METHODS IN CELL PHYSIOLOGY

An innovative intermittent hypoxia model for cell cultures allowing fast PO2 oscillations with minimal gas consumption

Mélanie Minoves,1,2 Jessica Morand,1,2 Frédéric Perriot,1,2 Morgane Chatard,3 Brigitte Gonthier,1,2 Emeline Lemarié,1,2 Jean-Baptiste Menut,4 Jan Polak,5 Jean-Louis Pépin,1,2,6 Diane Godin-Ribuot,1,2 and Anne Briançon-Marjollet1,2

1HP2 Laboratory, Université Grenoble Alpes, Grenoble, France; 2INSERM, U1042, Grenoble, France; 3Université de Lyon and Université Jean Monnet, Saint-Etienne, France; 4SMTEC, Chemin des Vignes, Nyon, Switzerland; 5Center for Research on Diabetes, Metabolism and Nutrition Third Faculty of Medicine, Charles University, Prague, Czech Republic; and 6Centre Hospitalier Universitaire, Grenoble, France

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An innovative intermittent hypoxia model for cell cultures allowing fast PO2 oscillations with minimal gas consumption. Am J Physiol Cell Physiol 313: C460–C468, 2017. First published July 26, 2017; doi:10.1152/ajpcell.00098.2017.—Performing hypoxia-reoxygenation cycles in cell culture with a cycle duration accurately reflecting what occurs in obstructive sleep apnea (OSA) patients is a difficult but crucial technical challenge. Our goal was to develop a novel device to expose multiple cell culture dishes to intermittent hypoxia (IH) cycles relevant to OSA with limited gas consumption. With gas flows as low as 200 ml/min, our combination of plate holders with gas-permeable cultureware generates rapid normoxia-hypoxia cycles. Cycles alternating 1 min at 20% O2 followed by 1 min at 2% O2 resulted in PO2 values ranging from 124 to 44 mmHg. Extending hypoxic and normoxic phases to 10 min allowed PO2 variations from 120 to 25 mmHg. The volume of culture medium or the presence of cells only modestly affected the PO2 variations. In contrast, the nadir of the hypoxia phase increased when measured at different heights above the membrane. We validated the physiological relevance of this model by showing that hypoxia inducible factor-1α expression was significantly increased by IH exposure in human aortic endothelial cells, murine breast carcinoma (4T1) cells as well as in a blood-brain barrier model (2.5-, 1.5-, and 6-fold increases, respectively). In conclusion, we have established a new device to perform rapid intermittent hypoxia cycles in cell cultures, with minimal gas consumption and the possibility to expose several culture dishes simultaneously. This device will allow functional studies of the consequences of IH and deciphering of the molecular biology of IH at the cellular level using oxygen cycles that are clinically relevant to OSA.

cell hypertrophy; diabetic nephropathy; fibrosis; microRNAs; mTOR complex 1

INTERMITTENT HYPOXIA (IH) is the hallmark of obstructive sleep apnea (OSA), a common chronic disease affecting 5–20% of the general population and characterized by recurrent collapses of the upper airway, leading to the repetitive occurrence of oxygen desaturationreoxygenation sequences (14). OSA is recognized as an important and independent risk factor for hypertension, coronary heart disease, and stroke and could also be associated with mild cognitive dysfunction (16). Moreover, recent studies suggest that the excess mortality in OSA could be at least partly due to an increased risk of cancer (5, 19). The molecular pathways underlying the deleterious consequences of OSA are under investigation (8, 10, 26) but cellular and molecular mechanisms remain poorly understood. Moreover, clinical research is limited by confounding factors that make it difficult to distinguish between the respective effects of intermittent hypoxia and comorbidities. Since stroke and coronary heart disease are common OSA-associated comorbidities, increased knowledge of the effects of IH exposure of endothelial cells and blood brain barrier appears to be crucial. Finally, IH is also observed in tumors and exposure of tumor microenvironment to IH might promote tumor growth and metastatic activity (1).

In this context, significant efforts were recently made in several leading laboratories in the field to obtain relevant models of IH in cell cultures. The achievement of rapid oxygen cycles in standard culture dishes is flawed by the very slow oxygen diffusion in culture medium in the absence of mixing (2). Moreover, thermally induced convective mixing of the media is not sufficient to ensure rapid oxygen equilibration across the height of medium (2, 17, 20) thus limiting the development of relevant IH systems. For instance, one of the first cellular IH models, based on air flushing in a Lucite chamber, generated cycles alternating 15 s of 1% O2 and 3 min of 21% O2 that allowed only limited (between 50 and 70 mmHg) fluctuations in PO2 in the culture medium (12). This highlights the challenge of oxygen diffusion for effective cell exposure to IH. Longer cycles have allowed cycling between 2% O2 and 15% O2, but with cycle durations of 1 h (11) and 1.5 h (28), that are not clinically relevant in the context of OSA-related IH. Another strategy has consisted in using preequilibrated culture medium (2, 24, 27). In these systems, the use of preconditioned medium does ensure instantaneous oxygen changes at the cell level. However, repeated changes in medium complicate the measurement of soluble factor secretion and can induce an important shear stress that could impact cell activity and metabolism. More recently, a team has proposed a system in which gas was...
directly injected in the culture flask (23). They alternated 5 min of 16% O2 and 5 min with 0% O2, resulting in six cycles per hour, which was the best compromise to achieve sufficient oxygen variation amplitude.

Finally, the last type of system described in the literature uses gas-permeable dishes to obtain rapid and accurate cycles at the cell level (18, 22). In this system, variations between 16% and 1% O2 lead to similar oxygen variations in the culture medium within minutes, allowing a frequency of six cycles per hour without any change in medium or bubbling. The major disadvantage of this elegant setting is the high gas consumption necessary to replace the air volume of the cabinet incubator hosting the dishes. Achievement of the control normoxic exposure also relies on expensive premixed gas bottles (16% O2, 5% CO2, and 79% N2). Recently, a variant of this system, based on air circulation underneath highly-permeable polydimethylsiloxane (PDMS) membranes for cell culture, achieved very fast oxygen cycles at the cell level in a system adapted for direct microscopy imaging (4). The major limitation of this model relies on its small size (4-mm-diameter dishes) and thus in the low number of exposed cells, preventing large-scale studies or the collection of cells for biochemical or molecular biology studies.

Therefore, our objective was to set up a cost-effective and rapid-cycling model producing IH cycles able to mimic the tissue oxygenation characteristics of OSA. We aimed at developing and characterizing a device that would allow rapid oxygen cycling and, minimal gas consumption, avoiding the use of expensive premixed gas bottles, and allowing exposure of multiwell plates and larger culture plates. The originality of this system is that it generates rapid cycles in culture media while minimizing gas consumption by using gas-permeable cell cultureware and custom-made holders in which the air is renewed only below the dishes.

MATERIALS AND METHODS

Gas-permeable dishes and plate holders. Gas-permeable dishes were either from Sarstedt (Lumox 55-mm dishes, 24- and 96-well plates, Sarstedt, Germany) or from Zell-Kontakt (Fluorocarbon Imaging plates, 24- and 96-well plates, Zell-Kontakt, Germany). The thickness of the membrane is 25 μm for Zell-Kontakt plates and Sarstedt dishes and 50 μm for Sarsted plates. Since poor cell adhesion was observed with both types of membranes, they were coated with type I collagen (0.2 mg/ml for 1 h at 37°C) before cells were seeded.

Transwells with either 0.4-μm or 8-μm pores (Corning) were placed in 24-well Zell-Kontakt plates. Transwells and plate wells were filled with 200 μl and 600 μl of culture medium, respectively.

The plate holders were custom-made (SMTEC, Nyon, Switzerland) to accommodate either 55-mm dishes or 24- or 96-well plates (Fig. 1). The dishes or plates are attached to the holders with clips to minimize air leakage. The air volume under the plate or dish is as low as 15–20 ml thus allowing very rapid flushing, even with flows as low as 200 ml/min, and very low gas consumption.

Gas supply. Compressed air and ≥98% nitrogen are provided by a nitrogen extractor and a gas compressor, and carbon dioxide is supplied by a gas bottle. The three gas inputs are connected to a gas blender (Gas Blender 100, MCQ Instruments, Rome, Italy) that can mix gases in any desired fraction with a maximum output flow of 250 ml/minute. The gas mixture is heated in a water bath set a 37°C and travels through a 5-m-long plastic tube to allow temperature equilibration before being transmitted to culture plate holders hosted in a standard cell culture incubator. Pressure equilibration is possible with air above the culture medium, thus the system operates under normobaric conditions.

Table 1. Gas settings and duration (in minutes) of the normoxic and hypoxic phases of the various protocols tested

<table>
<thead>
<tr>
<th>Protocol</th>
<th>20%</th>
<th>16%</th>
<th>3%</th>
<th>5%</th>
<th>2%</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% 30°–30’</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>2% 1°–1’</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>2% 2°–2’</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>2% 3°–3’</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>2% 3°–4’</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>2% 4°–5’</td>
<td>1</td>
<td>4</td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>5% 2°–2’</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>5% 3°–3’</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

In all the protocols except 2% 30°–30’ and 2% 1°–1’, a 20% O2 normoxic burst was used before stabilization at 16% O2 to decrease the duration of the ascending phase. Similarly, in 5% O2 protocols, a 3% O2 hypoxic burst was used before stabilization at 5% O2 to decrease the duration of the descending phase.
Oxygen measurements. Oxygen in air was measured with a gas analyzer (ADInstruments, Oxford, UK). Dissolved oxygen in culture medium was measured with precalibrated fluorescent oxygen sensors (PO2 E-Series Sensor) coupled to an Oxylite device (Oxford Optronix, Oxford, UK). The sensors have a 90% response time of 20 s and a precision of 0.1 mmHg. The PO2 sensors were maintained by a holder equipped with a micrometer screw and a binocular microscope, allowing the direct observation of the contact between the probe and the membrane, and the vertical movement of the sensor by 60-μm steps from the membrane level (pericellular environment) up to the top of the culture medium. In sealed dishes, the probe was inserted through a needle hole in the lid before sealing. Data were recorded using an acquisition system (Powerlab, ADInstruments, Oxford, UK).

Experimental intermittent hypoxia protocols. Different PO2 cycling protocols were programmed to characterize the behavior of the model (Table 1). The IH protocols alternated a 20% or 16% normoxic phase and a 5% or 2% hypoxic phase. The 16% oxygen value of normoxic phase was chosen according to the mean arterial PO2 value of healthy humans, as in other IH models (22, 23). We programmed IH protocols with 5% (5% 2'-2' and 3'-3') and 2% (2% 30''-30'', 1'-1', 2'-2', 3'-3', 3'-5' and 5'-5') hypoxic phases named according to the duration (in seconds or minutes) of the normoxic and hypoxic phases, respectively. Following hypoxia, a brief 20% O2 phase was used to accelerate the return towards the normoxic 16% plateau. Similarly, a 1-min burst of 3% PO2 was added to rapidly attain a stable 5% hypoxic plateau. No burst was used to attain the 2% hypoxic plateau, since 2% is the minimum oxygen contain of our gas supply (98% pure nitrogen). Carbon dioxide was always set at 5% in the gas mixture.

Cell culture and hypoxia-inducible factor-1α quantification. Human aortic endothelial cells (HAoEC, Cascade Biologics) were cultured in M200 medium, supplemented with large vessel endothelial

Fig. 2. Characterization of PO2 cycles at the membrane level in 55-mm dishes containing 5 ml of culture medium without cells. A–F: typical cycles measured with the following intermittent hypoxia protocols: 2% 30''-30'' (A), 2% 1'-1' (B), 2% 2'-2' (C), 5% 2'-2' (D), 2% 3'-3' (E), and 2% 5'-5' (F). G: peak and nadir PO2 values and amplitude of PO2 variation for each protocol. Data are from at least 3 independent measurements and for over 10 independent experiments for protocol 2% 5'-5'. H: cycles measured in three 55-mm dishes exposed simultaneously in holder to protocol 5% 2'-2'.
supplement (LVES) as recommended by the manufacturer, and used after 6–8 population doubling. Human breast carcinoma cells (4T1, graciously provided by V. Josserand, IAB, Grenoble, France) were cultured in RPMI medium supplemented with 10% fetal bovine serum and antibiotics. The blood-brain barrier (BBB) model is composed of bEnd.3 endothelial cells (ATCC) combined with C6 astrocytes (ATCC) cultured in transwells with 0.4-µm pores, as previously described (6).

H4oEC and 4T1 cells were exposed to 6 h of IH (protocol 2% 5′–5′). BBB model was exposed to 2 h of IH (protocol 2% 5′–5′) followed by 6 h of normoxia (16% O2), repeated 3 times for a total of 24 h.

At the end of IH exposure, supernatants were collected and frozen (−80°C), while the cells were fixed in 4% paraformaldehyde (PFA) for 10 min and kept at 4°C until use. Hypoxia-inducible factor-1α (HIF-1α) expression was measured with a cell-based Elisa assay (R&D Systems) on PFA-fixed cells.

Statistical analysis. Data were analyzed using GraphPad Prism 6 software (San Diego, CA). The various statistical tests performed are detailed in figure legends. Normality was determined using D’Agostino-Pearson normality tests as recommended by the GraphPad Prism software. Normal data are expressed as means ± SE. Statistical significance was set at \( P < 0.05 \).

RESULTS

Rapid intermittent hypoxia cycles with appropriate amplitude were achieved with the model. Figure 2 and Table 2 depict typical oxygen cycles observed in 55-mm dishes, filled with 5 ml of PBS, with the various protocols tested (see Table 1 for details of gas settings).

Short cycles composed of 30 s at 20% followed by 30 s at 2% resulted in oxygen cycling between 105.8 ± 1.3 and 58.5 ± 2.2 mmHg in the culture medium (Fig. 2A). Increasing the duration of the normoxic and hypoxic phases enhanced the nadir-to-peak amplitude, with cycles between 124.3 ± 2.4 and 44.3 ± 1.5 mmHg (nadir-to-peak amplitude: 79.9 ± 3.6 mmHg) with protocol 2% 1′–1′ (Fig. 2B), and between 118.8 ± 2.4 and 33.8 ± 0.3 mmHg (nadir-to-peak amplitude: 84.4 ± 1.9 mmHg) with protocol 2% 2′–2′ (Fig. 2C). Protocols with a 2-min normoxic phase resulted in a PO2 plateau around 120 mmHg and PO2 values >110 mmHg were achieved within a mean time of 52.1 ± 2.2 s. Protocols with 5% O2 generated a hypoxic plateau around 40 mmHg (Fig. 2D and Table 2). For protocols with 2% O2, a hypoxic plateau was more difficult to achieve, even with a 5-min hypoxia duration (Fig. 2, E and F) and time to reach PO2 values < 50 mmHg and < 40 mmHg was 47.2 ± 4.5 and 89.0 ± 9.1 s, respectively. PO2 values < 30 mmHg were reached after 3 min of hypoxia with protocols 2% 3′–3′ and 2% 5′–5′, while the minimum value of 25 mmHg could only be attained after 5 min of hypoxia (Fig. 2, F and G and Table 2). On the other hand, decreasing the duration of normoxia from 5 to 3 min resulted in identical peak and nadir PO2 values (Fig. 2F and Table 2).

Since our 55-mm dish holder is designed for three dishes, we measured the cycles in the three dishes and did not observe any difference in cycle pattern or PO2 range (Fig. 2H).

Effects of cultureware type and membrane thickness on intermittent hypoxia cycles. We compared the impact of support type and membrane thickness on the nadir-to-peak amplitude of the cycles generated by protocols 2% 3′–3′ and 2% 5′–5′. Whatever the protocol, we did not observe any difference on nadir-to-peak cycle amplitude between 25 µm-thick 24-well or 96-well plates and 55-mm dishes. Hence, typical cycles generated by protocol 2% 5′–5′ in 24-well plates were between 26 and 113 mmHg (Fig. 3A), similar to those observed in 96-well plates and 55-mm dishes. We serially connected three 96-well plates to compare the typical aspect of the PO2 cycles. We did not observe a difference in PO2 cycles between the three plates (Fig. 3B).

However, there was a significant difference in nadir values and nadir-to-peak amplitude of the cycles measured in 25-µm-thick and 50-µm-thick 24-well plates (Fig. 3C). Hence, nadir values were significantly lower in 25-µm-thick plates compared with 50-µm-thick plates (27.0 ± 1.9 vs. 55.4 ± 2.3 mmHg, respectively; \( P = 0.008 \)) while peak PO2 values were not affected (118.6 and 114.6 mmHg, respectively). Therefore, nadir-to-peak amplitude was 91.8 ± 2.9 mmHg in 25-µm-thick plates but only 59.2 ± 2.9 mmHg in 50-µm-thick plates (\( P = 0.008 \)).

Culture medium volume modestly alters the intermittent hypoxia cycles. Overall, culture medium volume had a significant impact on the cycles (\( P = 0.037 \)) (Table 3). In particular, the lowest nadir value was obtained with a volume of 5 ml (27.2 ± 2.4 mmHg vs. 36.7 ± 2.9 mmHg with a 3 ml volume, \( P = 0.019 \)). However, the nadir-to-peak amplitude was not significantly affected by culture medium volume (101.3 ± 2.9, 103.2 ± 1.6, and 104.7 ± 0.3 mmHg for 3, 5, and 7 ml, respectively).

Presence of cells decreases the amplitude of intermittent hypoxia cycles. The presence of cells induced a significant decrease in the nadir-to-peak amplitude of the IH cycles (from 95.6 ± 3.0 to 86.5 ± 1.5 mmHg, \( P = 0.004 \); Table 4). More precisely, the presence of cells decreased the maximum PO2 value (from 124.4 ± 0.9 to 113.1 ± 3.5 mmHg, \( P < 0.01 \)) without affecting the minimum value.

Amplitude of intermittent hypoxia cycles decreases from bottom to top of culture medium. To test the homogeneity of the cycles throughout the culture medium, we recorded oxygen cycles from the membrane up to the top of the liquid layer using protocols 2% 3′–3′ and 2% 5′–5′. To estimate the effect of gas exchange with ambient air we also measured PO2 gradient with protocol 2% 5′–5′ in dishes sealed with tape. Whatever the distance from the membrane and the IH protocol, the maximum PO2 value remained stable. However, as expected, the minimum PO2 value linearly increased when the O2 probe was moved away from the membrane and the nadir-to-peak amplitude therefore concomitantly decreased (Fig. 3D).

Table 2. Nadir and peak PO2 values and cycle amplitude (calculated delta value) measured in 55-mm dishes submitted to the intermittent hypoxia protocols depicted in Table 1

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Nadir</th>
<th>Peak</th>
<th>Delta</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% 3′–3′*</td>
<td>58.5 ± 2.2**</td>
<td>105.8 ± 1.3**</td>
<td>47.3 ± 3.0**</td>
</tr>
<tr>
<td>2% 1′–1′*</td>
<td>44.3 ± 1.5**</td>
<td>124.3 ± 2.4</td>
<td>79.9 ± 3.6</td>
</tr>
<tr>
<td>2% 2′–2′</td>
<td>33.8 ± 0.3</td>
<td>118.2 ± 2.4</td>
<td>84.4 ± 1.9</td>
</tr>
<tr>
<td>2% 3′–3′</td>
<td>30.3 ± 1.4</td>
<td>115.4 ± 4.4</td>
<td>85.1 ± 3.7</td>
</tr>
<tr>
<td>2% 3′–5′</td>
<td>24.9 ± 2.0</td>
<td>120.4 ± 4.5</td>
<td>95.5 ± 3.3</td>
</tr>
<tr>
<td>2% 5′–5′</td>
<td>25.5 ± 1.2</td>
<td>120.6 ± 1.9</td>
<td>95.1 ± 1.6</td>
</tr>
<tr>
<td>5% 2′–2′</td>
<td>38.0 ± 3.1*</td>
<td>123.3 ± 3.3</td>
<td>85.3 ± 6.4</td>
</tr>
<tr>
<td>5% 3′–3′</td>
<td>39.7 ± 5.5*</td>
<td>126.0 ± 8.0</td>
<td>86.3 ± 4.5</td>
</tr>
</tbody>
</table>

Data (in mmHg) are means ± SE. *\( P < 0.05 \) and **\( P < 0.01 \) vs. 2% 5′–5′ protocol (two-way ANOVA). \( P = 0.008 \) for delta 2% 2′–2′ vs. 2% 5′–5′.
Moreover, AUC obtained with sealed-plates was significantly higher than that recorded in unsealed plates ($P < 0.012$; Fig. 4A).

Finally, to evaluate the possibility of using transwells for cocultures or for permeability or migration assays under IH, we measured cycles in transwell inserts placed in 24-well permeable plates. The nadir-to-peak amplitude of IH cycles measured in transwells increased with pore diameter. Indeed, amplitudes of 49 mmHg (nadir and peak $P_{O2}$ values of 78 and 127 mmHg) and 59 mmHg (nadir and peak $P_{O2}$ values of 66 and 125 mmHg values; Fig. 4C) were measured in transwells with 0.4-$\mu$m and 8-$\mu$m pores, respectively.

**Gas-permeable dishes allow optimal cell oxygenation under normoxic conditions.** Baseline culture medium $P_{O2}$ values measured in plastic dishes with 4T1 cells were significantly lower than those measured in permeable dishes independently of cell confluence (Fig. 5). At 70% confluence, median $P_{O2}$ values of 120 and 137 mmHg were measured in plastic and permeable dishes, respectively ($P = 0.016$). At 100% confluence, median $P_{O2}$ values of 115 and 150 mmHg were measured in plastic and permeable dishes, respectively ($P = 0.008$). Interestingly, no difference between plastic and permeable dishes was observed with 100% confluent HAoEC cells.
Table 3. Impact of culture medium volume on intermittent hypoxia cycles

<table>
<thead>
<tr>
<th>Volume</th>
<th>Nadir (mmHg)</th>
<th>Peak (mmHg)</th>
<th>Delta (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3ml</td>
<td>36.7 ± 2.9*</td>
<td>138.0 ± 0.1</td>
<td>101.3 ± 2.9</td>
</tr>
<tr>
<td>5ml</td>
<td>27.2 ± 2.4</td>
<td>130.3 ± 0.9</td>
<td>103.2 ± 1.6</td>
</tr>
<tr>
<td>7ml</td>
<td>29.3 ± 3.0</td>
<td>134.0 ± 3.1</td>
<td>104.7 ± 0.3</td>
</tr>
</tbody>
</table>

Nadir and peak PO2 values and cycle amplitude (calculated delta value) measured in 55-mm dishes filled with different volumes of culture medium and submitted to protocol 2% 5’-5’. Data (in mmHg) are means ± SE; n = 3 independent experiments. Medium volume significantly alters the cycles (P = 0.037; two-way ANOVA). *P = 0.019 vs. nadir at 5 ml (two-way ANOVA). P = 0.07 for nadir 3 ml vs. 7 ml, P = 0.06 for peak 3 ml vs. 5 ml.

Intermittent hypoxia exposure increases HIF-1α expression in cells. Six hours of cell exposure to IH with protocol 2% 5’-5’ resulted in a significant increase in HIF-1α expression compared with normoxia (2.4- and 1.4-fold increase in HAoEC and 4T1 cells, respectively, P < 0.01; Fig. 6A).

Moreover, repeated IH exposure over 24 h of a cell BBB model resulted in a very significant increase in HIF-1α expression compared with normoxia (6.1-fold increase, P < 0.01; Fig. 6B).

DISCUSSION

In this paper, we describe an innovative system aiming to perform exposure of cells cultures to intermittent hypoxia with a pattern accurately mimicking that occurring in sleep apnea patients. This system is adapted from commercial gas-permeable dishes and uses custom-made plate holders. The small volume of air in the holders is easily renewed even with low gas flow, thus allowing low gas consumption and fast cycling. Our system is working with gas sources (air, CO2, and N2) either from commercial bottles (CO2) or produced by gas extractor and compressor, thus avoiding expensive premixed gas bottles described elsewhere (22). We were able to connect up to three holders in series, thus allowing the simultaneous exposure of three multiwell plates (24-well or 96-well) or up to nine 55-mm dishes, or combinations of multiwell plates and 55-mm dishes. This low-cost system effectively produced short IH cycles in culture medium and allowed the exposure of a large number of cells. This should help the field to address the molecular biology of IH exposure with a relevant physiological model.

We characterized the oxygen cycles obtained with various settings, with normoxic phases between 20% and 12% oxygen and hypoxic phases between 5% and 2%. With 16–2% cycling at a frequency of 6 to 8 cycles per hour, we obtained nadir-to-

Table 4. Impact of the presence of cells on intermittent hypoxia cycles

<table>
<thead>
<tr>
<th></th>
<th>Nadir (mmHg)</th>
<th>Peak (mmHg)</th>
<th>Delta (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without cells</td>
<td>28.8 ± 2.4</td>
<td>124.4 ± 0.9</td>
<td>95.6 ± 2.9</td>
</tr>
<tr>
<td>With cells</td>
<td>26.7 ± 2.7</td>
<td>113.1 ± 3.5**</td>
<td>86.5 ± 1.5**</td>
</tr>
</tbody>
</table>

Nadir and peak PO2 values and cycle amplitude (calculated delta value) measured in 55-mm dishes filled with 4 ml of culture medium, with or without a confluent layer of 4T1 cells, and exposed to protocol 2% 5’-5’. The presence of cells does not change nadir values but lowers peak and delta values. Data (in mmHg) are means ± SE; n = 3 independent experiments performed in duplicate. **P < 0.01 vs. corresponding values without cells (two-way ANOVA).
and C6 astrocytes after 24 h of IH (2 h of 2% 

normoxia (Mann-Whitney rank-sum test).

PO2 values were measured at the level of the cell layer in standard 

normoxia. Cultures were preequilibrated at 21% O2 in a standard incubator, 

cells (HAoEC) or 4T1 cells at different cell confluences and incubated under 

plastic or gas-permeable 55-mm dishes plated with human aortic endothelial 

be slightly higher than the mean value of arterial PO2 in healthy 

patients during hypoxic episodes (15). Similarly, the peak can 

roughly corresponds to the lowest arterial blood saturation 

level (60% oxygen saturation) recorded in severe sleep apnea 

the amount of IH and its deleterious or preconditioning out-

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peak amplitudes of ~95 mmHg, cycling between 25 and 30 

mmHg and 120 mmHg. Although a nadir value of 25–30 

mmHg is not as low as those attained with other systems (4, 

our system can reproduce patterns of hypoxia-reoxygenation rele-

ant to severe human OSA by cycling between 30 and 100– 

120 mmHg or to moderate OSA by cycling between 40 and 45 

mmHg and 100–120 mmHg. This is a major advantage to 

appropriately assess the dose-response relationship between 

the amount of IH and its deleterious or preconditioning out-

Although similar cycles were observed in Lumox dishes and 

in fluorocarbon plates which have 25-µm-thick membranes, 

cycles measured in plates with 50-µm-thick Lumox mem-

branes presented high nadir values and decreased nadir-to-peak 

amplitudes. This suggests that Lumox membrane thickness can 

alter the cycles measured in the culture medium (data not 

shown). We also observed that the presence of cells modestly 

decreased peak values and amplitude of the cycles, whereas 

nadir values were unchanged. The difference in cycles in the 

presence of cells could be due to oxygen consumption and/or 
to impaired gas diffusion through the cell layer. Nevertheless, 
since the impact of the presence of cells was modest (<10% 
decrease in cycle amplitude), we can conclude that the mem-

brane maintains good gas permeability even in the presence of a 

confluent cell monolayer.

Variations in culture medium volume also modestly affected 

the IH cycles. While peak values and nadir-to-peak amplitude 

were not significantly altered, nadir values measured in 55-mm 

dishes were lower at high (5 ml) compared with low (3 ml) 

medium volume. This could be explained by oxygen retrodif-

fusion from air above the medium since low volumes result in 

thin thickness (~1 mm with 3 ml vs. 2 mm with 5 ml in 55-mm 

dishes). Thus, it appears mandatory to always use the same 

amount of culture medium to ensure reproducibility of the IH 
cycles.

A drawback of some IH systems is the limited number of 

doors or plates that can be exposed simultaneously, resulting in 
in insufficient amount of cells for biochemical or molecular 

biology analysis. To minimize gas consumption, we did not choose to perform IH cycles directly in the cell incubator as in 

other systems (22), but we wanted to be able to expose at least 

several 55-mm dishes or multiwell plates. Our use of custom-
designed plate holders branched in series enabled us to obtain 
similar cycles in up to three multiwell plates or nine 55-mm 
dishes (or combinations of multiwell plates and 55-mm dishes) 
simultaneously exposed to IH. This setup ensures that several 

experiments can be performed simultaneously and that a suf-

ficient quantity of cells can be collected for biochemical analysis or molecular biology.

However, since gases are diffusing from below the plate, we 

expected that the cycles would perform well at the membrane 

level only. This is perfectly suitable for adherent cells but 

represents a limitation for nonadherent cell cultures, such as 

monocytes or peripheral blood mononuclear cells, or for co-

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Fig. 6. Intermittent hypoxia (IH) increases 

hypoxia-inducible factor-1α (HIF-1α) ex-

pression. A: expression of HIF-1α in HAoEC 

and 4T1 cells after 6 h of exposure to proto-

col 2% 5% CO2, expressed as a ratio of HIF-1α 

expression in cells exposed to 6 h of nor-

moxia (16% O2). *P < 0.05 vs. normoxia 

(Mann-Whitney rank-sum test). B: expres-

sion of HIF-1α in a blood-brain barrier 

model composed of bEnd.3 endothelial cells 

and C6 astrocytes after 24 h of IH (2 h of 2% 

5% CO2 and 6 h of normoxia, repeated 3 times).

Individual data and median are plotted. n = 3 

independent experiments; **P < 0.01 vs. 

normoxia (Mann-Whitney rank-sum test).
culture systems such as transwell inserts. Indeed, while the peak value was unchanged, we observed that the nadir of the IH cycles gradually increased when measured away from the membrane so that the cycles almost disappeared at the top of the liquid layer. However, adequate IH cycles oscillating between 50 and 110 mmHg were still measured 500 μm above the membrane. Moreover, cycles measured inside 8-μm-pore transwells, commonly used for cell migration or permeability assays, oscillated between 65 and 125 mmHg, suggesting that our system can be used to study the effects of moderate IH on cell migration. Furthermore, our system was successfully used to induce a clear increase of HIF-1 expression in cells cultured in 0.4-μm-pore transwell.

Indeed, HIF-1α expression was increased by more than sixfold in a BBB model composed of endothelial cells and astrocytes cultured in transwells and exposed to IH. In addition to transwells, we observed that HIF-1α expression was increased by 2.6- and 1.5-fold, in primary endothelial and in tumor cells, respectively, after 6 h of IH exposure. It should be stressed that HIF-1α expression might have been underestimated in our experimental conditions since it was assessed in PFA-fixed samples, in which PFA could have interfered with antigen reactivity. In accordance with various other studies, we chose to use HIF-1α expression as a marker of hypoxia sensing to validate IH exposure of our cells. Hence, Yuan et al. (31) reported an increase in HIF-1 protein level in nuclear extracts together with an increase in HIF-1 reporter genes in PC12 cells after 60 cycles of IH (4 min–30 s, 20–1.5%), and more recently Campillo et al. (4) observed a 1.5-fold increase in HIF-1α nuclear translocation in mesenchymal stem cells exposed to 4 h of IH (30 s–30 s, 20–1%). In a very different system, exposure of endothelial cells to long IH cycles (1 h–30 min, 20–1%) did not increase HIF-1 gene expression but increased its nuclear translocation and DNA binding activity (29). Nevertheless, other studies have failed to observe changes in HIF-1 expression or transcriptional activity with different paradigms of IH exposure (22–24). Thus, despite numerous studies in animal models showing that HIF-1 is activated by intermittent hypoxia in various organs (3, 7, 30), the determinants of HIF-1 activation in cells exposed to IH remain to be assessed, with particular emphasis on the characteristics of the IH cycles (minimal and maximal Po2, duration of normoxic and hypoxic phases, total duration of exposure, or number of cycles) and on the metabolic activity of the cells exposed.

Finally, we compared standard plastic dishes and permeable dishes under normoxic conditions. We measured a difference of up to 50 mmHg between standard plastic and semipermeable dishes, depending on cell type and confluence rate. Indeed, the biggest difference was observed for metabolically active cancer cells. These results are consistent with previous data showing different levels of culture medium hypoxia with more or less metabolically active cells (17, 22). In particular, Polak et al. (22) showed that HIF-1 activity was much higher in standard than in permeable plates, probably because of poor oxygenation of culture medium in plastic plates along with O2 consumption by cells. Despite the fact that oxygen tension during standard cell culture is often a neglected factor, these results suggest that it could be of interest to use gas-permeable plates even in routine use under normoxic conditions, to ensure real and reproducible normoxia at different stages of cell culture and confluence levels.

It should be pointed out that the Po2 measurements performed in the present study were recorded with probes having a 90% response time of 20 s (data provided by the manufacturer and experimentally verified in our laboratory, data not shown), which is not negligible compared with the rise and decay duration of our IH cycles. Therefore, one can assume that the oxygen cycles performed by our system are actually faster than those measured and that the rise and decay durations are overestimated. Moreover, Po2 values measured for short cycles that did not reach plateau values might be inaccurate so that the nadir-to-peak amplitude might actually be larger than reported. Nevertheless, we can suggest that the peak and nadir Po2 values measured for longer IH cycles (3’–3’ or 5’–5’) were estimated with a good accuracy since we were able to attain plateau phases at both cases. Carbon dioxide was set at 5% in all gas mixtures, and since CO2 has a very high diffusion coefficient it was theoretically stable in the culture medium throughout intermittent hypoxia exposure.

In summary, we have developed a new low-cost device to perform rapid intermittent hypoxia cycles at the cellular level characterized by very low gas consumption and good cell throughput for biochemistry or molecular biology analysis. This device can be used to mimic the cellular impact of OSA but also that of other pathological conditions in which rapid hypoxia-reoxygenation cycles are observed, such as ischemia-reperfusion events in heart, brain, or other organs, or tumor development where oxygen variations can be observed independently of OSA, due to structural abnormalities of the vascular network (9, 25). This device thus presents a new alternative to existing techniques for laboratories that plan to perform cellular IH or simply want to precisely control oxygen tension in their cell cultures.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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