Anaerobic respiration sustains mitochondrial membrane potential in a prolyl hydroxylase pathway-activated cancer cell line in a hypoxic microenvironment

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Takahashi E, Sato M. Anaerobic respiration sustains mitochondrial membrane potential in a prolyl hydroxylase pathway-activated cancer cell line in a hypoxic microenvironment. Am J Physiol Cell Physiol 306: C334–C342, 2014. First published September 18, 2013; doi:10.1152/ajpcell.00255.2013.—To elucidate how tumor cells produce energy in oxygen-depleted microenvironments, we studied the possibility of mitochondrial electron transport without oxygen. We produced well-controlled oxygen gradients (ΔO2) in monolayer-cultured cells. We then visualized oxygen levels and mitochondrial membrane potential (ΔΨm) in individual cells by using the red shift of green fluorescent protein (GFP) fluorescence and a cationic fluorescent dye, respectively. In this two-dimensional tissue model, ΔΨm was abolished in cells >500 μm from the oxygen source [the anoxic front (AF)], indicating limitations in diffusional oxygen delivery. This result perfectly matched GFP-determined ΔO2. In cells pretreated with dimethylaminoethylglycine (DMOG), a prolyl hydroxylase domain-containing protein (PHD) inhibitor, the AF was expanded to 1,500–2,000 μm from the source. In these cells, tissue ΔO2 was substantially decreased, indicating that PHD pathway activation suppressed mitochondrial respiration. The expansion of the AF and the reduction of ΔO2 were much more prominent in a cancer cell line (Hep3B) than in the equivalent fibroblast-like cell line (COS-7), indicating limitations in diffusional oxygen delivery. This result perfectly matched GFP-determined ΔO2. In cells pretreated with dimethylaminoethylglycine (DMOG), a prolyl hydroxylase domain-containing protein (PHD) inhibitor, the AF was expanded to 1,500–2,000 μm from the source. In these cells, tissue ΔO2 was substantially decreased, indicating that PHD pathway activation suppressed mitochondrial respiration. The expansion of the AF and the reduction of ΔO2 were much more prominent in a cancer cell line (Hep3B) than in the equivalent fibroblast-like cell line (COS-7). Hence, the results indicate that PHD pathway-activated cells sustain ΔΨm, despite significantly decreased electron flux to complex IV. Complex II inhibition abolished the effect of DMOG in expanding the AF, although tissue ΔO2 remained shallow. Separate experiments demonstrated that complex II plays a substantial role in sustaining ΔΨm in DMOG-pretreated Hep3B cells with complex III inhibition. From these results, we conclude that PHD pathway activation can sustain ΔΨm in an otherwise anoxic microenvironment by decreasing tissue ΔO2 while activating oxygen-independent electron transport in mitochondria.

hypoxia; cancer cell line; prolyl hydroxylase domain-containing protein; hypoxia-inducible factor; anaerobic respiration

For aerobic organisms, active decrease in oxygen demand is an ingenious way to cope with oxygen insufficiencies in the cell. Hypoxia-inducible factor (HIF)-1 has been implicated in this adaptation, because it mediates reprogramming of mitochondrial oxygen metabolism, leading to suppression of mitochondrial respiration (1, 10, 20). This is accomplished by decreasing the electron flow to cytochrome c oxidase by limiting the supplies of NADH to the respiratory chain or by inhibiting the respiratory enzymes. The former includes HIF-1-dependent inductions of pyruvate dehydrogenase kinase-1 and lactate dehydrogenase A (13, 18), which attenuate flux of glycolytically produced acetyl coenzyme A to the tricarboxylic acid (TCA) cycle. The latter includes the HIF-1 target gene product NADH dehydrogenase [ubiquinone] 1α subcomplex 4-like 2 (NDUFA4L2) (25), which directly inhibits complex I in the respiratory chain. HIF-1-driven microRNA, miR-210, may inhibit enzymes in the TCA cycle, as well as the respiratory enzymes (5, 6). In addition, HIF-1 mediates mitophagy via BCL2/adenovirus E1B 19-kDa protein-interacting protein 3 inductions (31) while repressing mitochondrial biogenesis through the peroxisome proliferator-activated receptor-γ coactivator 1β pathway (32). In fact, considerable downregulation of cellular oxygen consumption has been demonstrated with HIF-1 induction in cultured cells (4, 13, 18, 32) and neonatal cardiomyocytes (21, 22). Less pronounced reductions in mitochondrial oxygen consumption have been reported in skeletal muscle of prolyl hydroxylase domain-containing protein-1 (Phd1)-deficient mice (2).

Hypoxic tissue volume emerging from insufficient oxygen supply relative to cellular oxygen demand is the hallmark of solid tumors (29). Tumor hypoxia has been recognized as one of the important factors determining the pathophysiology of tumor tissue through induction of HIFs (19). In vivo, HIF-induced reduction of cellular oxygen consumption is expected to decrease the gradient of oxygen concentration from blood to mitochondria (Fick’s law of diffusion). Thus hypoxic induction of HIF-1α enhances delivery of oxygen to cells in a hypoxic microenvironment. Mitochondrial membrane potential (ΔΨm), the source of energy for mitochondrial ATP production, is generated by extrusion of H+ from the matrix to the intermembrane space in mitochondria as the reducing equivalent is transported to respiratory enzymes. Mitochondrial electron transport is tightly coupled with the reduction of oxygen to water at the terminal respiratory enzyme cytochrome c oxidase. Thus the shallow tissue oxygen gradients appear to be protective for ΔΨm in a hypoxic microenvironment.

However, this hypothesis is debatable, because the rate of electron transport (and consequent ΔΨm generation) is also determined, independently of the oxygen availability, by the electron supplies to the respiratory chain. If oxygen supply is nonlimiting, HIF-induced reduction of oxygen consumption would be indicative of decreases in electron flux in the respiratory chain. Overall, the expected outcome of hypoxic HIF-1 induction would be suppression of ΔΨm, despite increased oxygen delivery.

In the present study, however, we demonstrate that pharmacological inhibition of prolyl hydroxylase domain-containing protein (PHD), an enzyme essential to HIF-1α degradation (12, 16), sustains ΔΨm in cells in a hypoxic microenvironment. On
the basis of visualizations of oxygen gradients and ΔΦm in a two-dimensional tissue model of a hypoxic microenvironment, we address the mechanism whereby PHD pathway-activated cells can maintain ΔΦm while mitochondrial respiration is significantly decreased.1

METHODS

We used the fibroblast-like cell line COS-7 and the hepatoma cell line Hep3B. Cells were cultured on cover glasses (13-mm diameter, 0.15-mm thick; CultureCoverGlass, Matsunami) in DMEM (Sigma) supplemented with 10% FCS and antibiotics. At 60–100% confluence, the cells on the CultureCoverGlass were transferred to the airtight measuring cuvette in which humidified mixed gas (air + >99.999% N2) was supplied at 2 ml/min. Oxygen concentration in the mixed gas was controlled using computer-controlled mass flow controllers. The dead space of the measuring cuvette was 6.1 ml. The measuring cuvette was placed on the stage of an inverted microscope.

1 This article is the topic of an Editorial Focus by Hiroyasu Esumi (8a).

Fig. 2. Relationship between distance from CG edge and red shift of green fluorescent protein (GFP) fluorescence in Hep3B cells. Increases in ratio of red fluorescence intensity (565–625 nm, 1.8-s exposure) to the corresponding green fluorescence intensity (499–522 nm, 0.6-s exposure) (R/G). As reported previously (24), R/G values start to increase when atmospheric oxygen concentration is lowered below ~2%. First, we determined the R/G value after PA in anaerobic cells. R/G was 0.175 ± 0.013 (SD) before PA (n = 10) and increased to 1.16 ± 0.25 (n = 10) after PA. R/G was unchanged when PA was performed at 20% oxygen (0.171 ± 0.015, n = 22). Cell density was determined by counting the number of cells using the phase-contrast optics.

In separate experiments, ΔΦm in individual cells was assessed using tetramethylrhodamine methyl ester (TMRM), a cell-permeant cationic fluorescent dye that accumulates in mitochondria according to ΔΦm. Cells on the CultureCoverGlass were incubated with 500 nM TMRM for 20 min at 37°C. Cells were washed twice with HEPES-Tyrode buffer and, after placement of a CG, equilibrated in the measuring cuvette to 3% oxygen for 1 h. TMRM fluorescence was determined at 605 nm.

All images were acquired using a 16-bit charge-coupled device camera (model SV512, PixelVision) attached to the microscope and cell density was determined by counting the number of cells using the phase-contrast optics.
are presented in negative gray scale. Image analyses were performed offline using IPLab for Mac (Scanalytics) software. Fluorescence intensities are presented as means ± SD. Differences in the mean between two groups were judged by Student’s t-test. Differences in the variance and the mean among three groups (see Fig. 7) were judged by Bartlett’s test and the Kruskal-Wallis test, respectively, followed by Scheffé’s F test. P < 0.05 was considered significant.

RESULTS

Visualization of oxygen gradients and hypoxic microenvironment in the two-dimensional tissue model. In the tissue model using Hep3B cells, at 3% oxygen in the measuring cuvette, R/G values increased with distance from the CG edge, reaching a plateau (the anaerobic level) at ∼500-μm from the oxygen source (control, Fig. 2), which implies a steep oxygen gradient with a magnitude of 0.04 mmHg/μm (∼0.8 mmHg/cell, with the assumption of a 20-μm cell diameter).

In vivo, HIF-1 may be induced in a hypoxic microenvironment. However, in the present tissue model, HIF-1 was not detected in the anaerobic regions beneath the CG within 1 h of equilibration (immunofluorescent imaging; data not shown). To address the effect of HIF-1 on the oxygen gradient, cells were pretreated with 1 mM DMOG for >6 h. We found that HIF-1 induction by DMOG (immunofluorescent imaging; data not shown) significantly decreased oxygen gradients in this tissue model (DMOG, Fig. 2). Half-maximal change in R/G (between aerobic and anaerobic R/G) took place 370 and 1,250 μm from the oxygen source in the control and DMOG-pretreated cells, respectively. According to Fick’s law of diffusion, a greater than threefold prolongation of the oxygen diffusion length implies that cellular oxygen consumption in

![Fig. 3. Changes in tetramethylrhodamine methyl ester (TMRM) fluorescence in Hep3B cells. At 3% oxygen in the measuring cuvette (outside the CG), TMRM fluorescence in cells far inside the CG edge (B) disappeared within 60 min, whereas that in cells just inside the CG edge (A) did not show a significant difference. Deep cells retained TMRM fluorescence at 60 min (C, right) when oxygen concentration in the measuring cuvette was 20%.](http://ajpcell.physiology.org/)

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DMOG-pretreated cells was less than one-third of that in control cells.

Visualization of the anoxic front in the two-dimensional tissue model. Because generation of $\Delta \Psi_m$ strongly relies on oxygen availability at cytochrome $c$ oxidase, we also visualized $\Delta \Psi_m$ in this tissue model to determine the remotest regions from the oxygen source at which diffusional oxygen delivery could maintain $\Delta \Psi_m$ [the anoxic front (AF)]. In the present tissue model, TMRM fluorescence in cells near the CG edge was virtually unchanged for 1 h (Fig. 3A), whereas mitochondria-specific TMRM fluorescence in cells far from the oxygen source (1,200 $\mu$m inside the CG edge) was completely lost within 1 h (Fig. 3B). When the oxygen concentration in the measuring cuvette was elevated from 3% to 20%, TMRM fluorescence in cells deep inside the CG remained intact (Fig. 3C). These results indicate that oxygen gradients in this tissue model significantly affect $\Delta \Psi_m$.

We then attempted to define AF in this model. Figure 4 demonstrates changes in TMRM fluorescence after the CG was placed on almost-confluent (90–100%) COS-7 cells. Within 30 min, TMRM fluorescence in the cells >500 $\mu$m from the CG edge started to lose the mitochondria-specific TMRM fluorescence pattern. At 60 min, these cells appeared to have completely lost mitochondrial TMRM fluorescence. In cells near the CG edge, the magnitude and the pattern of the fluorescence remained unchanged for $\geq$1 h. We then drew a curve virtually dividing cells with intact and abolished mitochondrial TMRM fluorescence (Fig. 4, bottom right) and designated it the AF. We calculated the average distance from the CG edge to the AF.

Figure 5 summarizes the AF in COS-7 and Hep3B cells. According to Fick’s law of diffusion, oxygen flux ($J_O_2$) is represented by

$$J_O_2 = K_O_2 A \Delta P_O_2 / T$$  \hspace{1cm} (1)

and

$$J_O_2 = n \dot{Q}_O_2$$  \hspace{1cm} (2)

where $K_O_2$ is Krogh’s diffusion constant for oxygen, $A$ is area, $\Delta P_O_2$ is oxygen partial pressure gradient, $T$ is maximum diffusion distance (i.e., the AF), $n$ is number of cells, and $\dot{Q}_O_2$ is average oxygen consumption rate in a cell. These equations yield

$$T = (K_O_2 / \dot{Q}_O_2) \Delta P_O_2 (n/A)^{-1}$$  \hspace{1cm} (3)

$$\Delta P_O_2 / T = (\dot{Q}_O_2 / K_O_2) (n/A)$$  \hspace{1cm} (4)

The data in Fig. 5 were approximated as functions of the cell density ($n/A$), according to these theoretical formulations. At a standard confluence in the present experiment (500 cells/mm$^2$), the predicted AFs were 590 and 540 $\mu$m for COS-7 and Hep3B cells, respectively. If it is assumed that oxygen is completely depleted at the AF, the predicted tissue oxygen gradients are 0.039 and 0.045 mmHg/$\mu$m for COS-7 and Hep3B cells, respectively. The oxygen gradients thus predicted from the TMRM fluorescence perfectly agree with the value determined by the green fluorescent protein (GFP) oxygen measurement mentioned above. Hence, these results suggest that the AF is determined primarily by the availability of oxygen.
Next, we examined the effects of PHD pathway activation by prolyl hydroxylase inhibition at the AF in the same model. As clearly demonstrated in Fig. 6A, pretreatment with 1 mM DMOG substantially prolonged the AF. At a standard confluence in the present experiment (500 cells/mm²), the predicted AFs were 1,115 and 1,734 µm for COS-7 and Hep3B cells, respectively (DMOG, Fig. 5, A and B). The predicted gradients of oxygen were 0.024 and 0.015 mmHg/µm for COS-7 and Hep3B cells, respectively (DMOG, Fig. 5, C and D). The 1.9-fold (COS-7 cells) and 3.2-fold (Hep3B cells) prolongations of the AF in DMOG-pretreated cells are not inconsistent with the shallow oxygen gradients in these cells demonstrated by the GFP oxygen measurement (Fig. 2) and still suggest a linkage between oxygen availability and \( \Delta \Phi_m \). Thus activation of the PHD pathway may protect \( \Delta \Phi_m \) in a hypoxic microenvironment.

Elimination of expanded AF in DMOG-pretreated cells by complex II inhibition. If a unique relationship exists between \( \Delta \Phi_m \) and oxygen availability at cytochrome c oxidase, then protection of \( \Delta \Phi_m \) by DMOG could be explained by down-regulation of mitochondrial respiration, which decreases tissue oxygen gradients and restores diffusional oxygen delivery to mitochondria in a hypoxic microenvironment. However, in addition to the oxygen availability, \( \Delta \Phi_m \) also depends on NADH supply and successive transport of electrons in the respiratory chain. Although the shallow oxygen gradient in DMOG-pretreated cells reflects decreased oxygen consumption, it also indicates that the rate of electron transport to cytochrome c oxidase is certainly decreased. With significantly decreased respiration (approximately one-third of that of non-DMOG-treated cells), mitochondria would not produce sufficient \( \Delta \Phi_m \), unless a mechanism that allows H⁺ extrusion across the inner membrane without oxygen consumption is presumed.

In the present study, we considered the possibility of mitochondrial electron transport without oxygen consumption (anaerobic respiration) in DMOG-pretreated cells. Specifically, we examined the role of complex II in the respiratory chain. We used a competitive inhibitor of ubiquinone binding to the quinone reduction site in complex II, thenoyltrifluoroacetone (TTFA). As clearly demonstrated in Fig. 6B, the prolongation of AF in DMOG-pretreated cells was completely reversed by 400 µM TTFA, whereas oxygen gradients determined by the GFP red shift were not affected (Fig. 2; half-maximal change in R/G occurred at \( \sim 1,330 \) µm). In Hep3B cells with densities of 400–600 cells/mm², the average AF in cells treated with 400 µM TTFA (609 ± 130 µm, \( n = 5 \)) was not different from
that in cells treated with 400 μM TTFA + 1 mM DMOG (635 ± 115 μm, n = 7).

Role of complex II in protecting ΔΨm. To highlight the role of mitochondrial complex II in the maintenance of ΔΨm, we repeated TMRM fluorescence measurements in Hep3B cells in which electron transport at and downstream of complex III was inhibited by 10 μM antimycin A (AMA). This experiment was carried out at 20% oxygen in the measuring cuvette without a CG. In the control cell, AMA completely abolished the mitochondria-specific TMRM fluorescence pattern (data not shown). Thus TMRM fluorescence (34% of the untreated cells) indicates only residual, nonspecific fluorescence (Fig. 7A). Pretreatment with 1 mM DMOG sustained TMRM fluorescence in the AMA-treated cells at 69% of that of the untreated cells. On the other hand, the effects of DMOG on TMRM fluorescence were completely abolished in cells treated with 400 μM TTFA (Fig. 7B). The fluorescence level (31% of the untreated controls) was comparable with that of the AMA-treated control cells. TTFA alone slightly, but significantly, decreased TMRM fluorescence (83%), whereas the mitochondria-specific pattern of TMRM fluorescence appeared intact (data not shown).

DISCUSSION

Hypoxia in solid tumors is not a simple manifestation of an insufficient supply of oxygen to meet cellular demand. Functionally, hypoxia endows tumor cells with distinct phenotypes through induction of a wide variety of genes where the PHD-HIF pathway plays a central role (19). Importantly, at the same time, tumor cells must survive and continue to produce energy sufficient for biosynthesis of proteins required for such genetic responses. Consequently, we predict mechanisms by which tumor cells can survive and produce ATP in a hypoxic microenvironment. While enhanced glycolysis is the major source of ATP in deep hypoxia, we sought, in the present study, complementary mechanisms that permit energy production even in glucose- and oxygen-deprived tissue cores.

Two novel techniques employed in the present study may be worth noting. 1) We used the red shift of GFP fluorescence to assess oxygen levels in individual cells. As demonstrated previously (24), this technique is sensitive to <2% oxygen and facilitates production of an oxygen map in monolayer-cultured cells. 2) This technique was combined with another novel (but very simple) technique for producing consistent oxygen gradients in monolayer-cultured cells (23). In the present study, by placing the CG on top of the cultured cell layer, we demonstrated gradients of oxygen with a magnitude of ~0.04 mmHg/μm in almost-confluent Hep3B cells. Thus, at 3% oxygen in the measuring cuvette, cells located under the CG center were anoxic, whereas oxygen supply to the cells near the CG edge was almost unlimited.

Fig. 6. Expansion of the AF in DMOG-pretreated Hep3B cells (A) and its reversal by an inhibitor of complex II (B). TMRM fluorescence images were acquired 60 min after a CG was placed on monolayer Hep3B cells. Oxygen concentration in the measuring cuvette was 3%. Cell densities were 536 and 619 cells/mm² for A and B, respectively. ⊠, edge of the CG. Oxygen diffuses from right to left. Circles indicate the AF.
HIF-1 (8, 21). Thus hypoxia stabilizes HIF-1 mitochondrial respiration secondary to DMOG induction of interpretation these findings to be results of downregulation of port, and redefined the hypoxic microenvironment (Fig. 2). We Hep3B), expanded the limitation of diffusional oxygen trans-

ration secondary to HIF induction should exert a significant impact on mitochondrial energy production. In normoxic cells, ΔΨm is generated at complexes I, III, and IV in mitochondria as electrons are transported in these enzymes. The rate of electron transport to the terminal respiratory enzyme, complex IV, defines mitochondrial oxygen consumption if oxygen supply is nonlimiting. In DMOG-pretreated cells, mitochondrial respiration may be actively suppressed by HIF, primarily through reductions in electron entry to the respiratory chain (13, 18). If that was the case, DMOG should have suppressed ΔΨm. This theory is not compatible with the interpretation that the shallow oxygen gradient per se expanded the AF in DMOG-pretreated cells. Rather, this pseudocoherence in DMOG-pretreated cells may favor a possibility that a complementary mechanism supports ΔΨm, even with decreased electron flux/oxygen consumption.

In the present study, we highlighted the role of complex II in the respiratory chain as the key to oxygen-independent electron transport. In normoxic mitochondria, complex II oxidizes succinate to fumarate as it reduces ubiquinone to ubiquinol. Ubiquinol is subsequently transported to complex III, where cytochrome c is reduced. Finally, reduced cytochrome c is used to convert molecular oxygen to water in complex IV. Distinct from the oxygen-coupled electron transport in normoxic mammalian mitochondria outlined above, fumarate respiration is a form of anaerobic respiration documented in various anaerobic eukaryotes, including freshwater snails, mussels, lugworms, and oysters (28). The mechanism of fumarate respiration for providing energy in deep hypoxia has been extensively studied, particularly in adult Ascaris suum (14). In this organism, electrons are transported from NADH to rhodoquinone in complex I and, subsequently, used to reduce fumarate to convert molecular oxygen to water in complex IV. Distinct from the oxygen-coupled electron transport in normoxic mammalian mitochondria outlined above, fumarate respiration is a form of anaerobic respiration documented in various anaerobic eukaryotes, including freshwater snails, mussels, lugworms, and oysters (28). The mechanism of fumarate respiration for providing energy in deep hypoxia has been extensively studied, particularly in adult Ascaris suum (14). In this organism, electrons are transported from NADH to rhodoquinone in complex I and, subsequently, used to reduce fumarate to succinate in complex II. With complex II functioning as an electron acceptor, this process enables H+ extrusion in complex I without the need for oxygen.

The present results are consistent with the hypothesis that ΔΨm in a hypoxic microenvironment may be supported by the reverse action of complex II (fumarate respiration). To specifically address the role of complex II, electron transport in complexes III and IV was abolished by AMA in Hep3B cells (Fig. 7). As expected, the mitochondria-specific TMRM fluorescence was almost completely eliminated, indicating that
is an attractive hypothesis from the standpoint of hypoxic mitochondrial membrane potential. of hypoxic-hypoglycemic conditions and functions to generate (DLD-1, Panc-1, and HepG2), that fumarate respiration pre-
Finally, Tomitsuka et al. (26) demonstrated, in mitochondria in the hypoxic microenvironment (Fig. 6). These results indicate that the pseudocoherence between diffusional oxygen transport and \( \Delta \Phi_m \) in DMOG-pretreated cells may be built by oxygen-independent electron transport to complex II. Thus mitochondrial energy production in the hypoxic microenvironment is supported by the PHD pathway, presumably through the activity of complex II as an electron acceptor. Recent studies suggest that fumarate respiration may be active in mammalian cells in specific conditions; Weinberg et al. (30) demonstrated, in isolated rabbit kidney proximal tubules, that the provision of fumarate by combined \( \alpha \)-ketoglutarate and aspartate supplementation significantly protects \( \Delta \Phi_m \) in hypoxia by anaerobic electron transport in complexes I and II. Sridharan et al. (21) demonstrated that DMOG inhibition of PHDs confers significant protection on \( \Delta \Phi_m \) in neonatal cardiomyocytes against metabolic inhibitions including cyanide treatment, whereas the protective effect was eliminated by complex I inhibition by rotenone. Sridharan et al. (22) further demonstrated, by direct measurement of the TCA cycle intermediates by HPLC-mass spectrometry, the accumulation of succinate during metabolic inhibition in DMOG-pretreated neonatal cardiomyocytes. Together, these results strongly indicate that reduction of fumarate to succinate in complex II is a prerequisite for \( H^+ \) extrusions in complex I in the absence of electron transport to cytochrome \( c \) oxidase. Finally, Tomitsuka et al. (26) demonstrated, in mitochondria isolated from hypoxic-hypoglycemic human cancer cell lines (DLD-1, Panc-1, and HepG2), that fumarate respiration predominates over the normoxic electron transport within 5 days of hypoxic-hypoglycemic conditions and functions to generate mitochondrial membrane potential.

Induction of anaerobic respiration in oxygen-deficient cells is an attractive hypothesis from the standpoint of hypoxic adaptation. If complex II, with the reverse-mode operation, supports \( H^+ \) extrusions in complex I, what would be the trigger for this seemingly unusual respiration? Inductions of fumarate respiration have been inferred in hypoglycemic and hypoxic tumor cells (26). Furthermore, pharmacological inhibition of PHD elicits protection over \( \Delta \Phi_m \) against hypoxia (present study) and metabolic inhibition (21, 22), presumably through fumarate respiration. Therefore, direct effects of PHD and/or PHD-regulated HIFs on the enzymes in complex II may be plausible (27). Furthermore, hypoxia or hypoxic induction of the PHD-HIF pathway appears to favor reverse complex II operation. First, in hypoxia, one of the TCA cycle products, NADH, accumulates in mitochondria, whereas HIF-1-induced pyruvate dehydrogenase kinase-1 decreases acetyl coenzyme \( A \) entry into the TCA cycle. These may stoichiometrically antagonize normal oxidative processes in the TCA cycle. Second, one of the end products of PHD-driven hydroxylation of proline residues in HIF-1\( \alpha \), succinate (12, 16), may play a role. Because prolyl hydroxylation continues in normoxic conditions, conversion of the cosubstrate 2-oxoglutarate to succinate and \( CO_2 \) also proceeds continuously. Thus, in normoxia, succinate may accumulate in the cytoplasm, and some of the succinate may subsequently be transported to the matrix space in the mitochondria via a mitochondrial dicarboxylate carrier (Slc25a10) (15). Finally, succinate in the matrix might drive complex II (forward mode). In hypoxia, lack of oxygen molecules upsets HIF-1\( \alpha \) prolyl hydroxylation and 2-oxoglutarate conversions to succinate. In the matrix space, decreases in the succinate level, if any, would favor the reverse action of complex II. Thus inhibition of PHD activity, not necessarily HIF-1 accumulation, might be a factor that promotes fumarate respiration. It remains to be determined, however, whether succinate associated with HIF \( \alpha \) prolyl hydroxylation significantly affects the TCA cycle anaplerosis and, eventually, electron transport in mitochondria.

In summary, we used a novel technique to visualize oxygen levels in a novel two-dimensional tissue model of the hypoxic microenvironment to demonstrate the critical dependence of \( \Delta \Phi_m \) on diffusional oxygen supply. In cells in which the PHD pathway is activated by DMOG, diffusional oxygen delivery was substantially enhanced, while AF expanded in a similar manner. The relationship between \( \Delta \Phi_m \) and oxygen delivery in DMOG-pretreated cells is, however, pseudocoherence, because the shallow oxygen gradients in these cells should require an auxiliary system for electron transport, independent of oxygen availability, to maintain \( \Delta \Phi_m \). We have provided evidence that complex II plays a substantial role in this anaerobic electron transport. In PHD pathway-activated cells, shallow oxygen gradients decrease heterogeneity and elevate oxygen levels within tissue volume, while sustaining \( \Delta \Phi_m \) by anaerobic respiration, presumably to prevent cell death following permeability transitions in the mitochondrial inner membrane.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

E.T. is responsible for conception and design of the research; E.T. and M.S. performed the experiments; E.T. analyzed the data; E.T. and M.S. interpreted the results of the experiments; E.T. prepared the figures; E.T. drafted the manuscript; E.T. edited and revised the manuscript; E.T. and M.S. approved the final version of the manuscript.

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