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Retrograde Flow and Shear Rate Acutely Impair Endothelial Function in Humans

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Abstract—Changes in arterial shear stress induce functional and structural vasculature adaptations. Recent studies indicate that substantial retrograde flow and shear can occur through human conduit arteries. In animals, retrograde shear is associated with atherogenic effects. The aim of this study was to examine the impact of incremental levels of retrograde shear on endothelial function in vivo. On 3 separate days, we examined bilateral brachial artery flow-mediated dilation, an index of NO-mediated endothelial function, in healthy men (24±3 years) before and after a 30-minute intervention consisting of cuff inflation to 25, 50, or 75 mm Hg. Cuff inflations resulted in "dose"-dependent increases in retrograde shear rate, compared with the noncuffed arm, within subjects ($P<0.001$). Flow-mediated dilation in the cuffed arm did not change in response to the 25-mm Hg stimulus but decreased significantly after both the 50- and 75-mm Hg interventions ($P<0.05$). The decrease in flow-mediated dilation after the 75-mm Hg intervention was significantly larger than that observed after a 50-mm Hg intervention ($P=0.03$). In the noncuffed arm, no changes in shear rate or flow-mediated dilation were observed. These results demonstrate that an increase in retrograde shear rate induces a dose-dependent attenuation of endothelial function in humans. This finding contributes to our understanding regarding the possible detrimental effects of retrograde shear rate in vivo. (*Hypertension*. 2009;53:986-992.)

Key Words: retrograde shear stress ■ endothelial function ■ shear stress pattern ■ echo Doppler ■ oscillatory

Rubanyi et al[1] demonstrated that increases in shear stress lead to endothelium-dependent arterial dilation. More recent studies indicate that changes in shear stress on the endothelial cell membrane are a key stimulus for adaptation in both vascular function and remodeling.[2–6] Elevation in endothelial shear stress, such as that present during exercise, is a key stimulus to express antiatherogenic genes (eg, endothelial NO synthase)[7] and decrease proatherogenic genes (eg, endothelin 1).[8] Importantly, these changes in gene expression are associated with enhanced endothelial function in vivo.[9,10] Although changes in shear can clearly transduce beneficial arterial adaptations, it is also apparent from studies performed in vitro and in animals that oscillatory shear stress, characterized by high levels of retrograde shear, can increase the expression of proatherogenic, and decreases antiatherogenic, genes.[11] For example, oscillatory shear increases endothelin 1 expression[12] and adhesion molecules[13,14] and enhances the release of superoxide[15] and expression of reactive oxygen species–producing enzymes (ie, NADPH oxidase)[16,17] but decreases endothelial NO synthase expression.[16,17] However, the impact of changing retrograde shear stress has never been examined in humans.

The primary purpose of this study was to examine the impact of different magnitudes of retrograde shear stress on endothelial function in humans. To this end, we simultaneously examined brachial artery endothelial function in both arms of healthy subjects before and after 30-minute exposure to stepwise increases in retrograde shear induced by cuff inflation on 1 forearm to 25, 50, or 75 mm Hg. In this way, we constructed a dose-response curve relating the increase in retrograde shear stress to changes in brachial artery endothelial function in vivo.

Methods

Subjects
Ten healthy recreationally active men (23.9±2.9 years of age; body mass index: 23.5±2.2 kg/m^2) were recruited from the community. No subject reported having been diagnosed with cardiovascular disease or risk factors such as hypercholesterolemia or hypertension. Subjects who were on medications influencing the cardiovascular system were excluded. The study procedures were approved by the ethics committee of Liverpool John Moores University and adhered to the Declaration of Helsinki, and all of the subjects gave previous written consent.

Experimental Design
All of the subjects reported 3 times to the laboratory. Under standardized conditions, bilateral endothelial function was examined simultaneously in the brachial artery of both arms using the flow-mediated dilation (FMD) technique, before and after a 30-minute
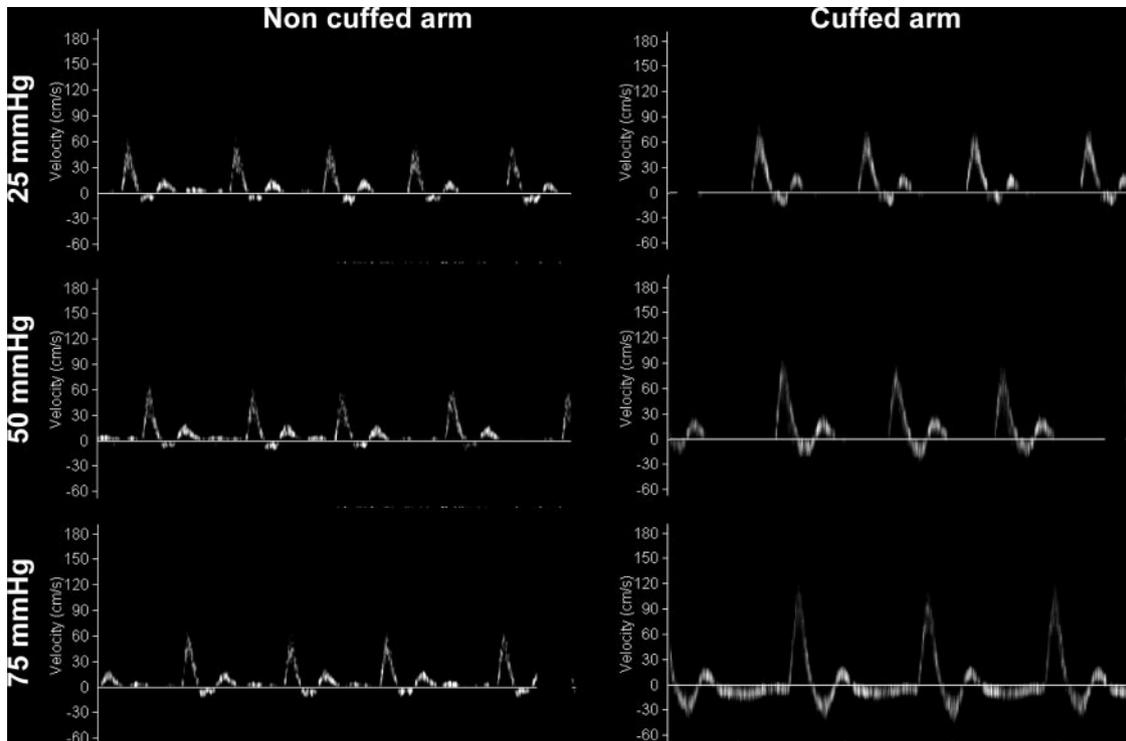
Figure 1. Screen captures of the Doppler velocity during each intervention (25 mm Hg, top; 50 mm Hg, middle; and 75 mm Hg, bottom) in the noncuffed (left) and cuffed (right) arms in a representative individual. Clearly, the retrograde SR in the cuffed arm increases when cuff pressure rises. Note that minimal changes in peak retrograde SR are present, whereas the area under the curve (ie, used to calculate retrograde SR) increases markedly.

intervention. The intervention consisted of inflation of an occluding cuff to 25, 50, or 75 mm Hg for 30 minutes around 1 forearm. Cuff inflation resulted in an increased retrograde shear rate (SR), without impacting the antegrade SR in the brachial artery (Figure 1). The level of cuff inflation was randomized across the 3 testing days.

Experimental Procedures

Vascular function assessments were conducted in a quiet, temperature-controlled environment. Repeated laboratory visits were conducted at the same time of day. Before each test, subjects were requested to fast for 6 hours, abstain from alcohol and caffeine for 18 hours, and avoid exercise for 24 hours.

Assessment of Flow-Mediated Endothelium-Dependent Vasodilator Function (FMD%)

Before and after each intervention, endothelium-dependent, largely NO-mediated, vasodilator function was assessed using the FMD approach.[18,19] Preintervention, subjects rested in the supine position for a period of $\geq$15 minutes to facilitate baseline assessment of heart rate and blood flow. Heart rate and mean arterial pressure were determined from an automated sphygmomanometer (Dinamap, GE Pro 300V2) placed around the left ankle.

To examine brachial artery FMD, both arms were extended and positioned at an angle of $\approx$80° from the torso. A rapid inflation and deflation pneumatic cuff (D.E. Hokanson, Bellevue, Wash) was positioned on both forearms of the imaged arm immediately distal to the olecranon process to provide a stimulus to forearm ischemia.[20] A 10-MHz multifrequency linear array probe attached to a high-resolution ultrasound machine (T3000, Terason) was used to image the brachial arteries in the distal third of the upper arm. When an optimal image was obtained, the probe was held stable, and the ultrasound parameters were set to optimize the longitudinal, B-mode images of the lumen-arterial wall interface. Continuous Doppler velocity assessment was simultaneously obtained using the ultrasound machine and was collected using the lowest possible insonation angle (always <60°), which did not vary during each study.

Baseline images were recorded for 1 minute. The forearm cuff was then inflated (>200 mm Hg) for 5 minutes. Diameter and blood flow recordings resumed 30 seconds before cuff deflation and continued for 3 minutes thereafter. These procedures for bilateral and simultaneous FMD assessment were repeated immediately after each intervention, and the same experienced sonographers were involved in each assessment for a given subject.

Retrograde Shear Interventions

Immediately after the initial bilateral FMD assessments, a 30-minute intervention was performed. A cuff was placed around 1 arm and inflated to 25, 50, or 75 mm Hg. Placement of this cuff around the left or right arm was randomized between subjects but always on the same arm for each subsequent intervention within subjects. The order of cuff intervention (25, 50, and 75 mm Hg) was randomized across the 3 testing days. Preliminary observations and pilot studies (n=2, healthy young subjects) revealed that each condition was sufficient to acutely alter the retrograde SR compared with the noncuffed, control arm. In addition, the pilot studies also revealed that changes in brachial artery blood flow and SR as a consequence of cuff inflation did not importantly alter across the 30-minute intervention period. Mean SR and the pattern of SR (antegrade versus retrograde) in both the cuffed and noncuffed arms were recorded during each intervention. Immediately after each intervention, the FMD assessment was performed. Preintervention and postintervention FMDs were examined simultaneously in both arms.

Brachial Artery Diameter, Blood Flow, and SR Analysis

Analysis of brachial artery diameters and SR before, during, and after the intervention was performed using custom-designed edge-detection and wall-tracking software, which is largely independent of investigator bias.[21] The initial phase of image analysis involves the identification of regions of interest (ROI) on the first frame of every ultrasound study. These ROIs allow automated calibration for diameters on the B-mode image and velocities on the Doppler strip.

Table 1.   Baseline Characteristics of Healthy Subjects Before and After Each Intervention

| Parameter | 25 mm Hg | | 50 mm Hg | | 75 mm Hg | | ANOVA *P* |
	Before	After	Before	After	Before	After	
SBP, mm Hg	122±17	124±15	124±6	125±9	120±9	124±10	0.47
DBP, mm Hg	58±9	58±10	61±9	61±7	58±7	61±7	0.13
HR, bpm	56±13	54±9	54±8	51±4	50±5	50±5	0.20

Values are mean±SD. *P* value refers to 1-way ANOVA between the preintervention values. No differences were found between preintervention values among the 3 testing days or between preintervention and postintervention values. SBP indicates systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate.

An ROI is drawn around the optimal area of the B-mode image, and within this ROI, a pixel-density algorithm automatically identifies the angle-corrected near and far-wall e-lines for every pixel column within the ROI. The near- and far-wall intimal edges are identified by a Rake routine. The position of the edge is established by determining the point where the pixel intensity changes most rapidly. Typical B-mode ROIs, therefore, contained ≈200 to 300 diameter measures per frame, the average of which is calculated and stored. This process occurs at 30 frames per second.[22]

Another ROI is drawn around the Doppler waveform, and the peak of the waveform is automatically detected. The mean diameter measures derived from within the B-mode diameter ROI (above) are automatically synchronized and combined with the velocity measures derived from the Doppler ROI, at 30 Hz. Ultimately, from this synchronized diameter and velocity data, blood flow (the product of lumen cross-sectional area and Doppler velocity [v]) and SR (4 times velocity divided by diameter)[23,24] are calculated at 30 Hz. All of the data are written to file and retrieved for analysis in a custom-designed analysis package (see below). We have shown that reproducibility of diameter measurements using this semiautomated software is significantly better than manual methods, reduces observer error significantly, and possesses an intraobserver coefficient of variation of 6.7%.[21] Furthermore, our method of blood flow assessment is closely correlated with actual blood flow through a "phantom" arterial flow system.[25]

The blood flow and SR analysis system described above was used for posthoc calculation of FMD% before and after each intervention using Terason (T3000, Terason) ultrasound machines. It was also used during each intervention to provide real time feedback during each of the 3 trials.

Data Analysis

Baseline diameter, blood flow, and SR were calculated as the mean of data acquired across the 1 minute preceding the cuff inflation period. Peak diameter after cuff deflation was automatically detected according to an algorithm that identified the maximum bracket of data subsequent to performance of a moving window-smoothing function.[22] FMD% was calculated as the percentage rise of this peak diameter from the preceding baseline diameter. The time to peak diameter (in seconds) was calculated from the point of cuff deflation to the maximum postdeflation diameter.[22] Calculation of FMD and time to peak were, therefore, observer independent and based on standardized algorithms applied to data that had undergone automated edge detection and wall tracking.[22] Using an older version of the edge-detection and wall-tracking software than that used in the present experiment, we have previously reported an FMD% coefficient of variation of 14.7% when the technique was repeated within 1 week.[21]

In accordance with recent findings,[22,24] we calculated the SR stimulus responsible for endothelium-dependent FMD after cuff deflation. The postdeflation SR data, derived from simultaneously acquired velocity and diameter measures at 30 Hz, were exported to a spreadsheet and the area under the SR curve (SR-AUC) calculated for data up to the point of maximal postdeflation diameter (FMD)[22] for each individual using the trapezoid rule.

The software described above was also used for analysis of SR, derived from simultaneously acquired velocity and diameter measures at 30 Hz, during the 3 interventions in both the cuffed and noncuffed arms. The patterns of SR were also assessed by calculating the area under the curve for all of the antegrade blood flow and shear and also the area under the retrograde blood flow and shear recordings.

Statistics

Statistical analyses were performed using SPSS 14.0 (SPSS) software. All of the data are reported as mean (SD) unless stated otherwise, whereas statistical significance was assumed at $P<0.05$. Assuming 80% power and an α of 0.05, ≥8 subjects would be required in an intervention study to detect an absolute 1.5% change in FMD using our observer-independent and automatic wall tracking system.[21] To control for possible dropout, we recruited 10 subjects.

An ANOVA (with intervention as independent factor) was used to assess differences among the 3 conditions for baseline characteristics and SR. A paired *t* test was used to compare SR between preintervention and during intervention and the preintervention versus postintervention FMDs during the different interventions. Posthoc *t* tests were performed when the ANOVA reported a significant main or interaction effect. Bonferroni's correction was used to correct for multiple comparisons. Pearson correlations were used to examine the relation between retrograde SR and FMD% and between the change in retrograde SR and the change in FMD% induced by the intervention. Coefficient of variation was calculated for the FMD% responses in the noncuffed arm for each intervention day.[26]

Results

Baseline systolic, diastolic, and mean blood pressures and heart rate were not different among the 3 interventions (Table 1).

Effect of SR Interventions on FMD% in the Cuffed Arm

Preintervention SR pattern and FMD% were not significantly different across the 3 testing days (Figure 2). Cuff inflation induced a dose-dependent change in retrograde SR; ie, a larger increase in cuff pressure induced a significantly larger increase in retrograde SR (Table 2; ANOVA, $P<0.001$; Figure 2A, 2C, and 2E). Because cuff inflation did not alter antegrade SR (ANOVA, $P=0.42$), mean SR was significantly reduced during cuff inflation to 50 and 75 mm Hg but not during inflation to 25 mm Hg (ANOVA, $P<0.001$; Figure 2).

Despite a small change in SR pattern during the 25-mm Hg intervention, FMD% was not significantly altered after the intervention (Figure 2). In contrast, the 50- and 75-mm Hg interventions induced significant decreases in FMD% in the cuffed arm ($P<0.05$; Figure 2). Moreover, a dose-dependent decrease in FMD% was observed after cuff inflation to 75 mm Hg compared with 50 mm Hg (Figure 3). Similar results were evident when FMD was presented in absolute terms (millimeters; Table 3). Preintervention SR-AUC was
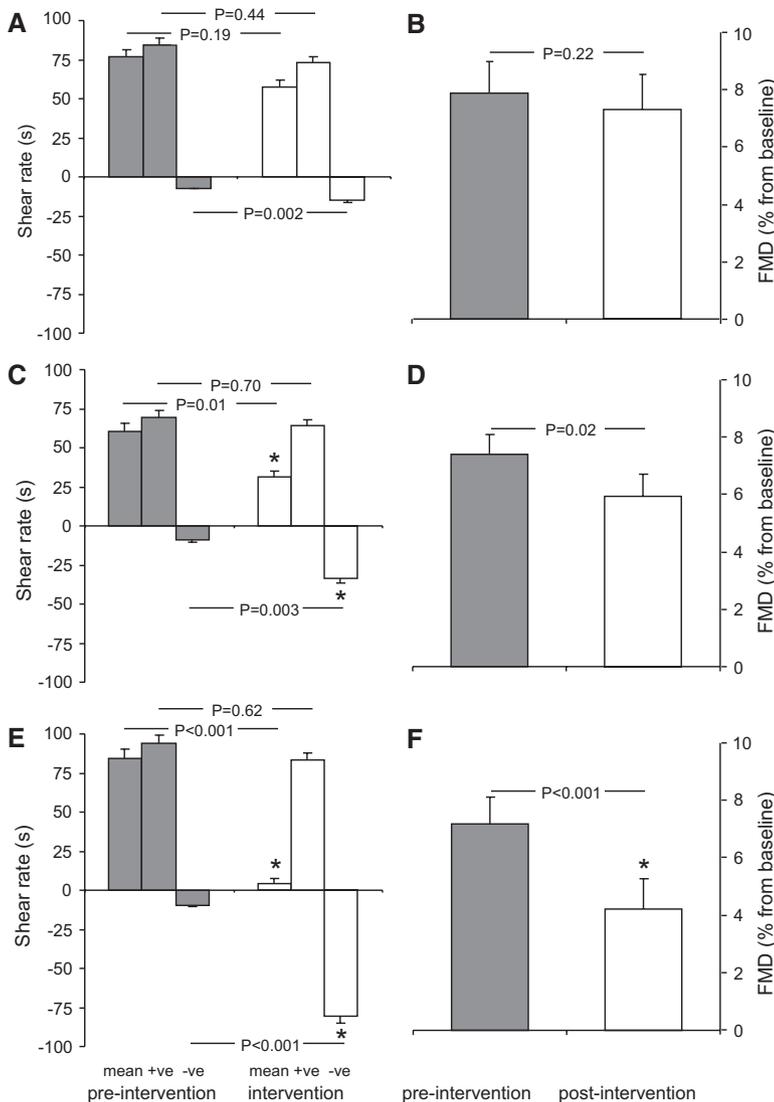
Figure 2. Mean SR and SR pattern before (▨) and during (□) the intervention (+ve, antegrade SR; −ve; retrograde SR) are presented for the cuffed arm (A, C, and E). In addition, on the right, FMD% before (▨) and after (□) each intervention are presented for the cuffed arm (B, D, and F). Data are presented for all 3 of the interventions with the cuff inflated to 25 mm Hg (A and B), 50 mm Hg (C and D), and 75 mm Hg (E and F) in healthy young men (n=10). Error bars represent SE.

not significantly different across the 3 days, whereas the intervention did not alter the SR-AUC (Table 3).

A modest correlation was found between baseline retrograde SR and FMD% ($r^2=0.10$; $P=0.045$). However, a strong, significant correlation was observed between the cuff inflation-induced change in retrograde SR and the change in FMD% ($r^2=0.26$; $P=0.006$; Figure 4).

FMD% Responses in the Noncuffed Arm
The baseline SR pattern was not significantly different among the 3 interventions (Figure 2). Inflation of the cuff around the contralateral limb had no effect on the mean, antegrade, and retrograde SRs in the noncuffed arm (Table 2). Brachial artery FMD, expressed in relative (percentage) and absolute terms (millimeters), was not different among the 3 testing days and did not change after inflation of the cuff around the contralateral forearm (Table 3). SR-AUC was not significantly different across the 3 testing days or between preintervention and postintervention (Table 3). The coefficients of variation for brachial artery FMD% on the 3 testing days (25, 50, and 75 mm Hg) were 6.7%, 10.9%, and 9.5%, respectively.

Table 2. Brachial Artery SR Characteristics of Healthy Subjects During the Intervention (25, 50, and 75 mm Hg) in the Cuffed and Noncuffed Arms

Parameter	25 mm Hg		50 mm Hg		75 mm Hg		ANOVA P
	Noncuffed	Cuffed	Noncuffed	Cuffed	Noncuffed	Cuffed	
Mean SR, s	61±36	58±12	61±47	31±13*	71±78	6±17*	0.03
Antegrade SR, s	71±31	73±12	74±40	65±11	81±72	88±13	0.76
Retrograde SR, s	−10±9	−15±−3*	−12±16	−34±9*	−10±8	−82±12*	<0.001

Values are mean±SD. P value refers to the interaction effect of a 2-way repeated-measures ANOVA with intervention as the independent factor and cuffed vs noncuffed arm as the dependent factor.

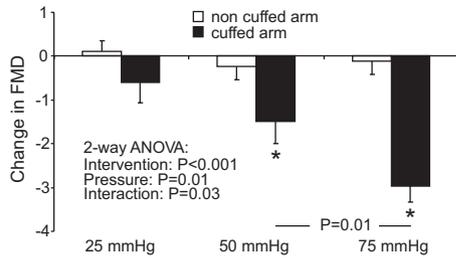*Posthoc was significantly different between arms.

Figure 3. The change in FMD (difference between preintervention and postintervention) in the noncuffed (□) and the cuffed (■) arms for all 3 interventions (25, 50, and 75 mm Hg) in healthy young men (n=10). *Posthoc significant from noncuffed arm. The *P* value refers to the posthoc test for the change in FMD in the cuffed arm during 50 vs 75 mm Hg. Error bars represent SE.

Figure 4. Correlation between the change in retrograde SR and the relative change in FMD% in healthy young subjects (n=10). Data are presented for all 3 of the interventions (25, ○; 50, ◉; and 75 mm Hg, ●) in healthy young men (n=10). The *P* value refers to the Pearson's correlation coefficient.

Discussion

Endothelial dysfunction is regarded as an early atherogenic event[27] and is present well before overt clinical manifestations of vascular disease.[28] A "low-flow" state is widely acknowledged to promote atherosclerosis,[29] with retrograde shear stress hypothesized as a potent contributing factor. However, to our knowledge, no studies have investigated the impact of retrograde flow and shear stress states on vascular function in humans. In the present study we induced a dose-dependent increase in retrograde shear by using cuff inflation, without impacting on antegrade shear, under circumstances where systemic and reflex parameters, eg, blood pressure and heart rate, were maintained at baseline levels. Our results indicate that FMD%, a measure of largely NO-dependent endothelial function,[18,30] was impaired in a dose-dependent manner in response to retrograde shear. Furthermore, higher cuff pressures, which resulted in larger increases in retrograde SRs, were also associated with greater impairment in FMD%. In the noncuffed arm, which served as an internal control, no changes were observed in the pattern of SR or in FMD%, thereby excluding the possibility of any systemic effect. These data indicate that increases in retrograde shear stress throug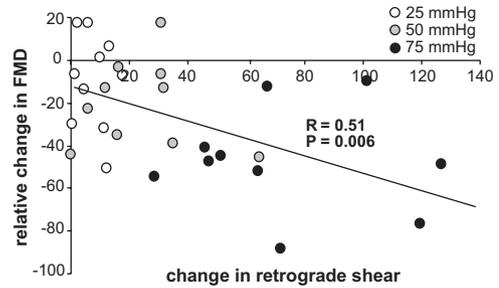h conduit arteries result in decreases in endothelial function in vivo. Given the established link between endothelial function and the atherosclerotic process,[28,31] these findings suggest a possible detrimental role for retrograde shear stress in terms of disease progression in humans.

We have previously observed substantial retrograde flow and shear in the brachial artery during leg cycling exercise in humans, and this was associated with the production of NO.[25,32–35] However, this model is importantly confounded by a concurrent increase in antegrade SR, heart rate, blood pressure, and other systemic changes associated with cycling exercise. In the present study, we increased retrograde SR in a dose-dependent manner without impacting on the antegrade SR. Moreover, the pattern of SR, heart rate, and blood pressure was not altered in the noncuffed arm. Using this novel experimental design, we observed a dose-dependent decrease in FMD% in the cuffed arm after short-term exposure to stepwise increased retrograde SR. An inverse correlation was also evident between retrograde SRs during each intervention and magnitude of impairments in FMD%. Taken together, these data demonstrate a dose-dependent decrease in endothelial function in vivo after short-term exposure to increases in retrograde SR.

Table 3. Brachial FMD% of Healthy Subjects Before and After Intervention (25, 50, and 75 mm Hg) in the Cuffed and Noncuffed Arms

Parameter	25 mm Hg		50 mm Hg		75 mm Hg		ANOVA *P*
	Before	After	Before	After	Before	After	
Noncuffed arm							
Baseline D, mm	4.3±0.5	4.3±0.4	4.3±0.6	4.4±0.7	4.2±0.6	4.2±0.4	0.56
BA FMD, mm	0.31±0.13	0.31±0.13	0.26±0.07	0.25±0.07	0.29±0.05	0.28±0.03	0.56
BA FMD, %	7.3±3.3	7.4±3.2	6.3±2.3	6.1±2.4	6.9±1.2	6.8±0.8	0.48
AUC$_{SR}$, s 10^4	18.9±13.9	16.3±10.7	16.4±10.5	15.5±6.7	17.6±12.7	15.3±14.5	0.75
Cuffed arm							
Baseline D, mm	4.3±0.4	4.3±0.3	4.2±0.3	4.2±0.3	4.2±0.3	4.2±0.3	0.39
BA FMD, mm	0.33±0.13	0.31±0.15	0.30±0.08	0.25±0.10*	0.30±0.12	0.18±0.14*	0.74
BA FMD, %	7.9±3.5	7.3±3.9	7.4±2.2	5.9±2.5*	7.2±3.0	4.2±3.4*	0.71
AUC$_{SR}$, s 10^4	15.1±11.5	15.7±7.5	13.9±7.3	11.1±7.3	14.0±8.0	15.0±6.0	0.92

Values are mean±SD. *P* value refers to a repeated-measures ANOVA between the preintervention values. No differences were found between the preintervention values among the 3 testing days. D indicates diameter; BA FMD, brachial artery FMD; AUC$_{SR}$, area under the shear rate curve.

*Data were significant between preintervention and postintervention.

Previous studies that have examined the effect of elevation in retrograde blood flow or SR have primarily been performed in vitro and/or using animal models.[12–17] These studies indicate that elevations in retrograde SR or blood flow, such as those present during oscillatory flow, result in elevated endothelin 1 expression,[12] expression of adhesion molecules[13,14] and reactive oxygen species–producing enzymes (ie, NADPH oxidase),[16,17] the release of superoxide anions,[15] and decreases in endothelial NO synthase expression.[16,17] These adaptations result in a proatherogenic phenotype in the vascular wall.[4] Accordingly, these changes may underlie the endothelial dysfunction after exposure to retrograde SR in our study. An interesting difference between these studies relates to the duration of the stimulus. Studies performed in animals typically expose the harvested endothelium for 4 to 24 hours to retrograde SR,[12–17] whereas we used a 30-minute intervention. This emphasizes the fact that retrograde SR is a highly potent stimulus to endothelial dysfunction in humans. Consistent with our findings, animal studies have also reported dose-dependent changes in reduced nicotinamide-adenine dinucleotide oxidase activity and reactive oxygen species generation in response to retrograde shear exposure.[15,16] Although the findings of the present study are largely consistent with the animal data, this is the first study that has directly manipulated retrograde flows and measured endothelial function responses in humans.

Although blood flow in a conduit artery is primarily in the antegrade direction, retrograde flow or SR is not necessarily a rare phenomenon in humans. Retrograde shear stress patterns may result from increased vascular tone in the downstream resistance vessel beds.[34] Advanced age,[36,37] obesity,[38] and hypertension[38–40] are characterized by elevated peripheral vascular tone, which may, as a consequence, be associated with elevated retrograde SR levels in the upstream conduit arteries. Interestingly, such conditions are also associated with an impaired endothelial function.[41–43] In addition, acute stimuli that elevate sympathetic nervous system tone and peripheral vascular tone can acutely impair brachial artery endothelial function.[44,45] Future studies should examine the links between retrograde SR and endothelial function in clinical conditions and also specifically in arteries prone to developing atherosclerosis.

Some limitations of the present study are germane. Although the sample size was relatively modest, it is in keeping with physiological studies of this nature, and the results were consistent between and within individuals. The clear dose-response relationship between the independent and dependent variables provides powerful evidence that our findings are robust. Therefore, recruiting a larger number of subjects would have not importantly changed the findings of the study. Although the subjects that we recruited were young and healthy with normal endothelial function, the impacts of cardiovascular disease, risk factors, and medications on the magnitude of retrograde SR and arterial function have not been addressed. Performing studies in these groups is a valid approach to further study the relevance of retrograde SR in humans. Finally, one may hypothesize that 30-minute cuff inflation to 75 mm Hg may induce ischemia-reperfusion injury, ie, a stimulus that attenuates brachial artery endothelial function.[46] However, cuff inflation to 75 mm Hg is not likely to be associated with ischemia, because 102 mm Hg was lowest systolic blood pressure observed in our subjects. Furthermore, preintervention and postintervention brachial artery SRs were similar for each intervention (data not shown), whereas ischemia-reperfusion should have resulted in prolonged (several minutes) increases in SR. We do not believe that ischemia-reperfusion injury explains our main findings.

Perspectives

By manipulating brachial artery retrograde blood flow and shear, without changing the antegrade SR or inducing hemodynamic or reflex changes, we observed a dose-dependent decrease in NO-mediated endothelial function in response to increases in the magnitude of retrograde shear. The impact of retrograde SR on endothelial function was reinforced by a strong correlation between the retrograde shear and decrease in endothelial function. No changes in the patterns of SR or FMD were observed in the contralateral arm, confirming that our findings are directly related to the elevated retrograde SR levels. Because these data demonstrate a dose-dependent decrease in human endothelial function in vivo after exposure to elevation in retrograde SR and blood flow, our findings may contribute to an understanding of the role of (retrograde) shear in the pathogenesis of endothelial dysfunction, a preclinical marker for atherosclerosis and a characteristic of hypertension.

Disclosures

None.

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