# SHEAR-MEDIATED DILATION OF THE INTERNAL CAROTID ARTERY OCCURS INDEPENDENT OF HYPERCAPNIA

Ryan L. Hoiland[1], Kurt J. Smith[2], Howard H. Carter[3], Nia C.S. Lewis[1], Michael M. Tymko[1], Kevin W. Wildfong[1], Anthony R. Bain[1,4], Daniel J. Green[2,5], Philip N. Ainslie[1]

[1]*Centre for Heart, Lung and Vascular Health, University of British Columbia – Okanagan Campus, School of Health and Exercise Sciences, Kelowna, B.C., Canada*
[2]*School of Sport Science, Exercise and Health, The University of Western Australia, Crawley, W.A., Australia*
[3]*Department of Nutrition, Exercise and Sports, University of Copenhagen, Denmark*
[4]*University of Colorado, Boulder, Department of Integrative Physiology, Integrative Vascular Biology Lab*
[5]*Reserch Institute for Sport and Exercise Sciences, Liverpool John Moores University, United Kingdom*

**Corresponding Author:**

Ryan L. Hoiland
Centre for Heart, Lung and Vascular Health, School of Health and Exercise Sciences, University of British Columbia – Okanagan
3333 University Way, Kelowna, British Columbia Canada, V1V 1V7
Email: ryanleohoiland@gmail.com

Word Count: 6352

**ABSTRACT**

Evidence for shear stress as a regulator of carotid artery dilation in response to increased arterial carbon dioxide was recently demonstrated in humans during sustained elevations in $CO_2$ (hypercapnia); however, the relative contributions of $CO_2$ and shear stress to this response remains unclear. We examined the hypothesis that, following a 30-second transient increase in arterial $CO_2$ tension and consequent increase in internal carotid artery shear stress, internal carotid artery diameter would increase, indicating shear-mediated dilation, in the absence of concurrent hypercapnia. In 27 healthy participants the partial pressures of end-tidal $O_2$ and $CO_2$, ventilation (pneumotachography), blood pressure (finger-photoplethysmography), heart-rate (electrocardiogram), internal carotid artery flow, diameter and shear stress (high resolution duplex ultrasound) and middle cerebral artery blood velocity (transcranial Doppler) were measured during 4-minute steady state and transient 30-second hypercapnic tests (both +9mmHg $CO_2$). Internal carotid artery dilation was lower in the transient, compared to the steady state hypercapnia (3.3±1.9% vs. 5.3±2.9%, respectively; $P<0.03$). Increases in internal carotid artery shear stress preceded increases in diameter in both the transient (time: 16.8±13.2s vs. 59.4±60.3s; $P<0.01$) and steady state (time: 18.2±14.2s vs. 110.3±79.6s; $P<0.01$) tests. Internal carotid artery dilation was positively correlated with shear rate area under the curve in the transient ($r^2=0.44$; $P<0.01$), but not steady state ($r^2=0.02$; $P=0.53$) trial. Collectively, these results suggest that hypercapnia induces shear-mediated dilation of the internal carotid artery in humans. This study further promotes the application and development of hypercapnia as a clinical strategy for the assessment of cerebrovascular vasodilatory function and health in humans.

**Key Words:** Cerebral blood flow; carbon dioxide; shear stress; flow-mediated dilation; transcranial Doppler; Ultrasound

**NEW AND NOTEWORTHY**

Shear stress dilates the internal carotid artery in humans. This vasodilatory response occurs independent of other physiological factors as demonstrated by our transient $CO_2$ test, and is strongly correlated to shear AUC. Assessing carotid shear-mediated dilation may provide a future avenue for assessing cerebrovascular health and risk of cerebrovascular events.

## INTRODUCTION

The endothelium is integral to the maintenance of vascular health and function in humans. Endothelial dysfunction is an early and integral event in the pathogenesis of atherosclerosis as well as cardio- and cerebro-vascular diseases (20). In the peripheral and coronary vasculature, flow mediated dilation (FMD) has been used as a functional bioassay to assess endothelial health. The FMD test strongly predicts cardiac events(18), potentially allowing for pre-clinical detection of future cardiovascular disease(12). While the conventional FMD test is based on the notion that a healthy endothelium produces autocoids (e.g. nitric oxide) that induce a quantifiable dilator response to an imposed increase in shear stress(17), there is currently no equivalent assessment of cerebrovascular function in humans. Although standard carbon dioxide ($CO_2$) reactivity tests using transcranial Doppler are predictive of cerebrovascular events (i.e. stroke) in individuals with pre-existing carotid stenosis(19), its use is limited as it does not provide significant predictive value pertaining to stroke risk in apparently healthy individuals(30). Other metrics of cerebrovascular function such as neurovascular coupling(27) and cerebral autoregulation(39, 44) are similarly impaired in clinical populations, but are not predictive of risk in apparently healthy individuals. Therefore, the development of an FMD-type test that could potentially provide an index of cerebrovascular endothelial function may prove to be clinically impactful.

Recently, our group has provided the first evidence in humans of shear-mediated dilation in the internal carotid artery (ICA)(10). Here, we demonstrated that vascular responsiveness to $CO_2$ was related to the rise in intra-arterial shear stress, leading to vasodilation with a similar time dependency to that of peripheral FMD. However, the carotid vasodilation observed in that study coincided with elevated arterial $PCO_2$ levels and we could not partition the effects of shear stress

from those occurring secondary to sustained hypercapnia [e.g. elevated blood pressure(33), cardiac output(7), chemoreceptor-mediated sympathoactivation(28), potential direct vasomotor effects of $CO_2$(21, 23, 47)]. As a result, the contribution of shear-dependent mechanisms to human CBF regulation and carotid dilator function requires further investigation *in vivo*.

Therefore, the primary purpose of this experiment was to extend our previous findings(10), using a test without sustained hypercapnia, to quantify the role that shear stress-dependent mechanisms play in carotid dilation in response to $CO_2$ reactivity. It was hypothesized that transient (30-second) increases in end-tidal $PCO_2$ ($P_{ET}CO_2$) would produce an ICA shear response similar to that of a *peripheral* FMD test (i.e. bell-curve). We further reasoned that upon return of $P_{ET}CO_2$ to baseline values, and consequent withdrawal of the direct effects of $CO_2$, a time dependent dilation of the ICA would occur (e.g. 30-60 second delay from shear onset), implying a shear-specific mechanism that is consistent with that observed in peripheral vessels of young healthy individuals(9).

## MATERIALS AND METHODS

### Ethical Approval

This study was approved by the University of British Columbia Clinical Research Ethics Board and the University of Western Australia Human Research Ethics Committee, and data was subsequently collected at both institutions. Prior to participation in the study, all participants completed written informed consent. All procedures conformed to the standards set by the Declaration of Helsinki.

**Research Participants**

Twenty-seven healthy (twenty two male, five female), young volunteers (mean±SD; 22±3 years; body mass index=22±2 kg/m$^2$) were recruited to participate in this study. Following written informed consent and familiarization the participants attended the laboratory on one occasion having fasted for ≥2 hours and refrained from exercise, alcohol and caffeine for 24 hours. During familiarization, participants were screened to ensure that reliable ICA ultrasound images and middle cerebral artery (MCA) signals could be obtained. Participants were familiarized with the remaining experimental equipment and procedures during this session. All participants were free of cardiovascular, respiratory, and cerebrovascular disease, were non-diabetic, and were not taking any prescription drugs (other than oral contraceptives; n=4) at their time of participation, as determined by a screening questionnaire. All females were tested in days 1-3 of their follicular phase. Of the 27 participants recruited, 24 completed both $CO_2$ protocols, while three only completed the transient $CO_2$ protocol.

**Experimental Measures**

*Cardiorespiratory Measures.*

All cardiorespiratory variables were sampled continuously at 1KHz via an analogue-to-digital converter (Powerlab, 16/30; ADInstruments, Colorado Springs, CO). Heart rate (HR) was measured by 3-lead electrocardiogram (ADI bioamp ML132), while beat-to-beat blood pressure was measured by finger photoplethysmography (Finometer PRO, Finapres Medical Systems, Amsterdam, Netherlands). The Finometer reconstructed brachial waveform was used for the calculation of mean arterial pressure (MAP) after values were back calibrated to the average of three automated brachial blood pressure measurements made over 5-minutes at rest (Tango+;

SunTech, Morrisville, NC). Both $P_{ET}CO_2$ and the end-tidal partial pressure of $O_2$ ($P_{ET}O_2$) were sampled at the mouth and recorded by a calibrated gas analyzer (model ML206, ADInstruments), while respiratory flow was measured by a pneumotachograph (model HR 800L, HansRudolph, Shawnee, KS) connected in series to a bacteriological filter. All data were interfaced with LabChart (Version 7).

*Dynamic End-Tidal Forcing.*

The $P_{ET}O_2$ and $P_{ET}CO_2$ were controlled by a portable dynamic end-tidal forcing system that has been previously described in detail(42, 43). Our end-tidal forcing system effectively controls end-tidal gases through wide ranges of $P_{ET}CO_2$ and $P_{ET}O_2$ independent of ventilation(42, 43) and has been previously used for similar $P_{ET}CO_2$ manipulations(21).

*Cerebrovascular Measures.*

Blood velocity in the right MCA (MCAv) was measured using a 2MHz transcranial Doppler ultrasound (TCD; Spencer Technologies, Seattle, WA). The TCD probe was fixed to a headpiece (model M600 bilateral head frame, Spencer Technologies) and secured into place. The MCA was insonated through the middle trans-temporal window, using previously described location and standardization techniques(46).

*Internal Carotid Artery Measurements*

Right ICA blood velocity (ICAv) and vessel diameter were measured using a 10MHz multi-frequency linear array duplex ultrasound (Terason T3200, Teratech, Burlington, MA). Specifically, B-mode imaging was used to measure arterial diameter, while pulse-wave mode was

used to concurrently measure peak blood velocity. Diameter and velocity of the ICA were measured at least 1.5 cm distal to the common carotid bifurcation to eliminate recordings of turbulent and retrograde flow and non-uniform shear. Great care was taken to ensure that the insonation angle (60°) was unchanged throughout each test. Further, upon acquisition of the first ultrasound image (i.e., resting baseline) there was no alteration of B-mode gain to avoid any artificial changes in arterial wall brightness/thickness. All recordings were made in accordance with published guidelines(41).

All recordings were screen captured and stored as video files for offline analysis. This analysis involved concurrent determination of arterial diameter and peak blood velocity at 30Hz, using customized edge detection and wall tracking software (BloodFlow Analysis, Version 5.1) designed to mitigate observer bias(49). Our *within* day coefficient of variation for the assessment of ICA diameter is 1.5% using this technique(21). Blood flow and shear rate were subsequently calculated as previously described(9, 21).

**Experimental Protocol**

*Carotid Shear-Mediated Dilation Tests*

Prior to commencing the study, pilot testing was ran to determine the optimal $P_{ET}CO_2$ change required to elicit a maximal transient shear stress response. Although it is well established that the cerebral blood flow response is linear with elevations in $P_{ET}CO_2$ (1), we determined that there was no appreciable difference in the transient test shear response between a targeted $P_{ET}CO_2$ stimuli of +9 or +15mmHg. Given that +15 leads to a greater extent of participant discomfort, and a larger ventilatory response, which would increase the difficulty of acquiring ultrasound recordings of the

ICA, we reasoned that a +9mmHg stimulus would be better suited to address our research question. In addition, the +9mmHg stimulus is roughly comparable to what would be induced by an $F_ICO_2$ of 0.05 – this level is commonly used in other studies (1).

Two separate $CO_2$ tests were utilized to assess the influence of shear and flow on ICA vascular tone: 1) a transient $CO_2$ test (+9 mmHg $P_{ET}CO_2$) to simulate the shear dynamics characteristic of a post occlusion brachial cuff release in standardized peripheral FMD tests(40) (Figures 1 & 2), and 2) a steady state $CO_2$ test to determine maximal reactivity/dilation to the same +9mmHg stimulus. The steady state $CO_2$ test was conducted fifteen minutes following the transient test.

1) *Transient $CO_2$ Test*: Participants lay supine, while breathing room air. Following five minutes of resting data collection, participants breathed simulated room air delivered from the end-tidal forcing system (i.e. $P_{ET}O_2$ & $P_{ET}CO_2$ did not change) for two minutes. Participants were then paced at 20 breaths per minute through verbal coaching and visual feedback while end-tidal values were maintained (i.e. did not change) for an additional two minutes. Breathing was maintained at 20 breaths per minute for the remainder of the trial. This approach was utilized to improve the end-tidal forcing systems ability to abruptly manipulate $P_{ET}CO_2$ in a controlled fashion, as a higher breathing rate allows for quicker gas manipulations. Further, this eliminated the potential for abrupt changes in $V_E$ due to $CO_2$ chemosensitivity, which could interfere with ultrasound imaging (e.g., large neck muscle movements) and act as a confounding physiological factor. At the two minute mark, $P_{ET}CO_2$ was abruptly (~five seconds) elevated to +9 mmHg above baseline for 30-seconds and then quickly returned to baseline values, which were maintained for three additional minutes. Reported data are from the last minute of hyperventilation onwards.

2) *Steady State $CO_2$ Test*: Participants lay supine, while breathing room air. Following five minutes of resting data collection, the participant breathed simulated room air on the end-tidal forcing system. Following two minutes of steady breathing, $P_{ET}CO_2$ was abruptly (~five seconds) elevated to +9 mmHg above baseline and maintained for four minutes. Following four minutes, participants returned to room air breathing.

**Data Analysis**

*Data Extraction and Carotid Shear-Mediated Dilation Software*

All data from LabChart (e.g. MCAv, MAP, $P_{ET}CO_2$ and $P_{ET}O_2$) were down sampled to 50Hz and exported into Microsoft Excel. Vascular data were interpolated at 50Hz and exported into the same excel spreadsheet and time-aligned with the LabChart data. The excel spreadsheet was re-opened as a LabChart data file and all cardiovascular/cerebrovascular data were exported to a new excel spreadsheet in beat-by-beat averages, while all respiratory variables were exported on a breath-by-breath basis. These variables were subsequently analyzed using custom designed shear-mediated dilation software (Labview®) as previously explained in detail(10). In brief, the data were filtered and the following variables were automatically detected and calculated: 1) Baseline values; 2) Peak responses, and; 3) Relative (%) changes. In addition, a threshold selection algorithm was applied to each data array (e.g., ICA shear, ICA diameter,), which identified threshold points for the increase in each variable following the onset of $CO_2$ administration. Once the software had automatically detected the threshold points, they were depicted on the filtered and raw data array and visually inspected to ensure they met the following criteria: a) the algorithm-detected threshold point occurred prior to the peak value and, b) the variable did not decrease below the algorithm-detected threshold point prior to the peak value occurring. Of the 240 responses

analyzed, 151 met the agreed criteria and their automatically detected points were accepted. In the remaining 89 cases (37%) the threshold points were manually adjusted by two independent investigators to a point where each deemed there was a clear deviation from baseline that met the above criteria. The mean of these manually assessed points were entered in the analysis. The coefficient of variation for the analysis of the threshold points detected using the above systematic approach was 4.2% overall, and 11.2% between the 89 manually adjusted files. Shear rate area under the curve (AUC) was also calculated for both the transient and steady state trials, in accordance with the analytical methods used for brachial FMD(9, 40) (i.e., from the onset of $CO_2$ stimulus until peak dilation occurred).

*Data Exclusion Criteria*

Of the subjects tested, four trials were excluded from analysis of the steady state $CO_2$ tests while seven were excluded from the transient $CO_2$ tests. Additionally, there were three subjects who only completed the transient $CO_2$ test (not both trials). Thus, the resulting samples sizes were n=20 for both the steady state $CO_2$ and transient $CO_2$ tests, with 13 subjects having repeated measures across both trials. Ultrasound videos were visually inspected prior to analyses and were excluded based upon the following criteria: 1) the occurrence of an overt angle change (n=2); 2) excessive movement of the vessel [e.g., due to high ventilation (n=3)]; and 3) overall poor image quality [e.g., blurry vessel walls (n=4)]. In two cases a test was excluded following video analysis due to low fidelity wall tracking despite the visual appearance of a clear vessel wall. Therefore, sample size is noted throughout the text for all statistical comparisons.

### Statistical Analyses

No statistical differences were found between male and female vascular responses in the current study; therefore, data were pooled for analyses. Sample sizes were based off of previous research by our group investigating vasomotion of the ICA (11, 21, 47). Comparisons between baseline and peak values within trial were made using two-tailed paired t-tests. Due to the differing participants for each trial (see *Data Exclusion Criteria*), between trial comparisons were made using a linear mixed model analysis with a compound symmetry covariance structure (Fixed factor: steady state vs. transient test) to account for this drop out. Pearson correlations were used to assess the relationship between vasodilatory responses during the transient and steady state $CO_2$ protocols in the subjects with repeated measures (n=13), and to determine the relationship between shear AUC and dilation in each trial (transient n=19; steady state n=20). For the shear AUC and dilation bivariate analysis, a statistical outlier was determined utilizing bagplot analysis(34). All statistical analyses were performed using SPSS (IBM statistics, Version 22.0).

## RESULTS

### Carotid Shear-Mediated Dilation

From the onset of elevated $P_{ET}CO_2$ (Figure 3), the delay for ICA shear to increase was 16.8±13.2s in the transient trial and 18.2±14.2s (P=0.61) in the steady state trial. Peak shear occurred at 42.2±9.9s in the transient trial and 163.6±87.5s in the steady state trial (P<0.01). The onset of dilation in the transient trial occurred 59.4±60.3s from onset of elevated $P_{ET}CO_2$, while the delay was 110.3±79.6s in the steady state trial (P=0.047). Peak dilation occurred 110.3±69.1s and 283.6±56.1s in the transient and steady state trial, respectively (P<0.01). In both the steady state

(18.2±14.2 vs. 110.3±79.6s; P<0.01) and transient test (16.8±13.2 vs. 59.4±60.3s; P<0.01) the increase in ICA shear preceded ICA dilation (Figure 3).

During the transient and steady state hypercapnic tests the ICA dilated by 3.3±1.9% (n=20; P<0.01) and 5.3±2.9% (n=20; P<0.01), respectively. The steady state elevation in $P_{ET}CO_2$ produced the greatest dilation as reflected in a 60% greater increase (P=0.03; Figure 4). Shear rate increased from 330.0±74.2$s^{-1}$ to a peak value of 448.5±111.7$s^{-1}$ (n=20; P<0.01) in the transient $CO_2$ trial and from 337.3±56.6$s^{-1}$ to 526.8±83.5$s^{-1}$ (n=20; P<0.01) in the steady state trial. The increase in shear rate during the steady state trial was greater than during the transient test (189.5±49.9$s^{-1}$ vs. 118.5±53.8$s^{-1}$; P<0.01). Shear rate AUC for the transient trial was 43,053±21,277$s^{-1}$ (n=19), and 131,476±30,880$s^{-1}$ (n=20) in the steady state trial (p<0.01; independent samples t-test). Shear rate AUC was correlated to the magnitude of vasodilation in the transient test ($r^2$=0.44; P<0.01; n=19); however, this relationship was not present in the steady state trial ($r^2$=0.02; P=0.53) (Figure 5).

**Intra- vs. Extra-Cranial Cerebral Hemodynamics**

Elevations in MCAv occurred at the same time as ICAv following the onset of hypercapnia in the transient (13.0±10.0 vs. 16.7±14.5; P=0.18) and steady state trials (15.1±14.9 vs. 16.9±13.9s; P=0.11), respectively (Figure 3). Peak MCAv and ICAv occurred at the same time in the transient (40.1±7.5 vs. 42.8±9.3s; P=0.09) and steady state (182.1±99.0 vs. 199.6±91.3s; P=0.24) trials. In the transient and steady state tests, peak ICA flow occurred 41.7±9.5s and 234.2±84.3s following the onset of $CO_2$ breathing, respectively.

**Blood Pressure Regulation**

During the transient $CO_2$ test, MAP increased from 94.6±12.5mmHg to peak value of 104.2±13.6mmHg (P<0.01), while the time of occurrence for peak MAP (81.2±73.1s) was not statistically different from the time of peak dilation (110.1±69.1s; P=0.20). However, the time to peak MAP and dilation were not related *within* subject ($r^2$=0.14; P=0.06), nor was the magnitude of dilation and change in MAP ($r^2$=0.09; P=0.22). During the steady state hypercapnia, MAP increased from 94.2±10.4mmHg to a peak value of 105.8±11.3mmHg (P<0.01), while the time of occurrence for peak MAP (169.0±96.3s) was earlier than that for peak dilation (283.6±56.1s; P<0.01). There was no relationship in the time to peak dilation and peak MAP ($R^2$=0.002; P=0.43).

**DISCUSSION**

The present study outlines a novel experimental design aimed at: 1) assessing shear-mediated dilation of cerebral conduit arteries without the confounding influence of sustained increases in $PaCO_2$, and 2) reinforcing the existence of shear mediated dilation of the ICA(10, 21). We observed dilation of the ICA in response to both transient and steady state hypercapnia. Under both conditions, increases in shear stress preceded dilation of the ICA in a time-dependent manner. Vasodilation of the ICA in the transient trial was strongly correlated to the increase in shear AUC, implicating shear as the primary stimulus for dilation, consistent with observations in the brachial, femoral, and coronary arteries(32).

The assessment of FMD in peripheral arteries dates back >20 years to the landmark study by Celermajer *et al.,* 1992(12). This technique evolved into a method for assessing the function and

health of endothelial cells in the general circulation(17). However, to date, there is no equivalent method of assessing cerebral conduit artery shear-mediated regulation. In humans, the ICA conveys blood flow to the brain and bilaterally this accounts for ~70% of global CBF(50). In addition to serving as cerebral conduit vessels, the ICAs regulate CBF(48) and provide the most accessible avenue(41) to assess cerebral endothelial function using duplex ultrasound. Therefore, the measurement of ICA diameter during a controlled and transient elevation in shear stress may provide a valid paradigm and for the specific assessment of *cerebral* conduit artery endothelial function (or dysfunction)(40). Whilst ischemia-induced hyperemia is utilized in *peripheral* FMD tests, this is obviously not possible in the cerebral vasculature(37). However, the present study indicates that hypercapnia-induced vasodilation, in particular utilizing transient exposure, induces ICA dilation following increases in intra-vascular shear stress of a similar manner and profile to post-occlusion cuff release(10). Given the congruent shear dynamics of a peripheral FMD test and the current carotid FMD test, in addition to the strong relationship between shear AUC and vasodilation, it appears that transient elevations in $CO_2$ provide a robust (and relatively specific) method to perturb shear-mediated regulation of CBF. We therefore propose a novel technique to assess "cerebrovascular FMD" in the present study; however, pharmacological inhibition of endothelial NO synthase is necessary to confirm the potential applicability of this technique. The lack of correlation between shear AUC and dilation in the steady state trial indicates that sustained hypercapnia is likely not an adequate method to assess shear-mediated regulation of the ICA due to a larger influence of confounding factors (increased blood pressure etc.,).

Our data implicate shear dependent mechanisms as key contributors to vasodilation in the ICA during a $CO_2$ reactivity test (Figures 4 & 5) in accordance with previous study (10). Considering

the transient $CO_2$ test elicited a more rapid, albeit lower, ICA dilation compared to the steady state test, our findings suggest that ICA dilation in the transient test is less influenced by extraneous factors (e.g., BP, sympathetic nervous activity, metabolism, direct effects of $CO_2$/pH etc.,) As depicted in figure 5, the relationship between shear AUC and dilation occurs within a range between 0-100,000$s^{-1}$, but in the steady state trial where shear AUC is >100,000$s^{-1}$ this relationship is lost. This may be due to two potential factors: 1) a ceiling effect of shear on ICA vasodilation, and 2) that as previously mentioned, sustained $CO_2$ engages other confounding physiological responses (e.g., elevated BP) that obscures the relationship between Shear AUC and vasodilation. Therefore, it appears the transient trial more closely resembles the NO-mediated FMD observed in the peripheral vasculature(17, 40), which is further supported by observations that other endothelial derived relaxing factors [e.g. prostaglandins(21)] do not possess an obligatory role in hypercapnic cerebral vasodilation. That the percent dilation was only modestly correlated *within* subjects between the transient and steady state $CO_2$ tests indicates that distinct mechanisms may be evoked differentially in response to varying stimuli (i.e. time of hypercapnia). This is consistent with findings from the peripheral FMD test, where five minutes of ischemia induces a largely NO-mediated dilation, whereas longer periods of ischemia induce compensatory redundant mechanisms resulting in less NO dependency(24). However, it must be stressed that studies pharmacologically inhibiting endothelial nitric oxide synthase activity have not consistently demonstrated a role for NO in hypercapnic vasodilation(22, 35, 45). These variable findings may be related to the use of velocity indices of cerebral blood flow (CBF) [i.e. TCD; discussed in(2)], which are unable to detect and quantify changes in vessel diameter. In addition, systemic administration of NO blockers such as L-NMMA have both direct, and indirect reflex, effects on

vasomotor tone and these are countervailing, making the impacts of such drugs difficult to interpret.

A delayed response (~60sec) to reach maximal steady state CBF for a given step change in $P_{ET}CO_2$ has been demonstrated previously(10, 36). Further, steady state hypercapnia is reported to elicit higher cerebrovascular reactivity than non-steady state tests [e.g., rebreathing(25)]. This disparity in reactivity may be explained by the time dependent nature of endothelial NO synthase upregulation and consequent NO mediated vasodilation, which may not be fully engaged at the termination of a progressive and dynamic stimulus. By quantifying the maximal dilation to a steady-state $CO_2$ stimulus in addition to the vasomotor reaction to a transient elevation in $CO_2$ and shear, we have provided insight into the percent contribution of shear-dependent mechanisms to total $CO_2$ reactivity. Further, this highlights that ~40% of $CO_2$ reactivity is not explained by shear, and may be partially attributable to changes in MAP(3, 26, 31), increases in cardiac output, and/or direct effects of $PaCO_2$/pH. The difference in time to peak dilation during the transient and steady-state $CO_2$ test likely indicates a different time course of these mechanism(s) on vasomotor tone. Therefore, while shear mechanisms likely acted in the same time course between tests, it is the additional stimuli that results in a further and delayed maximal dilation during steady state elevations in $CO_2$.

When considering the contribution of shear dependent mechanisms to cerebrovascular dilation one must also consider the potential for myogenic influences during hypercapnia-mediated increases in MAP(14). Increases in cerebral perfusion pressure have been shown to produce cerebral vasoconstriction(15); therefore, myogenic constriction may have masked vasodilation to some

degree (resulting in underestimation of shear-mediated vasodilation), and/or be related to the variable contribution of shear dependent mechanisms to hypercapnia mediated vasodilation. The opposing forces of shear-mediated dilation and myogenic constriction has been previously demonstrated in peripheral vessels(4, 29).

*Methodological considerations*

Recently it has become convention to normalize peripheral FMD tests via logarithmic transformation of baseline diameter, peak diameter, and shear rate AUC to allometrically scale for artery size and account for between subject variations in the shear stimulus (5, 6). The purpose of these corrections is to better estimate the endothelial responsiveness to a shear stimulus. Here, we have not scaled for baseline diameter and shear AUC between trials as the purpose of the current study was not to evoke and subsequently quantify a change in endothelial function, but to determine the experimental feasibility of a transient increase in ICA shear (via elevated $P_{ET}CO_2$) in eliciting a measureable change in arterial diameter. Indeed endothelial function should not be different between the two trials given no intervention was present.

While we have used dynamic end-tidal forcing to control $P_{ET}CO_2$, determining the efficacy of the Douglas bag technique for a 30-second manipulation of $CO_2$ may provide great utility, given the reduced technical demand and consequently easier transition into a clinical setting. Further determining the within and between-day variation in transient $CO_2$ mediated ICA vasodilation will help determine its potential translation into a clinical setting.

*Perspective*

Turbulent flow and non-uniform shear stress, which are characteristic of the carotid bulb and ICA, render the carotid vasculature a 'high risk' area for atherogenesis, wall thickening, and consequent vascular dysfunction(8, 16, 38). Therefore, the present carotid FMD test may possess future potential to detect early carotid and cerebral vascular dysfunction, before the development of overt wall thickening or intractable plaque deposition. Analogous to the peripheral FMD approaches, which predict future cardiovascular events(18), our carotid test may provide a useful clinical window into sub-clinical atherogenesis and also enable treat-to-target assessment of management efficacy. While steady state $CO_2$ reactivity tests have shown a strong ability to predict cerebral vascular events in clinical populations [e.g., carotid stenosis(19)], they have yet to demonstrate predictive value relative to cerebral vascular risk in healthy individuals(30). The inability to specifically predict cerebral vascular risk may be related to several factors including: 1) previous use of velocity indices of CBF and consequent inability to directly quantify vasomotion(2, 13), and 2) the confounding nature of the myriad physiological consequences secondary to sustained hypercapnia [i.e., elevated blood pressure (33) and cardiac output (7), chemoreceptor mediated sympathoactivation (28), and the potential for direct vasodilatory effects of $CO_2$/pH (21, 23, 47)]. Our approach mitigates these limitations by directly visualizing arterial diameter change and quantifying the magnitude and profile of the shear stimulus. It is tailored to perturb *cerebrovascular* endothelial function with reduced input from confounding physiological factors and couples concurrent measurement of cerebral conduit artery diameter, flow and shear at high temporal and spatial resolution. Future research should aim to 1) determine the influence of transiently elevated shear on ICA vasodilation prior to and following NO blockade to confirm endothelial involvement, and 2) determine the magnitude by which carotid shear-mediated dilation may be reflective of cerebral vascular health and risk of cerebrovascular events.

**SOURCES OF FUNDING**

Philip N. Ainslie was supported by a Canadian Research Chair in Cerebrovascular Physiology and a Natural Sciences and Engineering Research Council Discovery Grant. Ryan L. Hoiland was supported by the Constance-Livingstone Friedman Scholarship. Howard H Carter was supported by funding from the Novo Nordisk Foundation through the Danish Diabetes Academy.

Daniel J. Green is supported by a National Health and Medical Research Council (NHMRC) Principal Research Fellowship (APP1080914) and the portion of experimental work that was performed in his laboratory was supported by NHMRC project grant 1045204. Kurt J. Smith is a research associate funded by the Natural Sciences and Engineering Research Council of Canada. During this project, he was supported by an Australian Research Council Grant DP130103793.

**DISCLOSURES**

The authors declare no conflict of interest financial or otherwise.

## References


1. **Ainslie PN**, **Duffin J**. Integration of cerebrovascular CO2 reactivity and chemoreflex control of breathing: mechanisms of regulation, measurement, and interpretation. *Am J Physiol Regul Integr Comp Physiol* 296: R1473-95, 2009.
2. **Ainslie PN**, **Hoiland RL**. Transcranial Doppler Ultrasound: Valid, Invalid, or Both? *J Appl Physiol* 117: 1081–1083, 2014.
3. **Ainslie PN**, **Lucas SJE**, **Fan J-L**, **Thomas KN**, **Cotter JD**, **Tzeng YC**, **Burgess KR**. Influence of sympathoexcitation at high altitude on cerebrovascular function and ventilatory control in humans. *J Appl Physiol* 113: 1058–1067, 2012.
4. **Atkinson CL**, **Carter HH**, **Naylor LH**, **Dawson EA**, **Marusic P**, **Hering D**, **Schlaich MP**, **Thijssen DH**, **Green DJ**. Opposing effects of shear-mediated dilation and myogenic constriction on artery diameter in response to handgrip exercise in humans. *J Appl Physiol* 119: 858–864, 2015.
5. **Atkinson G**, **Batterham AM**. Allometric scaling of diameter change in the original flow-mediated dilation protocol. *Atherosclerosis* 226: 425–427, 2013.
6. **Atkinson G**, **Batterham AM**, **Thijssen DHJ**, **Green DJ**. A new approach to improve the specificity of flow-mediated dilation for indicating endothelial function in cardiovascular research. *J Hypertens* 31: 287–91, 2013.
7. **Balanos GM**, **Talbot NP**, **Dorrington KL**, **Robbins PA**. Human pulmonary vascular response to 4 h of hypercapnia and hypocapnia measured using Doppler echocardiography. *J Appl Physiol* 94: 1543–1551, 2003.
8. **Bijari PB**, **Wasserman BA**, **Steinman DA**. Carotid bifurcation geometry is an independent predictor of early wall thickening at the carotid bulb. *Stroke* 45: 473–478, 2014.
9. **Black MA**, **Cable NT**, **Thijssen DHJ**, **Green DJ**. Importance of measuring the time course of flow-mediated dilatation in humans. *Hypertension* 51: 203–10, 2008.
10. **Carter HH**, **Atkinson CL**, **Heinonen IHA**, **Haynes A**, **Robey E**, **Smith KJ**, **Ainslie PN**, **Hoiland RL**, **Green DJ**. Evidence for Shear Stress – Mediated Dilation of the Internal Carotid Artery in Humans. *Hypertension* 68: 1217–1224, 2016.
11. **Carter HH**, **Atkinson CL**, **Heinonen IHA**, **Haynes A**, **Robey E**, **Smith KJ**, **Ainslie PN**, **Hoiland RL**, **Green DJ**. Evidence for shear stress-mediated dilation of the internal carotid artery in humans. *Hypertension* 68: 1217–1224, 2016.
12. **Celermajer DS**, **Sorensen KE**, **Gooch VM**, **Spiegelhalter J**, **Miller OI**, **Sullivan ID**, **Lloyd JK**, **Deanfield JE**. Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. *Lancet* 340: 1111–1115, 1992.
13. **Coverdale NS**, **Gati JS**, **Opalevych O**, **Perrotta A**, **Shoemaker JK**. Cerebral blood flow velocity underestimates cerebral blood flow during modest hypercapnia and hypocapnia. *J Appl Physiol* 117: 1090–1096, 2014.
14. **Faraci FM**, **Baumbach GL**, **Heistad DD**. Myogenic mechanisms in the cerebral circulation. *J Hypertens* 7: S61–S64, 1989.
15. **Fog M**. Cerebral Circulation II: Reaction of Pial Arteries To Increase In Blood Pressure. *Arch Neurol Psychiatry* 41: 260–268, 1939.
16. **Glagov S**, **Zarins C**, **Giddens DP**, **Ku DN**. Hemodynamics and atherosclerosis. Insights and perspectives gained from studies of human arteries. *Arch. Pathol. Lab. Med.* 112: 1018–1031, 1988.

17. **Green DJ**, **Dawson EA**, **Groenewoud HMM**, **Jones H**, **Thijssen DHJ**. Is flow-mediated dilation nitric oxide mediated?: A meta-analysis. *Hypertension* 63: 376–382, 2014.
18. **Green DJ**, **Jones H**, **Thijssen D**, **Cable NT**, **Atkinson G**. Flow-Mediated Dilation and Cardiovascular Event Prediction: Does Nitric Oxide Matter ? *Hypertension* 57: 363–369, 2011.
19. **Gupta A**, **Chazen JL**, **Hartman M**, **Delgado D**, **Anumula N**, **Shao H**, **Mazumdar M**, **Segal AZ**, **Kamel H**, **Leifer D**, **Sanelli PC**. Cerebrovascular reserve and stroke risk in patients with carotid stenosis or occlusion: A systematic review and meta-analysis. *Stroke* 43: 2884–2891, 2012.
20. **Heitzer T**, **Schlinzig T**, **Krohn K**, **Meinertz T**, **Münzel T**. Endothelial Dysfunction, Oxidative Stress, and Risk of Cardiovascular Events in Patients With Coronary Artery Disease. *Circulation* 104: 2673–2678, 2001.
21. **Hoiland RL**, **Tymko MM**, **Bain AR**, **Wildfong KW**, **Monteleone B**, **Ainslie PN**. Carbon dioxide mediated vasomotion of extra-cranial cerebral arteries in humans: A role for prostaglandins? *J Physiol* 594: 3463–3481, 2016.
22. **Ide K**, **Worthley M**, **Anderson T**, **Poulin MJ**. Effects of the nitric oxide synthase inhibitor L-NMMA on cerebrovascular and cardiovascular responses to hypoxia and hypercapnia in humans. *J Physiol* 584: 321–332, 2007.
23. **Kontos HA**, **Raper AJ**, **Patterson JL**. Analysis of vasoactivity of local pH, PCO2 and bicarbonate on pial vessels. *Stroke* 8: 358–360, 1977.
24. **Mullen MJ**, **Kharbanda RK**, **Cross J**, **Donald AE**, **Taylor M**, **Vallance P**, **Deanfield JE**, **MacAllister RJ**. Heterogenous nature of flow-mediated dilatation in human conduit arteries in vivo: relevance to endothelial dysfunction in hypercholesterolemia. *Circ Res* 88: 145–151, 2001.
25. **Pandit JJ**, **Mohan RM**, **Paterson ND**, **Poulin MJ**. Cerebral blood flow sensitivity to CO2 measured with steady-state and Read's rebreathing methods. *Respir Physiol Neurobiol* 137: 1–10, 2003.
26. **Peebles KC**, **Ball OG**, **MacRae B a.**, **Horsman HM**, **Tzeng YC**. Sympathetic regulation of the human cerebrovascular response to carbon dioxide. *J Appl Physiol* 113: 700–706, 2012.
27. **Phillips AA**, **Chan FH**, **Zheng MMZ**, **Krassioukov A V**, **Ainslie PN**. Neurovascular coupling in humans: Physiology, methodological advances and clinical implications. *J Cereb Blood Flow Metab* 36: 647–664, 2015.
28. **Pitsikoulis C**, **Bartels MN**, **Gates G**, **Rebmann RA**, **Layton AM**, **De Meersman RE**. Sympathetic drive is modulated by central chemoreceptor activation. *Respir Physiol Neurobiol* 164: 373–379, 2008.
29. **Pohl U**, **Herlan K**, **Huang A**, **Bassenge E**. EDRF-mediated shear-induced dilation opposes myogenic vasoconstriction in small rabbit arteries. *Am J Physiol* 261: H2016-23, 1991.
30. **Portegies MLP**, **de Bruijn RFAG**, **Hofman A**, **Koudstaal PJ**, **Ikram MA**. Cerebral vasomotor reactivity and risk of mortality: the Rotterdam Study. *Stroke* 45: 42–7, 2014.
31. **Przybyłowski T**, **Bangash M-F**, **Reichmuth K**, **Morgan BJ**, **Skatrud JB**, **Dempsey JA**. Mechanisms of the cerebrovascular response to apnoea in humans. *J Physiol* 548: 323–332, 2003.
32. **Pyke KE**, **Tschakovsky ME**. Peak vs. total reactive hyperemia: which determines the magnitude of flow-mediated dilation? *J Appl Physiol* 102: 1510–1519, 2007.

33. **Regan RE**, **Fisher JA**, **Duffin J**. Factors affecting the determination of cerebrovascular reactivity. *Brain Behav* 4: 775–88, 2014.
34. **Rousseeuw PJ**, **Ruts I**, **Tukey JW**. The Bagplot: A Bivariate Boxplot. *Am Stat* 53: 382–387, 1999.
35. **Schmetterer L**, **Findl O**, **Strenn K**, **Graselli U**, **Kastner J**, **Eichler HG**, **Wolzt M**. Role of NO in the O2 and CO2 responsiveness of cerebral and ocular circulation in humans. *Am J Physiol* 273: R2005–R2012, 1997.
36. **Shapiro W**, **Wasserman AJ**, **Patterson JL**. Mechanism and Pattern of Human Cerebrovascular Regulation after Rapid Changes in Blood CO2 Tension. *J Clin Invest* 45, 1966.
37. **Smith BA**, **Clayton EW**, **Robertson D**. Experimental arrest of cerebral blood flow in human subjects: the red wing studies revisited. *Perspect Biol Med* 54: 121–31, 2011.
38. **Takeuchi S**, **Karino T**. Flow patterns and distributions of fluid velocity and wall shear stress in the human internal carotid and middle cerebral arteries. *World Neurosurg* 73: 174–185, 2010.
39. **Tang SC**, **Huang YW**, **Shieh JS**, **Huang SJ**, **Yip PK**, **Jeng JS**. Dynamic cerebral autoregulation in carotid stenosis before and after carotid stenting. *J Vasc Surg* 48: 88–92, 2008.
40. **Thijssen DHJ**, **Black MA**, **Pyke KE**, **Padilla J**, **Atkinson G**, **Harris R a**, **Parker B**, **Widlansky ME**, **Tschakovsky ME**, **Green DJ**. Assessment of flow-mediated dilation in humans: a methodological and physiological guideline. *Am J Physiol Heart Circ Physiol* 300: H2–H12, 2011.
41. **Thomas KN**, **Lewis NCS**, **Hill BG**, **Ainslie PN**. Technical recommendations for the use of carotid duplex ultrasound for the assessment of extracranial blood flow. *Am J Physiol Regul Integr Comp Physiol* 309: R707–R720, 2015.
42. **Tymko MM**, **Ainslie PN**, **Macleod DB**, **Willie CK**, **Foster GE**. End-tidal-to-arterial CO2 and O2 gas gradients at low- and high-altitude during dynamic end-tidal forcing. *Am J Physiol - Regul Integr Comp Physiol* 308: R895-906, 2015.
43. **Tymko MM**, **Hoiland RL**, **Kuca T**, **Boulet LM**, **Tremblay JC**, **Pinske BK**, **Williams AM**, **Foster GE**. Measuring the human ventilatory and cerebral blood flow response to CO2: a technical consideration for the end-tidal-to-arterial gas gradient. *J Appl Physiol* 120: 282–296, 2016.
44. **Vianna LC**, **Kim A**, **Fadel PJ**. Impaired dynamic cerebral autoregulation during isometric exercise in patients with type 2 diabetes. *FASEB J* 25, 2011.
45. **White RP**, **Deane C**, **Vallance P**, **Markus HS**. Nitric oxide synthase inhibition in humans reduces cerebral blood flow but not the hyperemic response to hypercapnia. *Stroke* 29: 467–472, 1998.
46. **Willie CK**, **Colino FL**, **Bailey DM**, **Tzeng YC**, **Binsted G**, **Jones LW**, **Haykowsky MJ**, **Bellapart J**, **Ogoh S**, **Smith KJ**, **Smirl JD**, **Day TA**, **Lucas SJ**, **Eller LK**, **Ainslie PN**. Utility of transcranial Doppler ultrasound for the integrative assessment of cerebrovascular function. *J Neurosci Methods* 196: 221–237, 2011.
47. **Willie CK**, **Macleod DB**, **Shaw AD**, **Smith KJ**, **Tzeng YC**, **Eves ND**, **Ikeda K**, **Graham J**, **Lewis NC**, **Day TA**, **Ainslie PN**. Regional brain blood flow in man during acute changes in arterial blood gases. *J Physiol* 59014: 3261–3275, 2012.
48. **Willie CK**, **Tzeng Y-C**, **Fisher JA**, **Ainslie PN**. Integrative regulation of human brain blood flow. *J Physiol* 592: 841–59, 2014.

49. **Woodman RJ**, **Playford D a**, **Watts GF**, **Cheetham C**, **Reed C**, **Taylor RR**, **Puddey IB**, **Beilin LJ**, **Burke V**, **Mori T a**, **Green D**. Improved analysis of brachial artery ultrasound using a novel edge-detection software system. *J Appl Physiol* 91: 929–937, 2001.
50. **Zarrinkoob L**, **Ambarki K**, **Wåhlin A**, **Birgander R**, **Eklund A**, **Malm J**. Blood flow distribution in cerebral arteries. *J Cereb Blood Flow & Metab* 35: 648–654, 2015.

**FIGURE LEGENDS**

**Figure 1. Representative traces for the transient and steady state $CO_2$ tests.** Panel A depicts a typical response for the transient $CO_2$ test and panel B depicts a typical response for the steady state $CO_2$ test. Each panel displays $P_{ET}CO_2$, $P_{ET}O_2$, MCAv, ICA shear rate, and ICA diameter, with the raw data represented by the open grey circles, and the smoothed data represented by the closed black circles. The circled X's indicate the response start and subsequent peak value of each hemodynamic variable, while the black bar denotes the duration of $CO_2$ manipulation.

**Figure 2. Shear rate and diameter during the transient $CO_2$ test.** This figure highlights the temporal dynamics of the 30-second transient $CO_2$ test in one subject. Time zero represents the onset of the 30-second $CO_2$ stimulus (+9mmHg $P_{ET}CO_2$). The increase in $CO_2$ produced an increase in CBF and therefore cerebral shear rate (grey circles) which mirrors that subsequent to brachial cuff release during a peripheral FMD test [see figure 2 in(40)]. Shear rate area under the curve is highlighted in grey. Following the transient increase in shear rate, ICA diameter (open squares) increased approximately 30 seconds after peak shear rate was reached (51-seconds from $CO_2$ onset). Peak diameter was reached approximately 60-seconds after peak shear occurred (80-seconds from $CO_2$ onset).

**Figure 3. The time course of cerebral vascular responses to steady state and transient $CO_2$ breathing.** The presented data represents the threshold at which middle cerebral artery blood velocity (MCAv), internal carotid artery (ICA) shear, and ICA diameter begin to deviate (increase) from baseline. * Denotes a significant difference between steady state CO2 and transient CO2

response times. † Denotes a significant difference in the time delay between ICA shear and ICA dilation *within* a trial.

**Figure 4. Peak internal carotid artery dilation during the steady state and transient $CO_2$ tests.** The left panel shows the change in diameter of the internal carotid artery (ICA) from baseline to peak dilation after a transient 30-second period of hypercapnia (+9mmHg) for 20 subjects. The right panel shows the change in diameter of the ICA from baseline to peak dilation during four minutes of steady state hypercapnia (+9mmHg) for 20 subjects. The %change in diameter in the steady state trial was greater than the %change in diameter in the transient 30-second trial (comparison of deltas between trials, P=0.03). Mean±SD is indicated for each trial by the filled circles (●) and error bars.

**Figure 5. The relationship between shear AUC and ICA dilation in the transient and steady state $CO_2$ trials.** The present data represent the stimulus-response relationship between shear area under the curve (AUC) and ICA vasodilation. In the transient trial, following removal of the statistical outlier (circled), shear area AUC was positively correlated with ICA vasodilation ($r^2$=0.44; P<0.01). No relationship was present between shear AUC and ICA vasodilation in the steady state trial ($r^2$=0.02;P=0.53).