Pietro Ghezzi
and
Anthony Cerami
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18. Using Plethysmography to Determine Erythropoietin's Impact on Neural Control of Ventilation

Tommy Seaborn [1], Max Gassmann [2] and Jorge Soliz [1]

(1) Faculty of Medicine, Department of Pediatrics, Centre de Recherche de l'Hôpital St-François d'Assise (CR-SFA), Centre Hospitalier Universitaire de Québec (CHUQ), Laval University, Québec, QC, Canada

(2) Institute of Veterinary Physiology, Vetsuisse Faculty and Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Zurich, Switzerland

Abstract

The evaluation of respiratory parameters often requires the use of anesthetics (that depress the neural network controlling respiration), and/or ways to restrain the animal's mobility (that produces a stress-dependent increase of respiration). Consequently, the establishment of plethysmography represented an invaluable technique in respiratory physiology. Plethysmography, indeed, allows the assessment of ventilatory parameters on living, unanesthetized, and unrestrained animals. The conception of the barometric plethysmography relies on the fact that an animal placed inside a hermetically closed chamber generates through its breathing a fluctuation of pressure in the chamber than can be recorded. Thus, the respiratory frequency and the tidal volume can be directly measured, while the animal's ventilation is calculated indirectly by the multiplication of these two parameters. In our hands, plethysmography was a key tool to investigate the impact of erythropoietin (Epo) on the neural control of hypoxic ventilation in mice.

Key words Lung capacity – Mouse – Respiration – Respiratory frequency – Tidal volume – Ventilation – Hypoxia

1 Introduction

Plethysmography is a reliable method to measure ventilation in several animal species, including mouse (*1*, *2*), rat (*3*), shrew (*4*), guinea pig (*5*), cat (*6*), dog (*7*), pig (*8*), sheep (*9*), horse (*10*), and primates (*11*). In humans, this technique is also commonly used as a clinical tool to determine the functional residual capacity as well as the lung's total capacity. Concerning small mammals in which the evaluation of gases in blood is challenging, plethysmography allows to obtain a subsampling of air that in turn allows the evaluation of metabolic parameters (O_2 consumption and CO_2 production). The metabolic assessment is essential in order to determine whether changes in ventilation and/or the ventilatory pattern are due to regulations on the neuronal network control system, or reflect only the alteration of metabolic parameters. The parameters directly provided by plethysmography are respiratory frequency (fR) and tidal volume (V_T), while ventilation must be calculated by multiplying fR with V_T. Plethysmography is technically demanding and requires a relatively complex set-up to accurately generate the desired air regimen and adequately acquire, register, and analyze data. Accordingly, the influence of several parameters, such as O_2 exposure regimen, air flow, ambient temperature within the chamber, evaporating water, and animal body temperature, must be taken into account. Plethysmography is an invaluable technique in respiratory physiology since it allows determination of the ventilatory parameters of an animal kept under physiological conditions (unanesthetized and unrestrained) during a relatively long period of time and inside a controlled environment. Once again, the concept underlying the barometric plethysmography system is to measure, in a hermetically closed chamber, the fluctuation of pressure produced by the respiration of the animal.

In our laboratory, plethysmography was a key method to determine that erythropoietin (Epo), is implicated in the modulation of the neural control system both at central (brainstem) as peripheral (carotid bodies) levels. We specifically demonstrated that Epo in adult mice improves the acute hypoxic ventilatory response, as well as the ventilatory acclimatization to hypoxia (*2*). Indeed, we have shown for the first time that neural respiratory and erythropoietic systems are tightly interconnected, thus playing a complementary role improving the tissue oxygenation upon hypoxia (*2*, *12*). Moreover, we have further expanded these findings showing that chronic hypoxic exposure produces a drastic down-regulation of the soluble Epo receptor (sEpoR—a truncated form of the Epo receptor, that binds and inactivates endogenous Epo) in the central nervous system in mice. Furthermore, when sEpoR was chronically infused in the mouse's central nervous system, the process of ventilatory acclimatization to chronic hypoxia was abolished. These results showed that neural regulation of Epo and its antagonist sEpoR play a contra-balancing role in the central nervous system in establishing ventilatory activity and ensuring systemic oxygen delivery under low O_2 conditions (*1*). Finally, still by using plethysmography, we demonstrated that the impact of

Epo on ventilation occurs in a sex-dependent manner. Keeping in mind that women are less susceptible to several respiratory diseases than men, these findings suggest that Epo plays a key role in sexually dimorphic hypoxic ventilation (*1*). All together, these results foresee that Epo has a potential therapeutic use as treatment for hypoxia-associated ventilatory diseases. This review describes how to measure basal ventilation and hypoxia ventilatory response in adult mice (*see* **Note 1**) under unrestrained conditions (*see* **Note 2**).

2 Materials

2.1 Plethysmography System Components

An overview of the plethysmography system as well as the connections between its components is schematized in Fig. 1.

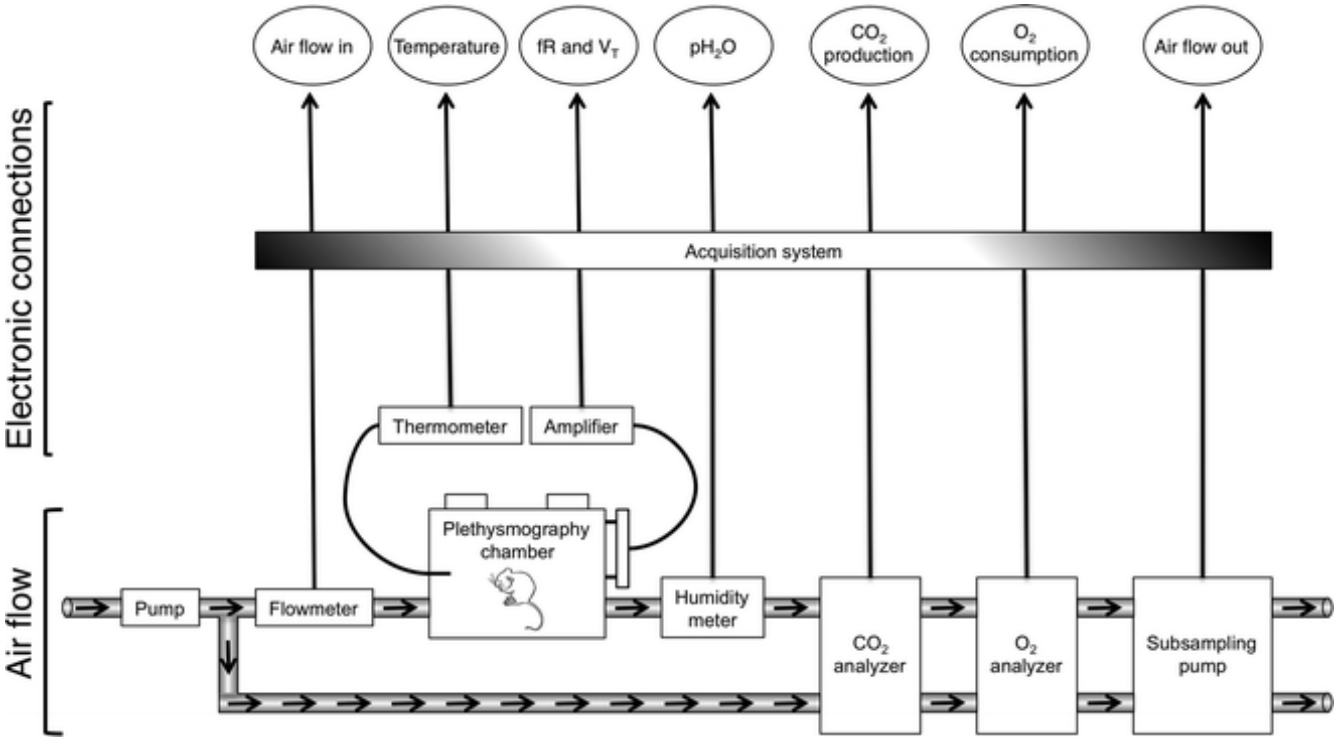

Fig. 1

Schematic overview of the system used for whole-body unrestrained plethysmography in adult mice. The tubings containing *arrows* represent the air path, while *dark lines* symbolize the electronic connections transporting analog data which are converted by the acquisition system into digital data to provide the experimental parameters included in the *ovals*

1. Pump for regulated air supply (EMKA Technologies) (*see* **Note 3**).

2. Flowmeter (E & E Process Instrumentation) (*see* **Note 4**).

3. Plethysmography chamber for whole-body plethysmography on unrestrained mice (EMKA Technologies).

4. Thermocouple monitoring thermometer with probe (*see* **Note 5**).

5. Differential pressure transducer (EMKA Technologies) (*see* **Note 6**).

6. Amplifier for differential pressure transducer's signal (EMKA Technologies).

7. Humidity meter (*see* **Note 7**).

8. Subsampling pump (Sable Systems International) (*see* **Note 8**).

9. CO_2 analyzer (Sable Systems International).

10. O_2 analyzer (Sable Systems International).

11. An acquisition system made up of an interface box (EMKA Technologies, Cat.# itf16) connected to an acquisition card (National Instruments, Cat.# PCI-6023E) inserted to a computer (*see* **Note 9**).

12. A computer with an installed acquisition, registering and processing software (iox version 1.8.9, EMKA Technologies) (*see* **Note 10**).

2.2 Experimental Gases

1. Calibrating cylinders for gas analyzers zero: 100% N_2, certified (*see* **Note 11**).

2. Calibrating cylinders for gas analyzers span: 21% O_2, 5% CO_2, and balance N_2, certified.

3. Cylinders containing 15, 10, and 6% O_2, respectively (each with 5% CO_2 and balance N_2).

4. Each gas cylinder should be equipped with a pressure regulator and a manometer corresponding to the gas contained in the cylinder.

5. Flexible PVC tubing nontoxic (conform to FDA standard) and nonporous (*see* **Note 12**).

6. Nylon connector kit (Harvard Apparatus) (*see* **Note 13**).

7. Desiccant membrane air dryer (Perma Pure) (*see* **Note 14**).

2.3 Mice and Epo

1. Sufficient number of adult mice (wild-type or transgenic) of a comparable age and of determined sex to generate reliable and reproducible mean group data (*see* **Note 15**).

2. Thermocouple monitoring thermometer and stainless steel rectal probe for mice (*see* **Note 16**).

3. In case of experiments involving injection of exogenous Epo (to wild type mice, typically), human recombinant Epo (*see* **Note 17**).

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3 Methods

3.1 Calibrations

1. Calibrate the air flow in to 700–800 ml/min by adjusting the air supplying pump (*see* **Note 18**).

2. Calibrate the air flow out to 600–700 ml/min by adjusting the subsampling pump (*see* **Note 19**).

3. Calibrate the CO_2 analyzer by using a 100% N_2 cylinder to calibrate the zero and a certified cylinder containing 5% CO_2 (with 21% O_2 and balance N_2) to calibrate the span.

4. Calibrate the O_2 analyzer by using a 100% N_2 cylinder to calibrate the zero and a certified cylinder containing a percentage of O_2 in the range of the experimental design (e.g., 21% O_2, 5% CO_2, balance N_2) to calibrate the span.

5. Calibrate the ventilation volume by injection of 1 ml of air in about 2–3 s into the animal chamber.

3.2 Effect of Epo on Basal Ventilatory State

1. Weight the mouse (*see* **Note 20**).

2. Put the mouse into the plethysmography chamber, close the chamber, and let the animal acclimatized until it reach a calm and quiet state reflected by a regular respiratory signal (typically between 30 and 60 min).

3. Open the plethysmography chamber and gently manipulate the animal to take body temperature with rectal probe (this is the initial body temperature) (*see* **Note 16**).

4. Put the mouse back to the plethysmography chamber and let it acclimatized again.

5. Once the mouse is calm, start to register the acquired data during 10 min with the animal exposed to room air (or to a cylinder containing 21% O_2, 5% CO_2, balance N_2 for more

accuracy). These data provide the baseline state to which the experimental state (severe hypoxia in this example) will be compared with.

3.3 Effect of Epo on Ventilatory Response to Severe Hypoxia (*See* **Note 21**)

1. In continuity with the basal state registering, turn open the gas cylinder containing 15% O_2, 5% CO_2, and balance N_2.

2. At this moment, a hand-made tag could be added on the recording stream to help to localize the O_2 regimen modification at the time of analysis.

3. Thereafter, a gradual decrease in O_2 exposure is generated by sequentially changing at each 5 min for a cylinder containing 10 and 6% O_2, respectively (with 5% CO_2 and balance N_2 in each case) (*see* **Note 22**). Tag each modification of O_2 regimen on the recording stream.

4. Continue to register the acquired data during 10 min with the animal exposed to hypoxia at 6% O_2. When compared with basal state, hypoxia should increase both frequency and amplitude of respiratory signal.

5. When hypoxia exposure is ended, turn off the gas cylinder generating the hypoxic air. This moment could be tagged on the stream as the end of hypoxia.

6. Continue to register the signal during 10 min with the animal exposed to room air (or to a cylinder containing 21% O_2, 5% CO_2, and balance N_2). These data characterized which is called the recovery period.

3.4 End of the Ventilation Measurements

1. Via the software control, stop to register the acquired data.

2. Rapidly open the plethysmography chamber and take body temperature with the rectal probe (this is the final body temperature) (*see* **Note 16**).

3. Return the mouse in its original cage or anesthetize the animal for euthanasy before harvesting tissues, depending on the experimental protocol.

4. Turn off the gas cylinder(s) if applicable, the air supply pump, the flowmeter, the thermometer, the humidity meter, and the subsampling pump (*see* **Note 23**).

5. Be sure all the acquired data have been saved on the computer for further analysis.

6. Turn off the acquisition system and the computer.

7. Carefully clean the plethysmography chamber (*see* **Note 24**).

3.5 Analyzing Ventilation and Metabolism Data (*See* **Note 25**)

For visual example purpose, typical results obtained by analysis of data from whole-body unrestrained plethysmography before and after hypoxia with mice injected or not with Epo are presented on Fig. 2.

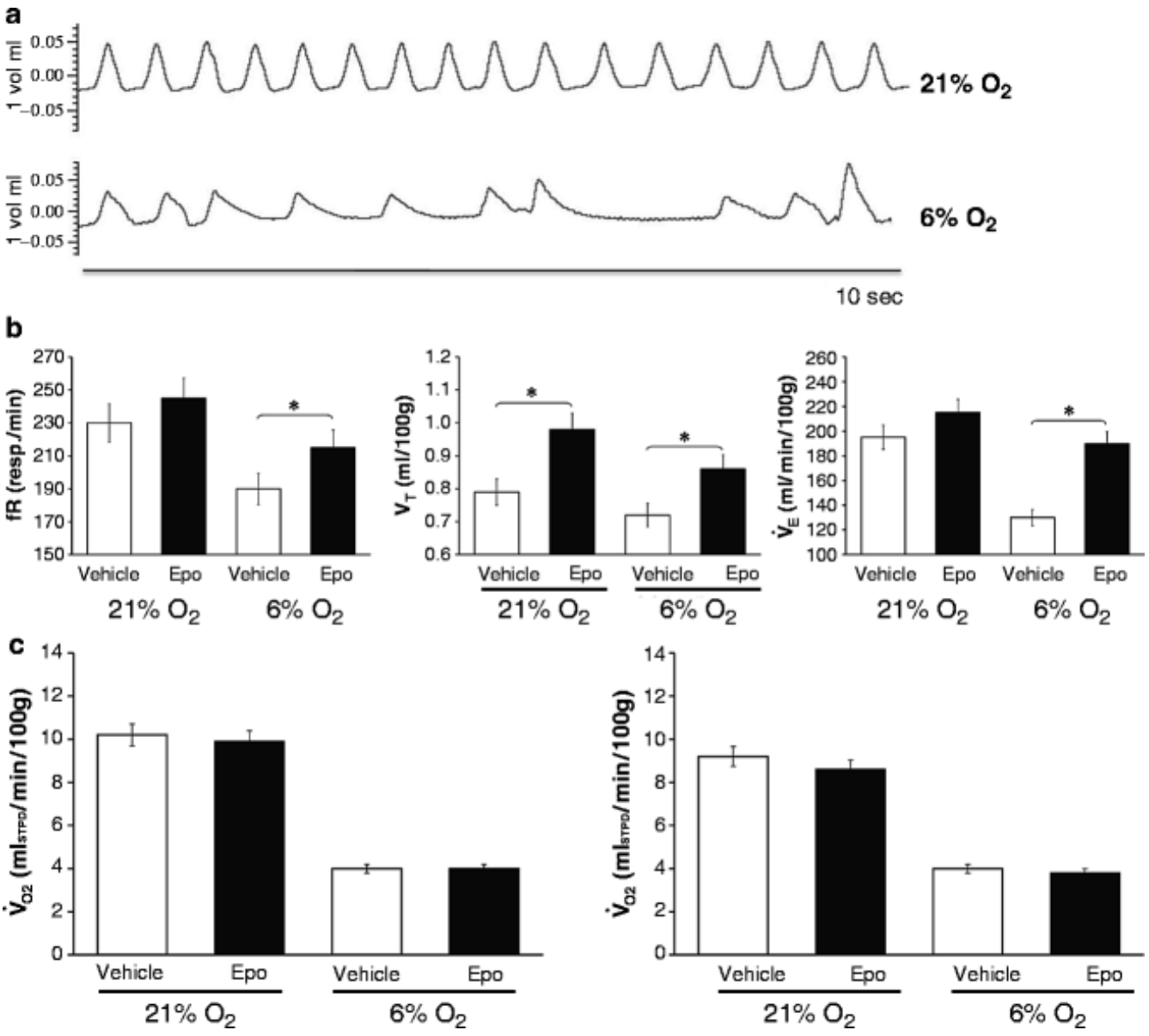

Fig. 2

Typical results obtained by analysis of data from whole-body unrestrained plethysmography achieved on female adult mice. (**a**) Representative examples of respiratory traces registered at 21% O_2 (normoxia) and at 6% O_2 (severe hypoxia). Example of results obtained for ventilatory (**b**) and metabolic (**c**) parameters in mice injected with Epo or vehicle (i.p.). Significant difference ($p < 0.05$) between vehicle and Epo groups are delineated by an *asterisk*

1. Respiratory frequency (fR) is directly evaluated as the number of respiratory cycles per minute (resp. per min).

2. Tidal volume (V_T) is obtained with previously described calculations (*13*) based on an equation firstly described by Drorbaugh and Fenn (*14*) in which V_T is expressed in ml per 100 g of body weight (ml per 100 g) in BTPS (body temperature and pressure, saturated) conditions.

3. Minute ventilation ($\dot{V}E$) is calculated as the product of fR and V_T and normalized to 100 g of body weight (ml/min/100 g).

4. O_2 consumption ($\dot{V}O_2$) is calculated as the difference between the fractions of O_2 before and after the plethysmography chamber corrected for STPD (standard temperature and pressure in air-dry) conditions and normalized for body weight (ml_{STPD}/min/100 g).

5. CO_2 production ($\dot{V}CO_2$) is calculated as the difference between the fractions of CO_2 after and before the plethysmography chamber corrected for STPD (standard temperature and pressure in air-dry) conditions and normalized for body weight (ml_{STPD}/min/100 g).

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4 Notes

1. It is well possible to perform similar experiments with younger mice, even from neonates, by using a specifically designed insert in the plethysmography chamber. Such an insert increases the relative impact of the small pressure fluctuations of the smaller animal by reducing the total air volume in the animal chamber. Moreover, controlling the temperature within the plethysmography chamber is necessary when experiments were carried out on younger animals that do not have yet the physiological capacity to thermoregulate adequately (<15 postnatal days in mice and rats).

2. Plethysmography can be achieved in a whole-body or double-chamber/head-out set-up. The whole-body system allows to make measurements on unrestrained and conscious animal during a long period of time without anesthetic bias. The relatively reasonable size of mice (and rats!) allows the use of this approach.

3. The pump is responsible to provide, at a constant and precise flow rate, the air mixture from the ambient air or gas cylinder(s) to the plethysmography chamber. There is a wide range of quality in the commercially available pumps, the main differences between them being the flow regulation. These parameters are very important to prevent any accumulation of CO_2 and evaporated water inside the chamber and to minimize the interaction of the provided air with the

animal's breathing pattern. The importance of the latter is increasing while the size of the tested animal is decreasing, becoming crucial in plethysmography experiments carried out on neonatal mice.

4. The flow meter is a device that directly and precisely measures, in a real-time manner, the flow of the air entering in the plethysmography chamber. It should be installed just before the arrival of the air to the chamber, in order to take into account all eventual air resistance upstream to the chamber. A precise flow meter is essential to a rigorous plethysmography system since it should provide an exact evaluation of the air flow the mice is exposed to, as well as a way to verify a constant flow in order to minimize the interaction with the animal's breathing pattern registered.

5. The ambient temperature in the plethysmography chamber should be monitored in order to make corrections for STPD conditions in the calculation of O_2 consumption and CO_2 production by the mice (cf. Subheading 3.5, **steps 4** and **5**).

6. The differential pressure transducer is a small device connected directly to the plethysmography chamber with one port exposed to the animal chamber and another one exposed to the reference chamber which is empty and subjected to the atmospheric pressure. Thanks to this configuration, the transducer can measure the pressure fluctuations within the plethysmography chamber which results from mice breathing.

7. The pressure odf the evaporated water in the plethysmography chamber should be monitored in order to make corrections for BTPS conditions in the calculation of V_T (cf. Subheading 3.5, **step 2**).

8. The subsampling pump helps to avoid the generation of a positive pressure within the plethysmography chamber and, most importantly, it allows obtaining the air composition before being modified by the animal breathing inside the chamber (Fig. 1). This subsampled air is essential to determine $\dot{V}O_2$ (cf. Subheading 3.5, **step 4**) and $\dot{V}CO_2$ (cf. Subheading 3.5, **step 5**).

9. A data acquisition system is necessary to receive analog data (electrical signals) from the measurements devices (flow meter, thermometer, amplifier of pressure transducer, humidity meter, CO_2 and O_2 analyzers) and to convert them into digital numeric values that can be processed by a computer. There are several acquisition systems commercially available and each of them has its pros and cons. The main concerns for the experimenter in a choice of acquisition system is the compatibility with analyzing software (especially in term of acquisition frequency) and measurement devices connections (usually BNC connectors).

10. Once converted by the acquisition system, the collected data should be registered and then

analyzed on a computer. Several comprehensive software are available for data acquisition and real-time analysis. Some software present two distinct modules, a first one which register the data inputs and a second one devoted to data analysis. Usually, the software used for plethysmography analysis generate both a stream file of the registered traces and data files containing numeric values obtained from the traces or calculated from them.

11. Although this is not necessary the gas content of the experimental cylinders to be certified, it would increase the homogeneity in the results (especially if one would compare experiments done by using more than one gas cylinder). If mixing the gases in the lab, we strongly recommend the use of an appropriate gas analyzer(s) located within the system to specifically assess the gas content of the air entering in the plethysmography chamber.

12. The caliber (internal and external diameter) of the tubing should be optimized as much as possible in order to avoid over-pressure and to limit the air dead volume within the air path. Also in order to limit the air dead volume, the length of the tubing should be kept at the necessary minimum.

13. A wide variety of connectors is necessary to properly and conveniently interconnect tubing together or with devices.

14. It is essential to dry the air before its arrival in the gas analyzers because evaporated water causes interference with CO_2 measurement which is carried out by infrared optical detection. For this reason, an air dryer should be inserted in the air path just before entering in the CO_2 analyzer.

15. Our experience teaches us that between 6 and 10 mice per group is usually sufficient to obtain satisfying and representative plethysmography results. Nevertheless, the sample size has to be determined empirically for each experimental design and increased until the standard errors are reasonable in comparison to means. If wild-type mice are used, the extrapolation and comparison with previous or published results should be carefully leaded since some differences in the breathing phenotype of different mice strains has been reported (*15*). Alternatively, transgenic mice over-expressing Epo only in the brain (Tg21 mice) or in both brain and plasma (Tg6 mice) have been generated (*16*, *17*) and they represent an interesting tool to study the effect of Epo on the respiratory control. In addition, since a sex difference exists in the ventilatory pattern, in the ventilatory response to hypoxia, and in Epo impact on this response, sex of the mice is an important variable and it should be determined and annotated. Indeed, the experimental groups should be designed in order to equally represent males and females or, ideally, to consider each gender separately, in both sampling and analysis.

16. The rectal temperature of mice should be measured before and after the experimental

protocol in order to make corrections for BTPS conditions in the calculation of V_T (cf. Subheading 3.5, **step 2**). The initial body temperature is considered to correct all the data, except the 5 last minutes (recovery period), which are corrected by using the mice temperature at the end of the protocol (the final body temperature).

17. Indeed, the dose- and time-effect of Epo should be firstly inferred from data available in the literature and/or determined specifically for the considered experimental context. The time of injection and the doses regimen of Epo are other parameters depending on the specific experimental design. Alternatively, other Epo derivatives are commercially available, some of them presenting only the non-erythropoietic characteristics of Epo and being a potential useful tool to study the impact of Epo on the control of respiration. Obviously, control animals should be injected with the vehicle used to resuspend the recombinant Epo.

18. The air flow in depends on the size of the animal and the value proposed here for adult mice has been determined empirically from our previous experiments.

19. The flow of the subsampling pump should always be a little bit lower than the air flow in to avoid the creation of a negative pressure within the plethysmography chamber.

20. In order to facilitate comparisons between mice, minute ventilation ($\dot{V}\mathrm{E}$), O_2 consumption ($\dot{V}\mathrm{O}_2$), and CO_2 production ($\dot{V}\mathrm{CO}_2$) are normalized to 100 g body weight. For this reason, mice should be weighted before the beginning of the experiment.

21. In addition to severe hypoxia as tested in this example, it is also possible to test by using a similar procedure the impact of other gas regimen on ventilation and/or of Epo treatment on ventilation (e.g., hyperoxia, normoxia, mild hypoxia, anoxia, hypercapnia, normocapnia, hypocapnia).

22. The fraction of O_2 in the plethysmography chamber should be gradually decreased from 21 to 6% O_2 during 15 min in order to avoid mice death due to a too rapid O_2 deprivation. However, if mice are exposed to 10% O_2 or higher during the hypoxia regimen, the decrease in the fraction of O_2 do not necessitate intermediate step(s) and could be done directly from 21 to 10% O_2 without risk of asphyxia for mice.

23. It is highly suggested to not turn off the O_2 and CO_2 analyzers and the amplifier after each utilization in order to maintain their functioning temperature as stable as possible.

24. Proceed as recommended by the supplier and, importantly, do not use any chemicals that may damage the plethysmograph chamber's integrity (such as ethanol) or influence respiration (or stress) of the next experimental mice.

25. There is several ways to analyze plethysmography data, depending on the experimental

context or the plethysmography set-up used. We present and propose here one of these possibilities, which is appropriate and relevant for the whole-body unrestrained plethysmography carried out on adult mice with the set-up we presented. However, this is not the only appropriate method to analyze this type of results.

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