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The Determinants of Oxygen Gradients in Respiring Samples and their Impact on Cellular Responses to Hypoxia

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Running title: Oxygen Gradients in Respiring Samples

Abstract

Changes in cellular O₂ levels elicit adaptive responses which can lead to the activation of oxygen-dependent transcription factors such as the hypoxia-inducible factor (HIF). However, our understanding of the determinants of these processes is still incomplete. Using the new intracellular O₂ sensing technique, we monitored O₂ gradients in static cultures of adherent PC12 cells exposed to graded atmospheric O₂ at rest and upon metabolic stimulation. Under high atmospheric O₂ (10-21%) the respiration of resting cells dictated that local O₂ was moderately reduced, and at a certain threshold (6% in galactose medium) cell layer became practically anoxic. Furthermore, cell stimulation triggered a major redistribution of O₂ and a prominent 'hypoxic overshoot' effect mediated by diffusion. The deep, prolonged cell deoxygenation upon stimulation was matched by an increase in nuclear HIF-1 α levels. In the presence of nitric oxide the hypoxic overshoot was truncated and HIF-1 α stabilization inhibited. Thus, the main determinants which impact upon cellular O₂ levels and oxygen-sensitive signaling pathways are the atmospheric O₂, sample geometry, cell density, respiration rate and its dynamics. Changes in any of these parameters can significantly alter the O₂ levels experienced by the cells and the subsequently activated signaling pathways.

Keywords: Localised Oxygen Gradients, Cell Respiration, Metabolic Stimulation, Phosphorescence Quenching Technique, Intracellular Oxygen-Sensitive Probe; Hypoxia, nitric oxide, HIF-1 α

Normal functioning of the cells in the body occurs at a range of pO_2 values. During the process of oxidative phosphorylation, molecular oxygen (O_2) provides the primary source of energy for aerobic organisms. Compared to ambient O_2 (20.9% or 147 mmHg), mammalian cells and tissues function under reduced O_2 levels which are maintained within narrow limits^{1,2}. For example, actively respiring brain tissue consumes a large fraction of available O_2 . It shows heterogeneity in activity and function, and requires stable and efficient O_2 supply from the blood. Alterations in either demand or supply of O_2 lead to redistribution of O_2 within the tissue, which may induce a state of hypoxia and lead to an energy crisis and ultimately cell death. To be able to cope with such threats, cells and tissues have developed metabolic, transcriptional and systemic responses to hypoxia directed towards survival and protection^{1,3}. Elaboration of these adaptive responses is important for the understanding and treatment of many (patho)physiological conditions, including ischemia/stroke, excitotoxicity, neurodegeneration, cancer and inflammation⁴.

Mimicking hypoxic conditions *in vitro* has helped elucidate the roles of key regulators of cellular responses to hypoxia including the hypoxia-inducible transcription factor HIF⁷⁻⁹, O_2 -dependent prolyl (PHD1-3) and asparaginyl (FIH) hydroxylases¹, AMP-activated kinase¹⁰, the activator of mitochondrial biogenesis PGC-1 α ^{11,12}, nitric oxide (NO)^{5,6} and reactive oxygen species (ROS)¹³. At the same time, many questions remain, particularly in relation to the primary O_2 chemosensor(s) and the thresholds of cellular oxygen for adaptive responses to be initiated in different tissues¹⁴. The interpretation of biological responses to hypoxia is often complicated by the fact that the actual levels of cell oxygenation in such experiments are not known or controlled precisely. Perfusion chambers and stirred measurement cells provide efficient mass exchange and uniform O_2 levels across the sample¹⁵, however they do not adequately reflect the conditions in respiring tissue where localized O_2 gradients and diffusion processes play important roles^{16,17}. Despite the advances in O_2 measurement, both in *in vivo*^{18,19} and *in vitro*²⁰⁻²² systems, the shape of localized O_2 gradients, their effects on cellular function and reliable O_2 maps in respiring objects (cells, tissue, vasculature) are still under active discussion^{2,18,23}. Their detailed knowledge can shed light on the mechanisms of adaptive responses to hypoxia and some common physiological and disease states.

A model commonly used in *in vitro* studies is a static cell culture maintained at a constant external (atmospheric) pO_2 . For such a system, O_2 consumption rates and diffusion processes are important factors which contribute to O_2 transport to the cells from gaseous macro-phase^{24,25}. Careful control of cellular O_2 in such experiments is still rare, and partial or complete deoxygenation and hypoxia-specific metabolic rearrangements may occur¹⁷ and contribute to the observed biological effects. In this work, using quenched-phosphorescence O_2 sensing technique which allows real-time monitoring of both intra- and extracellular O_2 concentration^{22,26,27} we investigated O_2 gradients in dense populations of neurosecretory pheochromocytoma PC12 cells cultured and differentiated under standard conditions in 96-well plates (dPC12). dPC12 cells possess active oxidative phosphorylation and glycolysis, produce robust responses to excitatory stimulation^{22,26}, and they represent a convenient model for studies of brain function and pathologies²⁸. For such a system with resting respiring dPC12 cells, steady state O_2 gradients at different atmospheric O_2 were modeled and measured experimentally. Subsequently, the effects of fast metabolic stimulation of the cells on these O_2 gradients were investigated. The main parameters determining the O_2 levels in such samples and their impact on the adaptive responses to hypoxia, particularly NO and HIF-1 α signaling, were assessed.

Results and Discussion

Model of the respiring sample. The samples under investigation represented for example by a well of 96-well plate (**Fig. 1A**) can be described as follows. A layer of dPC12 cells having effective thickness L_c [mm] and O_2 diffusion coefficient D_c [$mm^2 \cdot s^{-1}$] consumes dissolved O_2 at a specific rate k [$\mu mole \cdot ml^{-1} \cdot s^{-1}$]. The solution layer above the cells, which has a thickness L_s and O_2 diffusion coefficient D_s , is exposed to the gaseous atmosphere with constant pO_2 . The cell layer is acting as a sink generating O_2 gradient in the sample, and the solution layer – as a barrier through which O_2 diffuses to the cells from the gaseous macro-phase down the concentration gradient.

For a relatively wide sample and with a number of assumptions (the bottom of the well is O_2 -impermeable; the cells respire at a constant rate k ; negative O_2 levels generated by simulation are disregarded), this experimental model can be described by planar 1-D diffusion equations (see **Supplemental Material** and²⁹).

For such a system under steady state condition, O₂ profiles in the solution layer (*Cs*), and the cell layer (*Cc*) are described by the following equations:

$$Cs = C_0 - \frac{Lc}{Ds} k (X + Ls) \quad (1)$$

$$Cc = \frac{k}{2Dc} X^2 - k \frac{Lc}{Dc} X + H \left(C_0 - \frac{LcLs}{Ds} k \right) \quad (2)$$

where *C₀* is the O₂ concentration at the gas/solution interface (*X*=0) with given O₂ and temperature; *H* is the O₂ partition coefficient at the cells/solution interface ($Cc|_{X=Ls} = H Cs|_{X=Ls}$); *X* – distance from the interface. In other words, *Cs* changes as a linear function and *Cc* – as a quadratic function of the distance from the O₂ reservoir. Main parameters of the model which determine O₂ profile within the sample are defined in **Table 1**. Computer simulation of O₂ profiles for such a model is shown in **Fig. 1B**, for three different O₂ values (20.9%, 10% and 5%).

On the other hand, for a more general and physiological case cell respiration is dependent on O₂ concentration, i.e. *k* is a function of *Cc*³⁰, and this has an impact on both *Cc* and *Cs* profiles. Also when *k* is changing over time, mechanistic description of O₂ dynamics in different parts of the sample becomes more complicated than for the steady state with constant *k* (i.e. Eqs. 1,2 are not applicable anymore).

Measurement of O₂ gradients in samples with resting cells. To validate the above model, we measured the actual O₂ levels in experimental samples. Using the phosphorescent O₂-sensitive probe and time-resolved fluorescence reader placed in a hypoxia chamber with adjustable atmospheric O₂, both *Cc* and *Cs* levels can be assessed, when the O₂ probe is loaded into the cells or added to the medium²⁶, respectively. For our model, *Lc* was approximately 1000 times smaller than *Ls*, and measured *Cc* and *Cs* represented average values across the layer of cells and across the solution layer, respectively.

We examined the effects on *Cc* of the thickness and activity of the cell layer (i.e. *Lc* and *k*), atmospheric O₂, the height (*Ls*) and viscosity (*Ds*) of the solution layer. After the initial period of gas and temperature equilibration (20-60 min), the system gave stable fluorescent readings which corresponded to a steady state with characteristic intracellular O₂ values (**Fig. 2A**). For a confluent monolayer of dPC12 cells at 20.9% O₂ and 37°C, *Cc* was seen to be reduced moderately (by approximately 40 μM of O₂ compared to *C₀*). When more cells were added and allowed to settle, *Cc* was reduced further. With additional 6.75·10⁵ cells (roughly equivalent to 4 monolayers) the bottom layer of dPC12 cells became practically anoxic. The linear dependence between *Cc* and cell number (i.e. *Lc*) (**Fig. 2B**) supports the planar diffusion model (**Eqn. 2**).

When O₂ in the chamber was changed to 10% and 6%, the reduction in *Cc* relative to *C₀* increased (**Fig. 2C-E**). When glucose in the medium was replaced with galactose, *Cc* became significantly lower, such that at 6% O₂ the cells became practically anoxic. Such a reduction in *Cc* is because in galactose the glycolytic pathway no longer generates ATP, so to satisfy energy demand the cells increased oxidative phosphorylation and respiration rate *k*³¹. Increasing the O₂-barrier properties of the medium by adding Ficoll (higher viscosity, lower *Ds*) also decreased *Cc* (**Fig. 2E**). Conversely, inhibition of cell respiration with antimycin A (a large reduction in *k*) eliminated the O₂ gradient and brought *Cc* close to the *C₀* values (**Fig. 2C,D**).

These results illustrate that cells cultured under static conditions and separated from the gaseous macrophase with a few millimeters of medium can generate significant O₂ gradients even under 20.9% atmospheric O₂. Similar was reported for the other cells²⁴. When external O₂ is reduced, relative deoxygenation of the cells tends to increase such that at a certain *pO₂* threshold the cells create and maintain deeply hypoxic microenvironment. For a confluent monolayer of cells in galactose medium, this threshold is significantly higher (6% O₂) than what is normally used in ‘moderate hypoxia’ models (0.5-2% O₂). A similar dependence of the intracellular O₂ levels on external O₂ was reported for contracting myocytes³².

For the cells cultured in a vessel (flask or microplate), simple 1-dimensional model which accounts for diffusion and consumption of O₂ gives a satisfactory description of O₂ profiles under steady-state condition (resting cells). This was confirmed by direct measurement of *Cc* and *Cs* using the phosphorescent O₂ probe loaded into the cells or added to the medium, respectively. The O₂ gradients emerge at the cell layer and

propagate through the medium to the interface (**Fig. 3, C-E**). The degree of local deoxygenation depends on a number of parameters including respiratory activity and thickness of the cell layer, thickness and diffusion properties of the medium, external O₂ (**Fig. 2A,E, Fig. 3**). Knowledge of these determinants and their contribution is important for controlling cell oxygenation, especially under hypoxic macro-environment. Otherwise, cellular O₂ may fluctuate within broad limits, thus leading to misinterpretation of the observed biological effects and readout parameters. For slow respiring and highly glycolytic cell lines these effects may not be so pronounced, whereas for actively respiring cells that rely mostly on oxidative phosphorylation (e.g. dense populations of neurons, primary cells, tissue slices), large local gradients and reduced availability of O₂ may occur and lead to metabolic rearrangement, shift in bioenergetics, altered function and cell death.

O₂ dynamics upon cell stimulation. Following the analysis of steady state O₂ profiles, we applied to the resting dPC12 cells at 20.9% O₂ pharmacological treatments that alter their respiration. In particular, the addition of high extracellular K⁺ or depletion of extracellular Ca²⁺ by EGTA are known to induce rapid, transient spike in respiration of dPC12 cells due to increased energy requirements^{26,33}. Although modeling of this case is rather complex requiring extensive mathematical description, consideration of a number of additional parameters and solving the system of partial differential equations, real time changes in *Cc* and *Cs* induced by such stimulation can be monitored experimentally using the optical O₂ sensing technique.

Typical profiles of *Cc* upon EGTA addition shown in **Fig. 3A** reflect transition processes within the sample which are mediated by the changes in cell respiration and O₂ diffusion. In conditions when O₂ supply to the cells is controlled by the layer of medium (diffusion barrier for O₂), increased O₂ demand causes a rapid depletion of O₂ at the cell layer. The newly formed local O₂ gradient propagates outwards and, in turn, increases the flux of atmospheric O₂ to the cells. When increased O₂ consumption is balanced by increased O₂ influx, the system comes to a new steady state determined by the activity of stimulated cells, *k*. Altogether this produces a characteristic '*hypoxic overshoot*' - a transient dip in *Cc* due to the sudden imbalance between the demand and supply of atmospheric O₂ to the respiring cells/tissue, followed by re-oxygenation (partial or full, depending on the type of stimulation). Thus, after stimulation with EGTA the new steady state *Cc* is almost the same as before stimulation, indicating that the increase in cell respiration was transient. For the uncoupler FCCP²⁶, the new *Cc* was lower than before stimulation indicating a sustained increase in respiration by the drug (**Supplemental Material, Fig. S3**).

It can be anticipated from the model that the shape of hypoxic overshoot and changes in *Cc* upon cell stimulation depend on the barrier properties of the solution layer, namely *Ls* and *Ds*. Indeed, when we increased the volume of medium from 100 µL to 200 µL, the magnitude and duration of the response increased significantly (*p*<0.0001), resulting in deep and sustained deoxygenation of the cells (**Fig. 3A**). The effect of sample parameters on the response to cell stimulation was also analysed. Increased *Ls* made the responses to low doses of stimulant more pronounced (**Fig. 3B**), while increased viscosity of the barrier (*Ds*) made the deoxygenation phase faster and reoxygenation slower (**Supplemental Material, Fig. S4**). At higher cell numbers the response was increased (**Fig. 3E**). In agreement with diffusion model (**Fig. 1**), we also observed measurable changes in the solution layer (*Cs*) which were smaller in amplitude but similar in shape to those of the *Cc* (**Fig. 3C-E**). When antimycin A (inhibitor of respiration) was present in the medium, the respiratory response of dPC12 cells to both EGTA and FCCP was completely abolished (**Supplemental Material, Fig. S3**).

These results show that when the cells undergo metabolic stimulation, a number of additional factors come into play. A sudden imbalance between the demand and supply of O₂ induces a steep dip in local O₂ at cell layer making it deeply hypoxic or even anoxic. Even if the stimulation is not sustained, the cells and the sample still go through a relatively long transition (many minutes in our case) before establishing a new steady state with new *Cc* and *Cs*. As we demonstrate above, the processes underlying such hypoxic overshoot and its distinct phases have mostly diffusion nature. As a consequence of rapid metabolic stimulation and/or disbalance between O₂ demand and supply, deep and prolonged deoxygenation can occur, which primarily affects the layer of respiring cells but also propagates into remote areas of the sample. This effect is largely mediated by gas-barrier properties of the surrounding medium: process duration and O₂ fluxes are determined by diffusion time of the sample.

Since live tissue/blood vessel systems resemble our model, we anticipate that similar effects also occur *in vivo* and play a role in (patho)physiological conditions such as excitotoxicity, spontaneous firing of neurons, ischemia and tissue re-oxygenation. O₂ diffusion in tissue is thought to be close to water and extracellular fluid^{2, 34}, therefore the effects of local imbalance between O₂ utilization and supply should be similar, for example in the areas of the brain which undergo transient stimulation and which are remote from the source of O₂. It can well be that such long-distance gradients and waves of O₂ are part of feedback regulation in higher organisms providing chemical signals to vasculature and blood about altered respiration, increased demand or acute shortages in O₂ in remote areas of respiring tissue. However this still remains to be proven.

Physiological implications of local O₂ gradients. Pronounced O₂ gradients in the samples with respiring cells and their changes upon stimulation point to their importance for cell physiology. We investigated the links between these gradients and the key regulator of cellular responses to hypoxia, nitric oxide (NO), which competes with O₂ for the active site of cytochrome *c* oxidase. In hypoxia cells increase NO production through the increased expression of NO synthase and decreased NO metabolism by cytochrome *c* oxidase^{36, 37}. Thus, for isolated synaptosomes IC50 for NO drops from 270 nM at 145 μ M O₂ (close to arterial O₂) to 60 nM at 30 μ M O₂ (corresponds to tissue O₂)³⁵. NO inhibits O₂ consumption by endothelial cells (i.e. decreases effective *k*, see **Eqn. 1-2**) and acts on the vasculature causing vasodilation and enhanced distribution of O₂ to surrounding tissues³⁸.

Using dPC12 cells cultured at 20.9% of atmospheric O₂, we created different levels of NO in the medium (micromolar range) by the addition of DETA-NONOate (DETA) which provides controlled release of NO³⁹. The cells were then stimulated by EGTA while monitoring *Cc*. At low levels of DETA no significant changes in basal respiration and the shape of the respiratory response were seen, whereas at >0.4 mM DETA the response drastically changed (**Fig 4A, B**). In particular, NO released by DETA did not influence much the initial phase and total duration of the response, but it abruptly ‘switched off’ the process of cell deoxygenation at a certain *Cc* level which correlated with DETA/NO concentration. Thus, at 1.0 mM and 0.5 mM DETA in the medium *Cc* profile reached its minimum at 46 ± 7 μ M and 15 ± 5 μ M O₂, respectively (**Fig. 4C**). According to Pervin et al.³⁹ who used practically the same medium, 1 mM DETA maintains constant NO concentration of 0.5 μ M (arrow shown in **Fig. 4C**).

At very high DETA concentrations the response to EGTA was practically abolished, this coincided with a minor increase in basal *Cc*. For the cells relying on oxidative phosphorylation (in galactose medium), excitatory stimulation at ≥ 2 mM DETA caused rapid damage, since increased energy demand cannot be met by increased production of ATP. These cells quickly lost their integrity and died, as was evident from light microscopy. This was accompanied by a marked decrease in cellular ATP levels measured at 40 min and 90 min after stimulation (**Fig. 4D**), i.e. at peak and after termination of the respiratory response. Under the same conditions in glucose medium, the minimal *Cc* values were higher than in galactose, and the initial rates of deoxygenation were slower (3.5-4 and 8-9 μ M/min at 1.0 mM DETA, respectively). Despite the significant down-regulation of the respiratory response by DETA, cellular ATP levels in glucose medium did not change much (**Fig. 4D**). Again, these results agree with the diffusion model, kinetics of cell respiration (higher *k* in galactose) and the mode of action of NO via inhibition of cytochrome *c* oxidase.

From this we concluded that in conditions of strong excitatory stimulation, NO, if generated in sufficient quantities, robustly ‘switches off’ cell respiration at low O₂ concentrations, thus protecting the cells from becoming deeply hypoxic. NO truncates the original peak-shape response and produces a plateau region instead. When the cell deprived in glucose (non-glycolytic) received activatory stimulation, they were unable to maintain energy balance (**Fig. 4D**) and started to die. This is similar to neuronal excitotoxicity in glial cells triggered by the activation of iNOS⁴⁰ or by simultaneous increase in NO and decrease in O₂⁴¹.

Sustained cellular hypoxia is known to inhibit HIF-1 α proline hydroxylases and HIF-1 α degradation, thus increasing the levels of active HIF, a key event in triggering the ‘hypoxic’ rearrangement in gene expression¹. We examined how HIF-1 α levels can elevate upon excitatory stimulation under atmospheric normoxia (20.9% O₂), and what minimal dose of local cellular hypoxia is required for such elevation. Stimulation with EGTA which causes deep and sustained deoxygenation of a monolayer of dPC12 cells (residual *Cc* <5 μ M), increased

nuclear HIF-1 α levels already after 45 min ($p < 0.01$) (**Fig. 4E**). For a similar experiment performed in the presence of 1.0 mM DETA, such stabilization of HIF-1 α was not observed. A similar NO-dependent inhibition of cellular responses to hypoxia via HIF-dependent pathways was shown previously⁶.

When the respiration in dPC12 was not inhibited by DETA, metabolic stimulation with EGTA induced a strong respiratory response and activated HIF-1 α dependent regulatory pathway even under 20.9% of atmospheric O₂ (**Fig. 4E**). Detectable levels of HIF-1 α stabilization in PC12 cells were previously reported for significantly deeper (<2%) and prolonged (4 h) atmospheric hypoxia⁴². Similarly, PGC-1 α dependent increase of mitochondrial biogenesis and respiratory activity in C2C12 cells triggered HIF-1 α -regulated gene expression in normoxia¹².

Experimental

Cell Culture and O₂ Measurements. Rat pheochromocytoma PC12 cells (ATCC) were maintained in RPMI 1640 medium supplemented with NaHCO₃, 2 mM L-glutamine, 10% horse serum (HS), 5% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin (P/S), in 5% CO₂ at 37°C. The cells were seeded at 1-5 $\cdot$ 10⁴ cells/well in standard 96-well plates made of clear polystyrene (Sarstedt, Ireland) pre-coated with collagen IV. Unless specified otherwise, the cells were differentiated for 3-5 days in RPMI supplemented with 1% horse serum, P/S, and 100 ng/ml nerve growth factor to form a confluent monolayer (approximately 10⁵ cells/well or 3 $\cdot$ 10⁵ cells/cm²).

For the intracellular O₂ assay, cells were loaded by incubating them for 28 h in the differentiating conditions with 1.2 μ M of MitoXpress probe (Luxcel Biosciences, Ireland) and 6 μ M of Endo-Porter transfection reagent (Gene Tools). After that the wells with loaded dPC12 cells were washed three times with fresh medium and 50-200 μ L of air-saturated serum-free RPMI supplemented with 25 mM HEPES (pH=7.3-7.35), 1 mM pyruvate and 10 mM of either glucose or galactose (pre-heated at 37°C) were added to each sample well. The plate was then measured on a time-resolved fluorescence (TR-F) plate reader Victor 2 (PerkinElmer) at 37°C using 340nm excitation and 642nm emission filters. Probe phosphorescence was measured taking two TR-F intensity signals F₁ and F₂ at two delay times $t_1=30$ μ s and $t_2=50$ μ s. The plate was monitored over a period of 40-120 min taking readings in each well every 1-2 minutes. Measured TR-F signals were converted into phosphorescence lifetime, τ (μ s) as follows²²: $\tau = (t_2 - t_1) / \ln(F_1/F_2)$. The phosphorescence lifetime profiles were converted into O₂ concentration (C_c , μ M) using the following calibration function: $C_c = -0.0027\tau^3 + 0.5649\tau^2 - 40.104\tau + 972.23$. Representative raw intensity profiles and their conversion into lifetime and O₂ concentration profiles are shown in **Fig. S1 (Supplemental Material)**.

Calibration of the MitoXpress probe loaded in dPC12 cells was performed on the TR-F reader placed in a hypoxia chamber (Coy Scientific, USA) pre-set at different O₂ levels ranging between 20.9% and 1% O₂. Atmospheric O₂ values in the hypoxia chamber were set and maintained constant during the measurement with an accuracy of $\pm 0.1\%$ by the gas controller equipped with the calibrated electrochemical oxygen sensor (Coy Scientific). Atmospheric O₂ values were converted into dissolved O₂ concentration: 1% O₂ corresponds to 9.9 μ M. To eliminate the effect of respiration on intracellular O₂ concentration, Antimycin A (10 μ M) was added to the cells loaded with probe, the plate was placed in the TR-F reader pre-set at 37°C and measured over 30-90 min taking readings every 2.5 min. After reaching stable signals reflecting gas and temperature equilibration of samples, phosphorescence lifetime values were determined and used for the calibration. To obtain zero point on calibration, 100mM of D-(+)-glucose and 100 μ g/ml of glucose oxidase were added to the samples with cells (without antimycin A) and after the establishment of an O₂ free environment lifetime values were determined in a similar way. Calibration graph and its fitting with the above calibration function for C_c are shown in **Fig. S2 (Supplemental Material)**.

To determine average O₂ levels in the layer of medium, MitoXpress probe was added to the medium at a concentration 200 nM and measured as described above. To generate additional cell layers, trypsinized non-differentiated PC12 cells were applied on the top of the monolayer of dPC12 cells loaded with probe, left to settle for 30 min in CO₂ incubator and then measured. Measurements under normoxia (20.9% or 200 μ M O₂), 10% O₂ (97.6 μ M O₂), 6% O₂ (58.5 μ M O₂) were conducted by placing the TR-F reader in the hypoxia chamber (Coy Scientific) equilibrated at given O₂.

In the experiments with cell stimulation, the plate with cells pre-loaded with the probe was initially monitored on the reader for 10-20 min to obtain basal lifetime signals. Subsequently, the plate was withdrawn, effector stock solutions were applied gently on top of each sample (1/10 volume) and monitoring was resumed for further 60-120 min. To maintain constant levels of NO, NO donor DETA was added to the medium at 0.1-2 mM.

Analysis of HIF-1 α levels in resting and stimulated dPC12 cells was performed using the DNA-binding ELISA kit TransAM™ HIF-1 (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. At particular time intervals after stimulation, cells were collected from the microplate and nuclear extracts were prepared using Nuclear Extract Kit (Active Motif). For each time point, 20 identical samples/wells were pooled (~2·10⁶ cells), total protein concentration in nuclear extracts was determined with BCA™ Protein Assay kit (Pierce, Rockford, Ill) and normalized. The extracts were then applied on the 96-well plate (5 μ g/well) and analysed for HIF-1 α by ELISA.

Statistics. The data were evaluated for statistical difference using two-tailed Student t-test. The 0.01 level of confidence was accepted as statistically significant. Plate reader data are presented as average values $\pm$ standard deviation for 6-8 replicated samples (error bars on the plots). Each time, 24-30 samples/wells were analyzed simultaneously with all the controls, replicates and variables present on one plate. All the experiments were repeated 3-5 times to ensure consistency of results.

Conclusions

In this study we probed localized O₂ gradients in respiring samples and demonstrated their importance for cell physiology and responses to hypoxia. The phosphorescence based O₂ sensing technique has allowed direct monitoring of intracellular O₂ levels, dynamics of O₂ gradients, diffusion behavior of samples and metabolic responses of test cells, thus providing quantitative assessment of these processes and contributing factors. In particular, it helped to uncover and elaborate the prominent hypoxic overshoot and some new aspects of regulation of respiration by hypoxia, NO and HIF signaling. These processes may play a role in a number of common (patho)physiological conditions.

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Supporting Information Available

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Table. Symbols and definitions for the main parameters of the model.

Parameter	Definition
O_2	Atmospheric O_2 concentration
C_0	O_2 concentration at the interface with gaseous macro-phase
L_s	Thickness of the non-respiring solution layer
L_c	Thickness of the respiring layer with cells
k	Specific oxygen consumption rate
H	O_2 partition coefficient at the interface of the respiring and non-respiring layers
D_s, D_c	O_2 diffusion coefficients for solution and cell layer
C_s	O_2 concentration in the non-respiring solution layer
C_c	O_2 concentration in the respiring layer of cells
X	Distance from the gaseous macro-phase

Figure legends

Figure 1. Schematic representation of a respiring sample and simulated O₂ profiles under steady state.

A. Planar 1-D diffusion model with a layer of adherent cells cultured at the bottom of a vessel at constant atmospheric O₂. Growth medium above the cells acts as diffusion barrier. **B.** Simulated profiles of O₂ concentration at three different O₂ values: 20.9%, 10% and 5%. O₂ gradients are linear in the solution layer and quadratic in the cell layer. X – distance from the macro-phase with constant O₂. Diffusion coefficients for the respiring and non-respiring layers are considered the same and equal to $3.2 \cdot 10^{-3} \text{ mm}^2 \cdot \text{s}^{-1}$ ³⁴, and $H = 1$. Symbols show the main parameters of the sample, their description is given in the Table.

Figure 2. O₂ levels within the layer of resting dPC12 cells.

A. Under external normoxia (200 μM O₂), adding more PC12 cells on top of the monolayer (indicated as numbers from 0 to $6.75 \cdot 10^5$) decreases C_c . **B.** C_c in the monolayer of dPC12 cell has a linear dependence on the number of added cells. **C.** At 10% O₂ (100 μM O₂), steady-state C_c levels in galactose are lower than in glucose medium due to more active respiration. **D.** At 6% O₂ (60 μM O₂), the drop in C_c becomes greater and in galactose the cells become anoxic. **E.** At reduced O₂ relative deoxygenation of the cell layer increases and reaches anoxia. **F.** Increased viscosity of the medium by Ficoll addition decreases C_c (measured at 20.9% O₂). Sample volume 100 μL for all the experiments.

Figure 3. Dynamics of O₂ upon cell stimulation.

A. The duration and magnitude of the response (C_c) to 5 mM EGTA (depletion in extracellular Ca²⁺) is modulated by the sample volume/height. **B.** At higher O₂ barrier (200 μL of medium) even minor respiratory responses becomes more visible: the responses to $0.5 < \text{EGTA} < 1$ mM is significant in 200 μL ($p < 0.01$), but not seen in 100 μL samples. **C, D.** The shape of changes in C_s during cell stimulation are similar to those in C_c ; but the amplitude is smaller. **E.** Addition of more PC12 cells on top of the monolayer (100 μL of medium) increases the response to stimulation. Dotted lines show the time of temperature equilibration of samples. All the experiments, except **E**, were conducted in galactose medium.

Figure 4. Adaptive responses of dPC12 cells to local hypoxia induced by stimulation with EGTA under 20.9% O₂.

A, B. The response in galactose (A) and glucose (B) medium (sample volume 200 μL) is inhibited by NO in a concentration dependent manner. **C.** The relationship between NO concentration and threshold O₂ levels of ‘switching off’ cell deoxygenation in galactose medium. **D.** In the cells deprived of glucose and exposed to NO, ATP levels decrease significantly 40 min after stimulation, whereas in glucose medium the changes were insignificant. At the end of the response (90 min after stimulation) in galactose medium, ATP levels are partly restored. **E.** Deep deoxygenation induced by stimulation significantly elevates nuclear HIF-1α levels after 45 min ($p < 0.001$), and HIF-1α stabilization is inhibited by 0.5 μM NO. Sample volume 200 μL for all the experiments.

Figure 1

Figure 2

Figure 3

Figure 4