Elevated CO\textsubscript{2} Levels Cause Mitochondrial Dysfunction and Impair Cell Proliferation\textsuperscript{*[5]}

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**Background:** Cells are exposed to elevated levels of CO\textsubscript{2} (hypercapnia) in many diseases.

**Results:** Hypercapnia decreased cell proliferation, which was prevented with \(\alpha\)-ketoglutarate, IDH2 overexpression, and microRNA-183 inhibition.

**Conclusion:** Hypercapnia causes mitochondrial dysfunction by up-regulation of microRNA-183, which decreases the levels of IDH2.

**Significance:** Hypercapnia causes mitochondrial dysfunction, which is relevant for patients with lung diseases.

Elevated CO\textsubscript{2} concentrations (hypercapnia) occur in patients with severe lung diseases. Here, we provide evidence that high CO\textsubscript{2} levels decrease O\textsubscript{2} consumption and ATP production and impair cell proliferation independently of acidosis and hypoxia in fibroblasts (N12) and alveolar epithelial cells (A549). Cells exposed to elevated CO\textsubscript{2} died in galactose medium as well as when glucose-6-phosphate isomerase was knocked down, suggesting mitochondrial dysfunction. High CO\textsubscript{2} levels led to increased levels of microRNA-183 (miR-183), which in turn decreased expression of IDH2 (isocitrate dehydrogenase 2). The high CO\textsubscript{2}-induced decrease in cell proliferation was rescued by \(\alpha\)-ketoglutarate and overexpression of IDH2, whereas proliferation decreased in normocapnic cells transfected with siRNA for IDH2. Also, overexpression of miR-183 decreased IDH2 (mRNA and protein) as well as cell proliferation under normocapnic conditions, whereas inhibition of miR-183 rescued the normal proliferation phenotype in cells exposed to elevated levels of CO\textsubscript{2}. Accordingly, we provide evidence that high CO\textsubscript{2} induces miR-183, which down-regulates IDH2, thus impairing mitochondrial function and cell proliferation. These results are of relevance to patients with hypercapnia such as those with chronic obstructive pulmonary disease, asthma, cystic fibrosis, bronchopulmonary dysplasia, and muscular dystrophies.

Patients with diseases such as chronic obstructive pulmonary disease, cystic fibrosis, bronchopulmonary dysplasia, and muscular dystrophies may have elevated blood and tissue levels of CO\textsubscript{2} (hypercapnia) (1–3). Patients with cystic fibrosis or chronic obstructive pulmonary disease and hypercapnia have worse outcomes (2, 4–8). Some studies have suggested that elevated CO\textsubscript{2} may have deleterious effects on vascular and cardiac tissues (9–12).

Mitochondrial function plays an important role in the control of cell proliferation by generating cellular energy in the form of ATP (13–15). Mutations in mitochondrial respiratory chain genes cause a drop in intracellular ATP levels sufficient for cell survival, but not sufficient to allow progression through the cell cycle (16). Moreover, in higher plants, elevated atmospheric CO\textsubscript{2} concentrations suppress respiration (17). The TCA cycle is a key component of cell metabolism. It not only generates the reduction equivalents needed to generate ATP in the respiratory chain, but also its metabolites serve as intermediates for the synthesis of amino acids, therefore providing the essential building blocks for cell growth and proliferation (18). Isocitrate dehydrogenase (IDH)\textsuperscript{2} is a key enzyme of the TCA cycle that catalyzes the conversion of isocitrate to \(\alpha\)-ketoglutarate, generating one molecule of CO\textsubscript{2} and a reduction equivalent in the process. In mammals, three isoforms of IDH have been described: one cytosolic NAD\textsuperscript{+}-dependent form (IDH1) and two mitochondrial isoforms (IDH2 (NADP\textsuperscript{+}-dependent) and IDH3 (NAD\textsuperscript{3+}-dependent)). Recently, it has been suggested that IDH2 serves as the main catalyst for this reaction in the TCA cycle outside the retina (19). In more recent studies, a role for IDH2 in proliferation of cancer such as glioma or acute myeloid leukemia has been proposed (20, 21); however, the relationship of IDH and cell proliferation has not been elucidated.

MicroRNAs (miRNAs) are a large family of short RNA molecules (~22 bp) that regulate post-transcriptionally target mRNA by binding to complementary sequences in the UTR, usually resulting in translational repression (22). The human

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\textsuperscript{1} The abbreviations used are: IDH, isocitrate dehydrogenase; miRNA, microRNA; miR-183, microRNA-183; GPI, glucose-6-phosphate isomerase; qPCR, quantitative PCR; LDH, lactate dehydrogenase; aKG, \(\alpha\)-ketoglutarate.

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genome encodes >1000 miRNAs, which may control ~60% of mammalian genes (23), suggesting that miRNAs are involved in the regulation of most cellular processes (24).

In this study, we provide evidence that high CO\textsubscript{2} decreases cell proliferation (independently of pH and hypoxia) by causing mitochondrial dysfunction. This is due to induction of microRNA-183 (miR-183), which decreases IDH2 levels, leading to impaired cell proliferation and inability of the cells to survive during hypercapnia in galactose medium. Overexpression of IDH2 or inhibition of miR-183 ameliorates the effects of high CO\textsubscript{2} on cell proliferation and enables cells to grow in galactose medium. Accordingly, high CO\textsubscript{2} induces miR-183, which down-regulates IDH2, leading to mitochondrial dysfunction and decreased cell proliferation, which is of clinical relevance.

EXPERIMENTAL PROCEDURES

Reagents—All cell culture reagents were from Mediatech Inc. α-Ketoglutarate was from Sigma-Aldrich. Galactose was from Calbiochem. Anti-actin and anti-glucose-6-phosphate isomerase (GPI) antibodies were purchased from Sigma-Aldrich, polyclonal antibody directed against IDH2 (mitochondrial IDH) was purchased from Abcam and Santa Cruz Biotechnology, and anti-IDH3 antibody was purchased from Santa Cruz Biotechnology. HRP-conjugated goat anti-mouse secondary antibody was from Bio-Rad, donkey anti-goat secondary antibody was from Santa Cruz Biotechnology, and HRP-conjugated goat anti-rabbit antibody was from Cell Signaling. All other chemicals were purchased from Calbiochem and Sigma-Aldrich. Reagents for production of cDNA and quantitative (qPCR) assays were from Bio-Rad and Applied Biosystems. The mRNA isolation kit was from Qiagen. The miRNA assays for human hsa-miR-183 and the control assay for RNU48 were from Applied Biosystems. The mRNA isolation kit was from Qiagen. The miRNA assays for human hsa-miR-183 and the control assay for RNU48 were from Applied Biosystems. BrdU was from Pharmingen. Primers were purchased from Integrated DNA Technologies. Restriction endonucleases were obtained from Promega.

Cells Lines and Culture—A549 human cells (American Type Culture Collection CCL-185) and N12 human lung fibroblasts (gift from M. Selman, Instituto Nacional de Enfermedades Respiratorias, Mexico City, Mexico) (12, 25, 26) were grown in DMEM supplemented with 10% FBS (20% for N12 human lung fibroblasts), 100 units/ml penicillin, 100 μg/ml streptomycin, and 20 mM HEPES. Cells were incubated in a humidified atmosphere of 5% CO\textsubscript{2} and 95% air at 37 °C.

CO\textsubscript{2}, Medium and CO\textsubscript{2} Exposure—For the different experimental conditions, initial solutions were prepared with DMEM/Ham’s F-12 medium/Tris base/MOPS base (3:1:0.25:0.25) containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin for A549 cells and with DMEM medium/Tris base/MOPS base (4:0.25:0.25) containing 20% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin for N12 cells. The buffering capacity of the medium was modified by changing its initial pH with Tris and MOPS base to obtain a pH of 7.4 at the various CO\textsubscript{2} levels (pCO\textsubscript{2}, 40, 60, and 120 mm Hg). In some experiments modeling extracellular acidosis, an initial pH of 6.8 was used, resulting in a final pH of 7.2 and a pCO\textsubscript{2} of 40 mm Hg. The desired CO\textsubscript{2} and pH levels were achieved by equilibrating the medium overnight in a humidified chamber (C-Chamber, BioSpherix Ltd.). The atmosphere of the C-Chamber was controlled with a PROCO2 carbon dioxide controller (BioSpherix Ltd.). In this chamber, cells were exposed to the desired pCO\textsubscript{2} while maintaining 21% O\textsubscript{2} balanced with N\textsubscript{2}. Prior to and after CO\textsubscript{2} exposure, pH, pCO\textsubscript{2}, and pO\textsubscript{2} levels in the medium were measured using a Stat Profile pHOx blood gas analyzer (Nova Biomedical Corp.). Experiments were started by replacing the culture medium with the CO\textsubscript{2}-equilibrated medium and incubating in the C-Chamber for the desired time.

Cell Proliferation Assay—Cells were plated on a 60-mm plate at a density of 5 × 10\textsuperscript{4} cells/plate. After 1, 3, and 5 days, cells were trypsinized and resuspended in cell culture medium. Cells were counted using a hemocytometer and trypan blue exclusion. For the BrdU stain, a commercially available staining kit was used (Kamiya Biomedical Co.). The cells were grown on coverslips in a 35-mm dish (initially seeded at a density of ~1.5 × 10\textsuperscript{5}) for 3 days. BrdU was added at a final concentration of 10 μM 24 h before the cells were processed according to the manufacturer’s instructions.

Cell Death Assay—Cells were plated on a 60-mm plate at a density of 5 × 10\textsuperscript{4} cells/plate. After 1, 3, and 5 days, a lactate dehydrogenase (LDH) assay (Roche Applied Science) was performed according to the manufacturer’s instructions. Briefly, the culture supernatant was collected, and LDH activity was determined in a coupled enzymatic reaction in which 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride is reduced to formazan. The increase in supernatant LDH activity directly correlates with the amount of formazan formed over time. Percent cell death was calculated by dividing the percentage of LDH released by the maximal amount of LDH released.

Cell Cycle Analysis by Flow Cytometry—Cells were starved in serum-free medium for 2 days prior to exposure to high CO\textsubscript{2} to synchronize the cell cycle. After 3–5 days of exposure to the experimental conditions, cells (3–4 × 10\textsuperscript{6} cells/plate) were trypsinized, washed twice with PBS, and resuspended in 50 μl of PBS, and fixed with 450 μl of ice-cold 70% ethanol. Cells were centrifuged for 5 min at 13,000 rpm, and ethanol was aspirated. Cells were then washed twice with 2 ml of cold PBS and resuspended in 1 ml of propidium iodine staining solution (500 μl of 1 mg/ml propidium iodine in 10 ml of PBS, 2 mg of RNase A, and 100 μl of 10% Triton X-100 solution: final concentrations of 50 μg/ml propidium iodine and 0.1% Triton X-100). Cells were transferred to 5-m1 tubes, and cell cycle phases were measured by flow cytometry (Beckman Coulter Epics XL flow cytometer). Data were analyzed using ModFit LT software (Verity Software House) (27).

Measurement of O\textsubscript{2} Consumption—Cellular respiration was measured by high-resolution respirometry (Oxygraph-2k, OROBOROS Instruments Gmbh) as previously described (28). Cells were incubated with control (pCO\textsubscript{2}, 40 mm Hg, pH 7.4), buffered high CO\textsubscript{2} (pCO\textsubscript{2}, ~60 or ~120 mm Hg, pH 7.4 in both cases), or acidotic (pCO\textsubscript{2}, 40 mm Hg, pH 7.2) medium for 3 days. After 3 days, cell suspensions were transferred to the measurement chambers of the Oxygraph-2k device (kept at 37 °C), and respiration was measured in real time (1-s time intervals) and analyzed using DatLab software. Once reaching steady-state respiratory flux, endogenous respiration was measured, after
which the ATP synthase was inhibited with oligomycin (4 μg/ml), followed by uncoupling of oxidative phosphorylation by 0.5 μM stepwise titration of carbonyl cyanide p-trifluoromethoxyphenylhydrazone to assess maximal respiration.

**ATP Measurement**—Cells were plated on 60-mm dishes. After 1 and 3 days, the assay was performed according to the manufacturer’s instructions (Roche Applied Bioscience). Briefly, cells were lysed by boiling in 100 mM Tris and 4 mM EDTA (pH 7.75), and ATP concentration was assessed by measuring the emission of light at 562 nm produced by the reaction of ATP with r-luciferin.

**Real-time RT-qPCR—**IDH2 and IDH3 mRNA expression was determined by qPCR using SYBR Green chemistry. Total RNA was isolated after 1 and 3 days of exposure to high CO₂ (pCO₂ ~120 mm Hg). Total RNA was isolated with a Qiagen mini kit. cDNA was synthesized from 1 μg of total RNA using an iScript cDNA synthesis kit (Bio-Rad) with random decamer primers. For the miRNA, extracted total RNA was transcribed into cDNA using a TaqMan microRNA reverse transcription kit (Applied Biosystems), and qPCR was performed using cDNA with TaqMan Universal Master Mix II (Applied Biosystems) according to the manufacturer’s instructions. The assay numbers for endogenous control miRNA (RNU48) and target miRNA (human hasa-miR-183) were 001006 and Hs03302720_pri, respectively. cDNA was generated using a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems), and RT-qPCR was performed using an iCycler iQ system (Bio-Rad). Cycle threshold values were normalized for amplification of human mitochondrial RPL19 (ribosomal protein L19) (29).

**Construction of Plasmid Overexpressing IDH2—**IDH2 cDNA was obtained from the pCMV6-AC plasmid containing the human IDH2 gene (OriGene SC319226, RefSeq accession number NM_002168). The following PCR primers were used to amplify the IDH2 DNA from the construct pCMV6-AC-IDH2: 5′-CAG GAA GGT GTG CGT GGA GAC (sense) and CCG TGG GAT CCC TAC TGC CTG CCC AGG G (antisense); and human RPL19, ATC ATC CGC AAG CCT TGT TGT TCA GGA AGT (sense) and AGG AGG GCT GTG GGA TTC TGG TGT TCA GGA AGT (antisense); IDH3, ATC GGA GGT CTC CCT GTG GTG (sense) and AGG AGG GCT GTG GGA TTC TGG TGT TCA GGA AGT (antisense); and human RPL19, ATC ATC CGC AAG CCT GTG (sense) and TGA CCT TCT CGT GCA TTC (antisense).

**Statistics**—Data are expressed as means ± S.E. When comparisons were performed between two groups, significance was evaluated by Student’s t test, and when more than two groups were compared, analysis of variance was used, followed by Dunnett’s test. p < 0.05 was considered significant.

**RESULTS**

**Chronic Exposure to Elevated CO₂ Levels Leads to Decreased Cell Proliferation**—Cells grown in the presence of high CO₂ concentrations have been shown to have a reduced proliferation rate (24, 32, 33). To verify these observations, we used two different cell lines: one of cancer origin (A549 cells) and primary fibroblasts (N12 cells). We performed the experiments controlling the extracellular pH at a physiologic level of pH 7.35–7.45 and under normoxic conditions (pO₂ 100–120 mm Hg) to exclude the role of hypoxia and acidosis. As shown in Fig. 1, we found a decreased rate of proliferation (assessed as number of cells (Fig. 1, A and B) and BrdU incorporation (Fig. 1, C and D)), which became significant after 3 days of exposure to high levels of CO₂. Proliferation was decreased in a dose-dependent manner. It is also important to stress that the decreased proliferation was independent of extracellular pH.

**Decreased Cell Proliferation During Exposure to High CO₂ Levels Is Not Due to Increased Cell Death or Cell Cycle Arrest**—Hypercapnia has been described to lead to an increased expression of apoptotic proteins in cortical neurons of newborn piglets (34) and alveolar epithelial cells (35). Therefore, we measured release of LDH to investigate whether cell death was the cause for the reduced cell numbers seen after exposure to high CO₂ levels. Fig. 2 (A and B) shows that exposure to high levels of CO₂ did not lead to increased cell death compared with control conditions. We also excluded G1 cell arrest as a reason for the observed decreased in cell proliferation. Fig. 2 (C and D) shows the results from flow cytometry analysis of the cell cycle phases after the cells had been exposed to elevated CO₂ levels for 5 days. However, cells exposed to chronic elevated CO₂ levels showed a constantly decreased proliferation rate with an ∼30%
decreased population doubling time (Fig. 2, E and F). These results suggest that exposure to high CO₂ levels does not lead to cell death or G₁ cell cycle arrest but rather to a slower progression through all cell cycle phases compared with control conditions.

Exposure to High CO₂ Levels Leads to Mitochondrial Dysfunction in A549 and N12 Cells—After excluding cell death or cell cycle arrest as the reason for decreased proliferation, we hypothesized that exposure to high CO₂ levels leads to an alteration in cell metabolism. It is known that the inability to utilize galactose as the sole energy source by the mitochondrial oxidative phosphorylation pathway is attributed to altered mitochondria (18). We found that cells grown in elevated levels of CO₂ in the absence of glucose (galactose medium) had a decreased proliferation rate and an increased mortality rate measured as LDH release, suggestive of mitochondrial dysfunction (Fig. 3, A–D). We investigated further the role of glycolysis as the only source of energy by reducing GPI protein levels in cells stably transfected with a plasmid expressing GPI shRNA (18). As Fig. 3 (E and F) shows, both A549 and N12 cells in which GPI was reduced displayed increased cell death after 3 days of exposure to high CO₂. These findings suggest CO₂-induced mitochondrial dysfunction, as both cell lines were unable to survive in high CO₂ levels when glycolysis was impaired.

Mitochondrial dysfunction was confirmed by transmission electron microscopy (supplemental Fig. S1) and by measuring oxygen consumption. As shown in Fig. 3 (G and H), A549 and N12 cells displayed a dose-dependent decrease, independently of pH, in oxygen consumption when exposed to high CO₂. Also, uncoupling of the respiratory chain with carbonyl cyanide p-trifluoromethoxyphenylhydrazone demonstrated that the maximal respiration capacity was significantly decreased upon exposure to high CO₂. Also, ATP levels were decreased after 1 and 3 days of exposure to elevated CO₂ (Fig. 3, I and J).

Elevated CO₂ Impairs Mitochondrial Function by Down-regulating IDH2—Two CO₂ molecules are formed during the Krebs cycle: 1) conversion of isocitrate to α-ketoglutarate (αKG) and 2) conversion of αKG to succinyl-CoA. We hypothesized that exposure to high CO₂ levels impairs these reactions. To investigate whether the conversion of isocitrate to αKG was impaired, cells were incubated for 3 days in high CO₂ and culture medium supplemented with 7 mM αKG. As shown in Fig. 4 (A and B), the effect of high CO₂ on cell proliferation was prevented when cells were incubated in the presence of αKG. Furthermore, the addition of αKG prevented cell death when cells were grown in high CO₂ and glucose-free medium containing galactose (Fig. 4, C and D). Therefore, we set out to investigate the role of mitochondrial IDHs (IDH2 and IDH3). We found that exposure to high CO₂ levels lead to a significant decrease in mRNA levels for IDH2 (but not IDH3) as soon as 1 day (Fig. 4, E and F). To determine that exposure to high CO₂ does not lead to a general down-regulation of the TCA cycle, we also determined mRNA expression of other key enzymes of the TCA cycle such as mitochondrial aconitase and citrate synthase but found no change after up to 3 days of CO₂ exposure (supplemental Fig. S2). Consequently, protein levels of IDH2 (but
not IDH3) were decreased after 3 days of exposure to high CO\textsubscript{2} (Fig. 4, G and H).

To investigate further the role of IDH2 in cell proliferation, we conducted experiments either repressing IDH2 expression with siRNA or overexpressing IDH2. As shown in Fig. 5 (A and B), decreased expression of IDH2 in A549 and N12 cells led to decreased cell proliferation under control conditions. Conversely, overexpression of IDH2 by stably transfecting A549 cells with the p3XFLAG-CMV-10-IDH2 plasmid (Fig. 5C) enabled those cells to grow under high CO\textsubscript{2} conditions similarly to control cells, and these cells did not die when exposed to high CO\textsubscript{2} in galactose-free medium (Fig. 5D). To explore further the mechanism by which IDH2 controls cell proliferation, we cultured A549 and N12 cells in which IDH2 was silenced with siRNA in galactose-containing medium and found increased cell death (Fig. 5, E and F).

miR-183 Is Up-regulated in Cells Exposed to Elevated Levels of CO\textsubscript{2} and Mediates the Repression of IDH2 Expression—Multiple studies indicate that miRNAs are involved in the regulation of most cellular processes (24), and we found that miR-183 is a potential regulator of IDH2 (TargetScan). We performed quantitative PCR, and as shown in Fig. 6 (A and B), we found that miR-183 was up-regulated in both A549 and N12 cells after 24 h of exposure to high CO\textsubscript{2}. As shown in Fig. 6 (C–F), cells transfected with miR-183 inhibitor had restored levels of IDH2 mRNA and protein when exposed to elevated levels of CO\textsubscript{2}. Cells transfected with precursor miR-183 had decreased IDH2 mRNA and protein expression under control conditions. Furthermore, cells transfected with precursor miR-183 and grown under control conditions showed decreased cell proliferation similar to cells grown in high levels of CO\textsubscript{2} (Fig. 6, G and H). In the reverse experiment, cells transfected with miR-183 inhibitor grew comparably with those grown under control conditions when exposed to elevated levels of CO\textsubscript{2} (Fig. 6, I and J).
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A

B

cell count [relative units]

Time [days]

C

D

Time [days]

E

F

G

H

I

J

Wild type shGPI

GPI

actin

Oxygen flux [pmol/cm²]

pCO₂ 40 mmHg, pH 7.4

pCO₂ 40 mmHg, pH 7.2

pCO₂ 120 mmHg

Oxygen flux [pmol/cm²]

pCO₂ 40 mmHg, pH 7.4

pCO₂ 40 mmHg, pH 7.2

pCO₂ 120 mmHg

ATP [relative units]

CO₂ 1 day

CO₂ 3 days

ATP [relative units]

CO₂ 1 day

CO₂ 3 days
were able to survive in galactose medium. All if these effects of miR-183 are specific, as miR-30a, which is up-regulated under high CO2 conditions and targets IDH1, did not have any effect on IDH2 levels or on cell proliferation (supplemental Fig. S3).

DISCUSSION

Patients with chronic lung diseases such as cystic fibrosis and chronic obstructive pulmonary disease can have high CO2 levels (hypercapnia), which are associated with worse outcomes (2, 3).
The signaling pathways sensitive to changes in CO₂ levels in mammalian non-neuronal cells are incompletely understood (37, 38), but in plants and neuronal mammalian cells, many reports have identified conserved signaling pathways (39–41).

We studied the effects of high CO₂ on cell proliferation in both a lung cancer epithelial cell line (A549 cells) and a lung primary cell line (N12 fibroblasts) and found that the effects of high CO₂ were nearly identical in both cell lines. We observed decreased cell proliferation in cells exposed to high CO₂ levels within 3 days, which was dose-dependent and independent from acidosis and hypoxia (Fig. 1). In commercial cell culture reactors, high CO₂ was associated with decreased cell proliferation, which the authors attributed to increased osmolarity (32, 42). However, our data suggest that the effects on proliferation are independent of osmolarity (supplemental Fig. S4). We also carried out our experiments with exposure to rather high pCO₂ (120 mm Hg); therefore, we first set out to determine whether the decreased cell count observed could be attributed to cell death secondary to cytotoxic effects of CO₂. We found that exposure to high CO₂ did not result in increased cell death. We also did not find differences in the distribution of cell cycle phases in cells exposed to high CO₂, which rules out a cell cycle arrest as the cause for decreased proliferation. However, the cells had a decreased population doubling rate, indicating that cells exposed to high CO₂ have a prolonged cell cycle time. Cells exposed to high CO₂ had a slower proliferation rate of 25–30%, pointing to an alteration in cell metabolism, also manifested by decreased oxygen consumption and lower levels of ATP production (see Fig. 3).

Cells exposed to elevated CO₂ were unable to grow in glucose-free medium supplemented with galactose (Fig. 3), suggesting that high levels of aerobic glycolysis are required. We confirmed these data utilizing shRNA to suppress the levels of GPI. Cells expressing the GPI shRNA constructs did not survive in galactose medium, suggesting that high CO₂ levels cause mitochondrial dysfunction, rendering the cells dependent on glycolysis for survival. After completion of glycolysis, acetyl-CoA enters the TCA cycle, where the carbon skeleton gets further degraded to two molecules of carbon dioxide. We hypothesized that exposure to high CO₂ would impair those reactions.

**FIGURE 5. IDH2 regulates cell proliferation in A549 and N12 cells and restores the cell's ability to survive in galactose medium.** A549 (A) and N12 fibroblasts (B) were transfected with control (ctrl; scrambled) siRNA and siRNA against IDH2, and cell count was determined after 3 days. C, A549 cells were stably transfected with p3XFLAG-CMV plasmid overexpressing IDH2 and exposed for 3 days to control (pCO₂ 40 mm Hg, pH 7.4) and high CO₂ (pCO₂ 120 mm Hg, pH 7.4) conditions, and cell count was determined. D, A549 cells stably overexpressing IDH2 were cultured in glucose-free medium with 20 mM galactose, and cell death was assessed after 3 days. A549 (E) and N12 (F) cells transfected with siRNA against IDH2 were cultured in glucose-free medium with 20 mM galactose, and cell death was determined after 3 days. Error bars represent the mean ± S.E. (n = 4–9). *, p < 0.05; **, p < 0.01; ns, not statistically significant.
Studies performed over 30 years ago showed that bicarbonate levels alter the function of dehydrogenases (43, 44). The first CO2 molecule in the TCA cycle is produced when isocitrate is converted to αKG by the enzyme IDH. We found that the addition of αKG rescued the negative effect of high levels of CO2 on cell proliferation. This is in agreement with the suggestion of αKG as a potential pharmacological agent to improve some mitochondrial pathological disorders such as those with ATP synthase deficiency (45) and in patients undergoing lung resection to decrease oxidative stress (46).

Interestingly, upon investigation of mitochondrial IDH isoforms, we found that the NADP+-dependent IDH2 (but not the NAD+-dependent IDH3) is down-regulated and that cell proliferation and survival in glucose-free medium are dependent on IDH2. Cells in which IDH2 levels were decreased with siRNA had a slower proliferation rate and were unable to survive in glucose-free medium; conversely, A549 cells stably over-expressing IDH2 had an increased proliferation rate similar to control conditions when exposed to high CO2 and were able to survive in galactose-free medium when exposed to high CO2. These findings, together with insights gained from studies on otherwise healthy patients with retinitis pigmentosa due to IDH3 mutation (19), suggest that not IDH3 but IDH2 serves as the main catalyst of the conversion of isocitrate to αKG in the TCA cycle.

miR-183 is overexpressed in tumors such as medulloblastomas or breast cancer, and it has been found to negatively regulate proliferation and migration (47, 48). We found that the decrease in IDH2 is mediated by miR-183 and that inhibition of miR-183 would rescue our phenotype, whereas transfection of control cells with precursor miR-183 would mimic the results found in cells exposed to high CO2 (see Fig. 6).

In summary, as depicted schematically in Fig. 7, we have provided evidence that elevated CO2 levels induce miR-183, which in turn leads to mitochondrial dysfunction by down-regulating...
IDH2, leading to impaired proliferation of normal fibroblasts and epithelial cancer cells. These findings may explain how the cell resists a metabolic stress such as high CO2 by down-regulating cell metabolic activity and therefore proliferation and why in diseases such as chronic obstructive pulmonary disease and bronchopulmonary dysplasia there is significant failure to thrive.

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