Parabiotic model for differentiating local and systemic effects of continuous and intermittent hypoxia

Marta Torres,1,2 Mauricio Rojas,3,4 Noelia Campillo,1,5 Nayra Cardenes,3,4 Josep M. Montserrat,1,2,6 Daniel Navajas,1,5,7 and Ramon Farré1,5,6
1CIBER de Enfermedades Respiratorias, Bunyola, Spain; 2Sleep Laboratory, Hospital Clinic, Facultad de Medicina, Universitat de Barcelona, Barcelona, Spain; 3Dorothy P. & Richard P. Simmons Center for Interstitial Lung Disease, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania; 4McGowan Institute for Regenerative Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania; 5Unitat de Biofísica i Bioenginyeria, Facultat de Medicina, Universitat de Barcelona, Barcelona, Spain; 6Institut Investigacions Biomediques August Pi i Sunyer, Barcelona, Spain; and 7Institut de Bioenginyeria de Catalunya, Barcelona, Spain

Submitted 24 September 2014; accepted in final form 3 November 2014

Torres M, Rojas M, Campillo N, Cardenes N, Montserrat JM, Navajas D, Farré R. Parabiotic model for differentiating local and systemic effects of continuous and intermittent hypoxia. J Appl Physiol 118: 42–47, 2015. First published November 6, 2014; doi:10.1152/japplphysiol.00858.2014.—Hypoxia can be damaging either because cells are directly sensitive to low oxygen pressure in their local microenvironment and/or because they are exposed to circulating factors systemically secreted in response to hypoxia. The conventional hypoxia model, breathing hypoxic air, does not allow one to distinguish between these local and systemic effects. Here we propose and validate a model for differentially applying local and systemic hypoxic challenges in an animal. We used parabiosis, two mice sharing circulation by surgical union through the skin, and tested the hypothesis that when one of the parabionts breathes room air and the other one is subjected to hypoxic air, both mice share systemic circulation but remain normoxic and hypoxic, respectively. We tested two common hypoxic paradigms in 10 parabiotic pairs: continuous hypoxia (10% O2) mimicking chronic lung diseases, and intermittent hypoxia (40% 21% O2; 20 s, 5% O2) simulating sleep apnea. Arterial oxygen saturation and oxygen partial pressure at muscle tissue were measured in both parabionts. Effective cross-circulation was assessed by intraperitoneally injecting a dye in one of the parabionts and measuring blood dye concentration in both animals after 2 h. The results confirmed the hypothesis that tissues of the parabiont under room air were perfused with normally oxygenated blood and, at the same time, were exposed to all of the systemic mediators secreted by the other parabiont actually subjected to hypoxia. In conclusion, combination of parabiosis and hypoxic/normoxic air breathing is a novel approach to investigate the effects of local and systemic hypoxia in respiratory diseases.

parabiosis; animal model; local hypoxia; systemic hypoxia

Hypoxia, the condition where tissues are subjected to a low level of oxygenation, occurs normally at high altitude and under strenuous exercise. Moreover, hypoxia is a very relevant injurious challenge in several diseases. Indeed, although there are physiological mechanisms ready to respond to hypoxia (27), these natural defenses cannot be sufficiently effective in some severe pathologies, and the respiratory metabolism in the tissues of the patient is altered, with potential deleterious consequences (24). Usually, harmful hypoxia is directly originated by a respiratory system disease (32). For instance, impairment of ventilation and gas exchange in chronic obstructive pulmonary disease may result in continuous hypoxemia (16), and recurrent upper airway obstructions in sleep apnea induce intermittent tissue hypoxia (3). However, in other cases, hypoxia is the indirect consequence of another dysfunction, as in acute respiratory distress syndrome originally caused by sepsis, trauma, or cardiac failure (21). Hypoxia associated with pathological conditions is not always systemic, but restricted to some tissues, as in malignant tumors, where local hypoxia enhances their progression (4). Given the prevalence and severity of the diseases involving hypoxia, a great research effort is devoted to study the pathophysiological mechanisms involved at molecular, cellular, and systemic levels.

Animal research with a model approaching the clinical condition is, as in many pathologies, a powerful tool for investigating hypoxia. The most simple and useful experimental model of hypoxia consists of decreasing the oxygen fraction in the air breathed by the animal under test. Controlled alteration of ambient oxygen directly translates into the desired levels of hypoxemia and thus tissue oxygenation. This model realistically reproduces the conditions of continuous or intermittent hypoxemia experienced by patients and has been very useful in improving our understanding of hypoxia pathophysiology. Nevertheless, the model has an intrinsic limitation: it does not allow one to distinguish between the effects of local and systemic hypoxia. Indeed, hypoxia can induce dysfunctions, either because the cells in a tissue are directly sensitive to the low oxygen pressure in their local hypoxic microenvironment, and/or because they are exposed to the influence of circulating factors (e.g., inflammatory or immune mediators, progenitor cells) systemically secreted in response to hypoxia (24, 27). As these two potential contributions are acting simultaneously in animals subjected to the conventional hypoxic model, the effects cannot be isolated and identified. Accordingly, an in vivo setting, which could allow us to differentially control the application of local and systemic hypoxic challenges, would be very useful to investigate the consequences of hypoxia. In particular, a model where an animal that is normally oxygenated is simultaneously exposed to the whole systemic response induced by hypoxia would be of major interest.

The parabiotic model allows that two animals share the same blood circulation (5, 13, 14). The simplest implementation of parabiosis consists in surgically joining two rodents by the skin. Spontaneous anastomoses and de novo capillarization
around the wound area allow the establishment of cross-circulation after surgery. Given that parabiotic cross-circulation blood flow is small (14), circulating factors would be shared by both parabionts, depending on the half-life of the factor in the blood. Specifically, short-life factors such as oxygen (with a blood content continuously regulated by breathing) would be different in both parabionts in case that each one is breathing air with a different oxygen fraction. By contrast, long-life circulating factors secreted in response to hypoxia in one of the parabionts (e.g., inflammatory mediators, growth factors, immune cells) would be shared by both animals. The aim of this study was to validate the rationale of a novel application of the parabiotic model for studying continuous and intermittent hypoxia with a potentially more powerful approach. To this end, we tested the hypothesis that, when one parabiotic mouse breathes room air and the other one is subjected to hypoxic air, although both share blood circulation, the levels of blood and tissue oxygenation in each animal are those determined by the different air each animal is breathing.

METHODS

The study, which was approved by the Ethical Committee for Animal Research of the University of Barcelona, was conducted on 27 pathogen-free 15-wk-old female mice (C57BL/6; Charles River Laboratories, Saint Germain sur L’Arbresle, France) housed in standard cages and given tap water and food ad libitum. Two weeks before parabiotic surgery, 11 pairs of animals to be joined were housed together in an independent cage to promote their social interaction. The other five mice to be used as nonparabiotic controls were kept normally housed.

Eleven parabiotic pairs were established under intraperitoneal ketamine-xylacine anesthesia (100:10 mg/kg body wt) by surgical union of mice skin from the shoulder to the hip and the muscles of the abdominal wall. After shaving and sterilizing, a piece of skin from the shoulder to the hip was removed from opposite sides of each mouse, leaving visible the shoulder, the side of the rib cage, the side of the abdomen, and hip. Exterior shoulder and hip muscles from each mouse were sutured together with 4/0 sterile silk sutures. The skin was joined with a continuous 2/0 sterile silk suture. Immediately after surgery and the next 2 days, each mouse received 0.05 mg/kg body wt of buprenorphine as an analgesic. Each parabiotic pair was housed independently, with food pellets and water gel in the cage floor. The mice were supervised every day after surgery for 2 wk. One of the 11 parabiotic pairs did not survive the 2-wk period.

The parabiotic hypoxia/normoxia model was evaluated 2 wk after surgery. Each mouse in the parabiotic pair was anesthetized with intraperitoneal urethane (1 g/kg body wt). Two hypoxia paradigms were used to evaluate the model: intermittent (40 s, 21% O2 followed by 20 s, 5% O2) and continuous (10% O2) in six and four pairs of parabionts, respectively. In both cases the hypoxic challenge was applied for 2 h through a face mask to one of the parabionts, while the other pair was breathing room air. Arterial oxygen saturation (SpO2) in each parabiont was measured by pulse oximetry (MouseOx Plus, Starr Life Sciences). Moreover, in four of the parabiotic pairs subjected to intermittent hypoxia, tissue oxygen partial pressure (PtiO2) was measured in the skeletal muscle of both parabionts (3). To this end, an incision was made in the skin of the leg, and the muscle fascia removed to insert a fast-response Clark polarographic oxygen micro-electrode pipette (OX-50, Unisense A/S, Denmark; 50 μm diameter, 90% response time <2 s) around 3 mm below the tissue surface. PtiO2 was measured by the oxygen microelectrode connected to an amplified picooammeter (Unisense A/S, Denmark), previously calibrated in water at 100% O2, 21% O2, and oxygen-free solution (NaOH 0.1 M, sodium ascorbate 0.1 M) and recorded (MicOX software, Unisense A/S). After the 2-h hypoxia/normoxia test, room air breathing was resumed in the hypoxic parabiont.

Subsequently, effective cross-circulation was assessed by intraperitoneally injecting 200 μL of 0.5% Evans blue dye in one of the parabionts (7 pairs). Two hours later, blood from both parabionts was collected by cardiac puncture and centrifuged for 15 min at 3,000 rpm. The serum was diluted (1:50), and its absorbance at 620 nm was measured with a spectrophotometer (Multiskan FC Microplate Photometer, Thermo Scientific). The blood from the five nonparabiotic controls was identically processed.

Group data were computed as means ± SE, and statistical differences in SpO2 and PtiO2 between both parabionts along time were analyzed using two-way ANOVA. Differences between Evans blue dye concentrations values were assessed by the t-test. Statistical significance was considered for P < 0.05.

RESULTS

An illustrative example of SpO2 measured in both animals in a parabiotic pair when one of them was breathing normoxic air and the other one was subjected to intermittent hypoxic air is shown in Fig. 1. Whereas SpO2 remained unaltered at normal high values in the normoxic parabiont, its pair subjected to intermittent hypoxia experienced considerable recurrent swings in arterial oxygenation.

Figure 2 shows the group values of SpO2 and PtiO2 at the muscle in the parabionts subjected to intermittent hypoxia/normoxia. On average, SpO2 and muscle PtiO2 remained unchanged compared with the baseline (both parabionts under normoxia) in the mouse breathing room air, whereas its pair subjected to intermittent hypoxia experienced marked swings in SpO2 and, consequently, in PtiO2. The data corresponding to application of the normoxia-continuous hypoxia paradigm (Fig. 3) show that both parabionts had similar normal SpO2 at baseline, and that, after application of constant hypoxia to one of the animals, SpO2 remained unaltered in the parabiont with normoxic air and decreased and remained at a constant low value in the pair subjected to continuous hypoxia.

Parabiosis was effective in establishing cross-circulation between these investigated pairs, as indicated by the serum concentrations of Evans blue dye in Fig. 4. The concentration of this marker in the serum of both parabionts did not show statistically significant differences after 2-h dye injection in

---

Fig. 1. Example of arterial oxygen saturation (SpO2) measured in two parabiotic mice when one of them was breathing normoxic room air (dotted line) and its pair was breathing intermittent hypoxic air (solid line).

---

J Appl Physiol • doi:10.1152/japplphysiol.00858.2014 • www.jappl.org
one of the members of the pair, indicating almost full blood mixing caused by cross-circulation.

DISCUSSION

The results in this work confirm the hypothesis that, when two parabionts are breathing air with different oxygen fraction, both animals share the same blood but present different arterial oxygenation. Accordingly, the tissues of the parabiont under room air are perfused with normally oxygenated blood and, at the same time, are exposed to all of the systemic mediators secreted by the other parabiont in response to hypoxia.

The methodology we used is based on current models employed in animal research, and it is thus simple and easy to implement. The mouse parabiotic model is being progressively applied in recent years (9), particularly in research addressing ageing/rejuvenation (20, 25, 31) and repair/regeneration (1, 2, 17, 18, 28). After parabiosis surgery, we waited for 2 wk to ensure that cross-circulation was well established. In fact, data in the literature indicate that, although full interchange of some specific cell types could require up to 14 days (13), cross-circulation is effective after 2–7 days of parabiotic joining (14). This was confirmed in our mice, as indicated by the almost complete circulatory distribution of dye from one parabiont to the other in 2 h, which is fully consistent with distribution times observed when Evans blue dye injection is used as a routine check for cross-circulation (15, 19, 23). This relatively fast time of blood interchange is in keeping with data indicating that the cross-circulation blood flow amounts to 1–2% of each animal’s total blood volume exchanges each minute (14).

As regards hypoxic modeling, the two paradigms we used are among the most common in animal research for both continuous hypoxia mimicking chronic lung diseases (6, 30) and intermittent hypoxia simulating obstructive sleep apnea (4, 7). To test the rationale of the model, we implemented an acute setting where hypoxia was applied for only 2 h in anesthetized animals, but differential hypoxia in each parabiont could be chronically applied in awake animals by modifying existing experimental settings (10).

The parabiotic model has been used in recent years to investigate the molecular and cell communication between same-age animals under different pathological conditions [homochronic parabiosis (8, 15, 17, 18)] and to investigate how systemic soluble factors from a young animal modify the response of an old animal subjected to different challenges [heterochronic parabiosis (20, 25, 31)]. In the field of respiratory diseases, parabiosis has been employed to assess repair mechanisms in lung injury (1) and pulmonary fibrosis (28), but not to study hypoxia. Interestingly, the parabiotic model offersFig. 2. Group SpO2 and tissue partial pressure (P\text{tO}_2) at the muscle when one parabiont was breathing normoxic room air (dotted line) and its pair was breathing intermittent hypoxic air (20% to 5% O\text{2}; solid line). Values are means ± SE.

Fig. 3. Group SpO2, when one parabiont was breathing normoxic room air (dotted line) and its pair was continuously breathing hypoxic air (10% O\text{2}; solid line). Values are means ± SE.
a so far unexploited experimental approach to study local and systemic effects of hypoxia. However, it requires a perspective conceptually different from the one in previous applications of the model. Indeed, the rationale of parabiosis is that all circulating molecules and cells are shared by the two parabions because full cross-circulation between them homogenizes the blood of both animals (13). According to this perspective and given that oxygen is one of the most relevant factors transported by blood, in the case in which one parabiont breathes hypoxic air and the other one normoxic air, the same level of blood oxygenation is shared by both animals. Nevertheless, contrary to all other circulating factors (with the exception of CO₂), the amount of arterial oxygen in each animal is continuously reset to the level corresponding to that of its alveolar air, provided that gas exchange in the lungs works normally. Therefore, when one parabiont is breathing continuous or intermittent hypoxic air and the other one breathes normoxic air, the tissues of both animals are perfused with the same blood content, with the exception of oxygen (and CO₂). Accordingly, the organs of the later parabiont are normally oxygenated, but, at the same time, it is exposed to all of the soluble factors that hypoxia induces in its pair. Comparing the consequences of hypoxia/normoxia in both parabions and in corresponding nonparabiotic controls would allow us to assess the relative weight of the local and systemic effects of hypoxia in different tissues and organs.

The conventional application of parabiosis is based on cross-circulation through connection of the circulatory systems of both parabions, as represented by Fig. 5A. The diagram indicates that there is a capillary system connecting both parabiont’s circulation. Regardless of the complexity of this connecting system, each parabiont sees a fraction of its arterial blood (cross-circulation blood flow) derived from the other animal and that an equivalent amount of blood from its pair enters its venous system. Therefore, from the circulatory viewpoint of each parabiont, its pair could be represented by an equivalent circuit in parallel with its own systemic capillary circuit. Figure 5B illustrates how, if a bolus of a marker (for instance, Evans blue dye) is injected into one parabiont, this marker tends to be uniformly distributed within the whole blood system of both parabions. Figure 5C represents a typical setting in which the concentration of a given molecular (e.g., cytokine) or cell (e.g., progenitor) component in the blood of both parabions is negligible at baseline. When one parabiont is subjected to an experimental challenge that induces systemic secretion of this component, its concentration in the other parabiont follows the increase in its pair until the concentration is the same in both animals at steady state.

The rationale for applying the hypoxia/normoxia parabiotic model described here is based on the regulation of arterial oxygen pressure by the lungs. From the perspective of the mouse breathing room air, the effect of having its parabiotic pair connected is equivalent to the case that a fraction of its systemic capillaries would increase oxygen consumption, since cross-circulation blood entering from its hypoxic pair has lower oxygen content. Nevertheless, taking into account that cross-circulation is a very low percentage of total blood flow, the reserve in gas exchange capacity in the normal lung of the parabiont breathing room air will ensure full oxygenation of its arterial blood. The reverse situation is sensed from the perspective of the parabiont breathing hypoxic air. For this animal, being parabiosed with its pair is equivalent to a section of its systemic capillaries consuming less oxygen, negligibly increasing oxygenation of its venous blood. However, its arterial blood would be regulated to the oxygenation level corresponding to the hypoxic air breathed by this parabiont. Consequently, although there is cross-circulation and sharing of blood factors, the tissues of each animal are perfused with blood at the corresponding different levels of oxygenation, fully explaining the results obtained in the experimental test of the model.

The model described provides a novel approach to investigate the local and systemic modulatory effects of hypoxia in different tissues in conditions mimicking respiratory diseases in animals otherwise healthy. Interestingly, differential hypoxia can be combined with additional alterations found in patients with respiratory diseases (chronic obstructive pulmo-

![Figure 5](http://jap.physiology.org/Downloadedfrom)
nary disease, pulmonary hypertension, lung fibrosis, sleep apnea), diabetes (23), liver alterations (19), fat metabolism (22, 26), cardiac fibrosis (2), neovascularization (17), or cancer (8). Another potential new application of the parabiotic hypoxic/normoxic model is to investigate the interaction between oxygenation and aging: demyelinating diseases (25), cognitive function impairments and synaptic plasticity (31), muscle loss (29), or cardiac hypertrophy (20). Remarkably, according to its rationale, variants of the parabiotic paradigm described here can also be used to investigate local and systemic mechanisms in gas exchange problems other than hypoxia, specifically in models of hyperoxia (11) or hypercapnia (12).

In conclusion, this study shows that the combination of parabiosis and hypoxic/normoxic air breathing is a model offering a novel approach to investigate in more detail the effects of local and systemic hypoxia in the experimental context of a wide spectrum of respiratory diseases.

ACKNOWLEDGMENTS
The authors wish to thank Miguel A. Rodríguez and Maeba Polo for excellent technical assistance.

GRANTS
This work was supported in part by the Spanish Ministry of Economy and Competitiveness (Grants SAF2011-22576 and P11/01892).

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

AUTHOR CONTRIBUTIONS
Author contributions: M.T., M.R., and N. Campillo performed experiments; M.T., N. Campillo, N. Cardenes, J.M.M., D.N., and R.F. analyzed data; M.T.; prepared figures; M.T., M.R., and R.F. edited and revised manuscript; M.T., M.R., N. Campillo, N. Cardenes, J.M.M., D.N., and R.F. approved final version of manuscript; M.R., N. Cardenes, J.M.M., D.N., and R.F. interpreted results of experiments; R.F. conception and design of research; R.F. drafted manuscript.

REFERENCES
