A bioreactor for subjecting cultured cells to fast-rate intermittent hypoxia

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ARTICLE INFO

Article history:
Accepted 2 January 2012

Keywords:
Obstructive sleep apnea
Hypoxia–reoxygenation
Oxygen partial pressure

ABSTRACT

High frequency intermittent hypoxia is one of the most relevant injurious stimuli experienced by patients with obstructive sleep apnea (OSA). Given that the conventional setting for culturing cells under intermittent hypoxia conditions is limited by long equilibration times, we designed a simple bioreactor capable of effectively subjecting cultured cells to controlled high-frequency hypoxic/normoxic stimuli. The bioreactor’s operation is based on exposing cells to a medium that is bubbled with the appropriate mixture of gases into two separate containers, and from there it is directed to the cell culture dish with the aid of two bidirectional peristaltic pumps. The device was tested on human alveolar epithelial cells (A549) and mouse melanoma cells (B16-F10), subjecting them to patterns of intermittent hypoxia (20 s at 5% O2 and 50 s at 20% O2), which realistically mimic OSA of up to severe intensity as defined by the apnea hypopnea index. The proposed bioreactor can be easily and inexpensively assembled and is of practical use for investigating the effects of high-rate changes in oxygen concentration in the cell culture medium.

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1. Introduction

High frequency intermittent hypoxia is a significant biological challenge in obstructive sleep apnea (OSA). This syndrome, which is very prevalent in both adults and children (3–5% of population) (Lumeng and Chervin, 2008; Punjabi, 2008), is characterized by an abnormal increase in upper airway collapsibility during sleep (Farre et al., 2008a). As a result, OSA patients experience repeated apneic events with a frequency of up to more than one per minute. These recurrent apneas induce severe periodic desaturations of oxygen in arterial blood and, consequently, acute intermittent hypoxia at tissue level (Almendros et al., 2010). There is ample evidence in the literature that intermittent hypoxia is a main determinant of the increased morbidity (cognitive, metabolic and cardiovascular) and mortality observed in OSA patients (Gozal and Kheirandish-Gozal, 2008; Kheirandish-Gozal et al., 2010b; Levy et al., 2009).

Although the biological consequences of intermittent hypoxia have been thoroughly studied in animal and cell models of OSA (Farre et al., 2008b; Yuan et al., 2011), the information available on the effects of high frequency hypoxia–reoxygenation in cell stud-

ies is scarce. The main reason for this lack of data is the difficulty in subjecting cultured cells to a pattern of intermittent hypoxia that realistically mimics OSA. Indeed, the conventional oxygen chamber setting, based on changing the O2 content of the air, results in an excessively low rate of change of oxygen concentration at cell level because of the long time constant of gas diffusion from the air-medium interface to the cells cultured at the bottom of the culture well (Allen et al., 2001). Some alternative methods have been proposed to circumvent this problem (Oppegard et al., 2010, 2009; Polinkovsky et al., 2009). The application of these methods has been limited, however, because they require very specific equipment, beyond the reach of most labs interested in investigating the effects of OSA-mimicking intermittent hypoxia on different kind of cells.

To overcome the limitations imposed by the current experimental settings, we have designed and tested a readily available bioreactor for effectively subjecting cultured cells to oxygen partial pressures with controlled magnitude and time pattern. Our aim was specifically to simulate the high-frequency hypoxic stimulus characteristic of OSA. The bioreactor operates by exposing the cells, at accurately controlled intervals, to interchangeable media that have been previously equilibrated to the desired oxygen concentration. With our bioreactor prototype we achieved precise variations of oxygen concentration at cell culture level, from 20% to 5% in less than 10 s. Herein, we provide a detailed description of the prototype, as well as the results of feasibility tests with different cell types.

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doi:10.1016/j.resp.2012.01.001

2. Materials and methods

2.1. Description of the bioreactor

Fig. 1 shows a schematic diagram of the bioreactor that we have designed. Cells are cultured on 18 mm glass coverslips (not shown in the figure), which are placed at the bottom of conventional 12-well plates. Wells are slightly modified by drilling a small diameter (1 mm) hole in the peripheral area of their base to allow connection to a bifurcated tube. Each tube is connected to a culture medium container via a reversible peristaltic pump (WPL810; Williamson Pumps Ltd., UK) operating intermittently. The medium in each container is maintained at the desired oxygen concentration (supplemented with 5% CO₂) by continuously bubbling a gas mixture from an external source (37 °C and 100% humidity). The cyclic exchange of the culture medium in the well results in the exposure of the cells to intermittent oxygen concentration. One pump transfers the normoxic medium to the culture well and, after leaving it into contact with the cells for the desired time period, withdraws the medium back to its container. The other pump then drives the hypoxic medium to the well, leaves it to rest for an independently set time before withdrawing it. The whole cycle is subsequently repeated. The filling and emptying times are equal for both pumps (10 s for our particular experiments) but the resting times can be independently set by a timer that synchronizes and adjusts the frequency of the total cycle and the active period of one pump in relation to the other. The bioreactor setting is enclosed in a temperature-controlled chamber to maintain media and cell culture at 37 °C and 100% humidity. The low-voltage power supplies to the pumps (<12 V) were safely connected taking into account the ambient conditions within the chamber.

2.2. Measurement of oxygen partial pressure at cell level

A modified Clark’s polarographic fast-response oxygen micro-electrode pipette (OX-50, Unisense A/S, Denmark; 50 μm diameter, 90% response time <2 s) based on a gold cathode was used to measure the oxygen concentration at cell culture level during the application of the intermittent hypoxia patterns within the bioreactor. The oxygen of the culture medium was reduced on the surface of the cathode and an amplified picocomparator (Unisense A/S, Denmark) converted the resulting reduction current into a signal. At the beginning of each experimental setting, the sensor was calibrated (MicOX software, Unisense A/S, Denmark) using two reference points: 0 and 21% O₂ with an anoxic solution of sodium ascorbate and NaOH (both 0.1 M) and water equilibrated with ambient air, respectively.

2.3. Cell culture

The practical performance of the bioreactor for culturing cells was assessed by using human alveolar epithelial cells and mouse melanoma cell lines, both obtained from American Type Culture Collection (Manassas, VA). A549 human alveolar type II cells were cultured in RPMI 1640 medium supplemented with 10% inactivated fetal bovine serum, 1 mM l-glutamine (all from Gibco, Gaithersburg, MD), 100 U/ml penicillin, 100 mg/ml streptomycin and 2 μg/ml amphotericin B, and buffered with 1% HEPES (all from Sigma, St. Louis, MO). B16-F10 mouse melanoma cells were cultured in D-MEM medium (Gibco), supplemented with 10% inactivated fetal bovine serum (Gibco), 200 U/ml penicillin, 200 mg/ml streptomycin and 2 μg/ml amphotericin B (all Sigma). All cell cultures were maintained in a standard cell culture incubator (humidified air, 20% O₂, 75% N₂, 5% CO₂, 37 °C). One day before the experiment, the cells were dissociated by detaching the cells by rinsing the culture with 0.25% (w/v) Trypsin-EDTA (1X) solution (25200, Gibco) and plated on 18 mm glass coverslips (2 × 10⁵ and 10⁶ cells/cover slips for A549 and B16-F10, respectively). The samples were left overnight in the incubator and were transferred to the bioreactor the following morning.

2.4. Feasibility tests

Before the experiment, the bioreactor and the thermal chamber enclosing it were sterilized with ethanol and washed with distilled water. Each medium container (Fig. 1) was filled with 30 ml of cell culture medium, which was buffered with 1% HEPES (Sigma), and one reservoir was bubbled with normoxic air and the other with hypoxic air (5% CO₂ in both cases) (Fig. 1). Immediately after the transfer of the coverslips samples to the bioreactor’s culture dish, the bioreactor pumps were activated to subject the cells to a pattern of hypoxia (20 s, 5% O₂– normoxia (50 s) (Fig. 2) for 8 h. After their exposure to intermittent hypoxia, the cell cultures were observed with phase contrast images obtained with a b/w digital camera (AVT Marlin F-145B2, Allied Vision Technologies, Germany) coupled to an inverted microscope (Eclipse TE2000, Nikon, Japan). Cell number was quantified after staining with nuclear Hoechst dye 33342 (Invitrogen, 1 μg/ml diluted in PBS, 20 min incubation). Images were acquired with a 12 bit resolution cooled-CCD camera (Orca AG, Hamamatsu Photonics, Japan) attached to an inverted microscope (Ti -HUBC/A, Nikon, Japan) using the Metamorph software (Molecular Devices, Sunnyvale, CA). The number of nuclei was counted using the Fiji open-source image processing package and the average value was calculated from four randomly selected areas per sample. All the conditions were checked with duplicate coverslips. The experiment was repeated 4 days for the alveolar epithelial cells and 6 days for the melanoma cells.

In addition to the intermittent hypoxia experiments, two types of control measurements were simultaneously carried out. On the one hand, the cell coverslips were placed in a bioreactor identical to the one used for intermittent hypoxia but in this case the gas bubbled inside the two culture-medium containers was normoxic (dynamic normoxia). Accordingly, any change observed when comparing the data obtained from the two settings could be attributed to the intermittent hypoxia exclusively. On the other hand, we carried out a second control maintaining cell coverslips in a conventional normoxic incubator (static control). A comparison of the results obtained with the conventional incubator and those obtained with the bioreactor when using normoxic air would explain the differences induced by the dynamic vs. static conditions of the culture medium. The number of cells counted in each
condition was normalized to the number of cells at dynamic normoxia in order to compute an index independent of the initial number of seeded cells in the coverglass. The potential effects induced by the three culture conditions in both cell lines were assessed by two-way ANOVA. Data were considered significant for \( p < 0.05 \).

At the end of the intermittent hypoxia and dynamic normoxia experiments on the melanoma cells, the cultures were processed by immunostaining to assess the expression of vascular endothelial growth factor (VEGF) and of vascular cell adhesion molecule-1 (VCAM-1) as biomarkers. VEGF and VCAM-1, biomarkers involved in the response to intermittent hypoxia, are expressed by the B16-F10 melanoma cells (Brandon et al., 2009; Locatelli et al., 2011) and could potentially play a role in the increase in cancer progression recently observed in response to intermittent hypoxia mimicking OSA (Almendros et al., 2012). Samples were fixed with 4% P-formaldehyde (PFA) for 15 min, washed with PBS, blocked with blocking solution for 1 h (1% BSA, 6% fetal bovine serum, 0.5% triton in TBS) and incubated overnight with the primary antibodies. The primary antibodies used were anti-VEGF (Millipore, ABS82) and anti-VCAM-1 (H-276) (Santa Cruz Biotechnologies, sc-8304). After the overnight incubation, the samples were washed with 0.1% BSA, 6% fetal bovine serum and 0.1% triton in TBS and incubated with the secondary antibodies (Alexa Series, all from Jackson Immunoresearch). The samples were also counterstained with DAPI (Invitrogen, 21490). Images were taken using Leica SP5 confocal microscope, maintaining the same settings acquisition for all the samples. VEGF and VCAM-1 expression was indirectly assessed by measuring the intensity of the fluorescence signals using the ImageJ software. Briefly, five z-stack images were captured in five different regions of each sample, using a 40X (NA 1.25, oil) objective (Leica SP5 confocal microscope). For each z-stack image, the mean fluorescence intensity per cell was calculated by dividing the sum of the intensity of each section of the z-stack by the total cell number.

3. Results

Real-time measurement of the oxygen concentration inside the cell culture dish proved the bioreactor’s ability to generate fast-rate intermittent hypoxia patterns at cell level. Fig. 2 shows examples of the \( O_2 \) concentrations patterns measured at cell level when applying different settings (composition of the gas sources and timing of the pumps). Fig. 2A and B displays two oxygen patterns with a periodicity of 90 s, but with different durations of the hypoxic phase. In order to illustrate how the bioreactor can modify the oxygen concentration in the culture, Fig. 2C and D shows two intermittent hypoxic patterns measured at cell level when the gas bubbled in one of the reservoirs was modified to provide oxygen-enriched air.

When the cell cultures were analyzed by counting the cell numbers, non-significant differences, due either to cell type or to culture conditions, were found. For the alveolar epithelial cells, the cell number index was 1.00 ± 0.28 when subjected to dynamic normoxia, 1.07 ± 0.12 when cultured during intermittent hypoxia and 1.36 ± 0.20 in the static normoxic control. In the case of the melanoma cells, the data were 1.00 ± 0.23, 0.86 ± 0.47, and 1.31 ± 0.66, respectively. Fig. 3 shows an example illustrating how A549 and B16-F10 cell culture exhibited a similar pattern for the three culture conditions: static normoxia, dynamic normoxia and intermittent (dynamic) hypoxia.

Fig. 4 shows an example of the expression of VEGF and VCAM-1 when the melanoma cells were subjected to static control, dynamic normoxia and intermittent hypoxia. In comparison with static normoxia, both biomarkers increased their normalized fluorescent intensity when subjected to dynamic normoxia: from 1.00 ± 0.10 to 1.63 ± 0.32 (\( p < 0.05 \)) for VEGF and from 1.00 ± 0.31 to 1.95 ± 0.45 (\( p < 0.05 \)) for VCAM-1. VEGF and VCAM-1 fluorescence were 51% and 22% higher under intermittent hypoxia (2.46 ± 0.18; \( p < 0.05 \) and 2.38 ± 0.58; \( p < 0.05 \), respectively) than under dynamic normoxia.

4. Discussion

We designed a novel bioreactor for applying high-frequency intermittent hypoxia to cultured cells. The system, based on the exchange of culture media preconditioned with desirable oxygen concentrations, is able to subject cells to well-controlled changes in oxygen concentration with an amplitude range and variation rate mimicking the conditions characteristic of the hypoxic events experienced by patients with OSA.

Studying the pathophysiology of OSA at cellular level requires subjecting the cell cultures to cyclic hypoxic/normoxic events with...
a periodicity of around 1 min. This is the rate of change corresponding to an apnea–hipopnea index of 60 events/h, which is representative of patients with severe OSA. Whereas it is easy to investigate OSA in animal models by modifying the oxygen concentration in breathed gas (Farre et al., 2008b), it is more difficult to carry out cell studies under conditions of realistic intermittent hypoxia in vitro. In fact, the response of cells to intermittent hypoxia has been studied to date only at frequencies much lower than those found in OSA patients (Ryan et al., 2005; Polotsky et al., 2010; Yuan et al., 2005; Nanduri et al., 2009). The conventional setting, which involves cyclically changing the oxygen concentration in the gas above the interface between the air and the cell culture, has considerable limitations. Indeed, when the cell culture is placed inside an airtight chamber sealed and flushed with gas at the desired oxygen concentration, more than 2 h are needed for the oxygen concentration in the cell culture medium to equilibrate with that of the supplied gas (Allen et al., 2001). Accordingly, this setting is incapable of providing the decrease/recovery times, lasting only a few seconds, that are required to achieve cyclic periods of around 1 min. Short equilibration times are, therefore, an essential

**Fig. 3.** Example of (A) alveolar epithelial cells A549 and (B) mouse melanoma cells B16-F10 subjected for 8 h to: (i) intermittent hypoxia (cycles of 50 s at 20% $\text{O}_2$ and 20 s at 5% $\text{O}_2$), (ii) dynamic normoxia and (iii) conventional static normoxia. Scale bar equals 200 μm.

**Fig. 4.** Example of (A) VEGF and (B) VCAM-1 expression by B16-F10 cells subjected for 8 h to: (i) intermittent hypoxia, (ii) dynamic normoxia and (iii) conventional static normoxia. Scale bar equals 50 μm.
prerequisite for any in vitro experiment trying to mimic the intermittent hypoxia patterns observed in OSA patients’ arterial blood.

Recently, two new settings have been proposed to circumvent the problem associated with the slow oxygen diffusion time from the air–liquid interface to the cells at the bottom of the culture plate. These two approaches are based on miniaturizing the volume of the culture sample to allow fast changes in oxygen concentration at the cell level. One of these systems is based on micro-fabricated channels (Lam et al., 2009). The other approach involves building special casts for conventional well–culture plates so that the high permeability of a very narrow membrane separating the gas flow channel from the cell culture compartment permits fast adjustment of the oxygen concentration in the culture medium (Oppergard et al., 2010, 2009). Both techniques allow high rates of changes in oxygen concentration at the cell level. Nevertheless, the equipment used in these experimental approaches is not readily available to most labs because of the need for special technologies to construct the high-precision miniaturized cell culture settings. Moreover, it is worth noting that our approach, in contrast to bioreactors based on liquid convection through cell cultures inside capillary tubing (Baumgardner and Otto, 2003), presents the advantage that the cells are cultured on standard glass coverslips and are thus readily available for inspection with any microscopy technique or bio-analytical assay.

In contrast with previously proposed methods, the novel bioreactor described in this work is easily assembled since it requires simple commercially available components used in various kinds of bioreactors (Burdick and Vunjak-Novakovic, 2009). First, cell culture is carried out in common culture wells, the only special requirement being to drill a hole in their base in order to connect a narrow tube (Fig. 1). Second, the container, bubbling gas system and tubing are components that are easy to assemble from conventional lab material for cell culture, whether disposable or sterilizable. Third, the peristaltic pumps – which are not in direct contact with the culture media – are inexpensive and their cycling can be driven by an electronic timer system or, more easily, through a computer output. In addition to its simplicity of construction and low cost, the bioreactor in Fig. 1 is also easy to operate since the actual oxygen concentration in the cell culture is determined by adjusting the operating times in each pump and the composition of the hypoxic gas source. The use of a microsensor for measuring the actual oxygen concentration at cell level is not necessary for routinely operating the system once a given pattern has been set.

A potential limitation of the bioreactor we have devised comes from the fact that its operation is based on changing the culture medium to which cells are exposed. The cells are not surrounded by a static liquid, as in a conventional culture, and therefore during the periods of culture medium pumping they are exposed to a certain degree of shear stress. As it is well known that this mechanical stimulus can affect cell response, parallel control experiments are required under normoxic conditions, both static and dynamic. In our experiments to test the performance of the bioreactor in cells, we did not observe any significant effect of the culture conditions in the number of cells after 8 h of experiments (a duration equivalent to a full night of apneic events in an OSA patient). This finding is consistent with published data indicating that for such a short period of time, under constant or quasi-constant intermittent hypoxia, the number of alveolar epithelial cells (Botto et al., 2008) and melanoma cells (Rofstad et al., 2010) is not different from that of normoxic controls. However, this result does not exclude other relevant cell responses (e.g. oxidative stress, inflammation, and metabolism) being affected by potential shear stress during intermittent hypoxia, as suggested by the example in Fig. 4. Accordingly, the magnitude of medium flow during injection/to/withdrawal from the culture well should be minimized, as we did in our setting. Given that some degree of shear stress, however minute, is unavoidable, it is advisable to also carry out control measurements under the same pattern of dynamic hypoxia/normoxia, but with a different magnitude of flow generated by the pumps (easily achieved by controlling the latter’s voltage supply). Such a control experiment would make it possible to quantify any significant effect of shear stress on the investigated variable. In this regard, it is worth noting that any potential difference in cell response observed when comparing static normoxia and dynamic normoxia (e.g. in Fig. 4) could also be due to the fact that the cell culture is homogenized under dynamic conditions. Under static conditions, however, the culture medium in contact with the cell is not renewed, and so potential gradients of metabolite concentration within the medium can be established, despite natural convection in the static culture medium (Titmash et al., 2011). Accordingly, although conventional static cell culture conditions are the obvious laboratory gold standard, the cell environment in this experimental setting does not necessarily coincide with the in vivo cell surrounding.

The designed bioreactor can be applied to a wide variety of cells since virtually all the body cells are subjected to intermittent hypoxia in patients with OSA. The most obvious ones are the alveolar epithelial and arterial endothelial cells, since these are in the front line in sensing any alterations in oxygenation caused by apneas. In fact, some of the more clinically relevant consequences of OSA are cardiovascular events triggered by endothelial dysfunction (Yuan et al., 2011; Lavie and Polotsky, 2009). Moreover, markers of lung injury have been detected in OSA patients (Lederer et al., 2009). Furthermore, the cells in other body tissues experience intermittent hypoxia in OSA, as reported by recent measurements of local oxygen partial pressure in the brain, muscle and fat of rats subjected to realistic obstructive apneas (Almendros et al., 2010, 2011). It has also recently been shown that recurrent obstructive apneas activate and mobilize different types of adult stem cells residing in the bone marrow (Chariot et al., 2010; Carreras et al., 2009, 2010; Berger and Lavie, 2011; Kheirandish-Gozal et al., 2010a) and that intermittent hypoxia increases the growth rate of melanoma tumors in rats (Almendros et al., 2012). Although the design of the bioreactor described in this work has focused on intermittent hypoxia, by using CO2 enriched air in one of the gas sources, the device can also be used to study the effect of the intermittent hypercapnia that is associated with intermittent hypoxia during obstructive apneas. Although research in OSA has been focused on the effects of intermittent hypoxia (probably because this is a major injurious stimulus), the effects of changes in CO2 concentration have scarcely been studied. It seems, however, that intermittent hypercapnia should play a role in OSA, as recently suggested in the brain of rats subjected to intermittent hypoxia and obstructive apneas (hypoxia plus hypercapnia) (Almendros et al., 2011). It is interesting to note that the bioreactor we have devised can also be used to investigate basic aspects of the cell physiology in hypoxia. Specifically, the device could be a tool to implement the concept of external forced oscillation (widely used in respiratory mechanics (Bates et al., 2011)), to investigate the different cell responses to continuous or intermittent hypoxia at different frequencies. In fact, it has been reported that a central mechanism in hypoxia response such as the activation of Nf-κB depends on the frequency of the stimulus (Wang et al., 2011; Kobayashi and Kageyama, 2009) and that gene expression pathways are modulated by the frequency of the hypoxic stimulus (Wu et al., 2008).

5. Conclusions

The novel bioreactor described in this work subjects cells cultured in conventional glass coverslips to hypoxic patterns closely mimicking the ones experienced by cells in different tissues.

during OSA events. This setting is therefore a tool for enhancing the investigation of the pathophysiology of this disease at cell level.

Acknowledgments

The authors thank Miguel A. Rodríguez and Rocío Nieto for their help in the laboratory procedures. This work was supported in part by Ministerio de Ciencia e Innovación (SAF2009-2991 and PI081908). Dr. Elena Garreta was funded by a Marie Curie-IDIBAPS fellowship.

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