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Postischemic Oxidative Stress Promotes Mitochondrial Metabolic Failure in Neurons and Astrocytes

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Abstract

Oxidative stress and mitochondrial dysfunction have been closely associated in many subcellular, cellular, animal, and human studies of both acute brain injury and neurodegenerative diseases. Our animal models of brain injury caused by cardiac arrest illustrate this relationship and demonstrate that both oxidative molecular modifications and mitochondrial metabolic impairment are exacerbated by reoxygenation of the brain using 100% ventilatory O_2 compared to lower levels that maintain normoxemia. Numerous molecular mechanisms may be responsible for mitochondrial dysfunction caused by oxidative stress, including oxidation and inactivation of mitochondrial proteins, promotion of the mitochondrial membrane permeability transition, and consumption of metabolic cofactors and intermediates, e.g., NAD(H). Moreover, the relative contribution of these mechanisms to cell injury and death is likely different among different types of brain cells, e.g., neurons and astrocytes. In order to better understand these oxidative stress mechanisms and their relevance to neurologic disorders, we have undertaken studies with primary cultures of astrocytes and neurons exposed to O_2 and glucose deprivation and reoxygenation and compared the results of these studies to those using a rat model of neonatal asphyxic brain injury. These results support the hypothesis that release and or consumption of mitochondrial NAD(H) is at least partially responsible for respiratory inhibition, particularly in neurons.

Keywords

pyruvate dehydrogenase; respiration; nicotinamide adenine dinucleotide

Mitochondrial Dysfunction in Ischemic Brain Injury

A large body of evidence indicates that mitochondrial dysfunction plays a critical role in the pathophysiology of ischemic brain injury [1–6]. Consequences of mitochondrial dysfunction are numerous and include oxidative stress, loss of cellular Ca^{2+} homeostasis, promotion of apoptosis, and metabolic failure.

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There are many possible causes of mitochondrial metabolic impairment and most involve oxidative modifications to proteins, lipids, or DNA. Identification of the sites at which oxidative stress impairs respiration can guide the development of counteractive interventions with neuroprotective potential. Complex I of the electron transport chain (ETC), which catalyzes the oxidation of NADH and the reduction of ubiquinone, is particularly sensitive to inhibition by both oxidative stress and ischemia/reperfusion and is generally considered to be the rate-limiting component of the ETC [7-10]. Another cause of impaired ETC activity is the release of cytochrome c through the outer mitochondrial membrane into the cytosol, an event that is also often followed by caspase-dependent apoptosis [11]. Oxidative stress promotes cytochrome c release by several mechanisms, including those promoting translocation of Bax and Bak to the outer membrane where they form pores that allow for passive efflux of cytochrome c and other intermembrane proteins [12,13]. Oxidation of the mitochondria-specific phospholipid, cardiolipin, can also promote release of mitochondrial cytochrome c [14,15].

In addition to impaired ETC activities, oxidative phosphorylation can also be obstructed by inhibition of other mitochondrial enzymes and membrane transporters (Fig. 1). Thus, oxidative inactivation of mitochondrial matrix enzymes, e.g., pyruvate and α-ketoglutarate dehydrogenases and aconitase, are implicated in metabolic failure [16-18]. Evidence also suggests that mitochondrial oxidative stress impairs the adenine nucleotide translocase, necessary for influx of ADP and efflux of ATP[19]. In addition, much interest is currently focused on the availability of the metabolic cofactor NAD^+, necessary for dehydrogenases present within the mitochondrial matrix[20]. NAD^+ in its oxidized or reduced form (NADH) can be lost from the mitochondrial matrix following opening of the inner membrane permeability transition pore (PTP), which results in transmembrane equilibration of small ions and molecules of up to approximately 1500 Da[21,22] . The PTP is activated by abnormally high concentrations of Ca^{2+} and by oxidative stress[23]. Contribution of PTP opening to ischemic brain injury is supported by the neuroprotection observed with PTP inhibitors, e.g., cyclosporin drugs[24-27], that bind to cyclophilin D, the one well-established protein associated with pore opening. Cyclophilin D knock-out mice are also resistant to ischemic brain injury [28]. It is not known, however, if the PTP promotes ischemic neural cell death through chronic mitochondrial depolarization, and therefore uncoupling of oxidative phosphorylation, or through transient pore opening that releases sufficient NAD(H) to inhibit respiration and ATP synthesis by limiting the kinetics of dehydrogenase reactions.

NAD(H) Metabolism in Brain Injury

In addition to the multitude of redox reactions that either reduce or oxidize NAD(H), there are many reactions that consume NAD^+, including those catalyzed by poly(ADP-ribose) polymerases (PARPs) [29]. Under physiological conditions, the level of NAD^+ in the brain is primarily controlled by the NADase enzyme CD38 [30]. Under pathological conditions, e.g, during cerebral ischemia/reperfusion, oxidative stress, hypoglycemia, and glutamate excitotoxicity, PARP-1 appears to be the most potent NAD^+ consuming enzyme [31-35]. PARP-1 becomes activated due to its role in facilitating repair of DNA strand breaks, particularly those caused by oxidative modification [36]. Activated PARP-1 hydrolyzes NAD^+ and transfers the ADP-ribose moieties to form poly(ADP-ribose) on acceptor proteins [37]. This activity can result in a dramatic decline in cellular NAD^+, particularly under conditions where a decline in cellular ATP limits ATP-dependent NAD^+ biosynthesis. Once the NAD^+ concentration falls below the approximately 1 mM level necessary to sustain glycolysis in the cytosol or the TCA cycle in the mitochondrial matrix, ATP production is impaired, resulting in a vicious cycle that if not reversed, eventually results in permanent metabolic failure and necrotic cell death [38,39]. Reduced levels of ATP can also lead to impaired biosynthesis of NAD(H), at least in the cytosol [40], further accelerating the decline

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in energy metabolism. In addition to the effects of NAD^+ depletion on ATP production, loss of NAD(H) can induce necrosis and apoptosis by additional mechanisms, including promotion of PTP opening and modulation of NAD-dependent sirtuins, which regulate genetic cell death programs 41-43.

Mitochondrial NAD(H) Metabolism in Cellular and Animal Models of Brain Injury

Our interest in studying the effects of anoxia and reoxygenation on cellular and mitochondrial NAD(P)H fluorescence *in vitro* within neurons and astrocytes arose originally from observations made *in vivo* by Univ. of Miami investigators demonstrating a decrease in intrinsic NAD(P)H light absorbance on the surface of the cerebral cortex below baseline following global cerebral ischemia and reperfusion 44. This decrease and associated electrophysiologic abnormalities were ameliorated by the presence of antioxidants 45, and by perfusion with normoxic compared to hyperoxic gas-equilibrated media 46, suggesting that reactive oxygen species (ROS) mediate the decrease in light absorbance by NAD(P)H. While an apparent oxidized shift in the NAD(P)H redox state can be due to many factors, e.g., increased NADH utilization by stimulated respiration or impaired production of NADH from NAD^+, studies demonstrating net catabolism of NAD(H) in oxidative stress paradigms suggest an alternative explanation 47,48.

Experiments in our lab performed with primary cultures of rat cortical neurons exposed to glucose deprivation and either O_2 deprivation or chemical hypoxia (cyanide) support the concept that ischemia/reperfusion can result in rapid and extensive catabolism of cellular NAD(H), including that present in mitochondria, that is promoted by oxidative stress 49. Moreover, partial inhibition of NAD(H) catabolism under these conditions by cyclosporin A or by the PARP-1 inhibitor, 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2H)-isoquinolinone (DPQ), suggests that opening of the PTP triggers the release of mitochondrial NAD(H), allowing for its degradation by PARP-1 located in the nucleus or cytosol (Fig. 1) 50. Parallel experiments performed with primary cultures of cortical astrocytes indicated a relative resistance of these cells to NAD(H) catabolism induced by O_2 and glucose deprivation 49. While this resistance was attributed to the elevated reducing power afforded by glycogen present within astrocytes but not neurons, other factors, e.g., relative PARP activities, could also contribute to cell-selective vulnerability to NAD(H) degradation and metabolic failure.

Depletion of the total pool of NAD(H) has been reported for experiments performed *in vivo* using a rat model of cerebral ischemia/reperfusion 51. Our lab has extended such measurements using a neonatal rat hypoxic ischemia model and found that the mitochondrial pool of NAD(H) is partially depleted within 20 min of reperfusion after 75 min unilateral carotid arterial occlusion under 8% atmospheric O_2 52. Mitochondria from the ipsilateral hemispheres that were exposed to both ischemia and hypoxia contained 25% less NAD(H) compared to that present in mitochondria from the contralateral hemispheres that were exposed to hypoxia but no ischemia, as measured on perchloric acid extracts using an enzyme-linked fluorimetric assay 52. Furthermore, the loss of mitochondrial NAD(H) was accompanied by impairment of ADP-stimulated (state 3) O_2 consumption, observed in the presence of NAD(H)-dependent oxidizable substrates (malate plus glutamate) (Fig. 2A) but not with the NAD(H)-independent substrate succinate (not shown). Parallel experiments were performed under identical conditions measuring mitochondrial production of $^{14}CO_2$ over 60 min in the presence of 10 mM ^{14}C-glutamate. These experiments demonstrated a significant reduction of $^{14}CO_2$ generation by mitochondria from the hypoxic/ischemic hemisphere compared to the hypoxia alone hemisphere (25.7 ± 5.0 SEM vs 34.1 ± 6.7 nmol $^{14}CO_2$/mg protein. $n = 11$; $p < 0.05$ by two-tailed t test) 52. Most importantly, the depressed rate of state 3 respiration was completely reversed by adding 2.5 mM NAD^+ to

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the mitochondrial suspension, indicating that the loss of NAD(H) was responsible for the respiratory inhibition (Fig. 2B). Although not common knowledge, a few studies indicate that the mitochondrial inner membrane contains a transporter that mediates the uptake specifically of NAD^+ rather than NADH (Fig. 1)53‑55. The additional finding that NADH did not reverse the respiratory inhibition is consistent with this transport mechanism being responsible for mitochondrial NAD^+ uptake and provides an additional rationale for using extracellular or systemic addition of NAD^+ for neuroprotection, as shown in other models 36,56‑59. While the mechanism of mitochondrial NAD(H) depletion is at this juncture unknown, the cyclosporin A-sensitive PTP appears responsible for this phenomenon in the heart following cardiac ischemia/reperfusion 60. In the neonatal cerebral hypoxic ischemia model, respiration by the isolated mitochondria remains well-coupled, i.e., low state 4 (resting) respiration, despite the loss of NAD(H) and reduced state 3 respiration (Fig. 2). Thus it appears that if opening of the PTP was responsible for loss of NAD(H), the pore must have closed, either *in vivo* or during the mitochondrial isolation procedure. Another possible explanation for depletion of mitochondrial NAD(H) is direct consumption of NAD^+ by PARP-1 present within the mitochondrial matrix 61.

Promotion of Postischemic Oxidative Stress, Metabolic Dysfunction, and Neuronal Death by Hyperoxia

Our lab uses a clinically relevant canine cardiac arrest (CA) and resuscitation model to study molecular mechanisms of cell death and to identify targets for neuroprotection in global cerebral ischemia. A number of observations have been made using this model that illustrate the role of oxidative stress in postischemic metabolic dysfunction and demonstrate how both can be alleviated by simply avoiding abnormally high blood O_2 levels. One key observation was that the enzyme activity and immunoreactivity of the pyruvate dehydrogenase complex (PDHC) are both reduced in the brain as early as 2 hr of reperfusion, possibly explaining persistently high brain lactate levels 16,18,62. If decreased metabolic flux through PDHC were responsible for elevated lactate, the presence of an alternative source of fuel for aerobic energy metabolism might correct this abnormality. Indeed, postischemic intravenous administration of acetyl-L-carnitine reduces brain lactate levels and improves neurologic outcome 63. Since acetyl-L-carnitine can donate its acetyl group to coenzyme A, forming acetylCoA via the carnitine acetyltransferase reaction, neuroprotection by early postischemic infusion of this agent may be due to its aerobic metabolism and generation of reducing power to drive both ATP production and ROS detoxification.

When outcome measures for animals resuscitated for the first hour after CA using hyperoxic ventilation (100% O_2) were compared to those for animals resuscitated using normoxic ventilation, protein nitration, loss of PDHC, tissue lactic acidosis, impaired aerobic brain glucose metabolism, delayed neuronal death, and neurologic impairment are all significantly worse in the hyperoxic animals 16‑18,64,64‑66. Moreover, the differences in oxidative stress, PDHC, and aerobic energy metabolism observed within the first few hours after hyperoxic vs. normoxic resuscitation are evident in the hippocampus but not the frontal cortex, corresponding to the much greater vulnerability of the hippocampus to delayed neuronal cell death 18. While these results suggest that oxidative damage to PDHC may be responsible for impaired cerebral energy metabolism, measurements of respiration by isolated hippocampal mitochondria demonstrate a strong trend in respiratory inhibition by hyperoxic resuscitation using PDHC-independent oxidizable substrates (glutamate plus malate) as well as a significant inhibition using PDHC-dependent substrates (pyruvate plus malate) (Fig. 3) This finding implies that a mechanism in addition to, or other than inhibition of PDHC could be responsible for impaired postischemic cerebral energy metabolism. Based on the findings obtained with the neuronal O_2/glucose deprivation model and with the

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neonatal hypoxic ischemia model, we hypothesize that loss of mitochondrial NAD^+ is the additional or alternative mechanism.

Exacerbation of brain cell death by exposure to abnormally high O_2 concentrations after deprivation of O_2 and glucose has also been observed with primary cultures of neurons and astrocytes (Fig. 4) 67. Cells were placed in glucose free medium in an anaerobic chamber where the O_2 concentration in the medium was less than 1 μM, which effectively blocks the ability of cells to respire. Astrocytes and neurons were exposed to different periods of O_2 and glucose deprivation (4 hr and 30 min, respectively) that caused no immediate cell death but did result in significant delayed cell death following 24 hr reoxygenation. This pattern models the temporal course of cell death observed in the canine CA model. During reoxygenation in medium containing glucose, both cell types were exposed to either 20% O_2 (149 mm Hg), i.e., the normal 95% air / 5% CO_2 used in cell culture, or to 7% O_2 (52 mm Hg), which is far closer to the normal brain O_2 tension of around 30 mm Hg 68. The death of both astrocytes and neurons was significantly greater at 20% compared to 7% O_2. As expected, markers of oxidative stress, like protein nitration and DNA base oxidation, were elevated in cells under 20% compared to 7% O_2 within a few hours of reoxygenation67. Experiments using this model are in progress to determine how different reoxygenation O_2 tensions influence astrocyte and neuronal energy metabolism and whether changes in NAD(H) levels, PDHC activity, or other factors are responsible.

Development of Metabolism-Based Neuroprotective Interventions

Despite the explosion of available new information on how signal transduction mechanisms can promote cell survival or trigger pathologic apoptosis, mechanisms of metabolic failure and necrotic cell death continue to be critical targets for therapeutic intervention in global cerebral ischemia and other forms of neurodegeneration. The fact that neuroprotection has been observed with agents like creatine and ketone bodies or ketogenic diets in animal models of acute and chronic neurodegeneration 69-71 and that clinical trials based on these results are in progress are testaments to the therapeutic potential of correcting abnormal cerebral energy metabolism. Studies that identify the factor(s) that limit cerebral energy production and that delineate the time-course of metabolic disruption are necessary for strategic development of metabolic therapies. For instance, documentation that impaired PDHC activity limits aerobic energy metabolism at specific periods after global cerebral ischemia would provide the rationale for administering alternative metabolic fuels, e.g., acetyl-L-carnitine or ketone bodies, during these periods. Alternatively, should loss or catabolism of NAD^+ prove to be a limiting factor, this would support the pharmacologic use of exogenous NAD^+ or nicotinamide, an NAD^+ precursor, during the periods when they are needed. For instance, intranasal administration of NAD^+ at 10 mg/kg two hr after ischemic onset was recently shown to decrease infarct volume in a rat stroke model 56. Considering the heterogenous response of astrocytes, neurons, and neuronal subtypes to ischemia/ reperfusion, it is likely that the most effective metabolic therapies will involve combinations of agents, possibly designed selectively for gender and age. While these approaches are in development, further comparisons of metabolic outcome measures using hyperoxic and normoxic resuscitated animals may provide more support for avoiding hyperoxia early during reperfusion specifically after global cerebral ischemia.

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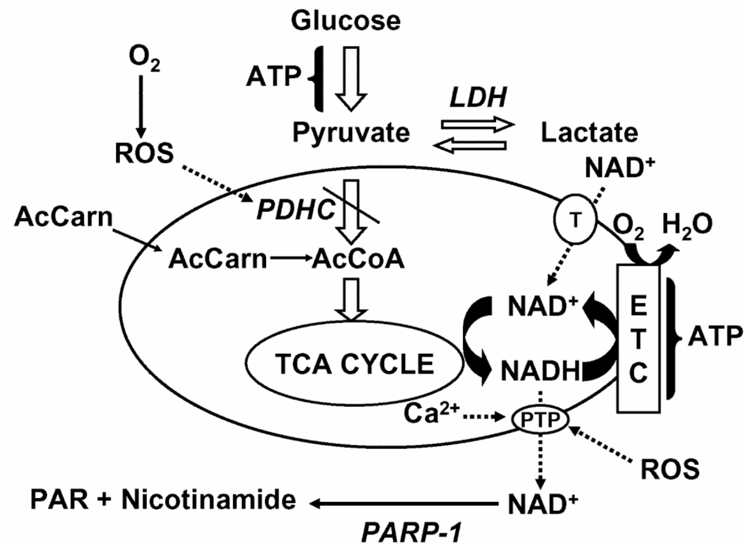

Fig. 1. Loss of pyruvate dehydrogenase activity and matrix NAD(H) as mitochondrial mechanisms of metabolic failure

Reactive O_2 and N_2 species can inhibit aerobic energy metabolism and promote lactate formation in several ways, including direct inhibition of PDHC and activation of the PTP, resulting in respiratory uncoupling and loss of NAD(H), that can then be consumed by PARP-1. Acetyl-carnitine can bypass the metabolic block at PDHC. Exogenous NAD^+ can enter intact mitochondria through a transporter (T), compensating for lost NAD(H), and providing the electron shuttle between dehydrogenases and the electron transport chain (ETC).

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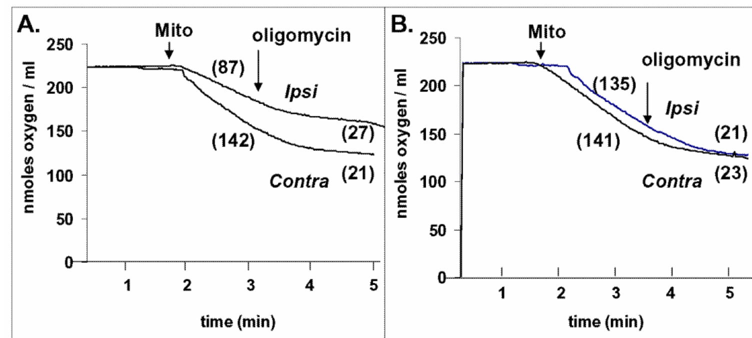

Fig. 2. Inhibition of respiration by brain mitochondria after neonatal hypoxic/ischemia and reversal of inhibition by NAD$^+$

Respiration was measured as described previously 7 at 37°C in medium containing 5 mM glutamate and 5 mM malate as substrates in the presence of 0.8 mM ADP and the absence (A) or presence (B) of 2.5 mM NAD$^+$. Numbers in parentheses represent rates of respiration in nmol O_2/min.mg protein. ADP-stimulated (state 3) respiration was lower for mitochondria from the ipsilateral, hypoxic/ischemic hemisphere (ipsi) compared to the contralateral, hypoxia only hemisphere (contra) (A), whereas both were very similar in the presence of NAD$^+$. Resting respiration (state 4) observed after addition of 1 μg/ml oligomycin was similar in all conditions. These tracings are representative of 7 different experiments.

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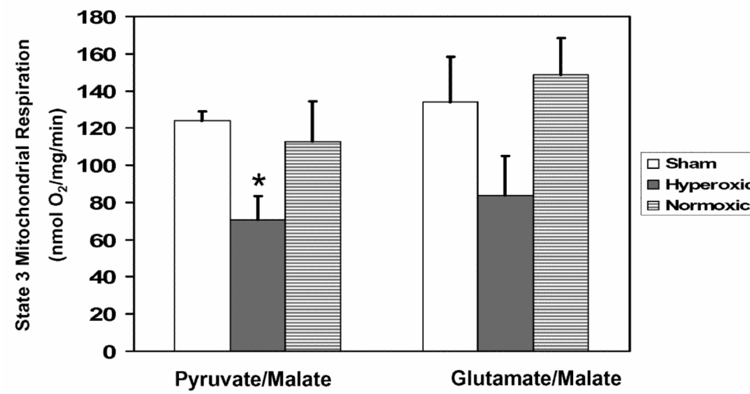

Fig. 3. Hyperoxic reperfusion after experimental cardiac arrest worsens mitochondrial respiration

Mitochondria were isolated from the hippocampi of dogs 2 hr after 10 min of cardiac arrest, using 1 hr of post-resuscitative mechanical ventilation on either 100% O_2 (hyperoxic) or room air (21% O_2; normoxic) 17. State 3 respiration was measured at 37°C in medium containing 0.1 mM malate plus either 5 mM pyruvate or 5 mM glutamate, as described previously6. Values represent the means ± SEM for 4 separate experiments. * Significantly different than non-ischemic, sham-operated controls; 1-way ANOVA with Tukey post hoc analysis, $p < 0.05$.

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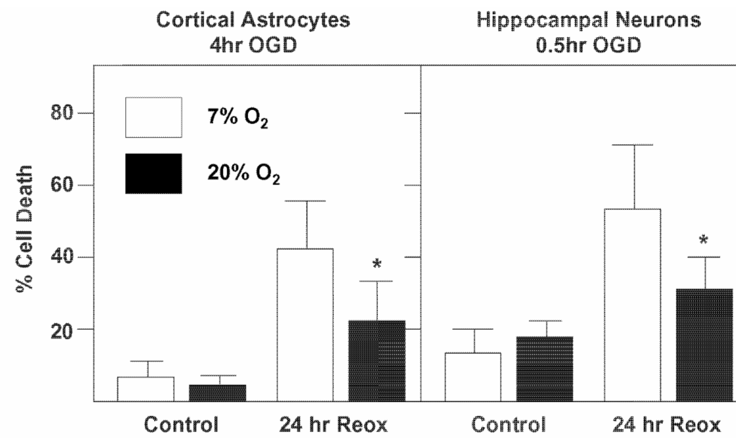

Fig. 4. Hyperoxia promotes death of astrocytes and neurons during rexoygenation after exposure to O_2 and glucose deprivation (OGD)

Rat cortical astrocytes (10 DIV) or murine hippocampal neurons (6 DIV) were exposed to OGD 4 hr and 0.5 hr, respectively, in serum-free “ischemic salts solution”, as described previously 72. The medium was then replaced with serum-free normal growth medium and the cells exposed to either 20% or 7% ambient O_2. Cell death was measured 24 hr later using the Hoescht/propidium iodide fluorescent assay for astrocytes and the Live/Dead Assay (Invitrogen) for neurons. See Danilov and Fiskum 67 for additional details. Values represent the means ± SEM for 6 - 8 separate experiments. * Significantly different from 20% O_2; t test, $p < 0.05$.