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Effects of Hypercapnia on Myocardial Blood Flow in Healthy Human Subjects

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ABSTRACT

Background: Elevation of end-tidal partial pressure of CO_2 ($P_{ET}CO_2$) increases cerebral and myocardial blood flow (MBF), suggesting that it may be a suitable alternative to pharmacological stress or exercise for myocardial perfusion imaging. The purpose of this study was to document the pharmacodynamics of CO_2 on MBF using prospective end-tidal targeting to precisely control arterial PCO_2 and use positron emission tomography (PET) to measure the outcome variable, MBF

Methods: 10 healthy males underwent serial ^{82}Rb PET/CT imaging. Imaging was performed at rest, and under 6 min hypercapnic plateaus ($P_{ET}CO_2$=baseline, 50, 55, 60, and repeated 60 mmHg, baseline). MBF was measured using ^{82}Rb injected 3 min after the beginning of hypercapnia, using a one-tissue-compartment model with flow-dependent extraction correction. Results were compared to those during an adenosine stress test (140 μg/kg/min)

Results: Baseline $P_{ET}CO_2$ was 38.9±0.8 (standard deviation, SD) mmHg (range 35 to 43 mmHg). All $P_{ET}CO_2$ targets were sustained with SD<1.5 mmHg. Heart rate, systolic blood pressure, rate pressure product (RPP), and respiratory frequency (*f*) increased with progressive hypercapnia. MBF increased significantly at each level of hypercapnia to 1.92 fold above baseline (0.86±0.24 vs 0.45±0.08 mL/min/g, p=0.002) at $P_{ET}CO_2$ of 60 mmHg. MBF following administration of adenosine was significantly greater than that during maximal hypercapnic stimuli (2.00 vs 0.86 mL/min/g, p<0.0001).

Conclusions: This is the first study to assess the response of MBF to different levels of hypercapnia in healthy humans with PET. MBF increases with increasing level of hypercapnia; at $P_{ET}CO_2$ 60 mmHg, MBF doubled compared to baseline.

Keywords: hypercapnia, myocardial blood flow, adenosine, myocardial perfusion imaging, rubidium, end-tidal partial pressure of CO_2

INTRODUCTION

Little is known about the effects of hypercapnia (arterial PCO_2 ($PaCO_2$)>45 mmHg) on MBF in humans. The effects of $PaCO_2$ on MBF were first studied more than 100 years ago in dogs, in which coronary blood flow increased with hypercapnia *(1,2)*. The authors attributed the increased MBF to smooth muscle relaxation leading to decreased coronary resistance. These results were reproduced using different MBF measurement techniques in different animal models *(3–6)*.

Yang et al. were the first to systematically examine the dose-response characteristics of controlled hypercapnia on absolute MBF in humans using precise CO_2 targeting *(7)*. They studied the effect of a single 10 mmHg increase in $P_{ET}CO_2$ on MBF in healthy humans using myocardial blood oxygen level-dependant magnetic resonance imaging, a surrogate of MBF *(7)*. They demonstrated that hypercapnia created a hyperemic response similar to that reported for adenosine infusion. Studies in canines demonstrated the dynamic response of MBF over a range between 30 and 60 mmHg. More recently, Yang et al. showed no significant difference between the responses to hypercapnia and adenosine infusion in canines without coronary stenosis, with MBF doubling compared to baseline *(8)*.

Other studies in humans investigating the effects of hypercapnia on MBF were performed with dissimilar protocols, and produced variable results. None provided the dynamic range of response of MBF to graded $P_{ET}CO_2$. In this study, we assessed the relationship between graded levels of $P_{ET}CO_2$ and increases in MBF as measured using PET imaging. The dose-response relationship between $P_{ET}CO_2$ and MBF is a prerequisite for employing CO_2 as a non-invasive stimulus in the measure of myocardial flow reserve (MFR).

MATERIALS AND METHODS

Study Population

Eleven healthy male volunteers, screened by a clinical history and physical examination, were recruited for this study. One recruited subject did not come on imaging day and was therefore excluded. Participants had no prior history of obstructive coronary artery disease (CAD) and had no symptoms of CAD or heart failure. Participants provided written informed consent and the study was approved by the Ottawa Health Science Network Research Ethics Board. Participants were instructed to avoid caffeinated drinks for 24 h before imaging.

PET Imaging

At present, MBF may be assessed non-invasively with several methods. Transthoracic echocardiography can estimate epicardial coronary artery flow reserve and magnetic resonance can approximate global and regional flow reserve. Nevertheless, PET imaging remains the clinical gold-standard to which other non-invasive techniques are compared and validated. Accuracy and repeatability of PET have made it the method of choice to quantify absolute MBF *(9)*.

All participants underwent a baseline rest ^{82}Rb PET scan to quantify MBF with low-dose CT for attenuation correction. Following this baseline study, serial ^{82}Rb PET scan using three levels of $P_{ET}CO_2$ (50 mmHg, 55 mmHg, and 60 mmHg), a suitable surrogate of $PaCO_2$ *(10)*, were acquired. Using sequential gas delivery, $P_{ET}CO_2$ are within ±1 mmHg of their arterial equivalents *(11)*. The 60 mmHg level was repeated following a rest period of at least 10 min. A second rest ^{82}Rb PET scan was performed after return to normocapnia. For every level of $P_{ET}CO_2$, hypercapnia was maintained for 6 min. ^{82}Rb infusion and PET acquisition were started 3 min after beginning of hypercapnia. This was followed by an adenosine stress ^{82}Rb PET scan at least 20 min later (Fig. 1). A total of seven ^{82}Rb PET scans per participant were performed within 90 min.

Heart rate (HR) and blood pressure (BP) were measured 1 to 3 min before stress, immediately before tracer injection, and 3 to 5 min post injection (Spot Vital Signs Device, Welch Allyn). Additionally, during pharmacological and hypercapnic stresses, HR was measured every minute using a 12-lead ECG

(CardioSoft Diagnostic System, GE). Rate pressure product (RPP) calculation was performed using the average HR and systolic blood pressure (SBP) at peak stress and post tracer infusion.

^{82}Rb PET imaging was performed according to the University of Ottawa Heart Institute standard clinical protocol previously described *(12)*. Briefly, participants were positioned in a 3D PET system (GE Discovery 690 PET/CT). The initial rest PET scan included a low-dose CT for attenuation correction (fast helical 1.5s, 120kVp with axial and angular mA modulation at noise-index=50). PET imaging was performed using 10 MBq/kg of ^{82}Rb, administered intravenously over 30s and imaging was performed over a period of 6 min. MBF was quantified using a one-tissue-compartment model with a flow-dependent extraction correction *(12)*. Polar-maps representing MBF were generated for each rest and stress state using validated software (FlowQuant©, Ottawa, Canada) *(13)*. Corrected MBF values were calculated using population average rest-RPP according to the following formula: Corrected MBF=(MBF×Average-RPP)/Subject-RPP. MFR was calculated by dividing MBF by rest-MBF. All presented MBF and MFR are unadjusted except where noted.

Hypercapnia

Until recently, precise modulation of $PaCO_2$ and arterial oxygen tension (PaO_2) levels could only be achieved by changing the concentrations of inspired gases. These fixed inspired gas methods are affected by variations in breathing patterns (frequency and tidal volume), leading to variability in end-tidal gas concentrations *(14)*. A new approach of breath-by-breath control of arterial blood gases using sequential gas delivery *(10,11,15)* enable the automated prospective control of arterial blood gases *(16)* independently of the subject's ventilation or pattern of breathing. Using sequential gas delivery, it is possible to maintain an euoxic-hypercapnic state at any level for several minutes with minimal discomfort for the subject *(17)*, conditions needed for precise assessment of MBF using PET imaging.

End-tidal CO_2 and O_2 partial pressures ($P_{ET}CO_2$ and $P_{ET}O_2$, respectively) targets were achieved using prospective end-tidal gas targeting, with previously described algorithms *(11)*. These algorithms were

applied by a computerized gas blender (RespirAct^{TM}; Thornhill Research, Toronto, Canada) with PCO_2 and PO_2 sensors and connected to a sequential gas delivery breathing circuit *(15)*. Pilot studies with the computerized gas blender showed that PCO_2 of 60 mmHg could tolerated consistently for several minutes. Therefore, this level was chosen as the upper PCO_2 target for the study, so that a dose-response relationship could be discerned and provide an indication whether the PCO_2-MBF response was plateauing as PCO_2 approached 60 mmHg. For this study, three levels of $P_{ET}CO_2$ were targeted (50, 55 and 60 mmHg), while maintaining euoxia ($P_{ET}O_2$ of 100 mmHg), and maintained over 6 min. Tidal PCO_2 and PO_2 were monitored continuously and recorded. Prior to imaging, the participants' PCO_2 was increased to 60 mmHg for 6 min to familiarize them with the sensation of hypercapnia and confirm their tolerance for the change. Baseline $P_{ET}CO_2$ levels were estimated using average $P_{ET}CO_2$ measurements after initial application of the mask, before hypercapnia. Baseline respiratory frequencies (*f*) were calculated after the patient was positioned under the camera and while wearing the mask.

Study tolerability

Subjects graded the discomfort associated with hypercapnia and adenosine on a subjective scale of 0 to 10, with 0 representing no discomfort and 10 representing intolerable discomfort.

Statistical Analysis

One-way repeated measures ANOVA were conducted prior to comparison of the different imaging states, using the first baseline study and first maximal hypercapnic stimuli (60 mmHg). When data for a participant were missing, the entire dataset of that participant was excluded from repeated measures ANOVA analyses. Post-hoc analyses were conducted using paired Student t-tests with Bonferroni-Holm sequential correction for multiple comparisons with the data set of all patients. A total of 8 comparisons were performed for the variables of interests (baseline vs 50 mmHg, baseline vs 60 mmHg, 50 mmHg vs 55 mmHg, 55 mmHg vs 60 mmHg, 60 mmHg vs repeated 60 mmHg, 60 mmHg vs adenosine, baseline vs repeated baseline, and repeated baseline vs adenosine). Tolerability was compared using paired-t test.

Average MFR was computed from the individual MFR values measured in each subject. Unless otherwise specified, results are presented as mean±SD. Values of $p<0.05$ were considered statistically significant. Statistical analysis was performed using GraphPad Prism version 6.01 for Windows (GraphPad Software, San Diego, USA) and MedCalc for Windows, version 12.2 (MedCalc Software, Ostend, Belgium).

RESULTS

Population

Participants' age, weight, height, and BMI were 30 ± 12y, 84.8 ± 11.2kg, 178 ± 8cm, and 26.9 ± 4.3kg/m^2 respectively. All participants had normal 12-leads ECG prior to entering the study. Measured baseline $P_{ET}CO_2$ was 38.9±0.8 mmHg (range 35 to 43 mmHg). For one participant, imaging at the 55 mmHg level was not acquired due to technical reasons; imaging at the 60 mmHg level was not acquired for another participant due to inability to maintain a stable $P_{ET}CO_2$. These two subjects were excluded from the repeated measure ANOVA analyses. Two other subjects did not have repeated 60 mmHg acquisitions.

Hypercapnic Stimulus

All $P_{ET}CO_2$ targets were attained with SD<1.5 mmHg (Table 1). A representative $P_{ET}CO_2$ tracing is presented in Fig. 2, showing a rapid increase in $P_{ET}CO_2$ to target followed by stable $P_{ET}CO_2$ at all target values.

Hemodynamic Parameters

HR, SBP, DBP, respiratory frequencies (*f*) and RPP increased with progressive levels of hypercapnia (Table 1, Fig. 3). There was a moderate correlation between RPP and MBF ($R^2 = 0.41$, $p<0.0001$, Fig. 4A). DBP did not change ($p_{ANOVA}=0.2$).

MBF and MFR

MBF increased 92% under maximal hypercapnic stimuli (0.86±0.24 vs 0.45±0.08 mL/min/g, p=0.002, Fig. 5). Increases in $P_{ET}CO_2$ and MFR were significantly correlated (R^2=0.43, p<0.0001, Fig. 4B). The dose-response relationship between MBF and $P_{ET}CO_2$ levels is shown in Fig. 4C. MBF was significantly greater at 50 mmHg compared to baseline (p=0.030), 55 mmHg compared to 50 mmHg (p=0.014), 60 mmHg compared to 55 mmHg (p=0.011), and 60 mmHg compared to baseline (p=0.002). There was a similar dose-response relationship with corrected MBF (Fig. 4C). Corrected MBF were significantly lower than uncorrected values at 50 mmHg (p=0.032), 55 mmHg (p=0.0174), and 60mmHg (p=0.002) levels. MFR was significantly higher at 55 mmHg compared to 50 mmHg (1.70 vs 1.38, p=0.005) and at 60 mmHg vs 55 mmHg (1.91 vs 1.70, p=0.005).

Multiple regression analyses were conducted to examine the relation between MBF and the two potential predictors, $P_{ET}CO_2$ and RPP. The multiple regression model with two predictor variables produced R^2=0.55, F(2,35)=21.23, p < 0.001. Both $P_{ET}CO_2$ (β=0.01285, p=0.0022) and RPP (β=0.00006, p=0.0014) had significant positive regression weights.

Adenosine

Adenosine increased MBF in all subjects, to 2.00 ± 0.34 mL/min/g, resulting in an MFR of 4.53 ± 0.70 (range 3.56 to 5.73). The adenosine-induced increase in MBF was significantly greater than that elicited by maximal hypercapnic stimuli of 60 mmHg (2.00 vs 0.86 mL/min/g, p<0.0001).

Reproducibility

The interval between first and second 60 mmHg acquisitions averaged 24.8 ± 8.3 min (range 12 - 41 min). MBF was not significantly different on the repeated 60 mmHg stimulus compared to the initial 60 mmHg one (0.87 vs 0.86 mL/min/g, p=0.051, Fig. 6A). This finding remained true when corrected for RPP (0.64 vs 0.59 mL/min/g, p=0.434). Finally, MFR was, on average, marginally greater on the repeated 60 mmHg level compared to the initial 60 mmHg stress (2.12 vs 1.97, p=0.019, Fig. 6B).

Study tolerability

For seven subjects, hypercapnia was more tolerable than adenosine while for two subjects adenosine was more tolerable than hypercapnia. In one, both stimuli were equally tolerable. Average tolerability scores for hypercapnic and adenosine stimuli did not differ (4.5 ± 2.0 vs 6.0 ± 2.5, p=0.15).

DISCUSSION

This study in healthy young men is the first documentation of a dose-response curve for CO2 and MBF with PET. MBF increased progressively with hypercapnia reaching a doubling of baseline MBF at $P_{ET}CO_2$ of 60 mmHg. Although some animal studies showed no change in MBF during hypercapnia *(18,19)*, our results are consistent with those of other animal studies reporting increase MBF with hypercapnia *(7,8,20,21)*. Beaudin et al. *(22)*, using cardiac magnetic resonance imaging, demonstrated an increase of 34% in coronary sinus flow with $PaCO_2$ of 45 mmHg. Tzou et al. *(23)* demonstrated that hypercapnia increased by ~40% the coronary blood flow velocity of the left anterior descending coronary artery measured with transthoracic Doppler echocardiography. Furthermore, this increase in blood flow velocity was greater at higher levels of inspired CO_2. Similar results were obtained with invasive measurements of coronary sinus flow *(24)*. However, other studies using Doppler echocardiography and PET showed little or no increase in MBF with hypercapnia *(25,26)*. These studies applied fixed inspired CO_2 concentrations that elicit unreliable increases in $PaCO_2$ *(14)*; indeed when measured, $PaCO_2$ barely changed, if at all *(25)*. However, these studies in humans were hampered by the inability to attain and maintain specific the PCO_2 levels needed to generate a MBF-PCO_2 relation, or separate the direct effects of CO_2 from the secondary effects of increases in myocardial work due to hypercapnia-induced increases in HR and SBP *(22,24,26)*. In our young subjects, these secondary effects were mild but contributed to the increase in MBF (Fig. 4).

We also found that hypercapnia elicited reproducible changes in MBF with a repeat $P_{ET}CO_2$ stimulus of 60 mmHg. This contrasts with intravenous agents, the pharmacokinetics of which result in

unpredictable blood levels and time courses of effects (see Fierstra et al. *(10)* for discussion). Hypercapnia in the range of 60 mmHg is high, but not out of the range commonly encountered by adults in the community. For example, in people with obstructive sleep apnea, such levels occur as often as 5-20 times per hour all night *(27)*. Hypercapnia does not cause irreversible adverse effects, even in patients with underlying morbidities *(28–30)*. A $P_{ET}CO_2$ of 60 mmHg was well tolerated by all subjects. As such hypercapnia may be considered a candidate in the search for a MBF stimulus that is non-invasive, tolerable, safe, rapidly reversible, and, most importantly, reproducible, in terms of both stimulus and response.

The increase in RPP, a proxy of cardiac work, during hypercapnia is associated with increased myocardial work and oxygen consumption *(31)*, leading to increased MBF. In our study, hypercapnia increased both HR and SBP, consistent with previous results in humans *(32,33)*. After correcting for RPP, there was still a significant increase in MBF. This was confirmed by a multiple variable regression demonstrating independent contributions of RPP and $P_{ET}CO_2$. These results suggest that the MBF increase can be partially, but not completely, attributed to increased cardiac work. Assuming the increase in MBF due to increase myocardial work was proportional to increase in RPP, approximately half of the MBF increase due to increased cardiac work. The other half is likely related to direct vasodilatation effect of hypercapnia. Indeed, prior studies have demonstrated that hypercapnia dilates the coronary arteries, a phenomenon mediated by endothelium-derived nitric oxide, adrenergic stimulus, and other mechanisms yet to be determined *(3,34–36)*. At similar $P_{ET}CO_2$ in young healthy subjects, Claassen et al. observed a 23% increase in mean arterial pressure with SBP up to 160 mmHg *(37)* which would increase RPP considerably more than in our study, providing a greater stress. Like them, we found hypertensive responses plateauing as $P_{ET}CO_2$ approached 60 mmHg.

In all subjects, adenosine injection increased MBF more than the maximal hypercapnic stimuli, with average MFR of 4.53 for adenosine stress. On the one hand, this is consistent with previously published results reporting MFR of 4 to 5 following adenosine stress and shows that our subjects had normal or

above normal coronary flow reserve *(38)*. On the other hand, this result differs from recently published observations by Yang et al., who compared MBF with hypercapnia ($PaCO_2$ increased by 25 mmHg) and adenosine stresses in canines without coronary stenosis, with non-flow limiting coronary stenosis, and following caffeine administration. In canines without coronary stenosis, increases in MBF did not differ in response to hypercapnia and adenosine, doubling compared to baseline *(8)*. Similar results were observed using myocardial blood oxygen level-dependant magnetic resonance imaging *(7)*. We observed similar MFR with hypercapnia but MFR was greater with adenosine. This could be related to the fact that the effects of adenosine on MBF are species-dependant and a standard dose of adenosine (140μg/kg/min) is insufficient to produce maximal hyperemia in canines *(39)*. Nevertheless, the increase in MBF under hypercapnia is similar in magnitude to previously reported increases during exercise stress (1.8 to 2.0) *(38,40)* and dipyridamole *(41,42)*. Our subject population overlaps the coronary stress test population, some of which also are young and have normal coronary anatomy. A definitive answer as to what extent hypercapnia can substitute for exercise and dipyridamole would require a head to head comparisons between exercise vs hypercapnia and dipyridamole vs hypercapnia in a coronary stress test population.

Finally, we showed that the effect of hypercapnia on MBF is reproducible; MBF and corrected MBF on the first and repeated 60 mmHg stimulus did not differ. The finding of MFR 8% greater on repeated measurements may be due to a combination of test-retest variability and effects of the intervening rest period of 12-41 minutes with free breathing at room air while the initial 60 mmHg acquisition was performed soon after the 50 mmHg and 55 mmHg acquisitions. Indeed, this finding could be partly attributable to the fact that the MBF response to an increase in $PaCO_2$ decreases during prolonged (> 10 min) hypercapnia *(3)*.

The main limitation of this study pertains to the studied population. Our subject were all healthy male volunteers with low resting MBF (Female participants were not purposely excluded. All of the volunteers were male). Whether similar results would be obtained in different populations, such as patients with CAD and subjects with high resting MBF, remains to be determined.

CONCLUSION

This is the first study in humans to assess changes in MBF in response to different levels of hypercapnia with PET. MBF increased at each incremental $P_{ET}CO_2$ level up to 60 mmHg. Approximately half of this increase can be attributed to increased cardiac work as indicated by the RPP. Under our maximal hypercapnic stimulus of 60 mmHg, MBF doubled, which is comparable to the increase in MBF obtained with exercise stress. Larger increases in RPP due to hypercapnia may increase MBF further.

Hypercapnia has additional characteristics to commend it for clinical investigation: it is readily available, inexpensive, and an intrinsic molecule that can be precisely, consistently and reproducibly administered by automated delivery systems. It is well tolerated at the effective partial pressure of 60 mmHg, and is safe even at multiple times its effective dose, giving it a large safety margin as a drug. The rapid onset and offset of PCO_2 levels and repeatability of MBF at repeated levels of hypercapnia commend it for repeat tests in a single session, as is sometimes required in clinical practice. To assess the potential clinical impact of hypercapnia in the diagnosis of CAD, further investigations comparing MBF with hypercapnia versus pharmacological stress or exercise in patients with CAD are warranted.

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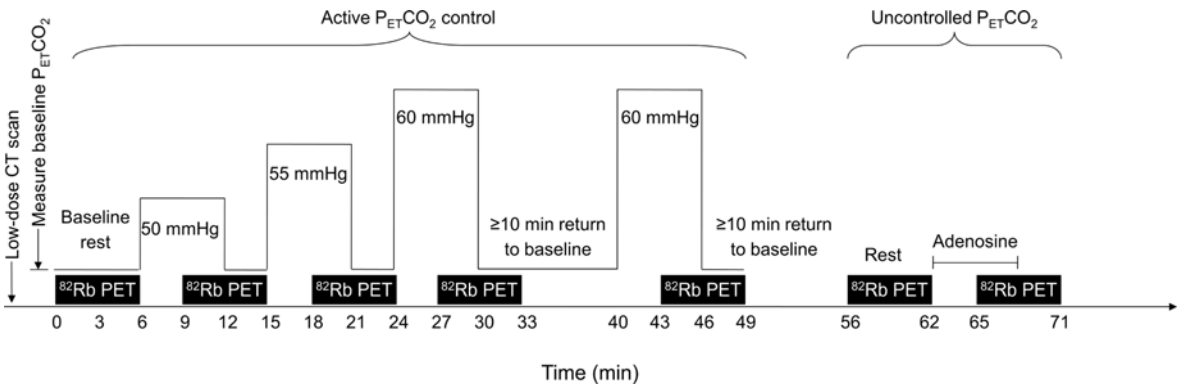

Figure 1: Sequence of imaging during a single visit.

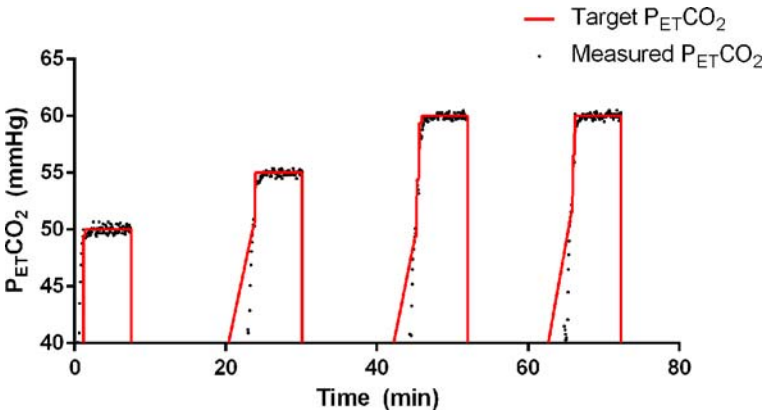

Figure 2: Representative curve of target $P_{ET}CO_2$ (red) and measured $PETCO_2$ (black dots) in a participant at the different levels of hypercapnia.

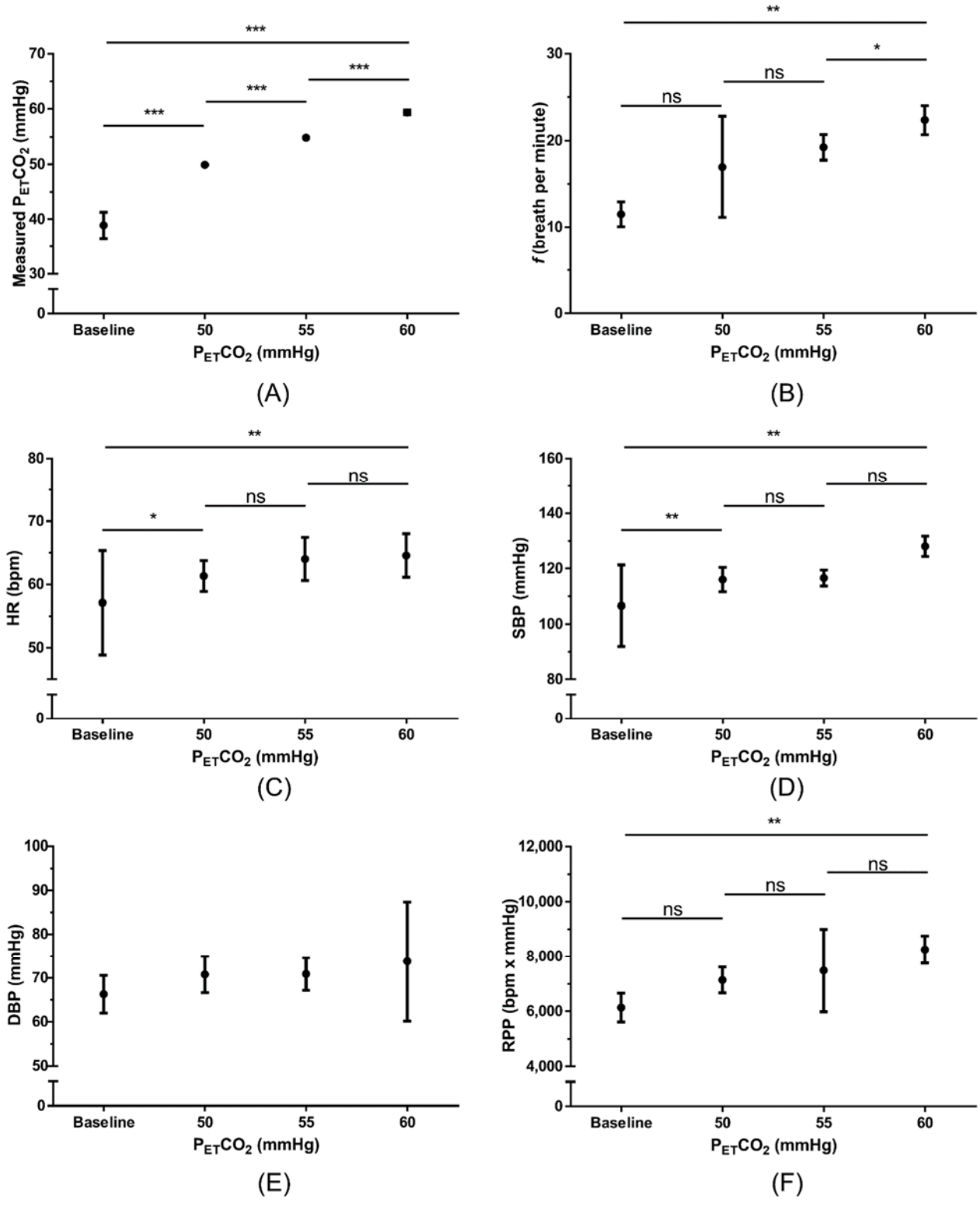

Figure 3: Measured end-tidal CO_2 pressure ($P_{ET}CO_2$, A), respiratory rate (*f*, B), heart rate (HR, C), systolic blood pressure (SBP, D), diastolic blood pressure (DBP, E), and rate pressure product (RPP, F) of

all participants at baseline and at different levels of hypercapnia. Results are presented as mean±SD. ns=p≥0.05, *p<0.05, **p<0.01, ***p<0.0001.

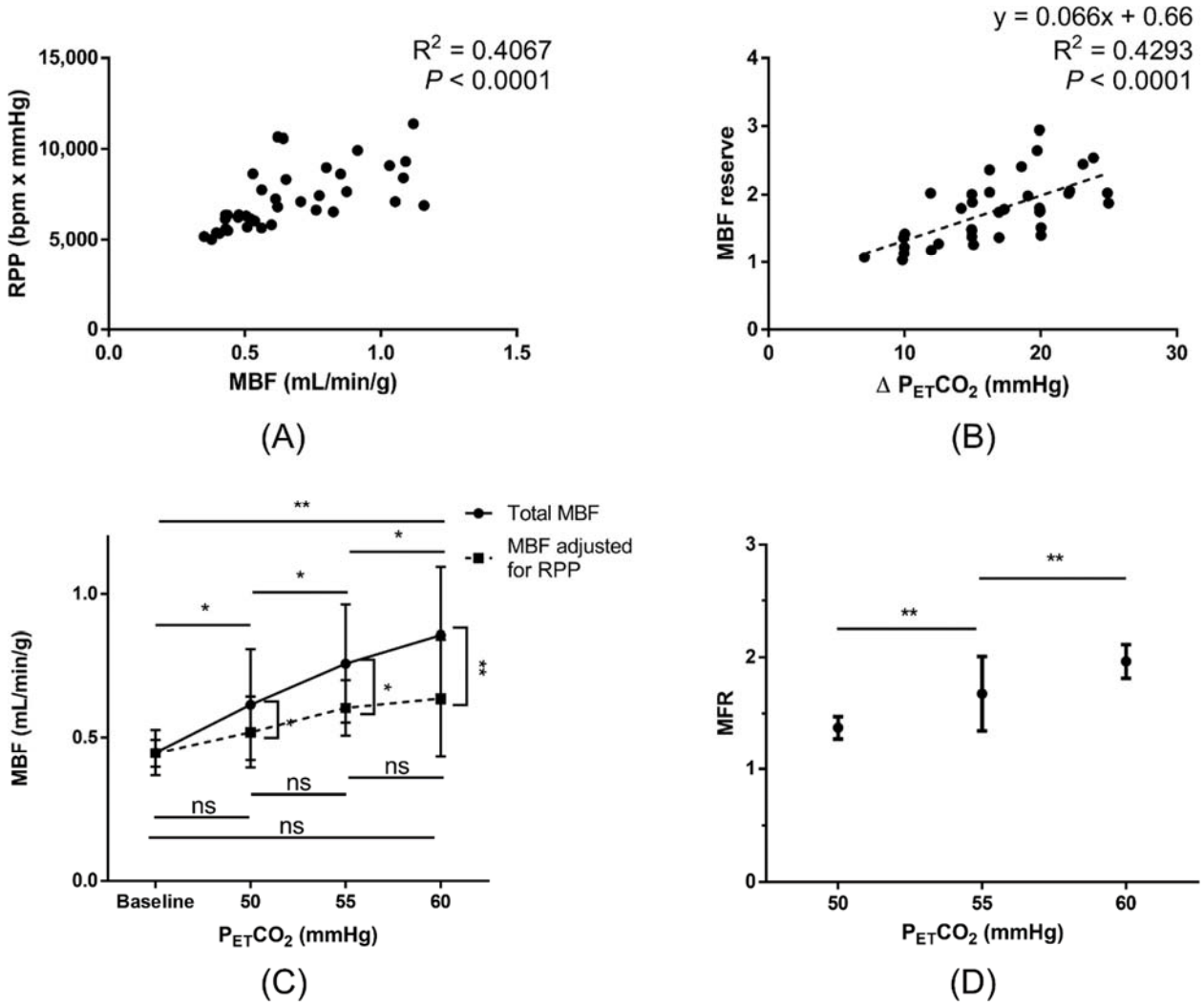

Figure 4: (A) Myocardial blood flow (MBF) vs rate pressure product (RPP). (B) Myocardial blood flow reserve (MFR) vs increase in end tidal partial pressure of CO_2 ($\Delta P_{ET}CO_2$). (C) Relation between myocardial blood flow (MBF) and adjusted MBF vs $P_{ET}CO_2$. (D) Relation between MFR and hypercapnia. Results are presented as mean±SD. *p<0.05, **p<0.01

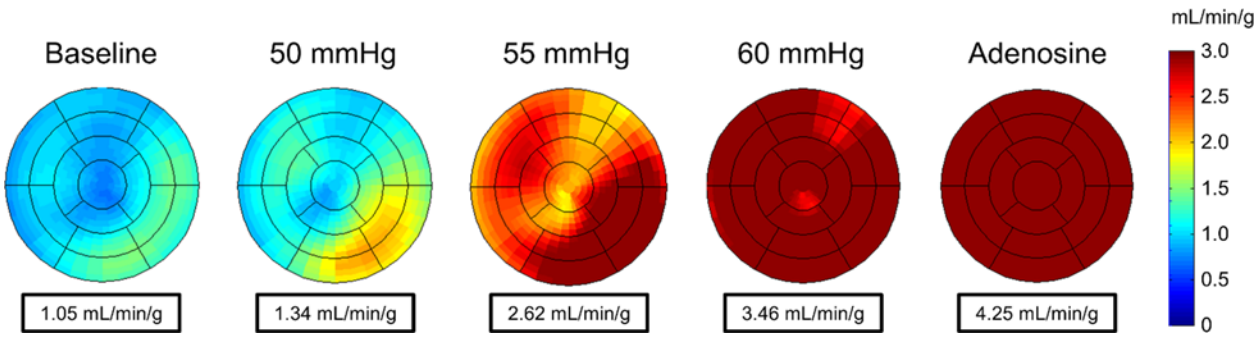

Figure 5: Myocardial blood flow polar maps of a representative subject with global MBF presented at baseline, during hypercapnic stimuli, and during adenosine stress.

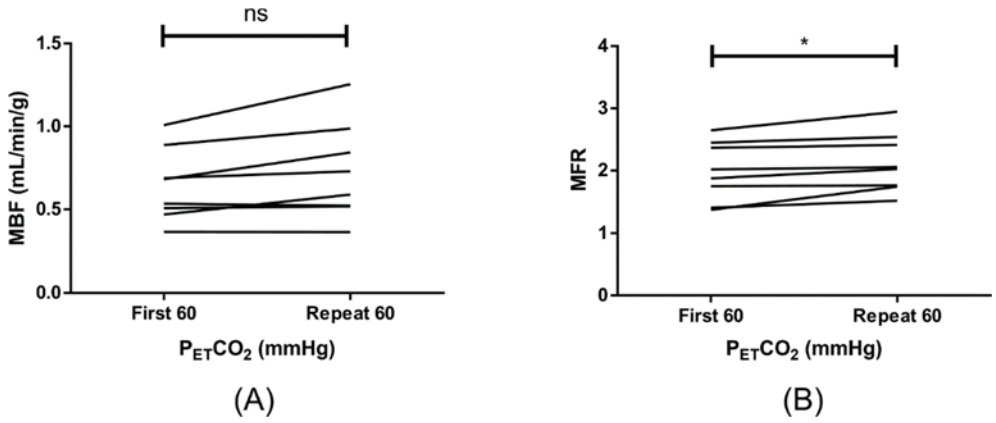

Figure 6: Average myocardial blood flow (MBF, A), as well as myocardial blood flow reserve (MFR, B) during the initial and second 60 mmHg $P_{ET}CO_2$ stress. Results are presented as mean±SD. ns=$p \geq 0.05$, *$p < 0.05$

	Baseline (n=10)	Baseline 2 (n=10)	50 mmHg (n=10)	55 mmHg (n=9)	60 mmHg (n=9)	60 mmHg (n=8)	Adenosine (n=10)	ANOVA p-value*
$P_{ET}CO_2$ (mmHg)	38.9±2.4	N/A	49.9±0.1	54.8±0.3	59.4±1.2	59.7±0.5	N/A	<0.0001
HR (beats/min)	57±8	59±8	62±7	66±9	68±10	69±8	76±10	<0.001
***f* (breaths/min)**	12±4	N/A	17±5	19±4	22±5	23±6	N/A	<0.001
SBP (mmHg)	109±13	117±13	119±15	123±11	131±12	125±8	121±13	<0.001
DBP (mmHg)	65±11	71±11	68±12	70±10	74±13	71±7	70±12	0.196
RPP (bpm × mmHg)	6135±1650	6900±1165	7140±1495	7487±1503	8256±1493	7666±1240	9123±1632	0.001
MBF (mL/min/g)	0.45±0.08	0.52±0.10	0.62±0.20	0.76±0.21	0.86±0.24	0.89±0.24	2.00±0.34	<0.0001
Corrected MBF (mL/min/g)	0.44±0.045	0.47±0.09	0.51±0.15	0.57±0.08	0.59±0.15	0.64±0.19	1.37±0.31	<0.0001
MFR	-	-	1.37±0.317	1.68±0.33	1.97±0.45	2.12±0.48	4.53±0.70	<0.0001

Table 1: Values presented as mean±SD. n, number of participants; $P_{ET}CO_2$, end tidal partial pressure of CO_2; HR, heart rate; *f*, respiratory frequency; SBP, systolic blood pressure; DBP, diastolic blood pressure; RPP, rate pressure product; MBF, myocardial blood flow; MFR, myocardial blood flow reserve; N/A, not available. *Three participants with missing data were excluded from the repeated-measure ANOVA.