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

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Abstract—In mammalian organs under normoxic conditions, O_2 concentration ranges from 12% to <0.5%, with O_2 ≈14% in arterial blood and <10% in the myocardium. During mild hypoxia, myocardial O_2 drops to ≈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 G_2/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-β1, (2) lowered telomerase activity, and (3) activation of transforming growth factor-β1 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).[1] P_{O_2} ranges from 90 to <3 mm Hg in mammalian organs under normoxic conditions, with arterial P_{O_2} of ≈100 mm Hg 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 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.[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.[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.[6] Moderate hypoxia is associated with a 30% to 60% decrease (≈1% to 3% O_2) in P_{O_2}.[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. More than 90% of the interstitial cells of the myocardium are fibroblasts,[8] which actively engage in crosstalk with myocytes to determine the quantity and quality of the ECM. The present

Original received November 25, 2002; revision received December 27, 2002; accepted January 7, 2003.

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Circulation Research **is available at http://www.circresaha.org** **DOI: 10.1161/01.RES.0000056770.30922.E6**

study rests on our striking observation that CFs isolated from adult murine ventricle and cultured in 10% or 21% O_2 (high O_2, relative to the P_{O_2} to which cells are adjusted in vivo), compared with 3% O_2 (mild hypoxia), exhibit reversible growth inhibition and a phenotype indicative of differentiation. The notion that marginal relative elevation in P_{O_2}, compared with the P_{O_2} 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 P_{O_2} 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 P_{O_2}.

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.[9] Ventricular P_{O_2} was directly determined using an OxyLite P_{O_2} monitoring needle probe and data acquisition systems[10] (Oxford Optronix).

Cell Counting

Cells were seeded at 5000 cells per well in 4-well plates (culture area per well 1.9 cm^2). 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[11] 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).[12]

Protein Oxidation

Proteins were derivatized with dinitrophenylhydrazine, separated by SDS-gel electrophoresis, and blotted with antibody (Zymed) against dinitrophenyl groups.[12]

Immunofluorescence 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 described previously using a Nikon E800 or Zeiss LSM510 multiphoton laser scanning microscope.[13]

Western Blot

Western blot was performed as described previously.[14] 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 RNase 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 SYBER Green-I and the following primer set: ACAGGAGCAAAGTGTGCCGTTGT and GCTCAGACACCAGAGTGCAAGACA.

Gel Contraction

Collagen lattices were prepared using type I collagen from rat-tail tendon as described.[15] 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.[16] 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).[17] 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 O_2, representing $<10\%$.[18–20] Using a needle probe,[10] we directly determined that mouse heart ventricular P_{O_2} is in the range of 5%. Thus, in the present study, 3% O_2, 10% O_2, and 21% O_2 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% O_2 were returned to the 3% O_2 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% O_2 for 3 to 5 days and subsequently maintained in 3% O_2 overnight respond to hyperoxia-induced growth arrest and other related effects as described below. Hyperoxia-induced reversible growth inhibition was associated with G_2/M arrest (Figure 1C). gp91 containing NADPH oxidases represents a significant source of ROS in the myocardial fibroblast[21] and is suggested to be a central player in O_2 sensing.[2] 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

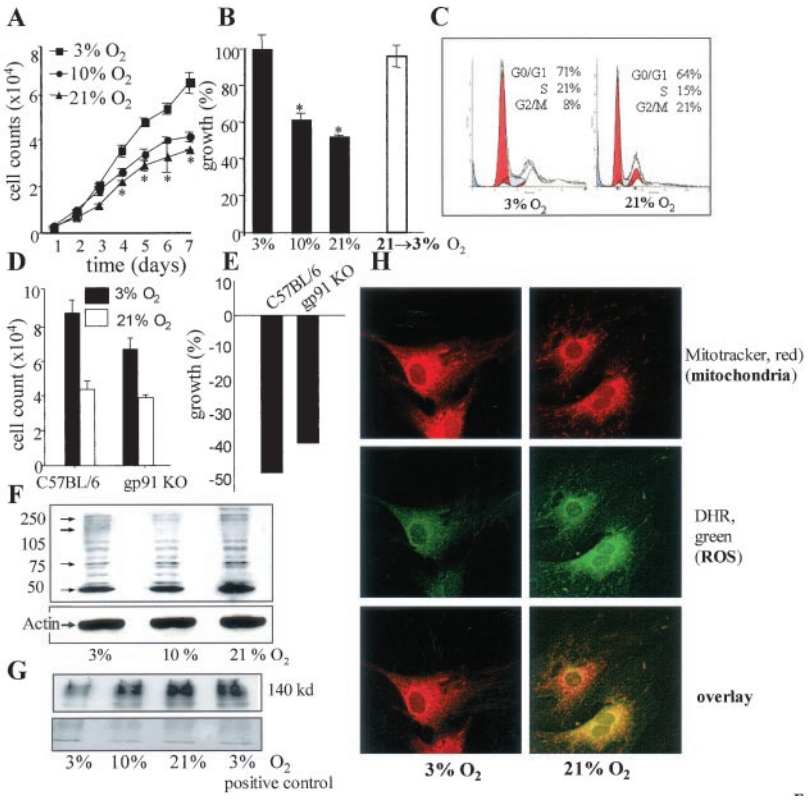

Figure 1. O_2-induced growth arrest and ROS production of primary CFs. A and B, Growth arrest. CFs isolated from C57BL/6 mouse (5 to 6 weeks old, male) heart ventricle were seeded at 3%, 10%, or 21% O_2 and cultured for 5 days. Cells were split, seeded at 5000 cells per well, and cultured in 3%, 10%, or 21% O_2 for the next 7 days. In panel B, 21→3% indicates cells cultured in 21% O_2 for 5 days that were split and cultured at 3% O_2 for 7 days (open bar). Panel A shows actual cell counts; panel B, percent change compared with 3% O_2 on day 7. *$P<0.05$ vs cell count at 3% O_2. C, Isolated CFs cultured for 5 days at 3% O_2 and then transferred to 3% or 21% O_2 for the next 3 days. Data show G_2/M arrest in cells cultured at 21% O_2. D and E, NADPH oxidase–deficient mice. Cells were isolated from mice with a wild-type background (C57BL/6) or from gp91phox knockout (KO) mice (see panels A and B for experimental conditions). Panel D shows total cell count on day 7; panel E, percent change compared with the cells isolated from C57BL/6 and cultured in 3% O_2 for 7 days. F through H, Cellular ROS formation and footprints of oxidation. Panel F shows Western blot detection of 4-HNE protein adducts from cell lysates; panel G, Western blot detection of oxidative protein modification (carbonyls) in lysates from cells grown under conditions of O_2 ambience shown. The positive control (far right) represents the same sample as in the far left lane treated with 1 mmol/L NaOCl for 15 minutes. Panel H, top, shows mitochondria stained using MitoTracker Red. Panel H, middle, shows ROS production visualized using dihydrorhodamine 123 (DHR). Panel H, bottom, is overlay of MitoTracker and DHR signals. CFs cultured at 21% O_2 clearly showed higher ROS (vs 3%).

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% O_2 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% O_2 (Figure 3C). To test whether

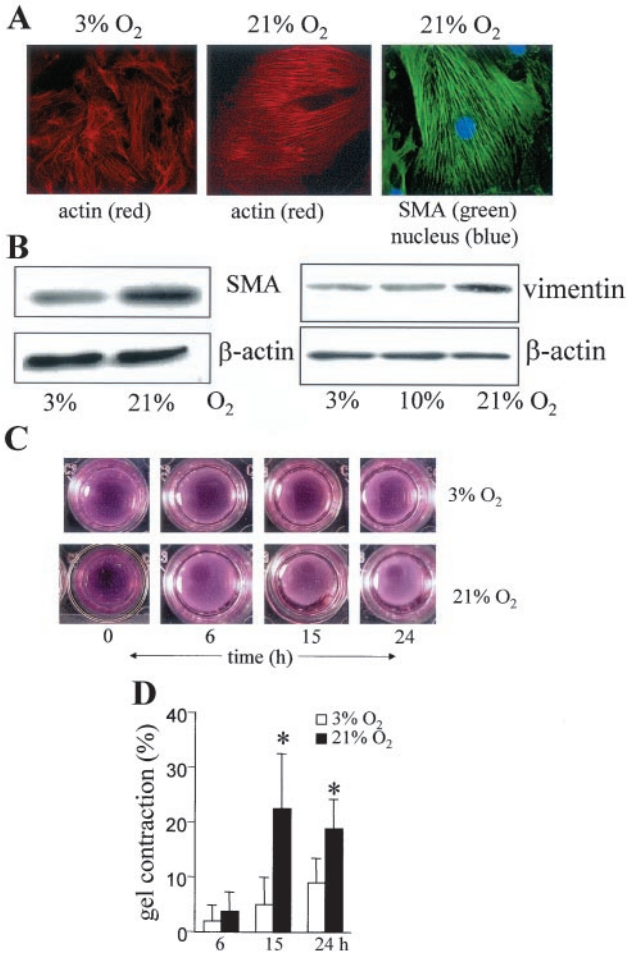

Figure 2. O_2-induced differentiation. After isolation, CFs were cultured at 3%, 10%, or 21% O_2 for 8 days. A, F-actin (phalloidin, red) and SMA (FITC, green) were detected using immunostaining and confocal microscopy. Nuclei were stained with DAPI (blue). B, SMA and vimentin were detected from cellular protein extracts using Western blot. C and D, Collagen gel contraction assay. Contraction of collagen gel in the dish is seen as a function of time; the contraction is more prominent in the bottom panel containing cells grown in 21% (C). Gel contraction was quantified as described in Materials and Methods and statistically treated (D). *$P<0.05$ vs CFs at 3% O_2.

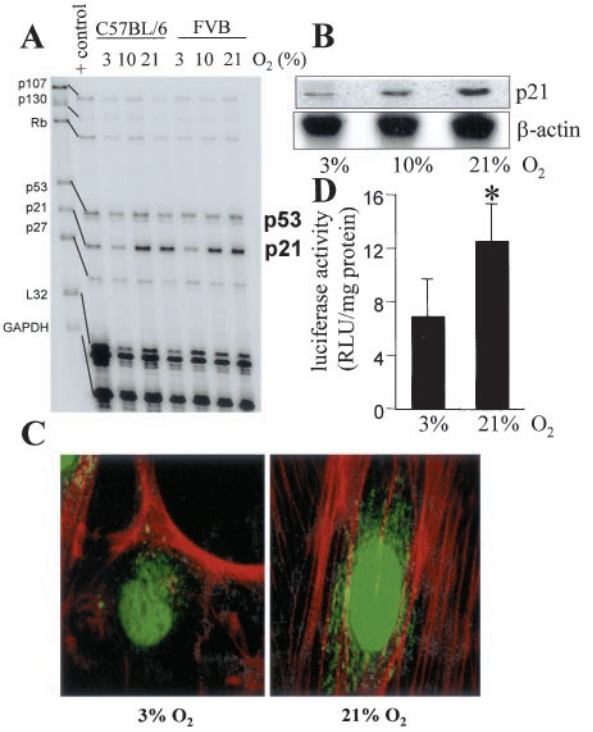

Figure 3. O_2-induced p21 expression. CFs isolated from mouse heart were seeded in a 3%, 10%, or 21% O_2 environment, where they were cultured for 8 days. A, p21 mRNA levels were determined using RPA. Results from two different murine strains (FVB/NJ and C57BL/6) are shown. Other data shown are from C57BL/6-derived CFs. B, Western blot of p21 protein. C, Nuclear localization of p21. Cells were stained with anti-p21 antibody (FITC, green) and phalloidin (red, actin filaments). D, CFs cultured at 3% O_2 for 5 days after isolation. CFs were transfected with p21 wild-type promoter-luciferase reporter construct plasmid and were further cultured at 3% O_2 for 18 hours. Next, cells were transferred to 21% O_2 or retained in 3% O_2. Luciferase activity was measured 18 hours later. Luciferase activities were normalized for the amount of the protein in cell lysates.

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% O_2 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 G_2/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 O_2-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 G_2/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 G_2/M arrest (Figure 1C). Fra-2 is a member of the Fos family of immediate-early

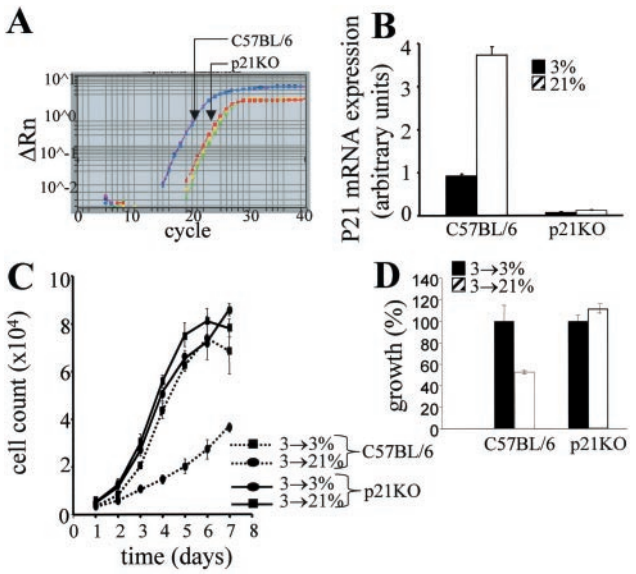

Figure 4. CFs from p21-deficient (p21KO) mice are resistant to O_2-induced growth arrest. CFs were isolated either from wild-type background (C57BL/6) or p21KO background. In panels A and B, RNA was extracted from cells cultured at 3% or 21% O_2 for 8 days. p21 mRNA expression was assessed using real-time 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% O_2 on day 7. ΔRn indicates delta normalized reporter intensity.

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% O_2 contains significantly higher amounts of active TGF-β1 than does the media of CFs grown at 3% O_2 (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

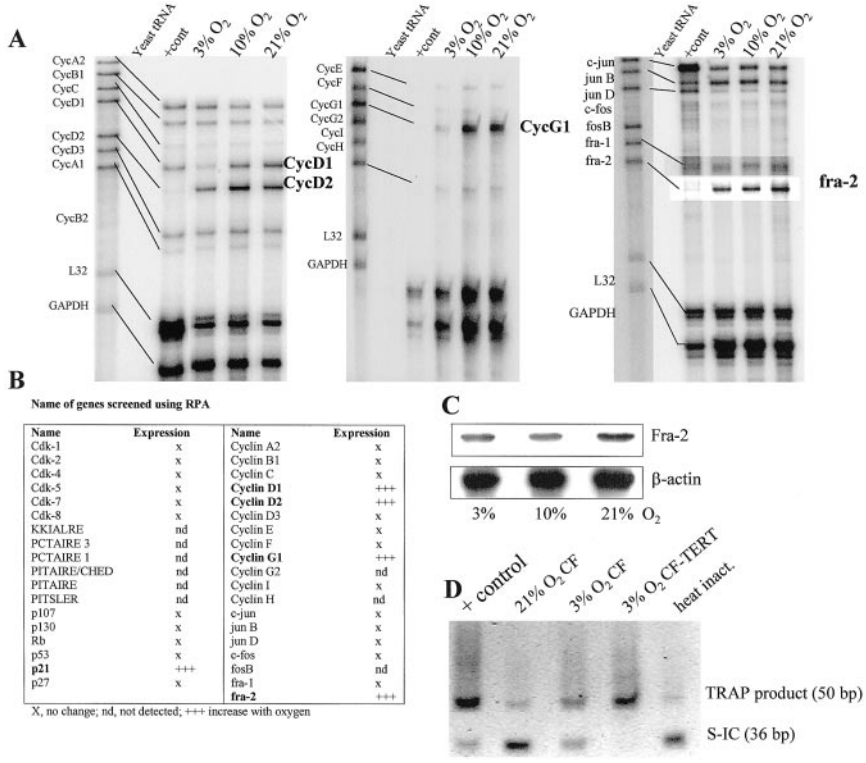

Figure 5. O_2-induced changes in cell cycle–related genes and telomerase activity. Isolated CFs were cultured at 3%, 10%, or 21% O_2 for 8 days. A, Quantitative RPA. Cyc indicates cyclin. B, List of genes screened using RPA. O_2-sensitive genes (21% versus 3%) are in bold. C, Fra-2 Western blot. D, Telomerase 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% O_2; 2, CF stably expressing TERT and cultured at 3% O_2; 3, CF cultured at 3% O_2; 4, CF cultured at 21% O_2; and 5, positive control (component of the kit; see Materials and Methods).

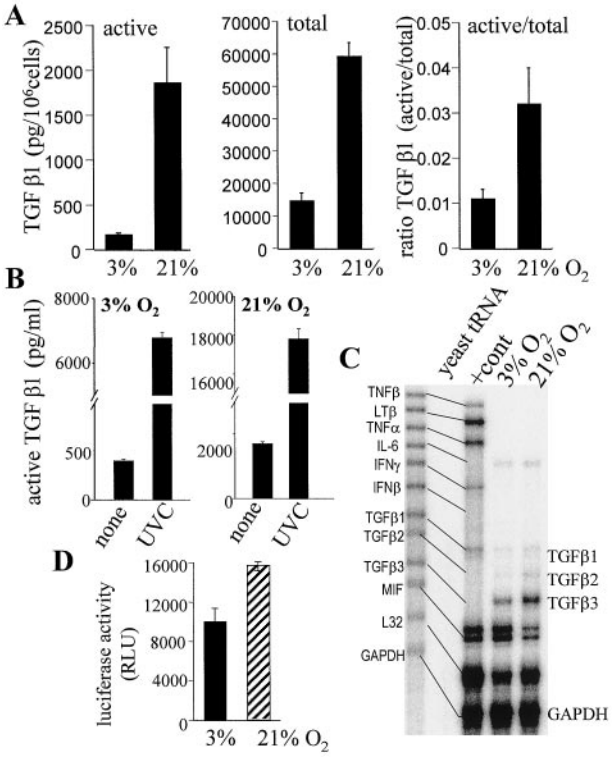

Figure 6. O_2-induced TGF-β1 activation and expression. Isolated CFs were cultured in 3% O_2 for 5 days and were seeded for experiments described below. A, Active or total TGF-β1 was measured from culture media by ELISA 7 days after splitting. B, Conditioned media from CFs (as in panel A) were treated for 20 minutes with the free radical source UVC. C, TGF-β transcription. RPA of TGF-β genes is shown. TNF indicates tumor necrosis factor; LT, leukotriene; IL, interleukin; MIF, macrophage migration inhibitory factor; and IFN, interferon. D, TGF-β1 promoter-driven reporter activity. CFs were transfected with a wild-type TGF-β1 promoter (nucleotides −453 to 11)–luciferase reporter construct and were further cultured at 3% O_2 for 18 hours. Next, cells were transferred to 21% O_2 or retained in 3% O_2. Luciferase activities were normalized for the amount of the protein in cell lysates.

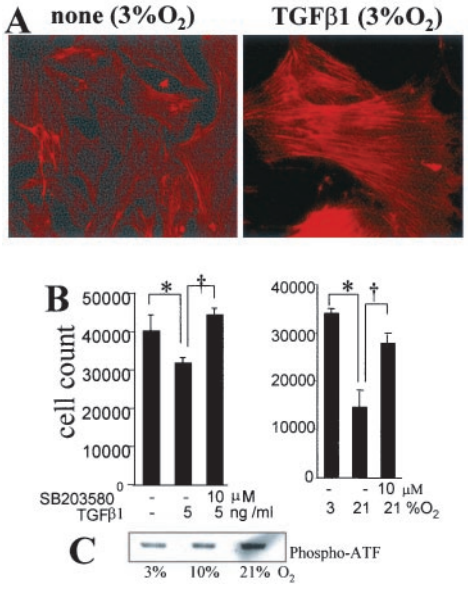

Figure 7. TGF-β1–induced changes in cell morphology are comparable to the effects of hyperoxia. A, Isolated CFs were cultured in 3% O_2 for 8 days. Cytoskeleton was visualized by staining F-actin filaments (phalloidin, red). B, Five days after isolation, cells were split and prepared as follows: (1) TGF-β1 (5 ng/mL) was added after 4 hours of seeding, and cells were cultured at 3% O_2 for 7 days or (2) transferred to 3% or 21% O_2. p38 MAPK inhibitor (SB203580, 10 μmol/L) was added to cells 5 minutes before TGF-β1. Cells were counted after 7 days. C, CFs were cultured at 3%, 10%, or 21% O_2 for 8 days. Activity of immunoprecipitated p38 MAPK was measured using ATF as the substrate.

NADH oxidase–dependent mechanism.[36] 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% O_2 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 O_2-induced growth inhibition as shown in Figure 1. We obtained direct evidence showing that the activation of p38 MAPK is sensitive to ambient O_2 (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 P_{O_2}, compared with P_{O_2} 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 P_{O_2} from $<$10% O_2 (in vivo normoxia) to 21% O_2 (widely interpreted as in vitro normoxia). This raises the vital broad-based issue of controlling ambient O_2 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 O_2 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.[37,38]

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 P_{O_2} 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.[25] Irreversible growth arrest is one of the hallmarks of senescence. The observation that O_2-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.[25,26] Under conditions of stress, the growth of cycling cells is arrested at one of two critical cell cycle check points, G_1-S or G_2-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 P_{O_2} 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.[27] Cyclin D2 expression is induced in multiple states of growth arrest.[28] Cyclin G1 is involved in G_2/M arrest.[29] This is consistent with our observation that CFs exposed to hyperoxia contain higher levels of cyclin G1 mRNA and are in G_2/M arrest.

TGF-β is a natural inducer of growth inhibition and differentiation in CFs.[34] The effects of TGF-β are associated with upregulation of the following Cdk inhibitors: p21, $p15^{Ink4B}$, and $p27^{Kip1}$.[39] p38 MAPK activation represents a prominent downstream target of TGF-β[40] and is critical for p21 induction[41] via p53 phosphorylation. Inhibition of p38 MAPK prevented TGF-β–induced differentiation of CFs to myofibroblasts, suggesting a key role of this signaling mediator.[34] Interestingly, ROS are involved in TGF-β–induced p38 MAPK activation.[42,43] 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 G_2/M.[44] 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.[35] 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.[45] 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 adenoviral 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 O_2 could well serve as a trigger initiating O_2-sensitive signaling pathways. This contention is consistent with a recent report demonstrating that molecular O_2 may indeed modulate signaling processes by an ROS-independent prolyl-hydroxylation reaction.[46]

That low O_2 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 O_2 environment is not limited to hypoxia. It demonstrates that marginal relative elevation in P_{O_2}, compared with P_{O_2} to which cells or tissues are adjusted, serves as a signal to trigger changes in the cellular phenotype relevant to tissue remodeling.

Acknowledgment

This study was supported by NIH grant GM-27345 to Dr Sen. M.A. and M.V. were on leave from the Department of Physiology, University of Kuopio, Finland.

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