Most cultured cells and intact animals die under hyperoxic conditions. However, a strain of HeLa cells which proliferates under 80% O\(_2\), termed 'HeLa-80', has been derived from wild-type HeLa cells ('HeLa-20') by selection for resistance to stepwise increases of oxygen partial pressure. The tolerance of HeLa-80 cells to hyperoxia is not associated with changes in antioxidant defenses or susceptibility to oxidant-mediated killing. Rather, under both 20% and 80% O\(_2\), mitochondrial reactive oxygen species (ROS) production is ~2-fold less in HeLa-80 cells, likely related to a significantly higher cytochrome c oxidase (COX) activity (~2-fold) which may act to deplete upstream electron-rich intermediates responsible for ROS generation. We now report that, in HeLa-80 cells, elevated COX activity is associated with a >2-fold increase in the regulatory subunit COX Vb whereas expression levels of other subunits are very close to wild-type. Small interfering RNA (siRNA) against Vb selectively lowers COX Vb expression in HeLa-80 cells, increases mitochondrial ROS generation, decreases COX activity 60-80% and diminishes viability under 80% (but not 20%) O\(_2\). In addition, overexpression of subunit Vb increases COX activity and decreases ROS production in wild-type HeLa-20 cells, along with some increase in tolerance to hyperoxia. Overall, our results indicate that it is possible to make cells tolerant of hyperoxia by manipulation of mitochondrial electron transport. These observations may suggest new pharmaceutical strategies to diminish oxygen-mediated cellular damage.

Although oxygen is essential to aerobic metabolism, excess oxygen is harmful. Hyperoxia-induced lung damage and retinopathy of prematurity occur frequently in premature infants given oxygen as therapy for pulmonary insufficiency (1). Hyperoxia also causes pulmonary damage in adults exposed to high partial pressures of inhaled O\(_2\) for more than 48 h. The symptoms include cough, shortness of breath, decreased vital capacity and increased alveolar-capillary permeability (2, 3) as well as damage to cardiovascular, nervous and gastrointestinal systems (4-8). However, the mechanisms involved in hyperoxic damage are still not completely understood.

It is commonly believed that free radicals play a central role in oxygen toxicity and cellular damage is probably mediated by increased production of ROS (9). This excessive production of ROS likely derives from the mitochondria which, under conditions of high oxygen, exhibit increased 'leak' from the electron transport chain (9, 10). Our previous results lend support to the importance of mitochondrial respiration and ROS generation in the etiology of hyperoxic cell damage. Using three strategies to diminish mitochondrial ROS production by HeLa cells (rho\(_-\) cells, chronic exposure to chloramphenicol and exposure to the protonophoric uncoupler of respiration, CCCP) we consistently found improved cell survival and growth under hyperoxic conditions (11).

In an effort to further understand the nature of hyperoxic damage, we focused on an oxygen tolerant strain of HeLa cells, which proliferates even under 80% O\(_2\) (HeLa-80). This strain was derived from wild-type HeLa cells (HeLa-20) by selection for resistance to stepwise increases of oxygen partial pressure (12). The oxygen tolerant HeLa-80 cells exhibit significantly decreased mitochondrial ROS generation (under both normoxia and hyperoxia). Furthermore, the lessened ROS production probably derives from enhanced activity of the terminal component of electron transport, cytochrome c oxidase (COX).
We earlier speculated that the net effect of this increased COX activity might be to deplete electron-rich intermediates (such as ubisemiquinone) in the electron transport chain, thereby diminishing the 'leak' of electrons into ROS. In partial support of this, preferential inhibition of COX by treatment with n-methyl protoporphyrin (which selectively diminishes synthesis of heme-a which is required for cytochrome c oxidase activity) enhances ROS production and abrogates the oxygen tolerance of the HeLa-80 cells (13).

Cytochrome c oxidase, (also known as complex IV), is the terminal complex of the mitochondrial respiratory chain and is comprised of 13 different subunits encoded by 3 mitochondrial genes (COX subunits I, II and III) and 10 nuclear genes (COX subunits IV, Va, Vb, V1α, V1b, V1c, V1IIa, V1IIb, V1IIc and VIII). Given the complex nature of COX, the reasons for increased activity in HeLa-80 cells were unclear; clearly, gain-of-function mutations in all 13 would be very unlikely but increased expression of some or all of the subunits of COX remained a possibility.

We now report that the elevated COX activity in oxygen tolerant HeLa-80 cells is associated with a >2-fold increase in the regulatory subunit COX Vb whereas expression levels of other subunits are very close to wild-type HeLa-20 cells. Transfection of COX Vb-specific small interfering RNA (siRNA) into HeLa-80 cells selectively lowers Vb expression, increases mitochondrial ROS generation, decreases COX activity 60-80% and diminishes viability under 80% (but not 20%) O₂. In addition, in wild-type HeLa-20 cells, over-expression of subunit Vb increases COX activity, decreases ROS production and increases tolerance to hyperoxia. We speculate that manipulations designed to similarly enhance the 'downhill' flow of electrons in the electron transport chain may have some utility in the suppression of cell damage caused by hyperoxia and other insults.

Experimental Procedures

**Cells and Reagents** - A wild-type strain of HeLa cells (HeLa-20) and an oxygen tolerant strain which grows normally under 80% oxygen (HeLa-80) were generously provided by Dr. Hans Joenje (VU University Medical Center, Amsterdam, The Netherlands). Unless otherwise noted, all reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco’s Modified Eagle’s Medium (DMEM), Opti-MEM, phosphate buffered saline (PBS), trypsin-EDTA and fetal bovine serum (FBS) were obtained from Gibco LifeTech (Grand Island, NY). Dihydoricholorfluorescein diacetate (DCFDA) and dihydroethidium were purchased from Molecular Probes (Eugene, OR). All antibodies, including the primary monoclonal antibodies (mouse anti-human) for cytochrome c oxidase subunits and a secondary antibody (goat anti-mouse IgG), were purchased from Molecular Probes. Oligofectamine was purchased from Invitrogen (Invitrogen, Carlsbad, CA). FuGENE 6 was obtained from Roche (Roche Molecular Biochemicals, Indianapolis, IN). The pcDNA3.1/V5-His TOPO-TA expression vector was kindly provided by Dr. Robert Mitchell (University of Louisville, Louisville, KY).

**Conditions of Cell Culture** - HeLa cells were routinely cultured in DMEM medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) under 20% O₂ (normoxia) or 80% O₂ (hyperoxia) with 5% CO₂ at 37°C. For routine passage, cells were washed with PBS and lifted with 0.05% trypsin, 0.02% EDTA in PBS. Stock cultures were grown in 10-cm cell culture dishes in a Forma Scientific incubator under an atmosphere of 20% O₂, 5% CO₂. Exposure to hyperoxia was performed with cells grown in 10-cm dishes under an atmosphere of 80% O₂, 15% N₂ and 5% CO₂ contained in a specially designed gas-tight modular incubator chamber (Billups-Rothenberg, Inc., Del Mar, CA). Because susceptibility to hyperoxia may vary with cell density (14), the initial cell number was routinely adjusted to ~1 x 10⁵ per dish in DMEM medium. The sealed chamber was placed in a standard tissue culture incubator and the gas was replenished every 48 h. The survival and growth of cells were assessed over a period of 10 days by counting cell numbers in marked sectors of the culture dishes using light microscopy.

It is important to note that the oxygen tolerance of HeLa-80 is a stable characteristic. Even after these cells have been continuously passed in normoxic culture for more than two months, they retain resistance to 80% O₂. To avoid
artifacts which might be introduced by the tendency of cell cultures to ‘drift’, cultures of both cell types were replenished from liquid nitrogen every 30 days and the oxygen tolerance of each new culture was checked.

*Estimation of ROS Production* - ROS production was assessed by the oxidation of dihydrodichlorofluorescein diacetate (DCFDA) or dihydroethidium. To estimate ROS production with DCFDA, cells were plated onto 48-well plates at an initial density of 2x10⁴ cells per well and grown for 3 days. After cells were >90% confluent, they were washed 3 times with HBSS, then 0.5 ml of HBSS was replaced per well. Following the addition of 20 µM DCFDA (final concentration), the appearance of DCF fluorescence was followed continuously using a thermostated plate reading spectrofluorometer (Molecular Devices Corp., Sunnyvale, CA), typically for 1 h, at ex486 nm and em530 nm. Cell protein in each well was measured using the bicinchoninic acid reaction (15) (Pierce, Rockford, IL) and the DCF fluorescence was corrected for variations in total protein between wells.

Generation of ROS was also evaluated under both 20% and 80% O₂ using the oxidation of dihydroethidium (16). Cells were plated and grown as for experiments with DCFDA (as mentioned above). When the cells were >90% confluent, fresh medium was added containing 100 µM dihydroethidium. Dihydroethidium oxidation permits estimates of ROS formation over longer periods of time in complete culture medium and the product, ethidium, is held within the cell via intercalation into nucleic acids. After incubation with dihydroethidium for 4 h, cells were washed 3 times with HBSS and ethidium fluorescence was detected at ex520 nm and em610 nm. Again, the relative fluorescence was corrected for variations in cell protein between individual wells.

**Real Time Quantitative RT-PCR** - Real time, quantitative RT-PCR measurements of the levels of mRNA for COX subunits along with control beta-actin mRNA were carried out. RNA was prepared as previously described (17). Sequence-specific oligonucleotide primers for the human genes have been tested and published (18). Results were obtained by measuring the cycle threshold (Cₚ), the first cycle in which there is significant increase in fluorescence above the background, and which correlates to the log-linear phase of PCR amplification. Generally, the runs were stopped at 8 cycles after Cₚ. The relative quantification in mRNA levels was evaluated by the ratio between the target gene and the housekeeping gene beta-actin. Data calculation was based on the ‘Delta-Delta method’, using the equation of \( R = 2^{-\Delta \Delta C_T} \) (19). The identity of the amplified DNA was confirmed by determination of melting temperature.

There are two different methods of analyzing data from real-time quantitative RT-PCR: absolute quantification or relative quantification. Absolute quantification can be done using a competitive oligonucleotide. Relative quantification involves measurement of the ratio between mRNA levels for the gene of interest versus a housekeeping gene. In the present instance, we used relative quantification because if mRNA levels for an appropriate housekeeping gene are used it is adequate for investigating proportionate changes in gene expression levels. The cautionary is that relative quantification relies on the assumption that the reference gene is unaffected by the experimental conditions.

**Western Blot Analysis** - General conditions of cell culture were as previously described (11). As a semi-quantitative method to determine the levels of COX subunits in HeLa-20 and HeLa-80 cell lines, we performed Western blot analysis on COX subunits from both cell types. Lysates were prepared from near-confluent cultures of HeLa cells using RIPA lysis buffer (Upstate Biochemicals, Charleottesville, VA) with protease inhibitors. Lysates were clarified by centrifugation at 7,000 x g for 4 min at 4°C. Twenty ug of total cellular protein was loaded on 10-20% SDS/PAGE gels and electro-transferred to PVDF membranes (Amersham Pharmacia). The membranes were blocked with 5% milk in phosphate buffered saline (PBS) containing 0.1% Tween-20 for 1 h, followed by incubation at room temperature 1 h with primary monoclonal antibodies (1:1000 dilution in PBS-T (0.1% Tween-20) buffer) against human COX subunits (Molecular Probes, Eugene, OR). For detection of immunoreactivity, the blots were incubated in a secondary antibody solution (horseradish peroxidase-conjugated goat anti-mouse diluted 1:4000 in PBS containing 0.1% Tween-20 buffer) and developed using an enhanced chemiluminescence, ECL™-Plus.
Western blotting detection kit (Amersham Pharmacia). Beta-actin was used as a loading control.

Cytochrome c Oxidase (COX) Assay - Lysates of fresh cells (directly counted and adjusted to 6 x 10^6 cells/ml) were prepared by suspension in 0.25 M sucrose, 40 mM potassium chloride, 2 mM EGTA, 1 mg/ml bovine serum albumin (BSA) and 20 mM Tris-HCl (pH 7.2) and disrupted by ten 1 s bursts from a microtip Fisher Model 100 Sonic Dismembrator at scale 3 (on a scale of 0-10 of the 100w maximum power output) (Fisher Scientific, Pittsburgh, PA). The lysate was centrifuged at 2,000 x g for 10 min. The pellet was discarded and the supernatant used for COX assays (20). Assays contained ~20 µg protein and were performed at 37°C in 200 µl reaction volumes. The assay involved the addition of 30 µM ferrocytochrome c in an isosmotic medium (10 mM KH_2PO_4, pH 6.5, 1 mg/ml BSA, 0.3 M sucrose) containing 2.5 mM n-dodecylmaltoside to permeabilize mitochondrial membranes (20). To confirm that this method specifically detects the oxidase activity, 10 µM of antimycin-A was added to a control group to block complex III. The activity was calculated from the rate of decrease in absorbance of ferrocytochrome c at 550 nm (ε=19.1 mM^-1cm^-1) (21) and results were normalized for protein.

Small Interfering RNA (siRNA) Transfection - Two siRNA duplexes that specifically target COX subunit Vb were designed and obtained from Dharmacon (Dharmacon, Inc., Lafayette, CO). A non-sense siRNA was used as control. We used two different siRNA duplexes (one against the open reading frame and one against an untranslated region) because some siRNAs may have multiple effects, may not work against the intended mRNA or may exhibit nonspecific silencing. The first target sequence was AAAGUAGGCUGCAUCUGUGAA. The corresponding siRNA duplex #1 (s1) was 21 nt. Its sense sequence was 5'-AGUAGGCUGCAUCUGUGAAdTdT-3'; the antisense sequence was 5'-UUCACAGAUGCAGCCUACUdTdT-3'. The second target sequence was AACAGUAAGACUAGGCCAUUG. The corresponding siRNA duplex #2 (s2) was also 21 nt. Its sense sequence was 5'-CAGUAAAGACUAGGCCAUUGdTdT-3'; the antisense sequence was 5'-CAAUGGCUAGUCUUACUGdTdT-3'.

One day before transfection, HeLa-80 cells were seeded at subconfluent density (8 x 10^4 cells per well in 6-well cell culture plates). During and after exposure to siRNA, the cultures were fed with enriched DMEM medium (10% FBS, supplemented with 4 g/L glucose, 100 µg/ml pyruvate and 50 µg/ml uridine) because substantial blockade of mitochondrial respiration might result in respiratory insufficiency and cell death. Control cultures were also fed with the same enriched medium. Transfection with siRNA was done with Oligofectamine (Invitrogen, Carlsbad, CA), following the manufacturer's guidelines with some modifications. We also carried out Oligofectamine controls with or without non-sense siRNA. Cells were further incubated for 48 h at 37°C after transfection. To ensure siRNA effectiveness, cells were processed for the evaluation of changes in COX subunit protein expression (Western blot) and mRNA by real time RT-PCR (as above).

Effects of Vb SiRNA on COX Activity and ROS Production - Two different concentrations of siRNA of COX Vb (20 nM and 100 nM) were used in the experiments along with Oligofectamine controls with or without non-sense siRNA. Cells were further incubated for 48 h at 37°C, 5% CO_2/20% O_2 after transfection. Then the cells were collected and processed as described above. COX activities were measured and ROS production was estimated in control cells and siRNA treated cells (as above).

Survival and Growth of SiRNA-transfected Cells under Normoxia and Hyperoxia - We cultured control cells and siRNA treated cells under both 20% O_2 and 80% O_2 for 6 days. Exposure to hyperoxia was performed as described above. Cells were seeded at a density of approximately 10% confluence and cell numbers were estimated by microscopy (as described above) as well as Alamar Blue reduction as previously described (13).

Transfection and Over-expression of COX Subunit Vb in Oxygen Sensitive HeLa-20 cells - Full length cDNA for COX subunit Vb was obtained by RT-PCR and the open reading frame (ORF) was ligated into the pcDNA3.1/V5-His TOPO-TA expression vector. For the PCR amplification...
reactions, Taq polymerase (Promega, Madison, WI) was employed: denaturation at 94°C, 30 s; annealing at 56°C, 30 s; and elongation at 72°C, 1 min; 30 cycles. A 10 min extension at 72°C was included after the last cycle to ensure that all PCR products were full length. The recombinant vector was transfected into TOP10 E. coli which was selected for ampicillin resistance. All PCR products and plasmids were analyzed and verified by restriction enzyme analysis and DNA sequencing for the presence and orientation of PCR inserts.

Using the TOPO cloning system, we employed the pcDNA3.1/V5-His TOPO TA expression system (Invitrogen, Carlsbad, CA) to over-express Vb in HeLa-20 cells. With this system, a Taq polymerase amplified PCR product is directly inserted into the expression vector. The vector contains a CMV promoter allowing high level expression. The TOPO vector contains both an ampicillin resistance gene (for selection in E. coli) and a neomycin (G418) resistance gene (for selection of stable transfectants). Cells were transfected with verified plasmids containing the entire open reading frame of COX subunit cDNA (2 µg per 1–3 x 10^5 cells) using FuGENE 6. As a control, cells were also transfected with empty vector pcDNA3.1/V5-His without an insert. The vector containing COX Vb sequence was transfected into oxygen sensitive HeLa-20 cells. To establish stable cell lines, transfected cells were cultured in a selection medium containing 400 µg/ml neomycin (G418) (Sigma Chemical Co., St. Louis, MO) for 2-4 weeks. Resulting colonies were further selected with 400 µg/ml G418 in order to obtain single stably transfected clones. These cells were cultured in the continuous presence of G418 (400 µg/ml). Several stable clones expressing selected COX subunit and non-expression clones containing empty vector were selected.

Functional Tests of Over-expression of COX Subunit Vb - The level of Vb expression of individual clones was analyzed by Western blot analysis using a specific antibody for COX subunit Vb as described above. COX activity was measured in these clones and several of these (in which Vb was over-expressed 2-4 fold) were selected for subsequent experiments. ROS production was detected in selected clones under both normoxic and hyperoxic conditions (as above). Survival and growth in hyperoxia were tested by exposure of clones to 80 % oxygen. A negative control of oxygen sensitive HeLa-20 cells and a positive control of oxygen tolerant HeLa-80 cells were also used in addition to cells treated with the empty vector.

Immunostaining of Proliferating Cell Nuclear Antigen (PCNA) – Cell proliferation was evaluated by detecting PCNA in cells grown in 24-well plates under either 20% or 80% oxygen. After 6 days of growth, cells were fixed with fresh 4% paraformaldehyde. After washing with PBS, cells were heated to 95°C for 30 minutes in citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6.0). After cooling and PBS washing, the cells were incubated with normal horse serum blocking solution for 30 minutes and, finally, a peroxidase blocking solution (3% H2O2 in PBS) was added for 10 minutes to eliminate unspecific peroxidase reaction. Immunostaining was performed by incubating the fixed cells with the primary antibody for 30 minutes (mouse anti-PCNA, IgG2a, PC10, Vector Lab. Inc.) and subsequently with the secondary antibody for 30 minutes (Biotinylated Universal Antibody from Vector Lab. Inc.). Finally, DAB peroxidase substrate solution (Sigma Chemical Co., St. Louis, MO) was added and samples were observed by light microscopy immediately.

Oxygen consumption - HeLa cell cultures in 10-cm dishes (>90% confluent) were washed with PBS, detached with trypsin/EDTA, and washed (1,000 x g x 8 min) in complete culture medium. Oxygen consumption was measured using a Gilson Oxygraph with a Clark electrode (Yellow Springs Instruments Co., Yellow Springs, OH). Respiration rates were measured using 2–3 x 10^6 cells suspended in a total volume of 3.0 ml of complete culture medium containing 10% FBS and supplemented with 17 mM glutamate at 37 °C. A starting O2 concentration of 240 µM was assumed based on O2 solubility at sea level at 37 °C (22).

Statistics Analysis - Analysis of differences between groups was done by unpaired two-tailed Student's t-test. Analyses between multiple groups were determined by one-way ANOVA. Results
RESULTS

COX subunit Vb is over-expressed in HeLa-80 cells

We previously reported that the elevated COX activity in oxygen tolerant HeLa-80 cells is associated with diminished mitochondrial ROS production and lessened hyperoxic damage (13). However, the mechanism(s) through which COX activity was increased remained unknown. COX consists of 13 subunits variously encoded by mitochondrial and nuclear DNA as described above. The larger subunits (I, II, III) are involved in the catalytic activity of COX and the smaller subunits are important in its regulation (23). In order to determine the reason(s) for the increased COX activity in HeLa-80 cells, we carried out analyses of the differential expression of COX subunits in HeLa-20 and HeLa-80 cells by Western blotting analyses. Western blots of 11 of the 13 subunits of COX (antibodies against two of these were not available) indicated that only COX subunit Vb was significantly increased (>2-fold) (Figure 1). This may be a critical observation because this subunit and its yeast homologue have been indicted as important in the regulation of overall COX activity and proper assembly of COX (24-27). Interestingly, however, the mRNA expression for each of the 13 subunits - including Vb - was similar (data not shown). This is surprising given the elevated levels of Vb protein and suggests either enhanced translation efficiency or increased stability of the protein product (although there are other possible explanations).

COX subunit Vb is selectively suppressed in HeLa-80 cells by siRNA

SiRNA transfection was used as a means to specifically and directly test whether the over-expressed COX Vb in HeLa-80 cells might lead to an overall increase in COX activity. The effects of siRNA on Vb expression were determined following 48 h exposure to siRNA. As shown in Figure 2, Western blots revealed significantly decreased COX Vb in siRNA treated HeLa-80 cells and the extent of inhibition of expression was dependent on the dose of siRNA. In similar experiments, we also determined by Western blot the expression levels of several other COX subunits (I and II which are mitochondrial products and Va and VIb which are nuclear products). No significant changes in the expression of I, Va and VIb were observed. However, levels of subunit II were decreased by approximately 20% (n=3, p<0.05) for presently unknown reasons (results not shown). For all of the above Western analyses, beta-actin was used as control and the results were calculated as the ratio of COX Vb/beta-actin (Figure 2A). Consistently, mRNA levels (with beta-actin used as a reference) were decreased as well, as shown by real time RT-PCR (Figure 2B).

COX activity is significantly decreased in subunit Vb siRNA treated HeLa-80 cells

After 4 days of Vb siRNA treatment in HeLa-80 cells, COX activities were significantly decreased by both siRNA constructs and the decrease in activity was roughly proportional to the doses of siRNA (Figure 3). The non-specific oligo had no effect on this activity.

Suppression of COX subunit Vb increases ROS production and abrogates the oxygen tolerance of HeLa-80 cells

If HeLa-80 cells are protected against the cytostatic and cytotoxic effects of hyperoxia by over-expression of subunit Vb, then suppression of COX Vb expression should (1) decrease COX activity, (2) increase ROS generation and (3) diminish tolerance to hyperoxia. Indeed, siRNA-mediated decreases in COX Vb expression in HeLa-80 not only decreased COX activity (above) but also increased ROS generation to levels at, or close to, wild type HeLa-20 cells (Figure 4). Our data indicate that the siRNA-mediated suppression lasts at least 5 days in normoxic culture (data not shown). Tests of changes in tolerance to hyperoxia revealed that siRNA transfected HeLa-80 cells showed diminished viability under 80% O₂, but not 20% O₂ (Figure 5).

Over-expression of COX subunit Vb significantly increases COX activity in HeLa-20 cells

If COX subunit Vb is associated with the enhanced COX activity, the over-expression of subunit Vb should increase COX activity, perhaps to levels near those of oxygen tolerant HeLa-80 cells. We employed the pcDNA3.1/V5-His TOPO...
TA expression system to over-express Vb in HeLa-20 cells. Clones of HeLa-20 cells over-expressing COX subunit Vb were successfully obtained. The over-expressed COX subunit Vb protein was detected by Western blot in all the clones with over-expressed COX Vb protein. The range of increase in COX activity was 50-80% in these clones compared to control HeLa-20 cells (Figure 7) although we did not obtain a clone with the same level of COX activity as HeLa-80 cells (which have 2-fold higher COX activity compared to control HeLa-20 cells). We should also note that the increases in COX activity did not correlate exactly with the level of Vb expression.

Over-expression of COX subunit Vb decreases ROS production and improves oxygen tolerance of HeLa-20 cells

Two selected Vb over-expressing clones (C2 and C5), along with both wild-type HeLa-20 and oxygen-tolerant HeLa-80 cells as controls, were cultured in 80% O2. ROS production was measured by DCF oxidation after 2 days of exposure to 80% O2. The generation of ROS was significantly decreased in both Vb over-expressing clones (Figure 8A). Similar results were found by following the oxidation of dihydroethidium (Figure 8B). Furthermore, following one week exposure of the test cells to 80% O2, the Vb over-expressing clones showed some degree of resistance to hyperoxia, although the degree of oxygen tolerance was not equivalent to that of HeLa-80 cells. In contrast, wild-type HeLa-20 cells stopped growing and progressively died after 5-6 days exposure to 80% O2 (Figure 9A).

PCNA expression is preserved in Vb over-expressing clones in hyperoxia

Proliferating Cell Nuclear Antigen (PCNA) is a co-factor of DNA polymerase δ expressed during the S phase of the cell cycle. The levels of PCNA were evaluated by immunostaining of the wild-type HeLa-20 cells, oxygen-tolerant HeLa-80 cells and Vb over-expressing clones after exposure to both 20% and 80% O2 for 6 days. Under 20% O2, PCNA was present in all the cells and the intensity of PCNA staining was similar among the different groups of the cells (Figure 9B). In contrast, under 80% O2, PCNA was almost completely absent in wild-type HeLa-20 cells whereas PCNA expression by the oxygen-tolerant HeLa-80 cells was unaffected. Most importantly, Vb over-expressing HeLa-20 cells exposed to 80% O2 maintained a significant level of PCNA expression (although somewhat lower than that in HeLa-80 cells under the same conditions) (Figure 9B). The increased level of PCNA expression in Vb over-expressing HeLa-20 clones indicates a higher rate of proliferation in the clones under hyperoxia. These results are consistent with the increased growth of Vb over-expressing HeLa-20 clones shown in Figure 9A.

Oxygen consumption is increased in Vb over-expressing clones in the presence of the mitochondrial uncoupler, CCCP

As described in our previous study, HeLa-80 cells have a highly significant increase in respiratory potential (13). Although steady-state oxygen consumption was similar between HeLa-20 and HeLa-80 cell lines, the rate of oxygen consumption was very much greater in HeLa-80 cells exposed to CCCP compared to that observed in HeLa-20 cells (Table 1). We hypothesized that the disproportionately increased O2 consumption in HeLa-80 cells might be due to the elevated COX activity which may be rate-limiting in electron transport and respiration (13). Interestingly, in our current study, the Vb over-expressing HeLa-20 clones show a similar pattern, with O2 consumption being significantly increased in the presence of CCCP. In addition, in Vb siRNA transfected HeLa-80 cells, O2 consumption was significant lower compared to HeLa-80 control and non-sense siRNA treated HeLa-80 cells in the absence and presence of CCCP (Table 1). These results suggest that subunit Vb might be a key regulator of COX activity and mitochondrial respiration.

DISCUSSION

Oxygen toxicity has been recognized for many decades. Hyperoxic cell and tissue injury are well appreciated and, in vivo, affect the respiratory, cardiovascular, nervous and gastrointestinal systems (4-8, 28). In fact, the toxic effect of oxygen was noticed shortly after its discovery by Priestley in the 1700’s. However, the clinical importance of oxygen toxicity was not
appreciated until, in the 1950's, an epidemic of retinopathy of prematurity (also known as retrolental fibroplasia) occurred in premature babies treated with oxygen for neonatal pulmonary insufficiency. Despite decades of research, the mechanisms involved in oxygen toxicity are still not completely understood.

The intracellular production of ROS by mitochondria under conditions of elevated O₂ pressures might be the most important mechanism of hyperoxic damage (11). The formation of excessive mitochondrial ROS in hyperoxic damage has also been noted by many other researchers (9, 10, 29). The increased ROS generation during exposure to hyperoxia can potentially overwhelm antioxidant defense mechanisms, eventuating in cell death (30). These earlier observations support the view that hyperoxic cell damage derives from increased mitochondrial ROS formation and the consequent cell damage done by these reactive species (11).

In an earlier study, we tentatively concluded that the oxygen tolerance of HeLa-80 cells might be due to decreased electron-rich intermediates in the mitochondrial electron transport chain (13). These intermediates (in particular, ubisemiquinone) are thought responsible for the majority of incidental mitochondrial ROS generation. HeLa-80 cells were found to have a >2-fold increase in the activity of the terminal electron transport complex, COX, raising the possibility that this increased activity had the net effect of decreasing both the electron-rich intermediates and associated ROS generation. However, the mechanism(s) through which COX activity was increased in HeLa-80 cells remained unknown.

In the present investigations, we have found that elevated COX activity in HeLa-80 cells is associated with a >2-fold increase in the regulatory subunit COX Vb. Suppression of COX Vb expression in HeLa-80 cells with siRNA leads to (i) a 60-80% decrease in COX activity, (ii) an increase in intracellular ROS generation and (iii) a parallel decrease in the growth of HeLa-80 cells under 80% (but not 20%) O₂. In further support of the importance of COX subunit Vb in these phenomena, over-expression of subunit Vb in wild-type HeLa-20 cells (i) increases COX activity, (ii) decreases ROS production and (iii) leads to increased (but not complete) tolerance to hyperoxia.

These results do not, however, rule out the possibility that changes in other COX subunits (or, for that matter, products not even associated with mitochondrial metabolism) might have an effect on oxygen tolerance. Indeed, this is partly supported by our observation that over-expression of Vb in normally hyperoxia-sensitive HeLa-20 cells does not completely protect against hyperoxia. This is not surprising in view of the fact that HeLa-80 cells were generated over a long (almost 2 year) period of selection by exposure to stepwise increases in oxygen partial pressure. Therefore, this line of HeLa cells - a cell type renowned for genetic instability - may have accumulated a number of additional (and presently unknown) mutations affecting oxygen tolerance.

Nonetheless, our results do support the view that COX subunit Vb and the level of COX activity play an important role in oxygen tolerance, at least in these lines of HeLa cells. Only subunit Vb - but none of the others - was increased >2-fold in HeLa-80 cells. This observation is probably not a coincidental finding because this subunit and its yeast homologue have been indicted as important in the regulation of overall COX activity and proper assembly of COX (24-27). In further support of the importance of Vb expression in oxygen tolerance, our preliminary results with a human telomerase (hTERT)-immortalized line of human bronchial epithelial cells indicate similar effects of Vb over-expression (i.e., elevated COX activity associated with increased resistance to oxygen toxicity).

Overall, our current results lend further support to the general idea that hyperoxic cell damage derives from enhanced leak of ROS from the mitochondrial electron transport chain. Our findings suggest that it should be possible to make cells tolerant of hyperoxia by depletion of electron-rich intermediates in this chain (particularly ubisemiquinone) and may suggest new pharmaceutical strategies to diminish cellular damage caused by hyperoxia and other insults.
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FOOTNOTES

1This work was supported in part by NIH DK58882 (J.W.E.) and by the Commonwealth of Kentucky Research Challenge Trust Fund. We are grateful to Dr. Hans Joenje for the provision of cell lines and helpful discussion.
The abbreviations used: COX, cytochrome c oxidase; DCFDA, dihydrodichlorofluorescein diacetate; DMEM, Dulbecco’s Modified Eagle’s Medium; HBSS, Hank’s balanced salt solution; HeLa-20, wild-type HeLa cells; HeLa-80, oxygen-tolerant HeLa cells; PBS, phosphate buffered saline; PCNA, Proliferating Cell Nuclear Antigen; ROS, reactive oxygen species; RT-PCR, real-time PCR; siRNA, small interfering RNA.

FIGURE LEGENDS

Figure 1. Western blot analyses of COX subunits in wild-type HeLa-20 cells and oxygen tolerant HeLa-80 cells. COX subunit Vb expression is increased in HeLa-80 cells whereas other COX subunits are close to HeLa-20. The gels were also probed with beta-actin as a loading control. (A) Western blots and (B) densitometric quantification (± 1S.D.) (n = 5, * p<0.05 for COX subunit Vb in HeLa-80 vs. HeLa-20 cells).

Figure 2. Small interfering RNA (siRNA) suppresses COX Vb expression in HeLa-80 cells. Significantly decreased COX Vb protein was found in siRNA transfected HeLa-80 cells by Western blot (A) and densitometric quantification (B). Markedly decreased mRNA levels for COX Vb were found in siRNA transfected HeLa-80 cells as detected by real time RT-PCR (C).

Figure 3. Cytochrome c oxidase (COX) activities are significantly decreased in siRNA transfected HeLa-80 cells treated with 20 or 100 nM of the two different constructs (S1 and S2) versus untreated (control) and non-sense siRNA treated cells (NS oligo). For all siRNA treated cells vs. control and NS oligo, * p<0.01, n=4.

Figure 4. ROS production was detected by the oxidation of the fluorescent probe dihydroethidium and DCF in HeLa-20 and HeLa-80 cells and in HeLa-80 cells treated with a non-sense siRNA (NS oligo) and Vb siRNA at two different doses. (A) The cells were exposed to 80% O₂ (4 h incubation with 100 µM dihydroethidium in complete culture medium). ROS generation increased in HeLa-80 cells treated with both doses of siRNA. Note that ROS levels in HeLa-80 cells transfected with 100 nM of siRNA were almost as the same as those in HeLa-20 cells (n = 8, * p<0.05 vs. untreated and NS oligo treated HeLa-80 cells). (B) Similarly, ROS production is increased in HeLa-80 cells treated with both doses of siRNA as estimated by the oxidation of DCF under 80% O₂ (n = 7, * p<0.05 vs. untreated and NS oligo treated HeLa-80 cells).

Figure 5. HeLa-80 cells were cultured in normoxic (20% O₂, open bars) and hyperoxic (80% O₂, solid bars) conditions. Cell cultures were started at 10% confluence (4x10⁴ cells per well in the 6-well plates) at day 0. Medium and atmosphere were replenished every 2 days. After exposure to 80% O₂ for 6 days, siRNA transfected HeLa-80 cells showed diminished growth under 80% O₂ (solid bars) but not 20% O₂ (open bars) (n = 4, * p<0.05 for siRNA transfected cells under 80% O₂ vs. both control/NS oligo under 20% and 80% O₂ and vs. siRNA transfected cells under 20% O₂).

Figure 6. Western analyses of COX Vb protein expression in HeLa-20 control cells and in 6 separate COX Vb over-expressing clones of HeLa-20 cells (A). Densitometric analyses of the Western blots (B) indicates that COX Vb protein levels in all Vb over-expressing clones is significantly higher than in the wild type HeLa-20 cells (n = 3, * p<0.05).

Figure 7. COX activities are significantly higher in COX Vb over-expressing HeLa-20 cell clones compared to the control HeLa-20 cells. For clones C1-C6 vs. HeLa-20 and for HeLa-20 vs. HeLa-80 cells, * p<0.05, n=5.
Figure 8. ROS production and cell growth under hyperoxia in wild-type HeLa-20 cells and Vb over-expressing HeLa-20 clones 2 and 5. (A) ROS production is decreased in the Vb over-expressing clones as estimated by the oxidation of DCF under 80% O₂. For C2 and C5 vs. wild-type HeLa-20, * p<0.05, n=6. (B) Similarly decreased ROS production is found in the Vb over-expressing clones 2 and 5 as estimated by the oxidation of dihydroethidium. The cells were exposed to 80% O₂ (4 h incubation with 100 μM dihydroethidium in complete culture medium). For C2 and C5 vs. wild-type HeLa-20, * p<0.05, n=4.

Figure 9. (A) Wild-type HeLa-20 cells, oxygen-tolerant HeLa-80 cells and Vb over-expressing HeLa-20 clones 2 and 5 were grown under 80% O₂ for 6 days. COX Vb over-expressing Hela-20 clones showed some increase in tolerance to hyperoxia. Cell cultures were started at 10% confluence (1x10⁵ cells per dish in the 10-cm dishes). For C2 and C5 vs. HeLa-20, * p<0.05, n=4; for HeLa-20 vs. HeLa-80 cells, ** p<0.01, n=4. (B) Immunostaining of PCNA in the HeLa cells after cells were grown under both 20% O₂ and 80% O₂ for 6 days. (a) HeLa-20 cells under 20% O₂; (b) HeLa-20 cells under 80% O₂; (c) HeLa-80 cells under 20% O₂; (d) HeLa-80 cells under 80% O₂; (e) Vb over-expressing HeLa-20 clone 5 under 20% O₂; (f) Vb over-expressing HeLa-20 clone 5 under 80% O₂. A very weak and diffuse cytoplasmic PCNA immuno-positivity is observed in HeLa-20 cells after exposure to 80% O₂ for 6 days whereas more immuno-positivity is apparent in Vb over-expressing HeLa-20 clone 5 under 80% O₂ (f).
Table 1. Oxygen consumption of HeLa cells (wild-type HeLa-20 cells, oxygen-tolerant HeLa-80 cells, siRNA treated HeLa-80 cells and Vb over-expressing HeLa-20 clones) was estimated in the absence and presence of 20 µM CCCP. Note that addition of CCCP increases oxygen consumption in all the cell lines but that the increases are significantly greater in the HeLa-80 cells, HeLa-80 NS oligo, and Vb over-expressing HeLa-20 clone 5 vs. control HeLa20 cells (* p<0.05, n=3). On the other hand, oxygen consumptions are significantly decreased in siRNA treated HeLa-80 cells in the absence and presence of CCCP. For all siRNA treated HeLa-80 cells vs. HeLa-80 control and NS oligo, # p<0.05, n=3. Results are expressed as means ± 1 SD of O₂ consumption (nmol/min/10⁷ cells). Statistical evaluation was performed using one way ANOVA.

| Cell line        | O₂ consumption (nmol/min/10⁷ cells) |
|------------------|------------------------------------|
|                  | Control          | + CCCP             |
| HeLa-20          | 21.48 ± 2.38     | 28.01 ± 3.12       |
| HeLa-80          | 22.93 ± 3.54     | 47.54 ± 6.72 *     |
| HeLa-80 NS oligo | 20.95 ± 2.33     | 43.81 ± 5.71 *     |
| HeLa-80 S1-20nM  | 19.71 ± 4.14     | 25.72 ± 3.68 #     |
| HeLa-80 S1-100nM | 12.57 ± 1.71 #   | 13.14 ± 2.43 #     |
| HeLa-20 Vector   | 21.60 ± 3.03     | 24.82 ± 2.55       |
| HeLa-20 Clone 5  | 24.00 ± 3.18     | 40.80 ± 4.66 *     |
Figure 1

A

B

COX subunit/β-actin (% of HeLa-20)

n: 5 4 3 3 4 5 3 4 3 3 3

*
Figure 2

A

COX Vb

β-actin

HeLa80 HeLa80 NS oligo S1-20nM S1-100nM S2-20nM S2-100nM

B

COX Vb / beta-actin

HeLa80 HeLa80 NS oligo S1-20nM S1-100nM S2-20nM S2-100nM

C

COX Vb mRNA (fold difference in Ct)

HeLa80 HeLa80 S1-20nM S1-100nM S2-20nM S2-100nM
Figure 3

[Bar chart showing COX activity in nmol/min/mg protein for different treatments: control, NS oligo, S1-20nM, S1-100nM, S2-20nM, S2-100nM. Asterisks indicate statistically significant differences.]
Figure 4

A

ROS generation (FU % of HeLa-80 cells)

HeLa20  HeLa80  HeLa80 NS oligo  HeLa80 S1-20nM  HeLa80 S1-100nM

B

ROS generation (FU % of HeLa-80 cells)

HeLa20  HeLa80  HeLa80 NS oligo  HeLa80 S1-20nM  HeLa80 S1-100nM
Figure 5

Cell number (% of control)

control  NS oligo  S1-20nM  S1-100nM  S2-20nM  S2-100nM

20% O₂  80% O₂
Figure 6
Figure 7

COX activity
nmol/min/mg protein

HeLa20  HeLa20-C1  HeLa20-C2  HeLa20-C3  HeLa20-C4  HeLa20-C5  HeLa20-C6

*
Figure 8

A

![Bar chart showing ROS generation in HeLa20, HeLa20-C2, and HeLa20-C5 cells.]

B

![Bar chart showing ROS generation in HeLa20, HeLa20-C2, and HeLa20-C5 cells.]
Figure 9

A

Cell number (% of HeLa-80 cells)

HeLa20  HeLa80  HeLa20-C2  HeLa20-C5

*  **  *  *

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