Oxygen Sensing by Primary Cardiac Fibroblasts
A Key Role of p21\textsuperscript{Waf1/Cip1/Sdi1}

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Abstract—In mammalian organs under normoxic conditions, $O_2$ concentration ranges from 12% to <0.5%, with $O_2 \approx 14\%$ in arterial blood and <10% in the myocardium. During mild hypoxia, myocardial $O_2$ drops to $\approx 1\%$ to 3% or lower. In response to chronic moderate hypoxia, cells adjust their normoxia set point such that reoxygenation-dependent relative elevation of $P_{O_2}$ results in perceived hyperoxia. We hypothesized that $O_2$, even in marginal relative excess of the $P_{O_2}$ to which cardiac cells are adjusted, results in activation of specific signal transduction pathways that alter the phenotype and function of these cells. To test this hypothesis, cardiac fibroblasts (CFs) isolated from adult murine ventricle were cultured in 10% or 21% $O_2$ (hyperoxia relative to the $P_{O_2}$ to which cells are adjusted in vivo) and were compared with those cultured in 3% $O_2$ (mild hypoxia). Compared with cells cultured in 3% $O_2$, cells that were cultured in 10% or 21% $O_2$ demonstrated remarkable reversible G2/M arrest and a phenotype indicative of differentiation to myofibroblasts. These effects were independent of NADPH oxidase function. CFs exposed to high $O_2$ exhibited higher levels of reactive oxygen species production. The molecular signature response to perceived hyperoxia included (1) induction of p21, cyclin D1, cyclin D2, cyclin G1, Fos-related antigen-2, and transforming growth factor-$\beta$1, (2) lowered telomerase activity, and (3) activation of transforming growth factor-$\beta$1 and p38 mitogen-activated protein kinase. CFs deficient in p21 were resistant to such $O_2$ sensitivity. This study raises the vital broad-based issue of controlling ambient $O_2$ during the culture of primary cells isolated from organs. (Circ Res. 2003;92:264-271.)

Key Words: redox ■ free radicals ■ heart ■ cell culture

Cellular $O_2$ concentrations are maintained within a narrow range (normoxia) because of the risk of oxidative damage from excess $O_2$ (hyperoxia) and of metabolic demise from insufficient $O_2$ (hypoxia).\textsuperscript{1} $P_{O_2}$ ranges from 90 to <3 mm Hg in mammalian organs under normoxic conditions, with arterial $P_{O_2}$ of $\approx 100$ mm Hg or $\approx 14\% O_2$.\textsuperscript{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 $P_{O_2}$. Current work in this field is almost exclusively focused on the study of hypoxia. Reoxygenation, on the other hand, has been mostly investigated in the context of oxidative injury. Over 25 years ago, it was observed that $P_{O_2}$ beyond the comfort of the “perceived normoxic range” is a significant stressor, leading to growth arrest.\textsuperscript{3} The molecular bases of such observations remain to be characterized in light of current knowledge of signal transduction.

During chronic hypoxia in the heart, cells adjust their normoxic set point such that the return to normoxic $P_{O_2}$ after chronic hypoxia is perceived as relative hyperoxia.\textsuperscript{4,5} We hypothesized that such challenge triggers changes in signal transduction processes. Although acute insult caused during reperfusion may be lethal to cells localized at the focus of insult, elevation of $O_2$ tension in the surrounding ischemic tissue triggers phenotypic changes in the surviving cells that may be associated with tissue remodeling.

Ischemia in the heart results in a hypoxic area containing a central focus of near-zero $O_2$ pressure bordered by tissue with diminished but nonzero $O_2$ pressures. These border zones extend for several millimeters from the hypoxic core, with the $O_2$ pressures progressively increasing from the focus to the normoxic region.\textsuperscript{6} Moderate hypoxia is associated with a 30% to 60% decrease ($\approx 1\%$ to 3% $O_2$) in $P_{O_2}$.\textsuperscript{7} Cardiac fibroblasts (CFs) are mainly responsible for the synthesis of major extracellular matrix (ECM) in the myocardium, including fibrillar collagen types I and III and fibronectin.\textsuperscript{8} More than 90% of the interstitial cells of the myocardium are fibroblasts,\textsuperscript{8} which actively engage in crosstalk with myocytes to determine the quantity and quality of the ECM. The present...
study rests on our striking observation that CFs isolated from adult murine ventricle and cultured in 10% or 21% O₂ (high O₂, relative to the P₂O to which cells are adjusted in vivo), compared with 3% O₂ (mild hypoxia), exhibit reversible growth inhibition and a phenotype indicative of differentiation. The notion that marginal relative elevation in PO₂, compared with the P₂O to which cells are adjusted during chronic moderate hypoxia, may serve as a signal to trigger CF differentiation and tissue remodeling is a novel concept relevant to fibrosis and tissue repair related to reperfusion injury. We hypothesized that in reoxygenated tissue, a sudden elevation of P₂O may profoundly influence the cellular phenotype even at those sites where oxidative injury may not be predominantly evident. Our objective was to characterize signal transduction pathways in the CFs that are sensitive to a sudden elevation of ambient P₂O.

Materials and Methods

CF Isolation and Culture
Experiments using primary CFs isolated from adult (5- to 6-week-old) mouse ventricles were performed using procedures described previously. Ventricular P₂O was directly determined using an OxyLite P₂O monitoring needle probe and data acquisition systems (Oxford Optronix).

Cell Counting
Cells were seeded at 5000 cells per well in 4-well plates (culture area per well 1.9 cm²). Before they were counted, the cells were trypsinized and resuspended in a single-cell suspension. Counting was performed using a Z1 series Coulter counter.

Cell Cycle
Cell cycle profiles were determined using a flow cytometer and CellQuest software (BD Biosciences).

Mitochondrial Staining, ROS, and Oxidative Stress Markers
Mitochondria were stained using MitoTracker Red (Molecular Probes). Cellular reactive oxygen species (ROS) were detected using dihydrorhodamine 123 (Molecular Probes).

Lipid Peroxidation
4-Hydroxynonenal (HNE) protein adduct was detected using a specific antibody (Upstate Biotech).

Protein Oxidation
Proteins were derivatized with dinitrophenylhydrazine, separated by SDS-gel electrophoresis, and blotted with antibody (Zymed) against dinitrophenyl groups.

Immunochemistry Microscopy
F-actin (phalloidin, dilution 1:40, Molecular Probes), α-smooth muscle actin (SMA, Sigma), and p21 (Santa Cruz) immunostaining and microscopy were performed as previously using a Nikon E800 or Zeiss LSM510 multiphoton laser scanning microscope.

Western Blot
Western blot was performed as described previously. Primary antibodies against vimentin (dilution 1:1000, Sigma), SMA (dilution 1:4000, Sigma), β-actin (dilution 1:5000, Sigma), p21 (dilution 1:200, Santa Cruz), and Fos-related antigen-2 (Fra-2; dilution 1:200, Santa Cruz) were used to detect these antigens.

ELISA
Transforming growth factor (TGF)-β1 ELISA was performed using a commercially available kit per the manufacturer’s recommendation (Promega).

mRNA Quantification
mRNAs were quantified by RNAase protection assay (RPA) using commercial DNA templates (BD RiboQuant Ribonuclease Protection Assay System). Real-time polymerase chain reaction (PCR) detection of p21 was performed using double-stranded DNA binding dye SYPER Green-I and the following primer set: ACAGGAGCAAAGTGTGCCGGTGT and GCCTCAGACACCAGTGCAAGACA.

Gel Contraction
Collagen lattices were prepared using type I collagen from rat-tail tendon as described. The degree of collagen gel contraction was determined after 6, 15, and 24 hours.

Luciferase and Chloramphenicol Acetyltransferase Reporter Assay
Luciferase activity was performed as described. A chloramphenicol acetyltransferase reporter assay (ELISA-based) was performed using a commercial kit (Promega). A TGF-β1-luciferase promoter construct was kindly provided by Dr Cora Weigert and Dr Erwin D. Schleicher of the University of Tuebingen, Tuebingen, Germany.

p38 MAPK Activity
Phosphorylation of ATF-2 (substrate) by p38 mitogen-activated protein kinase (MAPK) was determined using a commercial kit (Cell Signaling Inc).

Telomerase Activity
Telomerase activity was determined using telomeric repeat amplification protocol (TRAPeze assay, Intergen). Retroviral hTERT vectors were kindly provided by Dr Robert Weinberg, Whitehead Institute for Biomedical Research (Cambridge, Mass), and Dr Judith Campisi, Lawrence Berkeley National Laboratory (Berkeley, Calif).

Results
Under conditions of systemic normoxia, heart cells receive a limited supply of O₂, representing <10%. Using a needle probe, we directly determined that mouse heart ventricular P₂O is in the range of 5%. Thus, in the present study, 3% O₂, 10% O₂, and 21% O₂ are referred to as conditions representing mild hypoxia, mild hyperoxia, and acute hyperoxia, respectively. Using CFs isolated from adult murine ventricles, we observed that exposure to hyperoxia results in potent growth inhibition (Figure 1A). When cells grown in 21% O₂ were returned to the 3% O₂ condition, growth resumed, suggesting that hyperoxia-induced growth arrest is reversible (Figure 1B). We have observed that cell lines do not exhibit such responses to hyperoxia. However, CFs allowed to adjust in 21% O₂ for 3 to 5 days and subsequently maintained in 3% O₂ overnight respond to hyperoxia-induced growth arrest and other related effects as described below. Hyperoxia-induced reversible growth inhibition was associated with G/M arrest (Figure 1C). gp91 containing NADPH oxidases represents a significant source of ROS in the myocardial fibroblast and is suggested to be a central player in O₂ sensing. To test whether hyperoxia induces growth inhibition by generating ROS through a NADPH oxidase–dependent mechanism, we tested CFs from gp91-deficient mice. It was evident that CFs lacking NADPH oxidase activity were no different from those of the corresponding wild-type mice (Figures 1D and
1E). These results suggest that NADPH oxidase may not be a central player in conferring sensitivity to hyperoxia. However, it was clear that exposure of CFs to hyperoxic conditions resulted in an appreciable oxidative modification of proteins and lipids (Figures 1F and 1G). HNE is an end product of lipid peroxidation that can regulate fibrogenic signaling pathways in a variety of organs. Protein carbonyl formation is a reliable biological marker of ROS that is associated with cardiac fibrosis.22 Reoxygenation in the heart is known to elevate the levels of both HNE and protein carbonyl.23,24 Using a dual fluorescence staining approach, we were able to confirm that exposure to hyperoxia indeed enhances cellular oxidant production (Figure 1H).

Of importance, morphological characteristic of CFs grown at 3% O₂ remained stable during the course of culture. In contrast, cells grown under hyperoxic conditions exhibited changes of phenotype indicative of differentiation (Figure 2A). Under hyperoxic conditions, cell size increased substantially (3- to 6-fold), and the appearance of stress fibers was evident, as shown in Figure 2A. Such changes were associated with a reorganization of SMA with stress fibers, a distinct morphological characteristic of CF differentiation. Additional results confirmed our contention that the exposure of CFs to a hyperoxic condition induces differentiation of fibroblasts to myofibroblasts. Hyperoxia enhances vimentin and SMA expression (Figure 2B). Contractile phenotype is a major characteristic feature of myofibroblasts. We used the collagen matrix contraction assay to test the contractility of hyperoxia-induced myofibroblasts. Figure 2C clearly demonstrates that under conditions of hyperoxia, CFs assume a markedly contractile phenotype.

Hyperoxic exposure resulted in potent induction of p21 expression and marginal increases in p53 expression in CFs (Figure 3A). Increased p21 mRNA expression was indeed associated with higher levels of p21 protein in CFs exposed to hyperoxia (Figure 3B). Microscopic visualization of CFs consistently revealed increased nuclear localization of p21 in CFs exposed to 21% O₂ (Figure 3C). To test whether...
Exposure to hyperoxia resulted in the activation of p21 transcription, p21 promoter (kindly provided by Drs Toshiyuki Sakai and Yoshihiro Sowa, Kyoto Prefectural University of Medicine, Kyoto, Japan) studies were conducted. Results show that exposure of CFs to 21% O2 resulted in a significant increase in p21 promoter-driven luciferase reporter activity (Figure 3D).

p21 plays an important role in differentiation-associated growth arrest, and its expression is augmented in many differentiating cells.25,26 p21 is also known to play a major role in G2/M arrest, a response triggered by exposure to hyperoxia (Figure 1C). Therefore, we were led to hypothesize that p21 induction in response to hyperoxia plays a major role in growth inhibition and subsequent differentiation. Disruption of this gene will allow fibroblasts to bypass hyperoxia-induced growth arrest and differentiation response. To test this hypothesis, experiments using CFs isolated from p21-deficient mice were conducted (Figures 4A and 4B). We observed that CFs deficient in p21 were not sensitive to hyperoxia-induced growth arrest (Figures 4C and 4D).

Next, our focus was directed to the study of O2-sensitive cellular signal transduction processes that accounted for the observed responses of CFs to hyperoxic exposure. The quantitative RPA approach was used to screen for 37 genes that are known to be key regulators of the cell cycle (Figure 5A). A summary of the findings is listed (Figure 5B).

Exposure of CFs to hyperoxia resulted in marked induction of the following mediators of growth arrest/differentiation: cyclin D1, cyclin D2, cyclin G1, and Fra-2 (Figure 5A). Elevated expression of these candidates is associated not only with growth inhibition but also with differentiation. The D-type cyclins consist of cyclins D1, D2, and D3. Cyclin D1 synthesis is induced by p21.27 Cyclin D2 expression is induced in multiple states of growth arrest.28 Cyclin G1 is involved in G2/M arrest.29 This is consistent with our observation that CFs exposed to hyperoxia contain higher levels of cyclin G1 mRNA (Figure 5A) and are in G2/M arrest (Figure 1C). Fra-2 is a member of the Fos family of immediate-early genes.
genes, most of which are rapidly induced by second messengers. Although the role and biology of Fra-2 are less understood than those of its relatives, c-Fos, Fra-1, and FosB, it is evident that elevated Fra-2 is associated with cellular differentiation.30 In CFs exposed to hyperoxia, induction of Fra-2 mRNA was associated with higher levels of Fra-2 protein (Figure 5C). Telomerase, a ribonucleoprotein enzyme complex containing a catalytic reverse transcriptase component (hTERT) and an RNA template component, functions to add hexameric repeats of 5'-TTAGGG-3' to the end of telomeres to compensate for the progressive loss during cell division.31 Recently, it has been shown that telomerase activity in myofibroblasts is lower than that in fibroblasts.32 We observed that the exposure of CFs to hyperoxia results in the downregulation of telomerase activity (Figure 5D). It has been observed that compromised telomerase activity in proliferating cells leads to differentiation. In such cells, enhanced expression of p21 and p27 has been noted.33

TGF-β1 induces CF differentiation.34 We determined that both total and active TGF-β1 were substantially higher in CFs exposed to hyperoxia (Figure 6A). Experiments with conditioned cell culture media consistently support the notion that the media of CFs grown at 21% O2 contains significantly higher amounts of active TGF-β1 than does the media of CFs grown at 3% O2 (Figure 6A). In support of the hypothesis that ROS (Figures 1F through 1H) can trigger the displacement of latency-associated peptide (LAP) from TGF-β1,35 we observed that exposure to TGF-β1–containing conditioned culture media to oxidant challenge (UVC) significantly increased the levels of active TGF-β1 (Figure 6B). Therefore, it is plausible that ROS generated by cells under conditions of hyperoxia (Figures 1F through 1H) may contribute to the activation of TGF-β1. RPA results showed that TGF-β1, TGF-β2, and TGF-β3 are sensitive to hyperoxia (Figure 6C). That hyperoxia indeed induced TGF-β1 transcription was confirmed using a reporter assay (Figure 6D). Engagement of TGF-β1 to its receptor is known to generate ROS by an

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Figure 4. CFs from p21-deficient (p21KO) mice are resistant to O2-induced growth arrest. CFs were isolated either from wild-type background (C57BL/6) or p21KO background. In panels A and B, RNA was extracted from cells cultured at 3% or 21% O2 for 8 days. p21 mRNA expression was assessed using real-time RT-PCR. A, Real-time PCR graphs. B, Quantification of p21 mRNA expression (normalized against GAPDH). C, Actual cell count. D, Percent change vs 3% O2 on day 7. ΔRn indicates delta normalized reporter intensity.

Figure 5. O2-induced changes in cell cycle–related genes and telomerase activity. Isolated CFs were cultured at 3%, 10%, or 21% O2 for 8 days. A, Quantitative RPA. Cyc indicates cyclin. B, List of genes screened using RPA. O2-sensitive genes (21% versus 3%) are in bold. C, Fra-2 Western blot. D, Telomerase activity. Note that the 50-bp telomeric repeat amplification protocol (TRAP) product semiquantitatively competes with the 36-bp internal control (S-IC) band. Greater intensity of 50-bp product indicates higher processibility of telomerase. Lanes are as follows (right to left): 1, heat-inactivated sample from CF stably expressing the catalytic subunit of telomerase (TERT) and cultured at 3% O2; 2, CF stably expressing TERT and cultured at 3% O2; 3, CF cultured at 3% O2; 4, CF cultured at 21% O2; and 5, positive control (component of the kit; see Materials and Methods).
NADH oxidase–dependent mechanism. Thus, we sought to clarify whether elevated levels of TGF-β1 present under conditions of hyperoxia are responsible for higher oxidant production (Figure 1F through 1H). We observed that treatment of CFs with TGF-β1 triggers differentiation (Figure 7A) but does not elevate ROS generation (not shown) as determined in the present study.

The phenotypic characteristics of CFs observed in response to hyperoxia could be reproduced in CFs cultured at 3% O2 by treating cells with TGF-β1 (compare Figure 2A with Figure 7A). Strikingly, inhibition of p38 MAPK, a downstream mediator of TGF-β1 signaling, released both TGF-β1–induced as well as hyperoxia-induced growth inhibition. This result was obtained after tests of several known inhibitors of the signal transduction path; no other tested inhibitor shared the effect of SB203580. These data suggest a parallel between TGF-β1–induced and hyperoxia-induced changes in cellular responses (Figure 7B). On the basis of this observation, we hypothesized that exposure of CFs to hyperoxia induces the activation of p38 MAPK, which in turn plays a significant role in executing O2-induced growth inhibition as shown in Figure 1. We obtained direct evidence showing that the activation of p38 MAPK is sensitive to ambient O2 (Figure 7C).

Discussion

The present study provides the following significances: (1) It offers a fresh look at ischemia/reoxygenation biology. The notion that marginal relative elevation in PO2, compared with PO2 to which cells are adjusted during chronic moderate hypoxia, may serve as a signal to trigger CF differentiation and tissue remodeling is a novel concept relevant to fibrosis and tissue repair related to reperfusion injury. (2) It postulates that CFs isolated from the heart are not "blind" to a change of PO2 from 10% O2 (in vivo normoxia) to 21% O2 (widely interpreted as in vitro normoxia). This raises the vital broad-based issue of controlling ambient O2 during the culture of primary cells isolated from organs. (3) An in vitro model for the study of chronic moderate hypoxia followed by reoxygenation is offered. Although physiological adjustments in the face of reduced O2 supply have been studied in the myocardium, the cellular basis of such an adaptation and the signaling pathways involved in the process remain to be defined. It is acknowledged that stable maintenance of partial blood flow reduction is difficult to achieve in in vivo experimental models. A 1998 National Heart, Lung, and Blood Institute workshop identified the development of in vitro models of chronic moderate hypoxia as an area of priority.
Cardiac remodeling leads to changes in the ECM and, in some cases, to cardiac fibrosis, a process involving myofibroblasts as an active component. Myofibroblasts are larger and more stellate, proliferate slowly, and have the most prominent microfilament arrays. These cells are characterized by the presence of SMA-containing stress fibers, vinculin-containing fibronexus adhesion complexes, fibronectin fibrils containing the ED-A splice variant, and increased expression of vimentin. Therefore, our observation that exposure to a higher PO₂ relative to which cells are adjusted induces the differentiation of CF to myofibroblasts is of significant relevance to cardiac tissue remodeling in the face of reoxygenation.

In mammalian cells, differentiation and senescence are two common cellular processes that are initiated by cell cycle block. Irreversible growth arrest is one of the hallmarks of senescence. The observation that O₂-induced growth arrest of CFs is reversible indicates a specific commitment toward differentiation. Induction of p21, a cyclin-dependent kinase (Cdk) inhibitor, is a common mechanism of growth arrest and early differentiation. Under conditions of stress, the growth of cycling cells is arrested at one of two critical cell cycle check points, G₁-S or G₂-M. Our findings confirm that p21 induction in response to hyperoxia plays a major role in growth inhibition and subsequent differentiation. Disruption of this gene allows CFs to bypass hyperoxia-induced growth arrest and differentiation response.

The molecular signature of exposure to a higher PO₂ relative to which cells are adjusted included, in addition to p21, enhanced expression of other cell cycle inhibitors, such as cyclin D1, cyclin D2, cyclin G1, and Fra-2. These genes are associated not only with growth inhibition but also with differentiation. The D-type cyclins consist of cyclins D1, D2, and D3. Cyclin D1 synthesis is induced by p21. Cyclin D2 expression is induced in multiple stages of growth arrest. Cyclin G1 is involved in G₂/M arrest. This is consistent with our observation that CFs exposed to hyperoxia contain higher levels of cyclin G1 mRNA and are in G₂/M arrest. TGF-β is a natural inducer of growth inhibition and differentiation in CFs. The effects of TGF-β are associated with upregulation of the following Cdk inhibitors: p21, p15, and p27. MAPK activation represents a prominent downstream target of TGF-β and is critical for p21 induction via p53 phosphorylation. Inhibition of p38 MAPK prevented TGF-β-induced differentiation of CFs to myofibroblasts, suggesting a key role of this signaling mediator. Interestingly, ROS are involved in TGF-β-induced p38 MAPK activation. Note that in the TGF-β/p21 signaling axis, the activation of each key mediator (TGF-β, p38 MAPK, and cysteine-rich zinc-finger transcription factor Sp-1) is ROS inducible. Mild sublethal oxidative challenge selectively activates p38 MAPK, resulting in cell cycle arrest at G₂/M. Mechanisms controlling the activation of latent TGF-β are important for regulating the activity of TGF-β. Oxidation reactions disrupt the interaction between the LAP and TGF-β to release active TGF-β. Active TGF-β tightly regulates the production of ECM by balancing new synthesis and turnover of ECM components. In this capacity, TGF-β may provide a key role in physiological tissue remodeling or generate excess ECM to cause fibrosis. The effects of hyperoxic exposure reported in the present study are associated with elevated levels of ROS. We have failed to attenuate the effects by strategies involving adenosyl overexpression of catalase in CFs or treatment of cells with the commonly used antioxidant N-acetylcysteine. These observations led us to postulate that although a supporting role of ROS in signaling for hyperoxia may not be ruled out, molecular O₂ could well serve as a trigger initiating O₂-sensitive signaling pathways. This contention is consistent with a recent report demonstrating that molecular O₂ may indeed modulate signaling processes by an ROS-independent prolylhydroxylation reaction.

That low O₂ ambience serves as a cue to trigger angiogenesis is a well-accepted notion. The present study presents first evidence indicating that the sensing of the O₂ environment is not limited to hypoxia. It demonstrates that marginal relative elevation in PO₂, compared with PO₂ to which cells or tissues are adjusted, serves as a signal to trigger changes in the cellular phenotype relevant to tissue remodeling.

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References


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