

Molecular Mechanisms of Ischemia–Reperfusion Injury in Brain: Pivotal Role of the Mitochondrial Membrane Potential in Reactive Oxygen Species Generation

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Received: 30 April 2012 / Accepted: 27 August 2012 / Published online: 26 September 2012
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Abstract Stroke and circulatory arrest cause interferences in blood flow to the brain that result in considerable tissue damage. The primary method to reduce or prevent neurologic damage to patients suffering from brain ischemia is prompt restoration of blood flow to the ischemic tissue. However, paradoxically, restoration of blood flow causes additional damage and exacerbates neurocognitive deficits among patients who suffer a brain ischemic event. Mitochondria play a critical role in reperfusion injury by producing excessive reactive oxygen species (ROS) thereby damaging cellular components, and initiating cell death. In this review, we summarize our current understanding of the mechanisms of mitochondrial ROS generation during reperfusion, and specifically, the role the mitochondrial membrane potential plays in the pathology of cerebral ischemia/reperfusion. Additionally, we propose a temporal model of ROS generation in which posttranslational modifications of

key oxidative phosphorylation (OxPhos) proteins caused by ischemia induce a hyperactive state upon reintroduction of oxygen. Hyperactive OxPhos generates high mitochondrial membrane potentials, a condition known to generate excessive ROS. Such a state would lead to a “burst” of ROS upon reperfusion, thereby causing structural and functional damage to the mitochondria and inducing cell death signaling that eventually culminate in tissue damage. Finally, we propose that strategies aimed at modulating this maladaptive hyperpolarization of the mitochondrial membrane potential may be a novel therapeutic intervention and present specific studies demonstrating the cytoprotective effect of this treatment modality.

Keywords Brain · Reactive oxygen species · Ischemia · Reperfusion · Mitochondria · Oxidative phosphorylation

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Background: Neuropathology of Reperfusion Injury

Brain ischemia/reperfusion results in extensive injury and is a substantial medical burden because of the ensuing morbidity and mortality. In adults, ischemic insults to the brain typically result from stroke (caused by either thrombotic occlusion or rupture of a blood vessel) or cardiac arrest while, in infants, cerebral ischemia is initiated by complications during labor and delivery, resulting in neonatal hypoxic–ischemic encephalopathy. In both cohorts, restoring blood flow and thus reestablishing nutrient and oxygen delivery to the ischemic brain will undoubtedly salvage neurons. However, reperfusion itself causes additional, substantial brain damage termed “reperfusion injury.” The impact of cerebral ischemia/reperfusion injury on patient mortality is sizable irrespective of age or etiology.

Stroke

Stroke is the third leading cause of death and disability among Americans [1]. Strokes are most commonly ischemic in origin, caused by vascular obstruction in the cerebral circulation. If diagnosed in a timely manner, ischemic stroke can be reversed by administration of thrombolytic agents or, alternatively, the obstruction can be physically removed. Neurons that lie distal to the obstructed vessel, relying exclusively on blood supply from this vessel, die from the prolonged complete ischemia. These neurons comprise the infarcted region of the brain that is termed the “ischemic core.” Such neurons never regain function upon restoration of blood flow and are dead prior to any window of therapeutic intervention. Of greater clinical interest are the populations of neurons that die in a delayed manner after reperfusion is initiated. These neurons, surrounding the ischemic core—referred to as the “penumbra”—are in part perfused by collateral blood flow (i.e., are not fully reliant on blood flow from the obstructed vessel) and are therefore more resistant to ischemic damage. Although neurons of the penumbra do not succumb to the initial ischemia-induced cell death, they go on to die during reperfusion in a delayed manner, resembling an apoptotic pathway. The delay in death of these cells offers a window for therapeutic intervention, thus it is critical that treatment of the penumbra be considered prior to recanalization of the obstructed vessel.

Cardiac Arrest/Resuscitation

Another leading cause of brain ischemia/reperfusion injury is cardiac arrest followed by resuscitation. In contrast to a focal brain insult caused by stroke, cardiac arrest results in complete ischemia throughout the entire brain. The brain is an extremely sensitive organ, hence even short durations of global ischemia (beyond 10 min) can result in debilitating neurologic deficits [2]. This sensitivity to ischemia may result from reliance on oxidative phosphorylation (OxPhos) for energy production, a concept we will address in detail in subsequent sections.

Successful resuscitation can rapidly restore blood flow and oxygen delivery to the body, including the brain. While approximately 70,000 patients are resuscitated from cardiac arrest each year, only ~10–35 % of resuscitated patients survive to hospital discharge, likely as result of the severe brain damage caused by cerebral ischemia [2–4]. Whereas stroke results in a cerebral infarct, resuscitation from cardiac arrest results in neuronal death in select cell populations that are most sensitive to ischemic injury. The most sensitive populations of neurons, the CA1 hippocampal neurons [5, 6], die during the first days of reperfusion [7, 8]. The specific biochemical events that result in delayed neuronal death continue to be elucidated; however, overwhelming

evidence has identified reactive oxygen species (ROS) generation as a key damaging event that leads to death of neurons [9–12].

Neonatal Hypoxic–Ischemic Encephalopathy

Ischemic insults to the infant brain cause hypoxic–ischemic encephalopathy (HIE), which leads to long-term neurocognitive deficits including cerebral palsy and epilepsy [13]. With an occurrence of approximately 2–4 per 1,000 full-term in-hospital deliveries, HIE is a serious medical concern. The consequences of HIE are more severe among low birth weight and premature newborns [14, 15]. Various antepartum causes of HIE have been identified including maternal hypotension and fetal growth restriction, yet the most prevalent cause is prolapse or compression of the umbilical cord and placenta abruption [16–18]. There is extensive heterogeneity in neuropathology following HIE, primarily due to variation in the etiology and severity of the hypoxic/ischemic events [19]. However, as in the adult, reperfusion undoubtedly contributes significantly to the overall pathologic progression of HIE and thus is of therapeutic interest.

A Mitochondrial Perspective on Reperfusion Injury

Mitochondria have long been known to play a critical role in the pathogenesis of cerebral ischemia/reperfusion injury, via ROS generation, mitochondrial failure or dysfunction, and mitochondrial (type II) apoptosis. In fact, during brain ischemia/reperfusion, evidence exists for the three above deleterious events occurring within the mitochondria (see [20, 21]). In this review, we present a common link between these three events in mitochondrial pathologies seen during stroke, cardiac arrest, and HIE. In the “[Model of Ischemia/Reperfusion Injury](#)” section we extend this concept to unify these three events into a *novel paradigm that positions OxPhos as a central linchpin in the initiation and execution of cell death caused by ischemia/reperfusion*. However, before presenting this model of reperfusion injury, it is critical that we discuss: (1) how the OxPhos system functions, (2) the mechanism by which OxPhos generates and controls the mitochondrial membrane potential ($\Delta\Psi_m$), (3) control of OxPhos by phosphorylation, and finally (4) the role of the $\Delta\Psi_m$ in ROS generation.

The Electron Transport Chain and Oxidative Phosphorylation

The mitochondrial electrochemical gradient or proton motive force (Δp_m) is generated and utilized by the OxPhos system, composed of the electron transport chain (ETC) and ATP synthase. A primary function of the ETC is to execute

the transfer of electrons to the final electron acceptor O_2 . The molecular machinery that comprises the ETC includes two major sites for electron entry, complex I (NADH dehydrogenase), and complex II (succinate dehydrogenase). Electrons donated to either of these protein complexes are transferred to complex III (bc_1 complex) via the nonprotein electron carrier ubiquinone. Electrons are transferred from complex III to complex IV (cytochrome c oxidase, CcO) via the electron carrier cytochrome c (Cyt c). CcO catalyzes the final and proposed rate-limiting step in electron transfer: the donation of electrons to O_2 , allowing conversion of O_2 and H^+ to H_2O . The energy stored in the electrons is stepwise extracted by complexes I, III, and IV, which couple electron transfer along the ETC to pumping of H^+ across the inner mitochondrial membrane. This pumping of protons across the inner mitochondrial membrane constitutes the largest contributing force of the mitochondrial membrane potential ($\Delta\Psi_m$), representing the overall charge difference across the inner mitochondrial membrane (see “The Proton Motive Force and Mitochondrial Membrane Potential” section). In addition, these chemical protons that are taken up from the matrix site contribute to the generation of Δp_m . Finally, Δp_m is utilized by ATP synthase (complex V), which drives the conversion of ADP and Pi to ATP (Fig. 1, *normal oxidative phosphorylation*). This process provides the vast majority (90 %) of ATP in the brain under normal conditions.

Under nonstressed conditions, the transfer of electrons is a tightly regulated process. In fact, the vast majority of electrons that are donated to the ETC complete the entire reaction, culminating in reduction of O_2 . However, a small percentage of electrons escape the ETC and can react with O_2 to form superoxide (O_2^-), a potent ROS. The specific sites of ROS generation along the ETC are complexes I and III. Although CcO (complex IV) produces several radical intermediates

during the reduction of O_2 , no electrons are allowed to escape; as a result, CcO does not directly produce ROS. Under normal conditions, endogenous antioxidant systems are sufficient to scavenge the modest amounts of ROS generated and prevent cellular damage. However, under pathologic conditions, antioxidants become overwhelmed or exhausted, allowing the unopposed and uncontrolled production of ROS and resultant ROS-initiated damage to cellular proteins, lipids, nucleic acids, and polysaccharides in an indiscriminate fashion [22–26]. To fully understand how ROS generation occurs and how this is controlled in a normal physiologic context, an appreciation of Δp_m and its primary component, the mitochondrial membrane potential $\Delta\Psi_m$, is required.

The Proton Motive Force and Mitochondrial Membrane Potential

Δp_m consists of two components: (1) an electrical constituent, simply referred to as the mitochondrial membrane potential $\Delta\Psi_m$, and 2) a chemical constituent, the pH difference across the inner mitochondrial membrane. Their relationship is defined as $\Delta p_m = \Delta\Psi_m - 59 \Delta pH$. The electrical component, $\Delta\Psi_m$, represents the major portion of Δp_m in higher organisms.

In the traditional view, Δp_m (and $\Delta\Psi_m$) are determined by two basic components, substrate availability and respiratory control, which both act on the OxPhos complexes. The most basic means of mitochondrial OxPhos regulation is dependent on both availability of substrate (e.g., NADH, O_2 , ADP, Pi) and its product, ATP. ATP and ADP are allosteric inhibitors and activators of CcO, respectively, and this control mechanism was proposed to adjust ETC activity to energy demand [27, 28]. Another major OxPhos regulatory mechanism is provided by Δp_m itself, and is

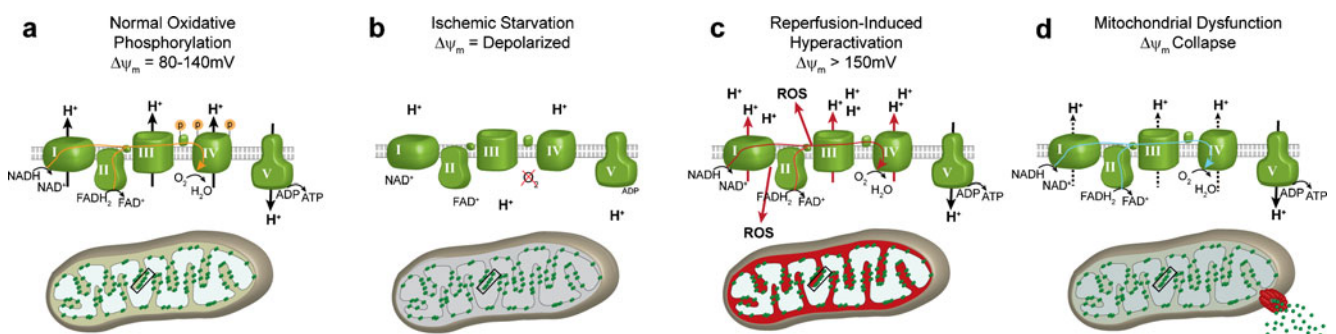


Fig. 1 Progression of ischemia/reperfusion injury. **a** Under normal, nonstressed conditions, OxPhos components are phosphorylated (as illustrated for Cyt c and CcO), promoting controlled electron transfer and maintaining the $\Delta\Psi_m$ in the 80–140 mV range. This respiratory state is conducive to maximal ATP production and minimal ROS generation. **b** Ischemia induces a starvation state where the ETC cannot function due to lack of O_2 . Dephosphorylation of OxPhos during ischemia renders these enzymes “primed” for hyperactivity, however they cannot operate due to lack of the terminal substrate for respiration,

O_2 . **c** Reintroduction of O_2 with reperfusion initiates electron transfer, proton pumping and ATP synthesis. However, in this hyperactive (dephosphorylated) state, OxPhos hyperpolarizes the $\Delta\Psi_m$, causing an exponential increase in ROS generation at complexes I and/or III. **d** This burst in ROS can act as a signal for triggering apoptosis. In addition, damage caused by ROS induces a mitochondrial dysfunction state, with reduced electron transfer kinetics and reduced $\Delta\Psi_m$ levels, which results in energetic failure

called respiratory control, as was demonstrated in isolated mitochondria more than five decades ago [29]. Respiratory control is a mechanism by which Δp_m causes inhibition of the ETC proton pumps when the proton gradient exceeds a threshold value, preventing further proton pumping at high Δp_m levels. When ATP synthase converts ADP to ATP by utilizing the proton gradient, the reduction in Δp_m allows the proton pumps (i.e., complexes I, III, and IV) to resume electron transfer and to pump protons across the inner membrane. In resting mitochondria, when the vast majority of ADP has been converted into ATP, synthesis of ATP slows and Δp_m increases, inhibiting the proton pumps and thus mitochondrial respiration. This feedback mechanism pairs the ETC activity to $\Delta\Psi_m$ and serves to maintain $\Delta\Psi_m$ at physiologic levels of 80–140 mV—a range in which ATP production is efficient and ROS generation is minimal.

Due to the difficulty of measuring absolute Δp_m in intact cells, most publications report $\Delta\Psi_m$ values, which constitute the majority of Δp_m . $\Delta\Psi_m$ can be monitored in living cells using voltage-dependent fluorescent probes such as the rhodamine dye TMRE (tetramethylrhodamine ethyl ester), and changes in fluorescence indicate relative changes in $\Delta\Psi_m$. Absolute $\Delta\Psi_m$ levels in millivolts can also be determined in isolated mitochondria by measuring the distribution of a membrane permeable cation such as tetraphenylphosphonium (TPP) with a TPP-sensitive electrode. In addition, absolute millivolt values for $\Delta\Psi_m$ can be determined in live cells by monitoring the redox states of the redox centers in bc_1 complex, thus allowing the precise calculation of $\Delta\Psi_m$ [30].

Since $\Delta\Psi_m$ can be measured readily, studies investigating $\Delta\Psi_m$ revealed an important difference of $\Delta\Psi_m$ levels observed in isolated mitochondria versus intact cells. In isolated mammalian mitochondria from liver and brain under state 4 conditions, $\Delta\Psi_m$ values were measured ≥ 150 mV, often exceeding 200 mV [31–38]. In contrast, the majority of studies performed in a more physiological context with a variety of intact mammalian cells or even intact organs showed lower $\Delta\Psi_m$ values in the range of 80 to 140 mV [39–43], with few studies reporting higher values between 140 and 161 mV [41, 43–47]. This discrepancy may be explained by differences in the regulation of OxPhos activity in higher organisms.

Respiratory control has traditionally been viewed as a key regulator of OxPhos. While this may be correct for OxPhos systems in bacteria, it appears that, in eukaryotes, additional regulatory mechanisms are in place. This idea is further supported by the fact that OxPhos enzymes are more complex in higher organisms. For example, CcO from bacteria contains 2 to 4 subunits whereas the mammalian enzyme is composed of 13 subunits per monomer and functions as a dimer, suggesting divergence with enhanced regulation [48, 49]. Although some differences between studies may be explained by different experimental

conditions and the use of cells from different species and tissues, the emerging picture is that $\Delta\Psi_m$ values in isolated mitochondria are higher compared to those in intact cells. Explanations for this discrepancy and the consequences of high $\Delta\Psi_m$ values will be discussed in the next two sections.

OxPhos Is Regulated by Reversible Phosphorylation

Higher $\Delta\Psi_m$ values observed in isolated mitochondria compared to intact cells suggest that the isolation procedure per se may induce modifications resulting in readings above the true physiologic range. Importantly, all mammalian OxPhos complexes are phosphorylated in vivo (reviewed in [50]), and we propose that these phosphorylations may be lost during traditional mitochondrial isolation. A recent study analyzing mitochondrial morphology and function showed that the structure of isolated mitochondria is clearly different compared to the morphology found in vivo [51]. The authors further demonstrated an approximately twofold increase in CcO activity. Calcium is a buffer component used in some traditional protocols to purify mitochondria, and is a highly potent physiological second messenger and activator of mitochondrial function [52]. Calcium was shown to trigger dephosphorylation of most mitochondrial proteins [53], which may be mediated by calcium-dependent phosphatases as we and others have postulated [50, 54, 55]. A similar scenario likely takes place during ischemic stress, and this will be the focus of the “Model of Ischemia/Reperfusion Injury” section.

We propose that phosphorylation of OxPhos complexes is a critical regulatory mechanism in higher organisms to maintain healthy respiration rates and to prevent hyperpolarization of $\Delta\Psi_m$. Using novel methods of mitochondrial and OxPhos protein isolation that preserve protein phosphorylation sites [56], we and others found that Cytc and CcO were reversibly phosphorylated at multiple residues [57–59]. Moreover, phosphorylation of these proteins altered their electron transfer kinetics and affected allosteric regulation by ATP and ADP [57]. Phosphorylation of Cytc at either Tyr48 and Tyr97 caused reduced reaction rates with CcO, and is proposed to lead to normal physiologic electron transfer rates [60–62]. In all tissue types investigated, Cytc was normally present in both the phosphorylated and dephosphorylated state. Upon cellular stress (specifically, cerebral ischemia), the enzyme is rapidly dephosphorylated [63]. Interestingly, activation of cell signaling cascades that promote cell survival, such as insulin signaling [64], induces Cytc phosphorylation [63]. We also identified a residue on CcO that is reversibly phosphorylated (Tyr304), leading to inhibition of CcO [54]. Thus, dephosphorylation of CcO results in higher basal respiration.

We further propose that mitochondrial isolation procedures or cellular stress, including ischemia, alters the physiological phosphorylation state of the OxPhos complexes.

This concept is supported by a study demonstrating hypoxia-induced changes in CcO phosphorylation in the heart [65]. In our model, phosphorylation of OxPhos proteins induces a healthy respiratory state where $\Delta\Psi_m$ values >140 mV inhibit further proton pumping, maintaining the 80–140 mV range where ROS production is minimal. In contrast, cellular stress in vivo and isolation of mitochondria in vitro cause changes and/or dephosphorylations of ETC complexes promoting maximal activity, and $\Delta\Psi_m$ only inhibits further proton pumping at very high $\Delta\Psi_m$ values; thus, in this state, $\Delta\Psi_m$ is hyperpolarized. Support of our model is provided by studies showing that phosphorylation of CcO and Cyt_c, as found in vivo, leads to partial inhibition and thus healthy cell respiration [54, 60, 66].

These studies demonstrate that stressful stimuli, such as ischemia, can result in changes in phosphorylation pattern of the OxPhos complexes, leading to ROS generation. Conversely, survival signaling promotes phosphorylation and “controlled” electron transfer rates. Our novel findings suggest a mechanism by which cell signaling cascades can regulate the overall basal activity rate of OxPhos [58]. Similar regulatory mechanisms may be discovered on other OxPhos complexes and functionally studied in the future. It is our hypothesis that mitochondria in intact cells under healthy conditions do not function at maximal capacity. This is reasonable because: (1) maximal rates of ATP synthesis by ATP synthase occur at $\Delta\Psi_m=100\text{--}120$ mV [67]; thus, a further increase in $\Delta\Psi_m$ will not result in more ATP production, and (2) as discussed in the “[Mitochondrial Membrane Potential Controls ROS Production](#)” section, high $\Delta\Psi_m$ levels cause excessive ROS production.

Mitochondrial Membrane Potential Controls ROS Production

Under normal conditions, over 90 % of oxygen is fully reduced to H₂O by CcO and only a small number of electrons “leak” and lead to partial reduction of O₂ to superoxide. This ROS production takes place proximal to CcO, at complexes I and III, which release superoxide on the matrix and intermembrane space sides, respectively [68]. In complex I, two major sites of electron escape have been proposed, the flavoprotein component of electron entry into complex I [69] or the iron–sulfur cluster [70]. For complex III, ROS is produced by inhibition of electron transfer through the Q cycle [71]. Electron transfer from the cytochrome b_L heme to the b_H heme is inhibited at high $\Delta\Psi_m$, resulting in ubisemiquinone radical generation [72]. Ubisemiquinone generated near the intermembrane space then reduces O₂ to form superoxide.

Electron leak occurs at complex I, complex III, or both, depending on the type of stressful stimuli and cell type, however one common mechanism exists. It has been clearly

demonstrated that ROS generation is dependent on $\Delta\Psi_m$. Specifically (1) the maintenance of physiologically optimal $\Delta\Psi_m$ values, not exceeding 140 mV, prevents generation of ROS, while providing the full capability to produce ATP [67]; (2) although there are some conditions where ROS can be generated at low $\Delta\Psi_m$ levels through different mechanisms [73], it is generally accepted that pathophysiologic levels of ROS are produced at high $\Delta\Psi_m$ values; and (3) hyperpolarization of $\Delta\Psi_m$ (exceeding 140 mV) causes an *exponential* increase in ROS generation at both complexes I and III [71, 74–76]. It was also shown that the $\Delta\Psi_m$ component alone, and not Δ pH of the proton motive force, determines ROS production at complex III [77]. High $\Delta\Psi_m$ levels extend the half-life of reaction intermediates of electron transfer at sites capable of single electron reduction of O₂, thus allowing electron escape.

Once partially reduced to superoxide, this oxygen radical reacts with other molecules such as H₂O or H⁺ to generate even more reactive species H₂O₂, HO₂, and OH. In addition, superoxide interacts with NO to form equally cytotoxic reactive nitrogen species. ROS generated in the mitochondria can freely cross mitochondrial membranes or exit via mitochondrial channels such as VDAC and, once released, can cause oxidative damage throughout the cell. Of note, ROS react quickly (half-life of seconds to nanoseconds) and irreversibly to damage cell constituents.

An important question remains: why do mitochondria have an excess capacity to generate higher $\Delta\Psi_m$ levels with potentially disastrous consequences for the cell? One reason is that mitochondria must have the capacity to adapt to varying energy demands. However, a more plausible explanation is their involvement in mitochondrial (type II) apoptosis. Numerous studies have demonstrated that induction of apoptosis, which is accompanied by stress signaling, can involve: (1) excessive calcium release; (2) transient hyperpolarization of $\Delta\Psi_m$; and (3) a burst in the production of ROS, which has been proposed as a key signal for committing a cell to apoptosis (reviewed in [78]). Accordingly, in the “[Model of Ischemia/Reperfusion Injury](#)” and “[Intervention at OxPhos or \$\Delta\Psi_m\$ as a Potent Method of Neuroprotection](#)” sections, we will integrate this mitochondrial-centric sequence of events leading to cellular demise into a model of ischemia/reperfusion injury, and discuss the concept that modulation of $\Delta\Psi_m$ may provide a novel strategy to attenuate brain damage caused by ischemia/reperfusion.

Model of Ischemia/Reperfusion Injury

The preceding sections have provided insight regarding the role of mitochondria in cell death caused by brain ischemia/reperfusion, and lead us to propose a model that focuses on changes of the phosphorylation state of mitochondrial

OxPhos and subsequent $\Delta\Psi_m$ hyperpolarization (Fig. 1). This model predicts that ischemic alterations of mitochondrial OxPhos prime the mitochondria for reperfusion-induced $\Delta\Psi_m$ hyperpolarization, an associated burst in mitochondrial ROS generation and loss of mitochondrial function, and subsequent delayed neuronal death. This progression is simplified into four main states that summarize the induction, progression, and execution of cell death during brain ischemia/reperfusion: (1) ischemic starvation, (2) reperfusion-induced hyperactivation, (3) mitochondrial dysfunction, and (4) delayed neuronal death (Fig. 1).

Ischemic–Starvation State: Ischemic OxPhos Complex Dephosphorylation and the Role of Calcium

A unique feature of the brain is that it is almost exclusively dependent on oxidative phosphorylation to generate energy. Therefore it is necessary to have a constant supply of oxygen to sustain functionality. As discussed in previous sections, even under normal conditions, oxidative phosphorylation results in low-level production of ROS which immediately react with antioxidants and do not cause measurable cellular damage. Brain ischemia of even short durations (on the order of seconds–minutes) causes cessation of electron transport, as OxPhos cannot proceed under anoxic conditions. Electron stalling occurs when the rate of entry of electrons into complex I exceeds the rate of transit through the slowest step of the chain. During ischemia, limiting electron transit through complex IV causes upstream build-up of electrons at complexes I and III, thus leading to reduced ETC.

Without electron transfer and proton pumping across the inner mitochondrial membrane, the proton gradient quickly dissipates, thereby disabling Δp_m -driven ATP generation by ATP synthase. Ischemia has been found to cause depolarization of $\Delta\Psi_m$ following simulated ischemia in vitro [79] and in vivo during experimental stroke [80]. If ischemia persists, this eventually leads to ATP depletion and failure of all energy-dependent processes in the mitochondria and throughout the cell [81, 82]. Depletion of ATP during ischemia would not allow ATP synthase to maintain $\Delta\Psi_m$ by operating in the reverse mode via ATP hydrolysis, a mechanism that operates under certain conditions such as in some cancer cells [83], where it is driven by high ATP flux through glycolysis. Of particular importance to ischemia/reperfusion injury is the equilibration of Ca^{2+} across the plasma membrane and subsequent accumulation of Ca^{2+} in the cytosol. At high cytosolic $[Ca^{2+}]$, mitochondria actively sequester Ca^{2+} to prevent pathologic increases in cytosolic $[Ca^{2+}]$. However, under the condition of ischemic depolarization, intramitochondrial $[Ca^{2+}]$ increases to pathologic levels with evidence (by electron microscopy) of severe mitochondrial swelling and accompanying hallmarks of cell death [84].

Calcium is a potent activation signal for mitochondrial phosphatases. For example, the calcium-dependent Ser/Thr phosphatase, calcineurin, can dephosphorylate proteins within the mitochondria [85]. Indeed, Ca^{2+} accumulation in the mitochondria induces dephosphorylation of multiple mitochondrial proteins [53, 57]. Moreover, it has been demonstrated that increased mitochondrial Ca^{2+} is a potent activation signal for mitochondrial respiration, leading to increased respiration and excessive ROS generation. This scenario is consistent with that suggested by McCormack and Denton who postulated that the main role of increased mitochondrial Ca^{2+} is to stimulate ATP production by activating enzymes involved in metabolism [86]. The effect of Ca^{2+} on mitochondrial respiration does not appear to be due to a direct action of Ca^{2+} on ETC components, suggesting an intermediate step exists that could be activated by Ca^{2+} . The recent discoveries that dephosphorylation of CcO and Cyt c lead to increased respiration rates provide a potential explanation for these early observations of the effect of Ca^{2+} on mitochondrial metabolism.

Our proposed model represents a pathologic alteration as a response to an imbalance between energy availability and energy demand. Under conditions of mild hypoxia and inadequate ATP, energy demand would trigger Ca^{2+} signaling to increase mitochondrial respiration to increase ATP production in order to correct the deficiencies [87]. In contrast, under ischemic conditions, attempts to increase OxPhos activity in response to inadequate ATP are futile, as the final electron acceptor, O_2 , is not present. One can speculate that this feed-forward mechanism caused by ischemia would eventually promote maximal activation of OxPhos. Indeed, others have questioned how a normal physiologic stimulus to increase energy production can lead to a pathologic increase in ROS generation [88]. When this process is viewed in the context of OxPhos dephosphorylation inducing subsequent hyperactivation of OxPhos, one can appreciate how ischemia could promote a mitochondrial state where substantial ROS can be generated upon reperfusion.

Reperfusion-Induced Hyperactivation State: OxPhos Hyperactivity, $\Delta\Psi_m$ Hyperpolarization, and ROS Generation

It is obvious that reperfusion of the ischemic brain is necessary for any attempt to salvage tissue. However, as we previously discussed, reperfusion per se contributes significantly to tissue damage. From the perspective of ischemic mitochondria, reperfusion is necessary to restore the terminal substrate for OxPhos and nutrients to reinitiate cellular respiration. However, ischemia promotes a mitochondrial state that is conducive to hyperactive electron transfer upon reperfusion (Fig. 2, *reperfusion-induced hyperactivation*).

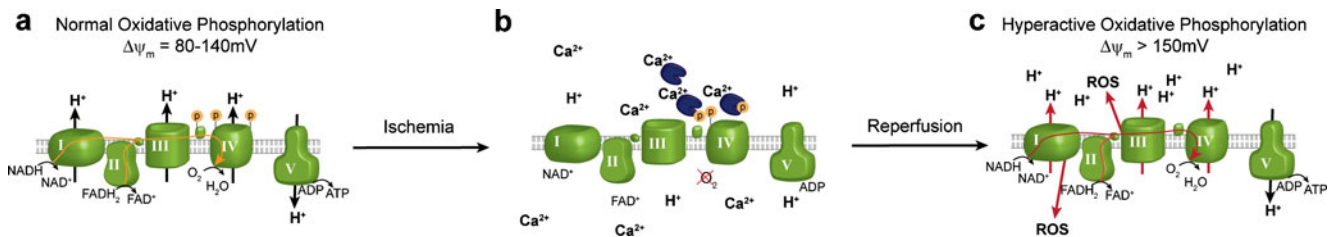


Fig. 2 Mechanism of ROS generation during reperfusion. During extended brain ischemia, increased intramitochondrial Ca^{2+} activates phosphatases that dephosphorylate OxPhos components, as shown for Cyt c and CcO in **b**. This promotes a state of OxPhos hyperactivity;

According to our model, ischemia evokes an increase in intermitochondrial Ca^{2+} [84, 89, 90], causing activation of mitochondrial phosphatases and dephosphorylation of OxPhos complexes [53], most notably Cyt c and CcO (Fig. 2). In addition to the effect on overall respiratory rate, dephosphorylation of CcO also leads to loss of allosteric inhibition by ATP [54, 55, 57, 60, 66]. Increased OxPhos activity alone could hyperpolarize $\Delta\Psi_m$ when reperfusion is initiated, and the loss of allosteric inhibition by ATP would further exacerbate this hyperpolarization. This effect could essentially “reset” respiratory control to a higher level, leading to loss of feedback inhibition between OxPhos and $\Delta\Psi_m$ at the normal 120–140 mV range. Under these conditions, hyperpolarization could be exacerbated and persist longer than otherwise possible without OxPhos complex dephosphorylation.

Enhanced and prolonged $\Delta\Psi_m$ hyperpolarization would have dramatic consequences. Liu et al. provided compelling evidence of the exponential nature of the relationship between ROS and $\Delta\Psi_m$ (reviewed in [71]). When $\Delta\Psi_m$ exceeds 140 mV, the exponential nature becomes clear, resulting in a 70–90 % increase in ROS generation with a modest 10-mV increase in $\Delta\Psi_m$ [71, 74]. Experimental measurements of $\Delta\Psi_m$ in this elevated range are plausible. In fact, as we previously discussed, traditional mitochondrial isolation methods that do not take into account preservation of complex OxPhos complex phosphorylation often result in $\Delta\Psi_m$ above 200 mV [31–38]. These studies suggest that OxPhos complex dephosphorylation during ischemia would have profound consequences when reperfusion is initiated.

During the initial moments of reperfusion, OxPhos complex dephosphorylation would serve to promote rapid reestablishment of $\Delta\Psi_m$ and restoration of cellular ATP levels. Indeed, following reversal of brain ischemia, $\Delta\Psi_m$ is restored to control levels within 1 min, and cellular ATP levels are restored in less than 15 min [80]. However, if normal respiratory control mechanisms are lost (including loss of allosteric inhibition by ATP), $\Delta\Psi_m$ would surpass normal levels, resulting in pathologic $\Delta\Psi_m$ hyperpolarization. In this regard, Liu et al. demonstrated that rapid restoration of $\Delta\Psi_m$ is quickly followed by hyperpolarization of $\Delta\Psi_m$. A study in neuronal cell culture exposed to simulated ischemia/

however, because O_2 is absent, electron transport cannot proceed. **c** Upon induction of reperfusion, OxPhos is allowed to operate at maximal activity, generating high $\Delta\Psi_m$ levels, which in turn promotes ROS generation

reperfusion injury demonstrated $\Delta\Psi_m$ hyperpolarization during the initial reperfusion stages [91]. Moreover, inhibition of $\Delta\Psi_m$ hyperpolarization by blocking complex I has been shown to prevent a stress-induced ROS burst and subsequent cell death [92]. Finally, this concept was extended by Starkov and Fiskum, who reported that mitochondria isolated from brain do indeed have an exponential relationship between $\Delta\Psi_m$ and ROS when assayed in vitro [74]. These studies suggest a pathologic condition where reperfusion results in $\Delta\Psi_m$ hyperpolarization which subsequently causes a significant ROS burst upon reperfusion.

Additional support for the OxPhos paradigm is provided by evidence demonstrating that the majority of ROS generation after brain ischemia occurs immediately upon reflow. For example, in the setting of global brain ischemia, ROS generation is most pronounced during the first 15 min of reperfusion [93]. Moreover, the predominant source of these ROS are the mitochondrial OxPhos complexes [11, 94, 95] and, in brain, escape of electrons from complex I appears to be responsible for most of the ROS produced [95–98].

As discussed in the “Mitochondrial Membrane Potential Controls ROS Production” section, the pivotal event that drives excessive electron escape and generation of ROS in vivo is $\Delta\Psi_m$ hyperpolarization. These data imply that interventions aimed at regulating $\Delta\Psi_m$ and preventing hyperpolarization may serve to attenuate ROS generation. Of particular interest is the dephosphorylation and hyperactivation of CcO, as CcO is the proposed rate-limiting step in OxPhos. Alternatively, direct targeting of $\Delta\Psi_m$ (for example, by using mitochondrial membrane uncoupling agents) may provide a feasible approach. However, before considering modulation of $\Delta\Psi_m$ as a therapeutic strategy (see the “Intervention at OxPhos or $\Delta\Psi_m$ as a Potent Method of Neuroprotection” section), we review the cytotoxic consequences of ROS generation.

Mitochondrial Dysfunction

Early studies into mitochondrial function following brain ischemia/reperfusion injury found that, by 30 min of reperfusion, mitochondrial respiration is dramatically decreased

in cell populations that are destined to die [99, 100]. More recent reports have demonstrated that global brain ischemia/reperfusion leads to a reduction in complex I and CcO activity at later stages of reperfusion. This respiratory inhibition occurs within the first hour of reperfusion for CcO and progresses more slowly for complex I (i.e., maximal inhibition by 24 h of reperfusion [101]). This loss of respiratory function is accompanied by a reduction and eventual collapse of $\Delta\Psi_m$, leading to cell death. Interestingly, while traditional studies have demonstrated reduced OxPhos activity during later reperfusion, recent evidence by Chomova and colleagues has shown that in the early minutes of reflow, OxPhos activities are increased over control levels [102]. These studies suggest that our current understanding of the responses of OxPhos to ischemia/reperfusion may be confounded by inappropriate mitochondrial extraction techniques employed in older reports.

Mitochondrial dysfunction during reperfusion has often been attributed to ROS-induced damage of mitochondrial components [70, 103]. The resulting OxPhos *hypo*-activation state (Fig. 1, mitochondrial dysfunction) results in impaired proton pumping and reduced electron transfer kinetics. The mitochondrial switch from a hyperactive to dysfunctional hypoactive state has been attributed to oxidative damage of OxPhos complexes and/or oxidative damage to lipids important to OxPhos function. In support of this concept, direct oxidative damage of mitochondria has been shown to be involved in cellular damage following brain ischemia/reperfusion [104, 105].

A critical mitochondrial lipid target of ROS damage is cardiolipin. This is a unique phospholipid in that the majority of cardiolipin is found in the inner mitochondrial membrane where it is in tight association with OxPhos components. Cardiolipin plays a critical role in membrane insertion and function of Cyt c , CcO, and other OxPhos complexes [106, 107], and there is growing evidence that cardiolipin plays a pivotal role in the regulation of mitochondrial bioenergetics [108, 109]. In fact, alterations in the structure and/or content of this phospholipid are responsible for mitochondrial dysfunction in a variety of pathological settings [110–113]; this concept is illustrated by the fact that disruption in the association of CcO with cardiolipin was accompanied by a ~50 % reduction in activity of the enzyme [109]. Upon peroxidation, cardiolipin undergoes redistribution to the outer mitochondrial membrane [114] where it is required for release of apoptotic proteins from mitochondria into the cytosol [115]. These effects could contribute to decreased CcO activity, impaired mitochondrial respiration, and mitochondrial failure.

Eventually, these pathologic alterations in mitochondrial function affect cellular functions and eventually lead to cell death. Alterations in mitochondrial function are potent signals for induction of cell death cascades. Additionally, ROS

has been implicated in directly activating cell death cascades. For example, under conditions of persistent impaired respiration, mitochondrial (type II) apoptosis is induced, with the release of apoptogenic factors (including Cyt c) from the mitochondria purportedly serving as the final and irreversible trigger of neuronal death.

Delayed Neuronal Death: an Apoptotic-Like Phenotype

Cell death is often classified as apoptotic or necrotic, however, following cerebral ischemia/reperfusion, cell death often proceeds in a manner distinct from traditional apoptosis or necrosis. Morphologic and biochemical analyses indicate that both necrotic and apoptotic events occur simultaneously [84, 116], and evidence linking various pathways suggests that a degree of cross talk exists that results in cell death occurring in a spectrum between apoptosis and necrosis [117]. The most common form of delayed, ischemia/reperfusion-induced neuronal cell death, and the type of insult that is the focus of our proposed mechanism, is characterized by an apoptotic-like phenotype. While all the specific characteristics of apoptosis may not be present, a key step—specifically, the release of apoptogenic factors from the mitochondria—appears critical in the initiation of cell death cascades [64, 118–120].

Many mechanisms have been proposed to explain the release of Cyt c from mitochondria. Historically, it was hypothesized that mitochondria simply swell and burst, thereby releasing their contents into the cytosol. More recently, a large body of work has focused on the Bcl-2 family of proteins and their interactions as important regulators of mitochondrial permeability to Cyt c . Of the Bcl-2 family, the primary candidates proposed to be involved in outer membrane permeabilization appear to be Bax and Bak; these proteins purportedly interact directly with mitochondria to promote the release of Cyt c and other apoptogenic proteins (e.g., AIF, Smac/Diablo) [121, 122]. In addition, other investigators have focused on elucidating the formation of so-called permeability transition pores that would facilitate Cyt c release.

The caveat in all of these studies is that they are based on the premise that Cyt c and other apoptogenic proteins are free within the mitochondria and, thus, could be released after pore formation or alterations in mitochondrial permeability. However, there is evidence that release of Cyt c is a two-step process, involving (1) the release of proteins usually tethered to inner membrane by cardiolipin, combined with (2) pore formation [123]. Indeed, Cyt c is among the proteins shown to be tethered to the inner mitochondrial membrane by cardiolipin [115, 124]. Exposure of mitochondria to ROS induces peroxidation followed by oxidation of cardiolipin, thereby releasing Cyt c into the intermitochondrial space [123]. Subsequently, upon pore formation or

alterations in permeability, the liberated Cyt_c is free to be released into the cytosol where it promotes formation of the apoptosome (a complex containing Cyt_c, caspase 9, and Apaf-1) that activates caspase 3. The apoptotic pathways activated following ischemia/reperfusion converge on caspase-3, the downstream cysteine protease, leading to the cleavage of hundreds of potential substrates within the brain and thus executing programmed cell death [125]. Indeed, activation of caspase-3, -8, and -9 have all been demonstrated to increase in the brain following ischemia/reperfusion [116, 126–128].

The aforementioned sequence of events identifies multiple points of possible therapeutic intervention that, if appropriately targeted, could stop the progression of delayed neuronal cell death and thereby salvage tissue from ischemia/reperfusion injury. For example, intervening at the level of apoptosis (including prevention of mitochondrial outer membrane pore formation, cardiolipin peroxidation, or caspase activation), should have therapeutic benefits. However, attempts to prevent apoptotic cell death have revealed that multiple concurrent and redundant cell death pathways can be activated, making specific targeting of individual mediators or single pathways of apoptosis difficult or ineffective. Therefore, a focus on upstream targets (that is, molecular events that precede Cyt_c release) may yield a more logical and robust therapeutic approach to neuroprotection.

Intervention at OxPhos or $\Delta\Psi_m$ as a Potent Method of Neuroprotection

Targeting ROS to reduce or prevent brain ischemia/reperfusion injury is one potential strategy to target an early cell death signal. However, this method has been met with numerous clinical failures. To understand this seemingly surprising lack of success, we must consider the traditional methodology for treatment of oxidative damage.

Current studies have shown that during reperfusion, ROS production exceeds the availability of endogenous antioxidants. Accordingly, previous attempts to design treatment modalities have focused on bolstering cellular antioxidant defenses to correct this imbalance. This strategy is primarily based on laboratory studies demonstrating robust neuroprotection in transgenic animals designed to overexpress endogenous mitochondrial antioxidants [104, 129–131]. These studies have provided substantial mechanistic insight into the pivotal role of ROS in cerebral ischemia/reperfusion injury. However, attempts to translate this concept and administer ROS scavengers as a clinical therapy have been futile. This discrepancy could be explained by the multitude of potential pitfalls inherent in delivery of pharmacologic scavengers and antioxidants to the brain during reperfusion, including difficulties in delivery, rapid reaction kinetics due to the short half-

life of ROS, multiple ROS generation sites, limited cellular drug uptake, and failure to cross the blood–brain barrier [132, 133]. Despite improvements in drug formulation and delivery, the efficacy of antioxidant strategies seen in animal studies has not been realized in human trials [134–137].

As an alternative to this antioxidant approach, we propose that interventions designed to *prevent* ROS generation (rather than scavenge ROS) will avoid many of the confounding issues associated with traditional scavenging techniques. In this regard, hyperpolarization of $\Delta\Psi_m$ is a critical regulatory step in multiple pathologies, including early reperfusion of multiple tissues [74, 80, 138]. Moreover, targeting of hyperpolarization has been shown to be a cytoprotective [91, 139, 140].

Uncoupling of Mitochondrial Membrane Potential

Cells express mitochondrial proteins, i.e., uncoupling proteins (UCPs) that allow H⁺ to cross the inner mitochondrial membrane down the proton gradient. This bypasses ATP synthase and thus does not produce ATP by utilizing the proton gradient. The physiologic role of these proton channels is typically associated with heat generation. Recent studies have, however, found that UCPs have additional functions in the cell, including stabilizing the $\Delta\Psi_m$, thereby limiting electron escape and thus partial reduction of O₂ [75]. Interestingly, when UCPs were investigated in tissues where heat generation is not a primary function (such as brain), it was found that these proteins render the brain resistant to ischemia/reperfusion injury. For example, Haines et al. demonstrated that knockout of uncoupling protein 2 (UCP2) resulted in dramatically larger infarcts after stroke [141]. The converse was also true: overexpression of UCP2 was associated with a decrease in ischemia/reperfusion-induced brain damage, ROS generation, and induction of apoptosis after stroke [142]. Finally, pivotal data from Teshima et al. demonstrated that increased expression of UCP2 prevented ROS-induced cell death by stabilizing $\Delta\Psi_m$ [143]. These findings suggest that uncoupling proteins may play an important role in mitochondria by stabilizing $\Delta\Psi_m$ to prevent hyperpolarization and ROS production under stress. As a result, these proteins may represent a potent target for therapeutic intervention.

In addition to the use of UCPs to stabilize the $\Delta\Psi_m$, exogenous chemicals that allow protons to cross the inner mitochondrial membrane have also been tested to prevent ischemia/reperfusion injury. Proton ionophores (agents that allow proton leak across the inner membrane) have been shown to be effective in multiple disease states. For example, mild mitochondrial membrane uncoupling reduced $\Delta\Psi_m$ hyperpolarization, prevented ROS, and reduced cell death in models of myocardial ischemia [140], traumatic brain injury [139], and peroxide-induced neuronal damage [92].

It is important to note that small concentrations of mitochondrial membrane uncoupling agents are profoundly protective, whereas, in contrast, higher concentrations *exacerbate* cellular damage [144]. These studies are consistent with our proposed mechanism of ischemia/reperfusion injury: mild membrane uncoupling will prevent the hyperpolarization of $\Delta\Psi_m$ during stressful conditions, while complete uncoupling would allow excessive proton escape across the inner membrane and result in an energetic crisis. This biphasic effect makes the use of these compounds potentially dangerous, as overdose of an uncoupling agent could dramatically compromise the ability to produce energy through OxPhos. These compounds would also need to be present in the mitochondria during the time window of $\Delta\Psi_m$ hyperpolarization. As the majority of studies suggest that this phenomenon occurs during the early seconds–minutes of reperfusion [93], delivery to tissue prior to reperfusion may pose a therapeutic barrier. However, as discussed in the following section, there are potential methods capable of attenuating $\Delta\Psi_m$ hyperpolarization that do not require pharmacologic delivery.

Ischemic Preconditioning

Several studies have demonstrated that $\Delta\Psi_m$ hyperpolarization during early reperfusion is a critical event in the generation of mitochondrial ROS. For example, Liu and Murphy utilized a customized laser scanning confocal microscope together with $\Delta\Psi_m$ -specific fluorescent probes for real-time analysis of $\Delta\Psi_m$ following relief of ischemia in a mouse model of stroke [80] and found that hyperpolarization of $\Delta\Psi_m$ was evident immediately following reflow. Moreover, the authors demonstrated that application of a robust neuroprotective strategy, ischemic preconditioning, prevented $\Delta\Psi_m$ hyperpolarization and dramatically reduced the extent of neurologic injury.

Of specific interest is the mechanism by which preconditioning can prevent $\Delta\Psi_m$ hyperpolarization at the onset of reperfusion. Dave et al. demonstrated that brief episodes of antecedent preconditioning ischemia triggered the activation of PKC ϵ and upregulated mitochondrial survival signaling [145]. Preconditioning applied prior to global brain ischemia provided multiple beneficial effects to the mitochondria, including phosphorylation of multiple OxPhos complexes, reduction of ROS generation, and preservation of mitochondrial respiration during late reperfusion (the mitochondrial dysfunction phase)—events that culminated in decreased Cyt c release, the putative trigger for apoptosis [145]. One could postulate that stimulation of cell signaling pathways that enhance phosphorylation of OxPhos complexes and limit Cyt c release could promote controlled respiration and stabilize $\Delta\Psi_m$. Alternatively, a sublethal ischemic event could induce OxPhos complex dephosphorylation on a small scale

and generate small amounts of ROS, thereby stimulating survival signaling responsible for maintaining phosphorylation. If the activation of these kinases was increased during the subsequent “lethal” ischemic event, this could provide protection from $\Delta\Psi_m$ hyperpolarization and ROS generation. In addition, preconditioning could induce the expression of UCPs, thereby maintaining lower basal $\Delta\Psi_m$ levels and limiting hyperpolarization. Indeed, Liu et al. demonstrated that preconditioning does increase UCP2 expression in brain [146]. However, whether this increase in expression in UCP2 contributes to preconditioning-induced neuroprotection—and, the precise mechanism by which preconditioning modulates $\Delta\Psi_m$ —remains unknown.

Induction of Cell Signaling to Induce OxPhos Complex Phosphorylation

Our current knowledge of Cyt c and CcO suggests that the primary role of the phosphorylation sites discovered to date is to sustain controlled respiratory rates and thereby prevent $\Delta\Psi_m$ hyperpolarization and ROS generation [54, 60, 62, 63, 66, 147]. Accordingly, it stands to reason that induction of cell signaling cascades that induce phosphorylation or prevent dephosphorylation would provide some protective effect. We have shown that phosphorylation of Cyt c at Tyr97 can be induced by insulin [63]. Moreover, insulin treatment was found to be neuroprotective in a model of global brain ischemia/reperfusion by preventing the release of Cyt c from mitochondria and inducing the upregulation of PI3K and other cell survival signaling cascades [64, 148]. Whether phosphorylation of Cyt c at Tyr97 contributes to insulin-induced neuroprotection by stabilizing $\Delta\Psi_m$ is a focus of current investigation by our group.

Conclusions

In this review, we present an overarching, mitochondrial-centric hypothesis describing the mechanisms that underlie mitochondrial ROS generation and cell death induced by reperfusion of ischemic brain tissue. There is evidence to support this injury mechanism in multiple scenarios of cerebral ischemia/reperfusion injury including stroke, cardiac arrest followed by resuscitation, and hypoxic–ischemic damage. The crux of the hypothesis is that $\Delta\Psi_m$ hyperpolarization and the ensuing ROS burst cause oxidative damage that precedes apoptosis in these pathologies. Specifically, we propose that: (1) ischemia induces dephosphorylation of OxPhos complexes, thereby (2) priming the mitochondria for hyperactive electron transport and $\Delta\Psi_m$ hyperpolarization upon reperfusion, (3) initiating a burst of ROS which overwhelms endogenous antioxidant systems that (4) damages cellular components and (5) culminates in the initiation of apoptotic-like cell death cascades.

Most notably, we propose that stabilization of $\Delta\Psi_m$ during early reperfusion provides a novel strategy to limit ROS generation and attenuate ischemia/reperfusion induced to the brain.

Acknowledgments This work was supported by the Department of Emergency Medicine, the Cardiovascular Research Institute, Wayne State University, Detroit, and grant GM089900 from the National Institutes of Health.

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