[8] Perceived Hyperoxia: Oxygen-Regulated Signal Transduction Pathways in the Heart

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Introduction

Cellular O_2 concentrations are maintained within a narrow range (perceived as ''normoxia'') due to the risk of oxidative damage from excess O_2 (hyperoxia) and of metabolic demise from insufficient O_2 (hypoxia).[1] pO_2 ranges from 90 to below 3 Torr in mammalian organs under normoxic conditions with an arterial pO_2 of about 100 Torr or ~14% O_2.[2] Thus, ''normoxia'' for cells is a variable that is dependent on the specific localization of the cell in organs and functional status of the specific tissue. O_2 sensing is required to adjust to physiological or pathophysiological variations in pO_2. Whereas acute responses often entail changes in the activity of preexisting proteins, chronic responses invariably involve O_2-sensitive changes in signal transduction and gene expression.[3] Several articles have highlighted the key significance of understanding the fundamentals of O_2 sensing.[4–14] Current work in this field is almost exclusively focused on the study of hypoxia. Reoxygenation, however, has been mostly investigated in the context of oxidative injury, and there is a clear paucity of data describing the O_2-sensitive signal transduction pathways under conditions of oxygenation that mildly or moderately exceed perceived normoxia. While acute insult caused during reperfusion may be lethal to cells localized at the focus of insult, elevation of O_2 tension in ischemic tissue is expected to trigger phenotypic changes in the surviving cells that may be associated with tissue remodeling (Fig. 1).

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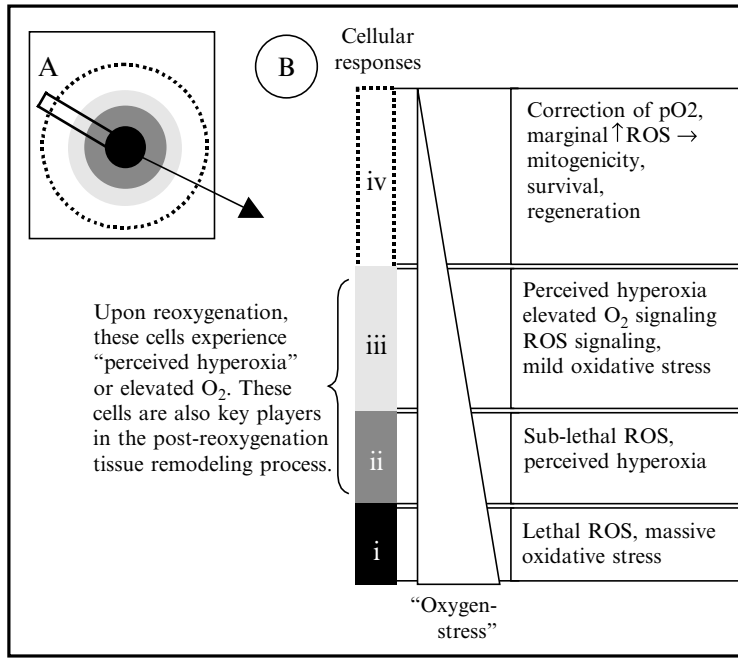

FIG. 1. Cellular responses to reoxygenation following chronic moderate hypoxia: A schematized concept. Chronic hypoxia results in cellular adjustments such that reoxygenation poses ''perceived hyperoxic insult'' as evident in part in the form of oxidative damage in numerous studies. (A) Diagram of hypoxia-reoxygenated tissue, with the focus of insult represented by the black center. (B) Cross section of A. Focal ischemia is known to be associated with graded oxygenation from near-zero status at focus to levels increasing with distance from focus. Important elements triggered by oxygen, molecular or reactive, during the course of reoxygenation-associated remodeling include (i) cell death or fatal injury at the focal point of insult, making room for regenerating tissues; (ii) nonfatal cellular injury, triggering reparative responses, and (iii) survival of phenotypically altered cells that favor remodeling (physiological or pathological/fibrogenic). Fibrosis denies room to regenerating healthy cells, and (iv) correction of pO_2 of mildly hypoxic cells localized beyond a critical distance from the focus of insult, favoring regeneration and restoration of physiologically functioning of the organ.

Oxygenation of the Heart

Under conditions of systemic normoxia, heart cells receive a limited supply of O_2 representing less than 10%.[15–17] We determined that the pO_2 of murine ventricular myocardium is in the range of 5%.[18] Moderate hypoxia is associated with a 30–60% decrease (~1–3% O_2) in pO_2.[19] In response to mild or moderate compromise in pO_2, adaptive processes in surviving cells allow for physiological functioning of the tissue. These

adjustments are evident, for example, in the hibernating myocardium where the organ maintains vital functions in the face of prolonged moderate hypoxia.[20] Although adjustments in metabolism and contractile function have been demonstrated to allow myocardial survival in the face of reduced O_2 supply, the cellular basis of adaptation and the signaling pathways involved in the process have yet to be defined.[21]

Perceived Hyperoxia

In response to chronic moderate hypoxia, cells adjust their normoxia set point such that reoxygenation-dependent relative elevation of pO_2 results in perceived hyperoxia.[18] We hypothesized that O_2, even in marginal relative excess of the pO_2 to which cardiac cells are adjusted, results in the activation of specific signal transduction pathways that alter the phenotype and function of these cells. While acute insult caused during reperfusion may be lethal to cells localized at the focus of insult, the elevation of O_2 tension in the surrounding ischemic tissue triggers phenotypic changes in the surviving cells that may be associated with tissue remodeling.

Oxygenation *In Vitro* Does Not Model *In Vivo* Conditions

Although cells are cultured in the laboratory at an ambient O_2 concentration of 21%, which corresponds to a pO_2 of approximately 150 mm Hg at sea level, cells in the human body are exposed to much lower O_2 concentrations, ranging from ~12% in the pulmonary alveoli to less than 6% (40 mm Hg) in most other organs of the body. Culturing cells at room air is generally considered to be a ''normoxic'' condition. Studies related to cellular effects of hyperoxia have focused on concentrations of O_2 much higher than 21%.[22] As demonstrated in 1977, human diploid fibroblasts grown at 10% O_2 have a longer life than cells grown at the routine 20.6% O_2.[23] Consistently, the development of preimplantation embryos clearly

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favors 7% O_2 over 20% O_2 ambience.[24] Ambient O_2 is dissolved readily in the cell culture medium.[25] If indeed the cells have signaling pathways sensitive to supraphysiological levels of O_2, then it is rational to assume that biology studied at 21% O_2 may not be expected to best represent cellular responses *in vivo*. The study of O_2-sensitive signal transduction pathways will enable us to examine the significance of controlling ambient O_2 conditions and to revisit results published from cells cultured in room air. In addition, we are cautiously optimistic that such studies will allow us to reconcile some of the differences between *in vitro* and *in vivo* biology and allow us to have more meaningful *in vitro* experimental models.

The experimental set-up described here was developed to control oxygen levels during all procedures of cell culture, as well as while performing live cell imaging (Fig. 2).

Cell Culture and Live Cell Imaging Under Controlled O_2 Environment

Some cellular processes (e.g., HIF1 stabilization)[26] have been shown to respond quickly (within minutes) to changes in O_2 tension. To control for this, we have the regulated O_2/CO_2 levels of cells not only in the culture incubators where cells will be grown, but also during all culture procedures (seeding, media change, etc.) and even during the isolation process. Such precise control on O_2 levels is achieved using a specially designed glove box where desired O_2 tension is maintained constantly (Fig. 2L). All cell culture procedures, including isolation, seeding, splitting, and media change, are performed in this sterile glove box. O_2 and CO_2 levels in this glove box are maintained using an electronic O_2 (PRO-OX) and CO_2 feedback regulator (PRO-CO_2, BioSpherix, Redfield, NY). These controllers are fitted with sensors that sense O_2/CO_2 levels in the glove box and adjust the level of these gases accordingly within seconds. Cells are maintained in culture incubators where the desired oxygen tension (0.1–99.9%) is maintained using The OxyCycler (BioSpherix), which is a fully automated device that provides oxygen profile control for accurately modeling chronic, acute, or intermittent hypoxia or hyperoxia. The oxygen environment inside the culture incubator is monitored using a gaseous oxygen sensor (BioSpherix). Continuous monitoring of the oxygen tension in cell culture media is done using a dissolved O_2 probe (BioSpherix).

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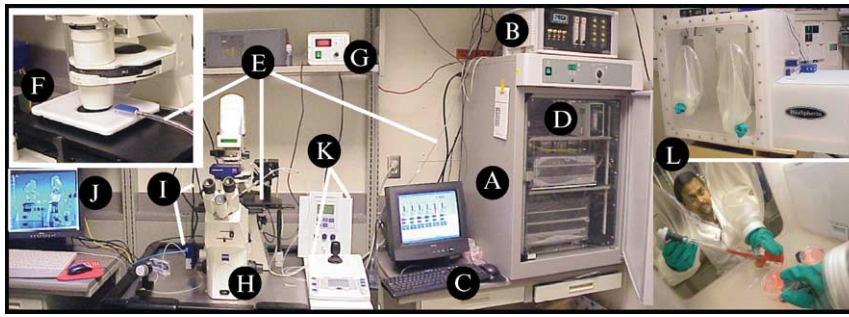

FIG. 2. Cell culture and live cell imaging under a controlled O_2 environment. Cells are grown in an incubator (A) where the gaseous environment can be regulated variably as a function of time using the OxyCycler (Biospherix, Redfield, NY) (B) controlled by PC software (C) capable of cycling (in minutes) and maintaining ambient O_2 and CO_2 concentrations based on feedback information from O_2 sensors installed in the incubator (A). Mixed gas is delivered by pump (D) via tubing (E) to a cell culture enclosure (f, zoomed inset), which is heated by g. Cells in f are imaged by an Axiovert 200 M (Zeiss) fully motorized fluorescence microscope (H) supported by dual (color and B/W) AxioCam digital cameras (I) and rested on an air table. Time-lapse images are collected and analyzed using Axiovision software (Zeiss) installed in a PC (J). The microscope contains necessary hardware/software to image cells grown on standard plastic culture plates (growing on glass coverslips is not necessary). A twin-tip micromanipulator (InjectMan NI2, Eppendorf) and microinjector (Femtojet, Eppendorf) system (K) is attached to the microscope to perform microinjections/manipulations as well as the collection of nuclear materials from single cells. For splitting/seeding of cells under a controlled gas environment, a specialized glove box (L) fitted with an O_2 controller (PRO-OX, Biospherix) is available. Six gas-controlled incubators are available for parallel experimentation.

The system used to perform live cell imaging (phase contrast as well as fluorescence) under controlled oxygen conditions has been illustrated (Fig. 2). In brief, the system includes an Axiovert 200 M (Zeiss) fully motorized fluorescence microscope supported by an AxiCam digital camera (color; multicolor tissue section imaging; B/W, high-resolution fluorescence imaging) and Axiovision software to perform live cell fluorescence imaging. The microscope is fitted with a chamber where temperature and gas (O_2 and CO_2) conditions can be regulated to observe live cell processes. In this chamber, O_2 concentration between 0 and 95% can be regulated. The microscope contains necessary hardware/software to perform fluorescence resonance energy transfer (FRET) analysis. Apotome (Zeiss), recently developed hardware that provides confocal capability, has been added to the microscope. A twin-tip micromanipulator (InjectMan NI2, Eppendorf) and microinjector (Femtojet, Eppendorf) system is attached to the microscope to perform microinjections/manipulations as well as collections of nuclear materials from single cells. The microscopy system is supported

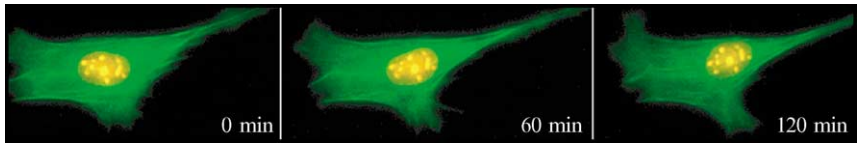

FIG. 3. Live cell imaging using fluorescence microscopy. Cotransfection of actin-EGFP (green fluorescence) and nuclear-targeted DS2-red (golden fluorescence) plasmids are used to visualize cytoskeletal and nuclear changes in live cells. Three images are shown after the indicated time from a 2-h time-lapse series where images were collected in a digital video format. Images were collected using the live cell microscopy system as described (Fig. 2).

by a high-end Celsius 670 workstation. Time-dependent changes in cell morphology and actin cytoskeleton in specific O_2 environments are imaged on a time-lapse basis and are calculated using Axiovision software.

Images shown in Fig. 3 were collected using the live cell microscopy system as described (Fig. 2). Three images shown in Fig. 3 are from a 2-h time-lapse series. To visualize cytoskeletal and nuclear changes in live cells under controlled oxygen conditions, murine adult cardiac fibroblasts (CF) were cotransfected with actin-EGFP (green fluorescence) and nuclear-targeted DS2-red (golden fluorescence) plasmids (Fig. 3).

Oxygen-Regulated Signal Transduction Pathways in Cardiac Cells

Using the system described (Fig. 2), we investigated oxygen-regulated signal transduction pathways in cardiac fibroblasts (CF). Variations in cell culture O_2 levels proposed later are based on an *in vivo* situation where cardiac cells are exposed to prolonged moderately hypoxic conditions followed by reperfusion/reoxygenation. As stated earlier, under these conditions, cardiac cells will adjust to low O_2 levels and, following reoxygenation, will experience ''perceived hyperoxia'' (Fig. 1). Based on our own data,[18] we chose 3% O_2 levels that can be considered moderately hypoxic, whereas 10% O_2 would be marginally hyperoxic relative to the pO_2 (5%) to which cells are adjusted *in vivo*. Additionally, the effect in room air, that is, 21% O_2, is also investigated because of the following reasons: (i) to magnify the O_2-sensitive cellular responses that are seen marginally in 10% O_2 and (ii) to compare our results with existing literature where room air (21%) has been considered normoxia.

To perform the experiments, primary cultures of adult murine cardiac fibroblasts are generated and cultured as described.[27] Briefly, ventricles are removed, minced, and incubated in Hank's buffer containing trypsin (0.1 mg/ml) and collagenase (50 units/ml) for 10 consecutive 10-min treatment periods at 37°. Cells from each digestion period are pooled,

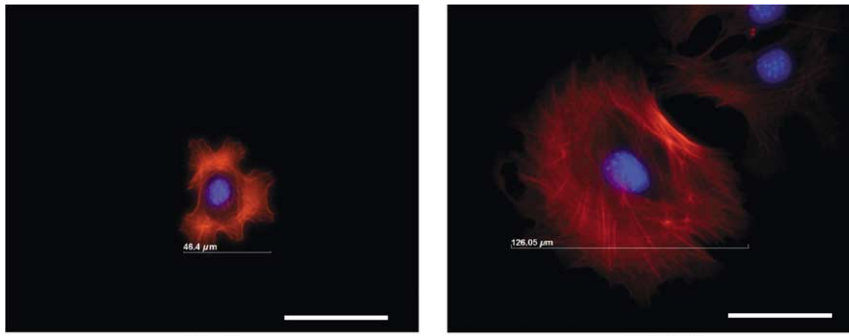

FIG. 4. Freshly isolated CF are phenotypically closer to CF cultured at 5% O_2 compared to CF cultured at higher O_2 tensions. After isolation, CF were cultured at 5 or 21% O_2 for 8 days. For morphologic comparisons, CF cultured at various O_2 tensions for 8 days were stained with phalloidin (actin, red) and nuclei were stained with DAPI (blue). Imaging was performed using a Zeiss microscope (Fig. 2). Scale bar: 50 μm.

resuspended in complete media (Dulbecco's modified Eagle medium containing 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin), and seeded in culture dishes and maintained under standard pH-buffered culture conditions and desired oxygen tensions. After 3 h, nonadherent debris is discarded and attached fibroblasts are maintained in complete media and culture conditions described earlier. Fibroblasts are stained for vimentin, a marker of fibroblast-like cells; the presence of contaminating vascular smooth muscle and endothelial cells is determined using desmin or factor VIII, respectively.

Compared to cells cultured at 3% O_2, remarkable reversible growth inhibition and a phenotype indicative of differentiation were observed in cells that were cultured in 10 or 21% O_2.[18] A representative figure of such a phenotype is presented in Fig. 4. CF exposed to high oxygen levels (10 or 21% O_2) exhibited higher levels of mitochondrial-reactive oxygen species production.[18] The molecular signature response to perceived hyperoxia included the induction of p21, cyclin D1, D2, and G1, Fra-2, transforming growth factor (TGF)β1, lowered telomerase activity, and activation of TGFβ1 and p38MAPK. CF deficient in p21 were resistant to such oxygen sensitivity.[18]

Acknowledgments

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