Detection of Reactive Oxygen Species via Endogenous Oxidative Pentose Phosphate Cycle Activity in Response to Oxygen Concentration

**IMPLICATIONS FOR THE MECHANISM OF HIF-1α STABILIZATION UNDER MODERATE HYPOXIA** *

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The oxidative pentose phosphate cycle (OPPC) is necessary to maintain cellular reducing capacity during periods of increased oxidative stress. Metabolic flux through the OPPC increases stoichiometrically in response to a broad range of chemical oxidants, including those that generate reactive oxygen species (ROS). Here, we show that OPPC sensitivity is sufficient to detect low levels of ROS produced metabolically as a function of the percentage of O₂. We observe a significant decrease in OPPC activity in cells incubated under severe and moderate hypoxia (ranging from <0.01 to 4% O₂), whereas hyperoxia (95% O₂) results in a significant increase in OPPC activity. These data indicate that metabolic ROS production is directly dependent on oxygen concentration. Moreover, we have found no evidence to suggest that ROS, produced by mitochondria, are needed to stabilize hypoxia-inducible factor 1α (HIF-1α) under moderate hypoxia. Myxothiazol, an inhibitor of mitochondrial electron transfer, did not prevent HIF-1α stabilization under moderate hypoxia. Moreover, the levels of HIF-1α that we observed after exposure to moderate hypoxia were comparable between ρ0 cells, which lack functional mitochondria, and the wild-type cells. Finally, we find no evidence for stabilization of HIF-1α in response to the non-toxic levels of H₂O₂ generated by the enzyme glucose oxidase. Therefore, we conclude that the oxygen dependence of the prolyl hydroxylase reaction is sufficient to mediate HIF-1α stability under moderate as well as severe hypoxia.

The primary role of the oxidative pentose phosphate cycle (OPPC) in mammalian cells is to maintain the [NADPH/NADP+] ratio, thereby helping to regulate the cellular redox equilibrium (1–3). Glucose-6-phosphate dehydrogenase (G6PD), the initial and rate-limiting enzyme of the OPPC, exists in a dimer-tetramer equilibrium with the tetramer being the catalytically active conformation. Each of four identical G6PD monomers contains a structural NADP+ binding site (4, 5). When NADP+ is bound at this site, formation of the active tetramer is favored. In n-stressed cells, the [NADPH]/[NADP+] ratio is very high (approaching 1000) and flux through the OPPC is minimal. However, even a slight increase in [NADP+] can increase the number of active G6PD tetramers. Therefore, G6PD activity, by regulating flux through the OPPC, is uniquely sensitive to reactive oxygen species (ROS) as well as other chemical oxidants.

The importance of the OPPC for the cellular response to ROS is evident from the elevated incidence of apoptosis that we observed in G6PD− Chinese hamster ovary cells following exposure to ionizing radiation (2). Likewise, Efferth et al. (6) observed an elevated incidence of oxidant-induced apoptosis in macrophages isolated from patients suffering from G6PD deficiency syndrome, while Fico et al. (42) observed H₂O₂-induced apoptosis in G6PD− mouse embryo fibroblasts. Notably, a 10-fold reduction in cloning efficiency was seen in G6PD− mouse embryo fibroblasts (MEFs) incubated in an atmosphere of 24% O₂ when compared with paired wild-type control MEFs. When these G6PD− MEFs were incubated under modest hypoxia (13% O₂), their cloning efficiency increased significantly relative to the wild-type controls (3). Recent evidence has also linked ROS production to the onset of replicative senescence in cultured primary rodent and human cells (7–9). The incidence of replicative senescence was much higher in MEFs grown at 20% O₂ than in parallel cultures grown at 3% O₂ (8). These data were attributed to the enhanced production of oxidative DNA damage due to the increased production of ROS at the higher oxygen tension. Notably, G6PD− primary human fibroblasts grown at 21% O₂ senesced more readily than wild-type cells due to their limited capacity to remove metabolically produced ROS (9). These studies suggest that metabolic pro-

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‡ The abbreviations used are: OPPC, oxidative pentose phosphate cycle; ROS, reactive oxygen species; HIF, hypoxia-inducible factor; G6PD, glucose-6-phosphate dehydrogenase; IR, ionizing radiation; MEM, modified Eagle’s medium; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propane sulfonate; EFS, [2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropro pyl)acetamide]; GO, glucose oxidase.

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duction of ROS is directly dependent on the $pO_2$, as was originally hypothesized by Boveris and Chance (10).

In addition to using G6PD-deficient cells to examine toxic effects of ROS, we have used metabolic flux through the OPPC to measure ROS produced during the metabolism of xenobiotic compounds or during exposure to ionizing radiation (IR) (1, 2, 11, 12). IR produces diverse ROS, including $H^+$, $HO^-$, $e_{aq}^-$, and $H_2O_2$ (12). IR produces these ROS under both aerobic and hypoxic conditions (13). However, $H^+$ or $e_{aq}^-$ can react with $O_2$, when present, to produce superoxide and hydroxyl radicals ($O_2^-$ and $HO^-$), which undergo dismutation to form additional $H_2O_2$. Upon investigating the effect of oxygen on IR-induced OPPC activity, we noticed a significant decrease in flux through the OPPC in the unirradiated control samples incubated under severe hypoxia compared with parallel cultures of cells irradiated in air. This suggested that OPPC activity could be used to measure metabolically produced ROS, providing us with means to directly test a recent hypothesis (14–16) that metabolic production of ROS is increased under moderate hypoxia ($\sim 1\% O_2$). This hypoxia-induced ROS model has led to the suggestion that ROS (specifically $H_2O_2$) arising at complex III of mitochondria are needed to stabilize HIF-1α. Hypoxia-inducible factor 1 (HIF-1) is a master transcription factor consisting of two subunits, the hypoxia-inducible $\alpha$ subunit and the constitutively expressed $\beta$ subunit (17, 18). HIF-1 binds to consensus sequences found in various hypoxia-inducible genes including vascular endothelial growth factor, glucose transporter 1, and several key glycolytic enzymes. Under normoxic conditions HIF-1α is hydroxylated on proline residues, which promotes binding to the von Hippel Lindau protein, leading to ubiquitination and ultimately degradation in the proteasome (19–22). Discovery of HIF-1α-specific proline hydroxylases explained the enhanced stability of HIF-1α under hypoxia, because these enzymes exhibit an absolute dependence on oxygen (23, 24). Although this is still accepted as the mechanism of HIF-1α stabilization under anoxia, it has been recently challenged by the theory that ROS are necessary for HIF-1α stabilization at more moderate levels of hypoxia. Our data using flux through the OPPC as a measure of ROS indicate that metabolic production of ROS is directly dependent on percentage of $O_2$ across a broad range, from near anoxia ($<0.015\%$) to hyperoxia (95%). We find no evidence for elevated ROS production under moderate hypoxia. Moreover, our data suggest that stabilization of HIF-1α does not depend on $O_2$ consumption and is not effected by respiratory inhibition. Finally, low level generation of $H_2O_2$ by glucose oxidase, did not increase HIF-1α protein levels.

**EXPERIMENTAL PROCEDURES**

**Culture Conditions**—HT1080 human fibrosarcoma and A549 human lung carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA) and were routinely checked and found free of mycoplasma. They were cultured at 37 °C in a humidified atmosphere of 95% air + 5% carbon dioxide in modified Eagle’s medium (MEM) (standard buffer sodium bicarbonate) supplemented with penicillin and streptomycin, 15% fetal calf serum, non-essential amino acids, and 1 mM pyruvate. For the experiments reported here, MEM was made from its individual components, with 2 mM glucose, 20 mM HEPES (i.e. no bicarbonate, to allow CO$_2$ release), without pyruvate (i.e. to prevent scavenging of H$_2$O$_2$).

**Thin Film Culture Method**—Percentage of $O_2$ is defined with respect to 1 atmosphere of dry gas at 37 °C (a partial pressure of 760 mm Hg). Because many of the experimental conditions cause changes in oxygen utilization or availability, oxygen control is a critical component of the experimental design. Thus, cells were plated onto the central area of 60-mm preconditioned glass Petri dishes and allowed to attach overnight (25, 26). For experiments, the medium was replaced by 1 ml of fresh medium, without pyruvate. Each dish was sealed in an aluminum chamber and the percentage of $O_2$ in the gas phases was decreased to the desired level by a series of precision evacuations of the chamber followed by replacement with nitrogen (gas exchanges). After warming, the chambers were shaken continuously at 37 °C to ensure that the $O_2$ in the gas phase was in equilibrium with the $O_2$ in the culture medium (25, 26). To measure macromolecular synthesis, a small amount of radioactive thymidine or leucine was added to standard medium and the amount incorporated into cellular macromolecules determined by rinsing followed by acid precipitation and liquid scintillation counting.

**14CO$_2$ Release Assay**—Cells were plated into preconditioned 25-mm glass scintillation vials. Although the surface of the vials was smaller than that of the 60-mm dishes, measurement of EF5 binding (see below) confirmed that the percentage of $O_2$ in situ was similar under these slightly divergent experimental conditions. OPPC and Krebs cycle activity was determined by incubating the cells in 1 ml of bicarbonate-free medium containing 2 mM [1-14C]- or [6-14C]glucose, respectively (supplemental Fig. 1) at a final specific activity of 100 μCi/mmol glucose. 14CO$_2$ released was trapped on a 1 × 0.5-cm Whatman GF/B glass fiber filter saturated with 0.1 ml of 5% KOH. Krebs cycle measurements, obtained in parallel samples incubated with [6-14C]glucose, were used to correct the OPPC measurements obtained from samples incubated with [1-14C]glucose (1). Each vial was completely sealed with a silicone serum stopper. To ensure the gas phase within each vial was in equilibrium with the gas phase in the aluminum chamber, a 25-gauge needle was inserted through the serum stopper. This effectively allowed slow evacuation and refilling to take place during the gas exchanges while maintaining an effective barrier against leakage of CO$_2$ from the vials during the incubation periods. This method does not allow instantaneous changes in the oxygen levels, but the metabolic impact of the gradual change in the percentage of $O_2$ in the gas and liquid phases was minimized by performing the gas exchanges at room temperature.

**Protein Collection and Western Blots**—To prevent postincubation changes in HIF-1α, the chambers, and all reagents, were cooled on ice for 20 min. When the chambers were opened, 100 μM deferoximine was added to the ice-cold medium and included in all subsequent steps. The cells were rinsed and scraped into versene, centrifuged, resuspended in 20 μl of phosphate-buffered saline, and lysed in hypotonic buffer with CHAPS, phenylmethylsulfonyl fluoride, and Sigma protease inhibitor (27). For whole cell extracts, urea was added followed by a high speed spin, and the supernatant was frozen. To separate nuclear from cytoplasmic proteins, the nuclei were centri-
ROS and HIF-1α Stabilization

FIGURE 1. OPPC activity in response to IR and O2 concentration. A, stimulation of OPPC activity by oxidative challenge. 10⁶ A549 cells were incubated at 37 °C for 1 h in 1 ml of MEM with 20 mM HEPES and 1% fetal calf serum with either [1-14C]glucose (to measure OPPC) or [6-14C]glucose (in parallel dishes to measure Krebs cycle activity; see supplemental Fig. S1). The radioactive glucose was added at a final specific activity of 50 mCi/mmol. Compared with untreated controls, cells were exposed to 100 μM H2O2, 1 mM tirapazamine (SR4233), 5 mM misonidazole (miso), 10 grays of γ-radiation (γ-irradiation), 100 μM diamide, or 1 mM hydroxyethyldisulfide. Ordinate represents nmols of CO2 released from samples incubated with [1-14C]glucose after correction for data obtained from samples incubated with [6-14C]glucose. B, OPPC stimulation in A549 cells exposed to ionizing radiation. 10⁶ cells were incubated in 1 ml of MEM with 20 mM Hepes and 1% fetal calf serum with [1-14C] or [6-14C]glucose at a specific activity of 50 μCi/mmol. Cells were incubated in an atmosphere containing 21 or 0.02% O2. Cells were exposed to IR from a cesium source. All steps were carried out at 37 °C, and the reactions were stopped after 1 h, including the time of irradiation. OPPC activity represents the data obtained with [1-14C]glucose after correction for data obtained with [6-14C]glucose. C, effect of percentage of O2 on OPPC and Krebs cycle activity. On the ordinate are plotted nmols of CO2 released from [1-14C]glucose (circles, OPPC activity) or [6-14C]glucose (squares, Krebs cycle activity) in A549 (open symbols) or HT1080 (closed symbols) cells. 10⁶ cells were incubated for 4 h at 37 °C in 1 ml of MEM containing labeled glucose at various O2 concentrations, ranging from 0.005% to 95%. (By t test, all oxygen groups of OPPC activity are significantly different except for 0.4 versus 1.6%. Mean ± S.D. of at least two duplicates of experiments done 2–5 times.) D, effect of percentage of O2 on OPPC and Krebs cycle activity. The combined data for A549 and HT1080 cells from panel C for 14CO2 released from [1-14C]glucose (circles, OPPC activity) or [6-14C]glucose (squares, Krebs cycle activity) were normalized to the effect observed at 20% O2. By reploting the data in this manner the oxygen dependence of respiration is more readily apparent. The correlation coefficient for log ([O2]) versus OPPC rate was = 0.98. Error bars represent the S.D. of data obtained with duplicate measurements in a minimum of two independent experiments.

fuged after lysis and the nuclear pellet and cytoplasmic supernatants treated individually as with whole lysed cells. These methods do not affect HIF-1α levels as determined by direct lysis of cells from suspension cultures (either aerobic or hypoxic) in sample buffer. HIF-1α antibody was from Transduction Laboratories; antibodies for β-tubulin and Ku86 were from Sigma.

Respiration—Cellular oxygen consumption was measured using a high sensitivity Clark-style polarographic sensor and spinner vial apparatus designed by one of the authors (25). Misonidazole was dissolved in dimethyl sulfoxide and added in a volume of <0.5% of the total.

Production of β Cells—β⁰ HT1080 cells were made by methods adapted from Desjardins et al. (28). Cells were seeded at low density (5000 cells/T75 flask) in supplemented MEM with 20 mg/ml ethidium bromide, 50 μg/ml uridine, and an additional 5 mM glucose. Over 2–3 weeks they grew slowly while losing their ability to consume oxygen. Furthermore, the residual oxygen consumption was insensitive to misonidazole.

EFS Binding—EFS is a 2-nitromidazole that forms covalent protein adducts in viable cells in a manner that is inversely proportional to the intracellular oxygen concentration, thus allowing for quantitative measurement (see Fig. 2 and supplemental Fig. S2). EFS binding and analysis of its hypoxia-dependent adducts has been published extensively (25, 30–32). In brief, the optical aspects of the flow cytometric evaluation are calibrated with a reference concentration of EFS adducts determined by radioactive drug uptake.

Reaction of α-Keto Acids with Hydrogen Peroxide—H2O2 and α-keto acids were added at equimolar concentration and the loss of H2O2 measured using a peroxide sensor (Yellow Springs). The reaction rate (K) was measured based upon the assumption that the reaction was stoichiometric (i.e. equal loss of peroxide and α-keto acid).

The data were fit to the equation: rate = K × [H2O2] × [Acid]n. The logarithms of the rate divided by peroxide concentration were plotted against logarithm acid concentration to determine n.

RESULTS

Respiration accounts for a small portion of the 14CO2 produced metabolically by cells incubated with [1-14C]glucose. However, an equivalent amount of 14CO2 is produced via respiration in cells incubated with [6-14C]glucose. In contrast, 14CO2 is not produced via the OPPC when cells are incubated with [6-14C]glucose. Therefore, by measuring 14CO2 released from replicate samples incubated with [1-14C]glucose or [6-14C]glucose, in situ OPPC activity can be accurately measured (see supplemental Fig. S1). OPPC activity in cultured A549 cells is stimulated by a variety of chemical oxidants (Fig. 1A). OPPC activity was exquisitely sensitive to H2O2 added directly to the cell culture as a bolus. Moreover, it was also extremely sensitive to continuous production of superoxide and H2O2 via drugs that undergo futile redox cycling, including misonidazole or tirapazamine (SR4233) (29). Thiol-specific oxidants, such as hydroxyethylisulfide or chemicals such as diamide, which can oxidize thiols but can also oxidize NADPH directly, (1, 33), also induce elevated OPPC activity.

We observed increased OPPC activity in cells exposed to IR (Fig. 1B). The slope of the dose-response curve obtained with...
ROS and HIF-1α Stabilization

We have used the classical method of measuring $^{14}$CO$_2$ released by cells incubated with $[1-^{14}$C]glucose or $[6-^{14}$C]glucose to determine OPPC and Krebs cycle activity as a function of percentage of O$_2$. OPPC activity increases in response to a broad range of chemicals that oxidize NADPH, directly or indirectly, as shown in Fig. 1A. For example, hydroxyethylisulphide is a thiol-specific oxidant; subsequently, NADPH acts as the electron donor to reduce the disulphides that are formed by hydroxyethylisulphide. The exquisite sensitivity of OPPC to ROS has been confirmed by measuring OPPC stimulation in cells exposed to low levels of ROS produced by IR ($H^+$, HO$, e_{aq}^-$, $H_2$O$_2$) and by drugs that undergo futile redox cycling.

cells irradiated in 21% O$_2$ was 2.3-fold higher than the slope obtained when cells were irradiated under 0.02% O$_2$, a direct reflection of the increased yield of H$_2$O$_2$ in irradiated aqueous solutions under aerobic conditions.

There was a significant decrease (1.8-fold) in OPPC activity in the unirradiated samples under hypoxic (20% O$_2$), compared with normoxic (20% O$_2$), conditions (Fig. 1B). Based on this observation, we performed a systematic measure of OPPC activity as a function of varying the percentage of O$_2$. Fig. 1C demonstrates that OPPC activity increases in both A549 and HT1080 tumor cells as a logarithmic function of percentage of O$_2$, ranging from near anoxia (<0.01%) to hyperoxia (95%) (see Fig. 1C). Although OPPC activity, measured at <0.01% O$_2$, was significantly higher (1.9-fold) in HT1080 versus A549 cells, the overall increase in OPPC activity that was observed in response to increasing the O$_2$ content was remarkably similar in both cell lines. These results suggest that the in situ production of ROS is directly dependent on the presence of O$_2$, as originally proposed in the classical work describing enhanced metabolic production of ROS in response to hyperoxia by Boveris and Chance (10).

Production of $^{14}$CO$_2$ by cells incubated with [6-14C]glucose is used to correct for glucose oxidation via respiration (Krebs cycle) in our measurements of OPPC activity (Fig. 1C). By normalizing these data to the effect observed at 20% O$_2$ we were also able to determine the oxygen dependence of cellular respiration (Fig. 1D).

In these experiments the percentage of O$_2$ that the cells experience is precisely controlled using a “thin film” culture method (25), with a slight modification in order to measure OPPC activity (see “Experimental Procedures”). The in situ O$_2$ percentage was confirmed using EF5, a 2-nitroimidazole that forms covalent protein adducts in viable hypoxic cells (31–33). The EF5 protein adducts are detected quantitatively via flow cytometry (supplemental Fig. S2). We used the thin film culture method to determine the O$_2$ dependence of EF5 binding in control, myxothiazol-treated, and $\rho^0$ HT1080 cells (Fig. 2). We then examined EF5 binding in the cells grown in the glass vials used to measure OPPC activity to verify that the percentage of O$_2$ in situ was as expected.

We then measured cellular oxygen consumption, using a Clark-style oxygen electrode (25). The data clearly demonstrate that 0.5 $\mu$M myxothiazol, a mitochondrial complex III inhibitor, was extremely effective at blocking cellular oxygen consumption (Fig. 3A). These data were confirmed by the ability of myxothiazol to inhibit the release of $^{14}$CO$_2$ from cells incubated in [6-14C]glucose. We also observed a pronounced increase in glucose consumption and lactate production in response to myxothiazol (data not shown). Myxothiazol had no effect on OPPC activity ($^{14}$CO$_2$ release from cells incubated in [1-14C]glucose), irrespective of the percentage of O$_2$ (Fig. 3B). Although myxothiazol has been reported to be cytotoxic, we observed little toxicity following a 4-h exposure to 0.5 $\mu$M, although protein and DNA synthesis were both inhibited by 0.5 mM myxothiazol (supplemental Fig. S3). $\rho^0$ HT1080 cells, which lack functional mitochondria, utilized O$_2$ at a much slower rate than wild-type HT1080 cells. Myxothiazol had no effect on the rate of O$_2$ consumption in two independently generated $\rho^0$ cells lines (Fig. 3A).

We next determined the effect of myxothiazol on HIF-1α stabilization under severe or moderate hypoxia (Fig. 3C). HIF-1α protein was not evident in the cytosolic or nuclear fractions from cells incubated in 21% O$_2$. As expected, HIF-1α protein levels increased in both the cytosolic and nuclear fraction, isolated from cells incubated under moderate (0.4% O$_2$) or severe (<0.01% O$_2$) hypoxia. No difference was observed in stabilization of HIF-1α at <0.01 or 0.4% O$_2$ in the presence or absence of 0.5 $\mu$M myxothiazol (Fig. 3C). Normal stabilization of HIF-1α protein was observed in HT1080 $\rho^0$ cells incubated at <0.01 or 0.4% O$_2$ (Fig. 4A).

To determine whether the generation of H$_2$O$_2$ affects the stability of HIF-1α, we used exogenous glucose oxidase (GO) to increase the steady state levels of H$_2$O$_2$ to which the cells were exposed. The addition of GO, at 0.02 and 0.1 units/ml, stimulated OPPC activity in both wild-type and $\rho^0$ HT1080 cells (Fig. 4B). OPPC stimulation in the presence of GO appeared to be entirely due to production of H$_2$O$_2$, because it was completely inhibited by the addition of excess catalase (data not shown). The low steady state levels of H$_2$O$_2$ produced at these GO concentrations had no effect on clonogenic survival (data not shown). Moreover, GO did not affect HIF-1α protein levels at any of the O$_2$ levels tested (0.01–20%) (Fig. 4C).

**DISCUSSION**

We have used the classical method of measuring $^{14}$CO$_2$ released by cells incubated with [1-14C]glucose or [6-14C]glucose to determine OPPC and Krebs cycle activity as a function of percentage of O$_2$. OPPC activity increases in response to a broad range of chemicals that oxidize NADPH, directly or indirectly, as shown in Fig. 1A. For example, hydroxyethylisulphide is a thiol-specific oxidant; subsequently, NADPH acts as the electron donor to reduce the disulphides that are formed by hydroxyethylisulphide. The exquisite sensitivity of OPPC to ROS has been confirmed by measuring OPPC stimulation in cells exposed to low levels of ROS produced by IR ($H^+$, HO$, e_{aq}^-$, $H_2$O$_2$) and by drugs that undergo futile redox cycling.
such as SR4223 and misonidazole (O$_2$). We recognize that these species react to form hydroperoxides within the cell; however, theoretically we cannot be sure that formation of hydroperoxides is necessary for all of the increase in OPPC activity that we observe. There was a significant decrease in OPPC activity observed in the unirradiated cells incubated under severe hypoxia. Based on these findings we set out to carefully examine the oxygen dependence of OPPC activity as an indicator of metabolically produced ROS. Such ROS have been suggested to arise from the mitochondrial electron transport chain, specifically at complex III. The resulting superoxide radical would rapidly be dismutated to H$_2$O$_2$ by manganese superoxide dismutase that is present in the mitochondrial matrix. We found that metabolic flux through the OPPC increases directly as the percentage of O$_2$ is progressively increased from near anoxia (0.01% O$_2$) to hyperoxia (95% O$_2$). By measuring oxidative damage to cellular DNA as an indicator of metabolic ROS production, other groups have come to the same conclusion (8, 9).

Previously, it was reported that functional mitochondria were not required for HIF-1α stabilization under hypoxia (35, 36).
However, these studies were performed using severe hypoxia. Hence, recent reports (14–16) have suggested that mitochondrial production of ROS is necessary for HIF-1α stabilization at more moderate levels of hypoxia but not at near anoxic conditions. Our data do not support this conclusion. In contrast to these reports (14–16), we observed no inhibition of HIF-1α stabilization by the mitochondrial complex III inhibitor myxothiazol (despite almost complete suppression of respiration) at any O2 percentage. This particular finding is also at odds with Hagen et al. (37), who found that myxothiazol inhibited HIF-1α stabilization during hypoxia but invoked a different explanation than ROS involvement, namely intracellular redistribution of oxygen toward prolyl hydroxylases. We found that ρ0 cells exhibited normal stabilization of HIF-1α in response to both severe and modest hypoxia. Conversely, production of non-toxic levels of H2O2 via the addition of GO had no effect on HIF-1α stabilization in these experiments.

Because this area of study has proven controversial, it is worthwhile considering possible differences in the experimental conditions as a potential explanation for the different outcomes. HT1080 and A549 cells (human sarcoma and lung cancer cell lines, respectively) were used in the experiments reported here. A549 cells were also used in the studies published by Brunelle et al. (16); therefore, cell line-specific differences cannot account for the discrepancy in the data. The culture medium used in the present experiments contained 2 mM glucose and was formulated without added pyruvate or bicarbonate. Previously we found that pyruvate at concentrations that are typically found in standard medium formulations (1–2.5 mM) reacts with H2O2, thereby protecting cells from spontaneous peroxide could be explained by consumption of one of the substrates needed for prolyl hydroxylase activity. However, it is important to reiterate that our data clearly show that peroxide is not induced in cells under moderate hypoxia.

A major difference between this and prior studies is the method used to measure ROS. The OPPC method is very accurate and sensitive and does not perturb the culture conditions in any way. However, its sensitivity depends directly on cell density. Thus, we have typically used at least 10⁶ cells/ml in the present experiments. The assay most commonly used for ROS measurements employs dyes whose fluorescence can be modified by redox change. In contrast to the OPPC method, they can be used at much lower cell densities (even single cells) but the fluorescence measurement involves a great perturbation in the culture environment, often poorly explained technically. In addition, the mechanism of fluorescence change is itself highly controversial; some reports have clearly shown that fluorescence can be modified by simple metabolism (i.e. not ROS-mediated) whereas others have shown that the dyes can themselves cause the formation of ROS (38–40). Therefore, Guzy et al. (15) used a novel fluorescence resonance energy transfer (FRET) sensor to suggest that ROS are produced by cells under moderate hypoxia. The FRET signal increased by <5% as the O2 level was reduced from 20 to 1%. Similarly, the FRET signal increased by ~3% after the addition of a relatively high concentration of GO. These two conditions, moderate hypoxia and the addition of GO, would likely produce H2O2 concentrations that are very different. Thus, it is difficult to understand why slight changes in FRET activity were observed in both experiments. Moreover, these authors demonstrate that a total of six bolus additions of 10 μM H2O2 over the course of a 2-h incubation were needed to stabilize HIF-1α to the same extent that was observed under hypoxia (15). Clearly, if H2O2 were produced intracellularly at similar levels under moderate hypoxia, it would have resulted in elevated OPPC activity.

This highlights what is likely the most substantial change in experimental conditions between the present and former studies, the precise measurement and control of O2. This problem has arisen repeatedly over the past few decades and has been extensively studied in the radiation biology literature (25). Assuming that the oxygen within a chamber can be accurately maintained at a specified level, this still does not guarantee that cells growing in dishes or flasks within the chamber are exposed to the same level of O2. Variations in the percentage of O2 at the cell surface arise due to numerous complicating factors, including metabolic activity of the cells, level of confluence, and O2 permeability of both the growth medium and the tissue culture plastic. In fact, Doege et al. (41) reported that inhibitors of respiration, including myxothiazol, could prevent stabilization of HIF-1α at 3% O2 when cells were cultured in conventional polystyrene dishes, whereas the same concentration of myxothiazol had little effect when cells were cultured on ultra-thin gas-permeable Teflon membranes. In the experiments presented in this work, we took great care to precisely control the O2 levels in situ by using the thin film culture technique (26). Moreover, the presumed percentage of O2 in situ was confirmed using EF5, a 2-nitroimidazole that forms covalent protein adducts in viable hypoxic cells. More than a decade of experience has confirmed that EF5 binding occurs in a manner that is inversely proportional to oxygen concentration throughout the hypoxic range (25, 30–32).

In summary, OPPC activity can be used as a sensitive measure of oxidative stress, including the formation of ROS in whole cells. To our knowledge, this is the first time that OPPC activity has been measured as a function of percentage of O2. Notably, we were also able to independently determine the oxygen dependence of respiration using the data from these experiments. We have found that OPPC activity increases propor-
tionately to the percentage of O$_2$ across a broad range from severe hypoxia (<0.01% O$_2$) to hyperoxia (95% O$_2$). Using this assay we found no evidence to support the hypothesis that ROS production increases under moderate hypoxia (0.4 – 1.6%). These observations are critically important to the current understanding of hypoxic adaptation that is mediated via stabilization of HIF-1α. Our data suggest that inhibition of proline hydroxylases that occurs when O$_2$ is removed is the mechanism for HIF-1α stabilization under both severe and moderate hypoxia. Using the OPPC to measure metabolically produced ROS may prove to be widely useful for understanding other biochemical effects where metabolically produced ROS have been hypothesized to play critical roles, for example, the more rapid onset of replicative senescence that is observed in primary cell lines grown at 21% O$_2$ when compared with the same cells maintained under moderate hypoxia (7–9).

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