GAS-FILLED MICROBUBBLES are highly echogenic and are readily observed via Doppler ultrasound because of their acoustic characteristics and capacity to enhance the backscattered signal (7). Saline contrast echocardiography has been used clinically for over 40 years (12) and takes advantage of the echogenic property of air-filled microbubbles to detect and diagnose intracardiac shunt (54). This anatomically based technique not only detects intracardiac right-to-left shunt but, because of the physical properties governing in vivo microbubble dynamics (4, 6, 8, 10, 16–18, 33, 34, 37, 38, 40, 44–46, 52, 55–58), can also identify large-diameter intrapulmonary arteriovenous (IPAV) anastomoses (3, 15, 41), which provide a breach in the “sieve” action of the pulmonary microcirculation (5, 34). Thus the appearance of saline contrast microbubbles in the left side of the heart in less than three heartbeats after saline contrast microbubbles fill the right side of the heart is indicative of an intracardiac shunt. However, the appearance of microbubbles in the left heart after a delay of greater than three heartbeats is indicative of an IPAV right-to-left shunt.

Critics of these findings have suggested that the in vivo partial pressure changes that result from breathing a differing inspired O₂ fraction (FIO₂) may explain the presence or absence of microbubbles in the left heart when room air is used as the gaseous component of the saline contrast microbubbles (19). They suggest that when breathing low or high levels of oxygen, the resulting external partial pressures interacting with the microbubbles will affect their size and/or life span. Accordingly, this could cause these room air microbubbles to either grow in size, becoming larger than patent IPAV anastomoses and thus get filtered out, or shrink in size and dissolve before reaching the left heart, regardless of the patency of IPAV anastomoses. In either case this would result in the absence of microbubbles in the left heart due to a change in microbubble dynamics and not due to the opening or closing of IPAV anastomoses as we have proposed (25).

The theoretical basis for this critique is that when breathing 100% O₂ the partial pressure of nitrogen (Pn₂) within the blood and tissues is significantly reduced compared with breathing room air or a hypoxic gas mixture (14). Therefore, the N₂ within room air microbubbles could very quickly diffuse out of the microbubbles and in this way they could dissolve down to...
Finally, during exercise in hyperoxia, saline contrast microbubbles created from room air do not appear in the left heart (28). The question, again, is whether or not this is due to either the altered in vivo external partial pressures creating a large “sink” for diffusion of N₂ out of the room air microbubbles or the closure of IPAV anastomoses. Following this logic, in addition to room air microbubbles, those created from 100% N₂, 100% CO₂, and 100% He should also rapidly dissolve and not be visualized in the left heart. However, microbubbles created from 100% O₂, where the diffusion gradient is essentially nonexistent, could survive long enough to traverse through patent IPAV anastomoses and be visualized in the left heart.

In summary, the in vivo dynamics of saline contrast microbubbles in subjects breathing normoxic, hypoxic, and hyperoxic gas mixtures is purely hypothetical at this time. Accordingly, we set out to definitively determine whether changing the partial pressures outside the microbubbles and/or the microbubbles’ internal gas composition impacts the detection of these microbubbles traveling through IPAV anastomoses in the left heart. We hypothesized that during submaximal exercise [60% peak O₂ consumption (V̇O₂peak)] the amount of saline contrast microbubbles traversing the pulmonary vasculature would remain the same for any FIO₂ (1.0, 0.21, 0.14), despite the use of microbubbles made with different gases.

METHODS

This study received approval from the University of Oregon Committee for the Protection of Human Subjects Biomedical Institutional Review Board, and each subject provided verbal and written informed consent before participation. All studies were performed according to the Declaration of Helsinki.

Subjects. Twenty-two healthy, nonsmoking volunteers (10 men, 12 women) aged 22–38 yr with no history of cardiopulmonary disease were recruited, and, after written informed consent was given, agreed to proceed with the study. All subjects completed a medical history and physical activity questionnaire before proceeding.

Cardiac screening. A registered diagnostic cardiac sonographer with >20 yr of experience conducted all ultrasound imaging (Philips Sonos 7500). An echocardiographic screening was performed to rule out any cardiac abnormalities including right ventricular outflow tract obstruction and pulmonary artery or aortic stenosis. Left ventricular function, all valves, great vessels, and the pericardium appeared normal. There were no signs of ischemia or congenital heart disease, except for one male subject with a bicuspid aortic valve, who was excluded from participation. Four subjects tested positive for a Val-salva-induced patent foramen ovale (1 man, 3 women) and were excluded from participation. Additionally, a non-Val-salva-dependent patent foramen ovale was discovered in one female subject, who was also excluded from participation. Four subjects (2 men, 2 women) had an echocardiogram suggestive of an intrapulmonary right-to-left shunt because of one or more microbubbles appearing in the left ventricle more than five heartbeats after opacification of the right ventricle at rest breathing room air. These subjects were excluded from participation. The remaining 12 subjects (6 men, 6 women) completed the study in its entirety.

Pulmonary function testing. Baseline pulmonary function testing included measures of forced vital capacity (FVC), forced expiratory volume in 1 s (FEV₁), and forced midexpiratory flows (FEF₂₅₋₇₅). Measurements were made with a computerized spirometry system (Ultima PFX, MedGraphics, St. Paul, MN) according to American Thoracic Society/European Respiratory Society (ATS/ERS) standards (35). Total lung capacity (TLC) was determined with whole body plethysmography (MedGraphics 1085D Series Plethysmograph) ac-

~20% of their original size (16, 17, 45, 57). Subsequently, other influences (e.g., surface tension, pressure, and flows) could further promote the rapid dissolution of microbubbles before they reach IPAV anastomoses. Thus the failure to see microbubbles appear in the left heart during exercise while subjects are breathing 100% O₂ could be due to microbubble disappearance rather than closure of IPAV anastomoses. However, these theoretical arguments have not been directly tested.

We designed a series of experiments in which we changed the internal gas composition as well as the external partial pressures interacting with the saline contrast microbubbles to test the validity of these critiques. The present study changed the FIO₂ with normoxic, hypoxic (FIO₂ = 0.14), or hyperoxic (FIO₂ = 1.0) gas mixtures to alter the partial pressures outside the microbubbles. Saline contrast microbubbles were created from five different gases (room air, 100% N₂, 100% O₂, 100% CO₂, and 100% He) by two separate methods to alter the microbubbles’ internal gas composition. Microbubbles created from gases other than room air have a different internal gas composition and, because of this, would possess different degrees of solubility and diffusivity and therefore should exhibit different life spans when exposed to different external partial pressures. In addition to FIO₂, microbubbles will encounter differing external partial pressures as they travel through the pulmonary circulation, from the partial pressures of gases within the mixed venous blood to the alveolar partial pressures of gases in the pulmonary veins. Thus the changes we created within the microbubbles’ internal and external partial pressures should produce results that would either refute or support our previous work. The possible outcomes for the three different exercise bouts, and therefore changes in the partial pressures surrounding the microbubbles, are described below.

During exercise in normoxia, saline contrast microbubbles created from room air have been used to demonstrate the opening of large-diameter IPAV anastomoses (9). However, if the microbubbles were instead created from 100% O₂, 100% CO₂, or 100% He they should rapidly dissolve because of the very large diffusion gradient favoring microbubble dissolution and not be visualized within the left heart. In contrast, microbubbles created from 100% N₂ would behave very similar to room air microbubbles because they would have very similar diffusion gradients and therefore would be visualized within the left heart to the same degree as microbubbles from room air.

During exercise in hypoxia, saline contrast microbubbles created from room air appear in the left heart at lower workloads compared with exercise in normoxia (27). However, if this was simply a result of the lower FIO₂ altering the partial pressures within the blood and tissues and subsequently stabilizing the room air microbubbles, then changing the gas composition of the microbubbles to 100% O₂, 100% CO₂, or 100% He (in which the altered in vivo external partial pressures would have little to no impact) should produce results different from those with room air microbubbles. Specifically, these microbubble gas compositions should rapidly dissolve and not be visualized in the left heart, whereas microbubbles created from room air and 100% N₂ should have a smaller diffusion gradient and thus maintain a life span long enough to be visualized in the left heart.
According to ATS/ERS standards (50). Lung diffusion capacity for carbon monoxide (DL_{CO}) was determined by the single-breath, breath-hold method according to ATS/ERS standards (30). The Jones and Meade method was used for timing and alveolar sample collection (MedGraphics Ultima PFX, Breeze v.6.3.006). Predicted values for pulmonary function and DL_{CO} were calculated as previously described (21, 22).

\( \text{V}_{\text{O}}\text{2peak testing} \). Subjects completed a progressive incremental exercise test to exhaustion in normoxia on a mechanically braked cycle ergometer (Lode Excalibur Sport). Each subject completed a 5- to 10-min warm-up at a self-selected workload. Individualized protocols were developed with the goal for all subjects to reach exhaustion between 9 and 14 min. These protocols started between 60 and 90 W and increased each minute by a constant workload of 15–30 W, depending on the subject. During the exercise protocol, subjects breathed through a low-resistance two-way nonrebreathing mouth-piece (model 2400, Hans Rudolph, Kansas City, MO), and pneumotachograph (MedGraphics PreVent). Although subjects were encouraged to continue until volitional exhaustion, a satisfactory test was determined if respiratory exchange ratio (RER) was \( >1.10 \), there was a plateau in oxygen consumption (\( \text{V}_{\text{O}}\text{2} \)), or a heart rate (HR) near the age-predicted maximum was achieved (1). Sixty percent of the wattage-current of the right atrium and ventricle (12, 40). Without right-to-left shunting, and because pulmonary capillaries never exceed 13 \( \mu \text{m} \) even when exposed to unphysiological pressures up to 100 \( \text{cmH}_{2}\text{O} \), saline contrast microbubbles larger than \( >12 \mu \text{m} \) should not appear in the left heart. Thus right-to-left shunting will only further accelerate the time to microbubble dissolution (44, 57, 58). For these reasons, small-diameter microbubbles are filtered out (5, 34). Assuming a surface tension of 50 dyn/cm \( ^{-1} \), a \( 8-\mu \text{m} \) microbubble should dissolve in 190–550 ms in static fluid, an 8-\( \mu \text{m} \) microbubble should dissolve in 190–550 ms in static fluid (34, 56, 57). Considering that the mean pulmonary capillary transit time from rest to maximal exercise (30 l/min) ranges from 750 to 450 ms, respectively, the significantly increased pressures and flows will only further accelerate the time to microbubble dissolution (44, 57, 58). For these reasons, small-diameter microbubbles rapidly collapse, while the sieve action of the pulmonary microcirculation and are filtered out (5, 34). Assuming a surface tension of 50 dyn/cm \( ^{-1} \) within whole blood (49), microbubbles of this size or smaller are subject to a very large pressure of at least 150,000 dyn/cm \( ^{-2} \) as indicated by the Laplace equation, making their time to dissolution very rapid. Depending on the degree of saturation of the surrounding fluid, an 8-\( \mu \text{m} \) microbubble should dissolve in 190–550 ms in static blood (34, 56, 57). Considering that the mean pulmonary capillary transit time from rest to maximal exercise (30 l/min) ranges from 750 to 450 ms, respectively, the significantly increased pressures and flows will only further accelerate the time to microbubble dissolution (44, 57, 58). For these reasons, small-diameter microbubbles rapidly collapse, while the sieve action of the pulmonary capillary bed filters out large-diameter microbubbles such that in the absence of any right-to-left shunt no microbubbles appear in the left heart. Thus observing the appearance of left-sided contrast following a peripheral hand-agitated saline contrast injection after a delay of three to five cardiac cycles (41, 54) during exercise demands that the microbubbles be significantly larger than pulmonary capillaries, and therefore must traverse the pulmonary circulation via large-diameter IPAV anastomoses (4, 5, 17, 18, 31, 33, 34, 40, 52, 57). The limitations with saline contrast echocardiography have previously been discussed in detail (9, 27).

It has been estimated that in order for a microbubble to remain stable enough, to survive long enough, to reach the left side of the heart, the minimum diameter necessary would be between 60 and 90 \( \mu \text{m} \) (9). Using saline contrast echocardiography, we cannot determine the range of microbubble diameters as they leave the syringe, traverse the pulmonary circulation, and ultimately end up in the left side of the heart. As stated above, based on the physical principles governing microbubble behavior, the microbubbles in the left heart would be expected to be \( >60 \mu \text{m} \). Furthermore, saline contrast echocardiography is generally accepted as the most sensitive noninvasive technique for detecting both intracardiac and IPAV shunt (53). Observation of even a single microbubble in the left heart in a subject without an intracardiac shunt has previously been defined as pathological shunt (34). Accordingly, this anatomically based technique allows us to determine the patency of these IPAV anastomoses.

For these reasons, we defined IPAV shunting as the appearance of saline contrast on the left side of the heart after a minimum of three cardiac cycles from the initial opacification of the right heart. After each injection, the subsequent 20 cardiac cycles immediately after opacification of the right atrium and ventricle are recorded at \( \geq 30 \) frames/s. The single frame in this recording with the maximum number of microbubbles present is used to assign a bubble score for that particular saline contrast injection. The previously published scoring system (23, 28) is based on a 0–5 scale using both the density and spatial distribution of microbubbles in the left ventricle. One bubble score was assigned for each contrast injection in the following manner: 0 = 0 microbubbles; 1 = 1–3 microbubbles; 2 = 4–12 microbubbles; 3 = more than 12 microbubbles in a bolus; 4 = more than 12 microbubbles heterogeneously distributed throughout the left ventricle; 5 = more than 12 microbubbles homogeneously distributed throughout the left ventricle.

This study employed the use of five different gases to create saline contrast microbubbles: room air, two inert gases (100% \( \text{N}_2 \) and 100% \( \text{He} \)), and two biologically active gases (100% \( \text{O}_2 \) and 100% \( \text{CO}_2 \)). Every gas, with the exception of room air, was medical grade (USP) and stored in a nondiffusing collection bag (series 6000, Hans Rudolph) during the study. Using two three-way stopcocks attached to the bag in series, we flushed the syringe and drew 10 ml of gas from each bag just before its use in creating the contrast microbubbles. In this way we avoided contaminating the purity of the gas within each collection bag during the study. One milliliter of pure gas was withdrawn from these bags and agitated with three milliliters of saline and stored in a nondiffusing collection bag (series 6000, Hans Rudolph) through a 22-gauge needle. Another modified solution set was connected to this bag. After attaching the syringe containing 10 ml of gas to the stopcock attached to the subject’s IV line, we reduced the volume down to 1 ml by expunging it through a stopcock into the room air before agitation with the 3 ml of saline.

Within each \( \text{F}_{\text{IO}} \), the order of the gas composition used for each saline contrast injection (room air, 100% \( \text{N}_2 \), 100% \( \text{O}_2 \), 100% \( \text{CO}_2 \), and 100% \( \text{He} \)) was exactly the same for all subjects. The decision not to randomize the order of saline contrast injections, but to perform the different exercise bouts in a random and balanced fashion, was made because there is no evidence that prior serial injections would influence any measurements taken during subsequent injections (9, 23, 27). Furthermore, the findings that exercise and \( \text{F}_{\text{IO}} \) are modulators of IPAV shunting have already been established, making it more important to know when each injection occurred for all subjects than to randomize the order of the injections.

Validation of microbubble gas composition. Microbubbles were formulated by two different methods. Method 1 was used in 12 subjects, for whom the different gases were stored in nondiffusing collection bags during the study. One milliliter of pure gas was withdrawn from these bags and agitated with three milliliters of normal saline to produce each saline contrast microbubble injection. Method 2 was an alternative method completed in 6 of the 12 subjects. In this method we equilibrated saline with each different gas for 1 h. Individual saline bags were equipped with a standard IV solution set that was then connected to the appropriate gas tank. Gas flowed through the solution set to the bottom of the saline bag, where it bubbled through the saline and pressurized the bag. Saline bags were relieved of pressure by puncturing the saline bag near the top with a 22-gauge needle. Another modified solution set was connected to this bag.
needle, which channeled the outflow gas into a beaker of water, such that there was no possibility for retrograde flow of room air. Subsequently, we combined 1 ml of pure gas from the nondiffusing bags with 3 ml of gas-equilibrated saline, and therefore eliminated the possibility for N2 or other gases to diffuse out of the saline and into the microbubbles during the agituation process.

We confirmed that the microbubbles created contained the desired gas composition by measuring the partial pressures immediately after the agituation process. To test this we performed standard saline contrast agitations using 1 ml of 100% N2, 100% O2, 100% CO2, or 100% He combined with 3 ml of either normal saline (method 1) or gas-equilibrated saline (method 2) and subsequently measured (in triplicate) the Po2 within each syringe with a tonometry corrected blood gas analyzer (Siemens RAPIDLAB 248 Blood Gas Analyzer). For method 1, with 1 ml of 100% O2 combined with normal saline the Po2 in the syringe was >749 Torr. With 1 ml of 100% N2, 100% CO2, or 100% He combined with normal saline the Po2 in the syringes was <60 Torr. For method 2, with 1 ml of 100% O2 combined with gas-equilibrated saline the Po2 in the syringe was >749 Torr. With 1 ml of 100% N2, 100% CO2, or 100% He combined with gas-equilibrated saline the Po2 in the syringes was <20 Torr.

Exercise protocol. Subjects performed three 11-min bouts of submaximal exercise (60% VO2peak) breathing room air (FIO2 = 0.21), a hypoxic gas mixture (FIO2 = 0.14), and a hyperoxic gas mixture (FIO2 = 1.0), in a random and balanced order. Each exercise bout was separated by a 15-min normoxic break. Before each exercise bout, subjects breathed normoxia or hypoxia for 7–10 min and hypoxia for at least 10 min while resting on the cycle ergometer. For all exercise bouts subjects breathed through a low-resistance, two-way nonrebreathing valve (model 2400, Hans Rudolph). At the end of each resting stabilization period, a saline contrast injection using room air was performed. Subsequent saline contrast injections created with each of the various gases were made at 2 min (room air), 4 min (100% N2), 6 min (100% O2), 8 min (100% CO2), and 10 min (100% He) during the exercise protocol. Metabolic rate was determined at rest and during exercise in hypoxia further increased the amount of contrast traversing the pulmonary vasculature compared with normoxic exercise (27). Changing the composition of gas used for each saline contrast injection did not alter the observed bubble score throughout the exercise bout (Fig. 1A). Bubble scores during normoxic exercise ranged from 2 to 4, with no significant difference between the different microbubble gas compositions (Fig. 2A). During the exercise bout metabolic data remained relatively stable, with only HR being significantly elevated at the fourth and fifth injections compared with the first injection.

Exercise in hypoxia: method 1. In support of previous work, exercise in normoxia induced shunting of saline contrast via the pulmonary vasculature (9, 27, 42, 43). Changing the composition of gas used for each saline contrast injection did not alter the observed bubble score throughout the exercise bout (Fig. 1B). Bubble scores during exercise ranged from 2 to 5, with no statistical difference between the different microbubble gas compositions (Fig. 2B). During the exercise bout metabolic data remained relatively stable, with only HR being significantly elevated at the third, fourth, and fifth injections, V˙E being significantly elevated at the fourth and fifth injections, and VO2 being significantly elevated at the fifth injection compared with the first injection.

Table 1. Anthropometric, pulmonary function, and VO2peak data

<table>
<thead>
<tr>
<th></th>
<th>Men (n = 6)</th>
<th>Women (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>27.5 ± 6.8</td>
<td>22.2 ± 1.6</td>
</tr>
<tr>
<td>Height, cm</td>
<td>179.2 ± 4.9</td>
<td>160.8 ± 5.9</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>85.7 ± 14.1</td>
<td>60.2 ± 5.6</td>
</tr>
<tr>
<td>FVC, l</td>
<td>5.5 ± 1.0 (97.6 ± 10.5)</td>
<td>4.1 ± 0.5 (106.1 ± 10.7)</td>
</tr>
<tr>
<td>FEV1, l</td>
<td>4.4 ± 0.6 (95.8 ± 6.3)</td>
<td>3.5 ± 0.6 (104.5 ± 15.4)</td>
</tr>
<tr>
<td>FEV1/FVC</td>
<td>0.81 ± 0.05 (98.3 ± 5.7)</td>
<td>0.85 ± 0.05 (97.8 ± 6.2)</td>
</tr>
<tr>
<td>FEF25-75, l/s</td>
<td>4.3 ± 0.9 (90.8 ± 14.8)</td>
<td>3.9 ± 1.0 (103.1 ± 26.6)</td>
</tr>
<tr>
<td>DLCO, ml·min⁻¹·Torr⁻¹</td>
<td>40.5 ± 3.8 (114.5 ± 5.1)</td>
<td>27.8 ± 3.8 (105.7 ± 5.5)</td>
</tr>
<tr>
<td>TLC, l</td>
<td>7.1 ± 1.1</td>
<td>5.4 ± 0.9</td>
</tr>
<tr>
<td>Power at V02peak, W</td>
<td>347 ± 53</td>
<td>223 ± 41.7</td>
</tr>
</tbody>
</table>

Values are means ± SD. Values in parentheses are % predicted. VO2peak, peak oxygen uptake; FVC, forced vital capacity; FEV1, forced expiratory volume in 1 s; FEF25-75, forced expiratory flow of midexpiratory volume; DLCO, diffusion capacity for carbon monoxide; TLC, total lung capacity; power at VO2peak, watts reached at peak oxygen uptake.
Exercise in hypoxia: method 1. In support of previous work, exercise in hypoxia reduced or eliminated the amount of contrast traversing the pulmonary vasculature compared with normoxic exercise (28). Changing the composition of gas used for each saline contrast injection did not alter the observed bubble score throughout the exercise bout (Fig. 1C). Bubble scores during exercise ranged from 0 to 1, with no statistical difference between the microbubble gas compositions (Fig. 2C). During the exercise bout metabolic data remained stable, with no significant change throughout the exercise bout (Table 2). Because of the high FIO2 (1.0) we were unable to acquire Ve, Vo2, or RER.

Data obtained with method 2. There were no significant differences in bubble scores compared with the data obtained by method 1 for any microbubble gas composition during exercise in normoxia, hypoxia, and hyperoxia (Table 3).

DISCUSSION

The purpose of this study was to determine whether room air is a viable gaseous component for use with saline contrast echocardiography to accurately detect the transpulmonary passage of saline contrast microbubbles regardless of FIO2. Bubble scores were not significantly different among the five different gases used to create the microbubbles within each FIO2, with both methods 1 and 2 in the present study. This suggests that changing the FIO2 does not appreciably alter microbubble life span in vivo but does affect the patency of IPAV anastomoses. Thus previous work examining exercise-induced IPAV shunt in subjects breathing normoxia (26, 42), hypoxygen (9, 27, 43), and hyperoxia (28) remains sound, because altered in vivo partial pressures do not explain differences in the appearance of microbubbles within the left ventricle.

Cardiopulmonary changes during exercise in normoxia, hypoxia, and hyperoxia. Each subject’s VO2peak was determined in normoxia and was used for each different FIO2 during the study. When subjects were breathing normoxia 60% represented a submaximal workload, and as such subjects were able to reach a steady state fairly quickly. Minimal changes were observed, except for a significantly elevated HR at the eighth and tenth minutes of exercise compared with the second minute of exercise (Table 2). This gradual increase in HR is typical of the “cardiovascular drift” associated with prolonged exercise (20). When subjects are breathing hypoxia, 60% would be a greater relative workload compared with normoxia and possibly above the lactate threshold in some subjects. For this reason we observed significantly elevated HR, Ve, and Vo2 during exercise in hypoxia (Table 2). These changes were within normal limits of the cardiorespiratory responses to exercise in hypoxia (27, 51). Indeed, Ve was greatest during hypoxia, and significantly different at the eighth and tenth minutes of exercise, corresponding to the 100% CO2 and 100% He injection, respectively. Furthermore, it is unlikely that the 100% CO2 injection caused any transient increase in Ve because ventilation was ~5 l/min greater after the injection of the bubbles made with the physiologically inert gas He.

In vivo microbubble dynamics. The majority of research concerning the behavior of gas microbubbles in the human body has stemmed from interest in decompression sickness (4, 18), iatrogenic events following surgery (2, 56), and the desire to produce a quantifiable contrast agent capable of traversing the pulmonary microvasculature (13, 38, 39). Estimations of microbubble behavior, especially in vivo, are limited to mathematical relationships and are largely constrained to extravascular fluids. Epstein and Plesset developed one of the first models (10), which approximates microbubble behavior in a quiescent aqueous solution, thereby neglecting the convective forces acting on the gas concentrations at the microbubble boundary. Intravascular microbubbles, such as those produced through hand-agitated saline contrast echocardiography, are assumed to follow the predictions of mass transfer between a gas phase and a moving liquid phase (17). As such they encounter a variety of additional influences, all of which lead toward their rapid and complete dissolution (44, 57).
In general, influences from surface tension and gas diffusion cause microbubbles to grow, shrink, or assume a quasi-equilibrium state (8, 18, 37, 38, 40, 52). The relative magnitudes that these influences have on the rate of microbubble dissolution depend largely on the microbubble's diameter as well as the partial pressures within and outside the microbubble. Relative to microbubble size, large-diameter microbubbles are primarily affected by gas diffusion while smaller microbubbles are predominantly affected by surface tension as defined by the Laplace equation. However, all intravascular microbubbles are affected by the aforementioned factors simultaneously, albeit to different relative degrees depending on the microbubble’s diameter and differences between the microbubble’s internal gas composition and the external partial pressures. It should be noted that the influence from surface tension always leads toward microbubble dissolution, while the effect of gas diffu-

Fig. 1. Representative 4-chamber apical echocardiogram images from a single female subject for each saline contrast injection. A: normoxia [inspired O₂ fraction (FIO₂) = 0.21]. B: hypoxia (FIO₂ = 0.14). C: hyperoxia (FIO₂ = 1.0). Pre-Ex, rest; Ex, exercise; Rm Air, room air; RV, right ventricle; LV, left ventricle; RA, right atrium; LA, left atrium. The number corresponding to each echocardiogram image represents the bubble score for that saline-contrast injection.
sion on microbubble size will depend on the partial pressure difference between the microbubble’s internal gas composition and the external partial pressures. Currently, even the best-known mathematical descriptors of gas microbubble behavior, such as the Rayleigh-Plesset equation (see Ref. 37), do not take into account the rheology of non-Newtonian human blood or potential “contaminates” acting as a surfactant on the microbubble surface and thus decreasing the rate of microbubble dissolution (16, 31). As a result of these and potentially other factors, the expected behavior of gas microbubbles in vivo may depart from theoretical predictions (24). Furthermore, changes in the microbubble surface film and its effect on microbubble diffusion properties are poorly understood but are likely to change as the microbubble diameter changes, the geometry of the surrounding environment changes, and/or the partial pressures outside the microbubble change (17, 24, 31).

Therefore, in order to examine whether the theoretical models of gas-filled microbubble dynamics hold true in vivo, this study utilized five different gases (room air, 100% N₂, 100% O₂, 100% CO₂, and 100% He) and two methods to produce saline contrast microbubbles. Additionally, we manipulated the in vivo partial pressures during exercise by changing the FIO₂. Therefore, during each exercise bout the external partial pressures were held constant, while we manipulated the microbubble’s internal gas composition. According to the theoretical models, the internal gas composition of the microbubble that most closely resembled the partial pressures within the blood and tissue would have been expected to survive the longest because of the minimization of the diffusion gradient.

**Theoretical microbubble stability and diffusion gradients in varying FIO₂.** We theorized that during exercise in normoxia microbubbles created from room air most closely resembled the partial pressures within the blood and tissue and therefore would be expected to survive the longest. Similarly, microbubbles created from 100% N₂ closely resembled room air microbubbles with PN₂ values of ~700 Torr and ~550 Torr, respectively. Alternatively, microbubbles created from 100% O₂, 100% CO₂, and 100% He injected into the venous blood in subjects breathing room air would all have much greater diffusion gradients and therefore were expected to have a shorter survival time. If this had been observed, these microbubbles would not have been visualized in either the right or the left heart even if IPAV anastomoses were inducible and patent when subjects were breathing normoxia. However, all five microbubble gas compositions were visualized within both the right and the left heart, and there was no difference in bubble score among the five different microbubble gas compositions during exercise in normoxia.

We theorized that during exercise in hypoxia, microbubbles created from either room air or 100% N₂ most closely resembled the partial pressures within the blood and tissue and therefore should have behaved similarly and survived the longest. Microbubbles created from 100% O₂, 100% CO₂, and 100% He were expected to survive for the shortest duration because of their large diffusion gradients. For this reason, these microbubbles should not have been visualized in either the right or the left heart even if IPAV anastomoses were inducible and patent when subjects were breathing hypoxia. However, all...
five microbubble gas compositions were visualized within both the right and left heart, and there was no difference in bubble score among the five different microbubble gas compositions during exercise in hypoxia.

We theorized that during hyperoxic exercise microbubbles created from 100% O₂ most closely resembled the partial pressures within the blood and tissue and therefore would have been expected to survive the longest. Thus, if IPAV anastomoses were patent during hyperoxic exercise, the 100% O₂ microbubbles would have the greatest probability of surviving long enough to traverse the pulmonary circulation and would have been visualized in the left heart, even if microbubbles created from other gases dissolved before entry into the left heart. Microbubbles created from room air, 100% N₂, 100% CO₂, and 100% He would have been expected to survive for very short durations because of their large diffusion gradients favoring microbubble dissolution. Despite these possibilities, we observed no difference in left-sided contrast across all microbubble gas compositions, again suggesting that IPAV anastomoses are closed during hyperoxic exercise.

Experimental observations of microbubble stability and diffusion gradients in varying FIO₂. There are several reasons why our experimental results deviated from predicted mathematical models. The explanations for these differences are outlined in detail below.

The first possible explanation is that during the agitation process between normal saline and a non-N₂ gas, N₂ could diffuse out of the saline and into these microbubbles so that microbubbles containing N₂ would be produced. As such, these microbubbles would behave similarly to the 100% N₂ and room air microbubbles despite differences in the initial gas compositions. However, method 2 used gas-equilibrated saline combined with the corresponding gas to ensure that microbubbles were pure in composition and without N₂ contamination from the saline. Both methods produced results that were not significantly different, eliminating this as a significant concern.

The second possible explanation is that changes in the external partial pressures could cause microbubbles to rapidly dissolve and never reach the right heart. However, the appearance and persistence of saline contrast microbubbles within the right atrium and ventricle after a peripheral venous injection provide important insights that help clarify and refute this concern. Each and every microbubble injection consistently appeared and remained unchanged within the right side of the heart during the subsequent 20 cardiac cycles after their initial appearance. For example, when subjects breathe 100% O₂ the PN₂ within the venous and arterial blood is reduced compared with breathing room air or hypoxia (14), yet despite the maximized diffusion gradient for microbubbles created from 100% N₂ these microbubbles were visualized in the right heart throughout the 20 cardiac cycles following their initial appearance. During exercise at 60% of V˙O₂peak, subject HRs were between 150 and 170 beats/min, meaning that microbubbles are persisting within the right side of the heart for roughly 6–8 s in duration, regardless of the FIO₂ breathed. This is important because it indicates that microbubbles with both large and small diffusion gradients do not immediately dissolve into the surrounding mixed venous blood and therefore they do in fact survive long enough to reach the left side of the heart.

The third possible explanation is that when subjects breathe any FIO₂, N₂ could diffuse out of the venous blood and into the

Table 3. Comparison of bubble scores for each subject between method 1 and method 2 to produce each saline contrast injection

<table>
<thead>
<tr>
<th>Subject 1</th>
<th>Subject 2</th>
<th>Subject 3</th>
<th>Subject 4</th>
<th>Subject 5</th>
<th>Subject 6</th>
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<tbody>
<tr>
<td>NS GS</td>
<td>NS GS</td>
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<td>NS GS</td>
<td>NS GS</td>
<td>NS GS</td>
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<tr>
<td>PreEx-Rm Air</td>
<td>0 0</td>
<td>0 0</td>
<td>0 0</td>
<td>0 0</td>
<td>0 0</td>
</tr>
<tr>
<td>Ex-Rm Air</td>
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<td>2 2</td>
<td>3 2</td>
<td>3 4</td>
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</tr>
<tr>
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<td>3 4</td>
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</tr>
<tr>
<td>Ex-O₂</td>
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<td>3 2</td>
<td>3 4</td>
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</tr>
<tr>
<td>Ex-CO₂</td>
<td>4 3</td>
<td>2 2</td>
<td>3 2</td>
<td>3 4</td>
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</tr>
<tr>
<td>Ex-He</td>
<td>4 3</td>
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</table>

Normoxia (FIO₂ = 0.21)

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<tr>
<th>Subject 1</th>
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<tr>
<td>PreEx-Rm Air</td>
<td>0 0</td>
<td>0 0</td>
<td>0 0</td>
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</tr>
<tr>
<td>Ex-Rm Air</td>
<td>4 3</td>
<td>2 2</td>
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<td>3 4</td>
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<tr>
<td>Ex-N₂</td>
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<td>Ex-O₂</td>
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<td>Ex-CO₂</td>
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</tr>
<tr>
<td>Ex-He</td>
<td>4 3</td>
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</tbody>
</table>

Hypoxia (FIO₂ = 0.14)

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<th>Subject 4</th>
<th>Subject 5</th>
<th>Subject 6</th>
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<td>NS GS</td>
<td>NS GS</td>
<td>NS GS</td>
<td>NS GS</td>
</tr>
<tr>
<td>PreEx-Rm Air</td>
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<td>0 0</td>
<td>0 0</td>
<td>0 0</td>
<td>0 0</td>
</tr>
<tr>
<td>Ex-Rm Air</td>
<td>4 3</td>
<td>2 2</td>
<td>3 2</td>
<td>3 4</td>
<td>4 4</td>
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<tr>
<td>Ex-N₂</td>
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<td>2 2</td>
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<td>3 4</td>
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</tr>
<tr>
<td>Ex-O₂</td>
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<td>3 2</td>
<td>3 4</td>
<td>4 4</td>
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<tr>
<td>Ex-CO₂</td>
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<tr>
<td>Ex-He</td>
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Hyperoxia (FIO₂ = 1.0)

Bubble scores in all 6 subjects measured by method 1 and method 2 for each gaseous saline contrast agent at rest (PreEx) and during exercise (Ex) at 60% of V˙O₂peak. NS, normal saline; GS, gas-equilibrated saline; Rm Air, room air. No statistical significance between gases during exercise within each FIO₂, Friedman’s test with a Dunn’s multiple comparison posttest.
non-$N_2$ microbubbles as they travel from the antecubital vein toward the right heart, which should theoretically promote their stability and decrease their rate of dissolution. Thus, once within the right heart, all of the non-$N_2$ microbubbles have taken on enough $N_2$ that they behave similarly to the room air and 100% $N_2$ microbubbles. The potential for $N_2$ to stabilize the non-$N_2$ microbubbles would be greatest during exercise in normoxia or hypoxia, as the $P_{N_2}$ within the venous blood is $\sim 600$ Torr. Conversely, in hyperoxia the $P_{N_2}$ within the venous blood is less than that in normoxia or hypoxia (14). Ultimately, the reduced $P_{N_2}$ within the venous blood during exercise in hyperoxia would reduce this possibility for $N_2$ to stabilize each non-$N_2$ microbubble. Therefore, while this is less likely to occur during exercise in hyperoxia, several lines of evidence argue against this possibility impacting on our results.

One line of evidence is that each microbubble gas composition produced bubbles that survived for similar durations of at least 6–8 s within the right heart regardless of the $F_{IO_2}$ breathed. Thus all microbubbles are surviving for similar durations despite the variable potential for $N_2$ diffusion to stabilize them. However, if $N_2$ were diffusing out of the blood and into the non-$N_2$ microbubbles during exercise in normoxia and hypoxia, and to some degree during exercise in hyperoxia, and stabilizing all the microbubbles, then we would expect to detect some degree of contrast within the left heart in every subject during exercise in hyperoxia. Instead, during exercise in hyperoxia with both methods 1 and 2 the vast majority of subjects failed to demonstrate the transpulmonary passage of even a single microbubble.

Therefore, the fourth and final possible location for bubble dynamics to affect IPAV shunt detection involves the changes in partial pressures occurring within the pulmonary venous circulation and left heart because of pulmonary gas exchange. It is clear that microbubbles survived in the mixed venous blood in the right heart for 6–8 s, regardless of their initial gas composition or the $F_{IO_2}$ breathed, despite potentially large and small diffusion gradients. Therefore, if bubbles do not appear in the left heart during hyperoxic exercise because of changes in bubble dynamics, the only remaining possible location for bubbles to collapse would be after they pass through patent IPAV anastomoses, come into contact with blood in the pulmonary vein, and dissolve before reaching the left heart. As microbubbles move through the systemic venous and pulmonary arterial circulation, all the non-$N_2$ microbubbles have a significant driving gradient favoring their non-$N_2$ gas to diffuse out of the microbubbles and into the blood. If their survival is only the result of small amounts of $N_2$ within the systemic venous and pulmonary arterial blood simultaneously diffusing into the microbubbles, then their stability would also be maintained in the pulmonary vein. Once within the pulmonary venous circulation the driving gradient would favor $N_2$ moving out of the microbubbles and into the blood, while a significant driving gradient favoring the diffusion of $O_2$ into the microbubbles would maintain their viability. Because the driving gradients for diffusion on the right side of the heart allow for microbubbles to persist for 6–8 s, there is no reason to believe that microbubbles would not be equally viable with similar diffusion gradients moving in opposite directions on the left side of the heart. Therefore, the lack of microbubbles in the left heart during exercise in hyperoxia is not explained by the collapse of microbubbles within the pulmonary vein.

Reconciling experimental and theoretical work. The present study provides direct and generalizable in vivo results with an established technique that appear to contradict theoretical work describing gas microbubble behavior (4, 17, 31, 37, 44–46, 52, 55–57). To explain the lack of any significant differences in bubble scores observed between microbubble gas compositions within varying partial pressure environments, several explanations exist that would reconcile our experimental work with the theoretical work. One explanation is that the average microbubble diameter is very large and thus mitigates the effect of surface tension and is able to withstand the changes in the external partial pressures. As we have already discussed, there is no way to know the diameter of the injected microbubbles. However, theoretical and experimental data would suggest that they are at least 60–90 $\mu$m in diameter if not greater, and recall that the larger the microbubble, the more stable it will be. Another explanation is that the external partial pressure changes are influencing microbubble life span and behavior. As detailed above, changes in the partial pressures outside the microbubbles do not explain our findings because the results are equivalent within each $F_{IO_2}$. Despite constant external partial pressures, injection of microbubbles of different internal gas compositions yielded identical results. A final explanation is that in vivo surface film alterations are occurring and influencing the microbubbles’ life span and behavior. If surface film alterations occurred in vivo and acted as a surfactant to retard the rate of microbubble dissolution, then these surface film alterations would be expected to occur to the same extent regardless of the $F_{IO_2}$ breathed. However, there is no explanation that we are aware of that would detail why this stabilizing surface film alteration would suddenly no longer exist, allowing for the destabilization of microbubbles before they reached the left heart during hyperoxia. Indeed, in vivo stabilization may very well be occurring, which would help to explain the persistence of contrast within the right heart (and likely the left heart when IPAV anastomoses are open), regardless of the initial microbubble gas composition.

Therefore, the reason we detect left-sided contrast during exercise in normoxia is likely because IPAV anastomoses are open. The reason we detect more left-sided contrast during exercise in hypoxia is likely because more IPAV anastomoses are open and/or more blood is flowing through them. And the reason we do not detect left-sided contrast during exercise in hyperoxia is likely because IPAV anastomoses are closed. These conclusions from our data obtained with saline contrast echocardiography are supported by data from Niden and Aviado (36), who used glass microspheres up to 420 $\mu$m to demonstrate that IPAV anastomoses exist in the dog lung. In this study, the number of beads collected from the pulmonary venous effluent increased when the lungs were ventilated with 10% oxygen while the number of beads collected decreased when the lungs were ventilated with 100% oxygen. In addition, Vogtizas et al. (47) have suggested that there would need to be “a major change in functional anatomy of the lungs caused by changing $F_{IO_2}$” in order to facilitate changes in microbubble transmission and to explain the contribution intrapulmonary shunt has on the total venous admixture recorded. Indeed, a study by Melsom et al. (32) demonstrated that both hypoxia and hyperoxia alter pulmonary perfusion in unanesthetized, upright sheep as measured with radiolabeled microspheres. Together, these data support the idea that functional changes
within the pulmonary circulation do occur in humans and animals breathing either hypoxia or hyperoxia.

Implications of the present work. The explanation that best balances the theoretical predictions of previous research and the experimental outcomes of the present study is that the detection of saline contrast microbubbles in the left heart indicates that the microbubbles have a relatively large average diameter, and must be traveling through large-diameter vessels during exercise in normoxia and hypoxia that are closed during exercise in hyperoxia. The present work provides experimental evidence that the behavior of gas-filled microbubbles in vivo deviates from theoretical calculations or observations with engineered systems designed to replicate internal environments. Microbubbles of differing internal gas compositions, and therefore solubilities and diffusivities, within conditions of constant partial pressures have differing rates of dissolution (16). Although we cannot quantify microbubble diameter, this is the first study to intravenously inject microbubbles of differing gas compositions and observe the presence or absence of contrast within the left heart by echocardiography. If we assume that the current theory describing gas microbubble behavior accurately reflects our in vivo results, then the most likely explanation for visualization of saline contrast microbubbles in the left heart is a large average microbubble diameter (≥60 to 90 μm). With an average microbubble diameter large enough to mitigate the influence of surface tension, even with differing gas diffusion gradients and therefore rates of diffusion, the microbubbles we observe in the echocardiogram images appear equivalent (Fig. 1). A more likely explanation is that both microbubble diameter and potential surface film alterations are acting together to combat the forces of surface tension and to reduce the rate of diffusion. This would result in equivalent results despite different microbubble gas compositions, in vivo partial pressure changes, and differing rates of dissolution.

Because of the lack of any difference observed in right-sided contrast across all microbubble gas compositions within each FIO2, we are confident that saline contrast microbubbles are traveling through IPAV anastomoses and not simply being altered by the external partial pressure environments. If these IPAV anastomoses participated in noncapillary gas exchange to any significant degree, the microbubbles that have the greatest diffusion gradient (e.g., 100% He with any FIO2) would be expected to rapidly dissolve as the intravascular partial pressure would rapidly diffuse down its partial pressure gradient and into the air spaces, further promoting microbubble collapse such that no microbubbles would be detected in the left heart. Despite this possibility, we observed no difference in bubble score with the use of any microbubble gas composition within each FIO2, suggesting that blood flowing through IPAV anastomoses is not likely participating in gas exchange to a significant degree, if at all.

Previous research using either the multiple inert gas elimination technique (MIGET) (48) or the 100% O2 technique (47) has determined that the degree of shunt calculated to contribute to the widened alveolar-arterial O2 difference (A-aDO2) observed during exercise is negligible. The 100% O2 technique cannot distinguish between intracardiac, bronchial, Thebesian, and intrapulmonary shunt sources and assumes that when subjects are breathing 100% O2 the elevated PO2 does not alter the patency of any IPAV anastomoses. The present research and our previous research (28) have shown that exercise-induced IPAV shunt is prevented in subjects breathing 100% O2. Accordingly, this traditional method of quantifying shunt through breathing 100% O2 would underestimate the contribution of inducible shunts if breathing a high FIO2, closed inducible IPAV anastomoses that would otherwise remain open. Therefore, the discrepancy between data obtained with these gas exchange-dependent methods (i.e., 100% O2 technique) and data obtained with anatomically dependent methods (i.e., saline contrast echocardiography) can be reconciled.

Summary. The results obtained in the present study support the conclusions reached in previous work using room air as the gaseous component of saline contrast microbubbles, suggesting that these inducible IPAV anastomoses are closed at rest and in exercising subjects breathing hyperoxia (28) but opened during exercise in normoxia (9, 43) and at rest in subjects breathing hypoxia (23, 27). Furthermore, the present work supports these previous studies as it demonstrates that the partial pressure changes associated with breathing a different FIO2 do not sufficiently alter the in vivo microbubble dynamics to an extent that would impact on our observations. Accordingly, the present findings support the idea that saline contrast microbubbles are traveling through anastomoses that do not participate in alveolar gas exchange to any significant degree. Discrepancies between theoretical and experimental work lead us to conclude that there may be additional, yet to be identified influences regulating the behavior of gas microbubbles in vivo.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


