Title: Hypoxia, not pulmonary vascular pressure induces blood flow through intrapulmonary arteriovenous anastomoses.

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Key points summary

- Blood flow through intrapulmonary arteriovenous anastomoses (IPAVA) is increased by acute hypoxia during rest by unknown mechanisms.
- Oral administration of acetazolamide blunts the pulmonary vascular pressure response to acute hypoxia, thus permitting the observation of IPAVA blood flow with minimal pulmonary pressure change.
- Hypoxic pulmonary vasoconstriction (HPV) was attenuated in humans following acetazolamide administration and partially restored with bicarbonate infusion, indicating that the effects of acetazolamide on HPV may involve an interaction between arterial pH and PCO_2.
- We observed that IPAVA blood flow during hypoxia was similar before and following acetazolamide administration, even after acid-base status correction, indicating pulmonary pressure, pH, and PCO_2 are unlikely regulators of IPAVA blood flow.

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Abstract

Blood flow through intrapulmonary arteriovenous anastomoses (IPAVA) is increased with exposure to acute hypoxia and has been associated with pulmonary artery systolic pressure (PASP). We aimed to determine the direct relationship between blood flow through IPAVA and PASP in 10 participants with no detectable intracardiac shunt by comparing: (1) isocapnic hypoxia (control); (2) isocapnic hypoxia with oral administration of acetazolamide (AZ; 250 mg, three times-a-day for 48 h) to prevent increases in PASP, and (3) isocapnic hypoxia with AZ and 8.4% $NaHCO_3$ infusion (AZ+HCO_3^-) to control for AZ-induced acidosis. Isocapnic hypoxia (20 min) was maintained by end-tidal forcing, blood flow through IPAVA was determined by agitated saline contrast echocardiography and PASP was estimated by Doppler ultrasound. Arterial blood samples were collected at rest before each isocapnic-hypoxia condition to determine pH, [HCO_3^-], and $PaCO_2$. AZ decreased pH (-0.08 ± 0.01), [HCO_3^-] (-7.1 ± 0.7 mmol/l), and $PaCO_2$ (-4.5 ± 1.4 mmHg; $p<0.01$), while intravenous $NaHCO_3$ restored arterial blood gas parameters to control levels. Although PASP increased from baseline in all three hypoxic conditions ($p<0.05$), a main effect of condition expressed an 11 ± 2% reduction in PASP from control ($p<0.001$) following AZ administration while intravenous $NaHCO_3$ partially restored the PASP response to isocapnic hypoxia. Blood flow through IPAVA increased during exposure to isocapnic hypoxia ($p<0.01$) and was unrelated to PASP, cardiac output and pulmonary vascular resistance for all conditions. In conclusion, isocapnic hypoxia induces blood flow through IPAVA independent of changes in PASP and the influence of AZ on the PASP response to isocapnic hypoxia is dependent upon the H^+ concentration or $PaCO_2$.

Abbreviations list: AZ, acetazolamide; FEV_1, forced expiratory volume in 1 second; F_IO_2, fraction of inspired oxygen; FVC, forced vital capacity; Hb, total haemoglobin; HPV, hypoxic pulmonary vasoconstriction; HR, heart rate; IPAVA, intrapulmonary arteriovenous anastomoses; MAP, mean arterial pressure; PASP, pulmonary artery systolic pressure; $P_{ET}CO_2$, end-tidal partial pressure of carbon dioxide; $P_{ET}O_2$, end-tidal partial pressure of oxygen; PFO, patent foramen ovale; PVR, pulmonary vascular resistance; $\dot{Q}_c$, cardiac output; RVOT, right ventricular outflow tract; S_{pO2},

oxyhaemoglobin saturation; SV, stroke volume; TRV, tricuspid regurgitant velocity; $\dot{V}_E$, minute ventilation; VTI, velocity-time integral

Introduction

The anatomical evidence of intrapulmonary arteriovenous anastomoses (IPAVA) is established in many mammals, including baboons, dogs, and humans (Tobin & Zariquiey, 1950; Tobin & Wilder, 1953; Tobin, 1966; Lovering *et al.*, 2007; Stickland *et al.*, 2007). In cadaveric human studies, IPAVA vessel diameter has been shown to be up to 200 μm in adults (Tobin, 1966), potentially indicating a transpulmonary pathway for erythrocytes that bypasses the alveoli. Indeed, this has been demonstrated in isolated human lungs; transpulmonary passage of microspheres exists, even if the microspheres are too large (50 μm) to pass through the alveolar capillaries (7-10 μm) (Lovering *et al.*, 2007). Similarly, blood flow through IPAVA can be detected in humans by agitated saline contrast echocardiography (Elliott *et al.*, 2011; Elliott *et al.*, 2013) or 99mTc macroaggregated albumin (Lovering *et al.*, 2009b; Bates *et al.*, 2014). Despite the anatomical and biophysical evidence, the functional significance of IPAVA *(if any)* is unclear.

Previous reports speculate that IPAVA act as a pressure release valve (Berk *et al.*, 1977; Stickland *et al.*, 2004), diverting blood flow away from the fragile microvessels of the lung and consequently reducing the risk of capillary stress failure and pulmonary oedema. The proposed protective mechanism has been supported most recently by Norris *et al.* (2014) who described a relationship between high magnitudes of blood flow through IPAVA, as estimated by microbubble passage, and total pulmonary resistance. Subjects who demonstrated a high amount of microbubble passage (i.e. bubble score ≥ 2) had lower total pulmonary resistance compared to those with less microbubble passage (i.e. bubble score < 2). On the other hand, while blood flow through IPAVA may serve to relieve capillary hydrostatic pressure it may also decrease gas exchange efficiency by allowing blood to bypass sites of pulmonary gas exchange. Indeed, a correlation ($r = 0.63 - 0.68$) between the change in 99mTc macroaggregates or the occurrence of saline contrast passage and the alveolar-arterial oxygen gradient has been observed during normoxic and hypoxic exercise (Stickland *et al.*, 2004; Bates *et al.*, 2014). Although its physiological contribution as an anatomical shunt remains a matter of debate (Hopkins *et al.*, 2009; Lovering *et al.*, 2009a), recent work by Bryan *et al.* (2012) and Elliott *et al.* (2014) indicate that blood flow through IPAVA does contribute to pulmonary gas exchange efficiency. Given these potential protective (i.e. reduced capillary stress) and detrimental (i.e. decreased gas

exchange efficiency) effects of blood flow through IPAVA, it is important to understand the stimuli or mechanisms responsible for regulating blood flow through IPAVA.

Blood flow through IPAVA is present in approximately 30% of healthy individuals without a patent foramen ovale (PFO) under normal resting physiological conditions (Elliott *et al.*, 2013). Both acute hypoxia and cycle exercise in normoxia have been utilised to recruit IPAVA pathways in nearly all participants (Eldridge *et al.*, 2004; Stickland *et al.*, 2004; Lovering *et al.*, 2006; Lovering *et al.*, 2008a; Laurie *et al.*, 2010; Kennedy *et al.*, 2012; Bates *et al.*, 2014; Foster *et al.*, 2014). Using participants without a PFO or other cardiac shunt, >90% of participants demonstrated intrapulmonary saline contrast passage during submaximal and maximal exertion (Eldridge *et al.*, 2004; Stickland *et al.*, 2004; Lovering *et al.*, 2008a). An increasing magnitude of bubble score with decreasing fraction of inspired oxygen (FIO_2) observed by Laurie *et al.* (2010) indicates that the mechanism of IPAVA recruitment likely owes to a common physiological mechanism found during both exercise and hypoxia.

The principal stimulus ultimately responsible for regulating IPAVA recruitment is unknown; however, the literature suggests that pulmonary blood flow, pressure, or alveolar hypoxia are likely mediators (Laurie *et al.*, 2010; Laurie *et al.*, 2012; Elliott *et al.*, 2014). In humans, progressive hypoxia appears to promote a graded increase in the magnitude of microbubble passage despite a small increase in pulmonary artery systolic pressure (PASP) (Laurie *et al.*, 2010), indicating that alveolar hypoxia may be more important than pulmonary vascular pressure. The results from studies using cycle exercise to increase blood flow through IPAVA illustrate that an increase in microvascular pressure secondary to the increase in flow recruits IPAVA and reduces pulmonary vascular resistance (PVR) (Stickland *et al.*, 2004). However, in a subsequent study, Stickland *et al.* (2006) found that microbubble passage through IPAVA was inconsistent with increasing mean pulmonary artery pressure, finding greater consistency with increased cardiac output ($\dot{Q}_c$), implicating pulmonary blood flow as the primary mediator of blood passage through IPAVA. Recent work by Elliott *et al.* (2014) suggest that increased $\dot{Q}_c$, independent of increased PASP, increases blood flow through IPAVA in subjects breathing room air and mild hyperoxia (40% O_2). The conflicting results between studies prompted us to determine the importance of pulmonary vascular pressure on hypoxia induced blood flow through IPAVA.

Acetazolamide (AZ) is a carbonic anhydrase inhibitor that induces an acidosis, a mild diuresis, can inhibit hypoxic pulmonary vasoconstriction (HPV) and is commonly used to treat and prevent acute mountain sickness (Swenson, 2006; Hohne *et al.*, 2007; Swenson, 2014). Previously AZ, has been demonstrated to reduce the pulmonary pressure response to isocapnic hypoxia in humans by approximately 57% (Teppema *et al.*, 2007). Therefore, administration of AZ permits the observation of blood flow through IPAVA during hypoxia with minimal changes in PASP.

Based on the above brief summary, the purpose of this investigation was two-fold: first, to determine if the stimulus for IPAVA recruitment in response to isocapnic hypoxia is hypoxia, elevated pulmonary arterial pressure, or arterial pH/PCO_2; and second, to determine if AZ inhibits HPV through metabolic acidosis. We hypothesized: (1) that exposure to isocapnic hypoxia would lead to an increase in PASP and blood flow through IPAVA; (2) that oral administration of AZ would blunt the PASP response to hypoxia and abolish blood flow through IPAVA independent of arterial pH.

Methods

Ethical approval. All experimental procedures and protocols were approved by the Clinical Research Ethics Board at the University of British Columbia, and conformed to the latest revision of the Declaration of Helsinki. All participants provided written informed consent prior to participation in this study.

Participants. Healthy adult human participants free from cardiopulmonary disease participated in this study. Participants were excluded if they were obese (body mass index > 30 kg m^{-2}), had a history of smoking (within the past year), were hypertensive (systolic blood pressure > 140 mmHg; diastolic blood pressure > 90mmHg) or had poor pulmonary function as determined by spirometry (i.e., $FEV_1/FVC < 0.75$). Since the purpose of this study was to investigate the mechanisms controlling blood flow through IPAVA, participants were excluded if they had a PFO at rest or with a provocative manoeuvre (see *Experimental Protocol*). Finally, participants were excluded if they had pulmonary hypertension (PASP > 35mmHg) determined by echocardiography (see *Pulmonary Haemodynamics*). These exclusion criteria ensured that the participants included in this study were healthy and free from cardiopulmonary disease. In total, 27 participants were screened, and 11 qualified for the study. An incomplete Doppler envelope of the tricuspid regurgitant jet necessitated the exclusion of an additional participant. The results presented, unless otherwise specified, are based on 10 participants (1 female) between the ages of 22 and 37 years.

Experimental Protocol. Participants attended the laboratory on three separate days to complete this study. Visits two and three were separated by a minimum of four days. Inclusion/exclusion criteria were assessed on the first visit and participants were familiarised with the breathing apparatus and echocardiography measurements. Height and weight were measured, spirometry was conducted in accordance with the standards outlined by the American Thoracic Society and the European Respiratory Society (Miller *et al.*, 2005), and participants were instrumented with an intravenous catheter at the antecubital fossa. Following 10 minutes of supine rest, three consecutive blood pressure measurements were obtained from the right upper arm by using a manual sphygmomanometer and stethoscope for auscultation of Korotkoff sounds. While in the supine position, collapse of the inferior vena cava during a rapid inspiration was imaged by ultrasound to estimate right atrial pressure (Yildirimturk *et al.*, 2011). The participant was then

positioned laterally on their left side and echocardiographic images were recorded to determine stroke volume (SV) and PASP (see *Pulmonary Haemodynamics*). Beat-to-beat blood pressure was measured by finger pulse photoplethysmography (Finometer PRO, Finapress Medical Systems, Amsterdam, Netherlands) and normalised to manual cuff measurements of the brachial artery. Mean arterial pressure (MAP) was calculated from the beat-to-beat recordings. Heart rate (HR) was measured by standard lead-II electrocardiogram. Next, an apical 4-chamber view of the heart was acquired and the presence or absence of intracardiac shunt or blood flow through IPAVA was determined by the technique of agitated saline contrast echocardiography (Kennedy *et al.*, 2012; Elliott *et al.*, 2013; Foster *et al.*, 2014). Agitated saline contrast echocardiography was repeated during the release of a Valsalva manoeuver as a provocative stimulus to ensure the patency of the foramen ovale was identified appropriately.

Participants identified as PFO negative (n = 11) returned within a week and breathed 20 minutes of isocapnic hypoxia (Control; $P_{ET}O_2$ = 47 mmHg) to determine pulmonary haemodynamics and blood flow through IPAVA during an acute hypoxic exposure in a supine position. This protocol was selected based on Laurie *et al.* (2010) who demonstrated an oxyhaemoglobin saturation (SpO_2) of 79.8 ± 4.7% with F_IO_2 of 0.12 and was sufficient to recruit IPAVA in all participants under resting hypoxic conditions. Pulmonary haemodynamics and blood flow through IPAVA were measured by echocardiogram during 5 minutes of rest and the last 5 minutes of hypoxia. Participants were then prescribed an oral dose of AZ (250 mg) every 8 hours for 2 days prior to their final laboratory visit. The final dose of AZ was taken one hour before experimentation. This pharmacological treatment is known to nearly abolish the pulmonary pressor response to hypoxia in humans and animals (Hohne *et al.*, 2007; Teppema *et al.*, 2007). The isocapnic hypoxia protocol was then repeated twice (separated by at least 30 minutes) without intravenous $NaHCO_3$ (AZ) and with intravenous $NaHCO_3^-$ (AZ+HCO_3^-). To control for the metabolic acidosis caused by AZ, an 8.4% intravenous $NaHCO_3^-$ solution (Hospira, Montreal, Quebec, Canada) was delivered over a 15-minute period to restore arterial pH to resting levels. Arterial blood samples were collected from the radial artery before each exposure to isocapnic hypoxia and, in a subgroup of participants (n = 7), following the AZ+HCO_3^- protocol to ensure that pH and [HCO_3^-] were maintained for the duration of the experimental protocol. The deficit in [HCO_3^-] was calculated from resting arterial [HCO_3^-] taken with and without AZ and using body mass to calculate the required dosage of $NaHCO_3$ (Kollef &

Isakow, 2012). Arterial blood gas samples were obtained after bicarbonate infusion to ensure sufficient normalisation. In the event that [HCO_3^-] was not completely restored to resting levels additional $NaHCO_3^-$ was infused and bicarbonate levels re-assessed prior to experimentation. The order of experiments was not randomized because of the lasting effects of AZ and $NaHCO_3$.

***Pulmonary Haemodynamics*.** All echocardiography measurements were performed using the same commercially available ultrasound system (Vivid Q, 3.5 MHz transducer, GE Healthcare) by the same experienced sonographer (M.S.). First, the diameter of the left ventricular outflow tract at the level of the aortic annulus was determined from the parasternal long axis view. Measurements were taken at the end of systole and the average of three cardiac cycles taken as the diameter of the aorta. Then, the velocity time integral (VTI) of the left ventricular outflow tract was obtained from an apical five-chamber view by placing a pulsed wave Doppler sample volume just within the aortic valve. SV was calculated as the product of the VTI and aortic area, and $\dot{Q}_C$ was obtained by multiplication with HR. These methods have been previously described and validated against thermodilution and direct Fick (Christie *et al.*, 1987). Tricuspid regurgitation peak velocity was identified using colour flow Doppler and measured by continuous-wave Doppler ultrasound from the apical four-chamber view. The pulmonary artery pressure gradient could then be estimated from the simplified Bernoulli equation and PASP could be estimated by addition of right atrial pressure (Vazquez de Prada *et al.*, 1987). The inferior vena cava diameter was measured from subcostal longitudinal images approximately 2 cm distal to the right atrial junction. The collapsibility index was calculated as the percentage of difference between maximal and minimal size of the inferior vena cava. Right atrial pressure was predicted using the collapsibility index as recommended by the American Society of Echocardiography (Rudski *et al.*, 2010). This method has been validated against right atrial pressure obtained directly by right heart catheterisation (Yildirimturk *et al.*, 2011). All pulmonary haemodynamic measurements were made on three cardiac cycles and averaged to provide a single value. Pulmonary vascular resistance was obtained from the ratio of the peak tricuspid valve regurgitation (TRV) to the right ventricular outflow tract time-velocity integral (TVI RVOT). This was subsequently converted to Woods units (wu) (PVR = TRV(TVI RVOT $\times$ 10 + 0.16)$^{-1}$). This noninvasive estimate of PVR correlates well with invasive measurements (Abbas *et al.*, 2003; Roule *et al.*, 2010).

Agitated Saline Contrast Echocardiography. The presence of intracardiac shunt (i.e., PFO) was determined under resting conditions and during a provocative stimulus (i.e., release from Valsalva) using the technique of agitated saline contrast echocardiography (Marriott *et al.*, 2013). Two 5-ml syringes were connected by three-way stopcocks and attached to the 22-gauge cannula placed in the antecubital vein. One syringe contained 4 ml of sterile saline and the other 0.5 ml of air. The two syringes were flushed back and forth forcefully to agitate the mixture prior to rapid injection. The agitated contrast was then visualised entering the right ventricle from an apical 4-chamber view of the heart. If no bubbles appeared in the left ventricle, a provocative manoeuver was used to further assess the patency of the foramen ovale. In this case, participants were instructed to Valsalva at the end of a normal expiration. Agitated contrast was subsequently injected and once visualised in the right atrium, the participants were asked to relax and breathe normally. A PFO was identified if contrast appeared in the left ventricle in $\leq$5 cardiac cycles after the contrast cloud filled the right atrium. After all contrast injections, a minimum of 20 cardiac cycles were recorded. All participants with a PFO were excluded from this study. Blood flow through an IPAVA was defined when contrast appeared in the left ventricle > 5 cardiac cycles after the contrast appeared in the right atrium. This technique has been used to investigate blood flow through IPAVA in participants during rest and exercise at sea level breathing room air and during acute normobaric hypoxia (Stickland *et al.*, 2004; Imray *et al.*, 2008; Kennedy *et al.*, 2012; Laurie *et al.*, 2012; Elliott *et al.*, 2013). A scoring system has been established to determine the severity of blood flow through IPAVA based on the greatest density and spatial distribution of microbubbles in the left ventricle of a single cardiac cycle during the subsequent 20 cardiac cycles (Lovering *et al.*, 2008b). This 0-5 scoring system assigns a '0' for no microbubbles; '1' for 1-3 microbubbles; '2' for 4-12 microbubbles; '3' for greater than 12 microbubbles bolus; '4' for greater than 12 microbubbles heterogeneously distributed; and a '5' for greater than 12 microbubbles homogeneously distributed. This scaling system is reproducible between independent blinded observers (Laurie *et al.*, 2010). All agitated saline contrast echocardiograms were reviewed and scored by the same experienced sonographer (M.S.)

Isocapnic Hypoxia. Respiratory parameters were acquired at 200 Hz using an analogue-to-digital converter (PL3504, ADInstruments, Colorado Springs, USA) interfaced with a personal computer and analysed using commercially available software (LabChart, ADInstruments).

During the isocapnic hypoxia protocol participants breathed through a mouthpiece and two-way non-rebreathing valve with a nose clip applied to obstruct the nasal passage. Respired gas pressures were sampled at the mouth and analysed for PO_2 and PCO_2 (ML206, ADInstruments). Respiratory flow was measured at the mouth using a pneumotachograph (HR 800L, Hans Rudolph, Shawnee, USA). $P_{ET}O_2$, $P_{ET}CO_2$, inspiratory and expiratory tidal volumes were determined for each breath online using custom designed software (LabView V13.0, National Instruments, Austin, USA). Oxyhaemoglobin saturation was measured continuously by pulse oximetry (ML320/F, ADInstruments). End-tidal oxygen was clamped at 47 mmHg and $P_{ET}CO_2$ was maintained at resting levels by using a portable end-tidal forcing system (AirForce, G.E.Foster, Kelowna, Canada) (Bain *et al.*, 2013; Querido *et al.*, 2013; Foster *et al.*, 2014).

Arterial Blood Gas Sampling. Arterial blood gas samples were collected from the radial artery using a 23-gauge self-filling pre-heparanised syringe (*safe*PICO syringes, Radiometer, Copenhagen, Denmark). Approximately 3 ml of blood was withdrawn, air bubbles were immediately evacuated from the syringe and blood gas analysis was performed within 30 s of sampling with a calibrated blood gas analyser (ABL90 FLEX, Radiometer, Copenhagen, Denmark). Arterial blood was analysed for pH, PaO_2, $PaCO_2$, haemoglobin ([Hb]) concentration, and $[HCO_3^-]$ was calculated from pH and $PaCO_2$. All samples were assumed to be collected at a resting body temperature of 37 degrees Celsius. All samples were withdrawn after the participant had been resting supine for 10 minutes and immediately prior to exposure to isocapnic hypoxia. In a subgroup of participants (n = 7), an additional sample was drawn after the AZ+HCO_3^- experimental condition to confirm $[HCO_3^-]$ and pH were normalised.

Statistical Analysis. Statistical comparisons and calculations were made using statistical software (Statistica v.7.0, Statsoft Inc., Tulsa, OK, USA). Participant resting characteristics were compared between the three conditions by one-way repeated measures analysis of variance (ANOVA). Cardiopulmonary parameters were compared using a three-by-two (pharmacological intervention by normoxia/hypoxia) repeated measures ANOVA. Blood sample parameters were compared using a one-way repeated measures ANOVA. When significant F-ratios were detected, Tukey's HSD was applied to determine where the differences lay. Since we were most concerned about the differences between PASP during isocapnic hypoxia we compared these by one-way ANOVA. Bubble scores were compared between groups by Friedman ANOVA, with

Dunn's post-test if appropriate. All data are presented as mean ± standard error (SE; unless otherwise noted) and statistical significance was set at $p<0.05$ for all comparisons. Where echocardiographic measurements were unsuccessful, a three-by-two factorial ANOVA was conservatively performed to enable comparison on groups of unequal subjects. Echocardiographic measurements were unsuccessful for two participants for SV, $\dot{Q}_c$ and PVR during normoxic AZ; one participant for SV and $\dot{Q}_c$ during hypoxic AZ; one participant for SV and $\dot{Q}_c$ and two participants for PVR during normoxic AZ+HCO_3^-.

Results

Participant characteristics

Ten healthy adults met our inclusion criteria, participated in this study (age = 30 ± 2 years, height = 178 ± 3 cm, weight = 83 ± 4 kg) and had pulmonary function within normal limits (FEV_1/FVC = 81 ± 2 % [104 ± 2 % predicted]). In adherence to the inclusion criteria, none of the participants demonstrated microbubble passage through a PFO during rest or following release of a Valsalva manoeuver in normoxic conditions.

Haematological measurements

Haematological measurements obtained at rest before each condition and following AZ+HCO_3^- are reported in Table 1. A significant main effect of the pharmacological intervention was detected for $PaCO_2$, pH and [HCO_3^-]. *Post hoc* analysis revealed a significant reduction in arterial pH and [HCO_3^-] following AZ administration ($p<0.001$) compared to control and AZ+HCO_3^-. $PaCO_2$ at rest was significantly reduced during AZ compared to control ($p<0.01$). In the subset of participants (n = 7) with blood samples taken following the AZ+HCO_3^- condition, arterial pH was reduced at this time point compared to AZ+HCO_3^- ($p<0.05$), but was still greater than AZ ($p<0.01$) and similar to control ($p = 0.06$). In addition, [HCO_3^-] was lower than control and AZ+HCO_3^- ($p<0.01$) but remained greater than AZ ($p<0.01$).

Cardiopulmonary parameters during normoxia and hypoxia

Figure 1 displays the group mean $P_{ET}O_2$, $P_{ET}CO_2$, and $\dot{V}_E$ in 15-second intervals during 2 minutes of baseline and throughout isocapnic hypoxia for all three conditions. Table 2 displays select cardiopulmonary parameters during normoxia and hypoxia for each condition. $P_{ET}CO_2$ was significantly decreased during AZ and AZ+HCO_3^- compared to control ($p<0.001$). The expected responses to hypoxia were similar in each condition for $\dot{V}_E$, MAP and HR (Table 2).

Effect of AZ and $NaHCO_3$ on the PASP, $\dot{Q}_c$, and PVR response to hypoxia

Figure 2 shows the individual and mean changes in PASP from normoxia to isocapnic hypoxia during each condition. All participants displayed an increase in PASP following hypoxic exposure, with the exception of a single individual during control, in whom PASP fell from 27.2

mmHg to 26.0 mmHg and another following AZ, who displayed a reduction in PASP in hypoxia, from 22.4 mmHg to 20.5 mmHg. Excluding the two instances of PASP reduction upon hypoxic exposure, PASP increased from 20.5 ± 0.5 mmHg to 26.1 ± 1.1 mmHg in control, 19.0 ± 0.7 mmHg to 21.4 ± 0.6 mmHg following AZ and 19.4 ± 0.6 mmHg to 23.7 ± 0.8 mmHg after $NaHCO_3$ infusion. A significant main effect of oxygen level was observed (i.e., normoxia vs hypoxia; $p<0.001$) such that PASP increased by 20 ± 2 % ($p<0.001$) during hypoxia. A main effect of pharmacological intervention (i.e., control/AZ/AZ+HCO_3^-; $p<0.01$) identified an 11 ± 2 % decrease in PASP from control to AZ, while AZ+HCO_3^- was similar to control ($p = 0.09$). No interaction effect on PASP was detected ($p = 0.22$). Cardiac output showed no effect of condition but increased by 0.9 ± 0.2 l min^{-1} with hypoxia ($p<0.01$). Pulmonary vascular resistance rose by 0.1 ± 0.03 wu in hypoxia ($p<0.05$). A main effect of condition on PVR was found ($p<0.001$), showing an elevation during AZ+HCO_3^- of 15 ± 4 % ($p<0.01$) compared to control and by 14 ± 3 % ($p<0.01$) compared to AZ (Table 2).

Blood flow through IPAVA

During normoxia, one participant demonstrated microbubble passage in the control condition and another during AZ (bubble score = 1). No participants displayed microbubble passage during normoxia in the AZ+HCO_3^-. Figure 4 displays the distribution of bubble scores for all conditions during hypoxia. Only one participant did not demonstrate any microbubble passage during hypoxia control, the remaining 9 participants had a bubble score of 1 (n = 4) or 2 (n = 5). During AZ and AZ+HCO_3^-, bubble scores were similar to control and included bubble scores of 1 (n = 4), 2 (n = 4) and 3 (n = 2). The two participants who scored a bubble score of 3 were the same in AZ and AZ+HCO_3^-. A significant increase in bubble score from normoxia to hypoxia ($p<0.001$) was found, but no effect of condition ($p = 0.22$). Responses to the conditions during isocapnic hypoxia varied between participants. Specifically, four participants displayed the same bubble score during all three interventions, AZ increased bubble score in four participants including one participant that increased from a score of 1 to 3, and sodium bicarbonate infusion decreased microbubble passage in a single participant while one participant demonstrated a lower bubble score during AZ that was reversed following AZ+HCO_3^-. Nevertheless, aside from one participant, all individual changes between conditions were only by a bubble score of one.

Relationship between changes in PASP, $\dot{Q}_C$, PVR and blood flow through IPAVA

Figure 4 displays the relationship between PASP, $\dot{Q}_c$ and PVR with bubble scores across the three conditions. Multiple linear regressions did not reveal any significant correlations with R^2 values of 0.008, 0.12 and 0.003 for PASP, $\dot{Q}_c$ and PVR, respectively. In addition, we conducted linear regression analysis to determine if the subjects with the largest change in PASP with isocapnic hypoxia had the largest blunting by AZ. We found a weak, non-significant correlation ($R^2 = 0.16$, $p = 0.19$) between these parameters suggesting that the subjects with the largest PASP response had the smallest PASP response with AZ.

Discussion

Main Findings

The purpose of this investigation was to determine if the stimulus for IPAVA recruitment in response to isocapnic hypoxia is alveolar hypoxia, elevated pulmonary arterial pressure, or arterial pH/PCO_2; and, to determine if AZ inhibits HPV through metabolic acidosis. Acute isocapnic hypoxia induces blood flow through IPAVA. By directly manipulating the pulmonary pressure response to isocapnic hypoxia with AZ we extend the findings of previous reports and illustrate that the magnitude of blood flow through IPAVA in response to isocapnic hypoxia is unrelated to changes in PASP, $\dot{Q}_c$, or PVR. We suggest that blood flow through IPAVA occurs independent of changes in pulmonary artery pressure and instead may be regulated by alveolar hypoxia. Similar to other reports we found that AZ blunts the PASP response to isocapnic hypoxia (Teppema *et al.*, 2007). However, we extend these findings by illustrating that the correction of arterial pH and [HCO_3^-] induced by AZ by intravenous $NaHCO_3$ infusion partially restored the PASP response to isocapnic hypoxia. These data indicate that oral AZ may reduce the PASP response to hypoxia by reducing arterial/alveolar PCO_2, since the correction of arterial pH and consequently $PaCO_2$ partially normalised the PASP response to hypoxia.

Hypoxia induces blood flow through IPAVA

With the exception of one participant during control, exposure to 20 minutes of isocapnic hypoxia induced blood flow through IPAVA. A unique finding was the absence of bubble scores of larger magnitude (i.e., >3). Employing a similar duration (i.e., 30 minutes) and intensity of hypoxia (i.e., alveolar $PO_2 = 47 \pm 1$ mmHg) as our investigation, Laurie *et al.* (2010) found that 50% of their participants achieved bubble scores greater than or equal to three, whereas only two participants from our study achieved a similar magnitude bubble score. Achieving even greater magnitudes of blood flow through IPAVA, Norris *et al.* (2014) observed bubble passage in all 16 young, healthy participants at $FIO_2 = 0.12$ with 12 of these achieving scores ≥ 4. Unlike the aforementioned studies, we maintained isocapnia throughout the hypoxic exposure to isolate the effects of hypoxia. Carbon dioxide is an important modulator of HPV, with hypocapnia and subsequent alkalosis blunting HPV (Balanos *et al.*, 2003; Ketabchi *et al.*, 2009). Alternatively, hypercapnia without accompanying pH changes may augment HPV (Brimioulle *et al.*, 1990).

Based upon this assessment, poikilocapnic hypoxia may be an important modulator of blood flow through IPAVA. Figure 5 displays the relationship between cardiac output and bubble score for our participants exposed to isocapnic hypoxia and the same relationship for participants exposed to poikilocapnic hypoxia from the study of Laurie *et al.* (2010). Despite a similar range in cardiac output there was no relationship between these two variables in response to isocapnic hypoxia. In summary, IPAVA appear to be recruited by hypoxia, while the use of isocapnic versus poikilocapnic hypoxia may have influenced the magnitude of blood passage through IPAVA. Studies of similar levels of hypoxia may display greater bubble scores due to the influences of poikilocapnia. Future research is needed to directly compare the magnitude of blood flow through IPAVA in response to isocapnic and poikilocapnic hypoxia in the same group of subjects.

Haemodynamic influence on blood flow through IPAVA

We successfully isolated acute isocapnic hypoxia from the consequent HPV by applying AZ to mitigate the rise in PASP during hypoxia. While AZ decreased PASP from 23.6 ± 0.7 to 20.8 ± 0.7 mmHg, it was not accompanied by a change in bubble score. In fact, no haemodynamic measurements correlated with bubble score (see Figure 4). Recent studies support an alveolar hypoxia-mediated recruitment of IPAVA. Bates *et al.* (2014) observed greater 99mTc macroaggregate transit through IPAVA during resting hypoxia compared to exercise at the same level of hypoxia, indicating that increased pulmonary perfusion did not further increase blood flow through IPAVA in hypoxia. Furthermore, Lozo *et al.* (2014) administered nitroglycerin, norepinephrine and aminophylline, to induce changes in $\dot{Q}_c$ and PASP in 10 trained technical divers. Nitroglycerin is a vasodilator that increases $\dot{Q}_c$ at higher doses, norepinephrine increases systemic and pulmonary pressures and aminophylline is a pulmonary vasodilator. None of these pharmacological agents induced blood flow through IPAVA despite increasing PASP from 21.6 ± 0.8 mmHg to 30.3 ± 1.6 mmHg following norepinephrine administration and significantly increasing $\dot{Q}$c from 5.4 ± 0.4 l min^{-1} to 6.0 ± 0.4 l min^{-1} with aminophylline. Further support for our finding of dissociation between haemodynamic parameters and bubble score comes from the absence of blood flow through IPAVA after acclimatization to high altitude. In our recent study comparing sea-level and high-altitude IPAVA responses, we found that blood flow through IPAVA following 3 weeks acclimatisation to 5050 m was undetectable in 7 of our 8 sea-level

dwellers during the same protocol that induced blood flow through IPAVA at sea level, despite elevated PASP at high altitude (33 vs. 20 mmHg) (Foster *et al.*, 2014). Interestingly, $\dot{Q}_c$ at high altitude was similar to normoxic conditions at sea level, indicating that blood flow through IPAVA is likely more closely related to changes in $\dot{Q}_c$ than PASP. This idea is consistent with the findings of Laurie *et al.* (2012) and Bryan *et al.* (2012) who showed that $\dot{Q}_c$ was related to bubble score during dopamine, epinephrine, and dobutamine infusion and more recently by Elliott *et al.* (2014) who demonstrated and increase in IPAVA blood flow with increased $\dot{Q}_c$ independent of increases in PASP. In the current study, the lack of relationship between $\dot{Q}_c$ and bubble score (see Figures 4 and 5) may be linked to isocapnia, as stated previously, but the fact that $\dot{Q}_c$ was similar between all three conditions may have also contributed to the lack of differences in bubble score between conditions. Based on this information, the primary mediator of IPAVA recruitment seems unlikely to be pressure-mediated and the contribution of pulmonary perfusion to blood flow through IPAVA cannot be entirely discounted.

Acetazolamide and Hypoxic Pulmonary Vasoconstriction

Acetazolamide led to a reduction in arterial pH, $[HCO_3^-]$, and $PaCO_2$ which can have a direct effect on pulmonary vessel tone. Alkalosis and hypocapnia are known to blunt HPV, while acidosis and hypercapnia normally augment it (Ketabchi *et al.*, 2009; Sylvester *et al.*, 2012). Our results show that HPV is blunted following oral AZ administration and is partially restored with correction of $[HCO_3^-]$ (see Figure 2). The correction of the PASP response to hypoxia with $[HCO_3^-]$ correction may be due to the different levels of $P_{ET}CO_2$ throughout each exposure as a result of the manipulation of arterial pH. End-tidal carbon dioxide was clamped at baseline levels, thus, $P_{ET}CO_2$ in the AZ condition was clamped at a lower level compared to control and AZ+HCO_3^-. A modest change in $P_{ET}CO_2$ can influence the degree of the pulmonary pressure response in hypoxia (Croft *et al.*, 2013). Thus, the reduced increase in PASP from baseline induced by AZ during hypoxia (2.7 ± 0.7 mmHg with AZ compared to 4.9 ± 1.1 mmHg in control) could be attributed to the 4.2 ± 0.7 mmHg lower $P_{ET}CO_2$ maintained throughout the isocapnic hypoxia protocol. According to Croft *et al.* (2013) this ~5 mmHg difference in $P_{ET}CO_2$ between conditions could account for approximately a 1.7 mmHg difference in PASP when $P_{ET}O_2$ was maintained at 50 mmHg. Although the acid-base status was not controlled in their study, the acute HPV appears to be similar in hypercapnic acidosis and hypercapnia when

pH is maintained at resting levels (Ketabchi *et al.*, 2009). Therefore, the differences we observed in PASP response between control and AZ may be the result of differences in $PaCO_2$, rather than the acid-base status. However, while the majority of participants displayed modest HPV, AZ treatment only partially alleviated the slight rise in PASP. Conversely, one participant had a substantial HPV response to hypoxia (PASP increased by 10.5 mmHg) and had complete abolishment of HPV following 48 hours of AZ administration.

In our study, intravenous $NaHCO_3$ normalised arterial pH, $[HCO_3^-]$ and $PaCO_2$ and partially restored the PASP response to hypoxia. While the mechanism whereby AZ attenuates HPV remains elusive, our data indicates that the reduction in $PaCO_2$, secondary to carbonic anhydrase inhibition, alleviates the rise in PASP. This is supported by a report demonstrating an inverse relationship between the hypoxic ventilatory response and HPV (Albert & Swenson, 2014). An augmented hypoxic ventilatory response would result in a greater reduction in $PaCO_2$ and a weakening of HPV. Additionally, hypocapnia reduces acute HPV in humans (Balanos *et al.*, 2003; Dorrington *et al.*, 2010; Croft *et al.*, 2013). However, recent work in dogs indicates that the reduction in HPV with AZ is independent of any carbonic anhydrase inhibition. Potent membrane-permeable and –impermeable carbonic anhydrase-inhibitors did not reduce the rise in pulmonary artery pressure upon exposure to hypoxia, though AZ did despite similar acid-base status, $PaCO_2$ and $\dot{V}_E$ across each intervention (Hohne *et al.*, 2007). This highlights that the mechanism of AZ on HPV reduction is independent of its known carbonic anhydrase-inhibiting effects in dogs. The exact mechanism of AZ on HPV reduction in humans remains obscure, but our data indicates that it reduces HPV during acute isocapnic hypoxia in a pH or PCO_2 dependent manner.

Finally, PVR increased in AZ+HCO_3^- compared to control and AZ. Such a response must be interpreted carefully. Although pH and bicarbonate levels have been temporarily normalised, AZ is still present in the system and acting on carbonic anhydrase. As such, PaO_2 and $PaCO_2$, while not statistically different from control are still partially elevated and decreased respectively. Perhaps a more important comparison is the relative hypercapnia with AZ+HCO_3^- compared with AZ. Since CO_2 has pulmonary vasoconstrictive effects it may be possible that this has contributed to a greater PVR compared to AZ independent of the normalisation of

[HCO_3^-] and pH. Indeed, respiratory acidosis increases PVR significantly (Fullerton *et al.*, 1993), with our results suggesting relative hypercapnia as the primary mediator.

Limitations

The limitations of using microbubble passage as an index of blood flow through IPAVA has been described extensively (Elliott *et al.*, 2011). Given the size and composition of the bubbles, the only pathway for agitated contrast to appear in the left atrium/ventricle in participants lacking an intracardiac shunt is through IPAVA or pulmonary arteriovenous malformations. It is unlikely that our participants had pulmonary arteriovenous malformations as resting arterial PO_2 and oxyhaemoglobin saturation were in the normal range. In addition, no participants were identified as having left ventricle contrast during our screening at rest.

The degree of HPV as indexed by PASP during hypoxia was smaller than expected. Although it is known to be a highly variable response, we employed similar techniques to Teppema *et al.* (2007) and found nearly a 50% smaller change in PASP in response to a similar isocapnic hypoxia protocol. Several differences between the two studies may contribute to the differences between the studies. Firstly, data were collected at slightly different altitudes; the study by Teppema *et al.* (2007) was performed at an elevation of 1100 m while the present study was conducted at lower altitudes (75 m (n = 3) and 344 m (n = 7)). Consequently, their participants had lower resting SpO_2, though we demonstrated greater desaturation following acute isocapnic hypoxia. Chronic exposure to this mild altitude may blunt peripheral chemosensitivity (Weil *et al.*, 1971), and this is suggested by the reduced resting ventilation seen in the participants tested at 1100 m. The difference in altitude may have contributed to a blunted peripheral chemoreceptor responsiveness and increased HPV (Albert & Swenson, 2014). Secondly, our group of participants did not have intracardiac shunts whereas the status of the participants in the study by Teppema *et al.* (2007) is not known. The presence of an intracardiac shunt could exaggerate arterial hypoxaemia leading to a greater degree of HPV (Levine *et al.*, 1991). Since an intracardiac shunt like a PFO is present in approximately a third of the general population (Elliott *et al.*, 2013; Marriott *et al.*, 2013), our exclusion criteria may have favoured the recruitment of low HPV-responders. Despite this, in the range of PASP measured in this study, we found no relationship between blood flow through IPAVA and PASP, $\dot{Q}_c$, or PVR. In addition, across our three conditions we can also infer that blood flow through IPAVA is not

affected by the humoral manipulations of arterial pH and PCO_2, at least over the range studied in this investigation.

Finally, it must be acknowledged that AZ is a mild diuretic (Kassamali & Sica, 2011). A non-significant increase in Hb (Table 1) supports such an action, which in turn may contribute to the lower PASP observed in the AZ condition. While infusion of $NaHCO_3$ did not exceed 220 ml in any participants, Hb did decrease by nearly 1 g dl^{-1} indicating possible volume expansion. However, the Hb concentration with $NaHCO_3$ was not different compared to control suggesting that pulmonary pressure may be slightly reduced at baseline with AZ as a result of its diuretic effect. We recognize that acute volume loading increases pulmonary pressure. However, a dose of less than 300 ml has minimal effect on pulmonary artery and capillary pressure (Doyle *et al.*, 1951) and our experimental trials did not exceed this value. On the other hand, the effect of increasing volume does not speak to the PASP response to hypoxia. In anesthetized and paralyzed dogs, Benumof and Wahrenbrock (1975) observed an inverse relationship between blood volume and hypoxic pulmonary vasoconstriction in dogs ($r = 0.69$; $p<0.01$). From this association, they suggest that increased blood volume reduces the pulmonary vasocontstrictive response to hypoxia due to increased intravascular pressures. Hence, the small change in fluid volume combined with the relative hypercapnia in our study (see Tables 1 & 2) could have contributed to the betwee-condition differences in pulmonary haemodynamics. To address this potential influence, future studies require an experimental condition with an equimolar sodium challenge with saline. Based on the conclusions of Benumof and Wahrenbrock (1975), the infusion of $NaHCO_3$ may have blunted hypoxic pulmonary vasoconstriction in our study. Therefore, had we accounted for the volume loading during control and AZ, restoration of acid-base status may have led to a complete, rather than partial, restoration of PASP and hypoxic pulmonary vasoconstriction. The haemoglobin changes identified above would lead to a 2% increase in haematocrit. Thus, a change in blood viscosity could contribute to the changes in pulmonary pressure. However, we calculated PVR for a standardized haematocrit of 45% (Faoro *et al.*, 2014). By accounting for this 2% difference in haematocrit we would observe a PVR of 1.38 and 1.40 in control and AZ. Thus, the small differences in haemoglobin have little effect on PVR suggesting that the PASP measurements between the three comparisons are unlikely to be attributable to changes in blood viscosity.

Conclusion

In conclusion, we found that manipulating pulmonary artery pressure during isocapnic hypoxia has no effect on the magnitude of blood flow through IPAVA. Oral dosing of AZ was effective in reducing PASP not only at rest but also during exposure to isocapnic hypoxia. For the first time, we have demonstrated that the reduction in PASP with AZ could be abolished by correction of bicarbonate, indicating that the effects of AZ on the pulmonary vasculature are related to pH or arterial/alveolar PCO_2. PASP did not influence blood flow through IPAVA, suggesting that the stimulus for their recruitment may be in response to acute hypoxia or pulmonary blood flow rather than pressure *per se*. The relatively lower magnitudes of blood flow through IPAVA and increases in PASP we observed compared to previous studies may indicate that there is an additive effect of hypoxic stimuli and elevated pressure in promoting blood passage through IPAVA.

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Additional Information.

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Tables.

Table 1. Resting haematological measurements made prior to each experimental condition (Control; AZ; AZ+HCO_3^-) and following AZ+HCO_3^-.

	Control	AZ	AZ+HCO_3^-	Post AZ+HCO_3^-
n	10	10	10	7
PaO_2, mmHg	89.0 ± 3.9	98.0 ± 2.4	93.9 ± 3.8	92.8 ± 2.9
$PaCO_2$, mmHg	40.3 ± 1.1	35.8 ± 1.0*	37.9 ± 0.8	36.7 ± 0.7
pH	7.43 ± 0.01	7.34 ± 0.01‡	7.43 ± 0.01	7.39 ± 0.01†
[HCO_3^-], mmol l^{-1}	26.0 ± 0.6	18.9 ± 0.4‡	24.7 ± 0.5	22.0 ± 0.6**
[Hb], g dl^{-1}	14.7 ± 0.3	15.4 ± 0.3	14.5 ± 0.3	15.1 ± 0.4

Values are means ± SE. *$p<0.01$ compared to control; $^{†}p<0.01$ compared to AZ and $p<0.05$ compared to AZ+HCO_3^- (n = 7); $^{‡}p<0.001$ compared to control and AZ+HCO_3^-. **$p<0.01$ compared to Control, AZ, and AZ+HCO_3^- (n = 7). Definition of abreviations: AZ = acetazolamide; PaO_2 = arterial partial pressure of oxygen; $PaCO_2$ = arterial partial pressure of carbon dioxide; [HCO_3^-] = concentration of bicarbonate; [Hb] = concentration of haemoglobin.

Table 2. Haemodynamic and cardiopulmonary response during control, AZ and AZ+HCO_3^- during normoxia and hypoxia.

		Control	AZ	AZ+HCO_3^-	Oxygen Level	Condition	Interaction
$P_{ET}O_2$, mmHg	Normoxia	92.9 ± 1.7	101.3 ± 2.0**	99.2 ± 1.6*	<0.001	<0.001	<0.001
	Hypoxia	46. 7 ± 0.7	46.8 ± 0.4	46.7 ± 0.4			
$P_{ET}CO_2$, mmHg	Normoxia	39.7 ± 0.5	35.0 ± 0.7**	36.5 ± 0.4**	<0.05	<0.001	0.200
	Hypoxia	39.9 ± 0.5	35.8 ± 0.7	36.9 ± 0.4			
$\dot{V}_E$, l·min^{-1}	Normoxia	11.9 ± 0.6	13.0 ± 0.6	12.7 ± 0.5	<0.05	0.971	0.341
	Hypoxia	21.2 ± 3.9	19.5 ± 2.5	20.1 ± 1.9			
SpO_2, %	Normoxia	97.7 ± 0.3	97.7 ± 0.4	98.1 ± 0.3	<0.001	0.566	0.501
	Hypoxia	80.4 ± 1.8	78.8 ± 1.1	79.5 ± 1.3			
HR, beats·min^{-1}	Normoxia	57 ± 2	55 ± 3	56 ± 3	<0.001	0.0596	0.609
	Hypoxia	68 ± 3	63 ± 4	67 ± 4			
SV, ml	Normoxia	77.9 ± 3.7	77.1 ± 5.0	76.6 ± 3.9	0.962	0.493	0.660
	Hypoxia	82.3 ± 4.2	74.7 ± 5.5	74.2 ± 4.2			
$\dot{Q}_c$, l·min^{-1}	Normoxia	4.0 ± 0.2	3.9 ± 0.3	4.2 ± 0.2	<0.001	0.700	0.565
	Hypoxia	5.2 ± 0.4	4.8 ± 0.4	4.8 ± 0.3			
MAP, mmHg	Normoxia	86 ± 2	84 ± 2	86 ± 2	<0.05	0.796	0.644
	Hypoxia	90 ± 3	91 ± 5	93 ± 4			
PVR, wu	Normoxia	1.35 ± 0.04	1.43 ± 0.05	1.62 ± 0.08†	0.065	<0.01	0.449
	Hypoxia	1.53 ± 0.06	1.48 ± 0.04	1.66 ± 0.06			

Values are means ± SE. *p<0.05 compared to control; **p<0.001 compared to control; †p<0.01 compared to AZ and control. The oxygen level, condition and interaction columns display the p-value for the effect of condition (control, AZ, AZ+HCO_3^-) and the effect of oxygen level (normoxia, hypoxia). Definition of abbreviations: AZ = acetazolamide; HR = heart rate; MAP = mean arterial pressure; $P_{ET}O_2$ = end-tidal partial pressure of oxygen; $P_{ET}CO_2$ = end-tidal partial pressure of carbon dioxide; PVR = pulmonary vascular resistance; SV = stroke volume; $\dot{Q}_c$ = cardiac output; $\dot{V}_E$ = minute ventilation

Figures and legends

Figure 1. End-tidal gases ($P_{ET}O_2$ and $P_{ET}CO_2$) and minute ventilation ($\dot{V}_E$) at baseline and throughout 20 min of isocapnic hypoxia for all participants (n = 10). Data points are 15 s mean ± SE. Definition of abbreviations: $P_{ET}O_2$: end-tidal partial pressure of oxygen; $P_{ET}CO_2$: end-tidal partial pressure of carbon dioxide; Control, isocapnic hypoxia intervention; AZ, acetazolamide intervention, AZ+HCO_3^-: bicarbonate correction intervention.

Figure 2. Individual and group mean pulmonary artery systolic pressure (PASP) during normoxia and isocapnic hypoxia during (A) control, (B) acetazolamide (AZ) and (C) AZ and intravenous $NaHCO_3$ (AZ+HCO_3^-). A significant main effect of hypoxia ($p<0.001$) and condition ($p<0.01$) were found. For the condition interaction, *post hoc* analysis revealed a difference between the control and AZ conditions. Means ± SE of each condition are provided above the x-axis. * = $p<0.001$, hypoxia compared to normoxia; † = $p<0.001$AZ compared to control. Control, isocapnic hypoxia intervention; AZ, acetazolamide intervention, AZ+HCO_3^-: bicarbonate correction intervention; PASP, pulmonary artery systolic pressure.

Figure 3. Bubble scores during the isocapnic hypoxia trial in each condition (n = 10). Each data point represents a participant with their corresponding bubble score on the y-axis. There were no significant differences between the conditions ($p>0.05$). Control, isocapnic hypoxia intervention; AZ, acetazolamide intervention; AZ+HCO_3^-, bicarbonate correction intervention.

Figure 4. Relationship between bubble score and (A), PASP (mmHg), (B), $\dot{Q}_c$ ($l \cdot min^{-1}$) and (C), PVR (wu) during hypoxia in each condition. Multiple linear regression revealed no significant linear relationships (R^2 = 0.008, 0.08 and 0.0006 for (A), (B), and (C) respectively; all $p>0.05$). Control, isocapnic hypoxia intervention; AZ, acetazolamide intervention, AZ+HCO_3^-: bicarbonate correction intervention; PASP, pulmonary artery systolic pressure; PVR, pulmonary vascular resistance; $\dot{Q}_c$, cardiac output.

Figure 5. Effect of isocapnic versus poikilocapnic hypoxia on the relationship between cardiac output ($\dot{Q}_c$) and bubble score. Bubble scores and $\dot{Q}_c$ obtained during the last minute of exposure

to 20 minutes of isocapnic hypoxia from the current study (n = 10) and poikilocapnic hypoxia from participants in the study by Laurie *et al.* (2010) (n = 12) breathing 12% O_2 for 30 minutes.