Effects of oxygen, growth state, and senescence on the antioxidant responses of WI-38 fibroblasts

Arthur K. Balin · Richard J. Reimer ·
Wende R. Reenstra · Steven M. Lilie · Ina Leong ·
Katherine Sullivan · Robert G. Allen

Abstract Mitotically active, growth-arrested cells and proliferatively senescent cultures of human fetal lung fibroblasts (WI-38) were exposed to six different oxygen tensions for various lengths of time and then analyzed to determine the responses of their antioxidant defense system. Glutathione (GSH) concentration increased as a function of ambient oxygen tension in early passage cultures; the effect was larger in exponentially growing cultures than in those in a state of contact-inhibited growth arrest, but was absent in senescent cells. Conversely, the activity of glutathione disulfide reductase was greater in growth-arrested cultures than in mitotically active cells irrespective of oxygen tension.

Glucose-6-phosphate dehydrogenase was lowest in log-phase cells exposed to different oxygen tensions for 24 h and in senescent cells. Both hypoxia and hyperoxia depressed selenium-dependent glutathione peroxidase activity in early passage cultures, while the activity of the enzyme progressively declined with increasing oxygen in senescent cells. The GSH S-transferase activity was unresponsive to changes in ambient oxygen tension in either young or senescent cultures. Manganese-containing superoxide dismutase (MnSOD) activity was unaffected by oxygen tension, but was elevated in young confluent cultures as compared with cultures in log-phase growth. MnSOD activity was significantly

A. K. Balin (✉) · R. J. Reimer · W. R. Reenstra ·
S. M. Lilie · I. Leong · R. G. Allen
Laboratory for Investigative Dermatology,
The Rockefeller University,
1230 York Avenue,
New York, NY 10021, USA
e-mail: akbalin@balin.com

Present Address:
A. K. Balin · K. Sullivan · R. G. Allen
Longevity Achievement Foundation,
110 Chesley Drive,
Media, PA 19063, USA

Present Address:
R. J. Reimer
Department of Neurology and Neurological Sciences,
Stanford University,
Stanford, CA 94305, USA

Present Address:
W. R. Reenstra
New England Inflammation and Tissue Protection Institute,
Departments of Biology and Pharmaceutical Sciences,
Northeastern University,
134 Mugar Building, 360 Huntington Avenue,
Boston, MA 02115, USA

Present Address:
S. M. Lilie
York Hospital,
1001S George St,
York, PA 17403, USA

higher in senescent cultures than in early passage cultures and was also responsive to increased oxygen tension in senescent cultures. Copper–zinc-containing superoxide dismutases activity was not affected by oxygen tension or the passage of time, but it declined in senescent cultures.

Introduction

At concentrations exceeding atmospheric level, oxygen is toxic to all organisms (Stogner and Payne 1992; Patel et al. 2003; Auten and Davis 2009). In fact, sub-atmospheric concentrations of oxygen are toxic at a cellular level (Balin et al. 1984, 2002b). Numerous studies have demonstrated oxygen toxicity in a broad spectrum of intact organisms as well as in a variety of cell culture models (for reviews, see Davies 1999, 2000; Greabu et al. 2008). An examination of the phenomenon at a cellular level reveals that specific points in cell cycle are oxygen sensitive (Balin et al. 1978; Rancourt et al. 2002). Both aerobic and anaerobic metabolism can be modulated profoundly by changes in ambient oxygen concentration (Balin et al. 1976). Aging of intact organisms is widely believed to be influenced by by-products of oxygen metabolism such as oxygen-centered free radicals and other non-radical reactive oxygen species (ROS; Allen and Balin 2003a).

Aging of cells in a culture environment manifests as limited proliferative capacity. Proliferative lifespan appears to be partly determined by loss of telomere DNA with each successive round of mitosis (Harley et al. 1990; Allsopp and Harley 1995). However, it has been reported that the rate of telomere shortening in various types of cells is affected by oxidative stress and hyperoxia (von Zglinicki et al. 1995, 2000; Parrinello et al. 2003). Furthermore, the proliferative capacity of various types of human cells can be modulated by changes in the ambient oxygen tension (Balin et al. 2002a).

The underlying causes of oxygen toxicity have not been clearly established. A widely held view is that oxygen toxicity arises from increased oxygen free radical generation in cells (Beckman and Ames 1998; Mattar and Haffor 2009), and it has been observed that exposure to hyperoxia accelerates the accumulation of oxidative by-products such as lipofuscin (von Zglinicki et al. 1995), which would seem to support the hypothesis.

Aerobic cells have antioxidant defenses that remove ROS before they can inflict damage. These include superoxide dismutases (SOD) that remove superoxide radicals (O_2^{-}) and produce hydrogen peroxide (H_2O_2; Allen 1998), and catalase and peroxidases that remove H_2O_2 (Keogh et al. 1996). The family of seleno-proteins referred to as GSH peroxidases (GPx) remove both H_2O_2 (Modrick et al. 2009) and lipid peroxides (Ran et al. 2006; Arsova-Sarafinovska et al. 2009). The most prominent of these, GPx-1, can metabolize both organic and inorganic substrates (Arsova-Sarafinovska et al. 2009). Enzymic defenses that remove highly reactive hydroxyl radicals (HO$^{\cdot}$) have never been identified, but non-enzymatic antioxidant defenses, such as tocopherol, urate, carotenoids, ascorbate, and glutathione (Keogh et al. 1996; Allen and Balin 2003a), can remove ROS including HO$^{\cdot}$ by reacting directly with them. Non-enzymic antioxidants can also break chain oxidation reactions (Beckman and Ames 1998). Of course, if ROS are the primary catalysts of oxygen toxicity, then antioxidant defenses should increase in response to increasing oxidant production resulting from elevated ambient oxygen tensions.

A variety of environmental factors, including ROS, are believed to contribute to senescence via mechanisms independent of telomere shortening, particularly in human cells (Cristofalo et al. 2000, 2004). The proliferative capacity of various types of human cells can be modulated by changes in the ambient oxygen tension (Balin et al. 2002a). The effects of oxygen on growth are also mediated by some culture-specific factors such as seeding density (Balin et al. 1984, 2002a).

The toxic effects of oxygen may be mediated by the balance between ROS generation and antioxidant response and may be influenced not only by the age of cultures, but also by their level of mitotic activity. The purpose of this study was to assess the influence of oxygen tension on the antioxidant defenses of fetal lung fibroblasts (WI-38) in both actively growing and quiescent growth states as well as in proliferatively senescent cultures.

Methods

Cell lines Human diploid embryonic lung fibroblast cell strains WI-38 were obtained from Dr. Vincent J. Cristofalo of the Lankenau Center for Medical Research, Wynnewood, PA, USA.

Cell culture Cells were grown in Dulbecco's modified Eagle's medium (1 g/l glucose: Gibco Laboratories, Grand Island, NY, USA). Immediately before use, the medium was supplemented with L-glutamine (2 mM; Flow Laboratories, Rockville, MD, USA) and fetal bovine serum (10% vol/vol; KC Biologicals). Antibiotics were not used. Cultures were grown at 37°C in 75-cm^2 sealed polystyrene flasks (No 3024; Falcon Labware, Oxnard, CA, USA) containing 40 ml medium 0.54 ml/cm^2 and with a gas phase volume of 233 cm^3. The cells were released from the plastic with trypsin (0.25%; Flow Laboratories) in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (M.A. Bioproducts, Walkersville, MD, USA). After suspension in medium containing 10% FBS, cell number was determined with a Coulter Counter (Coulter Electronics, Hialeah, FL, USA), and cells were then inoculated into appropriate vessels at a density of 10^4 cells/cm^2.

Population doublings (PD) were calculated by comparing cell counts per vessel at seeding with counts at confluence (Cristofalo et al. 2000). The percentage of labeled nuclei was determined autoradiographically (Cristofalo et al. 2000) and monitored for mycoplasma contamination by the methods of Schneider and Stanbridge (1975) and by incubation of cells and media on selective agar (Flow Laboratories). All young cultures had completed less than 60% of their proliferative lifespan and were free of mycoplasma contamination by these criteria. Cell viability was determined using Trypan Blue dye exclusion (Freshney 1994).

Senescent cells Cells were deemed senescent when the population failed to double 4 weeks and three refeedings after seeding. In the studies reported here, enough cells were collected to seed T175 tissue culture vessels for experimental analysis of the effects of oxygen tension. Senescent cells were seeded and maintained in medium equilibrated with 5% oxygen. After 7 days, cultures were refed with medium that contained 0.5% serum and transferred to the different oxygen tensions. Cultures were harvested for analysis after 48-h exposure.

Antioxidant defenses: cell culture Cultures used to study antioxidant defenses in log phase were seeded at 10^4 cells/cm^2 in T175 flasks, grown for 3 days in an atmosphere containing 5% CO$_2$, 5% O$_2$, and 90% N$_2$. The gas phase of the cultures was equilibrated with gas mixtures containing 5% CO$_2$, 0%, 5%, 20%, 35%, 50%, or 95% O$_2$, and the balance N$_2$, and transferred to one of six incubators (Heraeus, Tekmar Co., Cincinnati, OH, USA) maintained electronically at the oxygen tension used to equilibrate the flask. Each incubator was equipped with a Clark oxygen electrode and a CO$_2$ thermal conductivity detector to maintain the desired percentages of atmospheric oxygen and carbon dioxide. Incubators were calibrated each day by withdrawing a sample of gas and measuring the partial pressure of oxygen and carbon dioxide with a blood gas analyzer (model 113; Instrumentation Laboratory, Inc., Lexington, MA, USA). Cultures in log-phase growth were harvested for analysis at 24 or 48 h. Cultures grown for measurement of antioxidant defenses in stationary phase were seeded at 10^4 cells/cm^2 in T175 flasks and grown to confluence in an atmosphere containing 5% CO$_2$, 5% O$_2$, and 90% N$_2$. When cultures had reached a stationary growth phase (7 days), they were refed with fresh medium that contained 0.5% fetal bovine serum and the gas phase equilibrated with gas mixtures containing 5% CO$_2$, 0%, 5%, 20%, 35%, 50%, or 95% O$_2$ with the balance N$_2$. Cultures were harvested for analysis after 24-, 48-, 72-, 96-, or 168-h exposure. In all cases, a blood gas analyzer was used to determine the oxygen content of the flasks before harvest.

Biochemical analysis

Superoxide dismutase activity Superoxide dismutase activity was determined by a modification of the positive assay of Misra and Fridovich (1977) as previously described (Allen et al. 1995). Cells were suspended in 16.6 mM potassium phosphate buffer, pH 7.8, sonicated for 15 s, and then centrifuged at 20,000×g for 30 min. The supernatants were dialyzed overnight against 50 mM potassium phosphate buffer, pH 7.0. A 100-μl aliquot of homogenate (containing between 100 and 200 μg protein) was added to a

cuvette that contained 0.25 ml riboflavin (100 μM in 16.6 mM potassium phosphate buffer, pH 7.8); to this was added 1.65 ml of a 0.24 mM solution of *o*-dianisidine. The absorbance of the samples was then determined. The cuvettes were then illuminated 2 min with two 15-W Sylvania black lights, and the absorbance was read immediately. The difference in absorbencies before and after illumination minus a blank was proportional to SOD activity. In order to determine manganese-containing superoxide dismutase (MnSOD) activity, homogenate was added to a reaction mixture that contained 1.5 mM KCN (which inactivates copper–zinc SOD (Cu/Zn SOD)) about 5 min prior to illumination. The dianisidine assay was sensitive to the protein content of samples; the effect was linear at low protein concentrations, but undetected at higher protein concentrations. Therefore, an amount of bovine albumin equal to the average protein content of the samples was routinely added to all of the blanks. Also, addition of cyanide (to distinguish MnSOD) significantly increases the background change in absorbance; however, the color change observed with 1 unit MnSOD (minus the appropriate blank) was identical with or without cyanide. Hence, one unit of activity was taken to be the amount of SOD that yields a color change above background equal to the magnitude of the change observed in the blank used for total activity.

Glutathione (GSH + GSSG) The level of total glutathione was determined by the cycling method of Tietze (1969). In this procedure, homogenate (deproteinized with 10% perchlorate and neutralized with K_3PO_4), glutathione disulfide (GSSG) reductase, NADPH, and 5,5′-dithiobis-2-nitrobenzoic acid were mixed in a cuvette and the color change monitored at 412 nm. A standard curve was constructed with known amounts of GSSG and used to calculate the concentration of glutathione present in the homogenates. An attempt was made to determine GSSG concentration by first eliminating GSH with 2% 2-vinylpyridine according to the method of Griffith (1980) and then repeating the above assay. However, the concentration of GSSG in the fibroblast cell lines examined was low and often below the detection limits of the assay.

GSSG reductase A slight modification of the method of Carlberg and Mannervik (1985) was used to determine GSSG reductase (GR) activity. Homoge-

nate was added to a solution of 200 mM potassium phosphate buffer containing 0.2 mM NADPH and 2 mM GSSG. The rate of color loss monitored at 340 nm is proportional to GSSG reductase activity.

Glucose-6-phosphate dehydrogenase Glucose-6-phosphate dehydrogenase (G-6-PD) activity was quantified according to the method of Deutsch (1980), which is based on the formation of NADPH. Maleimide (1 mM) was added to block competing reactions. NADPH formation was determined spectrophotometrically at 340 nm.

GSH peroxidase (GPx-1) This assay couples the oxidation of GSH by GSH peroxidase to the oxidation of NADPH by GSH reductase as previously described (Keogh et al. 1996). The assay mixture contained 50 mM Tris HCl, pH 7.6; 1 unit/ml GSSG reductase, 0.25 mM GSH, 0.2 mM NADPH, and 3 mM KCN to increase stability. The reaction was initiated by the addition of 50 μl of a 12 mM H_2O_2 solution. This method detected predominantly type 1 GPx activity. One unit of activity is equal to consumption of 1 μM NADPH/min (monitored at 340 nm). Due to the relatively low GPx activity observed in fibroblasts, we report activity in milliunits.

GSH S-transferases Activity of *S*-transferases was determined using 1-chloro-2, 4-dinitrobenzene (CDNB) as a general substrate (Hazelton and Lang 1983). The assay mixture consisted of 50 μl of homogenate, 100 μl of 50 mM GSH, and 450 μl of CDNB (to a final concentration of 1.25 mM). The rate of increase in absorbance at 340 nm was used to calculate activity using 9.6 mM^{-1} cm^{-1} as an extinction coefficient. Units of activity are expressed as nmol min^{-1} mg $protein^{-1}$.

ROS generation The rate of ROS generation was determined using 2′,7′-dichlorofluorescin diacetate (DCFH-DA). Because it is non-polar, cell membranes are permeable to DCFH-DA (Bass et al. 1983); once in cells, the compound is desacetylated to DCFH. DCFH is polar and is thus trapped in cells. DCFH is oxidized predominantly by H_2O_2 to the highly fluorescent 2′,7′-dichlorofluorescein (Bass et al. 1983; Chiou and Tzeng 2000). The oxidation of DCFH to DCF has been widely used to determine H_2O_2 generation by flow cytometry and direct

visualization techniques (Rothe and Valet 1990, 1994; Carter et al. 1994). However, the indicator is not entirely specific to H_2O_2 (Liochev and Fridovich 2001); therefore, we shall refer to this measurement as ROS rather than H_2O_2 generation.

DCFH-DA was initially solubilized in 250 μl DMSO and then added to 150 ml phenyl red, serum-free MEM. WI-38 cells were seeded in a black 96-well viewplate (Perkin Elmer, Waltham, MA, USA) at 10,000 cells per well. The cells were washed once with phenyl red, serum-free MEM and then treated with 100 μl of DCFH-DA containing medium. The plates were incubated at 37°C for 1 h. Following incubation, the cells were washed once with the wash buffer and read (ex 494, em 518) with a Victor 2 Plate Reader (Perkin Elmer). Filters used were ex 485 (Perkin Elmer Filter 11440022) and em 535 (Perkin Elmer Filter 11440023). Protein in the samples was determined using Bradford protein assay reagent (Biorad). The reagent was diluted 1:5 with ultrapure water and filtered prior to treating samples. The Victor 2 plate reader was used to determine absorbance at 595 nm. Standard curves were prepared for each assay from known concentrations of DCF and protein.

Statistics All measurements were performed on triplicate samples in each experiment, and all experiments were repeated at least twice. Data were compared using analysis of variance (ANOVA). Least significant difference (LSD) was used to distinguish subgroups. In all cases, $p \leq 0.05$ was inferred to indicate significance.

Results

Superoxide dismutases

Copper–zinc SOD The average activity of copper–zinc SOD (Cu/Zn SOD) was relatively constant at 1.6 U/mg protein regardless of ambient oxygen tension or the duration of exposure. The activity of this enzyme was also independent of the growth state of the cells. Senescent cultures exhibited decreased Cu/Zn SOD activity of 0.7 U/mg protein. As in the case of early passage cells, the activity of this enzyme was not affected by increased oxygen tension.

Manganese superoxide dismutase The manganese-containing form of superoxide dismutase is mitochondrial and thus is pivotal to the removal of $O_2^{\cdot-}$. MnSOD was unaffected by oxygen tension in any of the groups of young cultures examined in this study (Fig. 1); however, growth state and proliferative age strongly influenced the activity of this enzyme. Log-phase cultures were statistically similar at all of the
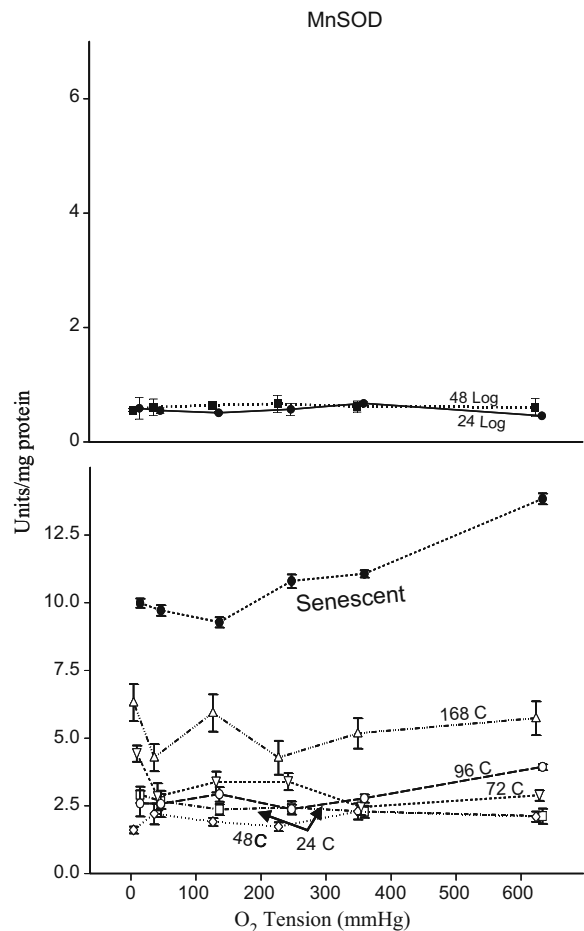

Fig. 1 MnSOD activity in WI-38 cultures exposed to different oxygen tensions for various periods of time. Mitotically active cultures were grown 3 days at under an ambient oxygen tension of 38 mm Hg then refed and transferred to the different oxygen tensions for 24 or 48 h (24 Log and 48 Log; *upper panel*). Non-growing cultures were grown to saturation density under 5% oxygen, refed with medium that contained 0.5% serum, and then transferred to different oxygen tensions. The non-proliferating, confluent (C) cultures were harvested and analyzed after 24, 48, 72, 96, and 168 h (24 C, 48 C, 72 C, 96 C, and 168 C; *lower panel*). Senescent cultures were harvested after 48-h exposure to different oxygen tensions (see "Methods")

oxygen tensions examined (ANOVA, LSD, $p \geq 0.7$ in all cases; Fig. 1, upper panel), but they exhibited significantly lower MnSOD activity than growth-arrested cultures maintained at corresponding oxygen tensions (ANOVA, LSD, $p \leq 0.00001$ in all comparisons). It was also observed that, in growth-arrested cultures, MnSOD activity accumulated with the passage of time, albeit the relationship was not linear (Fig. 1, lower panel). Confluent (C), growth-arrested cultures exposed to different oxygen tensions for 24 h (24 C) exhibited significantly greater MnSOD activity than similar cultures exposed for 48 h (48 C) at oxygen tensions of 9 and 242 mm Hg (ANOVA, LSD, $p = 0.00002$ and $p = 0.01$, respectively; Fig. 1, lower panel). The cultures maintained at other oxygen tensions in the 24 C group also tended to exhibit greater MnSOD activity than the corresponding cultures in the 48 C group, but the differences were not statistically significant. Growth-arrested cultures exposed to different oxygen tensions for 72 h (72 C) exhibited significantly greater MnSOD activity than observed in the 24 C or 48 C groups at all oxygen tensions ($p \leq 0.02$ in all cases) except 41 mm Hg where the 24 C group was similar (ANOVA, LSD, $p = 0.5$) and 354 mm Hg where both 24 and 48 C were similar to 72 C (ANOVA, LSD, $p \leq 0.5$ in both cases). Growth-arrested cultures exposed for 96 h (96 C) exhibited greater MnSOD at 9 (ANOVA, LSD, $p = 0.000001$), 242 (ANOVA, LSD, $p = 0.003$), and 628 mm Hg oxygen (ANOVA, LSD, $p = 0.0008$) than cultures in the 72 C group exposed to similar oxygen tensions. Cells maintained under different oxygen tensions for 168 h (168 C) exhibited significantly greater MnSOD activity than cultures maintained at corresponding oxygen tensions for shorter periods of time (ANOVA, LSD, $p \leq 0.0001$ in all cases; Fig. 1, lower panel). Since oxygen tension failed to stimulate enzyme activity in early passage cultures, it is inferred that MnSOD activity passively accumulated in quiescent cultures. Cultures of senescent cells exhibited higher MnSOD activity than proliferatively younger cultures at all oxygen tensions (ANOVA, $p < 0.0001$ in all cases). MnSOD activity was also induced by hyperoxia in senescent cultures ($pO_2 = 633$ nm; ANOVA, $p < 0.001$ in all comparisons with other oxygen tensions; Fig. 1, lower panel).

Glutathione Large volumes of medium surround cells in culture. Because cell membranes are partially permeable to oxidized glutathione (GSSG), it diffuses from cells into the relatively large volume of medium that surrounds them, leaving only extremely low concentrations of GSSG in normal cells. The concentration of GSSG was often near or below the limit of detection of the assay. Consequently, nearly all of the glutathione present in cells is in the reduced form (GSH). For the remainder of this discussion, we will refer to total glutathione as GSH.

In young cells, the concentration of GSH increased as a function of the ambient oxygen concentration; however, the effect was much more pronounced in log-phase cultures (Fig. 2, upper panel).

Hence, the greatest increase in GSH concentration was observed in log-phase cultures exposed to different O_2 partial pressures for 24 h (24 Log), and the next greatest was observed in log-phase cultures exposed to different O_2 partial pressures for 48 h (48 Log). The relative response to ambient oxygen tension was diminished in stationary phase (non-dividing) cultures. The least responsive cells were those in stationary phase cultures exposed to different oxygen tensions for only 24 h (24 C; see Fig. 2, lower panel). In all cases except the growth-arrested cultures exposed for 24 h (24 C), the GSH concentration present in cells maintained at 9 mm Hg was significantly lower than the concentration of GSH found in cells maintained at oxygen tensions of 131 mm Hg or greater (ANOVA, $p < 0.03$ in 48 C group and $p < 0.00001$ in all other cases). Cultures of proliferating cells maintained at oxygen partial pressure of 9 mm Hg for 24 h (24 Log) also tended to exhibit lower GSH than cultures maintained at 41 mm Hg oxygen than at 131 mm Hg oxygen (ANOVA, $p = 0.00001$). Conversely, confluent, growth-arrested cultures maintained under oxygen partial pressures of 9, 41, 131, and 242 mm Hg for 24 h (24 C) exhibited statistically similar concentrations of GSH (Fig. 2, lower panel). Furthermore, in all of the experiments in which it was examined (three experiments), the concentration of GSH was significantly lower ($p \leq 0.001$ in all cases) in growth-arrested cultures maintained at an oxygen partial pressure of 131, 242, 324, or 648 mm Hg for only 24 h (24 C) than in confluent cultures maintained at these oxygen tension for longer periods (Fig. 2, lower panel). These data suggest that proliferating cultures produce more GSH as the oxygen tension increases than cultures of non-dividing cells and that GSH concentration is lowest immediately after cultures reach confluence and the cells
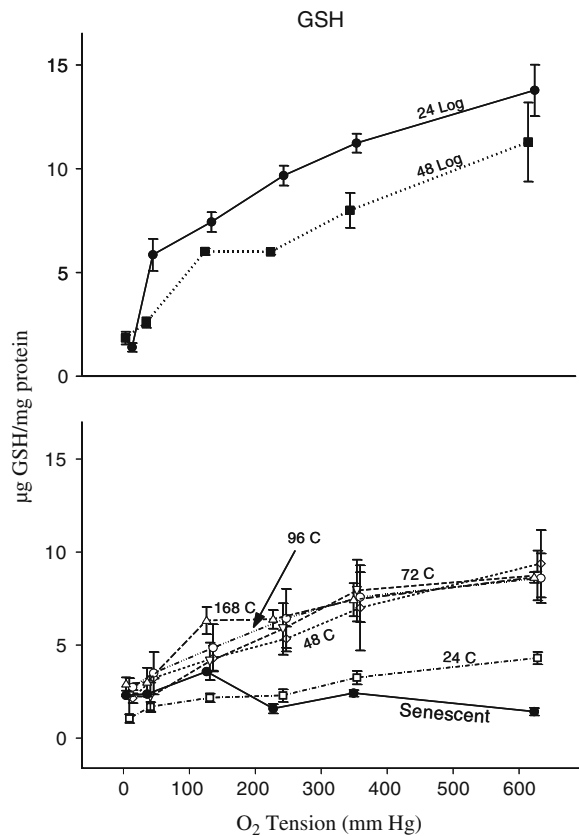
Fig. 2 GSH concentration in WI-38 cultures exposed to different oxygen tensions for various periods of time. Mitotically active cultures were grown 3 days under an ambient oxygen tension of 38 mm Hg then were refed and transferred to the different oxygen tensions for 24 or 48 h prior to harvest and analysis (24 Log and 48 Log). Exposures of non-growing, confluent cultures to different oxygen tensions was achieved by growing cells to saturation density under 38 mm Hg oxygen; the growth-arrested, confluent cultures (C) were then refed with medium that contained 0.5% serum and transferred to different oxygen tensions for 24, 48, 72, 96, or 168 h (24 C, 48 C, 72 C, 96 C, and 168 C; *lower panel*). Senescent cultures were harvested after 48-h exposure to different oxygen tensions (see "Methods")

enter a state of proliferative quiescence. Senescent cultures exhibited lower GSH concentrations at 247, 359, and 633 nm O₂ (ANOVA; $p<0.001$ in all comparisons with younger cultures; Fig. 2, lower panel). The results clearly indicate that unlike the results observed in early passage cultures, GSH concentration was not directly dependent on ambient O₂ concentration in senescent cultures (Fig. 2, lower panel).

GSSG reductase (GR) reduces GSSG to GSH using electrons obtained from NADPH. In vivo, the enzyme

plays an important role in maintaining redox balance. We examined whether GR activity could contribute to the large differences in GSH concentration that existed between actively growing and confluent, growth-arrested cultures by comparing both groups of actively growing cultures (24 Log and 48 Log) with the first two groups of growth-inhibited cultures (24 C and 48 C; Fig. 3). In contrast to GSH concentration (Fig. 2), GR activity was lower in log-phase cells. Although increasing the O₂ tension appeared to stimulate GR in growing cultures, there was no such effect in mitotically quiescent cells. Preliminary studies revealed no significant differences between mitotically quiescent cultures and senescent cells (at 134 nm Hg oxygen tension), and no further comparison of oxygen effects was made for senescent cultures. On the basis of these results, we infer that the changes in GSH concentration in both mitotically active and quiescent cells were independent of GR activity in all of the cultures examined.

Glucose-6-phosphate dehydrogenase (G-6-PD) is an enzyme in the pentose shunt that is sensitive to feedback activation by GSSG (Eggleston and Krebs 1974; Legan et al. 2008). It is important for proper
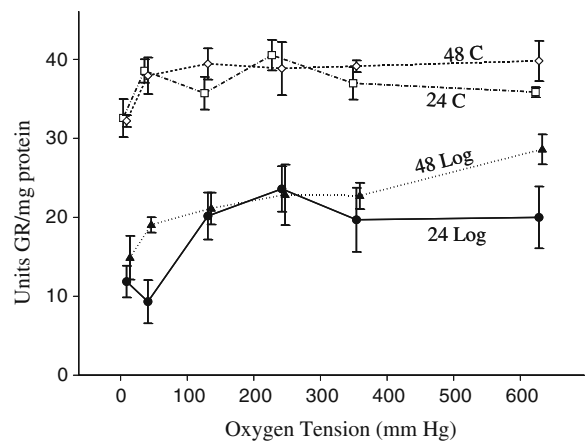

Fig. 3 GSSG reductase activity in WI-38 cultures exposed to different oxygen tensions for various periods of time. Mitotically active cultures were grown 3 days under an ambient oxygen tension of 38 mm Hg then were refed and transferred to the different oxygen tensions for 24 or 48 h (24 Log and 48 Log). Mitotically quiescent cultures were obtained by allowing them to grow to saturation density under 38 mm Hg oxygen for 7 days. The resulting stationary phase, confluent cultures (C) were refed with medium that contained 0.5% serum and then transferred to different oxygen tensions for 24 or 48 h prior to harvest and analysis (24 C, 48 C)

maintenance of GSH concentration in cells because it is involved in the synthesis of NADPH, the co-factor of GSSG reductase.

G-6-PD activity was greater in cells maintained under an oxygen tension of 324 mm Hg than in cells maintained at 9 mm Hg oxygen. The increase was statistically significant in the 48 Log (ANOVA, p=0.002; Fig. 4, upper panel), 72 C (AVOVA, LSD, p=0.003; Fig. 4, lower panel), 95 C (AVOVA, LSD p=0.02; Fig. 4, lower panel), and 168 C (AVOVA, LSD p=0.02; Fig. 4, lower panel) groups. G-6-PD activity also tended to be greater in growth-arrested cells
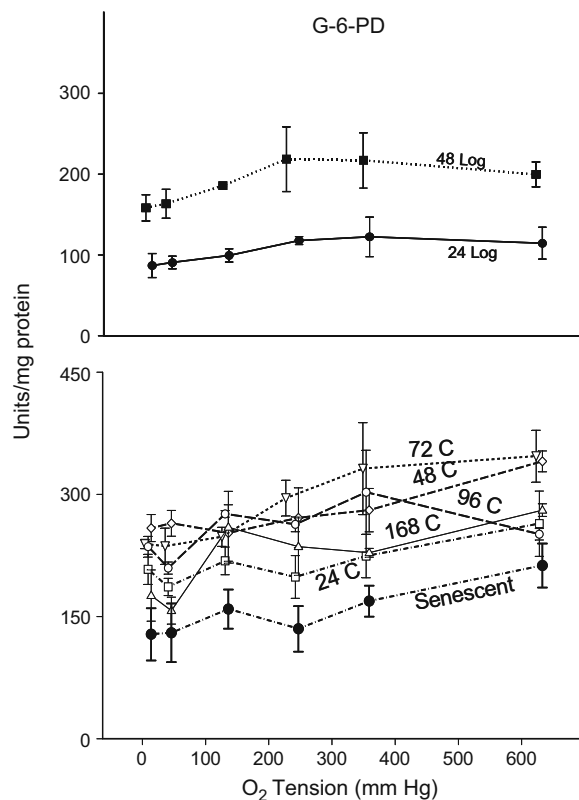

Fig. 4 G-6PD activity in WI-38 cultures exposed to different oxygen tensions for various periods of time. Mitotically active cultures were grown 3 days under an ambient oxygen tension of 5% then were refed and transferred to the different oxygen tensions for 24 or 48 h (24 Log and 48 Log; *upper panel*). Mitotically quiescent cultures were obtained by allowing them to grow to saturation density under 38 mm Hg oxygen for 7 days. The resulting stationary phase, confluent cultures (C) were refed with medium that contained 0.5% serum and then transferred to different oxygen tensions for 24, 48, 72, 96, or 168 h (24 C, 48 C, 72 C, 96 C, and 168 C; *lower panel*). Senescent cultures were harvested after 48-h exposure to different oxygen tensions (see "Methods")

maintained under an oxygen partial pressure of 648 mm Hg as compared to cultures maintained under 9 mm Hg oxygen in several of the groups, but the effect was less consistent than was observed in cells maintained under 324 mm Hg oxygen (Fig. 4, lower panel).

Significant elevations of G-6-PD at 648 mm Hg oxygen were observed in cultures from the 48 Log (ANOVA, LSD, p=0.04), 24 C (ANOVA, LSD, p=0.02), 72 C (ANOVA, LSD, p=0.002), and 168 C (ANOVA, LSD, p=0.00002) groups. G-6-PD tended to be lower in log-phase cells than in non-growing cultures. For example, G-6-PD activity was significantly lower in proliferating cultures exposed to different oxygen tensions for 24 h (Log 24) than in any other group of cultures (ANOVA, LSD, $p \leq 0.0001$ in all comparisons; Fig. 4, upper panel). The effect was far less pronounced in the Log 48 group, which exhibited similar G-6-PD activity to the 168 C group at 9, 41, and 324 mm Hg oxygen. The Log 48 group also exhibited higher activity than the 24 C group when maintained under 242 mm Hg oxygen. Nevertheless, the extremely low levels of G-6-PD in the Log 24 group and the fact that the Log 48 group exhibited activity that was relatively low as compared to most non-growing cultures suggested that the activity of the enzyme tended to become elevated as cells reached confluence and ceased to divide. Senescent cultures exhibited significantly lower (ANOVA, p<0.001; Fig. 4, lower panel) G-6-PD at all oxygen tensions except 14 and 46 nm O_2, where activity was similar between senescent cultures and cultures maintained at these oxygen tensions for 168 h (168 C). Activity was similar between the 24 C, 96 C, and senescent cultures at 633 mm Hg oxygen tension (ANOVA, p<0.09; p<0.1 respectively).

Glutathione peroxidase (GPx-1) Selenium-dependent GPx-1 plays an important role in the removal of H_2O_2 from cells. This is particularly true in fetal lung fibroblasts because they tend to have relatively low levels of catalase activity. Also, the K_M of catalase is high (Nicholls et al. 2001), which probably necessitates the involvement of other enzymes such as GPx-1 to effectively remove H_2O_2. Additionally, GPx-1 can remove lipid peroxides. The enzyme requires GSH as a co-factor. In general, GPx-1 activity was higher in cultures maintained at either 41 or 131 mm Hg oxygen as compared to cultures maintained under an oxygen partial pressure of 9 mm Hg (Fig. 5).
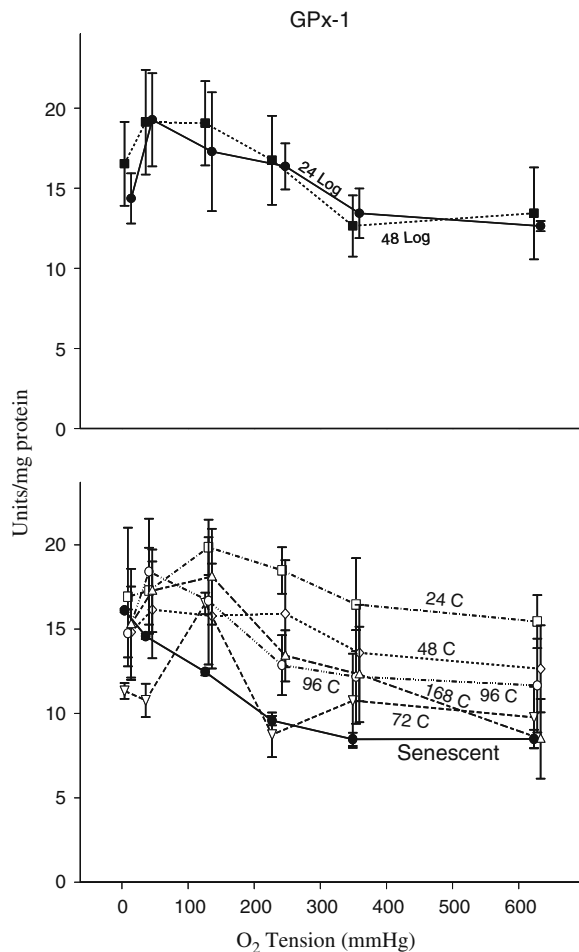
Fig. 5 GPx activity in WI-38 cultures exposed to different oxygen tensions for various periods of time. Mitotically active cultures were grown 3 days under an ambient oxygen tension of 5% then refed and transferred to the different oxygen tensions for 24 or 48 h (24 Log and 48 Log; *upper panel*). Analysis of non-growing cultures was performed by allowing them to grow to saturation density under 5% oxygen; the confluent (C) cultures were refed with medium that contained 0.5% serum and then transferred to different oxygen tensions for 24, 48, 72, 96, and 168 h (24 C, 48 C, 72 C, 96 C, and 168 C; *lower panel*). Senescent cultures were harvested after 48-h exposure to different oxygen tensions (see "Methods")

The difference was statistically significant in mitotically active cultures maintained at 9 and 41 mm Hg oxygen for 24 h (ANOVA, LSD, $p=0.005$), growth-arrested cultures maintained under oxygen partial pressures of 9 and 131 mm Hg for 72 h (ANOVA, LSD, $p=0.01$) and in growth-arrested cultures maintained under oxygen tensions of 9 and 41 mm Hg for 96 h (ANOVA, LSD, $p=0.03$). There

was also a tendency for high oxygen tension to slightly decrease GPx-1 activity. For example, actively growing cultures maintained at different oxygen tensions for 24 h exhibited a progressive loss of GPx-1 activity at oxygen tensions greater than 41 mm Hg. Furthermore, GPx-1 activity was significantly diminished at partial oxygen pressures of 324 and 648 mm Hg (ANOVA, LSD, $p=0.001$ and 0.00002, respectively) as compared to activity present under 41 mm Hg oxygen. A similar pattern was observed in all of the groups. Specifically, GPx-1 activity was lower in cells maintained at 324 and 648 mm Hg oxygen than in cultures maintained under 41 mm Hg oxygen in the 48 Log (ANOVA, LSD, $p=0.0003$, 0.001, respectively) and 24 C groups (ANOVA, LSD, $p=0.04$, 0.007, respectively). GPx-1 activity was significantly lower in the cultures of the 48 C group maintained at 648 mm Hg as compared to cultures maintained at 41 mm Hg oxygen. GPx-1 activity was lower in the 72 C group in cultures maintained at oxygen partial pressures of 242, 324, and 648 as compared with cultures maintained at 131 mm Hg oxygen (ANOVA, LSD, $p=0.003$, 0.007, 0.002, respectively). GPx-1 activity was also significantly lower in the 96 C and 168 C groups maintained at oxygen partial pressures of 242, 324, and 648 as compared with cultures maintained at 41 mm Hg oxygen (ANOVA, LSD, $p=0.0008$, 0.0002, 0.00006 and $p=0.02$, 0.003, 0.00001, respectively). On the basis of these observations, we infer that elevated oxygen tension decreased GPx-1 activity. Activity was significantly greater at 9 and 41 mm Hg oxygen in senescent cells than in the 72 C group (ANOVA, $p<0.0001$ in both cases). However, GPx-1 activity declined steadily in senescent cultures as oxygen tension was increased (Fig. 5, lower panel). At 648 mm Hg oxygen partial pressure, senescent cells exhibited GPx-1 activity that was significantly lower than all other groups except 72 C and 168 C (ANOVA, $p<0.08$; $p<0.9$ respectively).

Glutathione S-transferase The *S*-transferases are a family of enzymes that detoxify toxic substances by conjugating them with GSH. The most abundant *S*-transferase, ligandin, probably accounts for most of the activity we observed using CDNB as the substrate. Our results revealed that *S*-transferase activity was unaffected by oxygen tension and did not accumulate with the passage of time. The average activity of CDNB responsive *S*-transferase activity

was 215.9±40.6 mU/mg protein. We observed no significant differences in any of the activities recorded. *S*-transferase activity was slightly, though not significantly higher in senescent cells at 291± 48.8 mU/mg protein but, as in the case of young cultures, the activity of the enzyme was unresponsive to changes in ambient oxygen tension.

ROS generation In log-phase cultures, ROS levels were greatest at low oxygen tension (9 mm Hg; Fig. 6a). Addition of buthionine sulfoximine (BSO), which inhibits GSH synthesis, induced highly significant increases in ROS concentration at 9, 242, and 628 mm Hg O_2.

   Conversely, ROS concentration was generally higher in contact-inhibited stationary phase cultures, but addition of BSO produced little effect. The greatest ROS concentration in stationary phase cultures was observed at 131 mm Hg O_2, whether or not cells were pretreated with BSO.

Discussion

In this study, we examined the influence of oxygen on the antioxidant enzyme responses of normal human fibroblasts. The results reveal that GSH concentration was consistently stimulated by increased oxygen tension. The effect was larger in exponentially growing cultures than in those that were in a state of contact-inhibited growth arrest. The activities of enzymes involved in maintenance of GSH concentration such as GR and G-6-PD failed to correlate with GSH concentration. G-6-PD activity was lower in 24 Log than in confluent cultures, but was lowest in senescent cultures. Both hypoxia and hyperoxia depressed GPx-1 activity in proliferatively young cells, while GPx-1 activity declined progressively as a function of oxygen tension in senescent cells. Oxygen tension did not influence the activity of the CDNB-sensitive *S*-transferases in any of the cultures. MnSOD was elevated in young non-proliferating cultures as compared with actively growing cells; it also accumulated with the passage of time, but failed to respond to changes in ambient oxygen tension. Conversely, MnSOD activity was much more elevated in senescent cultures and could be stimulated by high oxygen partial pressure. Cu/Zn
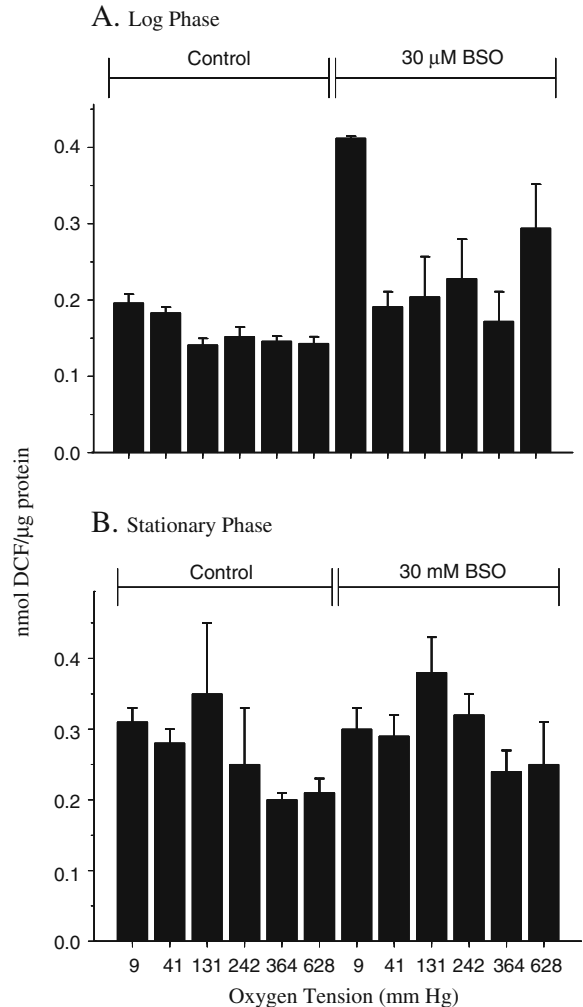

Fig. 6 ROS formation in WI-38 cultures exposed to different oxygen tensions for 24 h. The cells were washed once with phenyl red, serum-free MEM and then treated with 100 µl of DCFH-DA containing medium. The plates were incubated at 37°C for 1 h. Following incubation, the cells were washed once with the wash buffer and read (ex 494, em 518) with a Victor 2 Plate Reader (Perkin Elmer) to determine the amount of oxidized DCF in the cells. Filters used were ex 485 (Perkin Elmer Filter 11440022) and em 535 (Perkin Elmer Filter 11440023). Protein in the samples was determined using Bradford protein assay reagent (Biorad). The reagent was diluted 1:5 with ultrapure water and filtered prior to treating samples. The Victor 2 plate reader was used to determine absorbance at 595 nm. Each point is an average of eight determinations

SOD activity was constant in all of the cultures regardless of growth state or ambient oxygen tension. Cu/Zn SOD activity was diminished in senescent cultures; it remained insensitive to ambient oxygen tension.

Of the antioxidant defenses examined, GSH was clearly the most responsive to changes in ambient oxygen concentration, and the response was greatest in actively growing cultures. GSH concentration has been reported to increase in other types of cells, such as hepatocytes, when they are stimulated to divide (Huang et al. 1998). Furthermore, large increases in GSH in response to hyperoxia have also been observed in vivo (Rister and Wustrow 1985; Langley and Kelly 1993) and in vitro (Allen and Balin 2003b). The increase in GSH concentration that we observed was particularly important in view of the relative lack of change of other antioxidant defenses. GSH is sometimes viewed as reacting too slowly to protect against free radicals (Winterbourn and Metodiewa 1999; Jones et al. 2003); however, the observation that hyperoxia greatly increases GSSG in the intact lung tissues of human newborns suggests that it reacts either directly with oxygen or with oxygen-centered reactive species (Vento et al. 2002). It should be noted that in fetal guinea pig lung, other sulfhydryl-containing compounds such as N-acetylcysteine protected against hyperoxic damage without significantly increasing GSH concentration above the level induced by the hyperoxia (Langley and Kelly 1993). Presumably, this effect is additive with GSH concentration.

GSH concentration was lowest in log-phase cultures maintained at 9 mm Hg oxygen and ROS concentration was highest in these cells as compared with other cultures in log-phase growth. Also, GSH concentration was generally lower and oxidant concentration higher in contact-inhibited, growth-arrested cultures. The fact that the increase in GSH in log-phase cultures in response to increased O_2 was greater than observed in non-proliferating cultures may partly account for the failure of high oxygen tensions to produce large increases in ROS concentration. This hypothesis was further supported by the observation that inhibition of the GSH response with BSO resulted in a large increase in ROS concentration in log-phase cells, but not in stationary phase cultures, where the response of GSH to ambient oxygen tension is smaller. The decrease in GSH concentration observed in senescent cells maintained at oxygen tensions greater than 20% may partly explain the observation that MnSOD responded to high oxygen tension in these cells, but not in cultures that had increased GSH concentration in response to increasing oxygen tension.

In these studies, more fetal bovine serum was included in the growth medium than in the medium used to maintain stationary phase (10% as compared to 0.5% see "Methods" section), and this may have affected GSH results. Merely adding medium that contains 10% serum to growth-arrested cells temporarily doubles GSH concentration at 20% oxygen, although no effect is observed in senescent cells. Nevertheless, non-proliferating confluent cultures refed with medium containing 0.5% serum exhibited similar levels of GSH as cells maintained in 10% serum after 24-h exposure to 20% oxygen.

The large change in GSH concentration in response to elevation in oxygen tension may have also influenced other antioxidant capacities. For example, our result revealed no change in the CDNB-reactive form of the GSH S-transferases; however, the kinetics of the S-transferases is strongly dependent on the concentration of GSH. In the assay mixture, activity was proportional to GSH concentrations up to 4 mM. Similar observations have been reported in other organisms (van Hylckama Vlieg et al. 1999). In our standard assay, the 5 mM GSH concentration was in excess, and thus, GSH was never a limiting factor in the reaction. In intact cells, GSH concentration varied by more than an order of magnitude between hypoxic and hyperoxic environments and was well below the concentration that gave a plateau in S-transferase activity in vitro. Thus, while the amount of S-transferase protein was relatively constant, it is probable that S-transferase activity in intact cells varied dramatically as the ambient oxygen tension (and GSH concentration) was increased.

G-6-PD and GR activities both appeared to be independent of GSH concentration although the maintenance of GSH concentration is strongly dependent on these enzymes in vivo. The most probable explanation for this apparent discrepancy is the artificially low level of GSSG that remains in tissue culture cells. It is in fact the oxidized form of glutathione that activates both of these enzymes (Eggleston and Krebs 1974; Carlberg and Mannervik 1985). Since cell membranes are permeable to GSSG and since, in the culture environment, a large volume of medium surrounds the cells, simple diffusion keeps cellular GSSG at artificially low levels as compared to cells in intact tissues. Thus, cellular responses dependent on GSSG concentration should to some extent differ between in vivo and in vitro cellular environments.

We previously determined that catalase activity in fetal fibroblasts is very low and that it is strongly inhibited under hyperoxic conditions (Balin et al. 2002b). Due to its low activity and its relatively high K_M (Nicholls et al. 2001), catalase is probably not a significant factor in the removal of peroxides from proliferatively young cells. GSH peroxidase activity is also relatively low in human cells, but it exhibits a much lower K_M than catalase, which may increase its importance in elimination of endogenously produced hydrogen peroxide. Indeed, overexpression of GPx-1 has been found to increase resistance to oxygen toxicity (Ho and Howard 1992; Faucher et al. 2003; Modrick et al. 2009), and GPx activity is often stimulated by hyperoxia in the tissues of intact animals (White et al. 1988; Taylor and Bray 1991; Knickelbein et al. 1996). In young cultures, GPx-1 activity was suppressed by both hypoxia and hyperoxia. This suggests that at lower levels of oxygen (131 mm Hg), GPx-1 activity is inducible. Above 242 mm Hg oxygen, it decreased probably because of inactivation. The inhibition of H_2O_2 metabolizing enzymes by hyperoxia is consistent with increased superoxide generation since GPx contains several sensitive –SH groups needed for activity (Flohé 1982) that can be oxidized by superoxide (Blum and Fridovich 1985).

GPx activity declined as a function of ambient oxygen tension in senescent cells; however, catalase activity is elevated more than 3-fold in WI-38 cells that have senesced (Allen et al. 1999). This suggests that catalase may have a more significant role in the protection of senescent cells. Senescent cultures also produce 3-fold more H_2O_2 and 60% more superoxide than young cells (Allen et al. 1999), which further supports the hypothesis that lower GPx-1 activity in late passage cells results from inactivation rather than a decline in synthesis.

Hyperoxia has frequently been reported to increase SOD activity in animal tissues after prolonged exposure (Kimball et al. 1976; Housset and Junod 1982; Freeman et al. 1986; Webster et al. 1987; Taylor and Bray 1991; also see Allen and Balin 1989 for review). Increases in Cu/Zn SOD activity have Taylor and Bray 1991 also been reported in some types of human cell culture, such as human umbilical vein endothelial cells exposed to hyperoxia (Jornot and Junod 1992); however, neither form of SOD responded to variations in oxygen tension in the early passage WI-38 cultures examined. MnSOD activity was affected by growth state, proliferative age, and time. The fact that SOD activity was not induced by hyperoxia in young cell cultures raises the question of whether it is unable to respond due to factors in the culture environment or if oxygen toxicity in these cells is more dependent on factors other than increased superoxide generation. Previous studies have demonstrated that addition of the superoxide generating herbicide paraquat to medium increases MnSOD activity in both normal and virally transformed WI-38 cells (Allen and Balin 2003b), indicating that a significant increase in superoxide will stimulate SOD activity. The observation that oxygen fails to increase the activity of SOD in WI-38 cells even though SOD activity is clearly responsive to other treatments that increase superoxide generation leads us to infer that the toxicity of elevated partial pressures of oxygen are ameliorated at least partly by responses of other antioxidant defenses. Of course, in the case of WI-38 cells, the possibility remains that other factors that contribute to oxygen toxicity also block signaling pathways that lead to the induction of SOD activities. We previously observed that inhibition of GSH synthesis in WI-38 cells under normoxic conditions was sufficient to cause a modest increase in MnSOD activity (Allen and Balin 2003b). An examination of steady-state levels of ROS using DCF formation revealed greater concentrations of ROS in log-phase cultures maintained under lower oxygen tensions. This observation suggests that the electron transport chains of mitochondria are sufficiently reduced to produce oxidants only at lower oxygen tensions. At higher oxygen tensions, electrons may be stripped from cytochromes with the result that they exist in a predominantly oxidized state and thus generate fewer radicals (Boveris and Cadenas 1982; Forman and Boveris 1982). A complete analysis of hyperoxia on mitochondrial function was beyond the scope of this study but will be presented elsewhere.

Several studies have demonstrated a strong and nearly immediate effect of oxygen on the cell cycle of WI-38 cells (Balin et al. 1976, 1978, 1984; Balin and Pratt 2002). The observation that oxygen tensions that dramatically affect cell growth fail to induce the antioxidant defense enzymes suggests that the effects of oxygen on cell growth are the result of specific cellular interactions rather than random free radical interactions with cell components.

The results of this study revealed that GSH concentration changed dramatically in human fetal fibroblasts exposed to hyperoxia, while other antioxidant defenses tended to be relatively unresponsive under the conditions used. Several of the antioxidant defenses were far more responsive to growth state or proliferative age than to the ambient oxygen environment. The fact that the tissue culture environment tended to artificially decrease GSSG may account for the failure of some antioxidant defenses to respond to hyperoxia. The results of this and previous studies collectively demonstrate that antioxidant defense levels are more strongly modulated by growth state than by oxygen tension and that senescent cells, which exhibit an impaired GSH response, tend to be more oxidized than young cultures.

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