obtained immediately following this period (i.e., recorded between 20 and 30 min postexercise).

Measurement procedures. A 10-MHz multifrequency linear array probe attached to a high-resolution ultrasound machine (Acuson Aspen, Mountain View, CA) was used to image the brachial artery on the right arm. Conduit artery endothelium-dependent dilation was assessed via the response to FMD with the occluding cuff placed distal to ultrasound probe. Baseline scans for the assessment of resting vessel diameter and flow were recorded in the final minute of the initial rest period. The occluding cuff was then inflated to 220 mmHg for 5 min. Diameter and blood flow velocity recordings resumed 30 s prior to cuff deflation and continued for 3 min thereafter. Post-test analysis of conduit artery diameter was performed using customized edge-detection and wall-tracking software, as described previously (8, 11), which provides simultaneous and continuous measurements of arterial diameter and blood flow velocity. The assessment of blood flow velocity uses the edge detection algorithm to assess the peak velocity envelope from the Doppler gate, which is placed in the middle of the artery. From these data, the software calculates blood flow (the product of cross-sectional area and blood flow velocity) and SR (four times the velocity divided by the diameter) at 30 Hz. The edge-detection and wall-tracking software is semi-automated and provides diameter measurements that are considerably more repeatable (coefficient of variation = 6.7%) than manual methods and are associated with less observer error (45).

In accordance with recent recommendations (37–39), we also analyzed the FMD data after normalization for the SR stimulus. The postdeflation SR data, derived from simultaneously acquired velocity and diameter measured at 30 Hz, was exported to a spreadsheet, and the area under the SR curve (\( \text{SR}_{\text{AUC}} \)) was calculated for data up to the point of maximal postdeflation diameter (FMD) for each individual (\( \text{SR}_{\text{AUC}} \)). In this way an individual’s FMD was normalized to the area under his own SR curve between the point of deflation and maximal dilation for that individual.

Statistical analysis. Baseline (preexercise) and postexercise vascular and blood pressure measurements were analyzed using two-factor general linear models with repeated measures. The factors were time of day (which refers to morning or afternoon) and exercise (which refers to the preexercise or postexercise time point). The sphericity assumption in repeated measures is not relevant in this two-level situation. Statistically significant interactions were followed-up with multiple contrasts corrected for type I error rate using the Newman Keuls procedure. According to current opinion on normalization of FMD for differences in SR (20), we normalized FMD for SR using two statistical approaches; the simple ratio of FMD and SR, and an ANCOVA-based repeated-measures statistical model with SR entered as a changing covariate. In light of current debate about the general magnitude of correlation between SR and FMD in different research situations (4), we also calculated the within-subjects correlation coefficient between SR and FMD using appropriate methods, which partition the within-subject and between-subject influences properly (9). All data were analyzed using the STATISTICA software (Statsoft, Tulsa, OK). Data are presented in the text as means ± SD, and exact P values are cited (values of P of “0.000” provided by the statistics package are reported as “<0.0005”).

RESULTS

Brachial artery FMD and SR stimuli. A statistically significant interaction between time of day and exercise (referring to baseline/preexercise or postexercise) was evident for brachial FMD (P = 0.01). Follow-up contrasts indicated that baseline FMD was 7 ± 3% in the morning compared with 11 ± 6% in the afternoon (P = 0.01, Fig. 1). Nevertheless, the exercise effect depended on the time of day; following exercise in the afternoon, FMD was reduced to 7 ± 3% (P = 0.01), but no change in FMD was evident after exercise in the morning (P = 0.96).

SR was unaffected by time of day (time of day main effect: P = 0.33). SR was higher after exercise compared with baseline values at both times of day (exercise main effect: P < 0.005). The nonsignificant interaction between time of day and exercise (P = 0.41) indicated that the exercise-mediated increases of 12,011 ± 12,513 and 7,432 ± 7,138 AU in the morning and afternoon, respectively, were not significantly different (Fig. 2). Baseline and peak artery diameters, as well as the time-to-peak artery diameter, were not found to be influenced by time of day, exercise, or the interaction between these two factors (P > 0.23, Table 1).
Using appropriate statistical modeling (9), we found a low and nonsignificant within-subject correlation ($r = -0.02, P = 0.91$) between the measurements of SR and FMD in our experimental conditions. Regression lines are shown in Fig. 3 for individual subjects, and it can be seen that the slopes are very variable between subjects. We also found low ($r < 0.3$) and nonsignificant between-subject correlations between SR and FMD when examined in each of the four experimental conditions (preexercise and postexercise at the two times of day). Although this absence of a correlation between SR and FMD challenges the usefulness of normalization (4), we proceeded to examine the effects of two SR normalization approaches on our FMD data. When FMD was controlled for the effects of SR with the ANCOVA approach, the interaction between time of day and time was still statistically significant ($P = 0.01$). The covariate-adjusted mean FMD at preexercise/baseline was lower in the morning compared with the afternoon ($P = 0.005$). Exercise mediated a reduction in FMD but only in the afternoon so that postexercise FMD was not different between times of day ($P = 0.71$). When the FMD/SR ratio approach to normalization was adopted, similar trends in the nonnormalized and ANCOVA-normalized data were observed. FMD/SR was lower following exercise in the afternoon but not lower after exercise in the morning. Nevertheless, the $P$ value (0.08) for the interaction between time of day and exercise was higher for the FMD/SR data than the nonnormalized and ANCOVA-normalized data.

**Blood pressure.** Systolic and diastolic blood pressures were not different during the FMD test (i.e., pre-post occlusion) ($P > 0.11$). No interactive effects between time of day and exercise were evident for diastolic blood pressure ($P = 0.299$). There was evidence of an interaction between time of day and exercise for systolic blood pressure ($P = 0.06$, Fig. 4). Resting systolic blood pressure was $5 \pm 3$ mmHg greater in the afternoon compared with the morning ($P = 0.01$). Systolic blood pressure was reduced following exercise in the morning and afternoon ($P < 0.01$). The mean ± SD difference was $4 \pm 6$ and $10 \pm 6$ mmHg in the morning and afternoon, respectively.

**DISCUSSION**

In the present study, we employed the FMD technique to examine diurnal variation in endothelial-dependent FMD at rest and following a bout of intermittent exercise. Previous researchers have found that FMD is lower in the morning compared with other times of day (13, 30, 34), but these studies have not involved measurements of SR in parallel with measurements of FMD. In support of our primary hypothesis, our data indicate that lower FMD is not explained by differences in SR, since SR was not different in the morning and afternoon. This infers that intrinsic differences in the function of the endothelial NO-vasodilator system contribute to diurnal variation in FMD in arteries of similar size and function as epicardial coronary arteries (1, 41, 42). We also report, for the first time, that a bout of intermittent, submaximal exercise abolishes this diurnal variation in FMD, since FMD measured postexercise was not different between morning and afternoon test times.

**Diurnal variation in FMD.** Our data indicate that it is unlikely that variation in SR (37–39) is the explanation for the observed diurnal variation in FMD. Therefore, within the bounds of our measurement technique and study population, our data infer that intrinsic differences in the function of the endothelial NO-vasodilator system mediate the diurnal variation. This could be explained by cyclical changes in NO bioavailability or NO

### Table 1. Resting and peak artery diameters and the time-to-peak artery diameter values preexercise and postexercise in the morning and afternoon

<table>
<thead>
<tr>
<th></th>
<th>Morning</th>
<th>Posterior</th>
<th></th>
<th>Morning</th>
<th>Posterior</th>
<th></th>
<th>$P$ value TOD × Exercise</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline artery diameter, mm</td>
<td>4.43±0.37</td>
<td>4.49±0.37</td>
<td>4.35±0.28</td>
<td>4.43±0.37</td>
<td>$P=0.85$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak artery diameter, mm</td>
<td>4.75±0.44</td>
<td>4.79±0.44</td>
<td>4.82±0.34</td>
<td>4.66±0.28</td>
<td>$P=0.23$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time to Peak, s</td>
<td>67±28</td>
<td>75±44</td>
<td>68±41</td>
<td>86±34</td>
<td>$P=0.55$</td>
<td></td>
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</tr>
</tbody>
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Values are expressed as means ± SD, TOD, time of day.
synthase phosphorylation, which are thought to influence endothelial function (10, 18). Support for this notion comes from recent research data, which has linked disturbances in molecular biological oscillation driven by the circadian clock to endothelial dysfunction (3). Alternatively, or in combination with NO-mediated vasodilator function, cyclical variation in components opposing vasodilation, such as endothelin-1 (32) or even sympathetic nerve activity (SNA) (36), could be contributing to the diurnal variation in FMD (i.e., lower in the morning).

Normalization of FMD data. In keeping with recent advice (39) and as an added methodological angle to our study, we normalized our FMD data using the FMD/SR ratio and ANCOVA-based approaches. The effects of time of day and exercise on FMD were generally similar, irrespective of these types of analyses. This similarity is not surprising, since we could not detect any within-subjects correlation between changes in SR and FMD using appropriate data modeling techniques (9). Statistically, normalization for another variable (SR) would impact on specific study conclusions only if the observed correlation between that variable and the outcome variable (FMD) is at least moderate (4). Previous researchers, who directly manipulated SR using a pressure-cuff on the limb, reported moderate correlations between changes in SR and changes in FMD (23, 31, 35, 37), although data were pooled across subjects for the analyses in these studies. Interestingly, only low correlations were found between measured (but not manipulated directly) SR and FMD in a recent cross-sectional study on healthy people of different ages (43). In another cross-sectional study, the nature of the relationships between measured SR and FMD indicated that normalization might lead to erroneous conclusions (4). The magnitude of a correlation coefficient can also be influenced by the range of data collected, and we note that, in our repeated measures study, the biological variation in SR was only small between the two times of day studied. In addition, other unmeasured variables involved in the causal nexus between changes in SR and changes in FMD (e.g., the amount of vasodilator that arrives at the smooth muscle) could also have changed with time of day and exercise and may have influenced the observed magnitude of correlation.

**FMD technique.** In addition to the fact that we measured SR alongside FMD, the current study has several notable advantages over previous studies investigating diurnal variation in FMD in terms of the study design and methodological procedures. We employed a counterbalanced repeated-measures design on separate days rather than serial FMD measurements during the same day. We also adhered to the most appropriate methodology on young and healthy individuals to suggest a largely NO-mediated FMD response (22), which included both correct cuff positioning and postocclusion timing (8, 12). We have also complemented the exploration of diurnal variation in FMD, with the inclusion of postexercise FMD data. Our group has recently demonstrated that diurnal variation is evident in aspects of circulatory control following exercise (25–26).

**Fig. 4.** Systolic blood pressure values preexercise and postexercise in the morning and afternoon. Evidence of an interaction between time of day and exercise for systolic blood pressure ($P = 0.06$). *Significant difference between time of day pre-exercise. **Significant difference between preexercise and postexercise FMD at both times of day.
acute exercise such as that observed in the afternoon in the current study could potentially be explained by elevated oxidative stress (15, 19) or inflammation (2). Indeed, biomarkers of both oxidative stress and inflammation show diurnal variation at rest (29, 33). Peak concentrations of oxidative stress biomarkers are evident in the early evening (29). It might, therefore, be speculated that more oxidative stress in the afternoon impacts FMD. However, the diurnal variation oxidative stress at rest (i.e., higher in the afternoon) does not explain the resting FMD data in the current study, and no research to date has examined diurnal variation in such markers following exercise.

Only when baseline values of FMD were higher in the afternoon, did we observe an exercise-mediated reduction in FMD. A postexercise reduction in FMD is in agreement with some previous research (2, 11, 21) but not all (6, 15, 21). Generally, there is no consensus within the literature as to the direction of change in FMD immediately after exercise, and the discord is no consensus within the literature as to the direction of change in FMD immediately after exercise, and the discordance is evident in the early evening (29). It might, therefore, be speculated that more oxidative stress in the afternoon impacts FMD. However, the diurnal variation oxidative stress at rest (i.e., higher in the afternoon) does not explain the resting FMD data in the current study, and no research to date has examined diurnal variation in such markers following exercise.

Methodological limitations. One potential limitation of this study is the lack of assessment of diurnal variation in vascular smooth muscle function (i.e., endothelium-independent vasodilation). Previous researchers have suggested that there is no diurnal variation at all (14, 30, 31), or greater endothelium-independent vasodilation in the morning (5). However, all of the previous studies involving measurements of endothelium-independent vasodilation have utilized a 4-min assessment period, which may not have been long enough to fully observe the changes that occur with sublingual nitroglycerin administration (8). Therefore, further research is warranted to accurately quantify diurnal variation in endothelium-independent vasodilation. Another possible limitation of the present study relates to the lack of control of sleep prior to the two trials. Potentially, diurnal differences in resting FMD could be due to the longer period of inactivity (i.e., during sleep) or nocturnal sleep per se, prior to the morning measurements compared with the typical everyday activities, which participants undertook prior to the afternoon measurements. Although we have shown recently, using a protocol that controlled for the amount of prior sleep, that diurnal variation in blood pressure at rest and following exercise cannot be explained by sleep-related influences (26), FMD was not measured in this study. In addition, a long period of inactivity (3 days) has been shown to negatively affect FMD measurements (17).

Conclusion. The data from this study indicate that the diurnal variation in resting endothelium-dependent FMD is not explained by changes in SR. This suggests that intrinsic endothelial function mediates diurnal variation in FMD in conduit arteries. Diurnal variation in FMD was not evident following a bout of intermittent exercise, which suggests that diurnal variation in FMD is influenced by the rest-activity cycle over 24 h. To unravel the relative endogenous and exogenous influences on diurnal variation in FMD, it is clear from our data that the effects of physical activity should be taken into account.

Perspectives and Significance

A reduction in FMD in the morning has previously been implicated as a potential mechanism, which contributes to the morning peak in cardiovascular events (34). We present data that support some previous findings of diurnal variation in FMD and add the observation that this variation in FMD as a function of time of day is intrinsically mediated and not related to diurnal change in SR. Although our findings are specific to healthy normotensive males with no evidence of cardiovascular disease, lower FMD in the morning has been shown previously in individuals with cardiovascular disease at rest (30). Therefore, it can be reasonably assumed that a similar diurnal pattern in FMD will be evident in individuals with cardiovascular disease. Future research studies should focus upon determining the relative contribution of NO to the diurnal variation in endothelial function together with competing vasoconstrictor control mechanisms, ideally via pharmacological blockade.

DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


