MITOCHONDRIAL COMPLEX II PREVENTS HYPOXIC BUT NOT CALCIUM AND PRO-APOPTOTIC BCL-2 PROTEIN-INDUCED MITOCHONDRIAL MEMBRANE POTENTIAL LOSS

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Mitochondrial membrane potential loss has severe bioenergetic consequences and contributes to many human diseases including myocardial infarction, stroke, cancer, and neurodegeneration. However, despite its prominence and importance in cellular energy production, the basic mechanism whereby the mitochondrial membrane potential is established remains unclear. Our studies elucidate that complex II-driven electron flow is the primary means by which the mitochondrial membrane is polarized under hypoxic conditions, and that lack of the complex II substrate succinate resulted in reversible membrane potential loss that could be rapidly restored by succinate supplementation. Inhibition of mitochondrial complex I and F₀F₁ ATP synthase induced mitochondrial depolarization that was independent of the mitochondrial permeability transition pore, B-cell lymphoma 2 (Bcl-2) family proteins, or high amplitude swelling and could not be reversed by succinate. Importantly, succinate metabolism under hypoxic conditions restores membrane potential and ATP levels. Further, a reliance on complex II-mediated electron flow allows cells from mitochondrial disease patients devoid of a functional complex I to maintain a mitochondrial membrane potential that conveys both a mitochondrial structure and the ability to sequester agonist-induced calcium similar to that of normal cells. This finding is important as it sets the stage for complex II functional preservation as an attractive therapy to maintain mitochondrial function during hypoxia.

Mitochondria are multi-functional organelles involved in calcium buffering (1-3), apoptosis (4-9), necrosis (10,11), reactive oxygen species (ROS) production (12-15) and nuclear stress signaling (16-18). However, despite their importance in a number of cellular processes, the primary function of mitochondria remains that of cellular energy production. Although limited energy can be derived from cytosolic enzyme systems, the vast majority of cellular ATP generation is generated by the mitochondrial electrochemical gradient and ATP synthase complex. Structurally, the inner mitochondrial membrane (IMM) and the outer mitochondrial membrane (OMM) divide the organelle into two well-defined compartments, the matrix and the intermembrane space, respectively. Protein complexes within the highly selective IMM facilitate energetically favorable electron transfer from metabolic substrates to the terminal acceptor oxygen. These protein complexes utilize the energy derived from this electron transfer to pump protons across the IMM through the F₀F₁ ATP synthase molecular motor then drives the generation of ATP, a process described as the chemiosmotic theory (19).

While the chemiosmotic theory of mitochondrial energy production is widely accepted, the basic mechanism in which the mitochondria establish the ΔΨₘ remains poorly understood. Recent evidence has suggested that stable mitochondrial supercomplexes exist in a...
number of organisms (20,21), including a large assembly composed of complexes I, III, and IV in bovine heart mitochondria (22). Experimentally, inhibition of complex I or complex IV leads to dissipation of the mitochondrial membrane potential and serves as an effective means to mimic the effect of hypoxia on mitochondrial function (23,24). In contrast to the organized electron flow by respiratory supercomplexes, mitochondrial complex II (succinate dehydrogenase) can function as an independent enzyme whose activity is limited only by substrate availability (25). However, inhibition of mitochondrial complex II also leads to mitochondrial depolarization (26,27) and mimics hypoxia in cells (28). As a result, the discrete roles of complex I and II in the establishment and maintenance of the $\Delta \Psi_m$ both under normoxic and hypoxic conditions are unclear.

Here, we demonstrate that mitochondrial respiratory complex II is more efficient than complex I in establishing and maintaining $\Delta \Psi_m$ under chemical poisoning and hypoxic conditions. In support, complex II-mediated metabolism can generate a functional $\Delta \Psi_m$ in complex I-compromised mitochondrial disease patients at a decreased respiratory rate, and acute delivery of the complex II substrate Succ can maintain $\Delta \Psi_m$ and ATP levels during hypoxic conditions. This finding suggests that complex II-driven electron flow is the principle mechanism whereby the $\Delta \Psi_m$ is established during hypoxic conditions.

**Experimental Procedures**

*Cell isolation and Cell Culture* – Rat pulmonary microvascular endothelial cells (RPMVECs) were cultured in DMEM supplemented with 10% FCS, nonessential amino acids and antibiotics and endothelial cell growth supplement (ECGS). Murine pulmonary microvascular endothelial cells (MPMVEC) were isolated from lungs as previously described (29) and were cultured in media identical to that of RPMVECs. Human pulmonary microvascular ECs were cultured in M199 supplemented with 15% FCS, L-glutamine, ECGS, and antibiotics. Murine embryonic fibroblasts (MEFs) from both WT and $\text{bax}^{-/-}\text{bak}^{-/-}$ knockout lines were a generous gift of Dr. Craig Thompson (Abramson Family Cancer Center, University of Pennsylvania, Philadelphia, PA, USA) and were cultured in DMEM supplemented with 10% FCS and antibiotics. C2C12 were obtained from ATCC and cultured in High Glucose DMEM supplemented with 10% FCS and antibiotics. S2 Cells were cultured in Schneider’s Drosophila Medium supplemented with 10% FBS, L-Glutamine, and antibiotics. Human control (CF9), Leber Optic Atrophy (LHON) and Kearns-Sayre Syndrome (KSS) untransformed fibroblasts were obtained from the Coriell Institute for Medical Research and cultured in DMEM supplemented with 15% FBS, L-Glutamine and antibiotics. Primary Murine cardiomyocytes were isolated according to the method of Mitra and Morad from freshly harvested hearts (30). Briefly, excised hearts were cannulated by the aorta and retrograde perfused with Ca$^{2+}$-free Tyrode solution plus collagenase B and D. Once the heart was flaccid and pale, they were removed from the perfusion system, the ventricles teased apart with forceps and gently titurated to dissociate individual myocytes. The myocytes were then strained through a 70 µm mesh, allowed to settle, and re-equilibrated through increasing concentrations of [Ca$^{2+}$] in Tyrode solution up to 1.2 mM.

**Mitochondrial Membrane Potential ($\Delta \Psi_m$) Measurement** – Prior to permeabilization, cells were washed in an extracellular-like Ca$^{2+}$-free buffer (120 mM NaCl, 5 mM KCl, 1 mM KH$_2$PO$_4$, 0.2 mM MgCl$_2$, 0.1 mM EGTA, and 20 mM HEPES-NaOH pH 7.4) and stored on ice for at least 10 min. Following centrifugation, cells were transferred to an intracellular-like medium (permeabilization buffer; 120 mM KCl, 10 mM NaCl, 1 mM KH$_2$PO$_4$, 20 mM HEPES-Tris pH 7.2, protease inhibitors (EDTA-free cOmplete® Tablets, Roche Applied Science), and 2 µM thapsigargin). Digitonin was added to the experimental buffer to a concentration of 40 µg/ml (80 µg/ml digitonin was used for S2 cells and primary cardiomyocytes). In some experiments, the medium was also supplemented with either succinate (2 mM) or malate/glutamate (1 mM each) or an ATP regenerating system composed of 100 mM ATP, 5 mM phosphocreatine, 5 units/ml creatine kinase. Suspensions of cells (6-10×10$^6$ cells for RPMVECs, MPMVECs, HPMEVCs, MEFs, and C2C12; 20×10$^5$ for S2; 5×10$^5$ for...
primary cardiomyocytes), equivalent to ~2 mg of protein, were placed in fluorimeter cuvettes and permeabilized by gentle stirring. Following 20 sec (3 min for cardiomyocytes), the fluorescent dye JC-1 was added at a concentration of 800 nM for RPMVECs, MPMVECs, HPMVECs, MEFs, and C2C12 cells. S2 and cardiomyocytes required 1.2 mM JC1. Fluorescence was monitored in a temperature-controlled (37°C) multiwavelength-excitation dual wavelength-emission spectrofluorometer (Delta RAM, PTI) using 490-nm excitation/535-nm emission for the monomeric form and 570-nm excitation/595-nm emission for the J-aggregate of JC1. Recombinant Mouse truncated Bid (tBid) (Caspase-8-cleaved) was obtained from R & D Systems, Minneapolis, MN. ΔΨm is shown as a ratio of the fluorescence of J-aggregate (aqueous phase) and monomer (membrane-bound) forms of JC1. Additions are noted in the figures. Tracings are indicative of the JC-1 ratio of at least 3 independent experiments. Table values are represented as Mean ± S.E.M. of 3 independent experiments.

Hypoxia Experiments – Experimental buffers and media were made as described previously (31). Briefly, medium was saturated with a mixture of 95% N2/5% CO2 for 2 hours. Cell medium was replaced with glucose-containing, serum-free hypoxic medium and the indicated mitochondrial substrates. The cationic potentiometric fluorescent dye tetramethylrhodamine ethyl ester perchlorate (TMRE; 100 nM) was added to the hypoxic medium after 4.5 hours and allowed to incubate for a further 30 min. After dye loading, cells were imaged using the Bio-Rad Radiance 2000 imaging system (Carl Zeiss MicroImaging, Inc., Thornwood, NY) equipped with a Kr/Ar-ion laser source with excitation at 568 nm using a Nikon TE3000 inverted microscope with a 60x oil objective. Upon changes in ΔΨm, TMRE dissociates from the mitochondria. TMRE fluorescent changes were determined by perinuclear masking of all cells in the field and corrected to background nuclear fluorescence. Results indicate mean fluorescence of 3-4 independent experiments. ATP levels were assessed by ApoGlow® Assay Kit (Cambrex Bio Science Rockland, East Rutherford, NJ) according to the manufacturer’s protocol. Data are represented as mean ± S.E.M. of 3 independent experiments.

Western Blotting – Cytochrome c release was determined via methods used to determine ΔΨm. Briefly, C2C12 cells were permeabilized by digitonin (40 µg/ml). Following additions, samples were collected, centrifuged, and the supernatant (cytosolic fraction) collected. Cell pellets were resuspended in RIPA buffer (Upstate Biotechnology, Inc.) containing protease inhibitor cocktail, lysed by vigorous vortexing, and respun. The supernatant (membrane fraction) was collected and gel-loading buffer was added to both the cytosolic and membrane fractions. Similarly, C2C12 cells were lysed for determination of HIF-1α stabilization. Equal amounts of protein were separated by PAGE (4-12% BisTris for cytochrome c and 3-8% TrisAcetate for HIF-1α), transferred to a nitrocellulose membrane, and probed with anti-HIF-1α (Julian Lum and Celesta Simon, Cancer Biology Department, University of Pennsylvania) and anti-cytochrome c (BD Bioscience) antibodies as previously described (8).

Oxygen Consumption – C2C12 cells (8x106) were permeabilized by 0.01% digitonin in ICM buffer and placed into a MT200A MitoCell chamber and oxygen consumption measured via a Clark-type electrode (Strathkelvin Instruments). After the chamber was sealed, the following successive additions were made under continuous stirring; Malate+Pyruvate (5 mM each), Rotenone (100 nM), Succinate (10 mM), Antimycin A (50 nM), TMPD (0.2 mM) +Ascorbate (5 mM), and the uncoupler CCCP (1 µM) in 1 min intervals via a Hamilton syringe. Rate of oxygen consumption was measured and is displayed as nmol es oxygen/min/8x106 cells.

Specific activity of mitochondrial complexes – The activity of complex I was determined by monitoring the change in absorbance of cytochrome c in the presence of NADH at 550 nm (King &Howard, Meth Enzymology, 1967). Cardiomyocytes were incubated in hypoxic phosphate buffered saline as prepared in the Hypoxic Experiments section. After 3 hrs, cells were permeabilized with 80 µg/ml digitonin in intracellular-like medium, the mitochondrial pellet resuspended in glycylglycine experimental buffer, and the complex I activity measured as cytochrome c reduction at 550 nm. Complex II
activity was determined following hypoxic exposure by determining the conversion of 2-(p-iodophenyl)-3-(p-nitropenyl)-5-phenyl tetrazolium (INT) to formazan. Briefly, digitonin-permeabilized cells in phosphate buffered saline were incubated with Succ and INT at 37°C. After 15 min, the reaction was arrested with 10% TCA and formazan extracted with ethyl acetate. The absorbance of ethyl acetate-dissolved formazan was determined in a spectrophotometer at 490 nm.

RESULTS

Mitochondrial Complex II restores ΔΨm in response to complex I and ATP synthase inhibition but not calcium, tBid and alamethicin. Mitochondrial depolarization is a key event in both apoptosis and necrosis. During both apoptotic and necrotic cell death, mitochondrial swelling via opening of the permeability transition pore (PTP) (10,11), pore formation by apoptotic B-cell lymphoma 2 (Bcl-2) proteins (8,32,33), and microbial agents (34) leads to membrane rupture and collapse of membrane potential. Though anti-apoptotic Bcl-2 proteins and PTP blockers prevent mitochondrial permeabilization and swelling, mitochondrial depolarization can still occur under a number of cellular conditions including hypoxia. In fact, inhibition of mitochondrial respiratory complexes and ATP synthase are commonly employed to mimic the cellular hypoxic response during normoxia (35).

To induce chemical hypoxia, permeabilized cells were challenged with the complex I inhibitor rotenone (Rot) along with the F0F1-ATPase inhibitor oligomycin (Oligo) to eliminate electron flow originating from complex I and prevent mitochondrial ATP consumption and proton extrusion from the matrix (36). The permeabilized system is based on the isolated mitochondrial suspension and was used previously to assess mitochondrial depolarization in response to Ca2+ or after activation of pro-apoptotic Bcl-2 proteins (8,37,38). The permeabilized system has several advantages over the mitochondrial isolation model in that mitochondria are suspended in a cytoplasmic rather an artificial milieu that mimics the whole cell model. In addition, this model system does not require time-intensive manipulation that may alter mitochondrial function, and eliminates the need for supplementation of various exogenous molecules.

Permeabilized cells were loaded with the ratiometric mitochondrial membrane potential indicator JC-1 and then treated with the Rot/Oligo combination to induce ΔΨm loss. Rot/Oligo-induced mitochondrial depolarization was not prevented by the PTP blocker cyclosporine A (CsA), but subsequent addition of the complex II substrate succinate (Succ) substantially restored ΔΨm (Fig. 1A). Pretreatment with CsA also did not prevent Rot/Oligo-induced ΔΨm loss, although it significantly blunted Ca2+-dependent mitochondrial depolarization via the PTP open even in the presence of Succ (Fig. 1B), indicating that Succ cannot prevent opening of the PTP. In parallel, Succ supplementation did not prevent ΔΨm loss triggered by the mitochondrial swelling-inducing agent alamethicin (Fig. 1C). Next, it was of interest to determine whether Rot/Oligo-induced ΔΨm loss is similar to mitochondrial depolarization caused by pro-apoptotic Bcl-2 family proteins. A logical assumption would be that the mitochondrial proton gradient can be maintained if complex III and complex IV are intact and cytochrome c is present to enable electron transfer from complex III to IV. During apoptosis, cytochrome c is released from the intermembrane space via a Bax/Bak-dependent mechanism that precedes ΔΨm loss (9,39-41). As shown in Fig. 1D, Rot/Oligo-induced ΔΨm loss is a Bcl-2 independent phenomenon. ΔΨm loss by the pro-apoptotic protein tBid, an activator of Bax and Bak, is not rescued by Succ. This observation suggests that cytochrome c is essential for normal ΔΨm maintenance initiated by complex II. Similar to other cell types, WT and bax-/-bak-/- double KO fibroblasts underwent Succ-reversible ΔΨm loss even in the presence of CsA (Fig. 1E, F). Our data suggests that Rot/Oligo-induced mitochondrial depolarization is neither an apoptotic (Bcl-2-mediated) nor a necrotic (PTP opening) phenomenon, and thus should not exhibit mitochondrial cytochrome c release. Unlike tBid (100 nM), Rot/Oligo-induced depolarization did not trigger cytochrome c release after 20 min exposure (Fig. 1G). These results demonstrate that Rot/Oligo induces ΔΨm loss by mechanisms not directly dependent upon Bcl-2 or PTP opening and suggests that ΔΨm might be controlled during hypoxia via the availability of specific metabolic substrates.
The complex II pathway preserves the membrane potential in the absence of complex I activity. As Succ afforded protection from ΔΨ_m loss during pseudo-hypoxia, we next chose to investigate the discrete role of complex II in ΔΨ_m maintenance. To assess the contribution of individual mitochondrial complexes to the ΔΨ_m, permeabilized rat pulmonary microvascular endothelial cells (RPMVECs) were supplemented with complex-specific mitochondrial substrates either alone or in combination with mitochondrial complex II substrate succinate (Succ), but not the complex I substrates malate and glutamate (Mal/Glut), effectively maintained ΔΨ_m (Fig. 2A). Succ also prevented ΔΨ_m loss even in the presence of the F_0F_1-ATPase inhibitor Oligo, which expectedly blocked the protective effect of ATP on ΔΨ_m maintenance (Fig. 2B). Further, the effect of Succ on ΔΨ_m maintenance was not affected by the complex I inhibitor Rot (Fig. 2C), excluding the possibility that Succ initiates proton pumping via the reverse flow of electrons through complex I (42), and was completely abolished by the non-metabolizable complex II competitive inhibitor malonate (Mao) (Fig. 2D). However, Succ did not offer protection from complex II-mediated ΔΨ_m loss in the presence of the inhibitors myxothiazol and antimycin A (Fig. 2E and F). In particular, antimycin A induced immediate ΔΨ_m loss even in the combined presence of Succ and ATP, which hyperpolarizes the membrane (Fig. S1A), suggesting that complex II requires the proton pumping capability of mitochondrial complex III in order to establish ΔΨ_m. This initial observation raises the possibility that complex II pathway could establish ΔΨ_m in the absence of complex I.

To further characterize the role of complex II in the ΔΨ_m, we next assessed whether reintroduction of the complex II substrate Succ could restore ΔΨ_m after complete dissipation of the proton gradient (ΔΨ_m loss). Following permeabilization-induced ΔΨ_m loss, cells were supplemented with either a combination of complex I substrates (Mal/Glut or Mal/Pyr) or Succ. In contrast to Mal/Glut or Mal/Pyr, addition of Succ resulted in dramatic ΔΨ_m restoration (Fig. 3A). Because complex I substrates were ineffectual at maintaining ΔΨ_m, we developed a rapid chemical means to further test the efficacy of complex II in mitochondrial function and mimic hypoxia in normoxic cells. Cells were supplemented with Mal/Glut and then ΔΨ_m loss was induced by a combination of Rot/Oligo, completely eliminating the contribution of complex I (via secondary ATP production as in Fig. 2A) as well as intermembrane space proton loss through F_0F_1-ATPase. Cells were then treated with the complex II substrate Succ and extramitochondrial ATP alone or in combination. This approach (Succ versus ATP) allowed us to distinguish between proton gradient maintenance by Succ or glycolytic ATP, and to directly monitor ΔΨ_m recovery. The chemical combination of Rot/Oligo triggered rapid ΔΨ_m loss, which could be reversed by supplementation with Succ in several cell types that we examined (mouse, rat, and human pulmonary microvascular endothelial cells (MPMVEC, RPMVEC, and HPMVEC, respectively), C2C12 myoblast, and S2 drosophila cells) (Fig. 3B, C, F, Fig. S1C; Suppl Table 1) in a dose-dependent fashion (Fig. S2). As in Fig. 1, complex III inhibition resulted in ΔΨ_m loss that could not be rescued by the proton-pumping capabilities of complex I alone (Fig. S1B). While ATP in combination with Succ produced an additive effect on ΔΨ_m, ATP alone did not reverse ΔΨ_m loss (Fig. 3B). The fatty acid β-oxidation end-product acetyl CoA is a major fuel in some tissues, and ultimately enters the citric acid cycle where it will increase all TCA cycle metabolites, including Succ and Mal. However, Rot/Oligo-induced ΔΨ_m loss was not affected by acetyl CoA supplementation (Fig. 3C). Addition of the citric acid cycle metabolite α-ketoglutarate also did not restore ΔΨ_m, despite being a precursor of Succ in the TCA cycle (Fig. 3C). In addition to Rot/Oligo, ΔΨ_m loss also occurred following addition of the complex II competitive inhibitor Malonate (Mao). Subsequent addition of the complex I substrates Mal/Pyr and their essential cofactor NAD+ did not restore ΔΨ_m even at a concentration of 10 mM (Fig. 3D), indicating a rapid effect of Succ in the establishment of the ΔΨ_m.
Although our data demonstrate the restoration of ΔΨₐ by Succ following ΔΨₐ loss, there is a possibility that restoration of ΔΨₐ by Succ is actually due to either a difference in substrate delivery or to the secondary generation of NADH from mitochondria. To address this issue, we simultaneously measured both ΔΨₐ and NADH generation in permeabilized cells. Inhibition of complex I by Rot rapidly increased cellular NADH levels but did not affect ΔΨₐ as detected by TMRE fluorescence. However, subsequent addition of Succ rapidly increased ΔΨₐ (as indicated by a decrease in TMRE as more dye sequesters into the mitochondria) without an appreciable effect on NADH generation (Fig. 3E). Conversely, Mal/Grpd did not increase ΔΨₐ when complex II was inhibited by Mao despite a robust increase in cellular NADH levels. The rapid increase in NADH levels following addition of complex I substrates to permeabilized cells indicates sufficient delivery to the mitochondria, excluding the possibility that are findings are due to differences in mitochondrial loading of complex I versus II substrates. Because Succ-mediated electron transfer relies on complex III (Fig. 2E and F), we next assessed whether bypass of complex II/III by delivery of the complex IV substrates tetramethyl-p-phenylenediamine (TMPD) and ascorbate (Asc) could preserve ΔΨₐ in response to a combined complex I/FₒF₁-ATPase inhibition (Rot/Oligo). TMPD/Asc supplementation only partially restored ΔΨₐ (Fig. 3F). However, unlike the dose-dependent response seen with Succ (Fig. S2), addition of a higher concentration of TMPD/Asc was toxic to mitochondria and ΔΨₐ maintenance.

Establishment of ΔΨₐ in human fibroblasts from patients with mitochondrial disorders. Mitochondria are the only organelles outside the nucleus that contain DNA (mtDNA) and the machinery for synthesizing RNA and proteins. Mitochondrial complexes I, III and IV and FₒF₁-ATPase are multi-subunit protein complexes derived from both the nuclear and mitochondrial genome. In contrast, complex II polypeptides are derived solely from nuclear DNA (43). Many maternally inherited mitochondrial diseases arise from mutations in mitochondrial complexes I, III and IV and FₒF₁-ATPase, including Kearns-Sayre syndrome (KSS) – which results from a 1.9-kb mtDNA deletion spanning both complex I (ND1, ND2, ND4, NDL, and ND6 gene products) and FₒF₁-ATPase (ATP6 and ATP8 genes) (44) and Leber hereditary optic neuropathy (LHON), which affects subunit 4 of complex I (43). Primary fibroblasts from these patients therefore constitute an ideal model to identify the unique role of complex II in ΔΨₐ maintenance. Cells derived from LHON (Fig. 2B) and KSS (Fig. 2C) patients exhibited a similar ΔΨₐ response to Rot/Oligo as control fibroblasts (Fig. 2A) that was reversed by Succ. Intact CF9, LHON, and KSS cells also exhibited a similar phenotype with a predominance of filamentous mitochondria (Fig. 4D, E, F) and displayed a similar ΔΨₐ that was established principally by mitochondrial complex II (Fig. 4G – J). Because Mao addition resulted in ΔΨₐ loss in all three cell types, this evidence also suggests that LHON and KSS cells do not utilize a compensatory mechanism to generate basal ΔΨₐ, but rather, all three cells establish ΔΨₐ predominately through a mitochondrial complex II dependent pathway. An intact membrane potential generated by complex II also allows for LHON and KSS cells to sequester cytosolic calcium in an identical pattern to that of CF9 control cells (Fig. 4K), indicating that ΔΨₐ-dependent mitochondrial function is largely intact and dependent upon establishment of the IMM proton gradient by complex II. However, despite the ability to generate an intact membrane potential, complex I deficiencies lead to a compensatory glycolytic shift (45) and a decrease in ATP generation (46). Accordingly, LHON cells consumed significantly less oxygen than both KSS and CF9 cells (Fig. 4L). The fact that the LHON phenotype exhibited less oxygen utilization than KSS cells was surprising considering only complex I was affected, and led us to consider the possibility that establishment of the ΔΨₐ would be possible by diverting cells into succinate-driven metabolism during reduced oxygen availability.

Complex II substrate supplementation restores mitochondrial function during hypoxia. Our results demonstrate that Complex II-dependent ΔΨₐ maintenance is dependent upon electron flow through mitochondrial complexes II-IV in the permeabilized system. Further, our studies with
mitochondrial disease patients demonstrate that electron flow initiated by complex II can establish an intact ΔΨₘ even when complex I is nonfunctional. However, once electrons pass through the mitochondrial respiratory chain to complex IV, molecular oxygen is required as a terminal acceptor. Because LHON cells can generate a ΔΨₘ using less oxygen, we next focused on the idea that under basal conditions, complex II-driven electron flow may predominate as it consumes less oxygen than electron flow originating at complex I. Sequential addition of specific mitochondrial complex substrates (10 mM total substrate for each addition) and their respective inhibitors revealed that Succ reestablished a higher ΔΨₘ than either complex I or complex IV (Fig. 5A). However, while ΔΨₘ restoration was higher following supplementation of Succ, complex II-specific oxygen consumption was less than that of both complex I and complex IV (Fig. 5B). These data confirm that complex II-driven electron flow requires less oxygen than electron flow originating from complex I, and led us to hypothesize that ΔΨₘ could be maintained during hypoxic conditions via Succ metabolism.

To assess establishment of ΔΨₘ during hypoxic conditions, we chose to utilize freshly isolated murine cardiomyocytes (Fig. S3) as these cells are a primary target of hypoxic damage in humans during myocardial infarction. Chemical inhibition of complexes I and IV (Rot/Oligo) induced rapid ΔΨₘ loss in cardiomyocytes that was similar to other cell types (Fig. 1, 2, 3) that could be reversed by Succ supplementation (Fig. 6A). To directly assess the relationship between ΔΨₘ loss and hypoxia, ΔΨₘ was assessed following cell permeabilization in hypoxic experimental buffer. Marked ΔΨₘ loss was observed when cells were permeabilized in this buffer (Fig. 6B), the hypoxic status of which was verified by probing for HIF-1α stabilization after 5 hr (Fig. 6C). Succ but not Mal/Pyr supplementation restored ΔΨₘ in response to hypoxia (Fig. 6B). While it is possible that the nominal effect of complex I substrates on ΔΨₘ maintenance during hypoxia could be due the inactivation or enhancement of specific mitochondrial complexes, no differences in the activity of either complex I or complex II were noted (Fig. 6D). Similar to the permeabilized cell model, intact C2C12 and freshly isolated cardiomyocytes subjected to hypoxic conditions (Fig. 6E) exhibited a significant reduction in ΔΨₘ that was effectively restored by supplementation with complex II but not complex I substrates in the hypoxic medium (Fig. 6E, F, G, H). Active mitochondrial complexes support the generation of an electrochemical gradient across the IMM, which is vital for the efficient production of ATP (33). We therefore analyzed the role of complex II-dependent ΔΨₘ restoration in the maintenance of ATP levels under hypoxia. While less than that during normoxia, Succ supplementation resulted in significant ATP level maintenance, consistent with the restoration of ΔΨₘ by Succ in response to hypoxia (Fig. 6I).

**DISCUSSION**

Mitochondrial depolarization via Bcl-2-mediated membrane permeabilization, activation of the PTP complex, and toxin-induced mitochondrial pore formation are features of not only apoptotic, but also necrotic cell death (33) and are intimately involved in organ dysfunction during ischemia/reperfusion injury (47). Subsequently, anti-apoptotic Bcl-2 family proteins and the PTP component cyclophilin D tightly regulate mitochondrial permeabilization and preserve the ΔΨₘ. Although apoptotic and necrotic mitochondrial depolarization has been rescued by synthetic small molecules such as cyclosporine A, ΔΨₘ loss following mitochondrial substrate deprivation was not preventable (Fig. 1). Therefore, our present investigation demonstrates another mode of mitochondrial depolarization that is independent of OMM permeabilization and PTP opening. It is clear from Fig. 1 that rotenone plus oligomycin causes mitochondrial depolarization that is prevented by the complex II substrate Succ. This mitochondrial depolarization does not require complex I activity, because it is abolished when succinate is present (Fig. 2). ΔΨₘ preservation is dependent on complex II, III and IV, because it is eliminated by inhibitors of both complex II and III (Fig. 2). ΔΨₘ is also partially preserved when complex IV substrate is present, which directly donates protons. Rot/Oligo-induced mitochondrial depolarization is furthermore unrelated to OMM permeabilization or PTP opening, because
cytochrome c is retained in the mitochondria even after mitochondrial depolarization (Fig. 1G).

In an attempt to decipher the differential roles of complex I and II in mitochondrial function during hypoxia, we chose to optimize our permeabilized cell experiments. Surprisingly, we found that $\Delta \Psi_m$ could be maintained using complex II rather than complex I substrates, and that blockade of complex II would lead to membrane depolarization. This observation intrigued us, and led to the development of additional studies that supported the idea that basal $\Delta \Psi_m$ was a complex II-driven process. Indeed, supplementation of mitochondrial membrane-permeable NAD$^+$ or an increase in the cellular availability of NADH did not result in repolarization of the mitochondrial membrane following complex II inhibition (Fig. 3D and E), suggesting that electron flow initiating at complex II can more readily polarize the mitochondrial membrane than electron transfer originating from complex I. As complex I substrates can partially restore membrane potential when all mitochondrial respiratory chain enzymes are functioning properly (Fig. 3A), our findings suggest that complex I may contribute to overall $\Delta \Psi_m$. However, complex I substrates Mal/Glut did not retain $\Delta \Psi_m$ when FoF$\gamma$-ATPase was inhibited by oligomycin (Fig. 1B), making it unlikely that our results are simply due to a decreased $\Delta \Psi_m$ during ATP production. Further, proton pumping by complex I alone is not sufficient to reestablish the $\Delta \Psi_m$ when the respiratory chain is stalled downstream at complex III (Fig. S1B). Succ was effective in establishing and maintaining $\Delta \Psi_m$ in fibroblasts from patients with dysfunctional complex I and IV, indicating that a polarized mitochondrial membrane (Fig. 4H) can be preserved even when ATP production is compromised (48).

Mammalian succinate dehydrogenase is an enzyme complex composed of four nucleic-encoded subunits; two membrane-spanning anchoring proteins, and two matrix subunits that constitute the catalytic subunit of the enzyme complex (49). Functionally, oxidation of succinate to fumarate allows for the entry of electrons into the mitochondrial respiratory chain through coenzyme Q. However, unlike complexes I, III, and IV, succinate dehydrogenase does not directly couple electron transfer with proton pumping into the intermembrane space. Rather, complex II utilizes the labile properties of coenzyme Q within the IMM to transfer electrons to complex III, which can then act as a proton pump to contribute to the $\Delta \Psi_m$. Therefore, it is interesting that our studies uncovered a greater reliance on complex II to generate a membrane potential, as it does not directly contribute to membrane depolarization. The Solid-State Model proposes that ordered electron flow occurs through static enzyme complexes (50), and that the interaction between complex I and III allows for the efficient transfer of electrons through coenzyme Q, a process known as channeling (25). In support, isolated respiratory supercomplexes composed of complex I, III, and IV can form functional respiratory units (21), indicating that coenzyme Q is fixed within the supercomplex. In contrast to the Solid-State Model, the Random Collision Model describes electron transfer facilitated by the interaction between small diffusible molecules (cytochrome c and coenzyme Q) and independent enzymes (51). As the majority of coenzyme Q is not bound to complex I but rather is found free in the membrane bilayer (52) where it may not participate in supercomplex-mediated electron flow, the IMM coenzyme Q pool has a much greater capacity to store electrons under succinate (Succ) rather than NADH-dependent metabolism. Our findings suggest that increasing the cellular availability of Succ may essentially saturate the inner mitochondrial membrane with partially reduced (ubisemiquinone) or fully reduced (ubiquinol) coenzyme Q. While it is possible that Succ-mediated reduction of the membrane pool may displace oxidized coenzyme Q (ubiquinone) within the complex I-III supercomplex, our studies conducted under either chemical (rotenone) or genetic (LHON) complex I inhibition make it unlikely that Succ-mediated metabolism would alter ubiquinone channeling between complex I and III via reverse electron flow. Rather, as complex III and IV can exist either as a supercomplex with complex I or as separate units (21), our results suggest that complex II-mediated saturation of the mitochondrial inner membrane with ubiquinone or ubiquinol establishes the $\Delta \Psi_m$ through the labile movement of coenzyme Q to complex III and IV. This work therefore
supports the hypothesis that basal $\Delta \Psi_m$ is established primarily through the Random Collision Model as opposed to the Solid-State Model under both normal and reduced oxygen tensions. However, the presence of stable mitochondrial supercomplexes indicates that Solid-State Model electron flow is important for mitochondrial function. We postulate that it is the establishment of the $\Delta \Psi_m$ by complex II that allows the efficient electron transport between complex I and III and enhances ATP generation.

An important finding of our work was that supplementation of the complex II substrate Succ could maintain $\Delta \Psi_m$ during hypoxia. During normoxic glucose metabolism, pyruvate enters the mitochondria and generates ATP through the TCA cycle with molecular oxygen as the terminal electron acceptor. As oxygen tension decreases, pyruvate is converted to lactate rather than enter the TCA cycle, reducing mitochondrial substrate availability and the ability to generate $\Delta \Psi_m$ (53). However, we found that supplementing cells with exogenous Succ could restore $\Delta \Psi_m$ at low oxygen tensions. Lacking an innate proton pumping capacity, complex II requires complex III in order to transfer electrons and initiate establishment of the $\Delta \Psi_m$, and previous studies have described intact complex II/III function during hypoxia (54). Indeed, it is this ability to continue mitochondrial enzymatic activity during hypoxia is a hallmark of cancer cell metabolism (55), and alterations in succinate dehydrogynase have been implicated in certain forms of cancer (56). Our findings demonstrate that, unlike the aberrant metabolism observed during cancer, acute Succ metabolism in normal cells can maintain a $\Delta \Psi_m$ and acute ATP production during hypoxia.

In summary, our study demonstrates that mitochondrial depolarization either by inhibition of mitochondrial complex I and ATP synthase or during hypoxia was prevented by the complex II substrate Succ. However, irreversible $\Delta \Psi_m$ loss through PTP opening or activation of pro-apoptotic Bcl-2 family proteins could not be prevented by Succ. This indicates an important role for establishing the $\Delta \Psi_m$ via electron flow initiated by complex II. Strategies employing complex II–dependent preservation of mitochondrial function during hypoxia might therefore, constitute a promising tool for the treatment of ischemia-associated organ dysfunction.

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FOOTNOTES

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The abbreviations used are: \(\Delta \Psi_m\), Mitochondrial Membrane Potential; PTP, permeability transition pore; KSS, Kearns-Sayre syndrome; LHON, Leber Hereditary Optic Neuropathy; RPMVEC, rat pulmonary microvascular endothelial cells; Succ, Succinate; Pyr, pyruvate; Mal, Malate; Glut, glutamate; TMPD, tetramethyl-p-phenylenediamine; OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; CsA, cyclosporine A; tBid, truncated Bid; MEF, mouse embryonic fibroblasts; DKO, double knockout; ICM, Intracellular medium; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; TMRE, tetramethylrhodamine ethyl ester perchlorate;

FIGURE LEGENDS

Fig. 1. Complex II cannot restore mitochondrial function following irreversible \(\Delta \Psi_m\) loss. (A) Complex I/\(F_0F_1\)-ATPase inhibition (Rot/Oligo) triggered \(\Delta \Psi_m\) that was reversed by Succ (10 mM) but not the PTP blocker CsA (5 \(\mu\)M). (B) In contrast, Ca\(^{2+}\)-induced mitochondrial depolarization (200 \(\mu\)M) is blocked by CsA but not Succ. (C) Succ-dependent \(\Delta \Psi_m\) maintenance is abolished by mitochondrial swelling triggered by the microbial antibiotic alamethacin (20 \(\mu\)g/ml). Note: (B) and (C) were performed in the presence of 2 mM Succ. (D) Succ did not restore \(\Delta \Psi_m\) in response to Bcl-2 family protein tBid-induced (10 \(\mu\)g/ml) depolarization. Succ restored \(\Delta \Psi_m\) in both (E) wild type and (F) bax\(^{-/-}\)bak\(^{-/-}\) double-deficient murine embryonic fibroblasts following \(\Delta \Psi_m\) loss. (G) Mitochondrial cytochrome c release from the mitochondria in response to Ca\(^{2+}\), tBid, and the inhibitor combination of Rot/Oligo.

Fig. 2. Mitochondrial complex II establishes the membrane potential in permeabilized cells. Succinate (Succ; 2 mM) but not malate/glutamate (Mal/Glut; 1 mM/1 mM) maintained \(\Delta \Psi_m\) in digitonin-permeabilized RPMVECs under (A) control cells and (B) in the presence of the \(F_0F_1\)-ATPase inhibitor
oligomycin (Oligo; 20 µg/ml). ATP (100 µM) was effective only in the absence of oligomycin. (C) Complex I inhibition by rotenone (Rot; 20 µM) does not affect Succ-mediated \( \Delta \Psi_m \) maintenance but (D) the complex II inhibitor malonate (Mao; 2 µM) abolished Succ-dependent \( \Delta \Psi_m \). Succ afforded no protection from complex III inhibition by (E) myxothiazol (5 µM) or (F) antimycin A (20 µM). Arrows indicate the addition of different molecules at various intervals. The mitochondrial uncoupler CCCP was added at the indicated time to dissipate the membrane potential.

**Fig. 3.** Complex II substrate restores \( \Delta \Psi_m \) during complex I and \( \text{F}_0\text{F}_1\)-ATPase inhibition. (A) Differential effects of Complex II (Succ; 10 mM) and Complex I (Mal/Glut; 5 mM/5 mM, Mal/Pyr; 5 mM/5 mM) substrates on reestablishment of \( \Delta \Psi_m \) in murine myoblast (C2C12) cell line. (B) \( \Delta \Psi_m \) depolarization caused by complex I and \( \text{F}_0\text{F}_1\)-ATPase inhibition (Rot/Oligo; 20 µM/20 µg/ml) could be reversed by Succ (10 mM) in MPMVECs. (C) The citric acid entry substrate Acetyl CoA (AcetylCoA; 5 mM), and the Succ precursor \( \alpha \)-ketoglutarate did not restore \( \Delta \Psi_m \) in HPMVECs. (D) Mao-induced \( \Delta \Psi_m \) depolarization was not reversed by combination of the complex I substrates Mal/Pyr (5 mM/5 mM) and the cofactor NAD\(^+\) (100 µM). (E) An increase in NADH production in response to Mal/Glut (5 mM/5 mM) did not restore \( \Delta \Psi_m \) in response to complex II inhibition by Mao (2 mM). (F) The complex IV substrates TMPD/Asc do not effectively reestablish \( \Delta \Psi_m \) following Ros/Oligo-induced \( \Delta \Psi_m \) loss. Oligo was added at 80 sec in B, C, D, and F. Arrows indicate the addition of different molecules at various intervals.

**Fig. 4.** Establishment of \( \Delta \Psi_m \) in human fibroblasts from patients with mitochondrial disorders. (A) Control human fibroblasts (CF9) exhibit \( \Delta \Psi_m \) loss in response to Rot/Oligo that is reversed by Succ (10 mM). Similar responses were observed in human clinical samples from (B) LHON and (C) KSS patients. (D) CF9 control and (E) LHON and (F) KSS patient samples display a similar mitochondrial morphology as determined by the cationic dye TMRE. Mitochondrial depolarization, as detected by the presence of TMRE in the nucleus, occurs in response to Mao (5 mM) but not Rot (20 µM) in (G) CF9, (H) LHON, and (I) KSS cells. CCCP was used as a positive control. (J) Quantitation of nuclear TMRE fluorescence increase in response to Rot, Mao, and CCCP in CF9, LHON, and KSS cells. Values indicate mean ± s.e.m. (n=3). (K) Mitochondrial Ca\(^{2+}\) uptake is similar in all cell types in response to the protease-activated receptor agonist peptide TRAP (25 µM). (L) Whole cell mitochondrial oxygen consumption of CF9, LHON, and KSS cells (8x10\(^6\)). Stopper indicates when the oxygen consumption chamber was sealed. Sodium Azide (NaN\(_3\)) was used to inhibit cellular oxygen consumption at complex IV as a positive control. Values indicate oxygen consumption as nmols oxygen/min/8x10\(^6\) cells (mean ± s.e.m.; n=3).

**Fig. 5.** Differential effects of complex-specific metabolism on \( \Delta \Psi_m \) and oxygen consumption. (A) Successive assessment of the relative changes in \( \Delta \Psi_m \) following addition of the complex I substrates Mal/Pyr (inhibited by Rot), the complex II substrate Succ (inhibited by Myx), and the complex IV substrates TMPD/Asc. Values represent the mean value (±s.e.m.) of 10 data points immediately prior to addition of the respective inhibitor (n=4). (B) Oxygen measured via a Clark-type electrode following successive additions as indicated in permeabilized cells. Rate of oxygen consumption for complex II was less than both complexes I and IV. Rate of oxygen consumption was measured and is displayed as nmols oxygen/min/6x10\(^6\) cells (mean±s.e.m.; n=12). The trace is a single experiment that is representative of mean oxygen consumption.

**Fig. 6.** Preservation of mitochondrial function and energy metabolism by Succ-mediated metabolism during hypoxia. Both (A) chemical- and (B) hypoxic-induced \( \Delta \Psi_m \) loss is reversed by Succ (10 mM) but not Mal/Pyr (5 mM/5 mM) in freshly isolated permeabilized, mature murine cardiomyocytes and C2C12 cells, respectively. (C) Hypoxic status was confirmed by HIF-1α stabilization in C2C12 cells following 5
hr of hypoxia (NS; non specific). (D) Primary adult cardiomyocyte mitochondrial complex I and complex II activity were unaltered during hypoxia. (E) Intact C2C12 or (F) freshly isolated adult cardiomyocytes were subjected to hypoxic conditions for 5 hr and cells were loaded with the ΔΨ\textsubscript{m} indicator TMRE. Normoxic and Succ-supplemented hypoxic cells retained ΔΨ\textsubscript{m}. In contrast, hypoxia alone or supplemented with Mal/Pyr caused mitochondrial depolarization. (F) and (G) Average fluorescence intensity of mitochondrial TMRE retention as described in (F) and (G) following normoxic and hypoxic conditions. (H) ATP level maintenance by Succ supplementation in response to hypoxia. All values in D, G, H, and I indicate mean ± s.e.m. (n=3).
Figure 1
Figure 2

A. ATP

B. ATP

C. ATP

D. MCC

E. MCC

F. MCC
Figure 3

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Figure 5

A

\[ \Delta \Psi_m (R_{JC1}) \]

C2C12

\[ 1.80 \pm 0.38 \]

\[ 4.50 \pm 0.11 \]

\[ 0.88 \pm 0.08 \]

Time (sec)

B

\[ [\text{Oxygen}] \text{ (nmole/ml)} \]

\[ 31.6 \pm 0.008 \]

\[ 20.7 \pm 0.004 \]

\[ 157.0 \pm 0.01 \]

Mal/Pyr  Rot  Succ  Anti A  TMPD/Asc  Mal/Pyr  Rot  Succ  Anti A  TMPD/Asc  Mal/Pyr  Rot  Succ  Anti A  TMPD/Asc
Mitochondrial complex II prevents hypoxic but not calcium and pro-apoptotic BCL-2 protein-induced mitochondrial membrane potential loss

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