doi: 10.4149/gpb_2014043

# System for exposing cultured cells to intermittent hypoxia utilizing gas permeable cultureware

Jan Polak[1,2], Karen Studer-Rabeler[3], Holly McHugh[1], Mehboob A. Hussain[4] and Larissa A. Shimoda[1]

[1] *Division of Pulmonary and Critical Care Medicine, The Johns Hopkins University, School of Medicine, Baltimore, MD, USA*
[2] *Sport Medicine Department, Third Faculty of Medicine, Charles University in Prague, Ruska 87, Prague 100 00, Czech Republic*
[3] *Coy Laboratory Products Inc., Grass Lake, MI, USA*
[4] *Department of Pediatrics and Medicine, The Johns Hopkins University, School of Medicine, Baltimore, MD, USA*

**Abstract.** Tissue intermittent hypoxia (IH) occurs in obstructive sleep apnea, sickle cell anemia, physical exercise and other conditions. Poor gas solubility and slow diffusion through culture media hampers mimicking IH-induced transitions of $O_2$ *in vitro*. We aimed to develop a system enabling exposure of cultured cells to IH and to validate such exposure by real-time $O_2$ measurements and cellular responses. Standard 24-well culture plates and plates with bottoms made from a gas permeable film were placed in a heated cabinet. Desired cycling of $O_2$ levels was induced using programmable solenoids to purge mixtures of 95% $N_2$ + 5% $CO_2$ or 95% $O_2$ + 5% $CO_2$. Dissolved oxygen, gas pressure, temperature, and water evaporation were measured during cycling. IH-induced cellular effects were evaluated by hypoxia inducible factor (HIF) and NF-κB luciferase reporters in $HEK_{296}$ cells and by insulin secretion in rat insulinoma cells. Oxygen cycling in the cabinet was translated into identical changes of $O_2$ at the well bottom in gas permeable, but not in standard cultureware. Twenty-four hours of IH exposure increased HIF (112%), NF-κB (111%) and insulin secretion (44%). Described system enables reproducible and prolonged IH exposure in cultured cells while controlling for important environmental factors.

**Key words:** Oxygen — Cell culture — Insulin secretion — Hypoxia inducible factor 1 — NF-κB

## Introduction

Transient drops in blood oxygenation occur frequently throughout human life. For example, obstructive sleep apnea syndrome (OSA), affecting 5–15% of the general adult population (Young et al. 2002; Punjabi 2008) and as much as 30–40% of obese subjects (Valencia-Flores et al. 2000), is characterized by frequent occlusions of upper airways with subsequent hemoglobin desaturations and tissue intermittent hypoxia (IH) during sleep. Periods of tissue hypoxia also develop in organs affected by atherosclerotic narrowing of nutritive arteries, during physical exercise or during acclimation to high altitude (Roels et al. 2007; Mounier et al. 2009). Intermittent hypoxia has also been observed in rapidly growing tumors (Bhaskara et al. 2012), patients suffering from sickle cell anemia (Alexy et al. 2010) as well as in organs of premature infants (Poets et al. 1994; Stokowski 2005).

Although IH has been suggested to play a key role in the development of multiple disease states and adaptations including hypoxic preconditioning in cardiac and skeletal muscle, hypertension, atherosclerosis and metabolic impairments, the cellular mechanisms mediating these effects remain only partially elucidated (Cai et al. 2003; Baker-Herman et al. 2004; Sharp et al. 2004; Beguin et al. 2005, 2007; Brzecka 2005; Guo et al. 2009). *In vitro* investigations of the cellular effects of IH require reliable exposure of cultured cells to reproducible, frequent and rapid changes in pericellular $O_2$ concentration to mimic the *in vivo* situation. Unfortunately, the physical laws of gas diffusion in media can provide a significant limitation for the exposure of cultured cells to IH. Diffusion distance (i.e., media volume), molecular diffusivity and mixing of the

Correspondence to: Jan Polak, Sport Medicine Department, Third Faculty of Medicine, Charles University in Prague, Ruska 87, Prague 100 00, Czech Republic
E-mail: jan.polak@lf3.cuni.cz

media due to thermally-induced convective forces and/or mechanical vibrations are key factors determining the rapidity of $O_2$ transport from the gas phase above the surface of the culture media to the cellular level at the bottom of the culture plate (Metzen et al. 1995; Otto and Baumgardner 2001; Baumgardner et al. 2003; Zhdanov et al. 2010). Both mathematical models and empirical measurements of dissolved $O_2$ profiles in media showed that equilibration of the $O_2$ levels between the pericellular space and the gas phase above culture media can take upwards of 60 minutes (Metzen et al. 1995; Allen et al. 2001; Pettersen et al. 2005; Zhdanov et al. 2010) depending upon the depth of the media and other conditions. For example, it has been demonstrated that when using a standard cultureware, even extensive and rapid changes in ambient gas $O_2$ concentrations (150 to 0 mmHg) are insufficiently reflected in media $O_2$ changes at the pericellular level (80–50 mmHg) when equilibration time is 5 minutes (Yuan et al. 2004). Thus, physical laws of gas diffusion in liquids can significantly hamper cell culture *in-vitro* modeling of *in vivo* conditions where changes in $O_2$ levels typically appear in seconds or minutes (i.e. during an apneic episode in OSA).

Alternative approaches such as sustained shaking of cultureware, minimizing media volume, microfluidic techniques or forced flow-through systems have been developed to bypass some of the aforementioned limitations (Koch 1984; Lam et al. 2009; Polinkovsky et al. 2009; Oppegard et al. 2010; Lo et al. 2012; Tsapikouni et al. 2012). Furthermore, three-dimensional tissue models and microfluidic culture systems have been successfully used to model tissue ischemia in cancer cells and cardiomyocytes as well as to study cardiac ischemia/reperfusion injury (Khanal et al. 2011; Qiao and Ma 2013; Khanal et al. 2014; Mosadegh et al. 2014). However, several new limitations emerged with these techniques that hamper general application and limit their widespread employment. For example, undesirable shear stress or sub-optimal media volume and/or osmolarity might modify cell growth, responses to stimuli or limit the duration of exposure in certain cell types. Additionally, suggested techniques for IH exposure often require highly specialized equipment, development of modified protocols and typically offer only limited throughput.

In this study, we describe an experimental setup based on commercially available components enabling flexible, fast and reproducible exposure of cultured cells to IH for prolonged periods of time while also maintaining media volume, temperature, and pressure in desired ranges. The method is based on a cultureware where the bottom surface is composed of a gas permeable film supporting cultured cells and delivering $O_2$ transitions in the gas phase directly to the pericellular space. We describe this system, compare its physical characteristics to standard cultureware and provide biological evidence of IH exposure in adherent cells, with a special interest in modeling $O_2$ transitions occurring in OSA.

## Materials and Methods

### *Cell culture procedures*

Human embryonic kidney cells (HEK 293, purchased from ATTC, #CRL-1573) and rat insulinoma cells ($INS_{832/13}$ derived by Hohmeier (Hohmeier et al. 2000)) were expanded in T75 flasks (Corning Inc., Corning, NY, USA). HEK 293 cells were cultured in DMEM media (Corning Mediatech, Manassas, VA, USA) with 10% fetal bovine serum (FBS), while $INS_{832/13}$ were cultured in RPMI1640 media (Gibco, Life Technologies, Grand Island, NY, USA) containing 10% FBS and 11.1 mM glucose. Subsequently, cells were plated on a standard cultureware (Corning Inc., Corning, NY, USA), on 50 mm gas permeable culture dishes (Sarstedt AG & Co, Nümbrecht, Germany) or on 24-well gas permeable plates (Coy Laboratory Products, Grass Lake, MI, USA); 600,000 cells/dish) according to experimental protocols described below.

### *Dissolved $O_2$ measurements and media evaporation in cell culture plates*

Culture plates (24-well) with a gas permeable film bottom (product #'s 8602000 and 8602004-with adhesive on top of wells to attach film top seal, Coy Laboratory Products, MI, USA) were suspended on a holder to allow even gas flow below the dishes and compared to standard 24-well cultureware (product # 353047, Becton Dickinson Labware, NJ, USA). The plates were placed inside a heated incubation cabinet (interior size: 15 × 12 × 9.5 inches) in which gas levels could be precisely controlled. Due to differences in well diameter between the plates, the volume of media pipetted inside individual wells was calculated to yield an identical media depth of 2.8 mm (400 µl in gas permeable plates and 558 µl in standard plates). Evaporation of media was determined in permeable plates: a) without a lid; b) with a standard lid; c) with a standard lid that was sealed around the edges with vinyl tape (product #471, 3M, MN, USA) and in which a 1.3 mm ventilation hole was made in the center of the lid (referred to as edge-sealed); and d) sealed with a gas permeable film sheet (Product # 8602005, Coy Laboratory Products, MI, USA) covering all wells simultaneously (referred to as film-sealed; Figure 1A). $O_2$ in the media was measured at the pericellular level (Figure 1B) using a dip-in Clark-type electrode (MI-730 Oxygen Microelectrode Sensor, Microelectrodes, NH, USA) positioned at an angle to the bottom of the wells. Before dissolved $O_2$ measurements were performed, temperature and media $O_2$ levels were

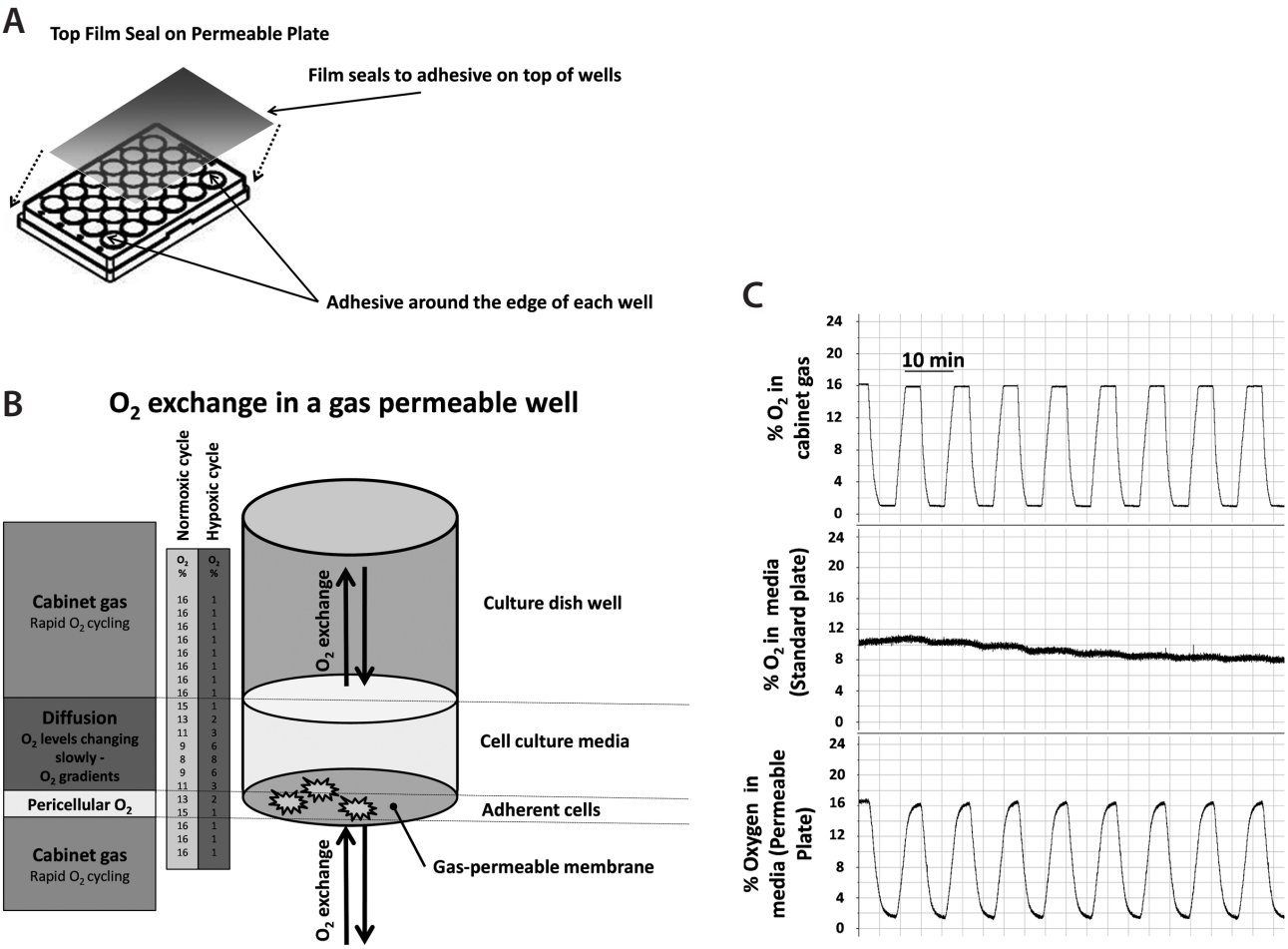


**Figure 1.** Transitions of $O_2$ in gas and media. **A.** Sealing of wells with a film top to prevent media evaporation. **B.** A schematic illustrating $O_2$ level transitions that occur in gas and media at the cell level during IH exposures. **C.** Representative recordings of $O_2$ measurements in cabinet gas (upper panel), in media at the cell level in the standard plate (middle panel) and in media at the cell level in a permeable plate (lower panel) during intermittent hypoxic exposure. For media measurements, the $O_2$ sensor was placed at the bottom of the well. In both types of cultureware, the media depth was 2.8 mm and they were open to the humidified gas (using bubblers).

allowed to equilibrate with the $O_2$ levels in the incubation cabinet (monitored by continuous $O_2$ measurements). Data were recorded for subsequent analysis using the PowerLab data acquisition system (AD Instruments, Colorado Springs, CO, USA).

*Controlling $O_2$ levels inside the incubation cabinet*

Cycling of $O_2$ concentration inside the cabinet ($O_2$ Control Cabinet, Coy Laboratory Products, MI, USA) was induced through repeated purging of gas mixtures (95% $O_2$ + 5% $CO_2$ and 95% $N_2$ + 5% $CO_2$) from dry gas cylinders. A programmable Model 2000 $O_2$ Controller with Ramp/Soak Upgrade (Coy Laboratory Products, MI, USA) regulated timing and flow of appropriate gases through servo-controlled solenoid valves based on real-time measurements of cabinet $O_2$ concentrations. In all experiments, the controller was programmed such that the $O_2$ concentration inside the cabinet changed from pre-set peak $O_2$ levels (16%, 8% or 4% $O_2$) to pre-set nadir $O_2$ levels (1%) within 2 min, followed by a 3 min soak period at the desired $O_2$ concentration. Subsequently, $O_2$ concentration was returned to pre-set peak $O_2$ level within 2 min and held at this level for 3 additional min. This protocol resulted in 6 full cycles of $O_2$ concentration *per* hour (duration of each cycle = 10 min) and is referred to as IH (intermittent hypoxia) exposure. For control experiments, exposures were performed in the same cabinet using a controller program enabling identical gas flow and timing as during the IH exposure, but the cabinet was purged with a gas mixture of 16% $O_2$; 5% $CO_2$; 79% $N_2$ throughout the whole cycle. The same $O_2$ controller with auxiliary flow meters was set to maintain desired $O_2$ levels

for sustained $O_2$ exposures. Additionally, this controller with auxiliary flow meters was used to maintain the gaseous $O_2$ level during the system equilibration for temperature, humidity and dissolved $O_2$ before exposures/measurements were begun.

*Humidity, temperature, and gas pressure*

In some experiments, we tested whether humidification of the gases in the cabinet altered the evaporation rate of media. When utilized, humidification of the gas inside the cabinet was achieved by running the gases through glass bottle bubblers filled with ~500 ml of distilled water and/or by water filled tray located inside the cabinet. The cabinet and water bubblers were enclosed in a polycarbonate glove box with temperature controlled by two microprocessor-controlled fan heaters (Coy Laboratory Products, MI, USA). Gas lines connecting the cabinet with gas tanks were pre-warmed using a heat exchanger or a water bath to maintain constant cabinet temperature during $O_2$ cycling. Temperature measurements in the cabinet gas were performed using a DirecTemp Thermistor (DTU6005-007-NC, Quality Thermistor, ID, USA distributed by Coy Laboratory Products, MI, USA) and the cabinet temperature for all experiments was maintained at 37°C (range ± 1°C) with the temperature varying by less than 0.3°C within an IH cycle. Inside the cabinet, relative humidity (RH) was measured using a DirectRH Probe (Quality Thermistor, ID, USA). The dry cabinet had ~0% RH. Humidification achieved by a water tray placed inside the cabinet provided a maximum of ~85% RH during the early IH exposure that trended downward an average of ~0.5–1.3% RH/hour with a variation of ~32–37% RH within an $O_2$ cycle. A scaled model of an individual well from a 24-well gas permeable plate (size ratio ~1 : 4.8 with the ratio of well top surface area to inner well void volume kept constant) was fabricated to enable recording (1 s intervals) of gas pressure inside the well (same plates used for the dissolved $O_2$ measurement as described above). All gas pressures (cabinet, plate, and well model) were measured using a Love digital differential manometer (HM28D3C10000, Dwyer Instruments, IN, USA).

*Transcription factor activity assays*

Dual luciferase reporter systems were used to evaluate changes in hypoxia-inducible factor (HIF) and nuclear factor-κB (NF-κB) transcriptional activity under sustained hypoxia or IH exposures in cells. HEK-293 cells were transfected in Opti-MEM I media (Invitrogen, Grand Island, NY, USA) with Attractene transfection reagent (Qiagen, Valencia, CA, USA) and DNA constructs containing a firefly luciferase reporter gene under transcriptional control of hypoxia response elements to evaluate HIF transcriptional activity (Cignal HIF Reporter Kit, Qiagen, Valencia, CA, USA) or NF-κB response elements to monitor transcriptional activity of NF-κB (Cignal NFκB Reporter Kit, Qiagen, Valencia, CA, USA) following manufacturer`s instructions. A constitutively expressed renilla luciferase construct under control of a cytomegalovirus promoter was co-transfected with firefly luciferase constructs and served as an internal control. Four hours after transfections, 24-well edge-sealed plates were placed in the dry cabinet and exposed to sustained $O_2$ levels (0%, 1%, 4% or 16% $O_2$), IH (cycling 16% to 1%, 8% to 1% or 4% to 1% $O_2$) or control conditions (16% $O_2$ with intermittent gas flow at flow rates matching those used for IH) for 24 hours. $CO_2$ concentration was maintained at 5% during all exposures. After exposures, cells were lysed following manufacturer`s instructions (Promega, Madison, WI, USA) and samples stored at –80°C until analysis. Firefly and renilla luciferase activity was measured following manufacturer`s instructions using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) and read *via* microplate luminometer (Veritas, Turner BioSystems, Sunnyvale, CA, USA). Transcriptional activity of HIF and NF-κB was expressed as the ratio of firefly to renilla luminescence.

*Glucose-induced insulin secretion assessment*

Biological effects of IH on cells were explored in $INS_{832/13}$ cells grown in 50 mm gas permeable culture dishes, as described above. After 24 hours in culture, media was changed and cells subsequently cultured for an additional 24 hours in serum-free RPMI1640 media containing 5.5 mM glucose prior to experimental exposures. Immediately before IH and control exposures, media was changed into serum-free RPMI1640 containing 3 mM glucose, and the dishes placed into the cabinet and exposed to IH (peak $O_2$ = 16%, nadir $O_2$ = 1%; 10 min cycles) or control conditions for 24 hours. The dish lids were tape-sealed and a venting hole was made using a hot 16G needle and placed into the dry (non-humidified) cabinet for IH exposures. Culture dishes rather than multiwell plates were used for these experiments because of the higher number of cells needed for the assay.

After exposures, cells were washed with HBSS buffer (HEPES-buffered salt solution) containing (in mM): 114 NaCl, 4.7 KCl, 1.2 $KH_2PO_4$, 1.16 $MgSO_4$, 20 HEPES, 2.5 $CaCl_2$ and 25.5 $NaHCO_3$ and 0.2% albumin. Cells were then incubated in 3 ml of HBSS buffer with 3, 8 or 16 mM glucose or with 16 mM glucose + 250 μM 3-isobutyl-1-methylxanthine (IBMX; phosphodiesterase inhibitor) for 2 hours to evaluate non-stimulated (3 mM glucose) and glucose-induced insulin secretion, respectively. After incubations, media was collected and stored at –80°C until analysis. Cells were then washed 3 times with PBS, lysed with T-PER lysis buffer (Thermo Scientific, Waltham, MA,

USA) and lysates stored at –80°C until analysis. The amount of DNA in each sample was determined spectrophotometrically and used to normalize insulin secretion to number of cells in each well. Secreted insulin was measured using an ELISA kit (Insulin (Rat) Ultrasensitive ELISA; Alpco, Salem, NH, USA).

*Statistical analysis*

Differences in $O_2$ cycling characteristics, pressure changes, media evaporation and transcriptional activity reporter assays between standard and permeable cultureware were analyzed using 2-way ANOVA or Repeated Measures ANOVA ($O_2$ cycling) using the SPSS software package (SPSS 13.0 for Windows, SPSS Inc., Chicago, IL, USA). The effect of IH on glucose-induced insulin secretion in $INS_{831/13}$ cells was evaluated using Repeated Measures ANOVA. Data are presented as mean ± SEM. A value of $p < 0.05$ was considered statistically significant.

## Results

*Pericellular $O_2$ level transitions in standard versus gas permeable cultureware*

Analysis of $O_2$ profiles revealed that the pericellular $O_2$ concentration in the gas permeable cultureware closely mirrored changes in $O_2$ concentrations in the gas phase inside the cabinet, as shown in Figure 1C. Average $O_2$ concentration at the end of the normoxic phase (Peak $O_2$) and at the end of the hypoxic phase of the cycle (Nadir $O_2$) was not statistically different between the inside of the cabinet and the pericellular space (Peak $O_2$ = 15.89 ± 0.01% *vs.* 15.31 ± 0.04% and Nadir $O_2$ = 0.99 ± 0.00% *vs.* 1.22 ± 0.01% for the gas phase inside the cabinet and pericellular space in media, respectively). Importantly, the pericellular peak and nadir $O_2$ concentrations of individual cycles remained unchanged throughout the measured period of 15 full cycles, as shown in Figure 2A-C. In contrast, pericellular peak and nadir $O_2$ con-

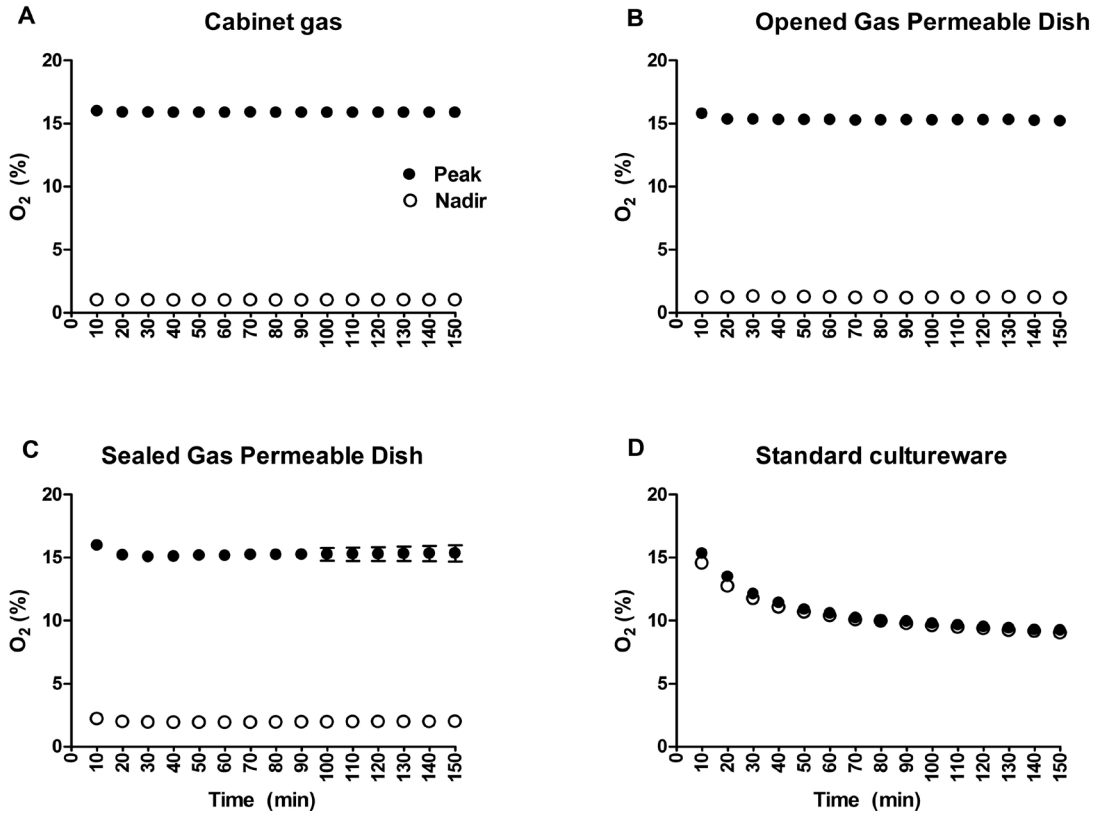


**Figure 2.** Peak and nadir $O_2$ levels during an intermittent hypoxic exposure. Individual peak and nadir $O_2$ levels were identified and graphed for 16 consecutive cycles. The following conditions are displayed: $O_2$ level in cabinet gas (**A**; $n = 11$); $O_2$ in media at the cell level in an open gas permeable plate (**B;** $n = 11$); $O_2$ in media at the cell level in a permeable plate with the top sealed with a gas permeable film over each well (**C**; $n = 3$) and $O_2$ in media at the cell level in an open standard plate (**D**; $n = 6$). Cycling timing: 2 minutes of gas-exchange phase was followed by 3 minutes exposure to constant pre-set $O_2$ levels (1% $O_2$), subsequently followed by 2 minutes of gas-exchange phase and 3 minutes of exposure to constant pre-set $O_2$ levels (16% $O_2$). Experiments presented in this figure were performed without growing cells in wells.

centrations in the standard cultureware continually drifted during the measured period of 15 cycles and averaged peak and nadir $O_2$ values were significantly different from cabinet gas phase $O_2$ concentrations (Peak $O_2$ = 15.89 ± 0.01% *vs.* 10.73 ± 0.45% and Nadir $O_2$ = 0.99 ± 0.00% *vs.* 10.45 ± 0.40% for the gas phase inside the cabinet and pericellular space in media, respectively; both $p < 0.001$). In fact, no effective cycling of $O_2$ levels was observed in the standard cultureware and pericellular $O_2$ continually decreased throughout the measured period (ANOVA; $p < 0.001$), ultimately leveling at ~8% $O_2$ (Figure 2D).

*Liquid evaporation and temperature control*

Because of the rapid gas exchange required to achieve $O_2$ cycling, media loss due to evaporation from individual wells of the culture plate represents a critical factor limiting prolonged cell exposures to IH. In open culture plates, 100% of initial media volume was lost to evaporation over 24 hours (Figure 3). Thus, we tested several methods to limit liquid evaporation during the development of the system. Sealing the whole plate around the edges using a vinyl tape and adding a small perforation in the lid to prevent thermal pressurization or using adhesive to seal the top of the plates

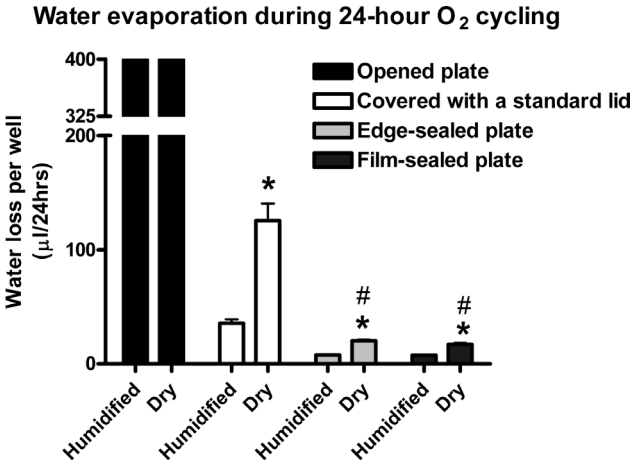


**Figure 3.** Media evaporation from a permeable plate during a 24 hour IH exposure. Media loss was quantified as evaporation of sterile water (400 µl/well) after 24 hour IH exposure (16% to 1% $O_2$ cycles; 6 cycles/hour). Using standard lid limited evaporation, while edge-sealing a vented lid or film-sealing the wells further decreased evaporation. * $p < 0.05$ for comparisons of humidified versus dry (0% RH) conditions (Student`s unpaired T-test), # $p < 0.05$ for comparison with a standard lid condition (2-way ANOVA). For the humidified cabinet, $n = 5$ for open plate; $n = 4$ for standard lid; $n = 8$ for edge-sealed standard lid with vent hole and $n = 7$ for film-sealed wells. For the dry cabinet, $n = 5$ for open plate; $n = 4$ for standard lid; $n = 6$ edge-sealed standard lid with vent hole and $n = 4$ film-sealed wells. Cycle duration: 10 minutes.

with gas permeable film significantly decreased liquid loss during 24 hours of IH exposure to 5% and 4% of the initial volume, respectively (Figure 3). The highest liquid loss for the standard lid was from the wells at the edges of the plates, whereas for the edge-sealed vented lid the greatest loss was from the wells adjacent to the vent hole. Furthermore, humidification of the cabinet`s atmosphere using a water tray placed inside the cabinet further decreased water evaporation to 1.8% of the initial volume for both edge- and film-sealed plates. However, temperature was harder to maintain during IH exposure in the humid condition due in large part to the cooling caused by the enthalpy of vaporization.

*Gas pressure changes within wells*

To determine whether sealing the plates with a gas permeable film caused changes in gas pressure inside individual sealed wells during temperature equilibration and $O_2$ cycling, we constructed an enlarged scaled model of an individual well and compared gas pressure recordings with those obtained in an edge-sealed permeable plate. As shown in Figure 4A and 4B, each transition between target $O_2$ levels was associated with a gas purge that increased gas pressure inside the cabinet (compared to the atmospheric pressure) by 2.673 ± 0.005 hPa and 1.465 ± 0.006 hPa for $N_2$ and $O_2$ purges, respectively, in a dry cabinet and by 2.388 ± 0.004 ($N_2$) and 0.885 ± 0.012 hPa ($O_2$) in a humidified cabinet (water tray). These pressure swings were fully transmitted to the interior of the permeable plate and film-sealed well model (Figure 4A and 4B). In addition to pressure changes induced when gas is purged into the cabinet, the sealed environment of an individual well is also subject to pressure changes due to heat-induced gas expansion and water vapor pressure when plates with media are moved from room temperature (mean ± SD: 20.2 ± 0.2°C) to the cabinet with temperature maintained at 37°C. Because of the venting hole introduced into the edge-sealed permeable plates, which enabled full pressure equilibration between the inside compartment of the plate and the outside environment of the cabinet, no heating-induced pressurization was detected (Figure 4C and 4D). In contrast, the film-sealed well exhibited a transient pressure increase inside the well, with maximum pressures reaching 0.65 hPa and 0.83 hPa in dry and humidified cabinets, respectively.

*HIF and NF-κB transcriptional activity under sustained and intermittent hypoxia*

After determining the physical characteristics of permeable plates during IH cycling and demonstrated cycling of $O_2$ concentration at the cellular level, we next wanted to describe the effects of sustained hypoxia and IH exposure on established hypoxia-regulated pathways in cultured cells (Yuan et

al. 2011; Prabhakar et al. 2012; Han et al. 2013). Edge-sealed permeable plates containing HEK 293 cells were placed in a dry cabinet. First, we compared the ability of increasing levels of sustained hypoxia to up-regulate the transcriptional activity of HIF in standard *versus* gas permeable plates. As shown in Figure 5, marked differences were observed between standard and permeable plates, with the transcriptional activity of HIF significantly higher in standard plates when cells are incubated under non-hypoxic (16% $O_2$) or moderately hypoxic (4% $O_2$) conditions. Furthermore, at 4% $O_2$, HIF transcriptional activity plateaued in cells grown in standard plates, suggesting severe cellular hypoxia, whereas no increase in HIF transcriptional activity was observed at this level of $O_2$ in gas permeable plates. In contrast, in cells grown on gas permeable plates, HIF activity levels did not increase until $O_2$ was reduced to 1% and reached maximum activation under anoxic conditions.

In HEK 293 cells exposed to IH cycling for 24 hours, no increase in HIF transcriptional activity was observed when cells were cycled between 16 and 1% $O_2$ or 8 and 1% $O_2$ (Figure 6A). However, when the peak $O_2$ level during cycling was reduced to 4% $O_2$ (cycling between 4 and 1% $O_2$), a 112% ($p < 0.05$) increase in HIF transcriptional activity became apparent. Similar results were observed using a reporter for NF-κB transcriptional activity (111% increase with IH exposure cycling between 4 and 1%, $p < 0.05$) (Figure 6B).

### *Effects of IH on insulin secretion in $INS_{832/13}$ cells*

We further investigated the effects of IH on cell function using INS cells. After 24 hours of IH (cycling between 16% and 1% $O_2$) or control exposures, 2 hours basal (non-stimulated) insulin secretion increased by 44% (Figure 7). As expected, in cells exposed to control conditions, stimulation with various concentrations of glucose (3 mM, 8 mM, 16 mM or 16 mM glucose + 250 μM IBMX) increased insulin secretion in a dose-dependent manner. Exposure to IH enhanced

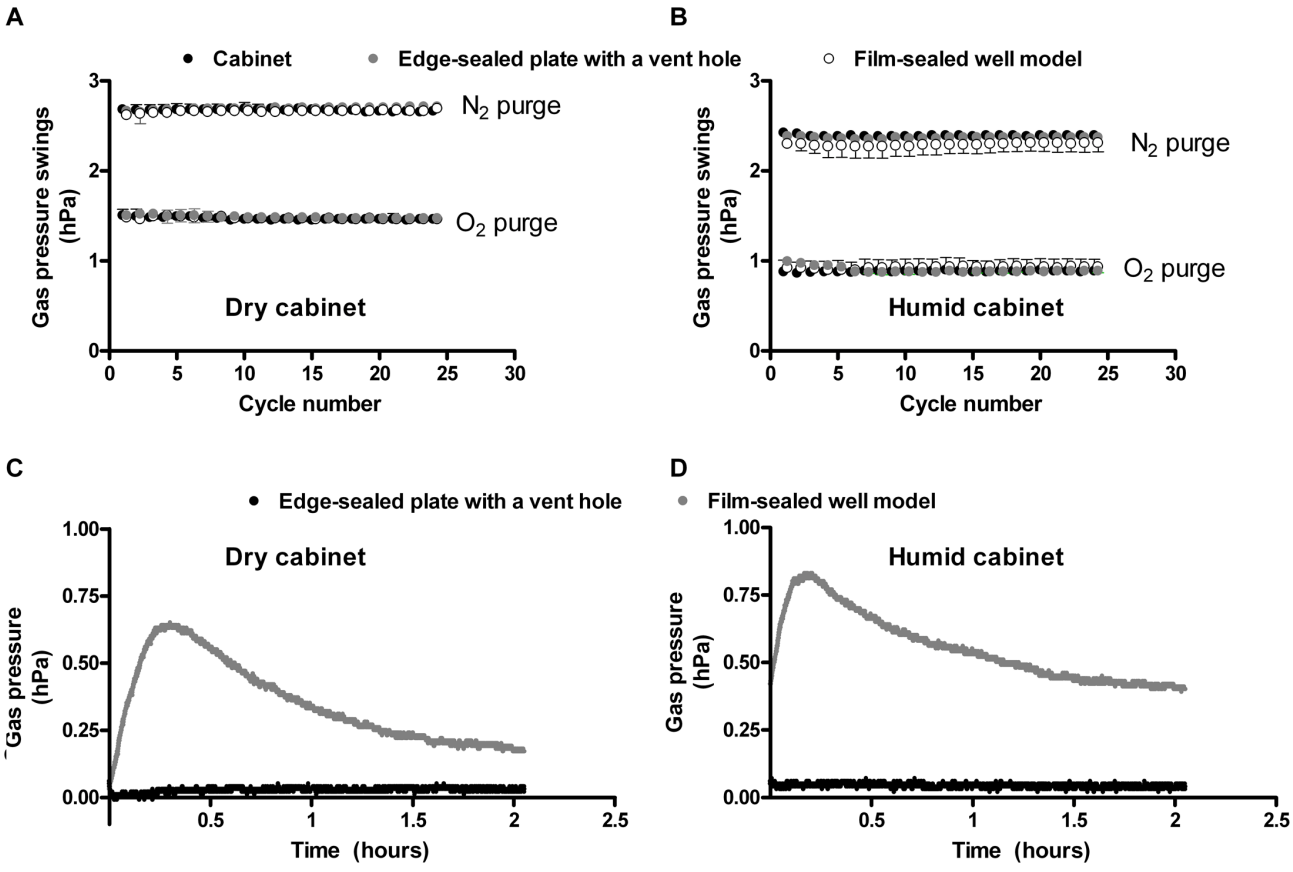


**Figure 4.** Pressure changes inside individual culture wells. Pressure swings in dry (**A**) or humidified (**B**) conditions inside vented edge-sealed permeable plates and inside the film-sealed well model during cycling between 16% and 1% $O_2$ (6 cycles/hour) ($n = 3$ each). Under dry or humidified cabinet conditions (no $O_2$ control), gas pressure build-up inside edge-sealed permeable plates and inside film-sealed well model during temperature equilibration from room temperature (mean ± SD: 20.2 ± 0.2°C) to 37°C (**C**, **D**). Traces are representative of 3 experiments *per* condition, $p = 0.053$ for interaction between exposure and glucose dose. Note: 1 hPa = 0.75 mmHg = 1.02 $cmH_2O$.

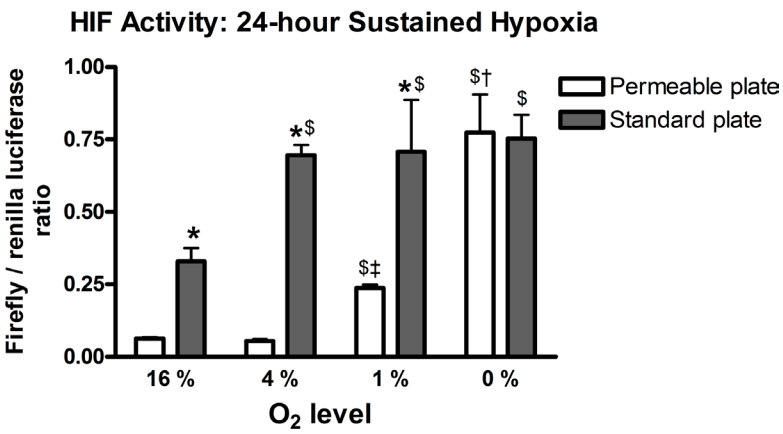


**Figure 5.** Comparison of HIF transcriptional activity during sustained hypoxia in standard and gas permeable plates. Bar graph shows relative HIF reporter activity in HEK-296 cells exposed to various levels of sustained $O_2$ levels for 24 h. * $p < 0.05$ for comparison between standard and permeable plates (2-way ANOVA, post-hoc tests), $^{\$}$ $p < 0.05$ for comparison with exposure to 16% $O_2$ within each cultureware type (ANOVA, post-hoc tests), $^{\ddagger}$ $p < 0.05$ for comparison with exposure to 4% $O_2$ within each cultureware type (ANOVA, post-hoc tests), $^{\dagger}$ $p < 0.05$ for comparison with exposure to 1% $O_2$ within each cultureware type (ANOVA, post-hoc tests). Permeable plate: $n = 9$ for 16% $O_2$, $n = 3$ for all other conditions; Standard plate: $n = 13$ for 16% $O_2$, $n = 4$ for 4% $O_2$, $n = 6$ for 1% $O_2$ and $n = 7$ for 0% $O_2$.

glucose-induced insulin secretion, although the difference did not quite reach statistical significance (ANOVA, $p = 0.053$ for interaction).

## Discussion

In this paper, we describe a system enabling exposure of adherent cells to reproducible cycles in pericellular $O_2$ concentration over prolonged periods of time while controlling multiple environmental factors including temperature, media evaporation and gas pressure changes. Using reporter assays for HIF and NF-κB, we verified that IH exposure was sensed by cultured cells. Finally, we investigated functional consequences of IH exposure in rat insulinoma cells ($INS_{832/13}$), finding that IH increased spontaneous and glucose-induced insulin secretion.

Diffusion of $O_2$ and other gasses from ambient gas through media to the cells grown at the bottom of the wells in standard cultureware represents a significant and often neglected factor in any cell culture experiment. In fact, it is rarely recognized that cells with higher metabolic rates grown in standard cultureware in a standard $CO_2$ incubator might actually live under much lower $O_2$ concentrations than is the $O_2$ level in the atmosphere of the ambient gas inside the incubator (Metzen et al. 1995). Additionally, it should be recognized that tissue $O_2$ levels in living organisms vary significantly and are typically lower than $O_2$ concentrations in the atmosphere of a standard $CO_2$ incubator. In the current study we confirmed these previous observations, showing that even under non-hypoxic conditions the activity of HIF, a key transcriptional factor regulated by hypoxia, was significantly higher in $HEK_{293}$ cells grown in standard plates compared to cells grown in gas permeable plates. HIF transcriptional activity was negligible in $HEK_{293}$ cells cultured in gas permeable plates at 16% or 4% $O_2$ and reached maximum when grown at or under 1% $O_2$. This observation is congruent with other reports documenting maximal HIF-1 stimulation between 1% and 0.5% $O_2$ (Jiang et al. 1996; Yu et al. 1998). In contrast, marked upregulation of HIF was observed in cells grown in a standard cultureware even at 4% $O_2$ which would appear to support the possibility that these cells may experience lower $O_2$ levels than is available in the gas surrounding the plate due to diffusion of $O_2$ not keeping up with its use in respiration. In this case, the resulting development of

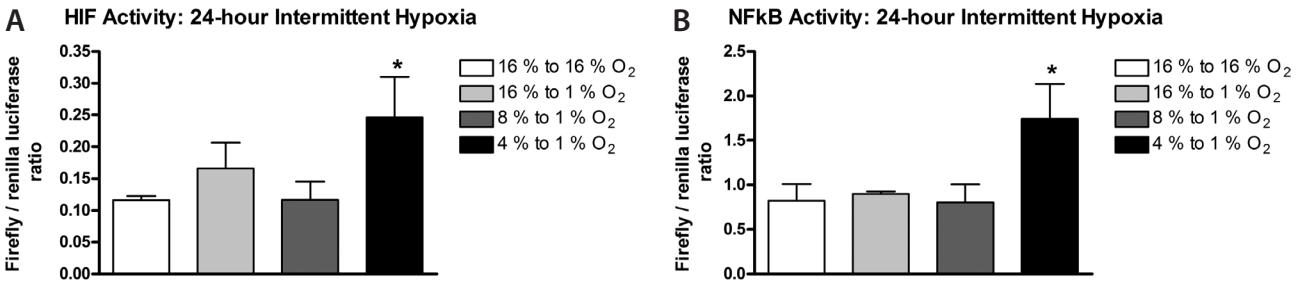


**Figure 6.** HIF and NFκB transcriptional activity under intermittent hypoxia exposure. Bar graphs showing relative HIF (**A**) and NF-kB (**B**) reporter activity in HEK-296 cells exposed to various levels of intermittent hypoxia for 24 hours. * $p < 0.05$ for comparison to a control exposure (16% intermittent normoxia), (ANOVA, post-hoc tests). For IH 4% to 1% $n = 3$; $n = 4$ for all other conditions.

hypoxia at the cellular level can be prevented by using the gas permeable plates.

Poor diffusivity of $O_2$ through media becomes even more of concern when IH exposure is considered. We verified $O_2$ transitions in media using direct real-time measurements; however, the ultimate question is whether such changes in $O_2$ availability induce any biological responses in cells and if so, what $O_2$ level and duration of hypoxia is required. Similar to others, we documented both HIF and NF-κB activation after IH exposure (Nanduri et al. 2008; Oliver et al. 2009; Prabhakar et al. 2010; Yuan et al. 2011; Quintero et al. 2013; Wang et al. 2013a), confirming thus possible molecular mechanisms for IH-related pro-inflammatory effects (Querido et al. 2012; Baessler et al. 2013; Murase et al. 2013; He et al. 2014). Furthermore, the severity of exposure required for NF-κB activation was of similar intensity to that required for HIF activation (cycling between 4% and 1% $O_2$), potentially suggesting shared intracellular pathways and mutual interactions between these two transcription factors, as suggested previously (Jung et al. 2003; Scholz et al. 2013).

We further observed that 24 hour intermittent hypoxic exposure administered through the described system increased basal/spontaneous insulin secretion in rat insulinoma cells. Although previous studies in animal models reported increased basal insulin secretion after IH exposure (Yokoe et al. 2008; Xu et al. 2009; Ota et al. 2013; Wang et al. 2013b), it remained to be elucidated whether such effects are mediated through whole-body endocrine regulations or whether they are mediated directly by repetitive drops in tissue oxygen levels. Using the system described in this paper allowed us to investigate direct effects of IH on insulin producing cells without interference of other changes occurring in the whole animal. Our results suggest that fluctuations in pancreatic $O_2$ levels increase basal and possibly also glucose-induced insulin secretion from β-cells. However, it is important to note that in the whole animal, IH exposure also leads to profound metabolic and endocrine changes determining the overall effect of IH on insulin secretion which might be different from direct effects of IH in the cells. In fact, studies describing impaired glucose-induced insulin secretion after exposure of animals IH (Ota et al. 2012; Sherwani et al. 2013; Pae and Kim 2014) demonstrate the importance of both approaches. Our data suggest that activation of pathways typically associated with sustained hypoxic exposure, such as HIF and NF-κB (Semenza et al. 2007; Culver et al. 2010), might also be involved under intermittent hypoxic conditions as recently reported Wang et al. (2013b). However, it should be noted that more severe intermittent hypoxic exposure was required for activation of HIF and NF-κB than for increased insulin secretion, suggesting that other mechanisms (e.g. reactive oxygen species generation) are probably involved (Ryan et al. 2007; Yuan et al. 2011) and/or that various cell types respond differently to intermittent hypoxia.

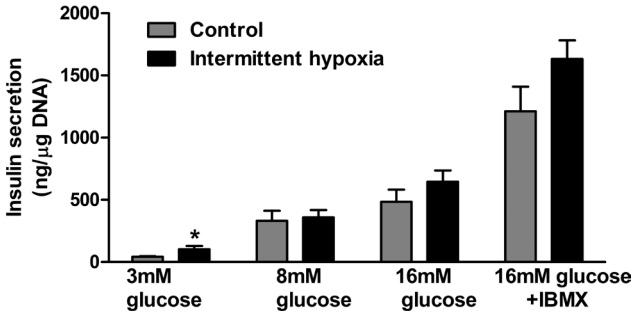


**Figure 7.** The effect of intermittent hypoxia on glucose-induced insulin secretion. Glucose-independent insulin secretion into media was measured in $INS_{831/13}$ cells during 2-hour incubations with 3 mM glucose, while glucose-induced insulin secretion is represented by incubations with increasing concentrations of glucose and IBMX (isobutyl-methyl-xanthine). Repeated measures ANOVA. $p = 0.053$ for interaction between exposure groups and glucose treatment; * $p < 0.05$ for comparison between exposure groups (Student`s unpaired T-test); $n = 16$ for all control exposure conditions and $n = 13$ for all intermittent hypoxic conditions.

Calculated and measured data suggest that the equilibration of $O_2$ concentrations across a 2.5 mm deep media layer requires over 60 min (Allen et al. 2001), which is in a sharp contrast to transitions in hemoglobin saturation observed in OSA, where shifts in pericellular $O_2$ happen in seconds to minutes (Reinke et al. 2011). Recently, alternative methods have been developed to overcome slow $O_2$ diffusion (administration of media pre-equilibrated at the desired $O_2$ levels, microfluidic technology, or custom-made well inserts (Koch 1984; Lam et al. 2009; Polinkovsky et al. 2009; Oppegard et al. 2010; Lo et al. 2012; Tsapikouni et al. 2012)); however, it should be noted that highly specialized technical equipment or custom modifications to available cultureware, with the added risk of biological contamination, are required to build and/or use the mentioned systems.

Several environmental factors (e.g. temperature, cabinet gas humidity and cabinet gas pressure) must be considered during IH exposure in addition to $O_2$ transitions. Temperature (absolute and variation during IH exposure) was difficult to maintain in a humidified cabinet due to the thermal effects from variability in water level in the bubbler, number and position of plates or dishes, gas flow rate, and/or water tray surface area. While temperature in a dry cabinet can be effectively controlled within a narrow range (mean ± SD: 37.1 ± 0.2°C during cycling, $n = 9$) by external heating of the cabinet and pre-heating the gas entering the cabinet to 37°C, media evaporation control and prevention of pressure build-up in the closed compartment of a culture plate during temperature equilibration required additional attention.

Despite substantial efforts to humidify the cabinet environment, as well as the gas entering the cabinet during transition between preset $O_2$ values, initial experiments revealed that relative humidity in the cabinet varied between ~60–90% during IH cycling (both mean humidity level *per* cycle that often trended downward over time and variation within a cycle could be affected by variables such as depth of water in bubblers, surface area of water tray, gas flow rate, and/or method of producing bubbling), resulting in unacceptable media evaporation during 24 hour IH exposures (144 hypoxic cycles). This limitation was solved by covering plates with 1) a standard lid supplied with the culture plate that was subsequently sealed to the edges of the plate with vinyl tape and perforated to create a vent hole or 2) gas permeable film material adhered to the rim of individual wells. Both methods of sealing reduced evaporation to acceptable levels (≤ 5% of initial volume) during 24 hour IH exposures, even in a non-humidified cabinet. Importantly, direct measurement of $O_2$ transitions at the pericellular level verified that $O_2$ profiles remained unchanged with sealing, further confirming that pertinent $O_2$ diffusion occurs through the permeable film at the bottom of the plate.

As temperature increases, any air-tight sealed compartment with liquid inside can be subject to considerable changes in gas pressure inside the compartment due to gas heat expansion and to vapor pressure resulting from evaporation. Such changes in temperature might occur at the beginning of experiments, when culture plates are moved from a room temperature environment (i.e, work in a tissue culture hood, media change, etc.) to the cabinet where temperature is regulated at 37°C. Making a vent hole in the lid completely prevented any pressure build-up inside the plate, whereas film-sealed individual wells demonstrated diminished, yet detectable, increases in gas pressure. Interestingly, the pressure inside the well showed 2-phase kinetics, with an initial increase followed by a gradual decrease. This phenomenon could be explained by the physical properties of the gas permeable film and adhesive used to seal the top of the wells, where pressure relief via a combination of gas diffusion through the film and micro-venting through the warm adhesive is not sufficient to keep up with the initial rapid pressure increase that slows near, and stops at, temperature equilibration. It should be emphasized that different approaches could be employed to avoid temperature changes at the beginning of the experiment. For example, pre-heating the plate and media in the cabinet or sealing the culture plates in a temperature-controlled environment (e.g. in a heated glove box) would reduce the build-up of pressure with heat-induced gas expansion; however, in this initial characterization of the system, we chose to model the worst-case scenario (i.e., no pre-equilibration of temperatures) and describe means to prevent/minimize pressure build-up.

In this paper, we have used 10-minute cycles, consisting of the 2-minute gas-exchange phase (e.g. 16% $O_2$ to 1% $O_2$) followed by 3-minute period of exposure to constant pre-set level of $O_2$ (e.g. 1% $O_2$). After this period, gas inside the cabinet was exchanged again within 2 minutes to reach pre-set level (e.g. 16% $O_2$) and cabinet $O_2$ concentration was kept constant at this level for 3 minutes (2 + 3 + 2 + 3 = 10 min). Various experimental setups requiring longer or shorter exposure periods can be achieved by prolonging or shortening the gas-exchange and/or the steady-state component of the IH cycle. However, it should be noted that physical laws of diffusion across the membrane (bottom of the culture well) represent limits on the minimal duration of the gas-exchange period, depending on the cycle amplitude. Using direct measurements of dissolved $O_2$, we have demonstrated nearly complete transitions of gas-phase $O_2$ levels in the cabinet to the pericellular space. However, we observed HIF-activation only when the amplitude of IH cycles was decreased to 4–1% $O_2$. Based on the work of other authors, demonstrating that HIF activity is up-regulated when pericellular $O_2$ levels reach a threshold of 4% or lower (Jiang et al. 1996), we speculate that IH exposure composed of cycling between 16–1% $O_2$ and 8–1% $O_2$ has not induced sufficient accumulative hypoxic exposure to up-regulate HIF-1, however, other mechanisms, including reactive oxygen species might be responsible for cellular effects of IH independently of HIF. Additionally, it is also possible that HIF is initially up-regulated during the hypoxic phase of the IH cycle, followed by subsequent rapid degradation during the normoxic phase of each cycle due to the direct regulation of prolyl-hydroxylases by $O_2$ levels. Although several aspects remain unresolved, we believe that available technologies, including the setup described in this paper will facilitate future research on this topic.

In summary, the ultimate goal of any system for IH exposures *in vitro* is to facilitate research on biological effects of such exposure in various cell types. In the presented system, adherent cells are grown on a commercially available gas permeable cultureware made of a thin gas permeable fluorocarbon film. Target pericellular peak and nadir $O_2$ concentrations are determined by $O_2$ concentrations inside the cabinet where the timing of gas flow into the cabinet is automatically regulated by a programmable feedback controller. It then becomes feasible to expose cells to virtually any pre-set $O_2$ concentrations with variable amplitude and frequency of IH cycling, with volume of the cabinet and gas flow rate defining the speed of $O_2$ transitions inside the cabinet. Importantly, plates should be edge-sealed or film-sealed to minimize evaporation. Finally, control experiments can be run mimicking all features of the IH exposure except the $O_2$ transitions, thus controlling for factors other than $O_2$ transitions. The major innovative contribution of our work is the ability to induce intermittent hypoxia (defined as rapid,

reproducible and repetitive changes of pericellular $O_2$ levels) in cultured cells. In contrast to other available solutions, including microfluidic approaches, our setup is not affected by a shear stress of flowing liquid and provides sufficient cell and culture media yield for standard downstream assays such as ELISA, Western-blotting, gene expression analysis or cell based assays including reporter assays. With the substantial advantages afforded by using this system, the major limitation and most challenging aspect in conducting experiments examining the effect of *in vitro* IH exposure becomes the lack of detailed knowledge regarding the precise $O_2$ levels that occur at the tissue level *in vivo* during sleep apnea or other clinical conditions characterized by IH.

**Acknowledgements**: The work was funded by an NIH grant NIH RC1 HL099952. Jan Polak was supported by the Fulbright Commission under the Fulbright-Masaryk Program and by The Czech Science Foundation grant GACR P304/13-27735S. Karen Studer-Rabeler is employed by Coy Laboratory Products, a manufacturer of some equipment used in the study. She participated in the design of the system and experiments but had no influence on data interpretation. She participated in pressure data collection and part of the evaporation data collection.

## References

Alexy T., Sangkatumvong S., Connes P., Pais E., Tripette J., Barthelemy J. C., Fisher T. C., Meiselman H. J., Khoo M. C., Coates T. D. (2010): Sickle cell disease: selected aspects of pathophysiology. Clin. Hemorheol. Microcirc. **44,** 155–166

Allen C. B., Schneider B. K., White C. W. (2001): Limitations to oxygen diffusion and equilibration in in vitro cell exposure systems in hyperoxia and hypoxia. Am. J. Physiol. Lung Cell Mol. Physiol. **281,** L1021–1027

Baessler A., Nadeem R., Harvey M., Madbouly E., Younus A., Sajid H., Naseem J., Asif A., Bawaadam H. (2013): Treatment for sleep apnea by continuous positive airway pressure improves levels of inflammatory markers - a meta-analysis. J. Inflamm. (Lond) **10,** 13 http://dx.doi.org/10.1186/1476-9255-10-13

Baker-Herman T. L., Fuller D. D., Bavis R. W., Zabka A. G., Golder F. J., Doperalski N. J., Johnson R. A., Watters J. J., Mitchell G. S. (2004): BDNF is necessary and sufficient for spinal respiratory plasticity following intermittent hypoxia. Nat. Neurosci. **7,** 48–55 http://dx.doi.org/10.1038/nn1166

Baumgardner J. E., Otto C. M. (2003): In vitro intermittent hypoxia: challenges for creating hypoxia in cell culture. Respir. Physiol. Neurobiol. **136,** 131–139 http://dx.doi.org/10.1016/S1569-9048(03)00077-6

Beguin P. C., Belaidi E., Godin-Ribuot D., Levy P., Ribuot C. (2007): Intermittent hypoxia-induced delayed cardioprotection is mediated by PKC and triggered by p38 MAP kinase and Erk1/2. J. Mol. Cell Cardiol. **42,** 343–351 http://dx.doi.org/10.1016/j.yjmcc.2006.11.008

Beguin P. C., Joyeux-Faure M., Godin-Ribuot D., Levy P., Ribuot C. (2005): Acute intermittent hypoxia improves rat myocardium tolerance to ischemia. J. Appl. Physiol. **99,** 1064–1069 http://dx.doi.org/10.1152/japplphysiol.00056.2005

Bhaskara V. K., Mohanam I., Rao J. S., Mohanam S. (2012): Intermittent hypoxia regulates stem-like characteristics and differentiation of neuroblastoma cells. PLoS ONE **7,** e30905 http://dx.doi.org/10.1371/journal.pone.0030905

Brzecka A. (2005): Brain preconditioning and obstructive sleep apnea syndrome. Acta Neurobiol. Exp. (Wars.) **65,** 213–220

Cai Z., Manalo D. J., Wei G., Rodriguez E. R., Fox-Talbot K., Lu H., Zweier J. L., Semenza G. L. (2003): Hearts from rodents exposed to intermittent hypoxia or erythropoietin are protected against ischemia-reperfusion injury. Circulation **108,** 79–85 http://dx.doi.org/10.1161/01.CIR.0000078635.89229.8A

Culver C., Sundqvist A., Mudie S., Melvin A., Xirodimas D., Rocha S. (2010): Mechanism of hypoxia-induced NF-kappaB. Mol. Cell Biol. **30,** 4901–4921 http://dx.doi.org/10.1128/MCB.00409-10

Guo H. C., Zhang Z., Zhang L. N., Xiong C., Feng C., Liu Q., Liu X., Shi X. L., Wang Y. L. (2009): Chronic intermittent hypobaric hypoxia protects the heart against ischemia/reperfusion injury through upregulation of antioxidant enzymes in adult guinea pigs. Acta Pharmacol. Sin. **30,** 947–955 http://dx.doi.org/10.1038/aps.2009.57

Han Q., Yeung S. C., Ip M. S., Mak J. C. (2013): Intermittent hypoxia-induced NF-kappaB and HO-1 regulation in human endothelial EA.hy926 cells. Cell Biochem. Biophys. **66,** 431–441 http://dx.doi.org/10.1007/s12013-012-9491-6

He Q., Yang Q. C., Zhou Q., Zhu H., Niu W. Y., Feng J., Wang Y., Cao J., Chen B. Y. (2014): Effects of varying degrees of intermittent hypoxia on proinflammatory cytokines and adipokines in rats and 3T3-L1 adipocytes. PLoS ONE **9,** e86326 http://dx.doi.org/10.1371/journal.pone.0086326

Hohmeier H. E., Mulder H., Chen G., Henkel-Rieger R., Prentki M., Newgard C. B. (2000): Isolation of INS-1-derived cell lines with robust ATP-sensitive K+ channel-dependent and -independent glucose-stimulated insulin secretion. Diabetes **49,** 424–430 http://dx.doi.org/10.2337/diabetes.49.3.424

Jiang B. H., Semenza G. L., Bauer C., Marti H. H. (1996): Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O2 tension. Am. J. Physiol. **271,** C1172–1180

Jung Y. J., Isaacs J. S., Lee S., Trepel J., Neckers L. (2003): IL-1-beta-mediated up-regulation of HIF-1alpha via an NFkappaB/COX-2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis. FASEB J. **17,** 2115–2117

Khanal G., Chung K., Solis-Wever X., Johnson B., Pappas D. (2011): Ischemia/reperfusion injury of primary porcine cardiomyocytes in a low-shear microfluidic culture and analysis device. Analyst. **136,** 3519–3526 http://dx.doi.org/10.1039/c0an00845a

Khanal G., Hiemstra S., Pappas D. (2014): Probing hypoxia-induced staurosporine resistance in prostate cancer cells with a microfluidic culture system. Analyst. **139,** 3274–3280 http://dx.doi.org/10.1039/C3AN02324A

Koch C. J. (1984): A thin-film culturing technique allowing rapid gas-liquid equilibration (6 sec) with no toxicity to mammalian cells. Radiat. Res. **97,** 434–442 http://dx.doi.org/10.2307/3576294

Lam R. H., Kim M. C., Thorsen T. (2009): Culturing aerobic and anaerobic bacteria and mammalian cells with a microfluidic differential oxygenator. Anal. Chem. **81,** 5918–5924 http://dx.doi.org/10.1021/ac9006864

Lo J. F., Wang Y., Blake A., Yu G., Harvat T. A., Jeon H., Oberholzer J., Eddington D. T. (2012): Islet preconditioning via multimodal microfluidic modulation of intermittent hypoxia. Anal. Chem. **84,** 1987–1993 http://dx.doi.org/10.1021/ac2030909

Metzen E., Wolff M., Fandrey J., Jelkmann W. (1995): Pericellular PO2 and O2 consumption in monolayer cell cultures. Respir. Physiol. **100,** 101–106 http://dx.doi.org/10.1016/0034-5687(94)00125-J

Mosadegh B., Dabiri B. E., Lockett M. R., Derda R., Campbell P., Parker K. K., Whitesides G. M. (2014): Three-dimensional paper-based model for cardiac ischemia. Adv. Healthc. Mater. **3,** 1036–1043 http://dx.doi.org/10.1002/adhm.201300575

Mounier R., Pialoux V., Roels B., Thomas C., Millet G., Mercier J., Coudert J., Fellmann N., Clottes E. (2009): Effect of intermittent hypoxic training on HIF gene expression in human skeletal muscle and leukocytes. Eur. J. Appl. Physiol. **105,** 515–524 http://dx.doi.org/10.1007/s00421-008-0928-y

Murase K., Mori K., Yoshimura C., Aihara K., Chihara Y., Azuma M., Harada Y., Toyama Y., Tanizawa K., Handa T., Hitomi T., Oga T., Mishima M., Chin K. (2013): Association between plasma neutrophil gelatinase associated lipocalin level and obstructive sleep apnea or nocturnal intermittent hypoxia. PLoS ONE **8,** e54184 http://dx.doi.org/10.1371/journal.pone.0054184

Nanduri J., Yuan G., Kumar G. K., Semenza G. L., Prabhakar N. R. (2008): Transcriptional responses to intermittent hypoxia. Respir. Physiol. Neurobiol. **164,** 277–281 http://dx.doi.org/10.1016/j.resp.2008.07.006

Oliver K. M., Garvey J. F., Ng C. T., Veale D. J., Fearon U., Cummins E. P., Taylor C. T. (2009): Hypoxia activates NF-kappaB-dependent gene expression through the canonical signaling pathway. Antioxid. Redox. Signal. **11,** 2057–2064 http://dx.doi.org/10.1089/ars.2008.2400

Oppegard S. C., Blake A. J., Williams J. C., Eddington D. T. (2010): Precise control over the oxygen conditions within the Boyden chamber using a microfabricated insert. Lab. Chip. **10,** 2366–2373 http://dx.doi.org/10.1039/c004856a

Ota H., Itaya-Hironaka A., Yamauchi A., Sakuramoto-Tsuchida S., Miyaoka T., Fujimura T., Tsujinaka H., Yoshimoto K., Nakagawara K., Tamaki S., Takasawa S., Kimura H. (2013): Pancreatic beta cell proliferation by intermittent hypoxia via up-regulation of Reg family genes and HGF gene. Life Sci. **93,** 664–672 http://dx.doi.org/10.1016/j.lfs.2013.09.001

Ota H., Tamaki S., Itaya-Hironaka A., Yamauchi A., Sakuramoto-Tsuchida S., Morioka T., Takasawa S., Kimura H. (2012): Attenuation of glucose-induced insulin secretion by intermittent hypoxia via down-regulation of CD38. Life Sci. **90,** 206–211 http://dx.doi.org/10.1016/j.lfs.2011.11.011

Otto C. M., Baumgardner J. E. (2001): Effect of culture PO2 on macrophage (RAW 264.7) nitric oxide production. Am. J. Physiol. Cell Physiol. **280,** C280–287

Pae E. K., Kim G. (2014): Insulin production hampered by intermittent hypoxia via impaired zinc homeostasis. PLoS ONE **9,** e90192 http://dx.doi.org/10.1371/journal.pone.0090192

Pettersen E. O., Larsen L. H., Ramsing N. B., Ebbesen P. (2005): Pericellular oxygen depletion during ordinary tissue culturing, measured with oxygen microsensors. Cell Prolif. **38,** 257–267 http://dx.doi.org/10.1111/j.1365-2184.2005.00345.x

Poets C. F., Samuels M. P., Southall D. P. (1994): Epidemiology and pathophysiology of apnoea of prematurity. Biol. Neonate **65,** 211–219 http://dx.doi.org/10.1159/000244055

Polinkovsky M., Gutierrez E., Levchenko A., Groisman A. (2009): Fine temporal control of the medium gas content and acidity and on-chip generation of series of oxygen concentrations for cell cultures. Lab. Chip. **9,** 1073–1084 http://dx.doi.org/10.1039/b816191g

Prabhakar N. R., Kumar G. K., Nanduri J. (2010): Intermittent hypoxia augments acute hypoxic sensing via HIF-mediated ROS. Respir. Physiol. Neurobiol. **174,** 230–234 http://dx.doi.org/10.1016/j.resp.2010.08.022

Prabhakar N. R., Semenza G. L. (2012): Adaptive and maladaptive cardiorespiratory responses to continuous and intermittent hypoxia mediated by hypoxia-inducible factors 1 and 2. Physiol. Rev. **92,** 967–1003 http://dx.doi.org/10.1152/physrev.00030.2011

Punjabi N. M. (2008): The epidemiology of adult obstructive sleep apnea. Proc. Am. Thorac. Soc. **5,** 136–143 http://dx.doi.org/10.1513/pats.200709-155MG

Qiao Y., Ma L. (2013): Predicting efficacy of cancer cell killing under hypoxic conditions with single cell DNA damage assay. Anal. Chem. **85,** 6953–6957 http://dx.doi.org/10.1021/ac401543t

Querido J. S., Sheel A. W., Cheema R., Van E. S., Mulgrew A. T., Ayas N. T. (2012): Effects of 10 days of modest intermittent hypoxia on circulating measures of inflammation in healthy humans. Sleep Breath. **16,** 657–662 http://dx.doi.org/10.1007/s11325-011-0555-4

Quintero M., Gonzalez-Martin M. C., Vega-Agapito V., Gonzalez C., Obeso A., Farre R., Agapito T., Yubero S. (2013): The effects of intermittent hypoxia on redox status, NF-kappaB activation, and plasma lipid levels are dependent on the lowest oxygen saturation. Free Radic. Biol. Med. **65,** 1143–1154 http://dx.doi.org/10.1016/j.freeradbiomed.2013.08.180

Reinke C., Bevans-Fonti S., Drager L. F., Shin M. K., Polotsky V. Y. (2011): Effects of different acute hypoxic regimens on tissue oxygen profiles and metabolic outcomes. J. Appl. Physiol. **111,** 881–890 http://dx.doi.org/10.1152/japplphysiol.00492.2011

Roels B., Bentley D. J., Coste O., Mercier J., Millet G. P. (2007): Effects of intermittent hypoxic training on cycling performance in well-trained athletes. Eur. J. Appl. Physiol. **101,** 359–368 http://dx.doi.org/10.1007/s00421-007-0506-8

Ryan S., McNicholas W. T., Taylor C. T. (2007): A critical role for p38 map kinase in NF-kappaB signaling during intermittent

hypoxia/reoxygenation. Biochem. Biophys. Res. Commun. **355,** 728–733
http://dx.doi.org/10.1016/j.bbrc.2007.02.015

Scholz C. C., Cavadas M. A., Tambuwala M. M., Hams E., Rodriguez J., von K. A. Cotter P., Bruning U., Fallon P. G., Cheong A., Cummins E. P., Taylor C. T. (2013): Regulation of IL-1-beta-induced NF-kappaB by hydroxylases links key hypoxic and inflammatory signaling pathways. Proc. Natl. Acad. Sci. USA **110,** 18490–18495
http://dx.doi.org/10.1073/pnas.1309718110

Semenza G. L., Prabhakar N. R. (2007): HIF-1-dependent respiratory, cardiovascular, and redox responses to chronic intermittent hypoxia. Antioxid. Redox. Signal. **9,** 1391–1396
http://dx.doi.org/10.1089/ars.2007.1691

Sharp F. R., Ran R., Lu A., Tang Y., Strauss K. I., Glass T., Ardizzone T., Bernaudin M. (2004): Hypoxic preconditioning protects against ischemic brain injury. NeuroRx **1,** 26–35
http://dx.doi.org/10.1602/neurorx.1.1.26

Sherwani S. I., Aldana C., Usmani S., Adin C., Kotha S., Khan M., Eubank T., Scherer P. E., Parinandi N., Magalang U. J. (2013): Intermittent hypoxia exacerbates pancreatic beta-cell dysfunction in A mouse model of diabetes mellitus. Sleep **36,** 1849–1858
http://dx.doi.org/10.5665/sleep.3214

Stokowski L. A. (2005): A primer on Apnea of prematurity. Adv. Neonatal Care **5,** 155–170
http://dx.doi.org/10.1016/j.adnc.2005.02.010

Tsapikouni T., Garreta E., Melo E., Navajas D., Farre R. (2012): A bioreactor for subjecting cultured cells to fast-rate intermittent hypoxia. Respir. Physiol. Neurobiol. **182,** 47–52
http://dx.doi.org/10.1016/j.resp.2012.01.001

Valencia-Flores M., Orea A., Castano V. A., Resendiz M., Rosales M., Rebollar V., Santiago V., Gallegos J., Campos R. M., Gonzalez J., Oseguera J., Garcia-Ramos G., Bliwise D. L. (2000): Prevalence of sleep apnea and electrocardiographic disturbances in morbidly obese patients. Obes. Res. **8,** 262–269
http://dx.doi.org/10.1038/oby.2000.31

Wang B., Yan B., Song D., Ye X., Liu S. F. (2013a): Chronic intermittent hypoxia down-regulates endothelial nitric oxide synthase expression by an NF-kappaB-dependent mechanism. Sleep Med. **14,** 165–171
http://dx.doi.org/10.1016/j.sleep.2012.10.020

Wang N., Khan S. A., Prabhakar N. R., Nanduri J. (2013b): Impairment of pancreatic beta-cell function by chronic intermittent hypoxia. Exp. Physiol **98,** 1376–1385
http://dx.doi.org/10.1113/expphysiol.2013.072454

Xu J., Long Y. S., Gozal D., Epstein P. N. (2009): Beta-cell death and proliferation after intermittent hypoxia: role of oxidative stress. Free Radic. Biol. Med. **46,** 783–790
http://dx.doi.org/10.1016/j.freeradbiomed.2008.11.026

Yokoe T., Alonso L. C., Romano L. C., Rosa T. C., O'Doherty R. M., Garcia-Ocana A., Minoguchi K., O'Donnell C. P. (2008): Intermittent hypoxia reverses the diurnal glucose rhythm and causes pancreatic beta-cell replication in mice. J. Physiol. **586,** 899–911
http://dx.doi.org/10.1113/jphysiol.2007.143586

Young T., Peppard P. E., Gottlieb D. J. (2002): Epidemiology of obstructive sleep apnea: a population health perspective. Am. J. Respir. Crit. Care Med. **165,** 1217–1239
http://dx.doi.org/10.1164/rccm.2109080

Yu A. Y., Frid M. G., Shimoda L. A., Wiener C. M., Stenmark K., Semenza G. L. (1998): Temporal, spatial, and oxygen-regulated expression of hypoxia-inducible factor-1 in the lung. Am. J. Physiol. **275,** L818–826

Yuan G., Adhikary G., McCormick A. A., Holcroft J. J., Kumar G. K., Prabhakar N. R. (2004): Role of oxidative stress in intermittent hypoxia-induced immediate early gene activation in rat PC12 cells. J. Physiol. **557,** 773–783
http://dx.doi.org/10.1113/jphysiol.2003.058503

Yuan G., Khan S. A., Luo W., Nanduri J., Semenza G. L., Prabhakar N. R. (2011): Hypoxia-inducible factor 1 mediates increased expression of NADPH oxidase-2 in response to intermittent hypoxia. J. Cell Physiol. **226,** 2925–2933
http://dx.doi.org/10.1002/jcp.22640

Zhdanov A. V., Ogurtsov V. I., Taylor C. T., Papkovsky D. B. (2010): Monitoring of cell oxygenation and responses to metabolic stimulation by intracellular oxyg en sensing technique. Integr. Biol. (Camb.) **2,** 443–451
http://dx.doi.org/10.1039/c0ib00021c

Received: October 5, 2014
Final version accepted: December 23, 2014
First published online: March 27, 2015